STUDIES ON THE CELLULAR FACTORS AND HORMONES CONTROLLING HIV-1 TRANSMISSION IN AN ORGAN CULTURE MODEL

by

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ABSTRACT

There is very little information available on how HIV-1 transmits through mucosal epithelial layer since it does not express HIV-1 receptor. Overall goal of the project is to elucidate the role of cellular factors and reproductive hormones on HIV-1 transmission across ectocervical and colonic mucosa in an organ culture model. We hypothesize that upon exposure to HIV-1, a complex signal transduction network is activated in epithelial cells, which leads to compromised barrier function by disrupted tight junctions and expression of immune mediators, which would recruit immune cells towards the epithelial layer for replication of virus. Furthermore, reproductive hormone might have an effect on HIV-1 acquisition risk. To test the hypothesis, we evaluated in context of tissue structure whether HIV-1 induces tight junction disruption in ectocervical and colon epithelial cells, examined the cellular factors, including inflammatory cytokines that are involved in HIV-1 transmission across ectocervical epithelia and evaluated the effect of reproductive hormone on HIV-1 transmission. Our results in aim 1 showed that after exposure to HIV-1, no significant changes in the tight junction/adherens junction protein expression were observed in ectocervical and colon epithelia. However, these tissues were infected after exposure to HIV-1. Our data thus indicate that HIV-1 transverses the ectocervical/colon mucosal epithelia without profoundly disrupting the tight junction/adherens junction between epithelial cell. In aim 2, we found that after HIV-1 exposure, the level of CXCL10 and CXCL11 messages in ectocervical epithelia were upregulated and such induction

of cytokines in ectocervical epithelia was dependent on HIV-1 infectivity. Furthermore, we measured the expression level of cellular factors in HIV-1 exposed ectocervical epithelia by next generation sequencing. Our results indicate that, cellular genes like IL36A, FMO2, CXCL10, MUC1, SAA1 and IL8 were differentially expressed in ectocervical epithelia exposed to HIV-1 compared to controls. These results suggest that exposure to HIV-1 induces cytokine production and other cellular factors in epithelial cells. In aim 3, we investigated the impact of reproductive hormones on the risk of HIV-1 acquisition by analyzing the susceptibility of ectocervical/vaginal tissues to HIV-1 infection and by comparing the epithelial thickness/tight junction protein expression in ectocervical/vaginal tissues at different phases of menstrual cycle. Our results showed no difference in HIV-1 susceptibility, epithelial layer thickness and tight junction/adherence junction protein expression levels in ectocervical/vaginal tissues at different stages of the menstrual cycle. Taken together, our results suggest that risk of HIV-1 infection in the ectocervical/vaginal region does not vary over the course of menstrual cycle. These findings are of public health importance because they expand our understanding on mechanism of atraumatic HIV-1 transmission in mucosal area that may be important for developing effective strategies for preventing HIV-1 transmission.

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1.0 INTRODUCTION

In 1981, increasing cases of opportunistic infections were documented in homosexual men and AIDS (acquired immune deficiency syndrome) was recognized as a new disease [1]. AIDS is caused by two lentiviruses, human immunodeficiency viruses (HIV) types 1 and 2 [2]. Both types of HIV likely originated by cross-species transmissions of simian immunodeficiency viruses (SIV), which are known to naturally infect African non-human primates [3]. HIV-2 infected people have longer incubation periods and lower morbidity [3, 4]. Due to the high error rate of the reverse transcriptase, HIV-1 is remarkably diverse. Thus HIV-1 has evolved over time into different groups and subtypes. HIV-1 strains can be classified into M, O and N groups [5]. Currently, there are millions of people living with HIV-1 in the world. HIV-1 transmission occurs through different routes like contaminated needle sharing, blood transfusion, sexual transmission and perinatal transmission [6]. However, sexual transmission through female reproductive tract and rectal regions accounts for majority of transmission in adults. HIV-1 traverses the epithelial layer of these mucosal regions through intercourse-induced mechanical microabrasion of the mucosal surface and also without mechanical tear. However, the exact mechanism of atraumatic transmission of HIV-1 through cervical and rectal mucosa during sexual transmission is not known. Elucidating the mechanism of atraumatic HIV-1 transmission through cervical and colon mucosa is very important to develop effective strategies for prevention of HIV-1 transmission.

1.1 HIV-1 EPIDEMIOLOGY

In 2013, UNAIDS estimated that globally 35.3 million people were living with HIV-1 in the world [119]. The numbers of people living with HIV-1 in Sub Saharan Africa, South East Asia, Latin America, Europe, North America, Middle East and North Africa were 25 million, 3.9 million, 0.88 million, 1.5 million, 0.86 million ,1.3 million, 0.26 million respectively (Figure 1) [126]. In 2013, 2.1 million people became newly infected with HIV-1 and about 1.5 million deaths occurred due to AIDS [126]. The majorities of HIV-1 viral strains are classified as group M which has various subtypes (A to K) [5] and are distributed worldwide. Subtype A infections are endemic to Central and East Africa as well as East European countries. Subtype B infections are endemic to West and Central Europe, the Americas, Australia, South America, and several southeast Asian countries. Subtype C infections are endemic to Sub Saharan Africa, India, and Brazil. Subtype D infections are endemic to North Africa and the Middle East. Subtype F infections are endemic to South and Southeast Asia. Subtype G infections are endemic to West and Central Africa. Subtype H, J, and K infections are endemic to Africa and the Middle East [7] [126].

Although currently there is no cure for HIV-1 infection, many studies have been carried out to understand the virus, its life cycle and pathogenesis leading to development of antiretroviral drugs that have helped to reduce viral transmission and disease progression. A study showed that the percentage of HIV-1 positive people receiving treatment was highest in Western Europe and North America (50%), Latin America (45%) and lowest in the Middle East and North Africa [127].

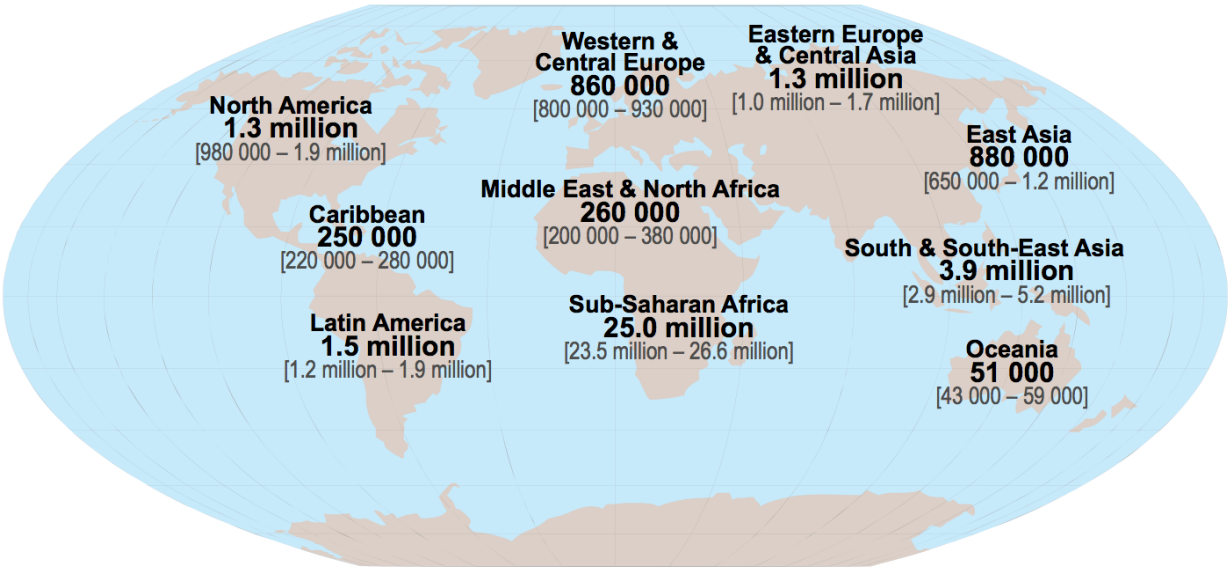


Figure 1. UNAIDS global report on adults and children to be living with HIV-1 in 2013

UNAIDS 2013 Report of the Global AIDS Epidemic. 2013. UNAIDS (Open access journal)

Epidemiological distribution of HIV-1 positive people in the world with highest number of people living with HIV-1 in Africa

1.2 HIV-1 LIFE CYCLE

1.2.1 Binding and Fusion

HIV-1 primarily infects CD4+ T cells and macrophages. HIV-1 lifecycle begins by binding of the gp120 subunit of HIV-1, the surface Env glycoprotein to the T cell surface receptor CD4. This causes conformational changes in gp120 and exposes the binding site for a co-receptor, CCR5 or CXCR4[8]. Furthermore, this causes conformational changes in the HIV-1 transmembrane protein gp41[9], which then penetrates into the cell membrane to fuse with the host cell membrane and release the viral core into the host cytoplasm. Studies have also shown

that HIV-1 can be endocytosed rapidly and fuse the viral envelope with the endosomal membrane, releasing the viral core into the cellular cytoplasm (Figure 2). [10].

1.2.2 Uncoating and Integration

The HIV-1 core is composed of the viral capsid (CA) proteins, the replication enzymes reverse transcriptase (RT), integrase (IN) and the viral genomic RNA. The viral core released into host cytoplasm undergoes morphological changes to dissociate the capsid protein subunit. Studies have shown that host factors like cyclophilin A are required for this process of uncoating [11, 12]. This is a very crucial step in the virus life cycle for subsequent nuclear import. Host factors like Tripartite motif-containing protein target the viral capsid protein and cause premature uncoating, thus impeding viral infection [13-15].

After the process of fusion and uncoating, the two strands of HIV-1 RNA genome with the proteins- protease, integrase and reverse transcriptase are released into the cytoplasm. Host proteins like transportin-SR2 and importin 7, and viral proteins like Vpr, aid nuclear import of this pre-integration complex that contains IN, viral DNA, cellular proteins [16, 17]. Reverse transcriptase consists of three subunits: a reverse transcriptase domain, a polymerase domain, and a RNAase H domain. The reverse transcriptase domain first converts ssRNA to ssDNA, followed by conversion of ssDNA to dsDNA using polymerase domain. The original RNA template is then cleaved and degraded by the RNase H domain. The low proofreading ability of reverse transcriptase results in high mutation rates of HIV-1. Viral enzyme integrase (IN) catalyzes the integration of viral dsDNA, mainly at sites of the host chromosome that are actively transcribed[18]. Integrase cleaves the 3’ viral DNA end and inserts it into host DNA. Once integration is complete and the retrovirus is in its ‘provirus’ form, this dsDNA contains the long

terminal repeats (LTR), which are made of U3, R, and U5. The LTR plays an important role in transcriptional regulation after integration of viral DNA into the host genome.

1.2.3 Transcription and Translation

Transcription of integrated proviral DNA takes place using the host RNA polymerase II and the viral transcription transctivator (Tat) [19]. Tat is unique sequence-specific activator of transcription and recognize an RNA element in nascent transcripts. Cellular proteins initiate transcription of proviral DNA in infected cells at some basal rate [19]. Tat proteins are the produced from the initial viral transcripts. These Tat proteins are imported into the nucleus and stimulate the transcription of proviral DNA. The viral RNA are produced and they undergo post-transcriptional modifications by 5' capping and 3' polyadenylation. The transcripts are either unspliced or multiple spliced. Proteins like Tat and Rev are produced from the multiply spliced transcripts. Some of the unspliced transcripts are used as the genetic material to be packaged into virions while the remaining unspliced transcripts are used for synthesis of polyprotein precursors like Gag and Gag-Pol. For the transport of the mRNA template to the cytoplasm, Rev shuttle back into the nucleus, bind to the Rev Response Element in *env* and transport the transcripts to the cytoplasm which are then translated to form viral proteins.

1.2.4 Assembly and Maturation

Once the viral proteins and viral RNA are synthesized, they are directed to the plasma membrane for assembly. Assembly of viral components takes place by myristylation of the N-terminal of Gag polyprotein followed by budding of the assembled viral components from the

plasma membrane [20]. Protease then cleaves Gag and Gag-Pol polyproteins for the formation of matured virion [21].

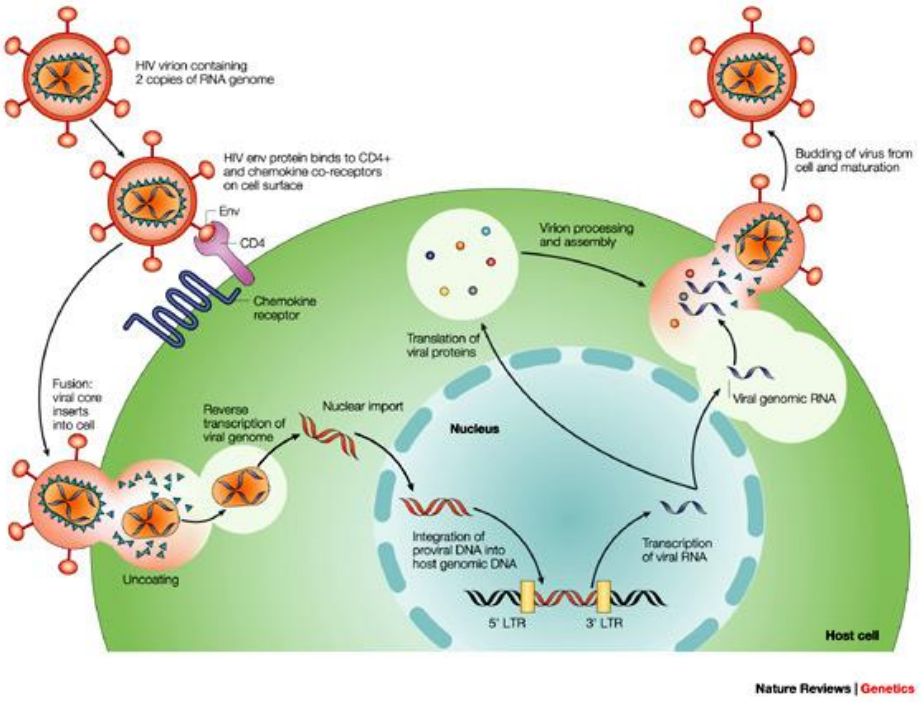


Figure 2. Key aspects of the HIV-1 life cycle

Figure taken from Rambaut, *et al.* Nature Reviews Genetics 5, 52-61, January 2004. Permission granted from rights holder, Nature Publishing Group.

1.3 NATURAL HISTORY OF HIV-1 INFECTION

HIV-1 can infect humans and disrupt the innate and adaptive immune systems. Based on CD4 counts, viral loads and clinical presentation, the course of HIV-1 infection is classified into three broad stages that include the acute stage, the chronic stage and the AIDS stage (Figure 3).

Acute phase of HIV-1 diseases is characterized by an initial high peak of plasma viremia and associated depletion of CD4+ T cells [8]. The reduction of CD4+ T cells is evident in the

peripheral blood, but is prominent and drastic at gut-associated lymphoid tissues due to HIV-1 targeting of CCR5+ CD4+ memory T cells that account for most mucosal CD4+ T cells [22]. Studies in rhesus macaques showed that 60% of mucosal memory CD4+ T cells were infected and 80% of the infected cells were depleted within four days of SIV infection[23, 24]. This observation has been consistent in HIV-1 infected humans. Studies have suggested that HIV-1 positive people will lose the majority of mucosal CD4+ T cells by the third week of infection and this depletion occurs more prominently in the initial few days of infection [8, 25, 26]. The neutralizing antibodies are produced by B cells a few weeks to months after infection. However their function in viral control has been controversial. Three to four weeks post infection is also characterized by development of adaptive immunity, giving rise to the initial HIV-1 specific CD8+T cell response that is temporally associated with initial drop on viremia. The CD8+ T cell response plays an important role in determining the plasma set point of HIV-1 and the subsequent slopes of CD4+ T cell decline [26].

A study by Fiebig *et al.* classified the acute phase of infection into six stages (Figure 3) based on HIV-1 replication and antibody responses [27]. During the first stage, there is an increase in viremia and only HIV-1 RNA can be detected in the blood. After about 7 days (Fiebig stage 2), HIV-1 RNA levels rise above 10,000 copies/mL and p24 antigen can be detected [27]. This stage is also characterized by intense inflammatory responses with high level of chemokines and cytokines. At stage 3, which is about one to two weeks after onset of symptoms, detectable levels of HIV-1 antibodies are seen in blood. Stages 4, 5 and 6 (7 days to 1 month after infection) is characterized by detection of intermediate and clear western blot band for defining HIV-1 infection.

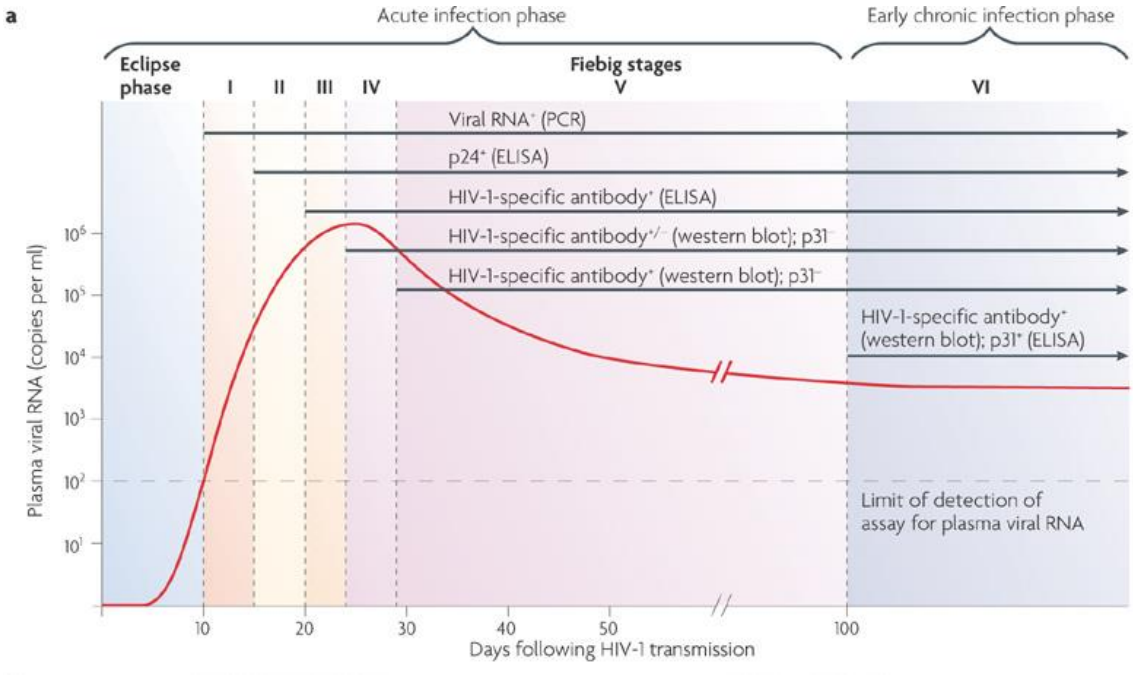


Figure 3. Fiebig Laboratory Staging of Acute HIV-1 Infection

Figure taken from McMicheal, *et al.* Nature Reviews Immunology 10, 11-23 (2010) Permission granted from rights holder (Nature publishing group).

While the acute phase of HIV-1 infection is marked by rapid increase in viral load and decrease in CD4+ T cell, the chronic phase demonstrates strikingly different viral and T cell dynamics. The chronic stage is characterized by reduced and stable viral load whereas CD4+T cell numbers rebound and are stable [25, 26]. However, there is a high level of immune activation. This immune activation leads to increase in the number of activated CD4+ T cells and consequently, the target cell numbers for HIV-1 infection [22]. Moreover, the number of Th17 CD4+ T cells is reduced in the gut, which leads to increased intestinal permeability and microbial translocation, which further leads to systemic activation of immune cells [28, 29]. Immune activation causes loss of CD4+T cells, which leads to apoptosis, and loss of CD8+ T cells and B cells during the latter stage of chronic infection [30, 31]. Previous studies have shown that this immune activation during the chronic stage is the main cause of HIV-1

immunopathogenesis [32]. Unlike humans and rhesus macaques, Sooty mangabeys can also be infected with SIV and undergo CD4+T cell loss, but do not exhibit immune activation and do not develop disease [33].

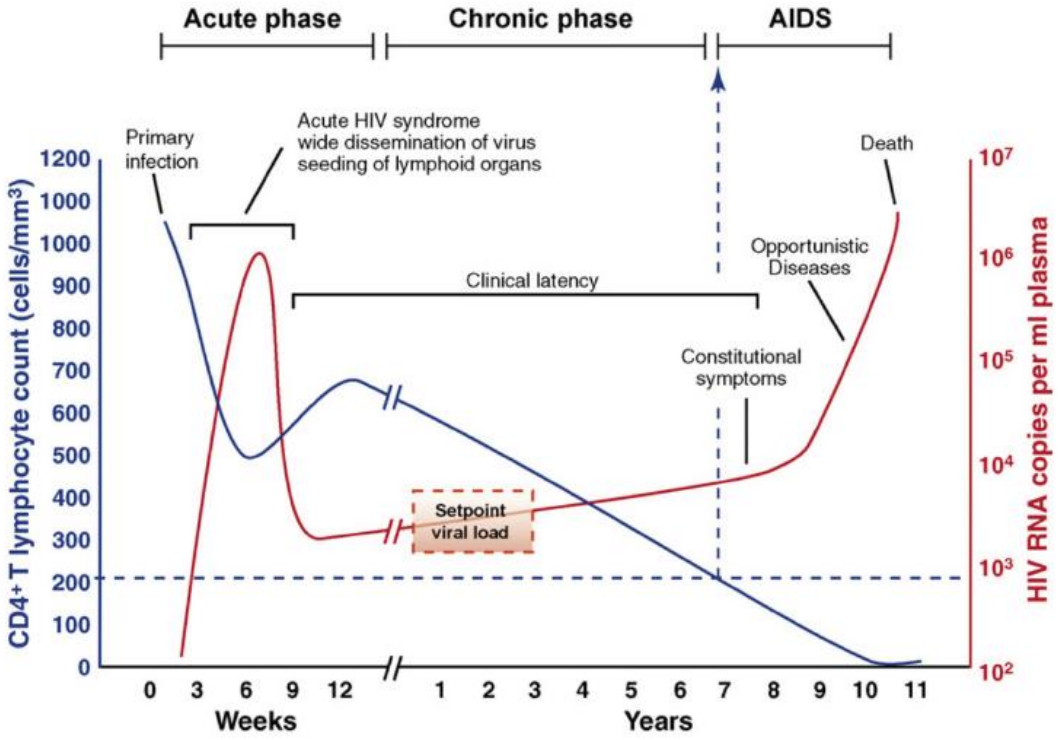


Figure 4. Natural course of HIV-1 infection

Figure taken from Ping An, *et al.* Trends in Genetics 26 (3): 119–131, March 2010. Permission granted from rights holder, Elsevier Publishing Group

The last stage of HIV-1 infection is AIDS (Figure 4). AIDS is characterized by CD4 T cell numbers of 200/mm^3 or lower[34]. Naïve T cell depletion, degradation of memory T cells and increased immune activation play an important role in disease progression[26]. Loss of CD4+ T cells leads to anergy in B cells [35]. Moreover, during AIDS the CD8+ T cells do not receive effective and appropriate co-stimulatory signals from CD4+ T cells leading to activation-

induced cell death of CD8+ T cells upon re-stimulation with antigen [36]. The resulting immunodeficiency leads to opportunistic infections such as kaposi's sarcoma, tuberculosis, pneumonia, cryptococcosis [26].

1.4 HIV-1 TRANSMISSION

HIV-1 transmission occurs through contaminated needle sharing, blood transfusion, mother-child transmission and during sexual intercourse[6]. HIV-1 prevalence is 28 times higher among drug users, 12 times higher in sex workers, 19 times higher in homosexual men and up to 49 times higher in transgender women and men compared to rest of the adult population [119]. HIV-1 spreads primarily through the genital and rectal mucosa during sexual intercourse [37], which accounts for the majority of HIV-1 transmission in adults. Based on non-human primate studies, virus can traverse through the mucosal barrier within hours after inoculation of SIV in both vaginal [38] and rectal areas followed by establishing small founder population of infected cells [39]. These founder viruses have been shown to be less diverse in newly infected HIV-1 positive people compared to the viruses isolated from the transmitter[40]. This genetic bottleneck of HIV-1 transmission has been confirmed in studies where viruses were sequenced in heterosexual transmission pair during acute infection [40, 41].

Within the first week of infection, local proliferation of the founder population takes place to produce viruses and virus-infected cells [38]. The local expansion of the viruses is crucial and causes dissemination of infection to draining lymph node [42, 43]. During the second week of infection, virus spreads to the lymphatic tissue [44]. These lymphatic regions have more HIV-1-target cells, which are distributed in close proximity compared to the regions at the portal

of entry [44]. Thus the virus replicates and spreads very rapidly once it reaches the lymphatic tissues. At the end of second week, the virus level in the tissues and blood peaks and by the fourth week of infection, viral levels decline to a steady lower level [44]. In these lymphatic tissues, the virus continues to replicate and the proviruses are hidden in latently infected cells[45]. Thus these lymphatic regions act as a reservoir for viruses and CD4+T cells in these regions undergo depletion, which eventually leads to disease progression [44].

Below is a detailed description of the initial events in transmission in ectocervical and rectal/colon mucosa. In order for HIV-1 to be transmitted through ectocervial and rectal/colon regions, the virus must cross the epithelial barrier of these mucosal tissues.

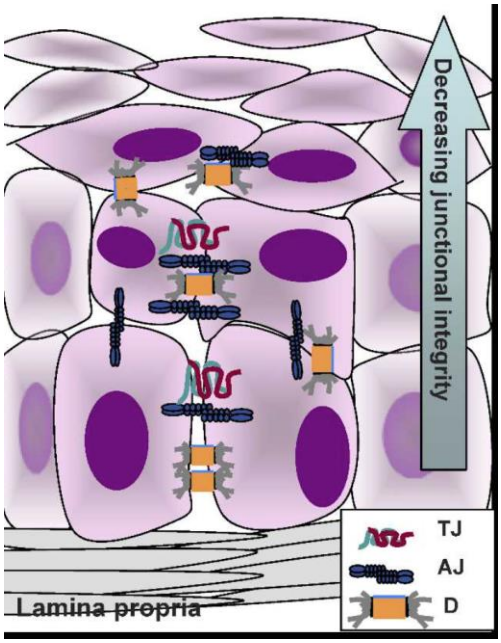
1.4.1 Epithelial barrier and HIV-1 transmission through ectocervical mucosa

1.4.1.1 Ectocervical mucosal barrier

Mucosal surfaces are the primary sites of HIV-1 transmission. Since the mucosal epithelia are the first cells that HIV-1 has to encounter before transmission through the mucosal region, physical and functional characteristics of the epithelia are important in determining the outcome of HIV-1 exposure [46]. The epithelial layer of vaginal and ectocervical mucosa is composed of stratified squamous epithelial cells. These epithelial cells are compactly bound, which in turn offer protection against entry of pathogens [47].

TJ are intercellular junctional structures that facilitate cell-to-cell adhesion and play an important role in epithelial cell function [48]. TJ are elaborate structures comprised of transmembrane proteins like claudins, occludin and cytosolic proteins such as the zona occludens (ZO) proteins[48]. There are about 24 different types of claudins. The transmembrane proteins occludin and claudin are made of two extracellular loops, four transmembrane domains, and two

intracellular domain which contribute to the tightness of paracellular barriers[48]. ZO proteins act as adapter molecules connecting the TJ proteins to the actin cytoskeleton (Figure 5) [47]. The adherens junction (AJ) on the other hand is composed of epithelial cadherin (E-cadherin), which is linked to the cytoskeleton via vinculin, and alpha and beta catenin [49]. AJs help in stabilizing cell-to-cell contact, help the formation of TJs, and also play a role in regulating barrier permeability. Studies have shown that loss of E-cadherin increased TJ permeability and also altered the localization of claudin-1,4 and ZO-1. TJ forms a barrier that restricts the transport of ions and nonelectrolytes through the extracellular clefts between cells (the 'gate' function)[50]. It also serves to maintain cell polarity forming a "fence" that restricts the diffusion of proteins and lipids between apical and basolateral surfaces [51, 52]. Na/K/ATPase expressed basolaterally plays a crucial role in development of epithelial polarity [51, 52]. Additionally, the continuous sloughing off of the superficial layers of ectocervical/ vaginal epithelium prevents the colonization and infection of many pathogens [53, 54]. However, large surface area of vaginal and ectocervical region increases the opportunity for HIV-1 to interact with and traverse through these mucosal epithelia.



TJ-tight junction, AJ- adherens junction, D- desmosomes.

Figure 5. Localization of interepithelial adhesion molecules between epithelial cells in the ectocervical tissues

Figure taken from Blaskewicz *et al*. Biology of Reproduction, 85(1):97-104. 2011. (Open access journal)

1.4.1.2 HIV-1 transmission through ectocervical mucosa

For successful HIV-1 transmission through vaginal or ectocervical mucosa, the virus must cross the epithelial barrier of these mucosal regions. The virus can traverse the epithelial layer through intercourse-induced mechanical micro abrasion of the mucosal surface and also without mechanical tear [55]. Studies using female macaques infected with SIV, humanized-mice and human cervical explants infected with HIV-1 have shown that SIV/HIV-1 infection can be established by both cell-free viruses and cell-associated viruses [53, 56]. Regardless whether HIV-1 virions are inoculated as cell free virus or cell-associated, the mechanism of HIV-1

transmission through the ectocervical mucosal layer, which does not express HIV-1 receptors, is not clear.

HIV-1 has been shown to bind epithelial cells via gp340, heparin sulfate, sulfated lactosylceramide, proteoglycans and syndecans, which are expressed on the genital mucosa epithelial cells [57, 58]. However, the relative contribution of these molecules to HIV-1 transmission in ectocervical and vaginal mucosa is uncertain. Studies using epithelial cells derived from the lower female genital tract have showed the binding, entry and the subsequent transfer of HIV-1 to susceptible CD4+ T cells. Virus transfer can take place by transcytosis in primary genital epithelial cells, where the viruses can traverse from the apical to the basal region, released from the cells and then infect susceptible target immune cells. However, only small percentages of viruses are able to pass through epithelial cells by transcytosis [53, 59]. Studies have also shown that cell-associated viruses appear to be more efficient in transcytosis than a cell-free virus [53, 60, 61]. Since cell-free virus seems to be more efficient in transmission than cell-associated HIV-1 and there are more viruses present in semen as cell-free than cell-associated virus [53, 60, 61], transmission seems to occur mostly via cell-free route. Therefore, transcytosis may not play an important role in the majority of sexual transmission.

The ectocervical and vaginal tissues have target cell population like dendritic cells and spatially distributed CD4+T cells just beneath the epithelial layer [53]. Thus once the virus traverses the mucosal barrier, they can easily access and infect the underlying target cells.

1.4.2 Mucosal barrier and HIV-1 transmission through rectal/colon mucosa

The colonic and rectal mucosa are the major site of HIV-1 transmission in unprotected heterosexual and homosexual transmission. Risk of HIV-1 transmission through unprotected rectal intercourse is 10-fold higher compared to vaginal intercourse [62, 63].

The colon and rectal mucosa are lined by a single layer of columnar epithelial cells, which are also compactly bound by intercellular junctional complexes including tight junction (TJ), adherence junction (AJ) that are crucial for preserving the integrity of the epithelial layer. Thus, gut mucosae with their intact TJ and AJ serves as the key barrier against the passage of macromolecules including pathogens. Apart from the tight junctions between the epithelial cells, the local secretion of mucin and antibodies on the epithelial surface help in preventing the entry of antigens and microorganisms [53]. A previous study has reported the presence of galactosyl ceramide on colorectal epithelial cell lines, which mediates virus attachment and entry[64]. However, the relative contribution of the galactosyl ceramide in HIV-1 transmission in colon mucosa is uncertain. Moreover, epithelial cells do not express CD4 receptors that are required for HIV-1 entry. Therefore, the mechanism of HIV-1 transmission across intact colon/rectal mucosa is still unclear.

HIV-1 transmission through rectal mucosa has been of particular importance because rectal mucosa is rich in lymphoid aggregates and follicles. The gut associated lymphoid tissue has dense populations of lymphocytes and antigen presenting cells that undergo continuous activation and proliferation by the luminal antigens. Thus, once the virus traverses through the mucosal barrier, this region serves as an important site for viral replication and persistence. SIV entry through rectal mucosa has been shown to be a rapid process and it takes four hours for the

virus to establish infection in the lamina propria, lymphoid aggregates [65]. In case of humans, studies using colon explants have shown HIV-1 replication in colon tissues after exposure to HIV-1 [66].

1.5 INFLAMMATORY RESPONSE IN MUCOSAL EPITHELIAL CELLS

Once HIV-1 crosses the epithelial layer, the virus establishes infection in small focal "hot spots"[58, 67]. Cervical and intestinal epithelial cells express TLRs 1to 5 &9 to recognize both bacterial and viral pathogenic motifs in the lumen[58]. TLR-mediated activation has been shown to induce cytokine production including CXCL10, IL-6, CXCL11, SDF-1, MIP-1α, MIP-1β, IL-8 TNFα, GM-CSF, Type 1 IFNs and RANTES, which play an important role in recruiting immune cells and enhancing their functions [58]. Various *in vitro* studies have shown that HIV-1 induces production of cytokines like IL8, MIP1 Beta, MCP-1, thymic stromal lymphopoietin (TSLP), TNFα, IL 6 in genital and intestinal cell lines [68, 69]. Brenchley *et al.* have shown that the degree of inflammation within the gastrointestinal tract was associated with viral replication and further observed that colonic mucosa from HIV-1 infected patients had significantly higher levels of pro-inflammatory cytokine expression (e.g. TNFα, IFN-γ, and IL-6) compared to control patients [22, 44]. Sastry *et al.* have reported up-regulation of TSLP in human cervical epithelial cell lines post-HIV-1 exposure that strongly activates human myeloid DC[70], while others have shown accumulation of HIV-1 RNA positive CD4+ T cells in the epithelial submucosal junction at six hours post-infection in human cervical tissue [55]. Therefore, epithelial cells may be the first target cells for HIV-1 to induce local immune activation, which enables the establishment of initial HIV-1 infection in the mucosal region.

CD4+ T cells are susceptible target cell population for SIV/HIV-1 infection and studies in humans have shown the presence of spatially dispersed populations of the target cells and other lymphocytes just beneath the epithelium in submucosa regions [53]. Studies have suggested that at the portal of entry, CD4+ T cells are the principle cell types to be infected and these infected founder population are important for the subsequent systemic infection [44, 53].

Based on a monkey study in which the local foci of SIV infection were mapped, the newly infected cells increased near the foci of infected founder cells, and the infection spread along the tracts of infiltrating inflammatory cells[71]. Exposure to SIV on the endocervical epithelium increased expression of MIP3α in the epithelial cells that in turn attracted pDCs (Plasmacytoid dendritic cells) [72]. These pDCs produce MIP-1β and other chemokines, which recruits CD4 T cells [44, 53]. Therefore, despite the presence of low density SIV target cells at the portal of entry, SIV is able to exploit the innate inflammatory responses which results in recruiting large number of target cells and establish infection in the mucosal region [44]. Thus the innate antiviral and inflammatory defense mechanism during SIV infection may facilitate SIV transmission. Another study has reported that exposure to semen caused secretion of chemokines and proinflammatory cytokines [73]. These cytokines are shown to recruits immune cells like DCs, macrophages and neutrophils beneath the cervical epithelium [73].

Epithelial cells are the first line of defense against various pathogen infections in mucosal area and the TJs present between the epithelial cells play a critical role in maintaining the epithelial barrier. Various studies have shown that cytokines play an important role in modulating the structures and functions of TJs between epithelial cells [74, 75]. Pro-inflammatory cytokines such as IL-1β, TNF-α and IFN-γ disrupt the TJs between cells [76-78].

Whereas, IL-10, IL17 and TGF-β prevent TJ disruption and also accelerate the development of a TJ barrier [79, 80]. Studies using endothelial cells and intestinal cell lines like T84, Caco2 have demonstrated that IFN-γ alters tight junction structure by translocating TJ protein occludin from TJ into endosome through macropinocytosis [81] and also by activation of PI3-kinase and NF-kB pathways [82]. Furthermore, TNF-α and IL-1β induces the expression of myosin light-chain kinase protein which triggers alteration in distribution of TJ proteins occludin, claudin 1, claudin 4 and JAM-1 in renal and intestinal epithelial cell line[83, 84]. These disruptions in TJ proteins lead to increase in paracellular permeability between epithelial cells and favor the entry of pathogens and macromolecules through the epithelial layer. Thus exposure to HIV on the mucosal epithelial cells may cause induction of cytokine from epithelial cells, which can recruit immune cells towards the epithelial layer and disrupt TJs between the epithelial cells, creating microenvironments conducive to viral transmission.

1.6 INFLUENCE OF REPRODUCTIVE HORMONES AND MENSTRUAL CYCLE ON HIV-1 TRANSMISSION THROUGH ECTOCERVICAL AND VAGINAL TISSUES:

Globally, there are about 17.3 million HIV-1 positive women and the majority of them are of reproductive age[85]. More than 100 million women use hormonal forms of contraception including oral contraceptive pills and the injectable Depot medroxy progesterone acetate (DMPA) [85]. Therefore, it is very important to understand the relationship between hormonal contraception and HIV-1 acquisition. The menstrual cycle of a woman is characterized by changes in level of reproductive hormone and divided into three phases: proliferative, ovulatory and secretory phase. During the proliferative phase, which is the first half of the menstrual cycle,

estradiol level is very low (Figure 6). The estradiol level then rises and peaks 2-3 days before ovulation followed by a rapid decline in estradiol level after ovulation (Figure 6). After 7-10 days, the estradiol and progesterone level increases (secretory phase) after which levels of both hormones decline, initiating menstruation [86]. These changes in hormonal level help in preparing the female genital tract for sperm survival and migration to fallopian tube for successful fertilization [53, 60, 61, 86, 87]. In addition, IgA, IgG and antimicrobials like SLPI, HBD2, human neutrophil peptide-1-3 levels in cervical-vaginal lavage decline during the mid-cycle and rise during the end of menstrual cycle[85, 86, 88]. The antiviral responses are regulated by type 1 interferons (IFNs) and IFN stimulated genes. IFNε is one of the type 1 IFNs which has antiviral activities. A study has also confirmed the role of IFNε by demonstrating significant increase in susceptibility of IFNε deficient mice to vaginal infection by HSV-2 and Chalmydia muridarum compared to the wild type mice[89]. Estrogen also induces the expression of IFNε and thus the level of IFNε is higher during the proliferative phase compared to the secretory phase of the menstrual cycle [89]. Therefore, hormones play a crucial role in antiviral defense in the female genital tract [89]. Therefore, natural fluctuation in hormone levels of estradiol and progesterone may play a key role in HIV-1 transmission, and consequently secretory phase has been postulated to provide a window of vulnerability to HIV-1 infection at a certain phase of menstrual cycle.

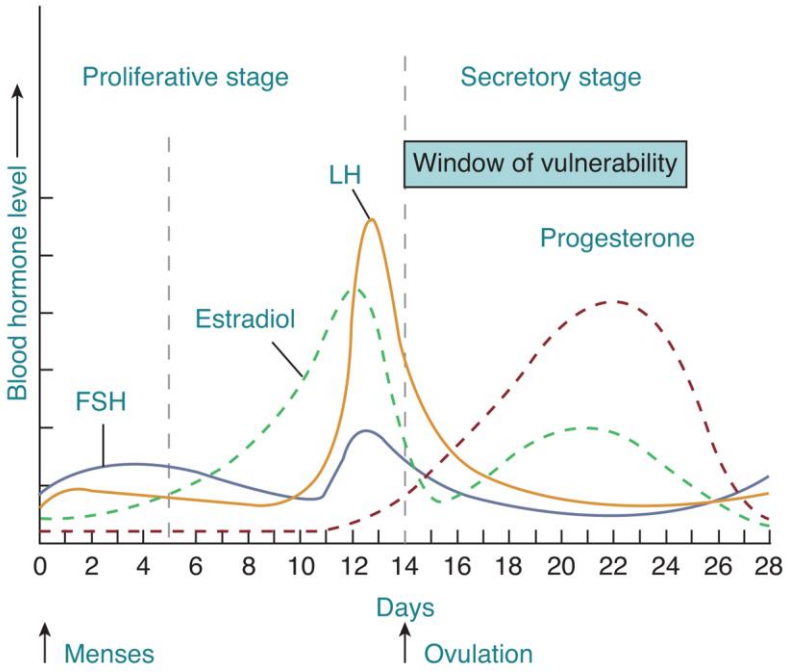


Figure 6. Relative changes in levels of estradiol and progesterone during the proliferative and secretory stages of the menstrual cycle

Figure taken from Wira *et al. AIDS*. 2008 October 1; 22(15): 1909–1917 (Open access journal).

A number of *in vivo* studies have been conducted to study the effect of hormones on HIV-1 transmission, but the results are controversial with studies presenting evidence in support and against hormonal effect on HIV-1 transmission. Studies in non-human primate models have shown that during the normal menstrual cycle, intravaginal inoculation of SIV in the secretory phase (progesterone dominant phase) had a higher infection rate than those inoculated during the proliferative phase (estrogen dominant phase) of menstrual cycle[90]. Furthermore, studies using injectable hormone in non-human primate models have shown that administration of DMPA, a progesterone based contraceptive, resulted in a 7.7-fold increase in SIV acquisition, increased viral levels and favored replication of the viruses that use CXCR4 co-receptor [91]. Another study by Abel *et al.* reported that protective effect of attenuated lentivirus against intravaginal

challenge with pathogenic SIV was abrogated in DMPA treated immunized macaques compared to DMPA untreated immunized macaques [92]. In contrast, Smith *et al.* showed that administration of estrogen in the form of subcutaneous implants or intravaginal cream protected ovariectomized female rhesus macaques against SIV infection through intravaginal inoculation [93]. However, the effect of hormones on HIV-1 transmission has not been consistent in humans. Polis *et al.* critically reviewed most of the epidemiological studies on the effect of different forms of hormonal contraceptive (oral, injectable and implants) on HIV-1 transmission[94]. This review found that while there are reports showing a significant increase in risk for HIV-1 transmission by using oral hormone based contraceptive (progesterone-only pills), there are an equal number of other reports showing non-significant increase in HIV-1 transmission by using oral hormone based contraceptive. In the case of injectable DMPA based contraceptives, only four out of nine studies reported significant association of DMPA and HIV-1 transmission [94]. The differences in outcome among these various studies is probably due to imprecise measurement of the timing of hormonal contraceptive use, differences in sexual behavior between contraception users and non-users, smaller sample sizes, infrequent measurement of contraception and HIV-1 infection [86, 94]. However, a study using *ex vivo* human endocervical tissues showed that productive HIV-1 infection of human endocervical tissue explants correlated with the secretory phase of the menstrual cycle [95].

Epithelial thickness and tight junction proteins play an important role in preventing transmission of various microorganisms through the cervical and vaginal mucosa. Although several studies using non-human primate models have showed that DMPA treatment can increase SIV transmission through vaginal region, the exact mechanisms for these effects have not been determined. Smith *et al.* showed that administration of estrogen caused thickening of

the vaginal stratified epithelium, which protected the macaques against intravaginal challenge with pathogenic SIV [93]. From these results they postulated that vaginal thinning by progesterone might be the reason for increased SIV transmission [91, 93]. In case of human, atrophic vaginitis is common in postmenopausal women due to estrogen deficiency and is characterized by a dry, thin epithelium, which bleeds after minimal trauma [96]. In a European study, a higher age of the women (greater than or equal to 45 years) was found to be associated with increased risk for male-female HIV-1 transmission that might be due to reduced estrogen level in the women suggesting that hormones also might influence HIV-1 acquisition in humans [94, 97]. Human studies on women using DMPA failed to show thinning of the vagina to the same extent seen in the NHP studies. However, it is difficult to compare the results from rhesus macaque studies to human studies due to the differences in anatomy and physiology of the reproductive tract of rhesus macaques and humans [86].

Hormones regulate the production of different cytokines from the epithelial cells in female genital tract. For example, estrogen induces secretion of different cytokines including IL-6, IL-10, TGFβ and IL-4 of epithelial cells of female genital tract [98-100]. Studies have shown that cytokines such as IL-1β, TNF-α, IFN-γ disrupt the TJs and cytokines such as IL-10, IL17, TGF-β have a role in development of a TJ barrier [76-80]. Another study reported that estrogen inhibits the production of IL-1α and TNF-α in vaginal epithelial cell lines, which can in-turn suppresses inflammation and also prevent TJ disruption [101]. In contrast, other in vitro studies have also shown that estrogen can alter the expression of TJ proteins like occludin and increase the permeability in cultured human ectocervical epithelial cells [102, 103]; progesterone but not estradiol increased expression of TJ proteins claudin 1,3,4, 7 and occludin in primary cultured human endometrial epithelial cells [104]. These studies suggest that TJ could be regulated by

hormones in the human female reproductive tract during menstrual cycle. Taken together, the effect of hormonal pressure on HIV-1 acquisition in human remains unclear. Due to the extremely high number of women using hormonal contraception, it is important to evaluate the effect of reproductive hormones on HIV-1 acquisition risk through the genital tract.

2.0 SPECIFIC AIMS

HIV-1 infection predominantly occurs through genital and rectal mucosa by sexual transmission. The mucosal lining of the female genital tract and intestine provides a robust barrier and these mucosal epithelial cells demonstrate no expression of CD4 receptor. However, HIV-1 passes through this protective layer and infects underlying CD4+ cells. The mechanism that allows HIV-1 to traverse the mucosal epithelia is not clearly understood. Studies on SIV infection in non-human primates demonstrate that when inoculated intravaginally with SIV, the virus crossed the epithelial layer within hours to generate sufficient virus and established infection in small foci within 48-72 hours [44]. The intercellular tight junctions and the intactness of apical membrane on the epithelial cells are very important for the preservation of the barrier function [47, 48]. The disruption of the tight junctions can increase the permeability between epithelial cells, leading to translocation of microbes, and inflammatory conditions in the mucosa [22, 44, 69]. Studies have shown that exposure to HIV-1 increases permeability in intestinal cell lines like Caco-2, T84 cells and primary endometrial epithelial cells [69, 105]. HIV-1 exposure also induced production of cytokines like IL8, MIP1 Beta, MCP-1, TSLP, TNFα, IL 6 in genital epithelial cells and T84 cells [68, 69]. All of these studies infer that HIV-1 can compromise the integrity of the mucosal barrier, induce inflammatory responses and increase target cell availability in the epithelial lining of the mucosal regions, which may facilitate the transmission of HIV-1. Furthermore, a number of studies have shown that during the course of

the menstrual cycle, the migration of immune cells like DCs, macrophages and neutrophils occurs in the lower female genital tract. Furthermore, estradiol and progesterone, whose levels fluctuate in the menstrual cycle, play a key role in regulating the physiology of FG. All these make the female genital tract uniquely susceptible to HIV-1 infection at specific times during the menstrual cycle. Although studies in macaques conclusively demonstrate that reproductive hormones influence SIV acquisition risk, epidemiological investigations in humans have been inconclusive. **We, therefore, hypothesized that exposure to HIV-1 increases the permeability of epithelia in the human mucosal tissue by disrupting the tight junctions (TJ) in epithelia, and stimulates the production of inflammatory cytokines, which promote HIV-1 transmission across the mucosal epithelium and facilitate infection in the ectocervical and rectal/colon area. Additionally, the susceptibility of the female genital tract to HIV-1 transmission/acquisition is altered by the dynamic changes of reproductive hormone levels at different stages of menstrual cycle.** To test our hypothesis, we propose the following specific aims:

Aim1: To study the effect of HIV-1 exposure on the integrity of tight junction and adherence junction in the epithelia of ectocervical and rectal/colon tissues.

Human ectocervical tissues and rectal/colon tissues were set in an established organ culture and the epithelial layer of the tissue were exposed to HIV-1. The epithelial layers of control/HIV-1 exposed tissue were examined for morphology and thickness by Haematoxylin and Eosin (H&E) staining. The quantity and distribution of tight junction proteins like ZO1, Claudin 4, adherens junction proteins like E-Cadherin in the epithelial cells of ectocervical and rectal/colon tissues were assessed by confocal immunofluorescence (IF) microscopy and by quantitating the images using the ImageJ and NS1 Element software.

Aim 2: To examine the cytokine expression profile in ectocervical epithelia and colon mucosa following exposure to HIV-1.

The mucosal layers of the control/HIV-1 exposed ectocervical and colon tissues were isolated followed by RNA extraction. The mRNA levels of various cytokines were measured by real time RT-PCR. To characterize the comprehensive profile of gene regulation in epithelial layer by HIV-1 exposure, next generation sequencing in an Ion Torrent technology platform was performed on extracted RNA samples from isolated human ectocervical epithelia following exposure to HIV-1 or control supernatant. To examine the effect of HIV-1 exposure on the distribution of immune cells, the presence of CD3+ CD8+cells` in the intraepithelial and subepithelial regions of the control and HIV-1 exposed tissue was investigated by IF.

Aim 3: To study the effect of reproductive hormone on the susceptibility to HIV-1 infection in human ectocervical /vaginal tissues.

Ectocervical/vaginal tissue biopsies were obtained from premenopausal, HIV-1 uninfected women during the proliferative, ovulatory and secretory phases of menstrual cycle. Susceptibility of the ectocervical/vaginal tissues to HIV-1 infection was evaluated using non-polarized organ culture by exposing the tissues to HIV-1 and quantifying the p24 production in culture supernatant at different time after exposure to HIV-1. To determine the HIV-1 acquisition risk at different phases of menstrual cycle, the TJ profile and thickness of epithelial layer were studied in these tissues at those phases of cycle.

3.0 MATERIALS AND METHODS

3.1 VIRUS CULTURE

PM1 cells, a T cell line that expresses CD4, CXCR4 and CCR5 were used to propagate R5 HIV-1. Briefly, PM1 cells were maintained in RPMI 1640 (Mediatech) containing 100 U/mL penicillin/0.1 mg/mL streptomycin, 20% fetal bovine serum (FBS) and 10 mM HEPES. PM1 cell were infected by R5-HIV-1 BAL-1 (NIH AIDS reagent Catalog # 11445) for 3 hours followed by washing to remove excess virus and then cultured for 15 days. Starting from day 3 post-infection, culture supernatant containing HIV-1 was collected every other day and filtered through an Amicon Ultra-15 filter device (Millipore, Billerica, US) to remove soluble cytokines. The control culture supernatant was prepared in the same way but the cells were not infected with HIV-1.

The filtered HIV-1 BAL viral stock was then quantified using HIV-1 p24 ELISA and its infectivity titer (TCID50/mL) was determined by titration on CD8 depleted PBMC from normal blood donors. Briefly, CD8 depleted PBMCs were treated with phytohemaglutinnin for three days to activate CD4+ cells followed by centrifugation at 500xg for 10 minutes. These CD8 depleted PBMCs were then seeded in a 96-well plate and incubated with cell-free HIV-1 BAL, which were serially diluted (1:5) in 10% RPMI media for 7 days at 37ºC. Culture supernatants at 1:10 dilution were harvested periodically and frozen at 70ºC. Frozen culture supernatants were

subsequently thawed and HIV-1 p24 was analyzed for infectivity titer according to the manufacturer's protocol. Aldrithiol-2 (AT2) inactivated HIV-1 ADA (R5 virus) was a gift from J. D. Lifson, National Cancer Institute, Frederick, Maryland. HIV-1 proteins gp120 envelope protein was obtained from NIH AIDS Reagent Program (catalog# 11784).

3.2 HUMAN ECTOCERVICAL/COLON MUCOSAL TISSUE CULTURE

3.2.1 Sources of ectocervical and colon tissues

Ectocervical tissues were obtained from premenopausal, HIV-1 negative patients with no history of sexually transmitted diseases who were undergoing hysterectomy for medical reasons unrelated to cervix at the Magee Women Hospital of the University of Pittsburgh Medical Center. The colon tissues were obtained from HIV-1 negative patients undergoing surgical resection of colon for medical reasons like cancer or non-inflammatory conditions at the University of Pittsburgh Medical Center. The Institutional Review Board of the University of Pittsburgh approved this study as an Exempt study. Informed consent from individuals was waived because this study used tissues that were procured through the Tissue Procurement Facility with only generalized patient information such as age and race of the patients.

3.2.2 Organ culture with ectocervical tissues

Ectocervical tissues were collected and processed within 2 hours of surgery. To study TJ/AJ proteins, the organ culture was performed with ectocervical tissues. Ectocervical tissues

were immersed in antibiotic solution (Penicillin-Streptomycin (20,000 U/ml), Fungizone (250 μg/ml) and Nystatin (120 U/ml) in PBS) for 5 minutes and then rinsed twice with RPMI media. The stromal side was then trimmed to about 2-3mm thick and the ectocervical punch biopsies (6mm diameter) were placed into a 12 well transwell (Becton Dickson, NJ, USA)) with the epithelial layer facing up and its edge was sealed with 3% agarose at room temperature. Cell-free HIV-1 ($TCID_{50}$ of 10^6) or control supernatant was added on the epithelial layer of the tissue in the upper chamber and complete IL-2 media (RPMI media, heat-inactivated fetal bovine serum (10%), and interleukin-2 (500 U)) was added to the bottom well. Cultures were incubated at 37ºC for 24 hours in a CO_2 incubator. To serve as a positive control for TJ/AJ disruption, ectocervical epithelia were exposed to 10 mM EDTA (Fisher Scientific International Inc., Hampton, NH) at 37ºC for 2 hours in a CO_2 incubator.

To study cytokine regulation in ectocervical tissues, the epithelial layer of ectocervical tissues was exposed to either cell-free HIV-1 BAL ($TCID_{50}$ of 10^6), GP120 0.8nM (0.1ug/ml), AT2 inactivated HIV-1 (71ng/ml) or control supernatant in an organ culture. Cultures were incubated at 37ºC for 24 hours in a CO_2 incubator. The tissues after organ culture were frozen down in OCT (Thermo Fisher. USA) at -80ºC and cryosectioned (7μm thickness) for subsequent studies.

To study HIV-1 transmission, ectocervical tissues were inoculated with cell-free HIV-1 BAL ($TCID_{50}$ of 10^6) supplemented in IL2 medium and cultured overnight in CO2 incubator at 37°C. Following the incubation, the biopsies were washed with PBS to remove excess virus and cultured again for additional 16 days. To monitor virus growth, HIV-1 p24 production was measured by ELISA (SAIC-Frederick) in the culture supernatant in every 3 days during the culture period.

3.2.3 Organ culture with colon tissue

Colon tissues were collected and processed within 2 hours of surgery. Similar to cervical tissues, colon tissues were immersed in a concentrated antibiotic solution and then rinsed with RPMI. The tissues were dissected into 6mm diameter biopsies and inoculated with 300µl of cell-free HIV-1 BAL ($TCID_{50}$ of 10^6) or control supernatant supplemented with RPMI with IL2 media in 12 well plates (Becton Dickson, NJ, USA)) for 6 hours in CO2 incubator at 37°C. To study the effect of HIV-1 exposure on cytokine production of epithelial cells, colon biopsies after HIV-1 exposure were then frozen down in OCT at -80'C. To study the effect of HIV-1 exposure on TJ/AJ, the tissues were fixed in safefix II (Fisher Scientific, MI, USA) for 2 hours, paraffin embedded and sectioned (7µm thickness) using a VibratomeTM.

To study virus transmission, colon biopsies were exposed to HIV-1 BAL ($TCID_{50}$ of 10^6) for 6 hours. The biopsies were then washed with PBS to remove excess virus and cultured for 16 days. HIV-1 p24 level was measured in the culture supernatant every 3 days by ELISA.

3.3 HUMAN ECTOCERVICAL AND VAGINAL TISSUES AT DIFFERENT PHASES OF MENSTRUAL CYCLE

This study is an ongoing collaborative study with the Brown University supported by a grant from NIAID. The recruitment of volunteers for the study and procurement of ectocervical, vaginal tissue biopsies was conducted at the Brown University according to Institutional Review Board of the Brown University. The ectocervical and vaginal biopsies were shipped at 4° overnight to our lab to perform organ culture and subsequent experiments.

3.3.1 Study population

Ectocervical and vaginal biopsies were obtained from women volunteers at different phases of menstrual cycle. The women in this study were not taking any form of exogenous hormones at

the time of enrollment and expressed their pre-defined interest in not starting any form of hormonal contraception during the study period.

Individuals who satisfied the following criteria were recruited to study the influence of reproductive hormone and stage of menstrual cycle on HIV-1 transmission: 18 years of age or older, HIV-1 negative, not pregnant, had normal pap smear reports in the last one year, did not have any history of sexually transmitted infections and did not have any acute illness, had not used any steroids, immunomodulatory drugs for last six months and were not immunized in the last one-month, agreed to give informed consent and follow-up visits.

Furthermore, baseline test for HIV-1, HSV-2, gonorrhea, chlamydia, trichomoniasis, bacterial vaginosis and pregnancy test were performed for all the women at their first visit.

To study the susceptibility of cervical/vaginal tissues to HIV-1 infection at different phases of menstrual cycle, two biopsies from cervical and vaginal regions were obtained from 21 donors at their proliferative phase, 6 donors at their ovulatory phase, 11 donors at their secretory phase. To study the effect of HIV-1 on cervical/vaginal epithelial layer thickness and TJ at different phases of menstrual cycle, cervical and vaginal biopsies from 7 donors at their proliferative, ovulatory and secretory phase were obtained.

3.3.2 Organ culture for ectocervical/vaginal tissues obtained from different phases of menstrual cycle

Ectocervical/vaginal tissues were exposed to HIV-1, washed and cultured in a non-polarized set up. Briefly, the tissues were exposed in a 96-well flat bottom plate to cell-free HIV-1 BAL ($TCID_{50}$ of 10^6) or control supernatant supplemented with IL2 media for 24 hours, washed with PBS, cultured for 16 days in IL2 media and virus production was monitored every 3 days by HIV-1 p24 measurement in the culture supernatant by ELISA.

To analyze the epithelial layer thickness and TJ protein expression between epithelial cells, the ectocervical tissues exposed to HIV-1 or control supernatant for 24 hours were washed and fixed in safefix II (Fisher Scientific, MI, USA) for 2 hours and then paraffin embedded and sectioned (7μm thickness) using a Vibratome TM.

3.4 HISTOLOGY AND IMAGE ANALYSIS

Ectocervical, vaginal and colon tissues sections were stained with hematoxylin and eosin (H&E) and examined by light microscopy to assess tissue morphology after exposure to control supernatant or HIV-1 over the culture period. After obtaining bright field images of H&E stained ectocervical/vaginal tissues, the thickness of epithelial layers was measured in three representative areas of mucosa from the basement membrane up to the surface using the Metamorph software. The mean of epithelial layer thickness was calculated for each biopsy.

3.5 IMMUNOFLUORESCENCE ANALYSIS OF CERVICAL AND COLON TISSUES

3.5.1 Antibodies

Rabbit polyclonal-α-ZO1(cat# 617300), rabbit polyclonal-α-Claudin-1((cat# 519000), mouse monoclonal-α-Claudin-4((cat# 329400), mouse monoclonal-α-E-cadherin(cat# 131700) were purchased from Invitrogen, Camarillo, CA, US. Mouse monoclonal-α-Na/ K/ATPase (05-369), rabbit polyclonal-α-CD3 (A0452), mouse polyclonal-α-CD68 (M0814)negative control rabbit immunoglobulin (x0936) were purchased from Millipore, Massachusetts, USA and Dako, Glostrup, Denmark respectively. Mouse IgG1 isotype (556648) was purchased from BD Pharmingen. Goat -α-mouse Alexa 488 and goat-α-rabbit Cy3 was purchased from Jackson Immunoresearch, West Groove, PA.

3.5.2 Immunofluorescence staining ectocervical and colon tissues

To study TJ/AJ proteins and distribution of CD3+/CD8+ cells, ectocervical, vaginal and colonic tissues were examined by immunofluorescence microscopy. Paraffin embedded ectocervical and colon tissue sections were deparaffinized and antigen retrieval was performed by heating sections in sodium citrate buffer (005000, Invitrogen, Frederick, MD, USA) in a microwave followed by cooling to room temperature for 10 min. Tissues were washed with PBS followed by permeabilizing with Triton X-100 and blocking with 2% BSA for 20 min. After washes, the tissue sections were then treated with one of the following primary antibodies: rabbit polyclonal anti ZO1, Claudin-1, mouse monoclonal anti Claudin-4, E-cadherin, Na/ K/ATPase at a dilution of 1:50, 1:75, 1:200 and 1:500 respectively for 60 min at room temperature. Tissue

sections were stained in parallel with an isotype control antibodies: negative control Rabbit Immunoglobulin, Mouse IgG1 isotype. Tissues were washed with 0.5% BSA and incubated with either goat anti-mouse Alexa 488 or goat anti-rabbit Cy3 at a dilution of 1:500 or 1:1000 respectively, in 0.5% BSA for 1 hour. Nuclear counterstaining was done with DAPI. All sections were mounted in gelvatol and coverslips were sealed on slides.

In the case of frozen ectocervical tissues, the tissue blocks were cryosectioned using cryostat (Microm HM550, Thermofisher). The cryosections were fixed with 2% PFA in PBS for 20 min and washed with 0.5% BSA. The tissue sections were then blocked with 2% BSA, treated with primary antibodies (ZO-1, Claudin-1, Claudin-4, E-Cadherin, Na/K/ATPase, CD3, CD8), secondary antibodies, DAPI and sealed using coverslip as mentioned earlier for colon tissues. Images were taken with Olympus Fluoview 1000 confocal microscope using a 20x or 40x oil objective. For acquiring images on confocal microscopy, the identical image acquisition setting was used for control and HIV-1 exposed tissues. For each experimental condition 12-20 separate, random images were obtained and analyzed using NSI Elements software.

3.5.3 Image analysis for tight junction and adherence junction expression

Images were analyzed using ImageJ program (http://rsb.info.nih.gov/ij/) or NSI Elements software. To examine ZO1 nuclear colocalization in ectocervical epithelia, 30 nuclei were randomly chosen per field and ZO 1 nuclear colocalization was analyzed using ImageJ program by maintaining the same threshold setting for the entire dataset. To study Claudin-4, E-cadherin, Na/K/ATPase protein expression in ectocervical epithelia, fluorescence intensity of the proteins in the epithelial region was measured respectively using NSI Elements software maintaining the

same threshold setting for the entire dataset and the fluorescence intensity was normalized to number of nuclei. In case of colon tissues, fluorescence intensity of ZO-1, Claudin-4, Ecadherin, Na/K/ATPase proteins was measured using NSI Elements software as mentioned for the ectocervical tissues.

3.5.4 Image analysis of immune cell distribution

The numbers of immune cells were measured using NSI Elements software maintaining the same threshold setting for an entire dataset. To study CD3+, CD8+ cell distribution in intraepithelial regions, the ratio between the number of immune cells in intraepithelial region and beneath the basolateral membrane, and the total number of immune cells in the field was analyzed and compared between control and HIV-1 exposed ectocervical tissues.

3.6 TRANSMISSION ELECTRON MICROSCOPY

Human colon tissues were exposed to control supernatant or HIV-1 for 6 hours and fixed in 2.5 % glutaraldehyde for 1 hour at room temperature. The biopsies were washed with PBS and processed as previously described[106]. Briefly, biopsies were post-fixed in aqueous 1% OsO4, 1% K3Fe(CN)6, dehydrated with ethanol series, infiltrated in 1:1 mixture of propylene oxide: Polybed 812 epon resin (EBS Sciences, East Grandy, CT) and epon for 1– 3 hours. This was further embedded in molds, cured and ultrathin (60-80nm) sections of the vitreous were collected on copper grids. Furthermore, they were stained with lead citrate, 2% uranyl acetate

and TEM images were acquired using a JEOL JEM 1011 TEM (Peabody, MA) at 80kV fitted with a side-mount AMT 2k digital camera (Advanced Microscopy Techniques, Danvers, MA).

3.7 MICRODISSECTION OF EPITHELIAL LAYER AND RNA EXTRACTION

The ectocervical and colon tissues exposed to control, HIV-1, AT2 or GP120 were cryosectioned followed by microdissection of the epithelial layer under microscope to minimize contamination from submucosa layer. RNA was extracted from the epithelial layer using RNA zol B (TEL-TEST, INC, Friendswood, TX).

3.8 TAQMAN® REAL-TIME PCR

RNA was isolated from microdissected ectocervical epithelial or colon mucosal layer using RNA Bee (TEL-TEST, INC) according to the manufacturer's instructions and treated with RNase free DNase (Roche Applied Science) for 30 min followed by RT-PCR. The cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen). The purity of the ectocervical epithelial regions obtained was assessed by the enrichment of cytokeratin 13 (Krt13) mRNA, which is predominately expressed in epithelial cells. The mRNA levels of cytokeratin 13 and cytokines of CXCL10, CXCL11, IL-6, IL1β, IL8, IL-10, TNFα and IFNγ were measured in the microdissected epithelial layers by real-time RT-PCR as described before[107]. Krt13 (endogenous control for epithelial cells), human CXCL10, CXCL11, IL-6, IL1β, IL8, IL-10, TNFα, IFNγ primers and probes labeled with FAM / MGB were purchased from Life Technology (Table 1).

Table 1. Real time RT PCR primers and probes gene assay ID

Gene	Primer and probe *
IL1β	Hs99999029_m1
IL-6	Hs99999032_m1
IL8	Hs99999034
IL-10	Hs00961622_m1
TNFα	Hs99999043
IFNγ	Hs00989291_m1
CXCL10	Hs00171042_m1
CXCL11	Hs00171138_m1
KRT13	**Hs00999762_m1**

*** Gene assay ID of primer and probe commercially purchased from Life Technology**

Real time RT-PCR with gene specific primers/probes was performed as described previously[108]. Briefly, RNA was reverse-transcribed with TaqMan® Reverse Transcription Reagents (Applied Biosystems) following manufacturer's protocols. A 25 µl PCR mixture consists of 20XTaqMan® Pre-Developed Assay Reagents (Applied Biosystems), 5 µl cDNA (20ng total RNA equivalent) and 2X TaqMan® Universal PCR Master Mix. Real-Time PCR was carried out using ABI Prism 7000 Sequence Detection System under the following cycling condition: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Results were expressed as fold-change relative to control.

3.9 NEXT GENERATION SEQUENCING USING ION TORRENT TECHNOLOGY

3.9.1 RNA extraction and library construction

RNA was extracted from microdissected epithelial layer of the tissue as described earlier. mRNAs were isolated from the total RNA with a commercially available kit (Dynabeads® mRNA DIRECT™ Micro Purification Kit, Life Technologies). This was followed by cDNA Library construction using Ion Torrent RNA-Seq Kit (Life Technologies) for whole transcriptome libraries. For individual sample Barcodes 1 through 8 were attached using Ion Xpress 1-16 barcoding kits. Quantitation of cDNA libraries was performed using the Ion Library Quantitation Kit (Life Technologies) to evaluate appropriate template dilution factor for subsequent emulsion PCR and sequencing. This was followed by next generation sequencing using the Ion Torrent platform according to manufacturer's protocols.

3.9.2 Data analysis

Raw sequencing reads were in FastQ format. CLC Genomics Bench 7 was used to assess the quality of raw sequencing reads. Reads were accepted based on the length (longer than 25 nucleotides) and number of ambiguous bases (Phred Quality score higher than 20). Quality trimming and adapter sequence clipping were performed prior to downstream analyses. The mean reads obtained after trimming in control ectocervical epithelia was 1.8 million and in HIV-1 exposed ectocervical epithelial was 1.7 million (Table 2). The trimmed reads were then mapped to Homo sapiens (hg19) mRNA sequence.

Table 2. Read count after trimming in control and HIV-1 exposed ectocervical epithelia used for mapping with Homo sapiens (hg19) sequence in Ion Torrent sequencing.

CONTROL/HIV-1 EXPOSED	NUMBER OF DONORS	MEAN TOTAL READS AFTER TRIM	TOTAL READS AFTER TRIM (RANGE)
Control	6	1857244.5	810930 to 2837978
HIV-1	6	1709218.5	733413 to 2380879

Bioconductor edgeR was employed to perform the differential expression analysis, and since it is a pairwise comparison, general linear model was used for the analysis. To make sure there were sufficient counts for each gene in the test, genes with mean read counts higher than 10 were retained in the analysis. Genes with Benjamini-Hochberg adjusted false discovery rate (FDR) <0.05 and absolute values of logFC greater than 1 were considered as significant genes.

3.9.3 Confirmation of Ion Torrent result by target-specific real time PCR

The expression level of the differentially expressed genes obtained from Ion Torrent data were evaluated again by real time PCR with specific primers and probes of IL36A, FMO2, CXCL10, MUC1, SAA1, IL8 as described earlier.

3.10 STATISTICAL ANALYSES

Data are presented as mean ± standard deviation. For analyzing mRNA expression levels, parametric single sample *t* test was used to determine the significance ($p<0.05$) for the fold change observed in HIV-1 treated group relative to controls. To determine significance ($p<0.05$) in fluorescence intensity of TJ proteins between HIV-1 treated group and controls, parametric paired student *t* test was applied. For comparisons of mRNA cytokine expression levels in the

ectocervical tissues treated with HIV-1, GP120, or AT2, T-Test Unequal Variance analysis was performed with significant level at $p<0.05$. To compare the susceptibility to HIV infection, levels of TJ protein expression and epithelial thickness in groups from different stages of menstrual cycle, ANOVA and Kruskal-Wallis analysis were performed with $p<0.05$.

4.0 SPECIFIC AIM 1: EFFECT OF HIV-1 EXPOSURE ON THE INTEGRITY OF TIGHT JUNCTION IN THE EPITHELIAL CELLS OF ECTOCERVICAL AND COLON TISSUES

4.1 INTRODUCTION

The disruption of tight junctions (TJ) can lead to microbial translocation and inflammation in the mucosa[69]. Various pathogenic organisms like rotavirus, astrovirus and *E.coli* have developed strategies to disrupt the TJ, leading to pathogenic conditions characterized by increased intestinal permeability[109, 110]. Studies on the effects of HIV-1 on tight junctions have been contradictory. Tugizov *et al.* demonstrated that incubation of fetal oral mucosa with HIV-1-infected lymphocytes and macrophages for 4 hours did not cause disruption of epithelial junctions [111]. Whereas another study suggested that HIV-1 disrupted TJs in polarized tonsil epithelial cells [112]. TJ disruption and decrease of transepithelial electrical resistance have also been observed in human primary endometrial cells and intestinal cell lines after exposure to HIV-1 [69]. Studies in rhesus macaques have demonstrated that during SIV infection, the integrity of epithelial barrier lining the GI tract is damaged and it is associated with microbial translocation and inflammation [44]. However, few data are available in the literature describing the effect of HIV-1 on epithelial tight junction in human cervical and rectal/colonic tissues.

We, therefore, hypothesized that **exposure to HIV-1 on the ectocervical and colon epithelium disrupts tight junctions and their functions, which increases the permeability of epithelial layers in ectocervical and rectal/colon tissues.**

4.2 RESULTS

4.2.1 Effect of HIV-1 exposure on the integrity of the cervical/colonic mucosal epithelia

The intactness of the apical cell membrane of epithelial cells and the intercellular TJ, AJ form the basis of the barrier function of mucosal epithelia[50]. We first performed histological examination of the epithelial layer of the ectocervical and colon tissues to determine the integrity of epithelial layer after incubation in organ culture. In the ectocervical tissues, hematoxylin and eosin (H&E) staining showed that the epithelium lining the ectocervical mucosa remained intact after cultivation for 24 hours and was characterized by multilayered stratified squamous epithelial cells, basal layer and submucosa. Histological examination of colon tissues showed retention of the epithelium and lamina propria cell integrity after 6 hours in culture, which was similar to that of healthy colon tissue without cultivation. However, when colon explants were cultured for more than 10 hours, shedding of the epithelial layer was detected (data not shown).

Next, to determine the effect of HIV-1 on the integrity of mucosal epithelia, ectocervical and colon tissues were exposed to HIV-1 for 24 hours and 6 hours, respectively, followed by H&E staining of fixed tissues. The morphology of epithelial layer and the basal layer in the ectocervical tissues remained largely unchanged after exposure to HIV-1 for 24 hours, which was similar to ectocervical tissues exposed to control supernatant (Figure 7A). Similarly,

colonic tissues exposed to HIV-1 for 6 hours also maintained an intact mucosal layer, resembling the morphology of the colonic tissues exposed to control supernatant (Figure 7B).

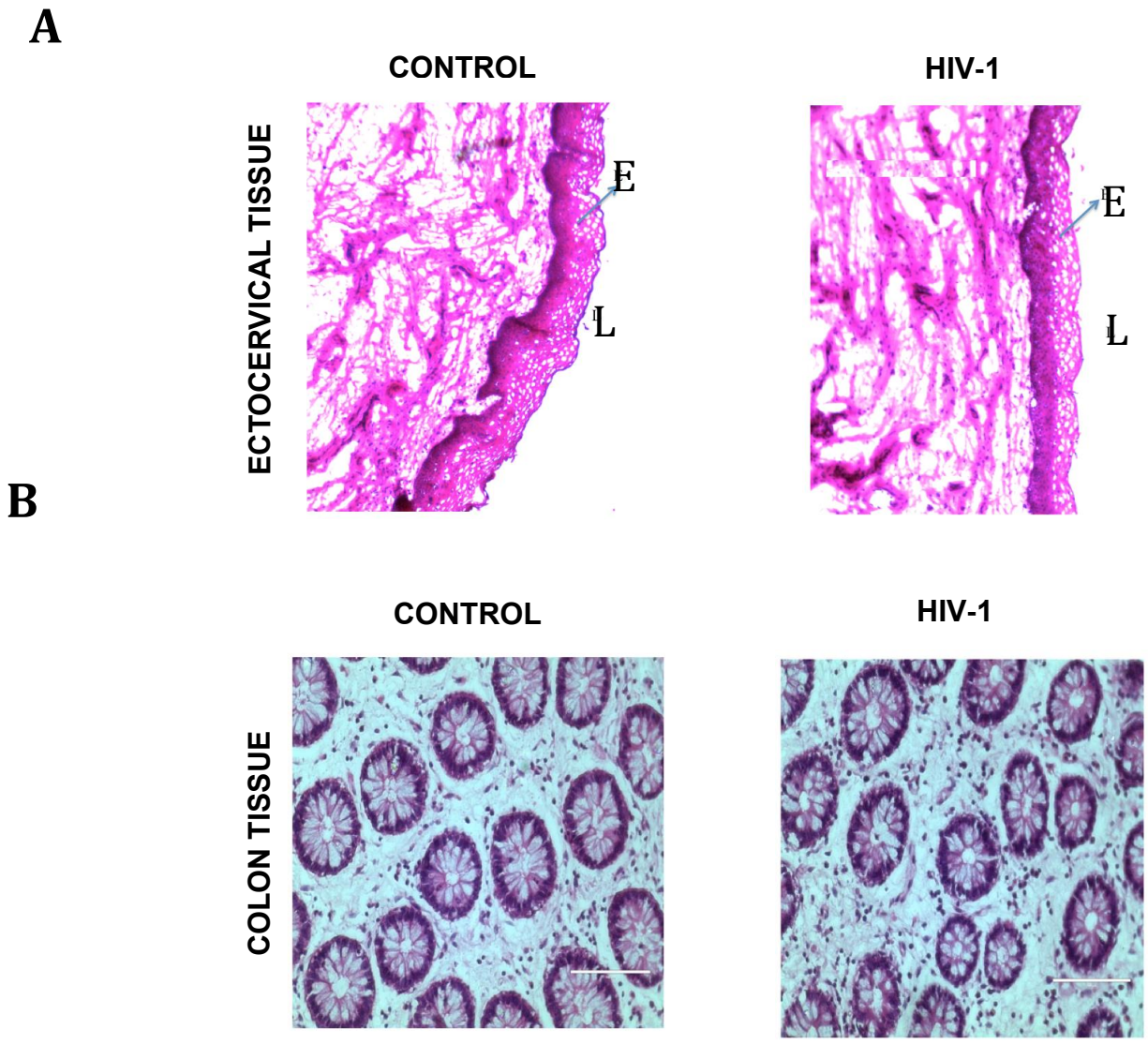


Figure 7. Effect of HIV-1 exposure on the integrity of the ectocervical/colon mucosal epithelia

H&E staining was performed on ectocervical tissues (A) and colon tissues (B) exposed to HIV-1 (10^6 infectious viral units) or control supernatant in the organ culture for 24 hours and 6 hours respectively. Images were obtained by bright field microscopy E: Epithelium; L: Lumen of ectocervix. Magnification in ectocervical tissues 10x and magnification in colon tissues 40x.

4.2.2 Characterization of tight junction and adherence junction in ectocervical tissues

The epithelial layer in ectocervical and colonic tissues provides a robust barrier against microorganisms. Various studies have shown the existence of TJ and AJ between epithelial cells in the colon tissue, but very little information is available regarding the structure and molecular composition of ectocervical epithelial junctions between the cells [47]. To determine whether TJ and AJ exist in multilayer ectocervical epithelium, we examined the profiles of a number of TJ proteins (ZO1, Claudin 1, Claudin 4), AJ protein (E-Cadherin) and Na/K ATPase in ectocervical epithelium.

TJs regulate the passage of ions and small molecules through the paracellular pathway and serves as a permeability barrier (gate function) [49]. To evaluate the gate function, we measured the paracellular permeability of ectocervical epithelia to solutes (3 kD fluorescent labeled dextran). Cervical organ culture was set up as described in Materials and Methods, and tissues were treated with or without EDTA (10mM) for 2 hours. EDTA treated tissues and the control tissues were then exposed to 3 kD fluorescent labeled dextran for 1 hour. Tissues were frozen down in OCT at -80°C and sectioned (7μm thickness) followed by confocal microscopy to monitor the distribution of dextran in the tissues. As shown in the Figure 8A, fluorescent-labeled dextran was not detected within the epithelial layer or the submucosal region of the control tissue, which suggests that the dextran penetration was prevented by the TJs of the ectocervical mucosal epithelium. In contrast when ectocervical tissues were exposed to EDTA, an agent known to disrupt TJ/AJ, fluorescently labeled dextran penetrated the epithelial layers into the epithelial layer of the ectocervical tissue (Figure 8B). Therefore, the epithelial junction in cervical tissue could “gate” the diffusion of dextran between epithelial cells. To study the TJ and AJ proteins, immunofluorescence staining of TJ and AJ proteins was performed on the

ectocervical and colon tissues exposed to control media or EDTA (10mM) for 2 hours. Immunofluorescence studies revealed distinct expression of interconnected ZO-1, Claudin-1 expression pattern that were located around the perimeter of each epithelial cell (Figure 8C) in control ectocervical tissues. However, discontinuous punctate distribution of ZO-1 and Claudin-1 between epithelial cells was detected in EDTA-treated ectocervical tissues (Figure 8C), indicating disruption of TJ/AJ.

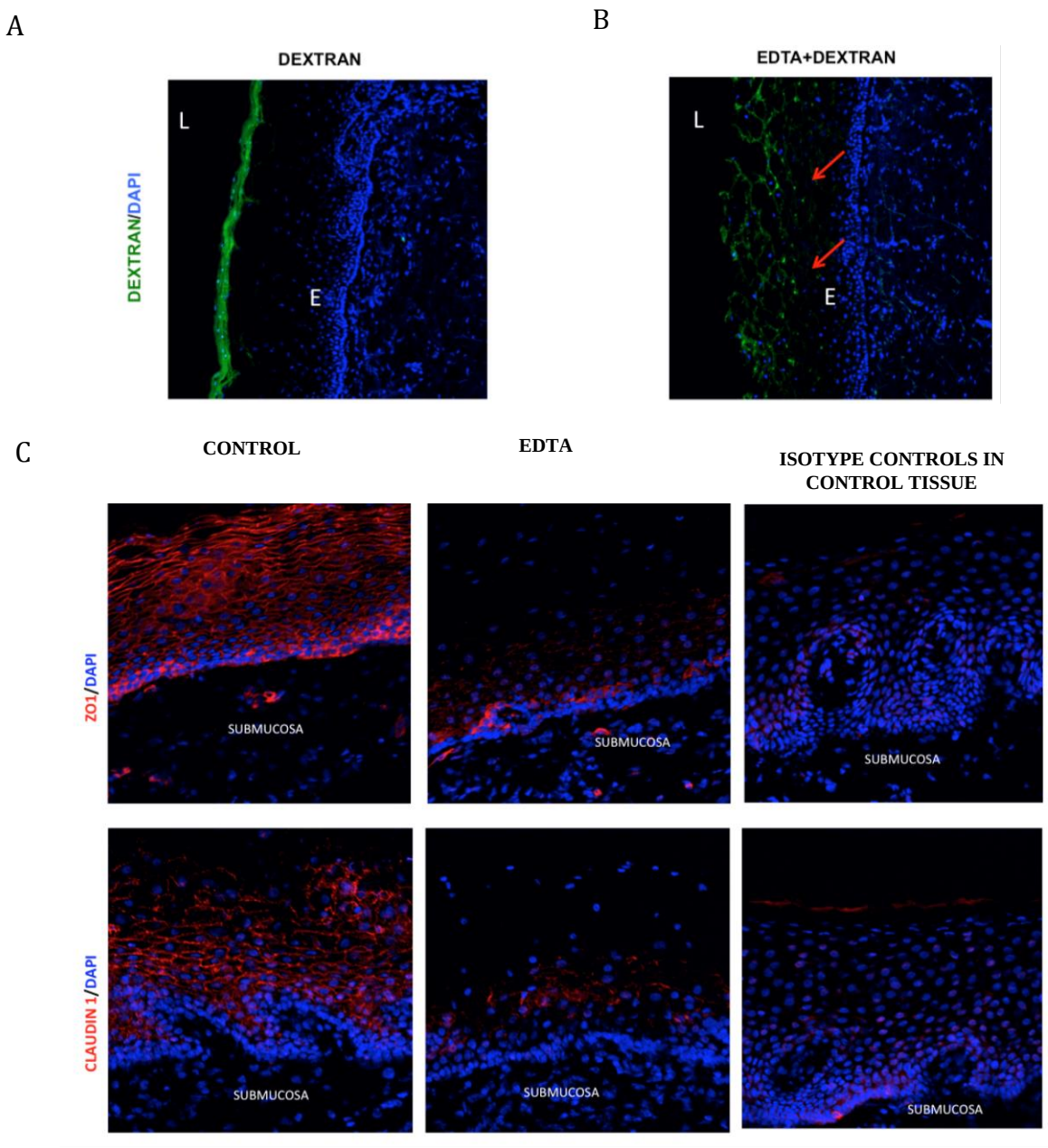


Figure 8. Characterization of tight junction and adherens junction proteins in ectocervical tissues

Ectocervical tissues incubated with fluorescent-labeled 3kD dextran (green) for 1 hour with (A)/without (B) prior exposure to EDTA (10mM) for 2 hours. Images were captured by confocal microscopy. The arrows on the right indicate that the dextran penetrated into epithelial layer of the ectocervical tissues exposed to EDTA. E: Epithelium; L: Lumen of ectocervix. (C) Ectocervical epithelia were either exposed to control media or EDTA (10mM) for 2 hours. Ectocervical tissue sections were stained using antibodies against either ZO-1 (red), Claudin-1 (red), or nonspecific IgG isotype control. Nuclei were stained with DAPI (blue). Images were captured by confocal microscope. Magnification 40X.

4.2.3 Effect of HIV-1 on tight junction and adherence junction proteins in ectocervical/colon tissues

Confocal scanning of ectocervical and colon tissues was performed, and images of mucosal epithelia were captured and quantitated. Control epithelia were characterized by well-defined and interconnected ZO-1, claudin 1, E-cadherin staining patterns located at the perimeter of each cell as a ring shape. Based on TJ protein distribution and expression in EDTA exposed ectocervical tissues (Figure 8C), if the TJ/AJ were disrupted in tissue epithelia, we expected the following characteristics of TJ/AJ proteins after exposure to HIV-1: discontinuous distribution pattern around the perimeter of cells, diffuse cytoplasmic localization of TJ/AJ proteins, presence of TJ/AJ protein in the apical and basal cell membranes, and reduced or complete inhibition of TJ/AJ protein expression. Contrary to our expectation, epithelial layers of ectocervical/colon tissues exposed to HIV-1 had similar distribution patterns of TJ proteins ZO-1, Claudin-1, Claudin-4 and E-cadherin compared to those in the control tissues (Figure 9,10,12). Quantitative analysis revealed that there was no significant reduction in fluorescent intensity of ZO-1, Claudin 4 and E Cadherin proteins in HIV-1 exposed tissues compared to control tissues indicating that there was no marked reduction in TJ/AJ proteins expression following exposure to HIV-1 (Figure 11, 13). Furthermore a number of studies suggest that ZO-1 nuclear translocation could occur by disruption of ZO-1 protein in epithelial cells [113]. Quantitative analysis of the presence of ZO-1 in nuclei also revealed that there was no significant increase in ZO-1 nuclear translocation in ectocervical epithelial cells after exposure to HIV-1 (Figure 11E). Thus, exposure to HIV-1 did not disrupt the distribution of TJ proteins and AJ protein between epithelial cells in both colon and ectocervical tissues.

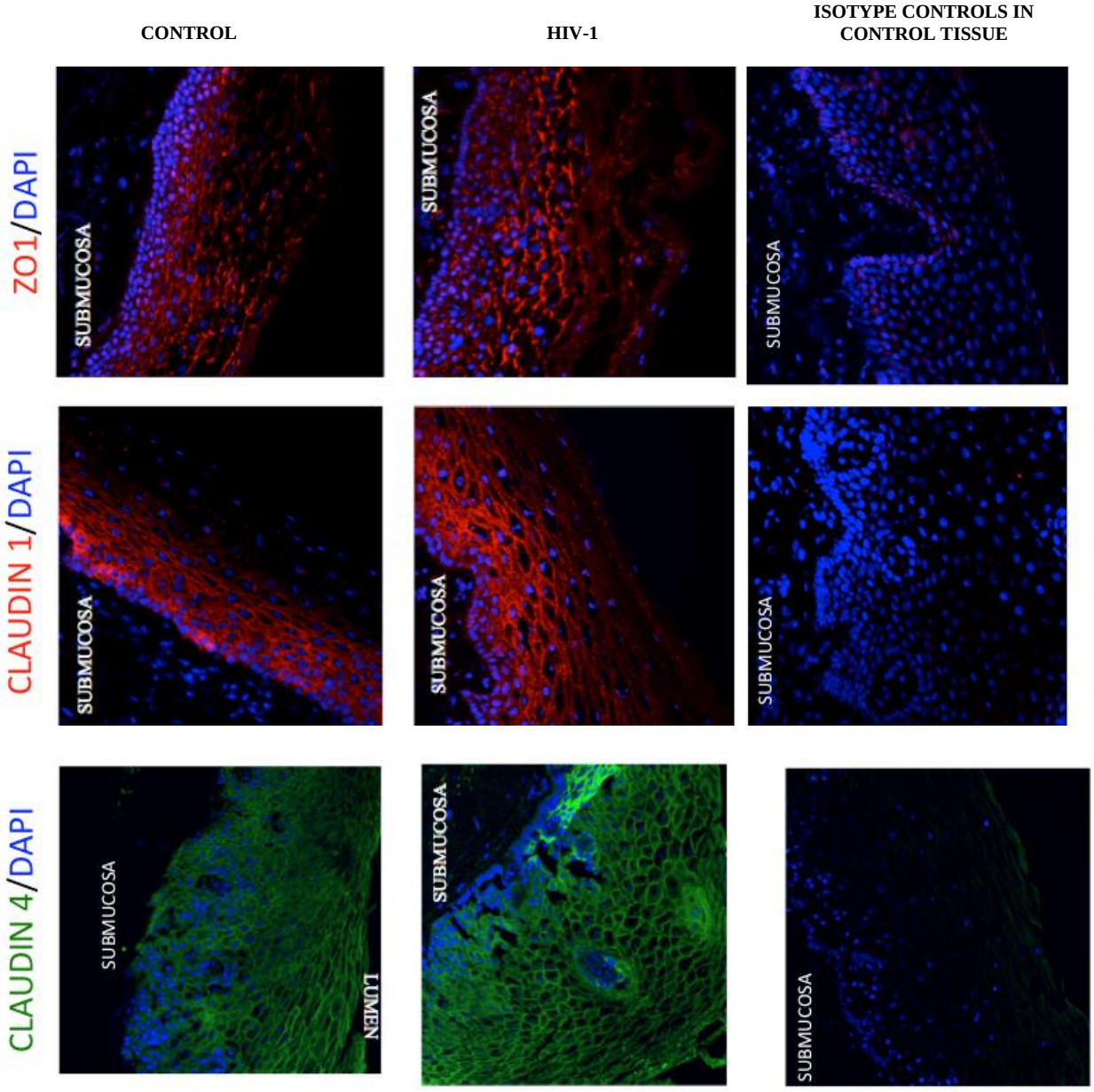


Figure 9. Effect of HIV-1 exposure on tight junction proteins in ectocervical tissues

Ectocervical epithelia were either exposed to HIV-1 (10^6 infectious viral units) or control supernatant for 24 hours in organ culture. Ectocervical tissue sections were stained using antibodies against ZO-1 (red), Claudin-1 (red), Claudin-4 (green) proteins or nonspecific IgG isotype control. Nuclei were stained with DAPI (blue). Five to ten images were captured from each biopsy by confocal microscopy. Magnification 40X. The images shown are representative of control/HIV-1 exposed biopsies from three different donors.

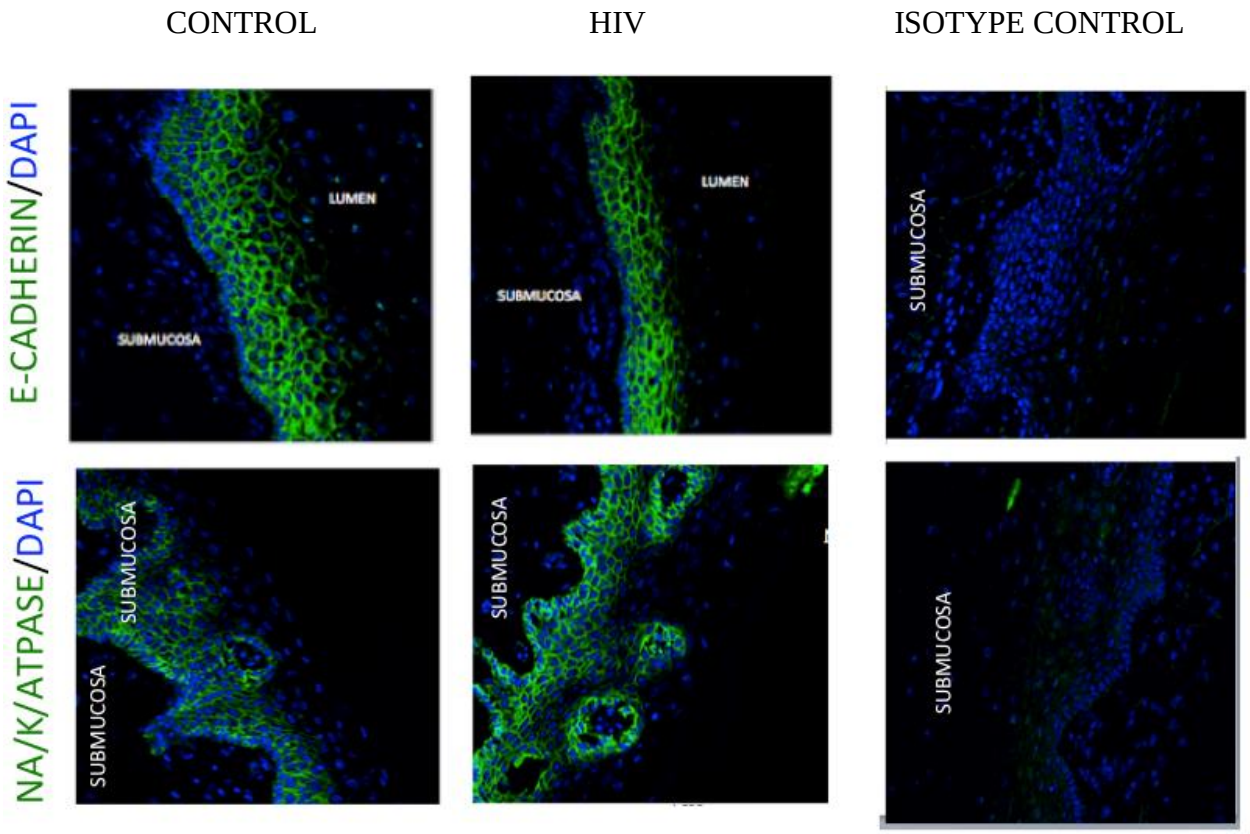


Figure 10. Effect of HIV-1 exposure on adherens junction protein and Na/K/ATPAase in ectocervical tissues

Ectocervical epithelia were either exposed to HIV-1 (10^6 infectious viral units) or control supernatant for 24 hours in organ culture. Ectocervical tissue sections were stained using antibodies against either E-Cadherin (green) or Na/K/ATPase (green) proteins or nonspecific IgG isotype control. Nuclei were stained with DAPI (blue). Five to ten images were captured from each biopsy by confocal microscopy. Magnification 40X. The images shown are representative of control/HIV-1 exposed biopsies from three different donors

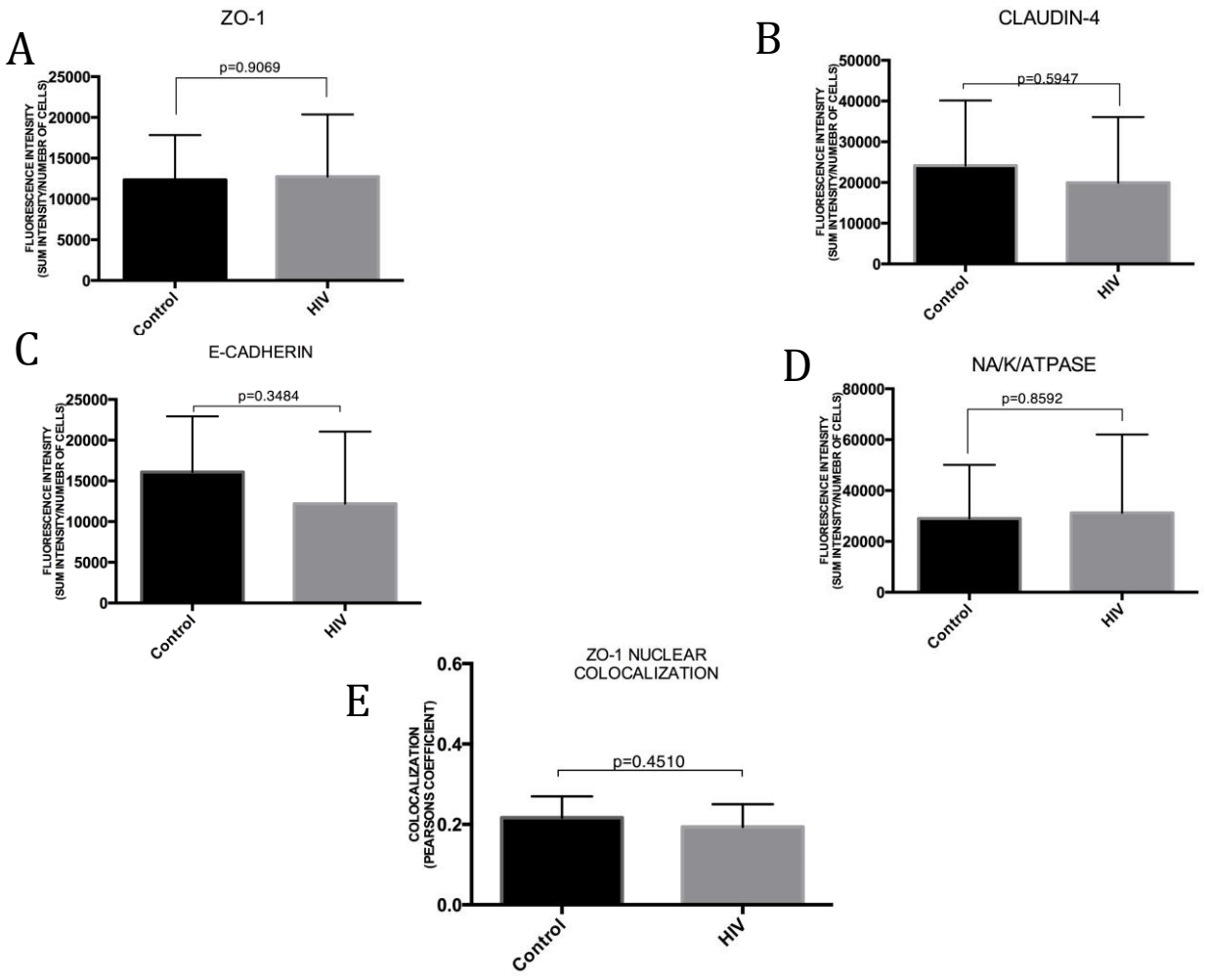


Figure 11. Quantitation of tight junction and adherens junction proteins in ectocervical tissues

Fluorescence intensity of ZO-1 (A), claudin-4 (B), E-Cadherin (C) or Na/K/ATPase (D) proteins in ectocervical epithelia was normalized based on number of nuclei in the selected fields. Data shown are the average of fluorescence intensity in epithelia exposed to control supernatant or HIV-1 and is presented as mean ± standard deviation of three independent experiments with different donors. (E) ZO-1 nuclear colocalization (shown as Pearson correlation coefficient between DAPI and ZO-1) in ectocervical epithelium. Data shown are the average of ZO-1 nuclear colocalization in epithelia exposed to control supernatant or HIV-1 and are presented as mean ± standard deviation of three independent experiments each with a different donor.

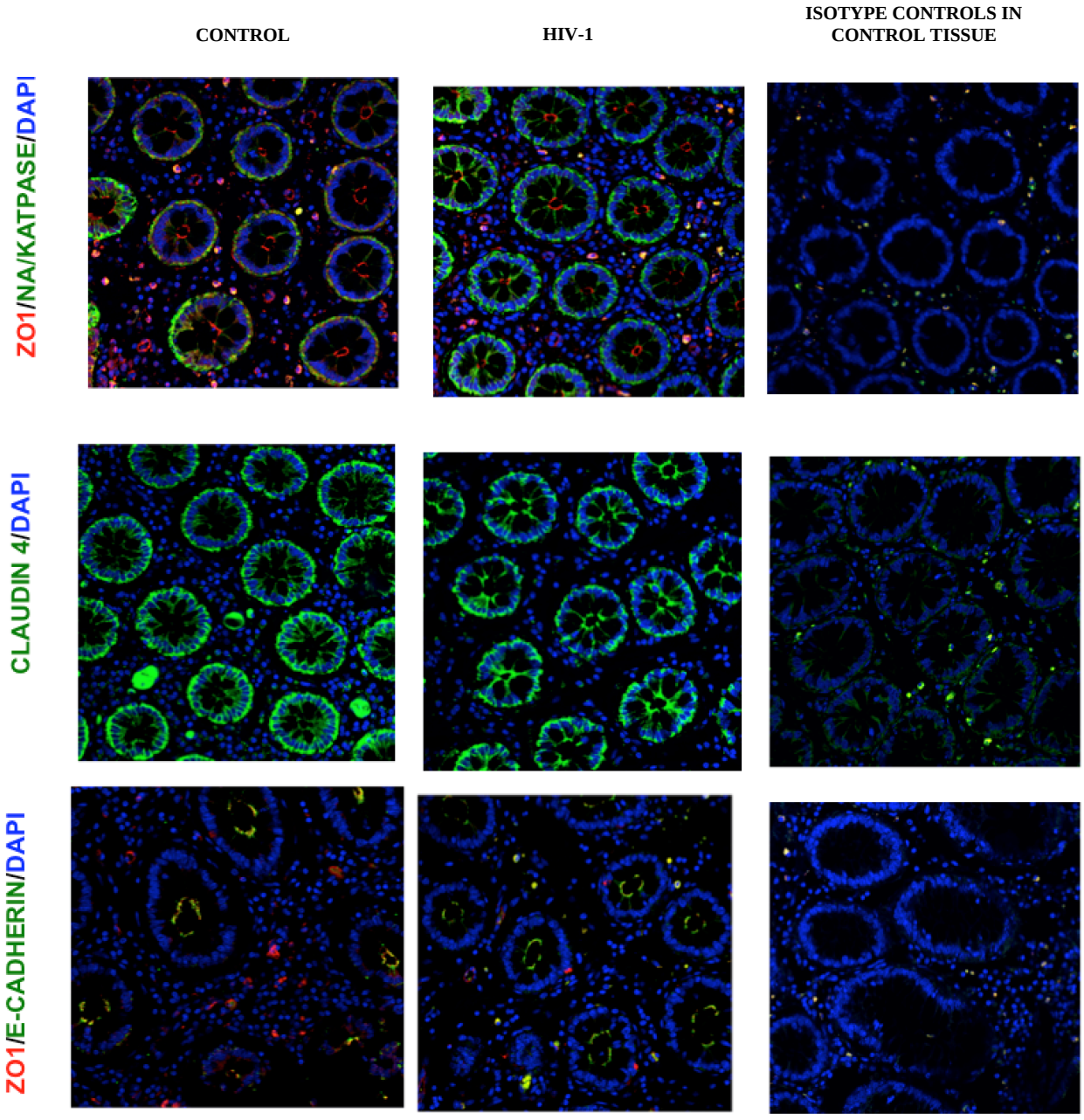


Figure 12. Effect of HIV-1 exposure on tight junction proteins and adherens junction proteins in colon tissues

Human colon tissues were either exposed to HIV-1 (10^6 infectious viral units) or control supernatant for 6 hours. Colon tissue sections were stained using antibodies against either ZO-1 (red), Claudin-4 (green), E-Cadherin (green), Na/K/ATPase (green) protein or nonspecific IgG isotype control. Nuclei were stained with DAPI (blue). Five to ten images were captured from each biopsy by confocal microscopy. Magnification 40X. The images shown are representative of control/HIV-1 exposed biopsies from three different donors

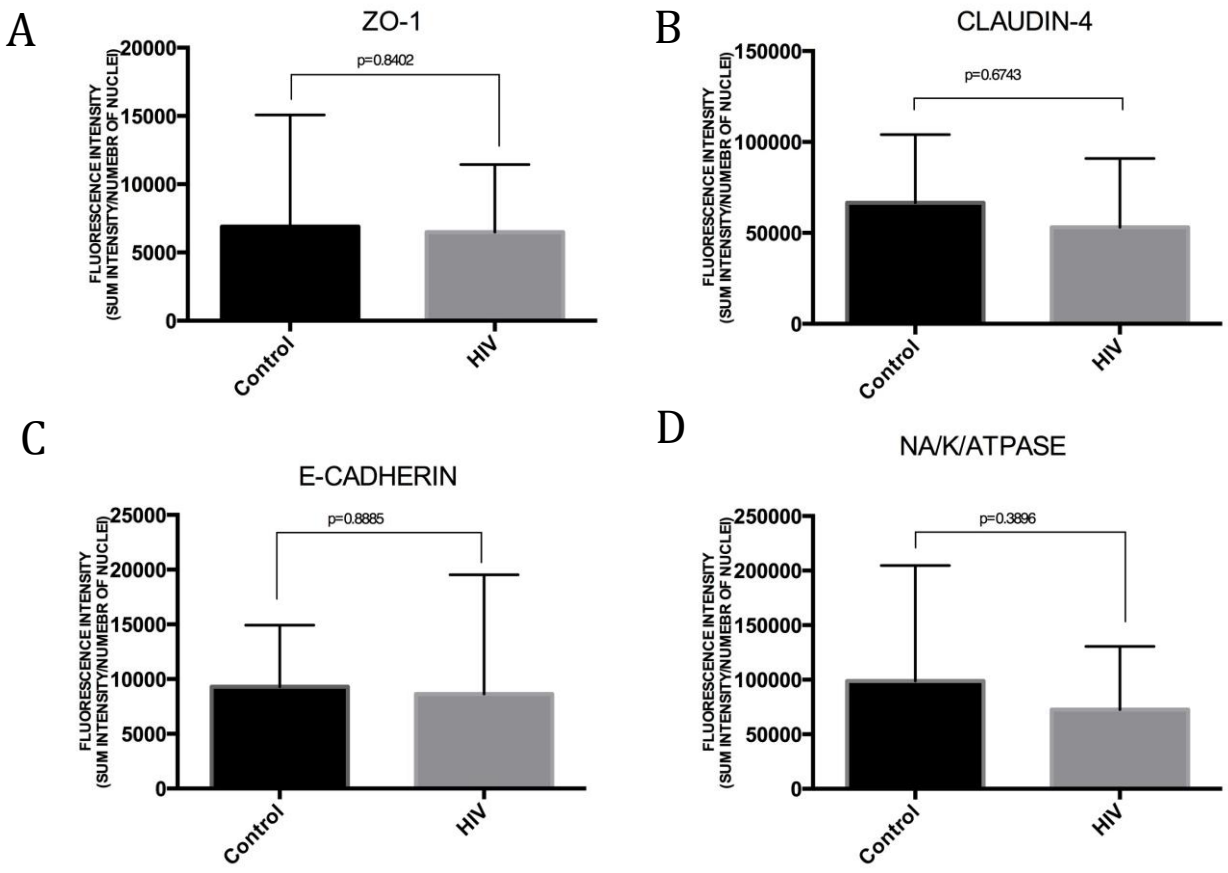


Figure 13. Quantitation of tight junction and adherens junction proteins in colon tissues

Fluorescence intensity of ZO-1 (A), Claudin-4 (B), E-Cadherin (C) or Na/K/ATPase (D) proteins in colon mucosa was normalized based on number of nuclei in the selected fields. Data shown are the average of fluorescence intensity in control supernatant or HIV-1 exposed tissues. Data are presented as mean ± standard deviation of three independent experiments each with a different donor.

4.2.4 Effect of HIV-1 on NA/K/ATPase in ectocervical/colon tissues

TJ maintains cell polarity by restricting the lateral diffusion of proteins and membrane lipids between the basolateral and apical compartments [51]. Studies have suggested that Na/K/ATPase, which is located in the basolateral membrane of the cell, plays an important role

in the formation of tight junctions and development of polarity [52]. To study the effect of HIV-1 on Na/K/ATPase expression pattern, the distribution of Na/K/ATPase between apical and basolateral membranes of epithelial cells was assessed in control or HIV-1 exposed ectocervical/colon tissues. In the control ectocervical/colon tissues, Na/K/ATPase staining was most intense in the basolateral regions and such distribution of Na/K/ATPase was not altered after exposure to HIV-1 (Figure 10,12). Furthermore, quantitative analysis of the expression of Na/K-ATPase also revealed that there was no significant difference in the Na/K/ATPase protein expression levels between HIV-1 exposed and control tissues (Figure 11,13). Thus, exposure to HIV-1 did not significantly disrupt Na/K/ATPase profiles in the epithelial cells in both colon and ectocervical tissues.

4.2.5 HIV-1 transmission through ectocervical and colon tissues

Since we did not observe any effect of HIV-1 exposure on TJ disruption, we investigated whether HIV-1 transmission did occur within 6 hours after exposure in colon and 24 hours after exposure to ectocervical tissue. For this purpose first we examined entry of HIV-1 across the epithelium layer within 6 hours of HIV-1 infection using transmission electron microscopy (TEM). Following exposure of colonic tissues to HIV-1 for 6 hours, TEM analysis demonstrated virus like particles inside and between the epithelial cells. Morphologically, the colon luminal surface exposed to both control supernatant and HIV-1 were characterized by presence of a compactly packed layer of epithelial cells with numerous microvilli (Figure 14A,B). Furthermore, the apical region of the colonic epithelial cells with and without HIV-1 exposure was characterized by intact epithelial junctions, which might prevent entry of luminal contents through the paracellular space.

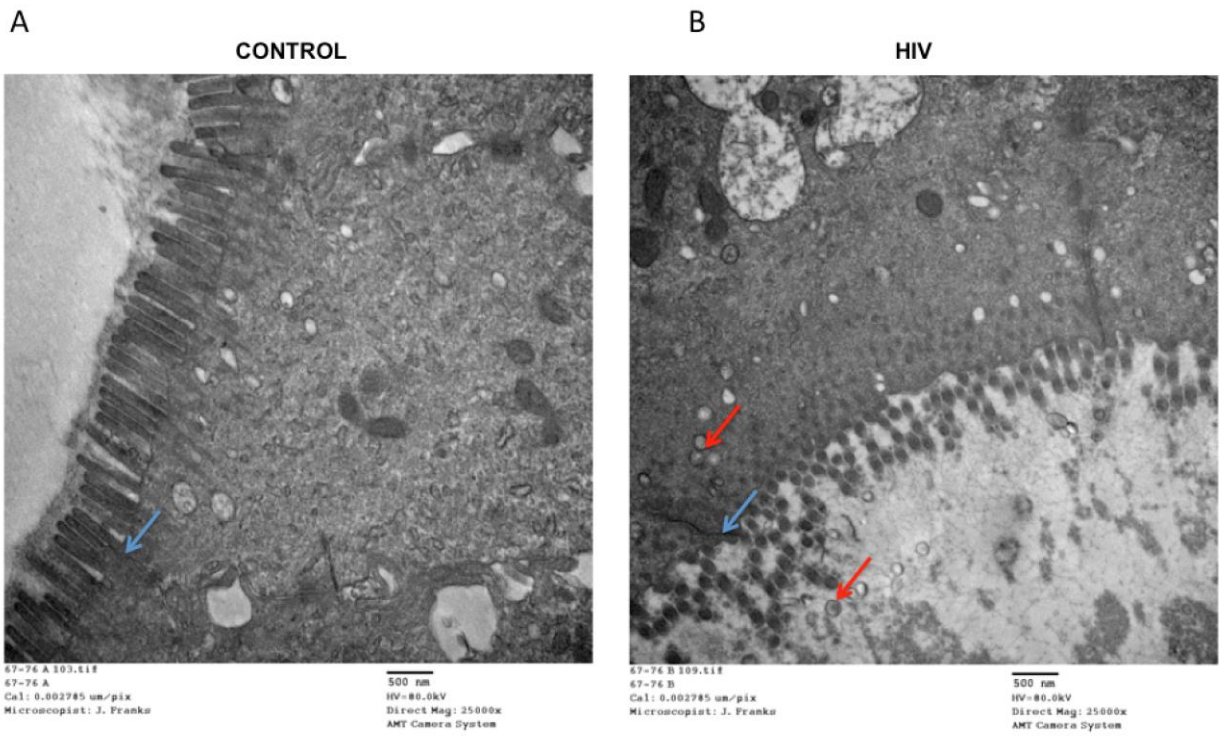


Figure 14. Visualization of epithelial junctions in the human colon tissues by transmission-electron microscopy

Colon biopsies obtained from one donor were exposed to control supernatant (A) or HIV-1 (10^6 infectious viral units) (B) for 6 hours followed by transmission electron microscopy of the tissue sections. Tissue processing and transmission electron microscopy were performed with the help of Biological Imaging EM Core, University of Pittsburgh. Blue arrow shows the epithelial junctions between colon columnar epithelial cells. Red arrow shows the HIV-like particles. Magnification 25000X

To examine further whether HIV-1 transmission occurs within 24 hours and 6 hours of exposure, transmission of HIV-1 in these tissues were examined. For this purpose colon and ectocervical tissues were exposed to HIV-1 for 6 or 24 hours respectively, after which the tissues were washed to remove the viral inoculum, and cultured in fresh medium for 16 days. HIV-1 transmission was detected by observing an increase in the level of HIV-1 p24 in the culture supernatant of colon (Figure15A) and ectocervical (Figure15B) tissues. At day 4 post-infection, the viral replication became evident and it increased over the course of the culture period in both

colonic and ectocervical culture. At day 16 post infection, the average p24 in culture supernatant was 1,160 pg/ml in colon tissue and 13,967 pg/ml in ectocervical tissue.

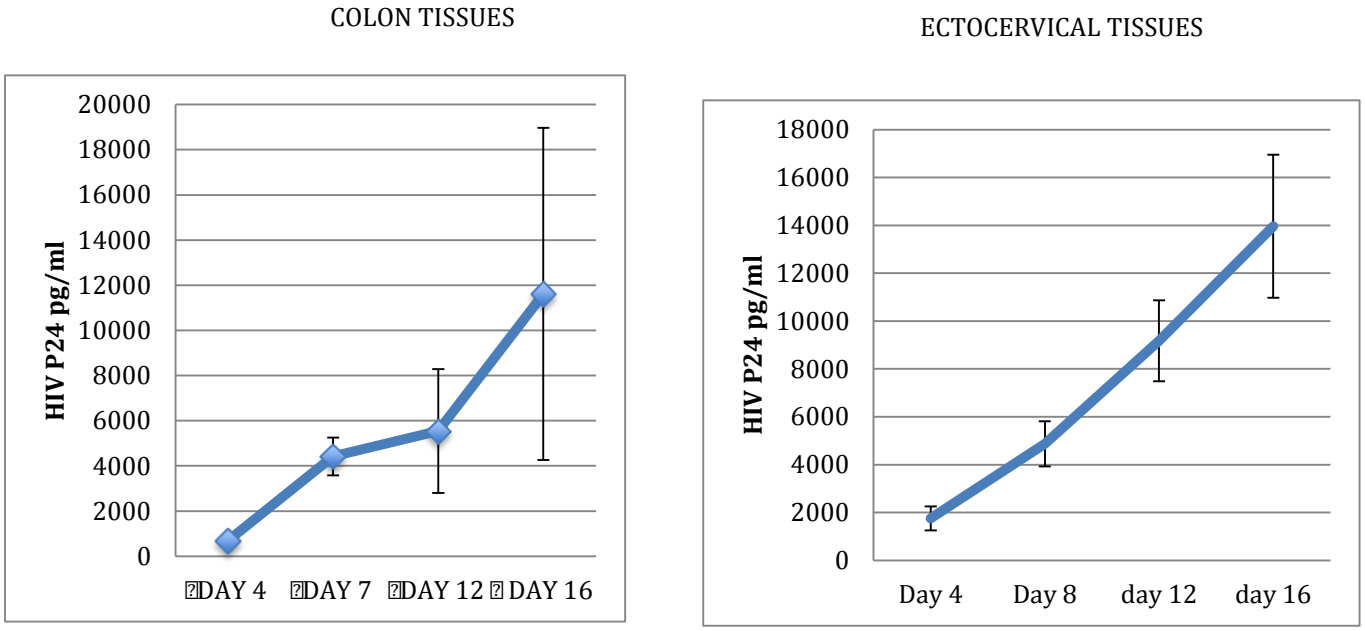


Figure 15. HIV-1 replication in colon/ectocervical tissues after exposure to HIV-1 in vitro

Human colon tissues (n=3) (A) or human ectocervical tissues (n=10) (B) were inoculated *ex vivo* with HIV-1 (10^6 infectious viral units) for 6 hours or 24 hours respectively, then washed and cultured for 16 days. The culture supernatant was tested for HIV-1 p24 antigen production at different time points. The ectocervical organ culture was performed with the help of Deena Ratner. Data are presented as mean ± standard deviation of independent experiments each with different donors (n=3 for colon tissues, n=10 for ectocervical tissues).

4.3 CONCLUSION

Our overall aim was to determine whether exposure to HIV-1 disrupts TJ/AJ between epithelial cells in the colon and ectocervical tissues as a mechanism of HIV-1 transmission through the mucosal epithelial barrier. Interestingly, our result so far indicate that exposure to HIV-1 does not disrupt the TJ/AJ between epithelial cells in ectocervical and colon tissues. We

also observed that Na/K/ATPase, which is an indicator of fence function, did not significantly alter in the HIV-1 exposed ectocervical and colon tissues. However, HIV-1 transmission was observed in colon and ectocervical tissues. These observations suggest that HIV-1 does not disrupt TJ/AJ between epithelial cells for successful transmission through ectocervical and colon mucosa.

5.0 SPECIFIC AIM 2: EVALUATION OF INFLAMMATORY RESPONSE IN EPITHELIAL CELLS FOLLOWING HIV-1 EXPOSURE IN THE ECTOCERVICAL AND RECTAL EPITHELIAL TISSUES

5.1 INTRODUCTION

After exposure to HIV-1, cervical and intestinal epithelial cell lines have been shown to secrete cytokine including IL8, MIP1 Beta, MCP-1, TSLP, TNFα, IL 6 which play an important role in recruiting immune cells [44, 58, 68]. Similarly, studies in rhesus macaques have demonstrated increases in expressions of cytokines like MIP3α, MIP-1β in the cervical epithelium which recruits immune cells like pDCs and CD4 T cells [44, 72]. Therefore, epithelial cells may be one of the first cell targets for HIV-1 to induce local inflammatory responses, which may facilitate HIV-1 transmission. However, these conclusions are drawn from *in vitro* human cell line studies and *in vivo* monkey studies focusing on early events of SIV transmission during the first few days of infection. Due to ethical and practical reasons, similar studies for understanding the early events of HIV-1 transmission in the first few days of infection have not been performed in humans. As mentioned by Haase *et al.*, within first few weeks of infection, SIV spreads to lymphatic tissues and establishes systemic infection [44]. Therefore to prevent the spread of HIV-1, understanding the early events of HIV-1 transmission is critical. We, therefore, examined the inflammatory responses caused by exposure to HIV-1 in ectocervical and colon

tissues using organ culture model. Based on the conclusions drawn from rhesus macaques' studies, we hypothesize that **interaction of HIV-1 with the ectocervical/rectal epithelium induces inflammatory gene expression in epithelial cells. Additionally, HIV-1 induced cytokines from epithelial cells will alter immune cell distribution in ectocervical tissues.**

5.2 RESULTS

5.2.1 Cytokine gene expression profile following HIV-1 exposure in epithelial cells of ectocervical and colon tissues

Previous studies have reported that following HIV-1 infection there is an upregulation of thymic stromal lymphopoietin (TSLP) that strongly activates human myeloid DCs, leading to robust induction of homeostatic proliferation of CD4+ T cells and promoting HIV-1 replication in the activated T cells [114]. Previous studies have also reported an increased production of cytokines like IL-2, IL-4, IL-5 and TNF-α in culture supernatants of HIV-1 exposed intestinal biopsies [115]. We therefore examined inflammatory response in the epithelial cells of the tissues exposed to HIV-1 since it may facilitate the establishment of local HIV-1 infection during HIV-1 transmission across the epithelial barrier. For this purpose, the mucosal epithelial layers from ectocervical tissues and mucosal layers from colonic tissues following HIV-1 exposure were harvested by microdissection followed by examination of the mRNA levels of different cytokines in epithelia.

The purity of microdissected ectocervical epithelia was assessed by measuring the cytokeratin 13 mRNA, which is mainly expressed in epithelial cells and less expressed in

submucosa [116, 117]. The mean CT value of cytokeratin 13 mRNA in microdissected epithelial regions was 10 CT less than that of sub-epithelial regions, which indicated that the epithelial cells were enriched at least by 1000 fold in the microdissected epithelia compared to sub-epithelial regions (data not shown). As shown in figure 16, the mean fold change of CXCL10 and IL-6 mRNA expression was 2 fold or higher in human colon mucosa exposed to HIV-1 compared to the tissues exposed to control supernatant. However, such increase was not statistically significant in HIV exposed colon mucosa. Significantly higher fold change of CXCL10 and CXCL11 mRNA expressions were detected in HIV-1 exposed ectocervical epithelia compared to the controls (Figure 17a).

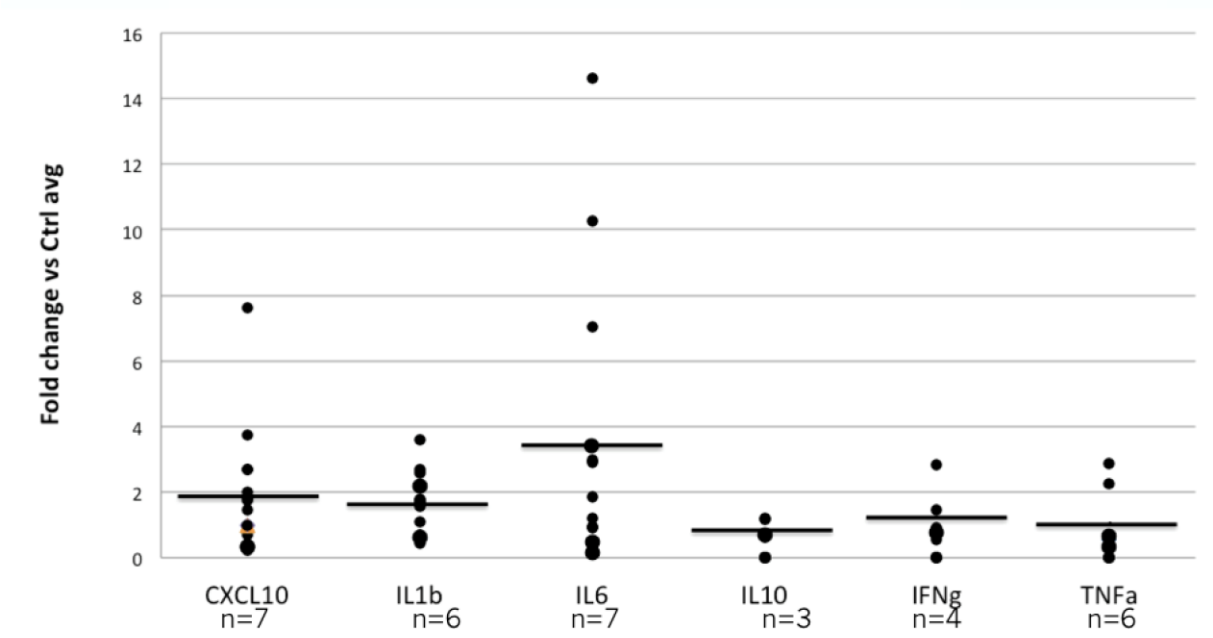


Figure 16. Cytokine gene expression in epithelia following HIV-1 exposure in the colon tissues

Colon tissues (n=3-7) were exposed to either HIV-1 (10^6 infectious viral units) or control supernatant for 6 hours. Total RNA was extracted from microdissected mucosal layers followed by real time RT-PCR for cytokine gene expression. GAPDH, a house-keeping gene, was measured for internal controls. Experiments were repeated in 7 different donors. Each donor had two biopsies per treatment. Each circle (•) represents the fold change of HIV-1 exposed mucosal layer compared to controls from each biopsy and horizontal lines represent mean values of the fold-change in the HIV exposed biopsies compared to controls

RNA extraction and Real-time RT PCR were performed with the help of Ming Ding.

To determine if infectious HIV-1 was required for such cytokine induction, ectocervical tissues were exposed to infectious HIV-1, AT2-inactivated HIV-1 or gp120 followed by microdissection of epithelial layer. As shown in Figure 17B and 17C, both AT2-inactivated HIV-1 and HIV-1 gp120 failed to increase cytokine expression in the ectocervical epithelial cells. These results indicate that infectious HIV-1 is required for cytokine induction in ectocervical epithelial cells.

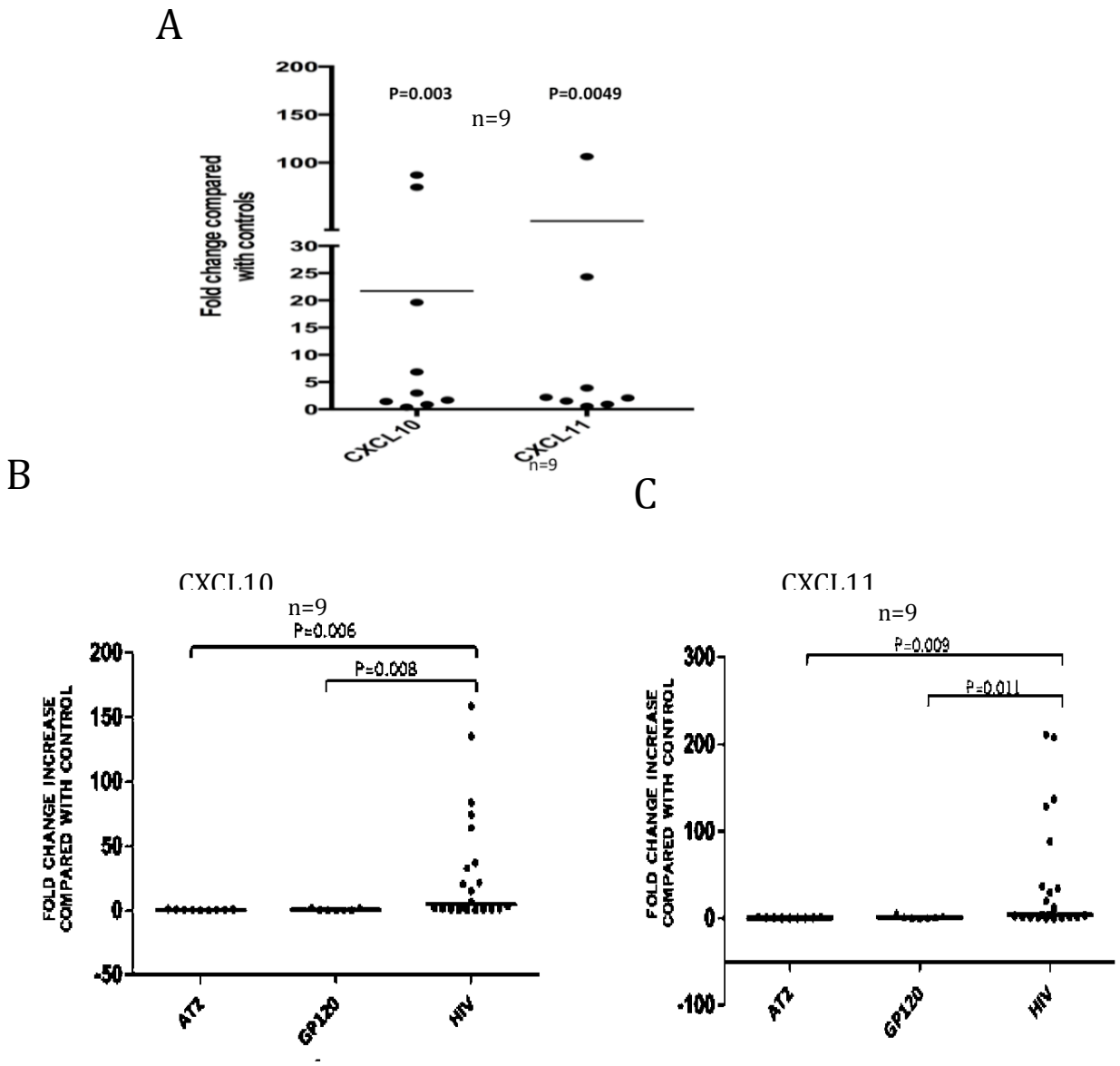


Figure 17. Cytokine gene expression in epithelia following HIV-1 exposure in the ectocervical tissues

Ectocervical tissues (n=9) were exposed to either control supernatant, HIV-1 (10^6 infectious viral units), AT2 inactivated HIV-1 or GP120 for 24 hours. Total RNA was extracted from microdissected epithelial layers followed by real time RT-PCR for cytokine gene expression. KRT 13, a house-keeping gene, was measured for internal controls. Experiments were repeated in 9 different donors. Each donor had two or more biopsies per treatment. Each circle (•) represents the fold change observed from each biopsy in the treated biopsies compared to controls. (A) Fold change observed in HIV-1 exposed ectocervial epithelia compared to control. (B) Fold change observed in HIV-1 or AT2 or GP120 exposed ectocervial epithelia compared to control. Horizontal lines represent mean values of the fold-change. RNA extraction and Real-time RT PCR was performed with the help of Ming Ding

5.2.2 Changes of gene expression profiles of cellular factors in ectocervical epithelia following HIV-1 exposure

Besides these cytokines, there might be other cellular factors that are involved in HIV-1 transmission through the epithelial layer and facilitate infection in the ectocervical tissues. To identify cellular factors in epithelia that are involved in HIV-1 transmission, a comprehensive transcriptome analysis by next generation sequencing using an Ion Torrent platform was performed with the RNA extracted from epithelia of ectocervical tissues (n=6) exposed to HIV-1 or control supernatant.

The mapped sequences based on mRNA database were analyzed to compare the expression levels of individual gene by statistical software R. Expression levels of about 52,000 genes were analyzed. The genes that had no expression or low read counts were excluded for further analysis. Expression levels of the remaining 10,711 gene were further analyzed based on false discover rate <0.05 to examine if their expression levels were significantly changed after exposure to HIV-1. Table 3 lists the top 10 most differentially expressed genes by fold change in HIV-1 exposed ectocervical epithelia compared to the controls. The top 10 most differentially expressed genes in HIV exposed ectocervical epithelia were IL36A, FMO2, CXCL10, MUC1, SAA1, IL8, WARS, RHOBYB3, SPRR2G, SECTM1. Amongst these genes, the expression level of IL36A, FMO2, CXCL10, MUC1, SAA1, IL8 were significantly higher ($p<0.05$) with 2 or higher fold change in HIV-1 exposed ectocervical epithelia compared to the control (Table 3, Figure 18). The genes, like CXCL10, IL8, SAA1, that were significantly upregulated after exposure to HIV-1 are known to play an important role in immune cell migration[72, 118-120], whereas MUC1 has a role in protecting against infection by preventing the binding of pathogens

to cells[121]. Thus up-regulation of these cellular factors such as CXCL10, IL8, SAA1 might increase target cell availability by recruiting immune cells towards the epithelial layer.

Table 3. Differentially expressed genes in HIV-1 exposed ectocervical epithelia compared to the controls as evaluated by next generation sequencing in an Ion Torrent platform

ID	Entrez Gene Name	Location	Type(s)	Log FC	P Value	FDR	Function
IL36A	Interleukin 36, alpha	Extracellular Space	Cytokine	2.6	1.77E-07	0.0015*	1. Plays an important role in innate and adaptive immunity. 2) IL-36 activates MAPK and NF-kB pathways and is produced by many different cells.
FMO2	flavin containing monooxygenase 2 (non-functional)	Cytoplasm	Enzyme	2.4	2.37E-07	0.0015*	Unknown
CXCL10	chemokine (C-X-C motif) ligand 10	Extracellular Space	Cytokine	2.5	3.87E-06	0.0167*	Plays an important role in chemoattraction of monocytes/macrophages,T cells, NK cells, and dendritic cells.
MUC1	mucin 1, cell surface associated	Plasma Membrane	Transmembrane receptor	2.7	9.55E-06	0.0277*	Protect the body from infection by pathogen binding to oligosaccharides
SAA1	serum amyloid A1	Extracellular Space	Transporter	3	1.07E-05	0.0277*	Promotes migration, and tissue infiltration of monocytes, neutrophils, lymphocytes.
IL8	chemokine (C-X-C motif) ligand 8	Extracellular Space	Cytokine	1.4	1.69E-05	0.036*	Induces chemotaxis in neutrophils and granulocytes.
WARS	tryptophanyl-tRNA synthetase	Cytoplasm	Enzyme	1.37328	5.13E-05	0.095	Promotes cell-to-cell signaling and cell-mediated immune esponse
RHOBTB3	Rho-related BTB domain containing 3	Cytoplasm	Enzyme	2.31804	8.03E-05	0.13	Involed in signal transduction
SPRR2G	small proline-rich protein 2G	Cytoplasm	Other	1.48239	1.08E-04	0.15	Is involved in process of keratinizatio and ; keratinocyte differentiation
SECTM1	secreted and transmembrane 1	Extracellular Space	Cytokine	1.64122	1.16E-04	0.15	Unknown

* Indicates statistically significant expressions.

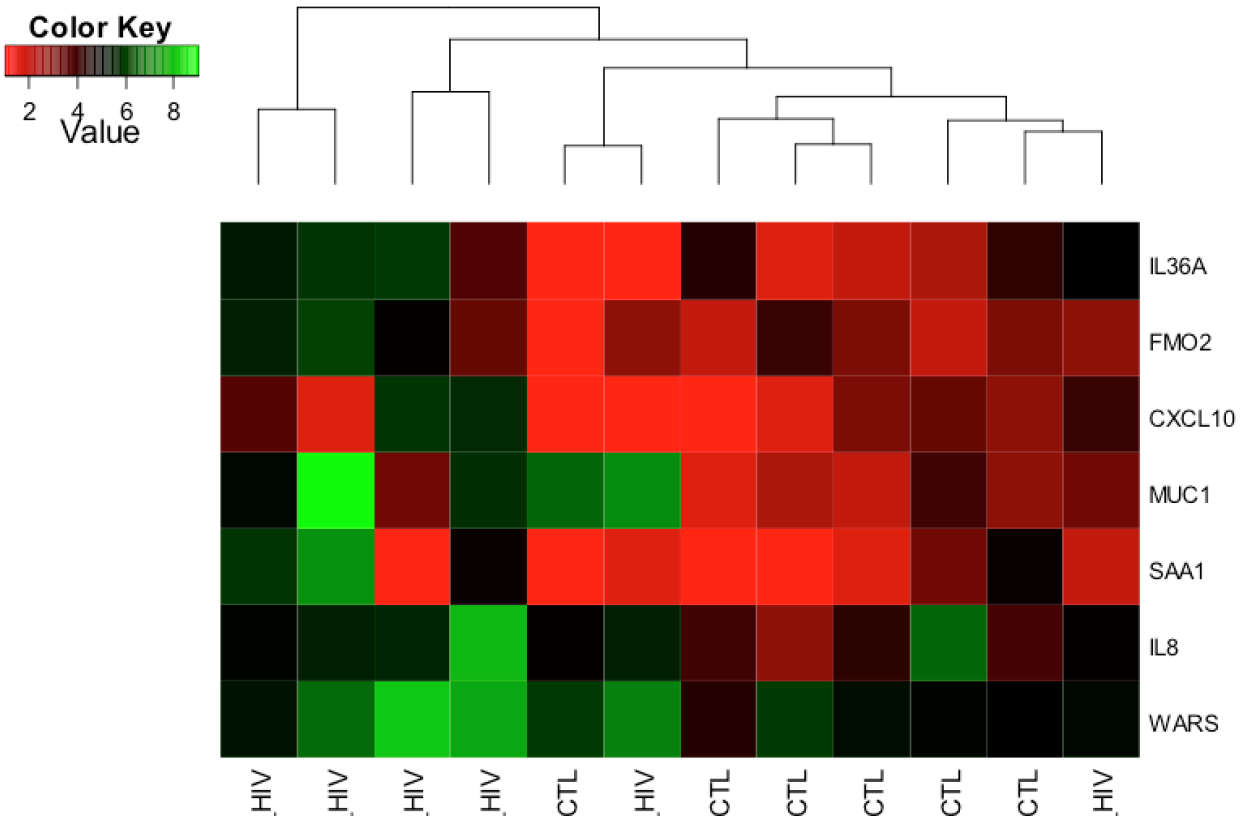


Figure 18. Heat map of differently expressed genes in HIV-1 exposed ectocervical epithelia compared to controls

Heat map of the genes that were significantly ($p<0.05$, fold change ≥ 2) upregulated in HIV-1 exposed ectocervical epithelia (n=6) compared to controls. CTL- Ectocervical tissues exposed to control supernatant; HIV: Ectocervical tissues exposed to HIV-1 for 24 hours. Ion torrent sequencing was performed with the help of Ming Ding and data analysis was performed with the help of Dr. Chengli Shen.

Differential expressions of these genes were further confirmed by real time RT-PCR of the 9 donors (Table 4). The mean values of fold changes of IL36A, FMO2, CXCL10, MUC1, SAA1, IL8 expression in HIV-1 exposed ectocervical epithelia relative to controls were 1.75 (SD±0.95), 2.09(SD±1.41), 11.32(SD±11.8), 2.29(SD±1.94), 2.64(SD±2.6), 2.12(SD±1.48) respectively.

Table 4. mRNA expression levels (fold change relative to control) of cytokines in HIV-1 exposed ectocervical epithelia by real time RT-PCR

GENES	FOLD CHANGE RANGE (n=9)	MEAN FOLD CHANGE (n=9)	P VALUE
CXCL10	1.4 to 40.22	11.32	0.0156
SAA1/SAA2	0.26 to 8.75	2.64	0.0509
MUC1	0.37 to 6.54	2.29	0.0411
IL36A	0.84 to 3.29	1.75	0.0233
FMO2	0.79 to 5.06	2.09	0.0247
IL8	0.99 to 5.39	2.12	0.0265

5.2.3 Study of immune cell migration in ectocervical tissues after exposure to HIV-1

Studies in rhesus macaques have suggested that inflammatory responses after SIV infection might facilitate SIV transmission by increasing target cell availability [44]. Since the immune cells are target cells for HIV-1, we assessed whether exposure to HIV-1 increased the number of immune cells in epithelial and subepithelial regions of ectocervical tissues. For this purpose, we examined the distribution of CD3+ and CD8+ cells in the intraepithelial and submucosal regions in control and HIV-1 exposed ectocervical tissues using immunofluorescence staining for CD3 and CD8 followed by confocal microscopy. Images acquired from 10 high power fields randomly chosen per slide were obtained for analysis. CD3+ or CD3+/CD8+ cells were detected throughout the intraepithelia and submucosal regions, both as single cells and in clusters (Figure 19A,B). Based on the current literature, we expected increases in CD3+/CD8+cell populations in the intraepithelial layer and just beneath the basement membrane in HIV-1 exposed ectocervical tissue. However, there was no significant

difference (p=0.53) in the ratio of number of CD3+ CD8+ cells in intraepithelial region to total number of CD3+ CD8+ in HIV-1 exposed ectocervical tissues compared to the control (Figure 19C).

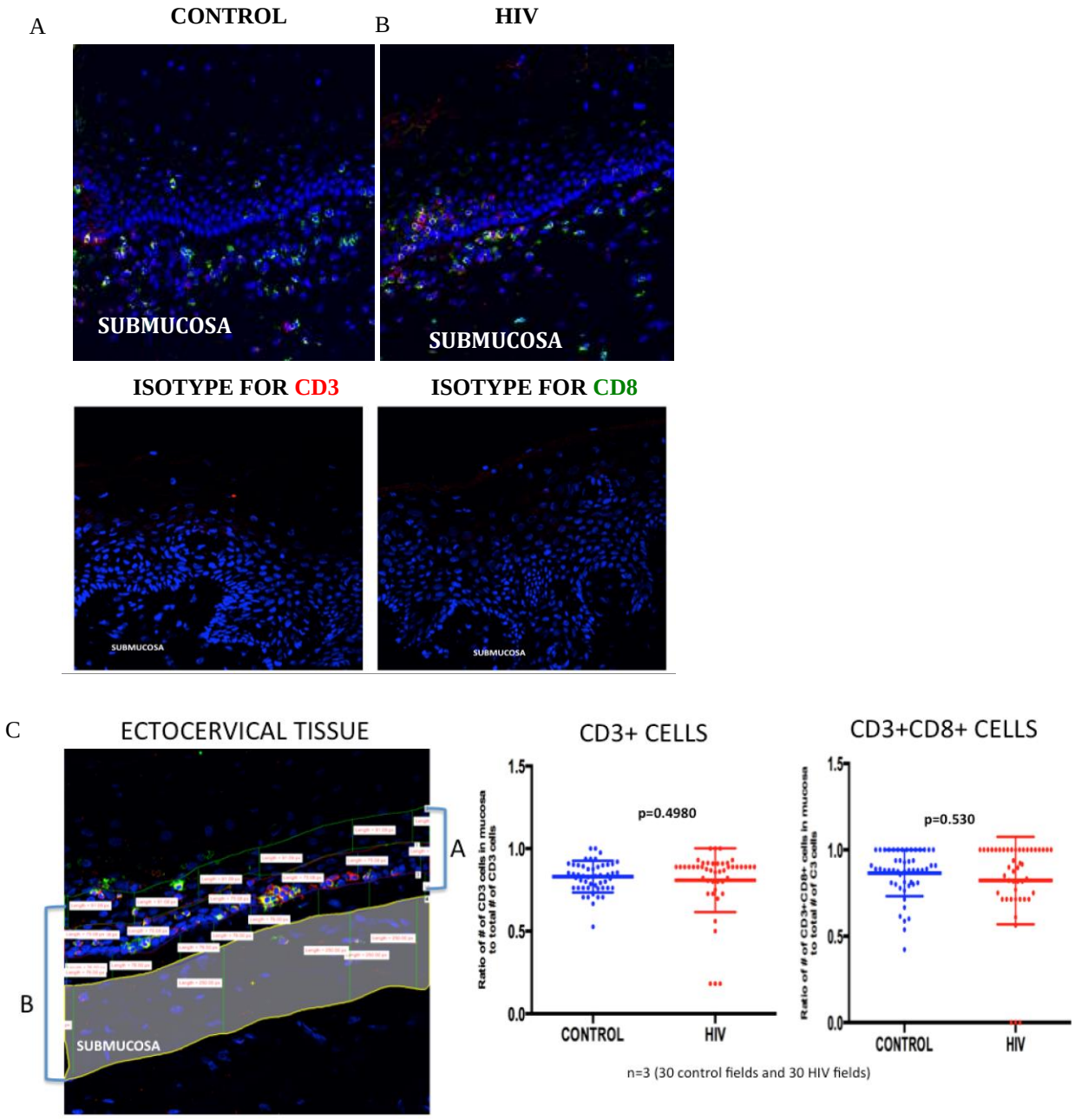


Figure 19. CD3+ CD8+ immune cell distribution in ectocervical tissues after exposure to HIV-1

Ectocervical tissues were either exposed to control supernatant (A) or HIV-1 (10^6 infectious viral units) (B) for 24 hours. Ectocervical tissue sections were stained using antibodies against CD3 (red), CD8 (green), isotype control for CD3 (red) or isotype control for CD8 (green). Nuclei were stained with DAPI (blue). Five to ten images were captured from each biopsy by confocal microscopy. Magnification 40X. The images shown are representative of control/HIV-1 exposed biopsies from three different donors. Each donor had two biopsies per treatment. (c) Ratio of the number of immune cells in the epithelial region and just

beneath the basolateral membrane (region A), and the total number of immune cells in mucosa and submucoa (region B) in each image. Each circle (□) represents ratio of region A to region B quantitated in each confocal image obtained from control/HIV-1 treated biopsies and horizontal lines represent mean value. Data are presented as mean ± standard deviation.

We also tried to examine the changes in the distribution of macrophages in ectocervical epithelia after exposure to HIV-1. Immunofluorescence staining for CD68 was performed on control and HIV-1 exposed human ectocervical tissues. As a positive control for CD68 staining, immunofluorescence staining of axillary lymph node tissues obtained from a rhesus macaque was performed at the same time. Axillary lymph node tissues were a gift from Dr. Todd Reinhart (University of Pittsburgh). Confocal microscopic images showed strong expression of CD68+ cells in rhesus macaque tissues (Figure 20). Unfortunately, no clear staining of CD68+ cells were observed in human ectocervical tissues (n=2) (Figure 20). Similarly, despite multiple attempts using antibodies from different companies, distinct staining of CD4+ cells was not observed in the ectocervical tissues (data not shown). This might be due to the quality of antibodies used in the study. Therefore, we were not able to determine the changes in distribution of CD4+ or CD68+ cells in ectocervical epithelia after exposure to HIV-1.

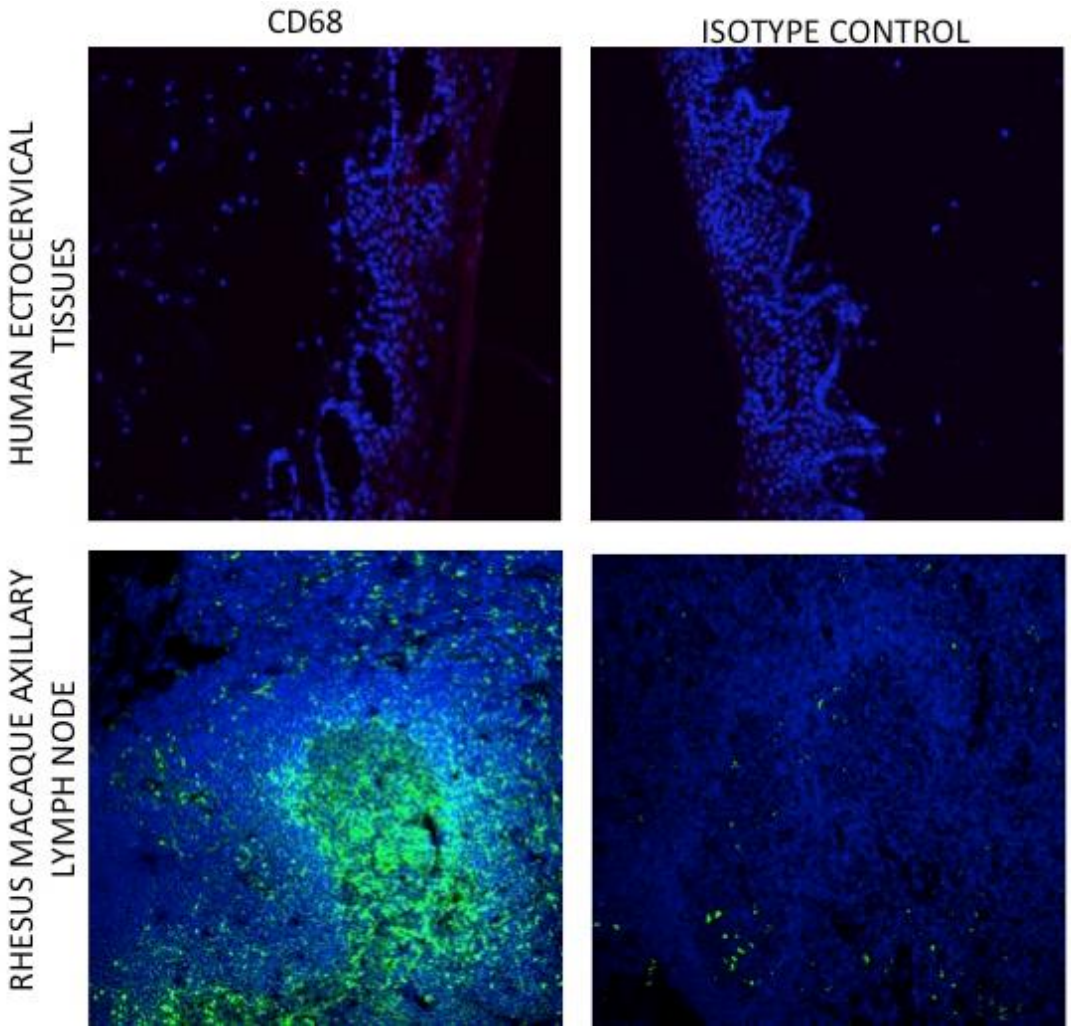


Figure 20. CD68+ immune cell staining in ectocervical tissues

Tissues sections from human ectocervical tissues and rhesus macaque axillary lymph node were stained for CD68 (red in human tissues, green in rhesus macaque tissues). Images were captured by confocal microscopy. DAPI-stained nuclei shown in blue.

5.3 CONCLUSION

We sought to determine the effect of HIV-1 on local inflammatory responses in epithelial cells, which may facilitate the establishment of local HIV-1 infection after HIV-1 crosses the epithelial barrier. We found that HIV-1 exposure to epithelium induced marginal CXCL10 and

IL6 expression in colonic tissue and CXCL 10 and CXCL11 in cervical tissues. Such induction of cytokines in ectocervical epithelia was dependent on infectious virus exposure. Furthermore, next generation sequencing of the mRNA isolated from ectocervical epithelial layers have identified certain cellular gene expression in epithelia following HIV-1 infection that may be important for HIV-1 transmission by recruiting immune cells for productive HIV-1 infection. However, there was no change in the distribution of CD3+ CD8+ immune cells in the intraepithelial and subepithelial region after HIV-1 infection.

6.0 SPECIFIC AIM 3: SUSCEPTIBILITY OF ECTOCERVICAL AND VAGINAL TISSUES TO HIV-1 INFECTION AT DIFFERENT STAGES OF MENSTRUAL CYCLE

6.1 INTRODUCTION

Natural fluctuations in levels of the hormones estradiol and progesterone play a crucial role in regulating physiology and function within the female genital tract, and may introduce a window of vulnerability to HIV-1 infection at specific times during the menstrual cycle [87]. Studies in non-human primate models have shown that progesterone treatment caused vaginal thinning and increased SIV acquisition in monkeys, whereas estrogen treatment protected female rhesus macaques against SIV infection [91, 122]. A number of epidemiological and laboratory studies have studied the influence of reproductive hormone on HIV-1 acquisition risk in women with few studies reporting a significant association between hormones and HIV-1 transmission while other studies failed to show such association [86, 94]. Moreover, studies have proposed multifactorial mechanisms of hormonal contraceptive use on HIV-1 infection including vaginal thinning, and evaluated the vaginal thickness in women using DMPA. However, conclusions from these studies have not been consistent so far. In vitro studies using human cervical cell lines have shown that reproductive hormones like estradiol altered the expression level of tight junction proteins and increase transepithelial resistance [102-104]. Thus, it remains uncertain if

and how reproductive hormones alter the risk of HIV-1 acquisition. **We, therefore, hypothesized that reproductive hormones might influence susceptibility to HIV-1 infection in human ectocervical and vaginal tissues by altering thickness of epithelial layer and disrupting tight junction proteins between epithelial cells**

6.2 RESULTS

6.2.1 Assessment of susceptibility to HIV-1 infection in ectocervical and vaginal tissues obtained at different stages of the menstrual cycle

Previous studies have postulated that during the menstrual cycle, there is a window of vulnerability in which the potential for viral infection in the female genital tract is increased[87]. Progesterone treatments in monkeys have been used to increase susceptibility to vaginal inoculation of SIV in studies of candidate HIV-1 vaccines[85, 86, 94, 123]. Therefore, we hypothesized that the cervical/vaginal tissues at different stages of menstrual cycle might be more susceptible to HIV-1 infection due to the elevation or depression of a reproductive hormone in a particular phase of menstrual cycle. To test this hypothesis, ectocervical and vaginal tissues obtained from women in proliferative (n=21), ovulatory (n=6) or secretory phases (n=11) were exposed to HIV-1 for 24 hours respectively. The tissues were then washed to remove the viral inoculum, and cultured in fresh medium for 16 days. HIV-1 transmission was monitored by measuring production of HIV-1 p24 periodically in the culture supernatant of ectocervical (Figure 21A, Table 5) and vaginal (Figure 21B, Table 6) tissues. On day 4 post-infection, viral replication became evident and increased during the course of culture period in

both ectocervical and vaginal tissue cultures (Figure 21A,B). On day 16 post-infection, the average p24 in culture supernatant of ectocervical tissues (Table 5) obtained during the proliferative phase, ovulatory phase and secretory phase was 10,979.2 (±10,031.8) pg/ml, 3,834.2 (±5,137.6) pg/ml, 7,637.9 (±8,873) pg/ml respectively. In vaginal tissues (Table 6), on day 16 post infection, the average p24 in culture supernatant of vaginal tissue obtained during proliferative phase, ovulatory phase and secretory phase was 4,003.5 (±3,791.2) pg/ml, 2,372.8 (±1,239.3) pg/ml, 4418.9 (±9,051.1) pg/ml respectively.

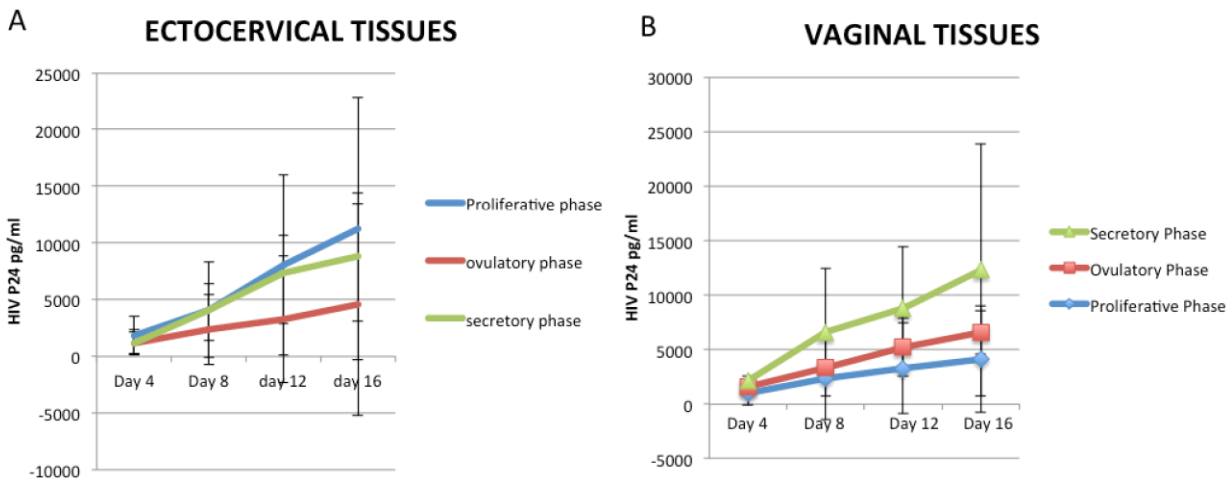


Figure 21. HIV-1 replication in ectocervical/vaginal tissues obtained from different phases of menstrual cycle

Human ectocervical tissues (n=6-21) (A) or human vaginal tissues (n=6-21) (B) obtained from different phases of menstrual cycle were exposed to HIV-1 (10^6 infectious viral units) for 24 hours, then washed and cultured for 16 days. The culture supernatant was tested for HIV-1 p24 antigen production at different time points. The ectocervical/vaginal organ culture was performed with the help of Deena Ratner. Data are presented as mean ± standard deviation of 6 to 21 independent experiments each with different donors.

Table 5. HIV-1 p24 (pg/ml) production in ectocervical tissues obtained from different phases of menstrual cycle

	Proliferative Phase	Ovulatory Phase	Secretory Phase	ANOVA P-Value	KW P-Value
Number of donors	21	6	11		
Day 04				0.282	0.141
Mean, SD	1833.9 (1487.3)	1169.3 (663.9)	1167.5 (948.9)		
Median, IQ Range	1513 (850)	1064 (590)	814 (1248)		
Day 08				0.518	0.509
Mean, SD	3995.9 (3418.0)	2379.5 (2086.3)	3717.4 (2530.1)		
Median, IQ Range	274 (3617)	1701 (2397)	4175 (3121)		
Day 12				0.254	0.335
Mean, SD	7859.4 (7041.6)	3274.3 (2863.9)	5697.8 (5443.2)		
Median, IQ Range	4936 (9262)	23 (345)	4465 (6778)		
Day 16				0.227	0.098
Mean, SD	10979.2 (10031.8)	3834.2 (5137.6)	7637.9 (8873.0)		
Median, IQ Range	7608 (10643)	2340 (920)	4622 (9650)		
Area under the curve				0.232	0.327
Mean, SD	72390.5 (56860.9)	32622.2 (19227.0)	55271.6 (48396.2)		
Median, IQ Range	45400 (94800)	35210 (23960)	52370 (38340)		
Peak				0.360	0.416
Mean, SD	11680.9 (10145.2)	6065.8 (4796.7)	8605.1 (8393.1)		
Median, IQ Range	7842 (10370)	5564 (4510)	5338 (7188)		

SD: Standard deviation

IQ range: Interquartile range

KW: Non parametric Kruskal-Wallis analysis

Table 6. HIV-1 p24 (pg/ml) production in vaginal tissues obtained from different phases of menstrual cycle

	Proliferative Phase	Ovulatory Phase	Secretory Phase	ANOVA P-Value	KW P-Value
Number of donors	20	6	11		
Day 04				0.338	0.505
Mean, SD	964.3 (1037.0)	578.9 (216.9)	560.8 (367.6)		
Median, Range	712 (394)	570 (385)	604 (794)		
Day 08				0.691	0.809
Mean, SD	2242.9 (2987.2)	1140.3 (776.4)	2689.6 (5051.5)		
Median, Range	1018 (2105)	812 (128)	580 (2192)		
Day 12				0.792	0.542
Mean, SD	2884.3 (2786.9)	1816.6 (1538.7)	2614.7 (4710.2)		
Median, Range	2442 (3048)	1400 (1579)	1106 (2417)		
Day 16				0.776	0.305
Mean, SD	4003.5 (3791.2)	2372.8 (1239.3)	4418.9 (9051.1)		
Median, Range	3401 (4032)	2418 (995)	1350 (1982)		
Area under the curve				0.761	0.510
Mean, SD	28180.8 (24457.9)	17731.0 (8702.5)	31176.6 (56392.5)		
Median, IQ Range	22010 (24458)	19570 (9260)	12860 (19924)		
Peak				0.721	0.416
Mean, SD	4650.7 (4054.1)	2673.0 (1422.7)	4896.0 (8951.6)		
Median, IQ Range	3662 (5758)	2495 (1215)	1415 (3402)		

SD: Standard deviation

IQ range: Interquartile range

KW: Non parametric Kruskal-Wallis analysis

To compare HIV-1 replication in ectocervical/vaginal tissues obtained from different phases of menstrual cycle, parametric and non parametric statistical analysis of p24 values were conducted at each time point (day 4, 8, 12) post HIV-1 infection for p24 produced in

cervical/vaginal tissues obtained from either proliferative, ovulatory or secretory phase of menstrual cycle. Statistical analyses by both parametric and non-parametric method demonstrated that irrespective of the time points after HIV-1 infection, there were no statistically significant differences (p=0.2) between the p24 produced in cervical/vaginal tissues obtained from either proliferative or ovulatory or secretory phase of menstrual cycle. To evaluate the overall virus production during the culture period, the area under the curve (AUC) was also calculated based on the p24 production on days 4, 8, 12, 16 post infection of cervical/vaginal tissues obtained from either proliferative or ovulatory or secretory phase. As shown in Table 5 the mean AUC for cervical tissues obtained at proliferative, ovulatory and secretory phase were 72,390.5, 32,622.2 and 55,271.6 pg/ml respectively. In case of vaginal tissues, AUC for the vaginal tissues obtained at proliferative, ovulatory and secretory phase were 28,180.8, 17,731.0 and 31,176.6 pg/ml respectively (Table 6). However, statistical analysis by both parametric and non-parametric methods showed there were no statistical differences (p=0.232) between AUC in cervical/vaginal tissues obtained at different phases of menstrual cycle. These results suggest that the overall susceptibility to HIV-1 infection in cervical/vaginal tissues was not significantly changed at different phases of menstrual cycle.

6.2.2 Epithelial thickness and tight junction protein expression at different phases of menstrual cycle

A decrease in epithelial thickness or epithelial junction protein expression could reduce the epithelial barrier function potentially favoring exposure of target cells to HIV-1 [124]. Studies in non-human primate models demonstrated that DMPA treatment reduced vaginal epithelial thickness and increased SIV transmission through vaginal mucosa [91]. In contrast,

estrogen treatment in non-human primates increased vaginal thickness and reduced vaginal SIV transmission [122]. Previous in vitro studies suggest that reproductive hormones like estrogen could regulate TJ proteins, transepithelial resistance in epithelial cells [87, 102-104], which might affect HIV-1 transmission.

Therefore, we investigated whether the epithelial thickness and TJ protein expression in vaginal and cervical tissues changed at different phases of menstrual cycle. Furthermore, although in Aim 1 we have shown that HIV-1 does not cause profound effects on epithelial morphology and TJ/AJ proteins in the epithelial layer following exposure to HIV-1, we evaluated the influence of reproductive hormones on epithelial thickness and TJ protein expression between epithelial cells in HIV-1 exposed human ectocervical/vaginal tissues. For this purpose human ectocervical (n=3 to 5) and vaginal tissues (n=3 to 5) obtained at different stages of menstrual cycle were exposed to control supernatant or HIV-1 for 24 hours followed by analysis of epithelial thickness and TJ proteins as mentioned in "Materials and Methods".

6.2.2.1 Analysis of epithelial thickness

In the control group of uninfected ectocervical tissues (n=5) (Figure 22A), the average epithelial thickness at the proliferative phase, ovulatory phase and secretory phase was 130.12 μm, 129.3μm and 110.3 μm respectively. Epithelial thickness in control uninfected vaginal tissues (n=5) (Figure 22B) tissues at proliferative phase, ovulatory phase and secretory phase was 201.7 μm, 151.1μm and 149.8 μm respectively. Statistical analysis showed that there were no significant differences (p=0.3 for cervical tissues, p=0.12 for vaginal tissues) in ectocervical/vaginal epithelial layer thicknesses between the tissues obtained from the three phases of menstrual cycle.

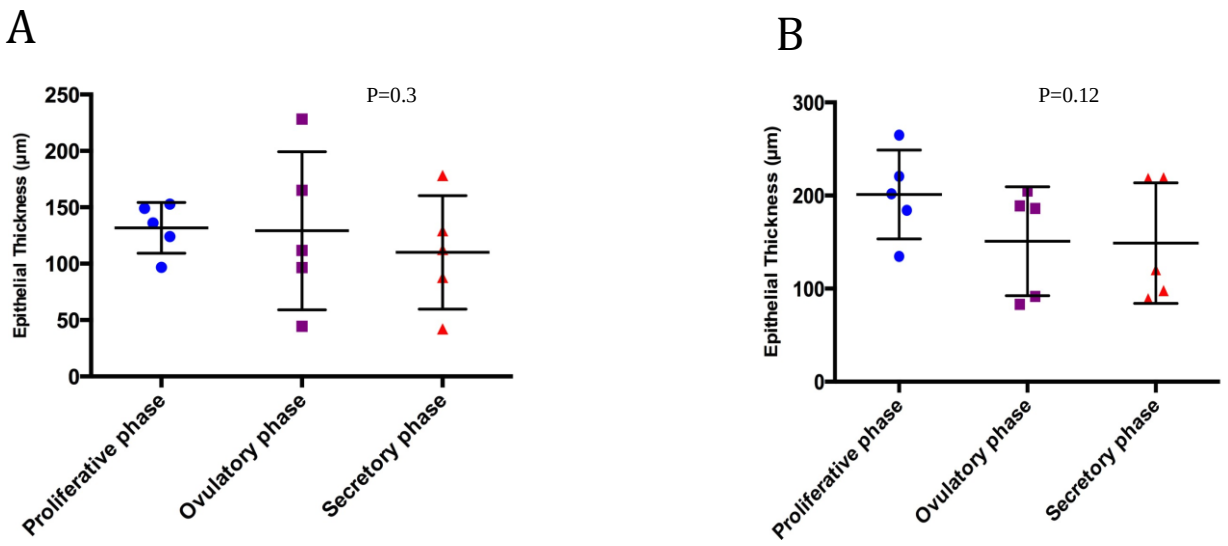


Figure 22. Epithelial thickness of ectocervical tissues and vaginal tissues obtained at different phases of menstrual cycle

Human ectocervical (A) and vaginal tissues (B) obtained from different phases of menstrual cycle were exposed to control supernatant for 24 hours. H&E staining of the tissue sections were performed and images were captured by bright field microscopy. Epithelial thickness was measured in three representative areas of mucosa from the basement membrane up to the surface and the mean of epithelial layer thickness was calculated for each biopsy. Experiments were repeated in ectocervical/vaginal tissues obtained from 5 different donors during different phase of menstrual cycle. Each circle (□) represents epithelial thickness in ectocervical or vaginal tissues from each donor obtained during proliferative phase. Similarly, each □, Δ represents epithelial thickness in ectocervical or vaginal tissues from each donor obtained during ovulatory and secretory phase respectively. Horizontal lines represent mean value of ectocervical or vaginal epithelial thickness from all five donors. Data are presented as mean ± standard deviation of epithelial thickness of all 5 different donors.

In case of HIV-1 exposed tissues, the average epithelial thickness in HIV-1 exposed ectocervical tissues (n=5) (Figure 23A), at proliferative phase, ovulatory phase and secretory phases was 145.04 µm, 83.6 µm and 81.49 µm, respectively. Whereas, in HIV-1 exposed vaginal tissues (Figure 23B), average epithelial thickness at proliferative phase, ovulatory phase and secretory phases was 208.4 µm, 143.32 µm and 147.9 µm respectively. Similar to the control

uninfected tissues, the epithelial thickness in HIV-1 exposed ectocervical/vaginal tissues was not statistically significantly different (p=0.24 for cervical tissues, p=0.32 for vaginal tissues).

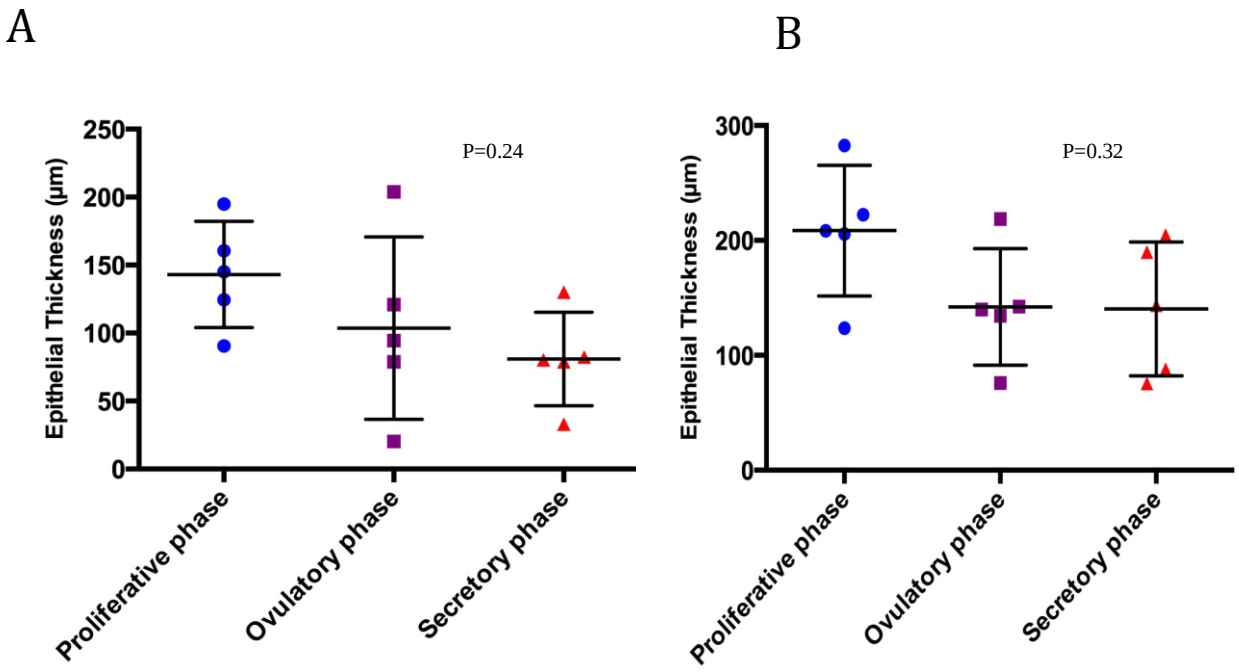


Figure 23. Epithelial thickness of HIV-1 exposed ectocervical tissues vaginal tissues obtained at different phases of menstrual cycle

Human ectocervical (A) and vaginal tissues (B) obtained from different phases of menstrual cycle were exposed to HIV-1 (10^6 infectious viral units) for 24 hours. H&E staining of the tissue sections were performed and images were captured by bright field microscopy. Epithelial thickness was measured in three representative areas of mucosa from the basement membrane up to the surface and the mean of epithelial layer thickness was calculated for each biopsy. Experiments were repeated in ectocervical/vaginal tissues obtained from 5 different donors during different phase of menstrual cycle. Each circle (□) represents epithelial thickness in ectocervical or vaginal tissues from each donor obtained during proliferative phase. Similarly, each □, Δ represents epithelial thickness in ectocervical or vaginal tissues from each donor obtained during ovulatory and secretory phase respectively. Horizontal lines represent mean value of ectocervical or vaginal epithelial thickness from all five donors. Data are presented as mean ± standard deviation of epithelial thickness of all 5 different donors.

6.2.2.2 Analysis of tight junction/adherens junction protein expression

To investigate the TJ/AJ protein expression in control/HIV-1 exposed ectocervical tissues obtained from different stages of menstrual cycle, ectocervical tissues were obtained during different phases of menstrual cycle and exposed to control supernatant or HIV-1 for 24 hours. These ectocervical tissues were stained for TJ protein (claudin-4), AJ protein (E-Cadherin) and Na/K/ATPase followed by confocal microscopy. Confocal images of mucosal epithelia were quantitated for fluorescence intensity of TJ, AJ proteins. Control uninfected and HIV-1 exposed ectocervical tissues (n=3) obtained from different stages of menstrual cycle revealed the presence of TJ and AJ molecules with well-defined, interconnected Claudin-4 (TJ), E-cadherin (AJ), Na/K/ATPase expression pattern in the perimeter of individual epithelial cells (Figure 24). Quantitative analysis showed no significant differences in the fluorescence intensity of Claudin-4 ($p=0.28$ for control, $p=0.42$ for HIV-1 exposed tissues), E-cadherin ($p=0.21$ for control, $p=0.32$ for HIV-1 exposed tissues) or Na/K/ATPase ($p=0.33$ for control, $p=0.43$ for HIV-1 exposed tissues) in the epithelial cells of control uninfected (Figure 25), or HIV-1 exposed ectocervical tissues (Figure 26) obtained during different phases of menstrual cycle. These results indicate that expression levels of epithelial TJ and AJ proteins are not significantly different at different stages of menstrual cycle regardless of whether they are HIV-1 exposed or not.

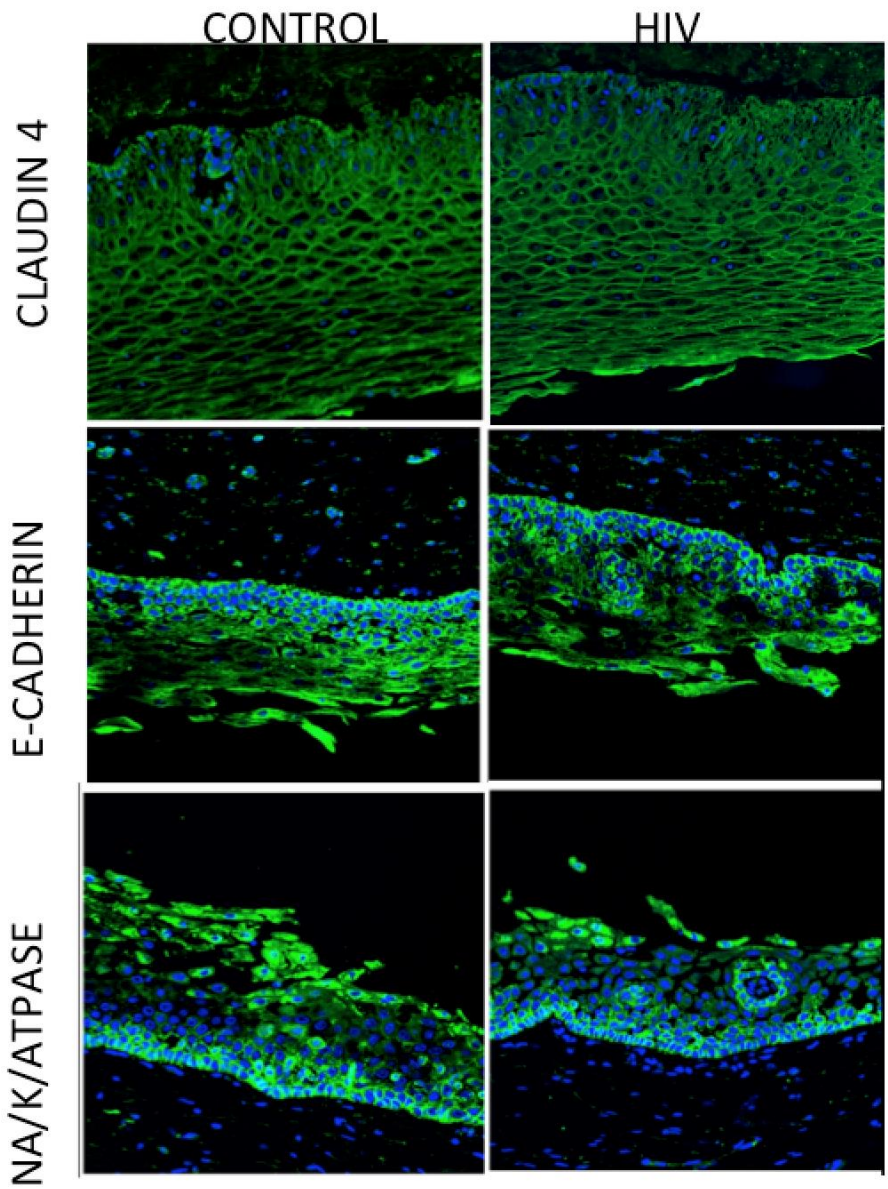


Figure 24. Expression of tight junction and adherens junction proteins in control or HIV-1 exposed ectocervical tissues obtained from proliferative phases of menstrual cycle

Human ectocervical tissues (n=3) obtained from different phases of menstrual cycle were either exposed to control supernatant or HIV-1 (10^6 infectious viral units) for 24 hours. Ectocervical tissue sections were stained using antibodies against either Claudin-4 (green), E-Cadherin (green) or Na/K/ATPase (green) proteins. Nuclei were stained with DAPI (blue). Three images were captured from each biopsy by confocal microscopy. Magnification 40X. The images shown are representative of control/HIV-1 exposed ectocervical biopsies obtained during different phases of menstrual cycle from three different donors.

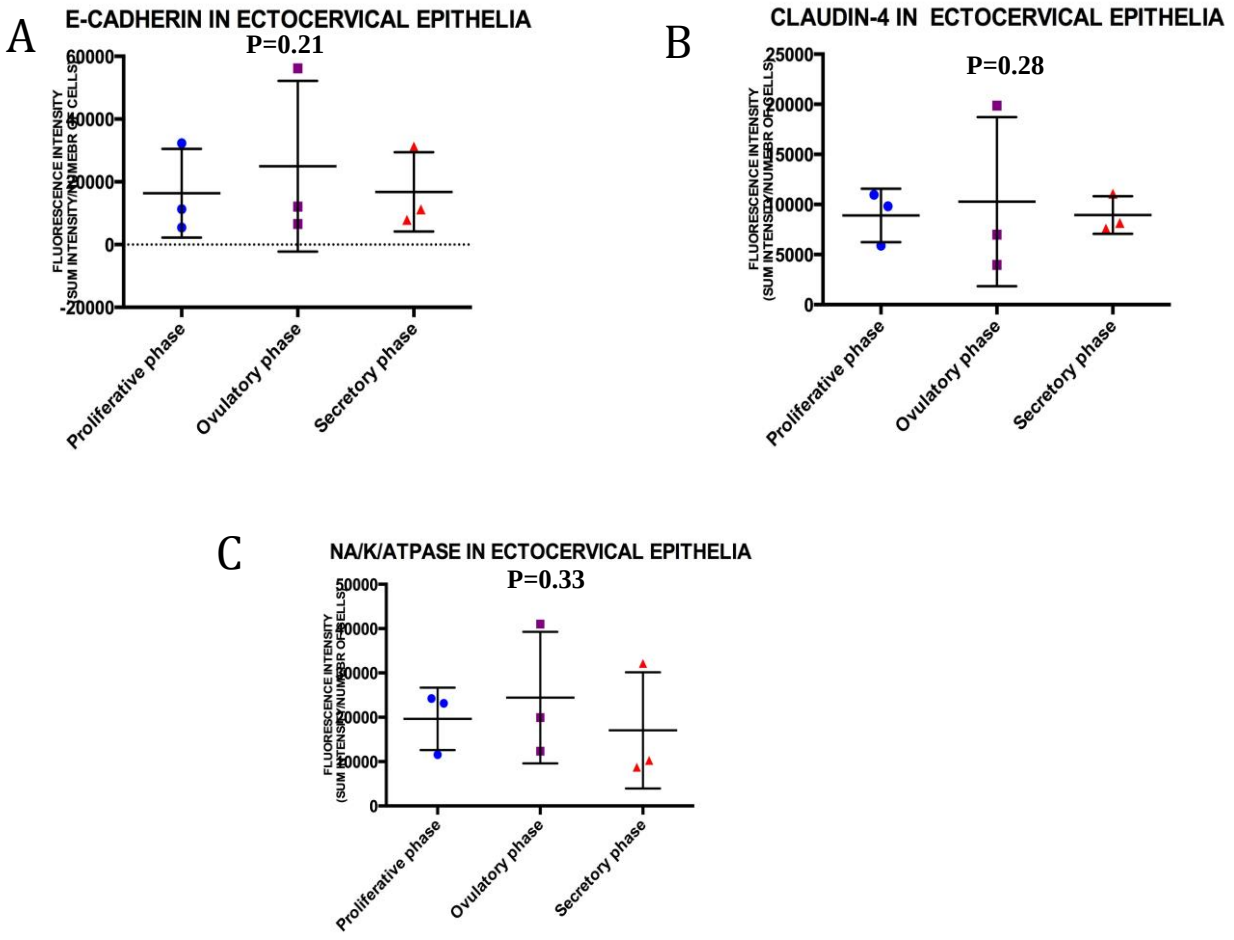


Figure 25. Quantitation of tight junction and adherens junction proteins in ectocervical tissues obtained at different phases of menstrual cycle

Ectocervical tissues were obtained from women at different phases of menstrual cycle and exposed to control supernatant for 24 hours. Fluorescence intensity of E-Cadherin (A), claudin-4 (B), Na/K/ATPase (C) proteins in ectocervical epithelia was normalized based on number of nuclei in the selected fields. Data are presented as mean ± standard deviation of 3 different donors for each phases of menstrual cycle.

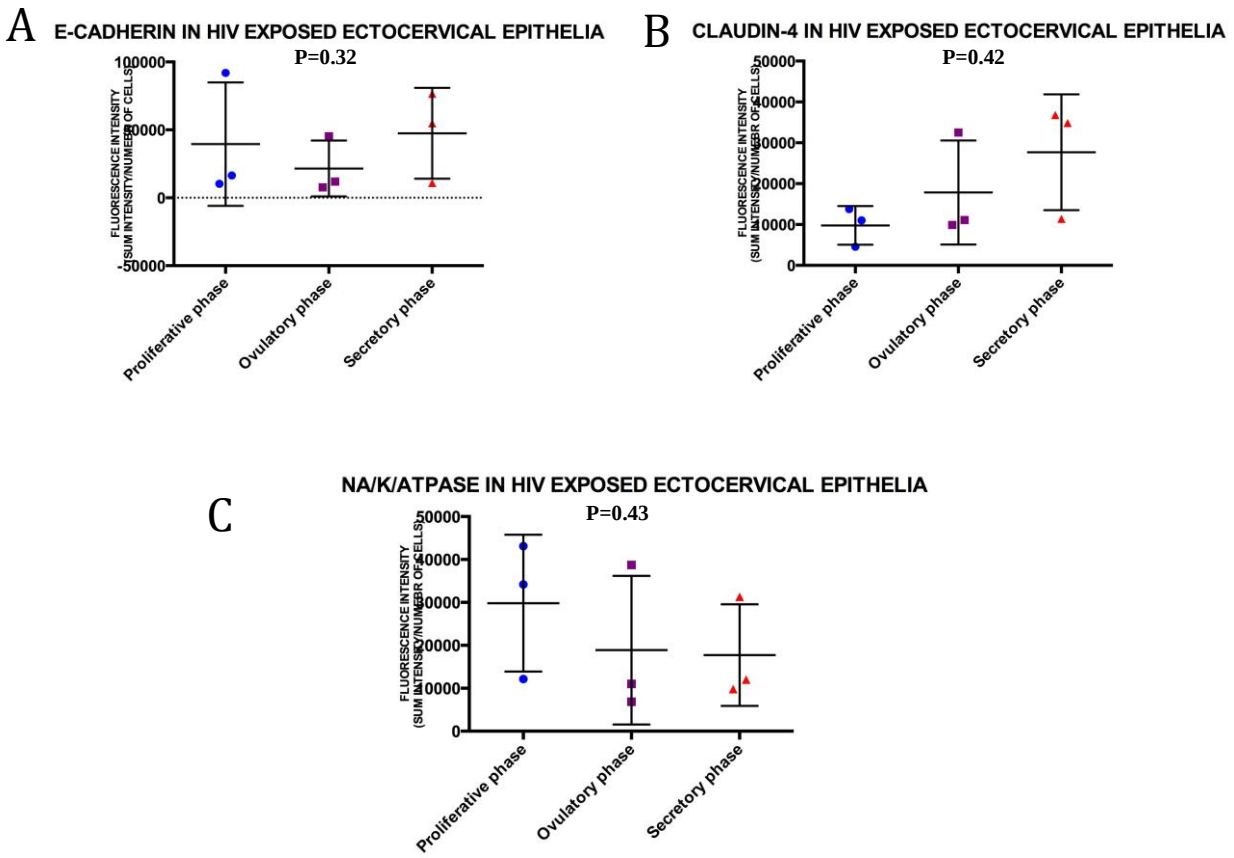


Figure 26. Quantitation of tight junction and adherens junction proteins in HIV-1 exposed ectocervical tissues obtained at different phases of menstrual cycle

Ectocervical tissues were obtained from women at different phases of menstrual cycle and exposed to HIV-1 (10^6 infectious viral units) for 24 hours. Fluorescence intensity of E-Cadherin (A), claudin-4 (B), Na/K/ATPase (C) proteins in ectocervical epithelia was normalized based on number of nuclei in the selected fields. Data are presented as mean ± standard deviation of 3 different donors for each phases of menstrual cycle.

6.3 CONCLUSION

Due to the controversies regarding the effects of hormonal contraceptive usage on susceptibility of HIV-1 infection, we investigated (a) the susceptibility of ectocervical/vaginal tissues to HIV-1 infection and (b) the effect of HIV-1 on epithelial thickness and tight junction

protein expression between epithelial cells in ectocervical/vaginal tissues obtained at different phases of menstrual cycle. Our results indicate that there were no significant differences in the susceptibility of ectocervical and vaginal tissues to HIV-1 infection among different phases of the menstrual cycle. Furthermore, epithelial layer thickness and epithelial TJ/AJ protein expression were not significantly different in the control (HIV-1 unexposed) or HIV-1 exposed ectocervical/vaginal tissues at the different stages of menstrual cycle, suggesting that epithelial barrier integrity did not change significantly during the course of the menstrual cycle regardless of whether they were subsequently HIV-1 exposed or not.

7.0 DISCUSSION

Mucosal surfaces are the primary sites for HIV-1 transmission during heterosexual or homosexual intercourse. Despite the importance of rectal and cervical transmission in the AIDS epidemic [125], current knowledge regarding the mechanism of HIV-1 entry across mucosa is limited to in vitro studies involving primary cervical cells and intestinal cell lines, which do not reproduce fully the complexity and specificity of the cervical/colon milieus. The purpose of the current study was to understand the mechanism of HIV-1 transmission across the mucosa layer in the ectocervical and colonic tissue. In this study, we utilized a previously reported ectocervical tissue-based organ culture model [126] and colon organ culture to determine the mechanism of transmission across mucosal epithelia. Unlike cell lines, the tissue in the organ culture model is comprised of the epithelial layer, subepithelial region containing immune cells including T cells, langerhans cells and macrophages, and allows for the evaluation of HIV-1 transmission across the mucosa [127]. The simple columnar epithelial layer lining the colon tissues are more fragile than the stratified squamous epithelial layer lining the ectocervical tissues, which poses a technical challenge in maintaining the epithelium layer intact for more than six hours in organ culture. In our studies, H&E staining of colon tissues at various times in culture showed that the epithelium in the colon mucosa remained largely unchanged for up to six hours. Therefore, the six hours culture duration was used in all subsequent experiments with colon tissues. The mucosal epithelium lining the ectocervical tissue consists of stratified multi-layered squamous

epithelium, but the existence of TJ in ectocervical epithelium remains controversial. There are limited reports in the literature on the characterization of epithelial intercellular junctions in human ectocervical tissue or cell lines [128]. In this study, we examined the epithelial TJ and AJ junction proteins in normal human ectocervical tissues. Our results confirm and extend the previous observation that TJ proteins ZO-1, claudin-4 and AJ protein E-cadherin are indeed present between the epithelial cells in the ectocervical [47] and colon/rectal tissues [129].

Various pathogenic organisms have been shown to traverse through the epithelial barrier at the mucosal surface by disrupting the TJ, which are characterized by defective TJ barrier and increased epithelium permeability [130, 131]. Recent studies have shown that HIV-1 disrupted the epithelial TJ in an intestinal cell line and primary endometrial epithelial cells [132, 133]. However, the direct effect of HIV-1 exposure on mucosal epithelium has not been studied in human ectocervical or colon tissues. The present study evaluated the effect of HIV-1 on TJ/AJ in cervical and rectal/colon epithelial layers in the context of mostly native tissue structure. Confocal microscopy analysis of stained TJ and AJ proteins suggest that the TJ/AJ structures in ectocervical/colon mucosal epithelia were not profoundly disrupted by exposure to HIV-1. To ascertain that HIV-1 indeed traversed through the epithelial layer without disrupting the TJ/AJ in the organ culture models, we examined HIV-1 transmission in the ectocervical and colon tissues. HIV-1 replication within the tissues was demonstrated in ectocervical and cultured colon tissues following exposure to HIV-1 for 24 hours and 6 hours respectively. These results suggest that HIV-1 transmission occurs through ectocervical tissues and colon tissues in the culture for 24 hours and 6 hours, respectively. This confirms results from a previous study showing the presence of HIV-1 RNA+ cells in the epithelial-submucosal junction of ectocervical tissue after 6 hours exposure to HIV-1 [55]. Our results also agree with previous reports showing rapid rectal

entry of SIV in a macaque model[39] and replication of HIV-1 in colon tissues after exposure to HIV-1[66]. These results indicate that HIV-1 transverses across the ectocervical/colon mucosal epithelia by other mechanisms instead of TJ/AJ disruption and TJ are not profoundly disrupted during the HIV-1 exposure period. Our result goes along with other study findings that TJ disruption occurs in colon tissues of both human [68], rhesus macaques[44] only at chronic stages of infection. Therefore HIV-1 transmission across epithelium in ectocervical/colon tissues might be mediated by other mechanisms like transcytosis, transmigration, by binding via an alternate receptor such as galactosyl ceramide, or by the aid of dendritic process from langerhan cells [53, 58]. In addition to these mechanisms, HIV-1 transmission in the colon/rectal region might also be mediated via M cells [134].

In response to HIV-1 infection, genital epithelial cell lines release TSLP hat strongly activates human myeloid DC, leading to robust induction of CD4+ T cell proliferation, promoting HIV-1 replication in these activated T cells [114]. Furthermore, studies have shown that accumulation of CD4+ T cells in human cervical tissue was detected within 6 hours post-HIV-1 infection [126, 135]. These results suggest that exposure to HIV-1 induces an innate inflammatory reaction in the mucosal area, which facilitates downstream amplification of virus in CD4+ T cells at the intraepithelial and submucosal layers. Previous studies have also reported increased production of cytokines like IL-2, IL-4, IL-5 and TNF-α in culture supernatant of HIV-1 exposed intestinal biopsies [115]. However, the cellular source of inflammatory cytokines in these mucosal regions remains unknown. To investigate the cytokine induction in mucosal epithelia post exposure to HIV-1, we analyzed the cytokine mRNA expression in microdissected mucosal epithelia of the control and HIV-1 exposed ectocervical/colon tissues, hypothesizing that epithelial cells are one of the primary sources of production of the inflammatory cytokines

after exposure to HIV-1. Our studies demonstrated that expression of the inflammatory cytokines like CXCL10 and CXCL11 is significantly higher in the ectocervical epithelia exposed to HIV-1 compared to those exposed to control supernatant. Furthermore, we observed that AT2 inactivated HIV-1 or purified HIV-1 gp120 did not upregulate cytokine expression. In the case of the colonic mucosal layer, HIV-1 induced higher expression of CXCL10 and IL6 after 6 hours exposure to HIV-1. These observations are in agreement with previous reports that intestinal cell lines and primary endocervical epithelial cells secreted TNF-α, IL-6, IL-8 and MCP-1 after exposure to HIV-1 [133]. Thus, results from our study suggest that exposure to HIV-1 induces cytokine responses in ectocervical and colon mucosa and, the epithelial cells might be one of the primary sources of inflammatory cytokines production. Furthermore, infectious HIV-1 is required to induce such cytokine response in ectocervical epithelia. The comprehensive analysis of gene expression in ectocervical epithelial after exposure to HIV-1 also demonstrated that exposure to HIV-1 upregulates expression of various cellular factors such as SAA1, IL36A, IL8, FMO2, MUC1 and CXCL10 in ectocervical epithelia. CXCL10 is an IFNs induced proteins [136, 137]. However, we did not detect increase in IFNs expression in ectocervical epithelia after exposure to HIV for 24 hours. This suggesting that dynamic changes occurs in the IFN expression pattern in these epithelial cells with rapid production of IFNs in epithelial cells following exposure to HIV-1 which induces CXCL10 expression, and the expression of IFNs is subsequently decreased. The cellular factors like CXCL10 CXCL11, SAA1, IL8 have been reported to contribute in the recruitment of T cells, macrophages and dendritic cells [138-140], where as IL36A is known to activate NFκB pathway and also play an important role in adaptive immune response [141]. Therefore, the observed HIV-1 mediated up regulation of cellular factors including proinflammatory cytokines may be responsible for increasing the target cell

availability by recruiting the immune cells towards the epithelial layer and also by activating these immune cells. This increase in target cell availability might play an important role in establishing infection in the ectocervical region during viral transmission.

Non-human primate studies suggest that exposure of endocervical epithelium to SIV results in the increased expression of cytokines like MIP-3α, MIP-1β which recruit pDCs and CD4 T cells [44, 72]. However the current knowledge on immune cell recruitment in human mucosa during HIV-1 transmission is limited. Understanding these early events of immune cell redistribution and establishment of initial infection in small founder population is critical for development of effective HIV-1 prevention approaches. Previous findings [53] and the results from the current study suggest that induction of cytokines in the mucosal epithelial cells post exposure to HIV-1 might be a strategy implemented by HIV-1 to prevent the hostile environment in the ectocervical/colonic mucosa through recruitment of HIV-1 target cells and establish initial infection. Although in our study we observed an increase in cytokine mRNA expression in the ectocervical epithelium after 24 hours exposure to HIV-1, we did not detect any change in CD3+ and CD8+ immune cell distribution in the ectocervical mucosa after the same period of HIV-1 exposure time. Our finding of no difference in immune cell distribution after exposure to HIV-1 may be due to the following technical limitations. First, the duration of HIV-1 exposure in organ culture is short. Indeed in non-human primate study, changes in immune cell distribution were observed after 4 days post exposure to SIV through rectal mucosa [65]. Therefore, it would be optimal to examine the immune cell distribution in ectocervical tissues after a longer exposure time to HIV-1. Second, the tissue biopsies are devoid of blood supply in the organ culture, which

is the major source for immune cell recruitments under *in vivo* conditions. Therefore, future experiments are needed to explore the dynamic changes of immune cell distributions in human mucosal area before and after HIV-1 exposure.

Changes in the level of reproductive hormone during the normal menstrual cycle in pre-menopausal women result in modulation of immune conditions and creates optimal condition for fertilization during the secretory phase[86]. These studies suggest that this immune condition may create a window of vulnerability during the secretory phase of the menstrual cycle and increase the likelihood of HIV-1 transmission in women [86, 87]. Monkey studies demonstrating that progesterone treatment increased the efficiency of SIV infection by intravaginal inoculation also highlight the role of reproductive hormones in sexual HIV-1 transmission [91]. This raises two important questions concerning women's health: 1) Are women more vulnerable to HIV-1 infection during the secretory phase of menstrual cycle compared to other phases of menstrual cycle. 2) Does hormonal contraceptive use increase the risk of HIV-1 acquisition?

Numerous studies have investigated the risk of HIV-1 transmission during usage of oral contraceptive DMPA [94]. However, the results from the previous studies are conflicting, with some studies reporting no significant association of hormone to HIV-1 acquisition and others showing significantly higher risk of HIV-1 acquisition [86, 94]. Most of the studies that demonstrated a higher risk of HIV-1 acquisition in DMPA users recruited high-risk women such as sex workers in their studies, which could create bias in their results [142, 143]. Moreover, studies have reported that women who use hormonal contraceptives have a tendency not to use condoms, which again represents another important bias [94, 144]. Therefore, further studies are required to determine whether women are more susceptible to HIV-1 infection during the secretory phase compared to the other two phases of the menstrual cycle.

In the present study we investigated the effect of reproductive hormones on HIV-1 transmission through ectocervical and vaginal mucosa at different stages of the menstrual cycle. However, we did not detect significant difference in the tissue susceptibility to HIV-1 infection between the ectocervical/vaginal tissues obtained from the proliferative phase, ovulatory phase or secretory phase of the menstrual cycle. These results suggest that the level of reproductive hormone at different phases of menstrual cycle might not alter the susceptibility of ectocervical/vaginal tissues to HIV-1 infection under the assay conditions used. In 2012, the World Health Organization reviewed epidemiological and biological data, and recommend no restriction on use of any method of hormonal contraceptives for women at high risk of HIV-1 [94]. In this regard, our results using *ex vivo* ectocervical/vaginal culture model are in agreement with both epidemiological and the biological evidence that reproductive hormones might not influence HIV-1 transmission.

There are a number of possible reasons for lack of correlation between HIV-1 susceptibility and hormonal levels during menstrual cycle. First, studies in non-human primates and women have shown that immune cell population including HIV-1 target cells like CD4+ DCs (dendritic cells) and T cells in vaginal tissues appeared to be stable throughout the menstrual cycle [97, 124]. Therefore, stable target cell population in cervical/vaginal tissues during all the phases of menstrual cycle might be one of the reasons for similar level of HIV-1 susceptibility of ectocervical/vaginal tissues at different phases of menstrual cycle. Second, in our organ culture the endogenous hormonal concentration in the tissues might be reduced or lost during the culture period, resulting in no difference in HIV-1 susceptibility of ectocervical/vaginal tissues at different phases of menstrual cycle. Therefore, *in vitro* organ culture might not be optimal for this type of study. Third, we might not be able to detect the

difference in HIV-1 susceptibility between the different phases of menstrual cycle due to the inter-individual variation in the present cross-sectional study. Further investigation using longitudinal study design might be necessary to observe the definite correlation between HIV-1 susceptibility and hormonal level during menstrual cycle.

Administration of progesterone to monkeys increases the risk of SIV transmission and this effect was thought to be associated to thinning of the vaginal mucosa [91]. Epithelial thickness of ectocervical/vaginal tissues during menstrual cycle has been investigated by a number of investigators, and different results reported by these investigators might be due to the differences in the observer bias, timing of tissue sampling, method of measurement, inter-individual variability and statistical analyses [86, 87, 94]. For example, Burgos *et al.* reported that the epithelial thickness of vaginal mucosa was 22 layers on day 10, 33 layers on day 19, and 23 layers on day 24 of the menstrual cycle [145]. However, the criteria for including the subjects in the study and the methods used for measurement were not stated. Findings from previous studies on the effect of DMPA on the epithelial thickness were also inconsistent, with two studies showing effect of DMPA on the vaginal epithelial thickness, while two other studies reported no change in the thickness of the vaginal tissue in women who use DMPA [94]. A study performed by Zondek *et al.* showed that the vaginal epithelial layer thickness was not significantly different in regularly menstruating women and women with primary/secondary amenorrhea (absence of a menstrual period) suggesting that thickness of the epithelial layer was not modified by changes in hormonal level during menstrual cycle [146]. We therefore investigated the epithelial thickness in human ectocervical and vaginal epithelium at different stages of menstrual cycle. We observed that the cervical/vaginal epithelial thickness was not significantly different during the secretory phase compared to the proliferative and ovulatory

phase of menstrual cycle. To further evaluate the effect of changes in hormonal levels during the menstrual cycle on epithelial integrity, we measured the density of intercellular epithelial tight junction protein Claudin 4, adherence junction protein E-Cadherin and Na/K/ATPase. It has been previously shown that estrogen reduced the tight junctional resistance and altered the tight junction protein concentration in vaginal and cervical epithelial cell lines [86, 87, 102-104]. In contrast to estrogen, progesterone had no effect on trans-epithelial resistance in endometrial cells [87, 102, 104]. However, limited data exist on the effect of reproductive hormone on epithelial tight junction in human ectocervical tissues at different phases of menstrual cycle. We demonstrated that there was no statistical difference on the expression of TJ/AJ proteins in ectocervical tissues at different phases of menstrual cycle. These results suggest that the epithelial thickness and expression of epithelial TJ/AJ proteins in ectocervical/vaginal tissues might not be highly modulated by the hormonal changes during a menstrual cycle. These results confirm previous report by Chandra *et al.* that the vaginal epithelial thickness and the density of tight junction proteins do not change over the course of the menstrual cycle [124]. However, it is unknown if a small decrease in epithelial thickness and TJ/AJ protein expression in ectocervical epithelia may facilitate access of virions to mucosal HIV-1 target cells.

Studies described in Aim 1 indicate that the exposure to HIV-1 did not exert profound effects on the morphology of epithelial layer and TJ/AJ protein profiles in the ectocervical epithelia. However, based on a previous study which suggests a window of vulnerability to infection at a certain phase of menstrual cycle [86], we hypothesized that exposure to HIV-1 may cause change in epithelial thickness and TJ/AJ under the influence of hormones. We therefore evaluated the effect of HIV-1 exposure on epithelial layer thickness and epithelial TJ/AJ protein in ectocervical/vaginal tissues at different stages of menstrual cycle. To the best of our

knowledge, this is the first study that evaluated the effect of HIV-1 on epithelial thickness and TJ/AJ proteins of vaginal/cervical tissues obtained from women at different stages of menstrual cycle. Furthermore, our data demonstrate that HIV-1 does not alter the thickness and TJ/AJ protein profiles in ectocervical tissues at all the three phases of menstrual cycle. These data suggest that the phase of menstrual cycle does not alter the effect of HIV-1 on the epithelial barrier integrity in ectocervical/vaginal tissues. These results confirm previous reports by Bahamondes *et al.* that uninterrupted usage of DMPA in women for 18 years did not cause thinning of vaginal epithelium [97]. We therefore believe that if reproductive hormones like progesterone can increase the risk of HIV-1 transmission, the mechanism is possibly not related to modification in ectocervical/vaginal epithelial thickness or ectocervical TJ/AJ expression.

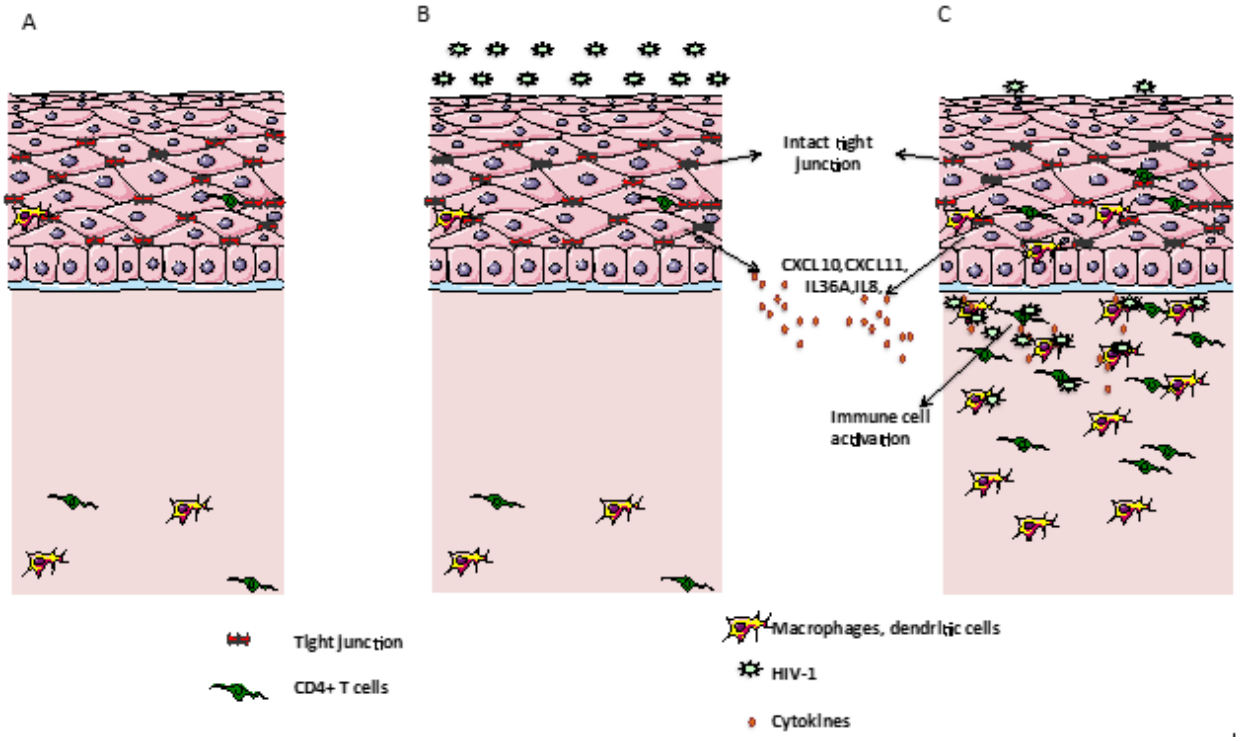


Figure 27. Schematic representation of HIV-1 transmission in ectocervical tissues

The ectocervical epithelium is made of multilayered squamous epithelial cells with tight junctions between the epithelial cells. Tight junctions play an important role in maintaining the barrier function of the epithelium. Transmission of cell-free HIV-1 across the intact

ectocervical epithelium occurs through unknown mechanism(s) without disrupting the tight junction. Exposure of ectocervical epithelium to HIV-1 up regulates expression of cellular factors such as CXCL10, CXCL11, IL8, IL36A and SAA1 in the epithelium. Cytokines like CXCL10, CXCL11, SAA1 and IL8 recruits immune cells towards the epithelial layer thus increasing the target cells availability for HIV-1 infection. Cellular factors such as IL36A activate the immune cells increasing the pool of target cells and fuel the local expansion that is necessary for establishment of infection in the ectocervical tissues once they cross the epithelial barrier.

In summary, the current study provides evidence that although the squamous ectocervical epithelium (Figure 27A) and columnar colon epithelium act as a physical barrier against pathogen entry, it is not impenetrable to HIV-1. HIV-1 traverses through the ectocervical/colon epithelia most probably by mechanisms other than TJ/AJ disruption. However, HIV-1 exposure at the ectocervical epithelium results in upregulation of cellular factors including inflammatory cytokine expression in the epithelial cells, which is a rapid response and dependent on HIV-1 infectivity (Figure 27B). These cellular factors recruit immune cells towards the epithelial layer and also activate these immune cells thus increasing the target cell availability for HIV-1 infection (Figure 27C). Thus, once the virus crosses the mucosal epithelial barrier they infect the recruited target cells and establish infection in the ectocervical tissues. Our current study on reproductive hormones and HIV-1 acquisition also demonstrate that during the course of the menstrual cycle, the epithelial layer thickness and epithelial TJ/AJ protein profile in the ectocervical/vaginal tissues do not alter significantly. Additionally, there does not appear to be an association between menstrual cycle and the effect of HIV-1 on the epithelial barrier integrity in the ectocervical/vaginal tissues. Finally, different phases of menstrual cycle do not influence the susceptibility of ectocervical/vaginal tissues to HIV-1 replication. Taken together, current evidence thus suggests that HIV-1 acquisition risk through the ectocervical/vaginal region is not altered by the presence of reproductive hormone during the course of menstrual cycle. However,

the debate on reproductive hormones and the risk of HIV-1 acquisition remains open and may be resolved with a longitudinal study with larger number of subjects.

8.0 PUBLIC HEALTH SIGNIFICANCE

HIV-1 infection is a global health concern. To develop a highly effective method to prevent HIV-1 transmission, it is crucial to gain a clear understanding of the mechanism of HIV-1 transmission across ectocervical and colonic mucosa. In the current study, we evaluated the possibility that HIV-1 crosses cervical and colonic mucosae by disrupting tight junction proteins in epithelia by measuring the tight junction/adherens junction proteins following HIV-1 exposure in the epithelia of ectocervical and colon tissues. An interesting finding of our study is that HIV-1 exposure did not profoundly disrupt epithelial junction/adherens junction in ectocervical and colon tissues indicating the HIV-1 transmission in mucosal region may not be mediated by epithelial tight junction damage, indicating involvement of other mechanisms of HIV-1 transmission. This work has public health significance because these findings will further enhance our understanding on the mechanism of HIV-1 transmission and also provide the basis for further inquiry on alternate routes for HIV-1 transmission.

By investigating cellular factors that are involved in HIV-1 transmission across ectocervical mucosa, we found that expression levels of certain cytokines including CXCL10, CXCL11 and other cellular factors including IL36A, FMO2, CXCL10, MUC1, SAA1 and IL8, were upregulated in ectocervical epithelia after exposure to HIV-1. These findings make important contribution to public health in a number of ways. First, these differently expressed cytokines and cellular factors in the epithelial cells could be developed as biomarkers to identify

HIV-1 exposed individuals. Second, after confirming the relevance of these genes in HIV-1 transmission, drugs that block the pathways involving these genes can be used to prevent HIV-1 transmission.

We also investigated the impact of reproductive hormones on HIV-1 transmission by measuring the susceptibility of ectocervical and vaginal tissues collected at different stages of the menstrual cycle to HIV-1 in an organ culture model. Our results indicate that there is no association between susceptibility to HIV-1 infection and the levels of reproductive hormone at different stages of menstrual cycle. These results indicate that there may not be a window of vulnerability during menstrual cycle where women could be more susceptible to HIV-1 infection. Furthermore, women who are using contraceptives may not be at any further risk of HIV-1 acquisition than women who do not use hormonal contraceptives. These findings have public health relevance in the context of contraceptive usage in women and risk of HIV-1 transmission.

APPENDIX: ABBREVIATIONS USED

AIDS: acquired immune deficiency syndrome

AJ: Adherens junction

DCs: Dendritic cells

DMPA: Depot medroxy progesterone acetate/ Depo-Provera

HIV: Human immunodeficiency viruses

IL: Interleukin

KRT 13: Cytokeratin 13

LC: Langerhans cells

mRNA: Messenger ribonucleic acid

pDCs: Plasmacytoid dendritic cells

RNA: Ribonucleic acid

RNA: Ribonucleic acid

SAA1: Serum amyloid A

SD: Standard Deviation

SEM: Standard error of the mean

SIV: Simian immunodeficiency virus

TEM: Transmission electron microscopy

TJ: Tight junctions

TLR: Toll like receptor

ZO-1: Zona occludens

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