STUDY OF HIV-1 TRANSMISSION ACROSS HUMAN CERVICAL TISSUE TO THE LYMPHOID TISSUE USING AN ORGAN CULTURE MODEL

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Submitted to the Graduate Faculty of

Graduate School of Public Health in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2008

UNIVERSITY OF PITTSBURGH

GRADUATE SCHOOL OF PUBLIC HEALTH

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The female genital tract is the main route for heterosexual transmission of HIV-1. Studies with SIV in macaque model indicate that after crossing the mucosa of cervix and vagina, SIV infected cells migrate to draining lymph nodes where the virus expands before it gets disseminated into the gut and other organs of the body. We have developed a combined organ culture system composed of cervical tissue together with tonsil tissue (surrogate lymphoid tissue for lymph node) to study HIV-1 transmission across the mucosal barrier and to evaluate the fate of HIV-1 and HIV-1 infected cells after crossing cervical mucosa and migrating to regional lymph nodes. To study the transmission of virus, a defined amount of pretitered cell-free or cell-associated HIV-1$_{BAL}$ or HIV-1$_{IIIB}$ was added to the top of the cervix tissue and incubated at 37°C in a CO_2 incubator. The top well was removed after 3-4 days and tonsil tissue or tonsil mononuclear cells in the bottom well were cultured for an additional 12 days. Replication of cell-free and cell-associated HIV-1$_{BAL}$ and HIV-1$_{IIIB}$ in tonsillar cells after crossing the cervix mucosa was confirmed by the presence of HIV-1 p24 in culture supernatant, the detection of HIV-1 DNA by real-time PCR, presence of p24 antigen by immunofluorescence assay and presence of HIV-1 RNA by simultaneous immunophenotyping and ultrasensitive fluorescence in situ hybridization. We have also characterized by immunofluorescence assay HIV-1 infected migratory cells exiting from the cervix tissue. CD4+ (T cells), CD11c+ (dendritic cells) and CD68+ (macrophages) cells

were found to migrate from cervix tissue and were positive for HIV-1. The public health relevance of this model is that we may use the combined cervix and tonsil tissue/cell model to determine the mechanism of sexual transmission of HIV-1 in women at the cellular and molecular level and to evaluate anti-HIV microbicides.

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1.0   INTRODUCTION

1.1   ORIGINS OF HIV-1

Viral transmission is governed by two universal models: the maintenance of infection in one species and the repeated infection by insects into vertebrate hosts (Flint, 2004, p. 523). However, viruses occasionally transfer from one species to another, including from animals to humans. Sometimes humans become a dead-end host following animal-to-human transmission while at other times the virus can further adapt to propagation in humans facilitating human-to-human transmission (Webby, 2004). RNA viruses are more frequently able to cross species. RNA-dependent RNA polymerases have no proofreading activity during replication and thus viral replication introduces mutations that create viruses that may become successfully transferred to susceptible hosts. Influenza (Kawaoka, 1989) and human immunodeficiency virus (HIV) are examples of this phenomenon in which an RNA virus typically found in animals has become a prevalent viral infection in humans.

Through genetic analysis, researchers have demonstrated that HIV-1 emerged from SIVcpz of chimpanzees whereas HIV-2 arose from SIVsm of sooty mangabeys (Lemey, 2003; Chen, 1995). Scientists believe that HIV-1, the major causative agent of acquired immune deficiency syndrome (AIDS), crossed to the human population when humans hunted the chimpanzees and were exposed to their infected blood (Hahn, 2000). It is estimated that HIV has been present in human populations since the 1930s (Hillis, 2000). Early evidence of HIV-1

infection has been found in a serum sample obtained in 1959 from a Bantu male in Kinshasa in the Democratic Republic of Congo (Sharp, 2002). However, HIV only appeared prevalent during the 1980s in the United States and Europe.

1.2   HIV EPIDEMICS

HIV-1 has spread to more than 150 countries on six continents (McCutchan, 2006). Approximately, 33 million people worldwide are currently infected with HIV, with 2.7 million new infections of HIV and 2 million HIV-related deaths estimated to have occurred in 2007 (UNAIDS/WHO (2008) Global Summary of AIDS Epidemic 2008; December 2007. www.unaids.gov).

The majority of HIV-1 new infections occur through heterosexual contact, even though injection drug use, sex between men and mother-to-child transmission make up a significant part of transmission groups in some countries (UNAIDS, 2008). The most affected group around the world is young women between ages 15-24. Women, among all people living with HIV worldwide, have remained at 50% for several years and almost 60% of HIV infection in sub-Saharan Africa (figure 1) (UNAIDS/WHO (2008), Report on the global AIDS epidemics: Status of the Global HIV Epidemics; December 2007 < www.unaids.gov>).
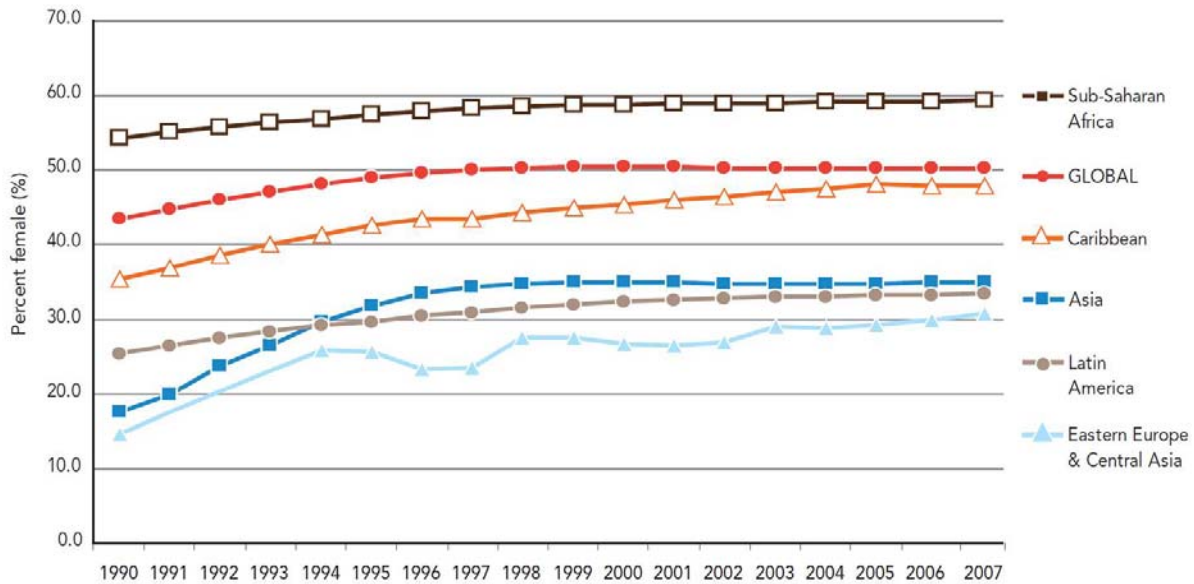
Figure 1: Percent of adults (15+) living with HIV who are female, 1990–2007.
Adapted from publicly available data from UNAIDS and WHO. <www.unaids.org>. Reproduced by kind permission of UNAIDS. © 2008 Report on the global AIDS epidemic.

1.3   HIV LIFECYCLE

HIV infection begins when the surface protein gp120 of virus particle binds to cellular glycoprotein CD4 (Moore, 1993), mainly expressed in CD4+ T cells, monocytes and macrophages. This initial binding facilitates further binding of gp120 to co-receptor molecules, the main two being chemokine receptors CCR5 (R5 tropic virus) and CXCR4 (X4 tropic virus) (Clapham, 2002). Binding of the co-receptors induces fusion of viral and cellular membranes and then the release of the viral core into the cell cytoplasm where the viral RNA genome is reverse transcribed into DNA by the viral reverse transcriptase (Hirsch, 1990, 4th ed.; Harrich, 2002.). The viral DNA, or pre-integration complex (PIC), is carried to the nucleus by HIV Vpr and other viral proteins and integrated into the host cell chromosome by the viral integrase (Stevenson,

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1990). Proviral transcription takes place when cellular RNA polymerase and various cellular factors bind to the viral long terminal repeat promoter which synthesizes genomic full-length copies of viral RNA and mRNA encoding viral proteins (El Kharroubi, 1998). During this time, Gag, Gag-Pol precursors and Env polyproteins move to the host cellular plasma membrane where they meet the viral RNA and start to assemble the virion components (Adamson, 2007; Orenstein, 1988). The new virus then buds from the plasma membrane as an immature virion (Ono, 2001; Nguyen, 2000). The virion matures and becomes infectious when, shortly after budding, the viral protease cleaves the Gag and Gag-Pol polyprotein (Lanman, 2002; Briggs, 2003).

1.4   NATURAL HISTORY OF HIV-1 INFECTION

Most people infected with HIV do not rapidly progress to AIDS. HIV is a lentivirus that belongs to the *Lentivirus* genus from the *Retroviridae* family. Lenti- means slow and for this reason these viruses have long incubation periods, therefore, HIV can take years to cause AIDS. It has been estimated that the mean incubation of disease development is 10 years (Muñoz, 1995; Hendriks, 1993). There are three distinct phases of natural history of HIV infection. First, acute infection, occurring between 2 to 6 weeks, described as influenza-like symptoms in 60-80% of cases. Symptoms typically consist of fever, sore throat, muscle ache and swollen lymph nodes (Levy, 2007, 3rd ed.). Second, asymptomatic/chronic infection, which can last for approximately 10 years, is characterized by nearly undetectable viral replication in the peripheral blood (Pantaleo, 1993). Third, the symptomatic period, classified as AIDS and defined with an increase in viral load, a fast decline in CD4+ T cell numbers (Pantaleo, 1993), and an onset of opportunistic infections, for example, oral candidiasis by *Candida* microbes and pneumonia by fungus *Pneumocystis carinni* (Kasper, 1998)*.*

The function of the lymph nodes is in setting an immune response to invading pathogens ensuing the formation of an adaptive immune response. During the acute phase of infection, upon HIV transmission, the virus is disseminated, primarily to the lymphoid tissues (Pantaleo 1993, Fox, 1991), which are replication sites for HIV-1. The immune system is activated after HIV has spread to the lymphatic tissues. Then, there is the stage of chronic activation in which viral load is maintained at low levels throughout the chronic phase of infection. A strong

adaptive immune response to HIV controls viremia by the cytolytic activity of virus-specific CD8+ T cells during the asymptomatic phase of infection (Betts, 2006; Kaul, 2000). The continuous action of CD8+ T cells and HIV specific neutralizing antibodies (Pantaleo, 1997) appears to be responsible of controlling virus levels in peripheral blood where viral loads can reach between 10^6 to 10^7 copies/ml (Daar, 1991). Therefore, during this period the virus levels decline while CD4 T cell counts recover, yet never reach to pre-infection levels. It is also during this time that HIV target cells, CCR5+CD4+ effector memory T cells mainly present at mucosal sites, i.e. gut and cervicovaginal tissue, are depleted from mucosal sites (Grossman, 2006). The numbers of infected CD4+ T cells that die per day are more than 10-100 million (Cavert, 1997), thus, CD4+ T cell level in the blood gradually declines. It is when the level of CD4+ T cells drops to approximately 200 cells/ml in blood that the host no longer is capable of controlling HIV replication. This happens because CD4+ T cells are essential in the development and maintenance of CD8+ T cell and antibody immune responses (Heeney, 2002). As CD4+ T cell levels decline, the host is not able to fight infection, the immune system is weakened and develop AIDS. Therefore, individuals with AIDS are vulnerable to opportunistic infections.

1.4.1   Role of the lymph node in HIV pathogenesis

Lymph nodes are the reservoirs and replication sites for HIV. This has been shown in asymptomatic individuals that demonstrated to have high viral load in lymphatic tissues with roughly no detectable viral replication in their peripheral blood (Pantaleo, 1993).

Lymph nodes are organized in a highly specialized and compartmentalized structure that comprise of follicular regions containing B cells and interfollicular regions that contain naïve and central memory T cells and antigen presenting cells, particularly dendritic cells. These antigen presenting cells carry antigens through the afferent lymphatics and into the T cell regions where presentation occurs for initiation of an immune response to the presented antigen (Veazey, 2003). Similarly, intra-epithelial dendritic cells and Langerhans cells in the vaginal mucosa are considered targets for HIV transmission through intact mucosal tissue. Langerhans cells express CD4, CXCR4 and CCR5 (Weissman, 1997; Zaitseva, 1997), can be infected by HIV (Kawamura, 2000) and presumably migrate from the epithelium to the afferent lymphatic vessels leading to the genital lymph nodes. The virus is amplified in these lymph nodes, spreading among T cells and antigen presenting cells. Lymph nodes in acute HIV infected individual are enlarged at this stage of infection (Schacker, 1996; Tindall, 1998). As the disease progresses, the tissue architecture breaks down and follicular dendritic cells are destroyed. The function of the lymph node is interrupted as it can no longer support strong T cell and B cell responses (Collier, 1988; Kroon, 1994), possibly by the infected host's inability to expand naïve and memory T cells during chronic HIV-1 infection (Sieg, 2001). Cell-free and cell-associated HIV exits the lymph nodes through the efferent lymphatics to the systemic circulation raising the viral load in the blood (Miller, 1992). In general, individuals with advanced HIV infection and AIDS have lymph nodes that are small, fibrotic, and profoundly depleted of lymphocyte populations (Biberfeld, 1987). At this stage, AIDS is imminent.

1.5   HIV TRANSMISSION

Newly acquired HIV-1 infections occur by virus transmission across mucosal surfaces (Hu, 2004; Belyakov, 2004; Lehner, 2003) with unprotected heterosexual intercourse being the major route of transmission.

1.5.1   Female genital tract cellular structure

Heterosexual sex accounts for 80% of new infections (Poonia B, 2006). During unprotected heterosexual intercourse, the cervix and vagina are the main sites of HIV acquisition (Miller, 1998, Gupta, 1997). The lower female genital tract consists of the vaginal mucosa, the ectocervix and the endocervix (van Herrewege, 2007). The vaginal mucosa and the ectocervix comprise of several layers of lined nonkeratinizing stratified squamous epithelium that form a natural barrier to block HIV infection. The transformation zone, a multilayered squamocolumnar junction, is where the ectocervix transitions to a single layer of mucin-secreting columnar epithelium called the endocervix (Shattock , 2003).

1.5.2   Mechanisms of sexual transmission

Unbreached vaginal and cervical epithelium provides a considerable barrier to HIV-1 infection (Greenhead, 2000), so it is unclear how cell-free or cell-associated HIV-1 enters the intact mucosa to start an infection through the sexual route (Miller, 1992). In general, establishing an infection with HIV-1 through the sexual route is not efficient. The rate of HIV-1 transmission in

a single exposure from an infected male to an uninfected female is 0.3% (Miller, 1994; Lehner, 2003; Belyakov, 2004). Some factors affecting low transmissibility of HIV-1 in women include the menstrual cycle stage, vaginal pH, endocrine hormone levels and contraceptive use being menstruation and vaginal pH to mainly affect the vaginal viral load (Benki, 2004; Schwebke, 2005; Quinn, 2005). Furthermore, HIV-1 virions and infected lymphocytes are inactivated by the low pH present in vaginal secretions. Vaginal pH can be raised to 7.0 by presence of semen and blood in the vagina either during the menstrual cycle or intercourse. Conversely, inflammation and trauma to the vaginal epithelia, including that caused by other sexually transmitted diseases, and hormonal contraceptives can increase the risk of HIV transmission and allow the immigration of the virus to susceptible cells in the submucosa (Lavreys, 2004; Sagar, 2004).

Certain cell surface receptors can influence HIV-1 binding and entry into the mucosal epithelium. Association of HIV-1 with dendritic cells through the binding of C-type lectins such as dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) can either confine the virus in the lumen (Pope, 2003), or become infected and transfer the virus to susceptible cells. A recent study demonstrated that gp340, expressed on the cervicovaginal epithelium, specifically bind to HIV envelope and can enhance the entry of HIV through the epithelium (Stoddard, 2007). Figure 2a illustrates the cell receptors that have been implicated for HIV-1 binding and infection. Moreover, several HIV-1 entry mechanisms have been described (figure 2b): a) entry of cell-free virus into epithelial cell gaps that is then captured and internalized by intraepithelial Langerhans cells; b) fusion of virus into intraepithelial CD4+ T cells productively infecting these cells; c) migration of cell-free or cell-associated virus through unbreached vaginal epithelium into the mucosal stroma where it can be taken up by lymphatic

or venous vessels and carried to local lymph nodes or the blood circulation, respectively; d) transcytosis of cell-free virus through epithelial cells (Hladik, 2008). Regardless of the mechanism of HIV transmission, virus successfully crosses the cervicovaginal mucosa and has access to underlying susceptible cells.
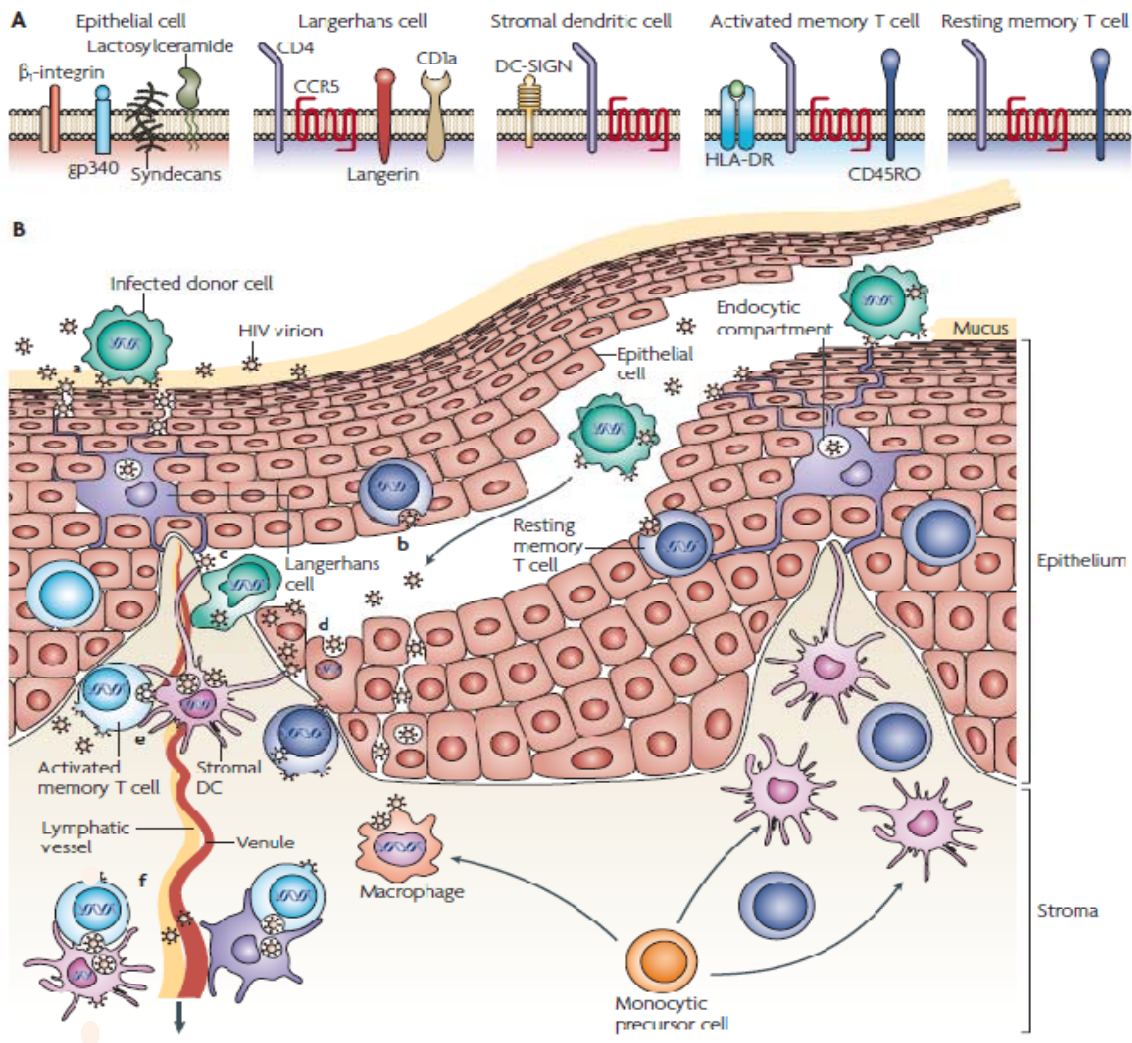
Figure 2: Pathways of HIV invasion in the mucosa of the vagina and uterine ectocervix.
A) Different cell receptors relevant for HIV binding and infection. B) Possible mechanisms for HIV entry: a) entry of cell-free virus into epithelial cell gaps; b) fusion of virus into intraepithelial CD4+ T cells; c) migration of cell-free or cell-associated virus through unbreached vaginal epithelium into the mucosal stroma; d) transcytosis of cell-free virus through epithelial cells. Reprinted by permission Macmillan Publishers Ltd. from Hladik and M. Juliana McElrath, 2008. Setting the stage: host invasion by HIV. Nat Rev Immunol 8(6): 447-457. Copyright © 2008 Nature Publishing Group. <www.nature.com>.

1.5.3   Cellular targets of HIV infection

To determine the cellular targets that first become infected in the cervicovaginal mucosa, a SIV/rhesus macaque model and in situ PCR amplification of HIV-1 DNA was used. In this model, SIV-infected cells were detected first in the lamina propria immediately adjacent to the vaginal mucosa but not in the epithelium itself. According to morphology and immunostaining techniques, the initial SIV-infected cells appeared to be Langerhans cells present in the lamina propria although infected Langerhans cells was not confirmed (Spira, 1996). In comparison, Zhang et al. (1999) found by in situ hybridization and immunohistochemical staining of macrophages, dendritic cells and T cells, that the largest group of cells to first become productively infected following 3 days of SIV intravaginal inoculation on monkeys was CD4+ T cells. More importantly, these CD4+ T cells comprised of both activated and resting T cells. Hu et al (Hu, 2000) showed that most of the cells that were infected within 60 minutes of intravaginal exposure to SIV were intraepithelial dendritic cells that subsequently migrated to the draining lymph nodes within 18 hours of vaginal exposure.

In cervical tissue from chronically HIV-1-infected women, T cells, macrophages and Langerhans cells were found to be the cell types infected with HIV-1 (Pomerantz, 1988). There are no data to designate the cell types initially infected in the reproductive tract of women. Our lab have developed a human cervical tissue-based organ culture in which we have shown that the first cells to become infected with HIV-1 within 6-24 hr of viral exposure are activated T cells located immediately below the epithelial cells. HIV-1-infected dendritic cells and macrophages were detected after 3 days of infection (Greenhead, 2000). Interestingly, a most recent study by Hladik et al (Hladik, 2007) showed, by using an organ culture of separated

vaginal epithelium, that HIV-1 enters intra-epithelial CD4+ T cells almost exclusively without requiring passage from Langerhans cells. However, it is not clear how the infection is transmitted after crossing cervical mucosa to regional lymph nodes.

1.5.4   Role of viral phenotypes in HIV-1 infection

The mucosal columnar epithelium of the endocervix contain a vast number of activated CD4+ T cells that express high levels of CCR5 (Stahl-Hennig, 1999; Veazey, 2003). Even as three HIV-1 phenotypes, R5, X4 and dual-tropic (R5X4), have been described for many HIV-1 subtypes (Delwart, 1998; Pillai, 2005; Scarlatti, 1993), most of the virus associated with acute infection are R5 tropic, despite the route of transmission (Zhu, 1993; van't Wout, 1994). This is seen in individuals with a 32 base pair deletion of the CCR5 gene which results in nearly full resistance to HIV infection and slow progression to AIDS when compared to individuals with wild type CCR5 (Huang, 1996; Liu, 1996). A selective transmission of homogeneous R5 tropic virus is seen in acutely infected men during homosexual transmission (Zhu T. N.-J., 1996). However, data from Long et al (Long, 2000) indicate that selective transmission of HIV-1 may not occur in women. For this reason, the role of viral phenotype in sexual transmission in women needs to be elucidated.

The mechanism of HIV-1 transmission may well depend if the transmitting virus is cell-free or cell associated as semen contains both cell-free and cell-associated HIV-1 (Vernazza, 1996; Gupta, 1997). It is suggested that HIV-1 transmission mostly occurs via cell-free virus since low levels of HIV-1- infected cells and high levels of cell-free HIV-1 have been detected in semen during all stages of disease (Gray, 2001). Data from the SIV/rhesus macaque model

indicates that cell-free SIV is more efficient than cell-associated SIV in vaginal transmission (Miller, 1992, 1994). However, it is still unclear which HIV-1 phenotype, if cell-free or cell-associated, is the most significant in male to female transmission.

1.6   ORGAN CULTURE TO STUDY TRANSMISSION

Studies with vaginal epithelial sheets have shown that HIV is taken into the cell's cytosol and endocytic compartments (Hladik, 2007) and that new viruses produced from epithelial sheets are able to infect susceptible leukocytes (Bomsel, 1997; Bobardt, 2007). Our laboratory has developed an organ culture model using ectocervical tissues to study HIV-1 transmission across the female genital tract (Collins, 2000) and has demonstrated viral transmission from cell-free and cell-associated HIV-1. Unlike other studies, the cervix tissue in our model includes the epithelial layer and the stroma papillae. The stroma side is enriched with dendritic cells which virions can infect or be internalized by and then passed on to CD4+ T cells. This model is useful to study HIV-1 transmission across the mucosal barrier as it provides the natural tissue architecture, including epithelial cells, submucosa, and immune cells such as T cells, macrophages and Langerhans cells that allows for the evaluation of transmission of cell-free and cell-associated infectious HIV-1. The major infected cell types detected in the submucosa were activated CD4+ T cells and the co-receptor usage of virus in infected tissue displayed the phenotypic properties of infecting virus (Gupta, 2002). Transmission in our organ culture model has proven to be reproducible from tissue to tissue and requires infectious virus. A number of investigators have confirmed the utility of cervical and anal tissue based organ culture to study

HIV-1 transmission and evaluate anti-HIV-1 activity of microbicides (Abner, 2005; Collins, 2000; Greenhead, 2000; Gupta, 2002; Narimatsu, 2005; Patterson, 2002).

The acquisition of HIV through the sexual route and spread to the lymph nodes has been continuously studied in macaques. However, transmission of HIV infection to the regional lymph nodes after crossing cervical mucosa in humans is not clear. Because human lymph nodes are difficult to assess routinely for studies of HIV infection, we used palatine tonsil tissues as a surrogate for lymph node tissues.

My thesis describes the development of a combined organ culture system with cervical tissue and tonsil tissue to mimic migration of virus and infected cells after crossing cervical mucosa to regional lymph nodes. The palatine tonsil is a secondary lymphoid organ that is routinely used as surrogate for lymph nodes. The external surface of the tonsil consists of stratified squamous epithelium, with invaginations known as tonsillar crypts. The crypts are lined by reticulated epithelium populated mainly by epithelial cells and leukocytes and has been suggested to function on the constant process of antigen sampling in the oral cavity (Nave, 2001; Perry, 1998). Well established tonsil organ culture to study HIV infection on lymph nodes have been described (Glushakova, 1995, 1999; Zhao, 2004, Southern, 2004). Maher et al (Maher, 2005) showed that cell-free HIV can establish infection in tonsil tissue when virus was applied directly to the tissue block.

2.0   HYPOTHESIS AND SPECIFIC AIMS

Rationale

Very little is known about how virus gets disseminated into the body after crossing cervical/vaginal mucosa. The macaque model used by Miller et al. established that the first 4 days after exposure the virus stays underneath the cervicovaginal epithelium and very little virus has been transported to the systemic lymphoid organs. However, 5-6 days later the small founder population under the cervicovaginal epithelium amplifies enough to travel to the systemic lymphoid organs (Miller, 2005). These results reveal that migration of cell-free virus and infected cells to the regional lymph nodes is an important source for systemic dissemination of HIV-1. However, it is difficult to study the fate of virus and virus infected cells after crossing cervical mucosa in women. Therefore, an in vitro model is required to study the process of viral migration to the lymph nodes. A combined cervical and tonsil tissue/cell model may offer an ideal *ex vivo* system to study the fate of HIV-1 and HIV-1-infected cells after crossing the cervical mucosa and migrating into the regional lymph node.

Hypothesis

The migration of virus and virus infected cells after crossing the cervical mucosa into regional lymph node can be studied in an organ culture system employing cervical and tonsil tissue.

Specific Aims

This hypothesis is addressed by the following two Specific Aims:

1. Develop a combined cervical and tonsil tissue model to study migration of HIV and HIV-infected cells to regional lymph nodes.

2. Characterize the phenotypes of HIV-infected cervical cells migrating out from cervical tissue.

3.0   MATERIALS AND METHODS

3.1   Sources of tissues

Ectocervical tissues were obtained from the Tissue Procurement Program at Magee Women's Hospital from HIV-1 negative, premenopausal women aged 50 or below undergoing hysterectomy with no history of sexually transmitted diseases.

Tonsil tissues were obtained from routine tonsillectomy performed at Children's Hospital of the University of Pittsburgh Medical Center, Pittsburgh, PA. Tonsil tissues with obvious abnormal sections were not considered for the study.

All tissues were processed in the laboratory within 1-5 hours post surgery.

Use of tissues for this study has been approved by the IRB of the University of Pittsburgh.

3.2   CD8-depleted peripheral blood mononuclear cells (PBMCs)

Separation of PBMCs was obtained from HIV-1 seronegative whole blood on lymphocyte separation medium gradients. Cells were washed twice with Hanks' buffered saline solution (HBSS) (Gibco BRL), resuspended in complete IL-2 medium (RPMI-1640 supplemented with

10% heat-inactivated fetal bovine serum (FBS), 500 U of IL-2 and 100 μg/ml Normocin^{TM}).
CD8+ lymphocytes were depleted from PBMCs by attachment to immunomagnetic beads –
negative selection (Dynabeads®CD8; Invitrogen). CD8+ beads were added to a 15 ml conical
tube, washed twice with cold phosphate-buffered saline (PBS) containing 2% FBS (PBS-
2%FBS) and incubated with PBMCs at a bead-to-target-cell ratio of 1:5 in cold PBS-2%FBS at
4°C on a rotating rocking platform for 1h. The immunomagnetic captured cells were then
separated by placing the tube in a magnetic apparatus for 5 minutes. The unbound cells were
removed by the removal of supernatant and cultured at a concentration of 1 x 10^6 cells per ml in
a T75 flask at 37°C in a 5% CO_2 incubator. All incubations at 37°C were performed in a 5%
CO_2 incubator unless otherwise mentioned.

3.3   Virus stocks

To prepare cell-free virus stock, CD8-depleted PBMCs at a concentration of 1 x 10^6/ml in 10%
RPMI 1640 medium (10% FBS and 100 μg/ml Normocin^{TM} (Amaxa)) were stimulated with
5μg/ml phytohemagglutinin (PHA) in a T75 flask for 48-72 hours at 37°C and subsequently
cultured for one hour in complete RPMI with 5μg/ml polybrene in a T25. Following incubation,
cells were transferred to a 15 ml conical tube and pelleted by centrifugation for 5 minutes at 500
x g. Supernatant was discarded and cell-free HIV-1$_{BAL}$ or HIV-1$_{IIIB}$ virus was added directly to
10 x 10^6 pelleted cells at a volume of 1 ml of 50% tissue culture infectious dose of 10^6 (10^6
TCID$_{50}$) and incubated for 2 hours at 37°C. Samples were shaken every 15 minutes during the 2
hour incubation period. Subsequently, cells were washed twice with 10 ml of 10% RPMI,

resuspended in complete IL-2 medium at a concentration of 1×10^6/ml. Cells were cultured at 37ºC. Culture supernatants were harvested on day 7 in a 50ml conical tube, centrifuged for 5 minutes at 500 x g and 1ml aliquots of supernatant were stored at -70ºC. Virus stock was used as cell-free virus in tissue/ cell culture experiments. HIV-1$_{BAL}$ strain was used as R5 tropic virus and HIV-1$_{IIIB}$ as X4 tropic virus.

3.4 Acutely infected HIV-1 cells

Fifty million CD8-depleted PBMCs were incubated in 10% RPMI containing 5μg of polybrene per ml for 1 h at 37ºC. Subsequently, cells were centrifuged at 500 x g for 10 minutes, resuspended in 1 ml of cell-free HIV-1$_{BAL}$ or HIV-1$_{IIIB}$ and incubated for 1 h at 37ºC. The cells were washed twice with HBSS and cultured at 37ºC with complete IL-2 medium for 2 days. Following incubation, cells were harvested, washed twice in HBSS and one ml aliquots of 5×10^6 cells were frozen with 10%DMSO/90%FBS at -70ºC overnight and then transferred to -140ºC.

Acutely infected CD8-depleted PBMCs were used as cell-associated virus in tissue and cell culture experiments.

3.5   Cell-free HIV-1 titration assay

CD8-depleted PBMCs that have been treated overnight with PHA were centrifuged at 500 x g for 10 minutes and resuspended in 10% RPMI. Cells were treated for 1 h with 10% RPMI containing 5μg/ml polybrene. Following incubation, cells were pelleted and resuspended in 10% RPMI. Two hundred thousand cells were seeded in a 96-well plate and serial dilutions of 1:5 thawed cell-free HIV-1$_{BAL}$ or HIV-1$_{IIIB}$ in 10% RPMI media was added to each row containing the pre-seeded cells and incubated at 37°C for 7 days. Subsequently, 1:10 dilutions of culture supernatants were harvested and frozen at -70°C. Thawed aliquots were measured for infectivity titer by an HIV p24 ELISA kit (PerkinElmer Life Sciences, Inc., MA, USA) according to the manufacturer's protocol.

3.6   Titration of cell-associated HIV-1

Thawed acutely infected cells were cultured in complete IL-2 medium at a concentration of 1 x 10^6 in a T25 flask for at least 4 hours. Following incubation, cells were pelleted and resuspended in complete IL-2 medium. 1×10^6, 5×10^5, 2×10^5, and 1×10^5 cells were seeded in a 48-well plate and cultured for 3 days at 37°C. Culture supernatant was then harvested at 1:5 dilutions in complete IL-2 medium and frozen for at least 24 h at -70°C. Cell-associated culture supernatants were tested for infectivity titer by an HIV p24 ELISA kit. Cell concentrations with the highest p24 levels were used as cell-associated virus, i.e., 2×10^6 cells.

3.7   Organ Culture with combined cervical and tonsil tissue/cells

Cervical tissues were immersed for 5 minutes in a concentrated antibiotic solution containing 20,000 U/ml Penicillin/Streptomycin, 120 U/ml Nystatin and 250 μg/ml Fungizone in PBS and then rinsed twice with Dulbecco's Modified Eagle's Medium (DMEM). The stromal side was cut to approximately 2-3mm thick, and a 6.0mm-diameter punch biopsy of cervical tissue was placed on the top chamber of a 12-well Transwell^{TM} (3μm membrane pore size) with the epithelial layer facing upwards (figure 8). The area surrounding the cervical tissue was sealed with 3% agarose (SeaKem Le Agarose).

Tonsil tissues were soaked in HBSS containing 100μg/ml Normocin^{TM} and 5% FBS for 5 minutes before cutting into 2-4mm^3 pieces. Three pieces of tonsil tissues were placed on a collagen support (Surgifoam; Pitt SHS Pharmacy, Pittsburgh, PA) at the gaseous/liquid interface in complete IL-2 medium on the bottom chamber of the Transwell^{TM}. To study the transmission of virus, 300μl of cell free HIV-1$_{BAL}$ or HIV-1$_{IIIB}$ of 10^7 TCID$_{50}$ or, for cell-associated HIV, 2 x 10^5 of pre-tittered HIV-1 infected cells, was added to the cervix tissue on the top chamber and incubated for 3 days at 37°C. Transwells^{TM} were removed on day 3 and tonsil tissues remained in culture for 12 additional days. Culture medium was removed and replaced with fresh complete IL-2 medium every three days. At least duplicate tissue samples from each patient were used. A Transwell^{TM} with agarose only was used as a negative control and a Transwell^{TM} only served as a positive control for virus replication in tonsil.

To examine the integrity of the cervix organ culture and any leakiness in the system, the virus was removed from the top chamber on day 3. The Transwell^{TM} was transferred to a new 12-well plate containing 1ml of complete IL-2 media. 300μl of 1% filter-sterilized blue dextran

in complete IL-2 media was added to the Transwells^{TM} containing cervix, the agarose and positive control wells and cultured for another 24 hours at 37⁰C. The supernatant in the bottom well was placed in a plastic cuvette then measured using a spectrophotometer at a wavelength of 620nm.

Alternatively, tonsil-derived mononuclear cells were used in the bottom well instead of intact tonsil tissue for the combined cervix and tonsil cell model. Tonsil cells were isolated by mechanical disaggregation of the tissue, pelleted by centrifugation at 500 x g for 5 minutes and resuspended in complete IL-2 medium. $4x10^6$ cells were seeded per well in 1ml and used as freshly isolated cells. Otherwise, cells were frozen at -70⁰C overnight and transferred to -140⁰C and used as frozen-thawed cells.

Viral replication was assessed every three days by production of HIV-1 p24 in culture supernatant. Several techniques were used to detect HIV-1 in tonsil tissues or cells after the 12 day incubation period. Tonsil cells were rinsed twice with HBSS, pelleted and stored at -80ºC to be analyzed for proviral DNA by TaqMan real-time PCR; tonsil tissues were frozen in optimum cutting temperature (OCT) medium on dry ice and cells were placed on slides to be analyzed by immunofluorescence; cells were frozen in 10%DMSO/90%FBS and stored at -140ºC for simultaneous immunophenotyping and HIV-1 gag-pol RNA detection by flow.

3.8   Measurement of viral replication

Samples were taken every 3 days to monitor viral replication in tonsil tissue/cells to be analyzed by an end-point HIV p24 ELISA assay. Aliquots if 50µl or 250µl diluted in complete IL-2 medium (1:5 or 1:10 and 1:0, respectively) were taken from culture supernatants of tonsil tissue/cells and stored at -70°C. Once p24 sample collection was obtained fresh complete medium was replenished in the culture supernatant.

3.9   Histology

Tonsil tissues were fixed in Streck Tissue Fixative, paraffin embedded and cut into 7µm thick sections using a Vibratome[TM], adhered to positive charged microscope slides (Fisherbrand) and deparaffinized through xylenes and graded alcohols. The slides were then stained with hematoxylin and eosin and examined by light microscopy to assess tissue architecture and necrosis over the culture period. Slides analysis was performed by Dr. Ronal Jaffe at Children's Hospital of the University of Pittsburgh Medical Center, Pittsburgh, PA.

3.10 Antibodies

Mouse Alexa Fluor 647-α-CD4 (RPA-T4), PE-α-CD68 (Y1/82A), PE-α-CD45RO, PE-α-CD68, APC-α-CD11c against human antigens were purchased from BD Pharmingen; mouse FITC-α-CD20 (CAT-13.6E12), PE-α-CD11c (BU15) against human antigens from Invitrogen (Caltag^{TM}); and mouse anti human PC5-α-CD4 and FITC-α-p24 (KC57) antibody against HIV-1 antigen from Beckman Coulter.

3.11 Immunofluorescence

Tonsil cells were washed twice in cold HBSS and placed on positive charged microscope slides (Fisherbrand) for 1 hour at room temperature to allow the cells to settle. Cells were fixed 15 minutes with 2% paraformaldehyde (formalin solution, neutral buffered, 10%; Sigma) in PBS (PFA/PBS) and surface stained 1 hour with CD4 (1:20) and CD11c (1:20) antibodies. Subsequently, samples were washed with PBS, fixed for 10 minutes with 1% PFA/PBS, permeabilized 10 minutes with 0.5% Triton X-100 in PBS, blocked 1 hour with 1% BSA in PBS and stained 1 hour for detection of intracellular marker with antibody against CD68 (1:5) and viral proteins with monoclonal p24 antibody diluted with PBS.

Tonsil tissues blocks were fixed on harvest day in 4% PFA/PBS and incubated at 37°C for 5 hours. Following incubation, the solution was changed as follow: 4% PFA/PBS was removed; PBS was added and incubated at 4⁰C. Day 2, PBS from the previous day was removed and fresh PBS was added and incubated at 4⁰C. Day 3, PBS was removed and 10%

sucrose in PBS was added. Day 4, removed 10% sucrose/PBS and added 20% sucrose/PBS. Tissues were frozen in OCT medium and stored at -80°C. Frozen tissues were cut into 7μm thick sections using a cryostat and adhered to positively charged microscope slides. Tissue sections on microscope slides were stored at -20°C when not immediately used.

To prepare the frozen tissue sections for staining, slides with frozen tissue sections were set to room temperature for 5 minutes, post fixed with 1% PFA/PBS, washed twice in PBS, permeabilized with 0.5% Triton X-100, blocked 1 hour with 2% BSA/ PBS and stained with p24 (1:50) antibody diluted in PBS. Slides were washed three times with PBS after staining. Nuclear counterstain Hoechst (Sigma) was used for both cell and tissue samples after surface and/or intracellular staining. All incubations were done at room temperature in a dark humidified compartment. Slides were mounted with Gel Mount™ Aqueous Mounting Medium (Sigma) for the subsequent addition of the coverslip. Samples were documented using a magnification of 40x and 60x using a Nikon Eclipse E600 microscope and a CRI Nuance spectral analyzer (CRI Inc., Woburn, MA). Emission filters used were 420 long pass (LP) for Hoechst, 520 LP for FITC, and 590 LP for Alexa Fluor 647 and PE.

3.12   Flow Cytometry

Tonsil cells were washed twice with cold HBSS and surface stained for CD20, CD4, and CD11c, and permeabilized with 0.1% Triton X-100 for intracellular staining of CD68 for 1 hour at 4°C. After staining, cells were fixed with 2.5% PFA/PBS and collected using a BD Canto

flow cytometer. Cells were gated on the lymphocyte gate. FCS files were analyzed using FlowJo version 7.2.5 (Tree Star, Inc.).

3.13   TaqMan Real Time PCR

DNA was extracted from frozen cell pellets as per manufacture's protocol (PUREGENE® DNA Purification kit). DNA pellet was resuspended to 20μl of nuclease free water. A 30μl TaqMan® PCR was performed by mixing 5μl DNA with TaqMan® Universal PCR Master Mix (AppliedBiosystems), 333nM each of forward and reverse primer and 250nM FAM/ MGB labeled probe. The specific primers for HIV-1 BAL-F (5'-TGG GTT ATG AAC TCC ATC CTG AT-3') and BAL-R (5'-TGT CAT TGA CAG TCC AGC TGT CT-3') were designed by Dr. Bruce Patterson (Patterson, 2005). TaqMan probe was BAL-P (5'-FAM-AAT GGA CAG TAC AGC CTA TAG TGC TGC CAG AA-TAMRA-3'). ABI Prism 7000 Sequence Detection System was used to carry out Real-Time PCR using the following cycling condition: 50 ºC for 2 min, 95 ºC for 10 min, 50 cycles of 95 ºC for 15 sec and 60 ºC for 1 min. Serial diluted pNL4-3 plasmid DNA ranging from 1 to 10^6 copies were applied to each PCR assay for a standard curve. No Template Control was included in each assay to guard against cross contamination. Each sample was run in triplicate. ABS Prism 7000 SDS Software (Applied Biosystems) was used for PCR data analysis and HIV copy number estimation.

3.14 Simultaneous Immunophenotyping and Ultrasensitive fluorescence in situ Hybridization

Tonsil cells were washed twice in HBSS, resuspended with 10% DMSO/90% FBS, and stored at -80ºC. Cells were thawed, rinsed, pelleted by centrifugation and stained for cell markers with CD4 and CD68. For the detection of HIV-1 mRNA, cells were treated with ViroTect VR in-cell HIV-1 detection system (Invirion Diagnostics). Flow cytometry was performed in a FC500 flow cytometer (Beckman-Coulter). This procedure was done by Dr. Bruce Patterson, Stanford University, Stanford, CA.

4.0   RESULTS

4.1   AIM 1

4.1.1   Replication of HIV in tonsil tissue

Before we set up the combined cervical and tonsil tissue model, we initiated a study to standardize the condition of HIV infection in tonsil tissue itself. To assess HIV infectivity in tonsil, 3 pieces of tissue cut into 2-4mm^3 were placed on a collagen support at the gaseous/liquid medium interface. Tonsil tissues were infected by carefully applying 5 µl of serial dilutions (TCID$_{50}$ 1:4, 1:16, 1:64; starting TCID$_{50}$ of 10^7-10^8) of cell-free HIV-1$_{BAL}$ directly to the tissue surface. The virus inoculums were washed away on day 3 and the tissue culture was continued for 12 days with culture medium replaced every 3 days.

HIV replication was monitored by measuring HIV p24 in culture supernatant. A dose dependent HIV replication was observed by HIV p24 production in culture supernatant (figure 3). Evidence of viral replication on the 1:64 virus dilutions is important for the subsequent use of virus in the combined tissue model, because approximately 5-10% of HIV-1 gets transmitted across the female genital mucosal barrier on our organ culture model.   Figure 4 shows expression of p24 antigen in tonsil tissues at day 12 post infection as evidenced by HIV-1 p24

specific immunocytochemical staining in tonsil tissues. These data demonstrate that HIV-1 replicated in tonsil tissue.
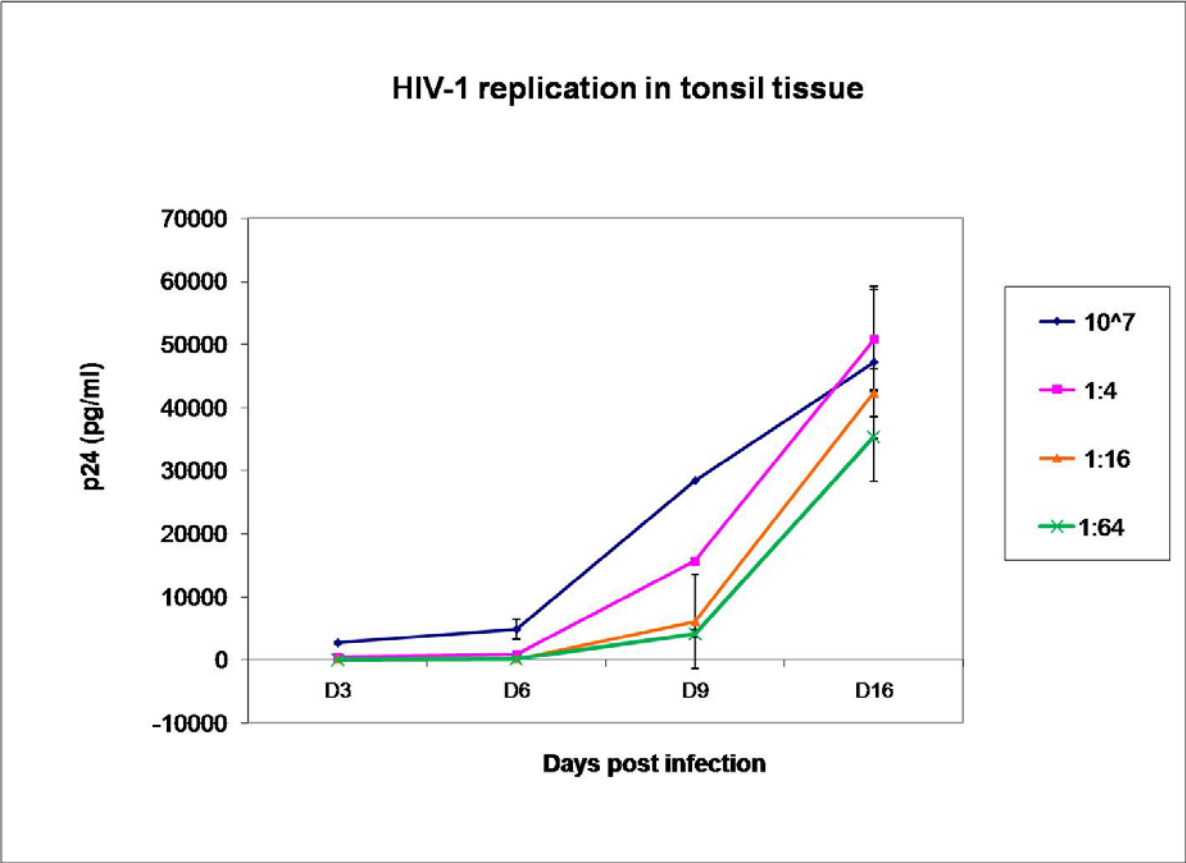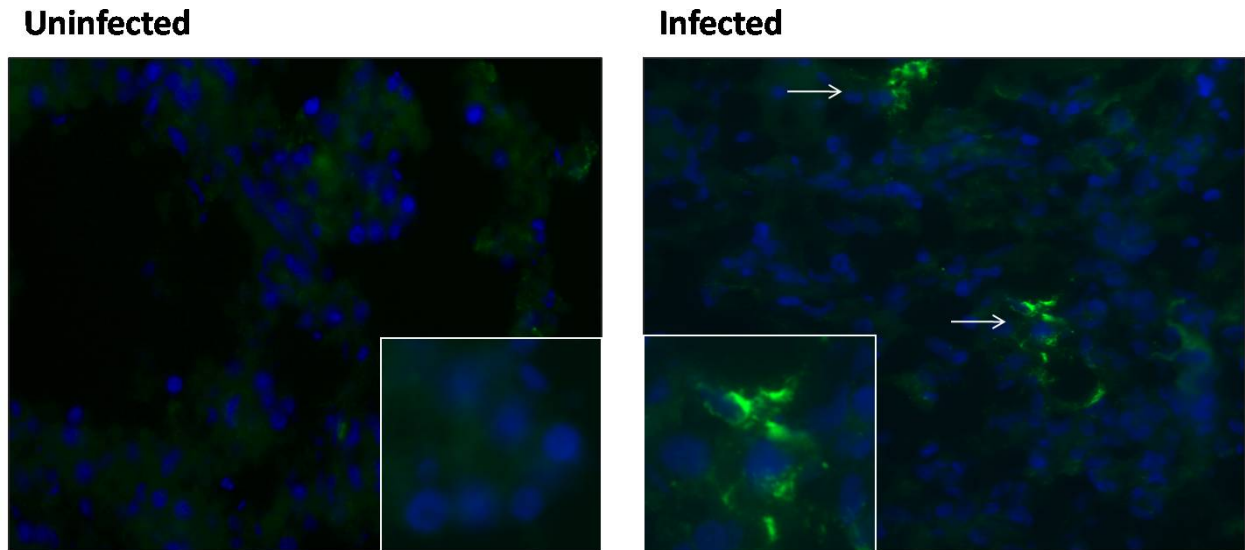

Figure 3: Dose-dependent response to HIV-1 infection in tonsil tissue.
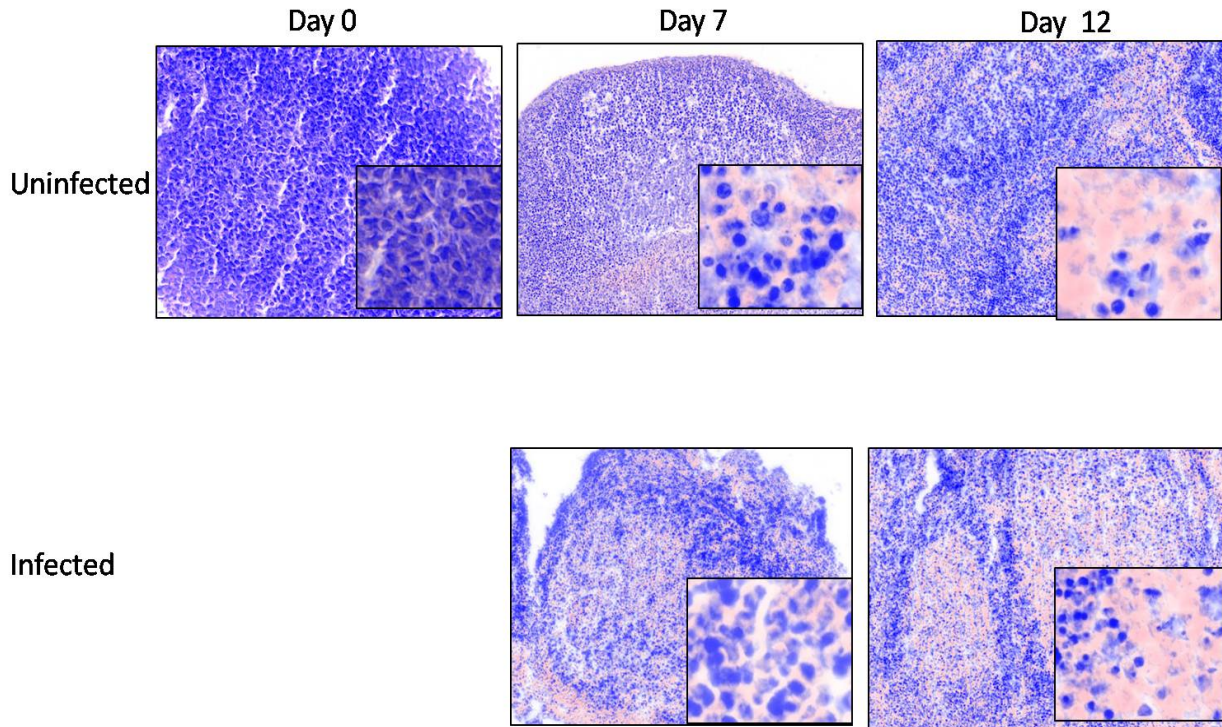
Uninfected

Infected

Figure 4: Single label immunofluorescence detection of HIV-1 p24 positive tonsil cells in tonsil tissue.
Green: p24 antigen; Blue: cell nuclear stain. Original magnification x40.

To evaluate tonsil tissue integrity, tissues were examined at day 0, day 7 and day 12 post infection by hematoxylin and eosin staining (figure 5). On day 0, cell nuclei detail was observed at a high power. On day 7, approximately two thirds of the cells in uninfected tonsil tissue remained viable as compared to cells observed in the lymphoid compartment of infected tonsil tissues in which an estimated 10% of the cells remained viable. Most of the nuclei observed were pycnotic. Namely, chromatin condensation representative of cellular apoptosis was seen by the dark purple stain and smaller nuclei, whereas a larger nuclear size was seen on day 0. Tonsil tissues on day 12 post infection had a lymphoid compartment that was largely destroyed despite the observed intact tonsillar epithelium and germinal mantle zones. Therefore, tissue disintegration could be due to the effect of HIV-1 in the lymph nodes, representative of a

similar situation that exists in vivo in which the lymph node architecture is destroyed due to the damaging effects of the immune activation.

Figure 5: Hematoxylin and eosin staining of paraffin embedded infected tonsil tissues at day 0, 7 and 12.
Inset: an enlarged section showing microanatomical regions. Original magnification: x20, inset magnification: x40.

4.1.2   Replication of HIV in tonsil cells

As an alternate to tonsil tissue, we considered using tonsil cells in our combined model. First, we determined the phenotype of cells present in mechanically disaggregated tonsil cell suspension by flow cytometry. These cells consist primarily of CD20 (B cells) (Table 1). A significant number of CD4+ and CD11c+ (dendritic cells) and, in lesser amount CD68+

(monocytes) were found in tonsil cells which are target cells for HIV-1 infection. Following 12 days in culture, tonsil cells still carried good amount of these cell types, even though their relative levels changed somewhat during culture and infection.

Table 1: Percentage expression of tonsil cells markers.

	CD20	CD11C	CD68	CD4
Uninfected Day 1	86.7%	27.7%	0.8%	26.6%
Uninfected Day 12	5.4%	2.3%	1.4%	87.2%
HIV-1 BAL Day 12	11.6%	16.3%	0.8%	58.1%

The susceptibility of single-tonsillar cell suspension to HIV infection was then examined. The literature has described different isolation methods for tonsil cells, thus, we investigated different isolation methods to determine which one most efficiently supports viral replication. To evaluate tonsil cell infectivity tonsil cells were isolated by three different methods: 1) pressing small pieces of tissue through a 60um sieve with a syringe plunger and lymphocyte separation by using Ficoll-hypaque density-gradient centrifugation, 2) sieve separation only, and 3) mechanically disaggregation of the tissue, namely, minced tonsil tissue cells. No further Ficoll-hypaque density-gradient to separate the lymphocyte population was used on method 2 nor 3. We compared freshly isolated and frozen-thawed cells because it would be beneficial if we standardize the system to use frozen-thawed cells as they would

pertain to one donor eliminating variability from experiment to experiment. Cells were exposed for 2 hours to serially diluted cell free HIV-1$_{BAL}$ and assessed for viral replication by the presence of HIV-1 p24 in culture supernatant (figure 6). All three methods, as well as frozen-thawed cells, were found to support viral replication. However, HIV replication in freshly isolated cells was higher when compared to frozen-thawed tonsil cells. Regardless of fresh or frozen, mechanically disaggregated tonsil tissue cells support the highest level of HIV replication. This may be attributed to a higher cell diversity population present in mechanically disaggregated tonsil tissue cells which are susceptible to HIV infection. Included in disaggregated tonsil tissue cells are epithelial cells that may contribute to a strong, though non-specific binding of HIV virions to tonsillar epithelial cells (Maher, 2004). Therefore, mechanically disaggregated tonsil tissue cells were used for subsequent experiments.
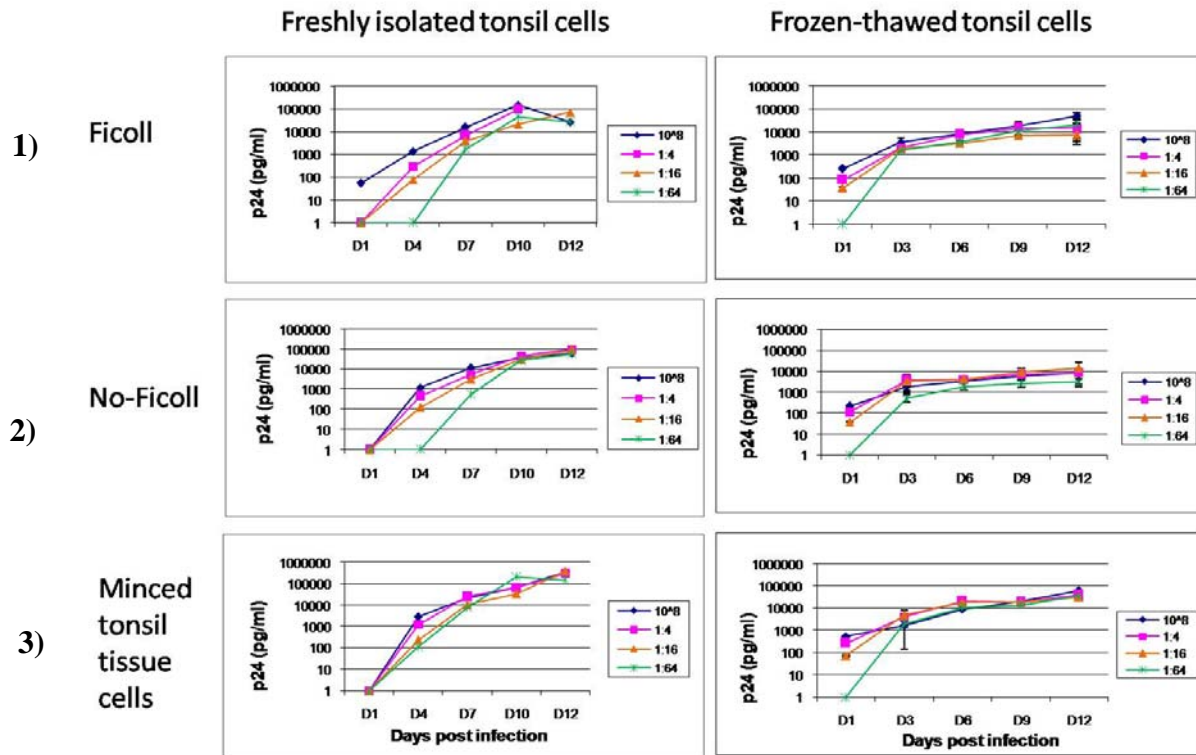
Figure 6: Viral replication of both freshly isolated tonsillar cells and frozen-thawed cells in suspension.
Tonsil cells isolated by: 1) 60 μm sieve and Ficoll-hypaque density-gradient centrifugation; 2) 60μm sieve without Ficoll-hypaque density-gradient centrifugation; 3) mechanically disaggregated tonsil tissue cells without any further separation, namely, minced tonsil tissue cells.

Intracellular p24 antigen was also detected in these HIV-1 infected tonsil tissue derived cells by using an immunofluorescence assay (figure 7). A qualitative analysis of the cell types that were positive to p24 antigen were identified to be CD4+, CD11c+, and CD68+ detected by double-staining of cell markers (red) and intracellular p24 (green) as compared to the single staining of p24. Viral replication was also confirmed by the detection of proviral DNA in tonsil cells as shown in figure 12, panel C and D.
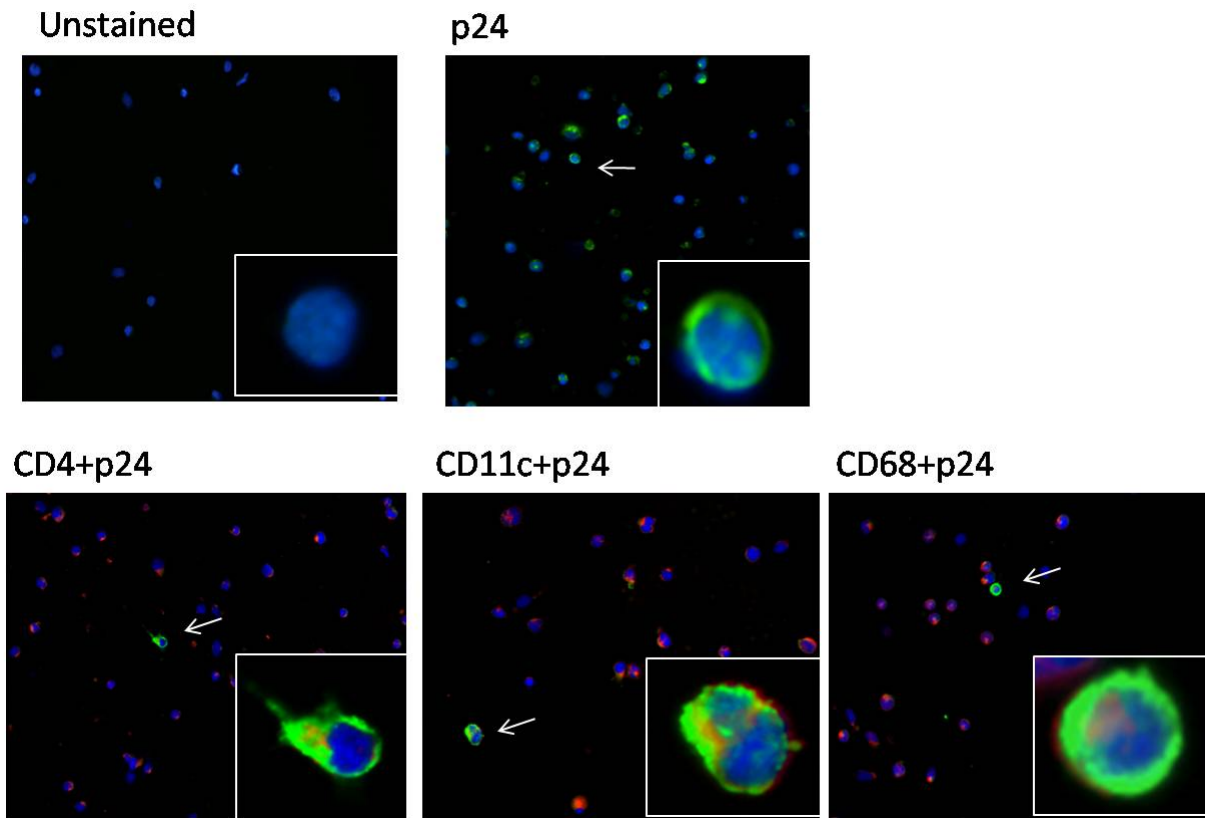
Figure 7: Expression of HIV-1 p24 in tonsil cell following infection with HIV-1 BAL

4.1.3 Combined cervical tissue and tonsil tissues

To evaluate HIV-1 transmission after crossing the cervix mucosa and migrating to regional lymph nodes, a combined cervix and tonsil tissue-derived organ culture system was used with cervical tissue at the top well and tonsil tissue on the bottom well (figure 8). HIV-1$_{BAL}$ or HIV-1$_{IIIB}$ was added onto the cervical tissue and incubated for 3 days. Following incubation, the top well with cervical tissue was removed and tonsil tissues on the bottom well were cultured for an

additional 12 days. Culture media was removed and replaced with fresh complete IL-2 medium every 3 days. Virus replication was monitored every 3 days by measuring HIV-1 p24 in the culture supernatant. Since very low HIV p24 was detected in culture supernatant from the bottom well with cervical tissue in the top well and only media in the bottom well, an increase in HIV p24 in bottom well containing tonsil cells of the combined model is considered as an indication of viral replication in tonsil tissue/cells. However, very little sustained replication was observed in combined tissue model with tonsil tissue in the bottom well in which very low HIV replication after day 6 was observed from the culture supernatant of tonsil tissue (figure 9). However, intracellular HIV p24 was detected in tonsil tissue on day 8 after infection by immunofluorescence assay (figure 10). Tonsil tissue alone infected with HIV-1 was used as a positive control and shows the presence of HIV-1 p24 positive cells. Even though we see intracellular p24 in tonsil tissue in the combined tissue model, these results were not very reproducible.
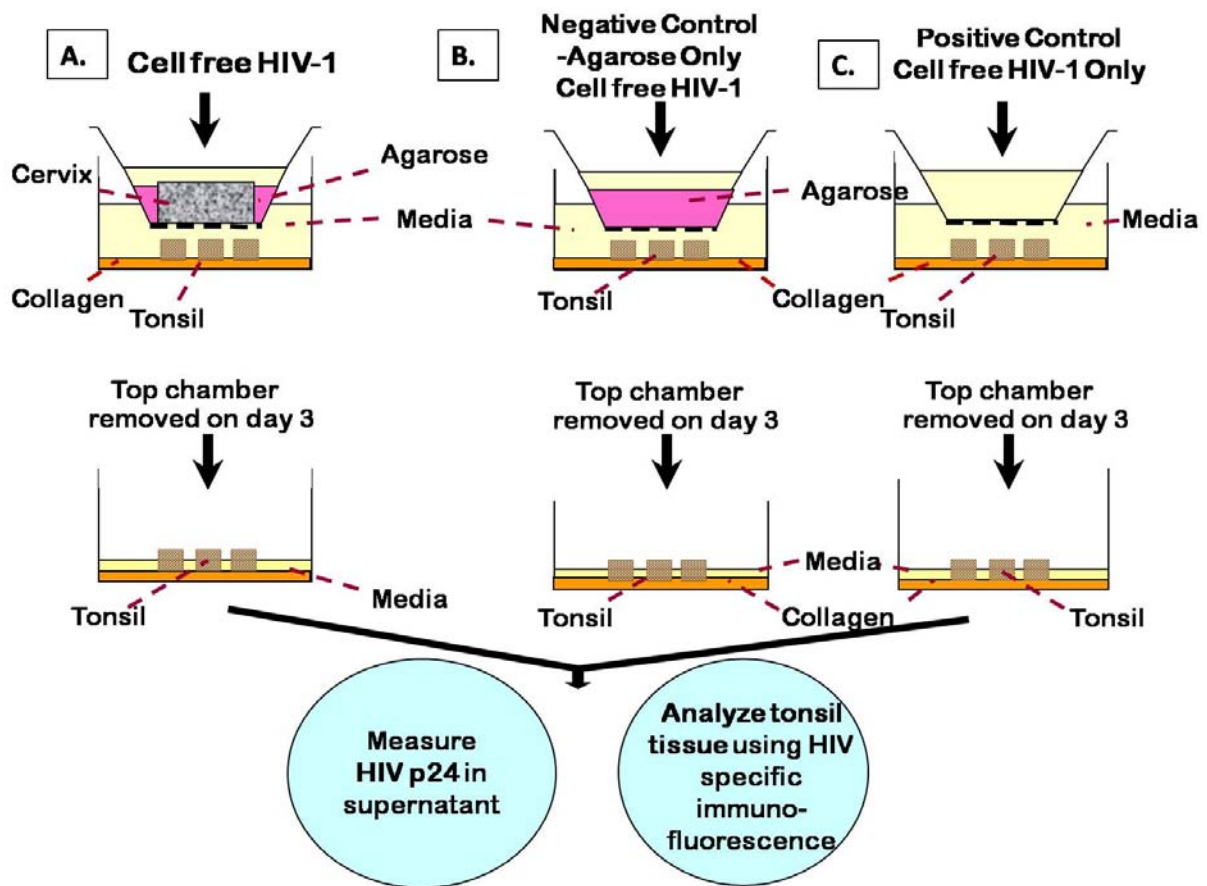
Figure 8: Diagram of the Transwell^{TM} utilized combined organ culture model of HIV-1 transmission across the cervical mucosa into lymphoid tissue.
A. Transwell^{TM} with cervix tissue surrounded with agarose B. Agarose only – negative control. C. Membrane alone – positive control. Tonsil tissue on collagen support on the bottom well.
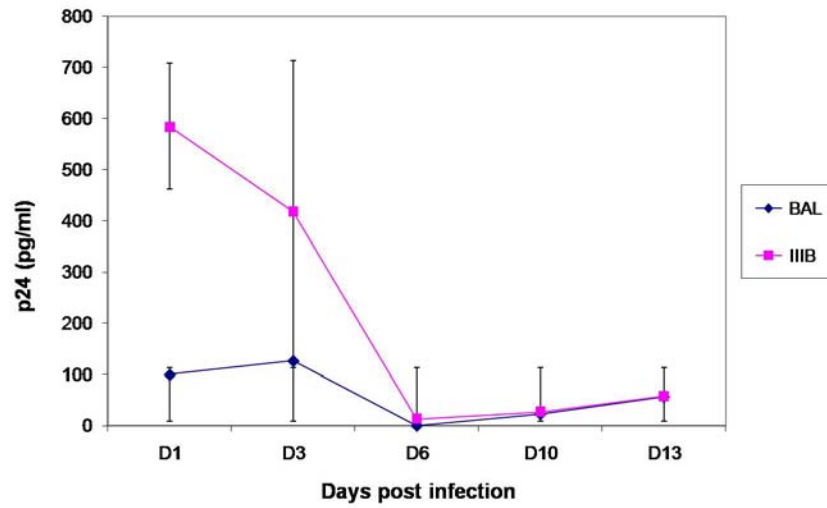
Figure 9: HIV-1$_{BAL}$ and HIV-1$_{IIIB}$ replication on tonsil tissue after exiting the cervix tissue.
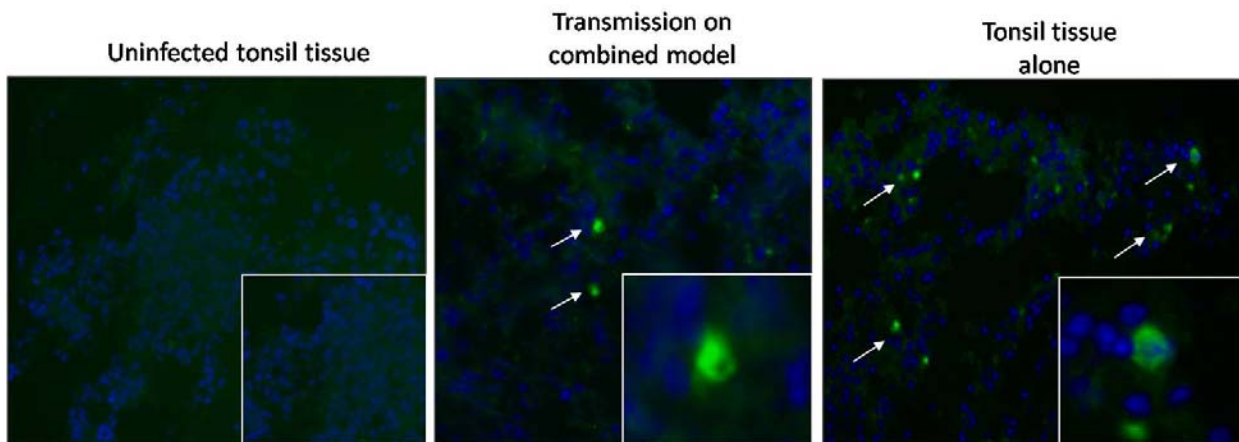
Figure 10: HIV-1 p24 expression of day 8 tonsil tissue cultured on the bottom well of a combined organ culture system.

Therefore, a combined organ culture was established with tonsil cells in the bottom well without the use of a collagen support. Cell-free HIV-1$_{BAL}$ or HIV-1$_{IIIB}$ (TCID$_{50}$ of 10^7) was used as inoculums to the cervix tissue. Replication in tonsil cells after transmission through cervical mucosa was confirmed by measuring of HIV-1 p24 in culture supernatant (Figure 11). HIV-1 p24 production in culture supernatant increased after day 6 of infection with both HIV-1$_{BAL}$ and HIV-1$_{IIIB}$. However, there seems to be higher level of replication with HIV-1$_{IIIB}$ as compared to HIV-1$_{BAL.}$ Similarly, higher replication of HIV-1$_{IIIB}$ compared to HIV-1BAL was observed in tonsil cells alone. Furthermore, analysis of HIV-1 proviral DNA in tonsil cells in the bottom well from virus crossing the cervical mucosa (figure 12, A and B) confirmed replication of HIV-1 in tonsil cells. Tonsil cells infected with HIV-1$_{IIIB}$ had higher copy levels of HIV-1 proviral DNA per nanogram when compared to tonsil cells infected with HIV-1$_{BAL}$ supporting p24 production data shown in figure 10. The low levels of HIV DNA present in tonsil cells alone (figure 12, C and D) could be due to a higher dose of virus present in tonsil cells alone causing cell death at earlier time points, thus, less infected cells containing HIV-1 DNA on day 12. Conversely, a low dose of virus crosses the cervix mucosa during the three days the cervix tissue is in culture with the tonsil cells and is probably less cytopathic than tonsil cells alone infected with HIV-1. HIV-1 replication in tonsil cells was further documented in an immunofluorescence assay by the presence of p24 antigen in tonsil cells (figure 13). Analysis of cell types that are harboring p24 antigen indicates that CD4+, CD11c+, and CD68+ were infected with HIV-1 after transmission from the cervix tissue (figure 13). A flow cytometric simultaneous immunophenotyping and ultrasensitive fluorescence in situ hybridization assay was performed to determine the tonsil cell types that become infected after virus has crossed the cervix mucosa (Figure 15, A).
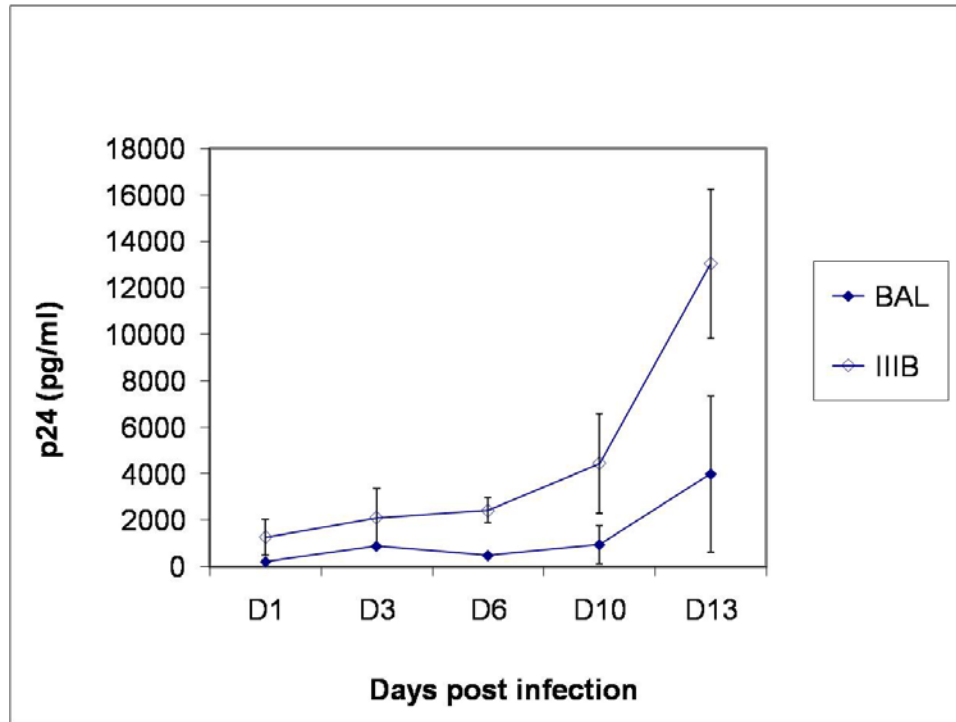
Figure 11: Viral replication from tonsil cells from combined cervix and tonsil cells.
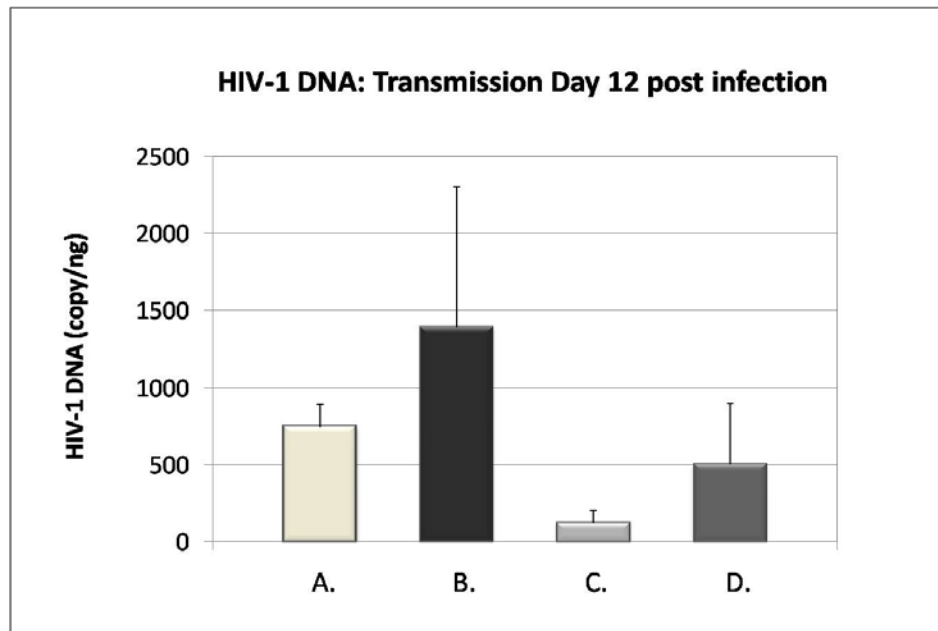
Figure 12: Qualitative analysis of integrated HIV-1 DNA.
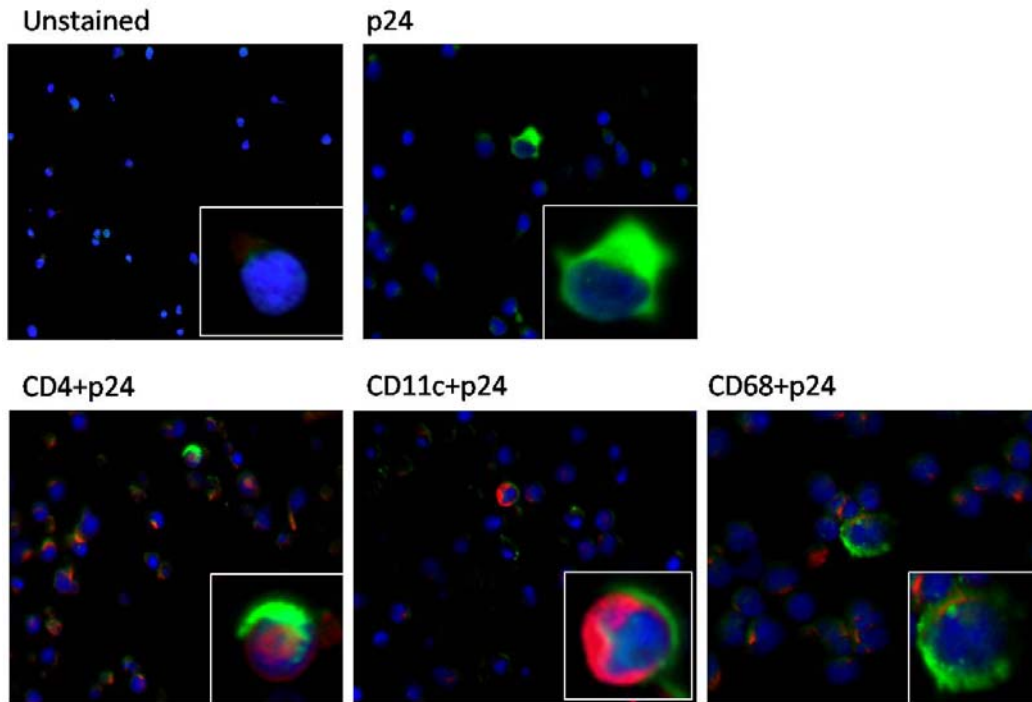
Figure 13: Expression of HIV-1 p24 in tonsil cells after crossing the cervix mucosa.

After 12 days in culture, tonsil cells were stained with antibodies to CD4 and CD45RO for activated T cells, CD11c for dendritic cells and CD68 for macrophages. After labeling, ultrasensitive fluorescence in situ hybridization was performed and the cells were examined by flow cytometry. Non-specific immunoglobins controls were used to adjust the dot plot quadrants (data not shown). Cells were gated according to the forward and side characteristics of light scatter. In tonsil cells that have been infected with HIV-1 after crossing the cervix mucosa, 3.4% of CD45RO+ T cells were detected to express HIV-1 RNA from HIV-1$_{BAL}$ and 8.9% from HIV-1$_{IIIB}$ when compared to 0.1% cells from the negative (uninfected) control. CD11c data is pending. These results indicate that cell-free HIV-1$_{BAL}$ and HIV-1$_{IIIB}$ crosses the cervix mucosa and is able to replicate in tonsil cells.

Next, cell-associated HIV-1$_{BAL}$ and HIV-1$_{IIIB}$ were evaluated for transmission across the cervix tissue since semen contains both cell-free and/or cell-associated HIV-1. Therefore, transmission of cell-associated HIV-1 could also be important in HIV-1 transmission in the female genital tract. HIV-1$_{BAL}$ or HIV-1$_{IIIB}$ infected CD8 depleted PBMCs (TCID$_{50}$ of 4x10^3) was added onto the cervix tissue as described for cell-free virus. Figure 14 shows replication of both cell-associated HIV-1$_{BAL}$ and HIV-1$_{IIIB}$ in tonsil cells, although, higher levels of HIV-1 p24 was obtained from HIV-1$_{IIIB}$. Simultaneous immunophenotyping and in situ hybridization was performed (figure 15, B). Approximately 3.0% of CD45RO+ T cells expressed HIV-1 RNA on HIV-1$_{BAL}$ and 10.0% on HIV-1$_{IIIB}$, whereas, CD68+ did not express HIV-1 RNA when compared to the negative control. CD11c data is also pending for the cell-associated in situ hybridization.
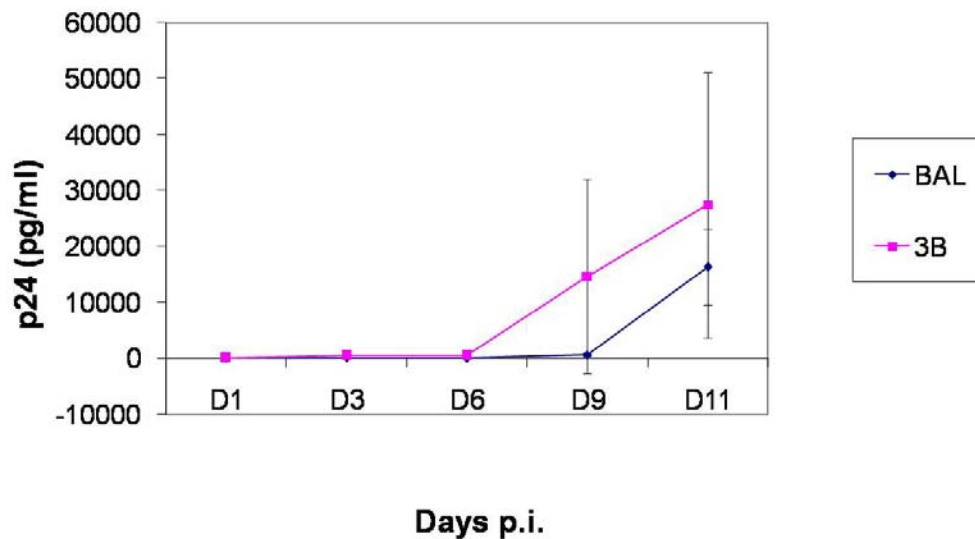
Figure 14: HIV-p24 in culture supernatant from combined cervix and tonsil cells from cell-associated virus.
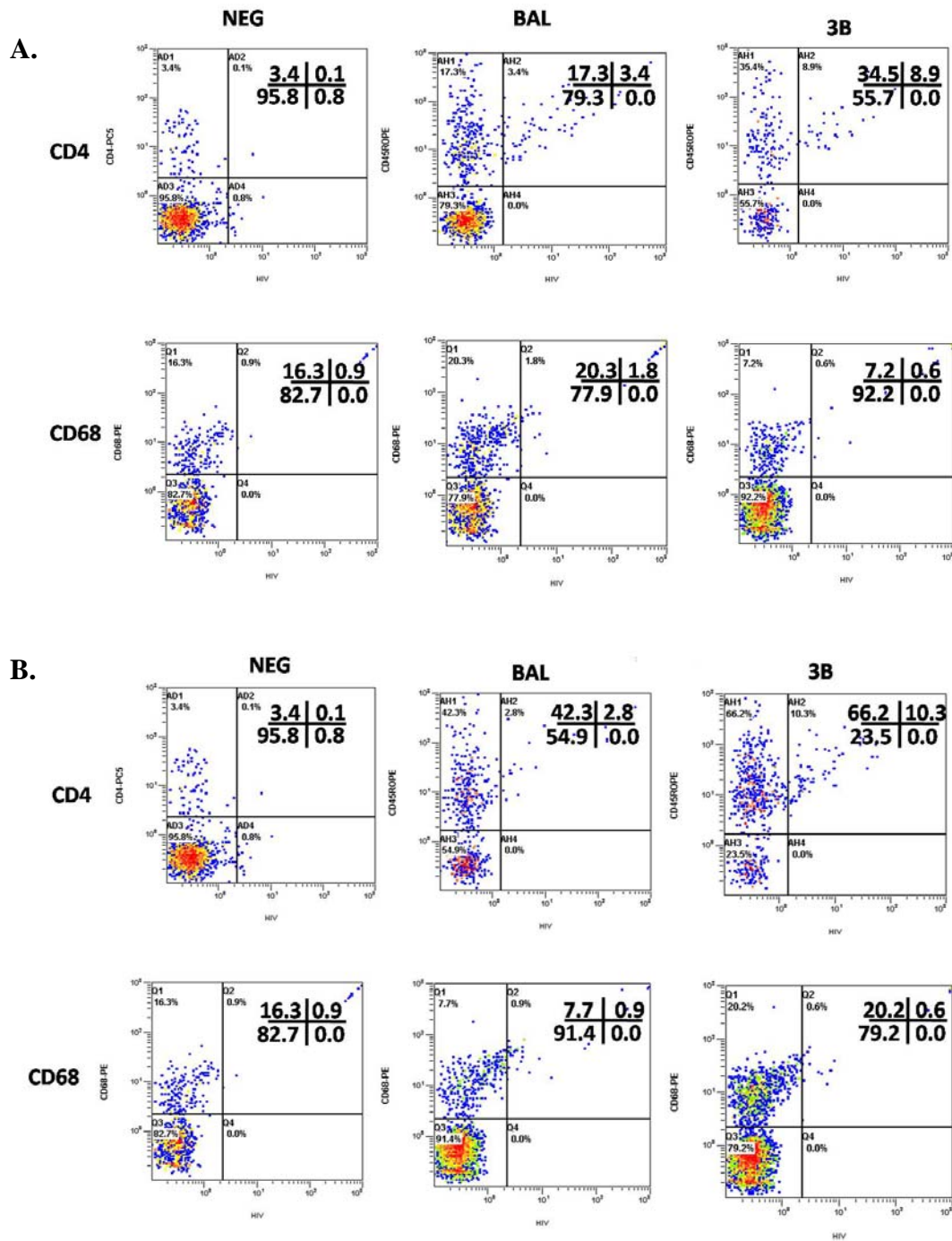
Figure 15: Dot plots representing the phenotype of productively infected cells analyzed by simultaneous immunophenotyping and ultrasensitive fluorescence in situ hybridization.
A) Cell-free HIV-1, B) cell-associated virus in tonsil cells after crossing the cervix mucosa.

4.2   AIM 2

4.2.1   Characterization HIV-infected cervical cells after migrating from cervix tissue

To characterize HIV-1 infected migratory cervical cells after exiting from the cervix tissue a cervical tissue derived organ culture (figure 9) was used without tonsil cells on the bottom well. A Transwell^{TM} with a 12μm pore size membrane was used to allow cells to migrate out from the cervical tissue to the bottom well containing complete IL-2 medium. The cervical tissue was not sealed with agarose to allow the passage of cells to the bottom well since agarose constricts the movement of cells from cervix tissue to the bottom. Five microliters of HIV-1$_{BAL}$ (TCID$_{50}$ of 10^7) or control medium was carefully added directly to the top of the cervix tissue and incubated for 3 days at 37°C. Migrating cells were harvested from the bottom well on day 3, washed twice with HBSS, placed on a positive charged microscope slide and stained with antibodies against intracellular p24, and cell markers CD4 (T cells), CD11c (dendritic cells), and CD68 (macrophages). These cells were chosen because they are the immune cell types that have been shown to be infected by HIV-1. Cells were analyzed by fluorescent microscopy (figure 16). An immunofluorescence assay with double staining of intracellular HIV-1 p24 and cell markers indicated that CD4+, CD11c+ and CD68+ were positive for HIV-1$_{BAL}$.
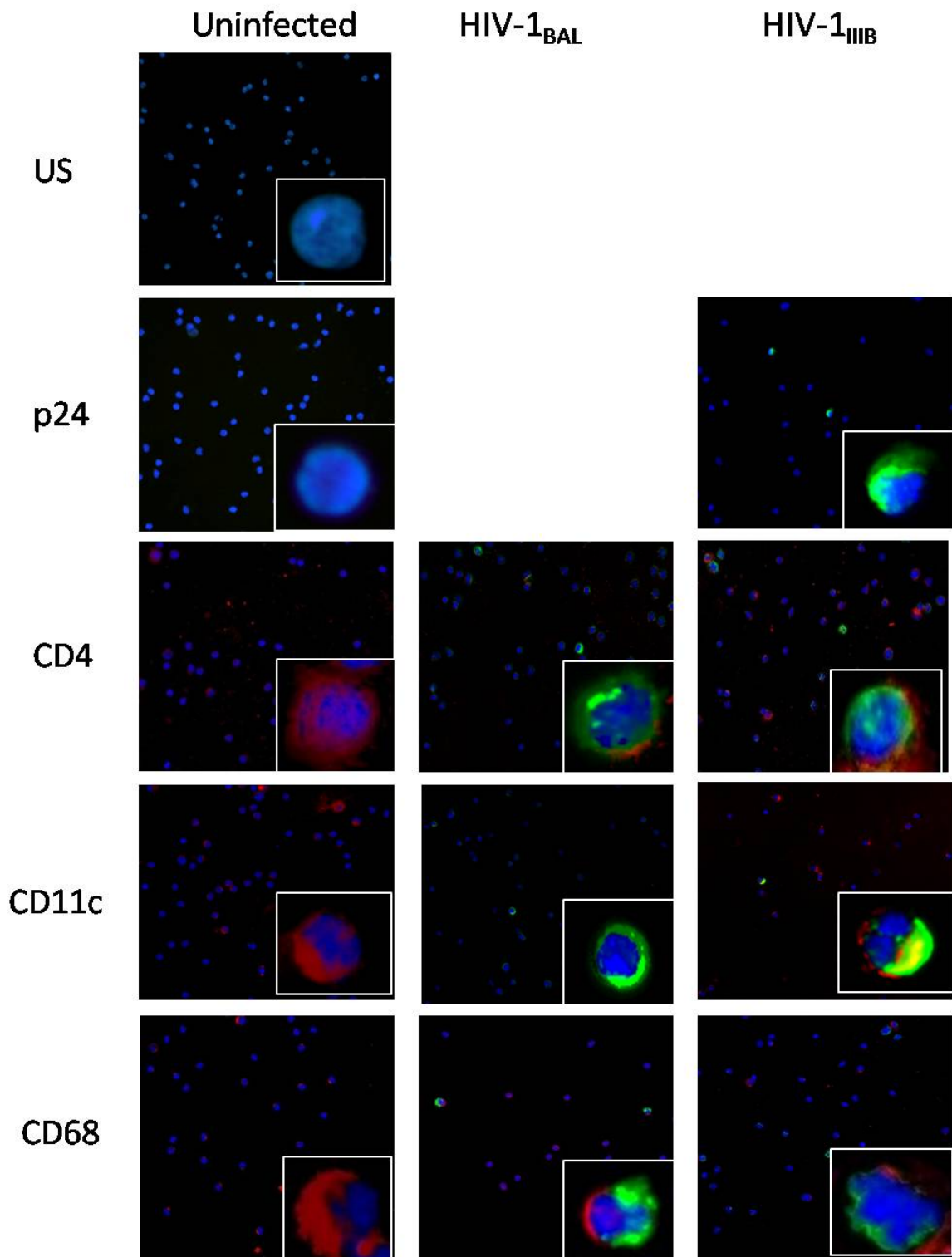
Figure 16: Migratory cells after exiting from cervix tissue.

5.0   DISCUSSION

This thesis describes a combined cervical tissue derived-organ culture and tonsil derived lymphoid cells to study the migration of virus and infected cells after crossing cervical mucosa to regional lymph nodes. Previously, our laboratory has developed a cervical tissue derived organ culture to study HIV-1 transmission in humans. The benefit of this model is that it includes all of the natural architecture of the *in vivo* conditions, that is, the cervix tissue contains stratified squamous epithelium, the submucosa, and immune cells. It was determined that the cervix tissue preserved its structural integrity without indications of necrosis after 1 week of cultivation and that transmission required infectious HIV-1.

The combined human cervical and tonsil tissue is an extension of the cervical tissue model to investigate HIV-1 transmission from the female genital tract to the regional lymph nodes. Our combined tissue system does not have afferent lymphatics to transport infected cells exiting from cervix to the regional lymph nodes that are present *in vivo*. However, the proximity of the cervical tissue with tonsil cells in our combined tissue model can serve as the afferent lymphatics *in vitro*. In the present study we provided evidence that infectious HIV-1 virus crosses the cervix mucosa and replicates in tonsil tissue/mononuclear cells, although replication in tonsil tissue is not robust. Part of the reason of low level of replication in tonsil tissue may be because tonsil tissue pieces were not placed at the gaseous/liquid interface medium as done

when assessing infectivity of tonsil tissue alone (section 4.1.1.). The space between the Transwell^{TM} membrane and the bottom of the well is approximately 1mm, whereas tonsil tissue pieces were approximately 3mm^3. O-rings were placed on the plate to elevate the Transwell^{TM} to extend the space between the Transwell^{TM} and the bottom well. Enough medium was added to the bottom chamber to keep the basolateral surface of the cervix tissue on the Transwell^{TM} moist. Even under these conditions, tonsil tissue integrity was not maintained for 12 days in culture. Hematoxylin and eosin staining of uninfected and infected tonsil tissue showed that the tissue compartments were to some extent maintained up to day 7, although infected tissues showed more disintegration compared to uninfected. A much more dramatic loss of tissue integrity was observed in the tissue lymphoid compartments of HIV-1$_{BAL}$ infected tonsil tissue on day 12 compared to uninfected tissue. This data confirms the comparable characteristics to those seen in acutely HIV-1 infected individuals. The lymph node germinal center, rich in B cells, T cells and dendritic cells, is destroyed (figure 5), possibly by a strong immune response that releases proinflammatory cytokines and chemokines to fight HIV-1 infection.

We have shown viral infected cells in tonsil tissue from the combined organ culture model by immunofluorescence assay, but were not able to detect viral replication from HIV-1 p24 in culture supernatant (figure 9). The low levels of p24 detected from the culture supernatant could be due to focal infection in the tonsil tissue (Miller, 2005), thus, the production of HIV-1 p24 may not be enough to be detected by ELISA.

While standardizing the combined organ culture model we used tonsil cells as a complimentary system instead of tonsil tissue. Use of tonsil-isolated cells is beneficial because they can be frozen and used in many experiments, eliminating variability from experiment to experiment. However, tonsil-isolated cells do not mimic the in vivo situation like intact tonsil

tissue does. Other investigators have used tonsillar cells as a model to study HIV-1 infection in the oral cavity (Maher, 2004; Maher, 2005). We used three methods of tonsil cell isolation, i.e., lightly pressing tissue through a 60 μm mesh followed by Ficoll-hypaque density gradient centrifugation, lightly pressing tissue through a 60 μm mesh gradient without Ficoll gradient centrifugation, and cells obtained directly from minced tonsil tissue. While tonsil cells isolated by all three methods supported HIV-1 replication, minced tonsil tissue cells supported the highest level of replication. This may be due to the presence of other non-lymphoid cells, for instance, epithelial cells in minced tonsil tissue cells that may contribute to strong non-specific binding of HIV virions. Virions may not necessarily infect these cells but may remain active during longer periods of times in endocytic compartment of cells. Moreover, although both fresh and frozen-thawed cells supported HIV-1 replication, higher level of HIV-1 replication was noted with freshly isolated cells. Flow cytometric analysis of minced tonsil tissue tonsil cells showed that they contain appreciable levels of CD4+, CD11c+ and CD68+ cells that are known to be the target cells for HIV-1 infection. Tonsil cells on day 1 of cultivation showed that the majority of tonsil cells were CD20 (B cells) (86.7%), and that a substantial quantity of cells were CD4+ cells (26.6%) and CD11c+ (27.7%). Very low levels of CD68+ cells was present in tonsil cell population on day 1. The number of CD20+ and CD11c+ uninfected cells decreases at day 12 (5.4% and 2.3%, respectively) possibly due to the absence of antigen (HIV-1) or cytokines present in the medium, whereas, CD4+ cells increases to 87.2% as a result of IL-2 present in the culture medium. The levels of CD20+ and CD11c+ on day 12 post infection are higher (11.6% and 16.3%, respectively) as compared to uninfected cells on day 12, most likely due to the presence of HIV antigens in the culture medium providing a sub-optimal stimulation

to cells. Moreover, tonsil cell population on HIV-1$_{BAL}$ infected cells is considered to carry a good proportion of CD4+ (58.1%) and CD11+ (16.3%) cells following 12 days of cultivation.

Results from the combined cervical and tonsil cells demonstrated that viral replication is comparable to data seen in vivo from monkeys that have received intravaginal inoculation of SIV (Miller, 1994). For example, in the macaque model, the virus is replicating on cervical tissue before exiting to the lymphatic tissues the first 3-6 days (Miller, 2005; Collins, 2000), whereas, in our combined cervix-tissue model virus particles measured by HIV-1 p24 in culture supernatant were very low the first 6 days of infection. HIV-1 p24 in culture supernatant was always higher after days 6-12.

The tonsil cell types that become infected after transmission from cervical tissue were CD4+, CD11c+, and CD68+ as detected by immunofluorescence. More specifically, simultaneous immunophenotyping and ultrasensitive fluorescence in situ hybridization assay showed that 3.4% and 8.9% of CD4+ T cells expressed HIV-1 RNA on HIV-1$_{BAL}$ and HIV-1$_{IIIB}$, respectively. CD11+ results are pending.

Transmission of cell-associated virus was also examined since semen contains both free infectious HIV-1 and HIV-1 infected cells. Therefore, transmission through the cervix mucosa could occur by both cell-free and cell-associated HIV-1. We have shown viral replication in tonsil cells of cell-associated HIV-1$_{BAL}$ and HIV-1$_{IIIB}$. HIV-1 p24 levels in culture supernatant were approximately 100-fold lower than cell-free HIV-1$_{BAL}$ and HIV-1$_{IIIB}$. Therefore, cell-associated HIV-1 was transmitted less efficiently than cell-free HIV-1. However, similar number of CD4+ T cells (2.8% and 10.3% of CD4+ T cells from HIV-1$_{BAL}$ and HIV-1$_{IIIB}$, respectively) was detected to express HIV-1 RNA when compared in situ data of cell-free HIV-1 after crossing the cervix mucosa. These results are comparable to data from the rhesus

macaque in which cell-free virus was better transmitted than cell-associated SIV (Sodora, 1998). HIV-1$_{BAL}$ is most effciecntly transmitted across the cervix mucosa (Collins, 2000), however, tthese data show that HIV-1$_{IIIB}$ is more efficient at producing virus in tonsil cells than HIV-1$_{BAL}$ after crossing the cervix tissue. The preference for HIV-1$_{IIIB}$ in tonsil cells could be explained because tonsil cells express higher levels of CXCR4 coreceptor, thus, higher susceptibility to HIV-1$_{IIIB}$ (Grivel, 1999).

Identification of infected migratory cells was performed by a qualitative immunofluorescence assay which showed that migrating cells have the CD4, CD11c, and CD68 receptors and all of these cells carried HIV. A study by Hu et al (Hu, 2004) demonstrated by flow cytometry and immunfluorescence assay that the major cells migrating out of the cervix tissue were CD3+HLA-DR- and CD3$^-$ HLA-DR +, and that 90% of HLA-DR+ cells disseminated HIV-1. We were not able to study relative proportion of the principle cell types to be involved in HIV-1 transmission from cervix to the regional lymph nodes. However, future studies will use simultaneous in situ hybridization for HIV-1 gag/pol RNA and cellular immunophenotyping to quantify the major migratory cells from cervix tissue.

6.0   FUTURE DIRECTIONS

Future studies using the combined cervical and tonsil cell model will utilize the simultaneous immunophenotyping and ultrasensitive fluorescence in situ hybridization assay to determine if CD45RO (activated T cells), CD68 (macrophages), and CD11c (dendritic cells) express HIV-1 RNA, since we have only included data of CD4+ and CD68+ cells. Both cell-free and cell associated HIV-1$_{BAL}$ and HIV-1$_{IIIB}$ will be evaluated to determine coreceptor use of these HIV-1 RNA positive cells by staining for CCR5 and CXCR4.

To further characterize the HIV-1-infected migratory cells from cervical tissue, an EGFP reporter HIV-1 may be added to the cervical tissue in the combined cervix tissue-tonsil cell system. The migratory cells can be analyzed by both immunofluorescence assay and flow cytometric simultaneous immunophenotyping and ultrasensitive fluorescence in situ hybridization. The relative proportion of HIV-1 infected cells and their specific phenotype, determined by either technique, will indicate the major cells that may be involved of viral transmission and infection from cervix to the regional lymph nodes.

Proinflammatory cytokines stimulate HIV-1 replication by activating NF-κB, a cellular factor that activates HIV-1 transcription (Pantaleo, 1993). In contrast, chemokines can affect HIV-1 expression and transmission in a positive or negative manner. For example, chemokines such as MIP1-α, MIP1-β and RANTES can block HIV-1 infection by obstructing attachment of

the viral envelope protein to the viral co-receptor (Deng, 1996) while some chemokines, such as CCL21, CCL19 and RANTES, affect the migration of infected DC to draining lymph node. Thus, the organ culture model could be suitably used to study the effect of cytokines and chemokines on viral replication in tonsil tissue/cells once the virus crosses the cervical mucosa. The migration of leukocytes from cervical tissue may be tested on the effect that chemokines (MIP-1α, CCL21, CCL19, and RANTES) and cytokines (IL-4, IL1-β, TNFα, IL-6 and IL-8) have on viral replication in tonsil tissue. Other cytokines (IL-2, IL-7, IL-8, IL-10, IL-12p70, IFNγ) could also be tested to assess their involvement in migration of leukocytes from cervical tissues.

7.0   PUBLIC HEALTH SIGNIFICANCE

The study of HIV-1 transmission in humans is challenging because there is no appropriate in vitro model to study HIV-1 transmission across the cervicovaginal mucosa to its lymphatic distribution. Therefore, a combined cervical and tonsil (surrogate for lymph node) tissue-derived organ culture model will be used to study the mechanisms of viral transmission in the female genital tract. Moreover, it will be useful to examine the migration of virus and virus-infected cells to and replication in the regional lymph node after crossing the cervical mucosa. The benefits of this model are that it uses primary, intact tissues rather than a monolayer of cells, either primary or transformed, which provides the natural tissue architecture with epithelial cells, submucosa, and immune cells, such as T cells, macrophages and Langerhans cells, thus mimicking the in vivo situation.

Another important utility of the combined cervix tissue and tonsil tissue/cells model is its use for screening candidate topical microbicides. Microbicides are gels and creams that can kill or neutralize the virus when applied to the vagina. Therefore, microbicides may block the entry of HIV across the mucosal barrier to prevent HIV infection.

This model can also be used to determine the role of host factors such as semen, vaginal fluid, cytokines and chemokines produced by sexually transmitted infection-related microorganisms on such migration. Furthermore, the model can be used to determine the role of

virologic factors, for example, the dependency of HIV-1 transmission on virus phenotypes and subtypes.

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