IDENTIFICATION OF CELLULAR FACTORS INVOLVED IN NEISSERIA GONORRHOEA INDUCED ENHANCED HIV-1 TRANSMISSION IN A CERVICAL TISSUE BASED ORGAN CULTURE MODEL

by

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ABSTRACT

The presence of sexually transmitted infections (STI) such as *Neisseria gonorrhoeae (NG)* can enhance the transmission of HIV-1. Our goal in this study is to elucidate mechanism by which NG induces enhanced HIV-1 transmission. A cervical tissue based organ culture system was developed to study the interaction between NG and HIV-1 which provided a unique opportunity to elucidate mechanism of NG induced enhanced HIV-1 transmission across cervical mucosa. We demonstrated that the NG exposure on the cervical tissue in the organ culture model showed physical characteristics of NG infection and induced high levels of inflammatory cytokines IL-1β and TNF-α that have been observed during *in-vivo* NG infection in the cervix. In elucidating the mechanism of NG induced enhancement of HIV-1 transmission, we demonstrated that NG not by itself but the culture fluids from NG exposed tissues (reminiscent of cervical milieu) increased HIV-1 transcription from the HIV- LTR in TZM-bl cells, induced full length HIV-1 from latently infected U1 and ACH2 cells and increased transmission of HIV-1 across cervical mucosa. The whole genome transcriptome analysis using second-generation sequencing of the micro-dissected epithelial layer of the tissues exposed to NG and HIV-1 identified with high statistical significance differentially expressed genes in NG exposed and HIV-1 exposed tissues, and identified common cellular factors as well as pathways involved in cell activation, migration and stimulation expressed in the epithelia.

Out of these only two differentially expressed genes that were common between tissues exposed to both NG and HIV-1 are CXCL10 and IL8. Addition of inhibitors of CXCL10 and IL8 suppressed HIV-1 transmission, while addition of CXCL10 and IL8 increased HIV-1 transmission indicating that CXCL10 and IL8 could be involved in HIV-1 transmission across cervical epithelia. IL-1β also increased CXCL10 and IL8 expression in cervical tissues and enhanced HIV-1 transmission Altogether these data are consistent with a model (Figure 42) for NG induced enhanced HIV-1 transmission: NG infection secretes IL-1β which induces increased production of epithelial CXCL10 and IL-8 causing the migration of HIV-1 target cells CD3+T cells and macrophages towards intraepithelial region that fuels HIV-1 replication in submucosa and consequently enhances HIV-1 transmission.

Taken together, our results confirm that the risk of acquisition of HIV-1 infection in the ecto-cervical region increases with prior NG infection. From the public health perspective, identification of IL-1β and its target cellular proteins in NG induced enhanced HIV-1 transmission could be useful in development of drugs that impair HIV transmission. Further work in nonhuman primates or humanized mouse models could provide confirmation of the role of CXCL10 and IL-8 in HIV transmission and its modulation by NG secreted proteins like IL-1β in vivo.

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DEDICATION

To my Family

Jagabandhu, Anuradha and Aditya

with Love

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1.0 INTRODUCTION

1.1 OVERVIEW

Sexually Transmitted Infections (STI) are spread mainly by sexual contact, which include vaginal, anal and oral sex but some STIs can also be spread through blood or blood products. Many STIs—including HSV-1, *Neisseria gonorrhea*, *Chlamydia sp*, Syphilis, Hepatitis B and most importantly HIV-1 are STIs which are alarming to the growing population of the world. STI can also be asymptomatic in the earlier infection process, which makes it difficult for diagnosis and study of diseases and is an obvious problem in developing and underdeveloped countries where diagnosis is still dependent upon earlier ways of symptomatic diagnostic approach, which disables the treatment to start from the onset of the diseases. It is reported by many international agencies that more than 1 million STIs are acquired every day. STIs can have serious consequences beyond the immediate impact of the infection itself.

Out of the millions of STIs occurring every year, gonorrhea caused by *Neisseria gonorrhea* (NG) is responsible for a huge (78 million) part of it. Accurate diagnostic tests for STIs are widely used in high-income countries, which can be useful for the diagnosis of asymptomatic infections like NG in females, but in low- and middle-income countries, diagnostic tests are largely unavailable. Antibiotic resistance in particular to NG has increased rapidly in recent years and has reduced treatment options. The emergence of decreased

susceptibility of gonorrhea to oral and injectable cephalosporin as treatment options together with antimicrobial resistance already shown to penicillin, sulphonamides, tetracycline etc. make NG a multidrug-resistant organism. It is also difficult to design proper vaccines and implement them in underdeveloped and developing countries due to the high cost associated with it thus making it more important to study this STI causing microorganisms and its molecular functions to design cost and user effective vaccine and treatment options.

One of the greatest problems in today's world is human immunodeficiency virus type 1 (HIV-1) infection that is one of the most emergent STI in both developing as well as developed nations and is causing concern for public health organizations around the world. In the absence of an effective vaccine, microbicides are now becoming important tools for HIV-1 prevention not only in poorer nations but worldwide. But most of all, NG can increase the risk of acquisition of other STIs to many folds [1-5]. Understanding the mechanism of enhanced HIV-1acquisition by NG is important and is the major goal of the project. It is difficult to study directly the role of NG on increased HIV-1 acquisition in females due to ethical reasons and lack of proper models as we discuss with the flow of the project report. Therefore in this study, we used a cervical tissue based organ culture that has earlier been extensively used by our group to study viral transmission from cell-free and cell-associated HIV-1[6, 7] to examine the effect of NG on HIV-1 transmission and investigate cellular factors, which may be responsible for this enhanced acquisition. We also evaluated the antiviral activity of some microbicides against HIV-1 in the presence of NG [7-9].

1.2 NEISSERIA GONORRHOEA (NG)

1.2.1 Background and Epidemiology

Neisseria gonorrhoeae (NG), a human obligate gram negative, diplococcal bacterium (Figure 1), is the causative agent of gonorrhea which is one of the oldest human diseases recorded. NG does not infect other animals or experimental animals and does not survive freely in the environment. The gonococcal infection occurs in the upper or lower tract, pharynx, ophthalmic area, rectum, and bloodstream. It still remains a major global health problem affecting an approximate of more than 80 million individuals throughout the world. Over 300,000 cases of infection have been reported in USA alone annually which makes NG infection the second most common STI in USA[10, 11]. Teenagers and adults are the most highly effected by this disease demographically [11]. Though initially the infection was believed to be a threat in men but it is greater in females and more adverse as seen in recent statistics.

Both sexually active men and women are susceptible to NG infection irrespective of races, ages, and socioeconomic backgrounds. However, according to CDC, 80% of the females and 10% of the males are asymptomatic out of the infected population. Males tend to display symptoms of swelling in the urethra, painful and more frequent urination, and abnormal penal discharge of a thick yellow exudate after five to seven days post exposure and infection. Females also experience chronic abdominal pain; inflammation of the cervix, painful urination, bleeding or irregular menstrual cycles, fever and increased vaginal yellow discharge but in most cases this happens after the infection is chronic in the system. Females also display 60- 90% higher risk of being infected after a single sexual encounter since epithelium of the female cervix are rich in receptors for this bacteria.

The significance of gonorrhea is further highlighted by the findings that gonococcal infection increases the risk of HIV-1 infection and co-infections of other sexually transmitted pathogens. Teenagers and young adults are at especially high risk for infection, which is troubling since there is an increased risk associated with gonorrhea for infection with HIV-1[12, 13]. It has been reported that there is an acute inflammatory response generated with NG infection and disease progression in patients which is later followed by loss of mucosal integrity which is then associated with the increased susceptibility to HIV-1 type 1 [14]. Ascending gonococcal infection occurs in up to 45% of infected women.

1.2.2 Symptoms and infection

NG infection primarily infects the urogenital epithelia of (urethral cells in men cervical cells in females) and humans. In males this is usually a more symptomatic infection, which enables early diagnosis and treatment. In contrast, the female infection is more asymptomatic and usually cannot be detected till late in the chronic stage. This prevents early diagnosis and treatment and also the study of the molecular mechanisms of the disease and host cellular responses to it. This late diagnosis causes extended colonization and chronic infection, which may lead to PID. This leads to scarring of the reproductive organs, resulting in chronic pelvic pan, ectopic pregnancy and/or infertility. The highest incidence areas included Africa and the Western Pacific (including China and Australia) regions and in less well developed countries.

Many virulence factors have been identified for NG that allows it to successfully adapt to variable microenvironments within its sole human host. This human adaptability and the repeated phase and antigenic change displayed by the gonococcus have hampered vaccine development. Many of the major virulence and adherence factors undergo constant phase and/or

antigenic variation[15, 16], which include the Pilus (Pil) [17, 18] and the Opacity-associated (Opa) outer membrane proteins [17, 19, 20]. The lipooligosaccharide (LOS) [21-24] has been seen to have some degree of phase variation but the Porin protein expression is stable for any NG strain. Women tend to be more asymptomatic based on a complement receptor 3 (CR3), localized in cervical epithelial cells, which does not activate an inflammatory response. An alternate effect for subdued inflammatory response is a ruffling of cell membranes when *NG* infects the cervical epithelium. The CR3 is absent in the male urogenital tract and when infected with NG there is no membrane ruffling. In contrast, infected males have an inflammatory response in the urethra.

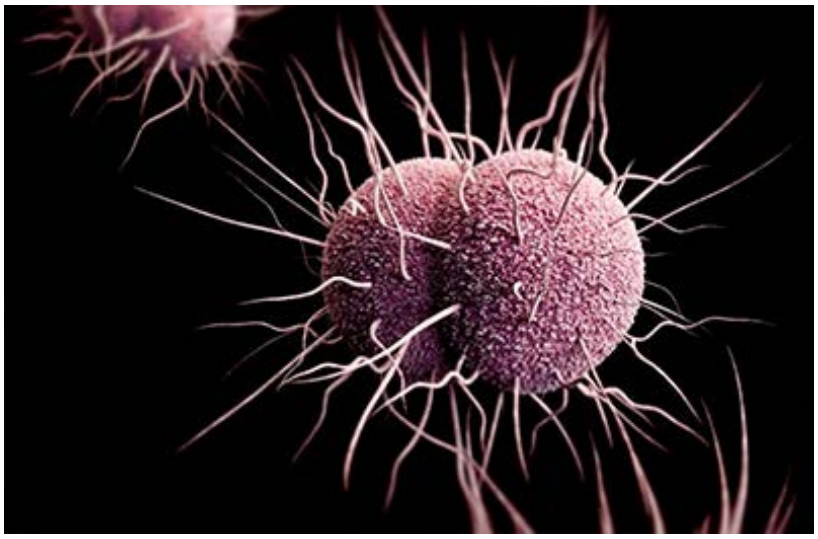


Figure 1: Computer generated structure of *Neisseria gonorrhea* (NG)

1.2.3 Transmission

Close sexual contact with an infected individual serves as the primary mode of *NG* transmission but pregnant women with gonococcal disease can transmit this bacterium to their children during delivery. NG can be transmitted more efficiently from an infected man to a woman independent

of number of exposures than from an infected woman to a man mainly due to the anatomical differences in the genitalia of males and females [25]. Transmission is common through anal, vaginal, and oral sex. Core group members that have multiple sex partners are at high risk for acquiring and transmitting gonorrhea. Vertical transmission occurs from mother to child during pregnancy or birth. Consequently, the child contracts gonorrhea in the eyes, which can lead to blindness.

1.2.4 Entry

NG does not thrive in the environment and grows in a CO_2 environment making the urethra, cervix, rectum, and throat the main sites of infection. After the NG enters into the mucous membrane of these areas, they migrate to their primary target, which are the columnar non-ciliated epithelial cells. NG uses a type IV pilus to anchor itself at the infection site and has been found to be the main factor for adherence to epithelial cells. Pilus mutants have been found to have defect in filament assembly, and thus adhere less to human epithelial cells[26].

To establish the infection, NG interacts with the epithelial cells of the genital tract using the outer surface molecules. The main surface components of NG are pili, porin, opacity-associated (Opa) proteins, and lipooligosaccharide (LOS). The type IV pili complex, consisting of the major protein Pil E and minor protein Pil C, is responsible for initiating interaction with the host cell[27, 28]. NG Opa protein in different antigenic variations also binds up to four different (CEACAM) receptors [29]. Most human cells encountered by NG usually express a combination of all these CEACAM receptors which also help in the entry of NG into the cells, Fcγ, CEACAM, HSPG, and integrin receptors present on the cell surface may potentially all play a role in NG adherence and/or internalization [30]. Eleven different Opa proteins, encoded by

eleven different genes, are responsible for interactions of NG with the host cells. Porin, the most abundant and the only non-variance surface protein on the NG surface, forms anion selective channels in the host and is required for the NG survival (Figure 2). These porin can also be transferred from the bacteria to human epithelial cells, which has been shown to induce transient calcium influx[31]. The LOS can be sialylated in the host system protecting it from recognition by the host immune factors, which include the bactericidal antibodies and complement system and allowes the NG to survive and spread in the host [32].

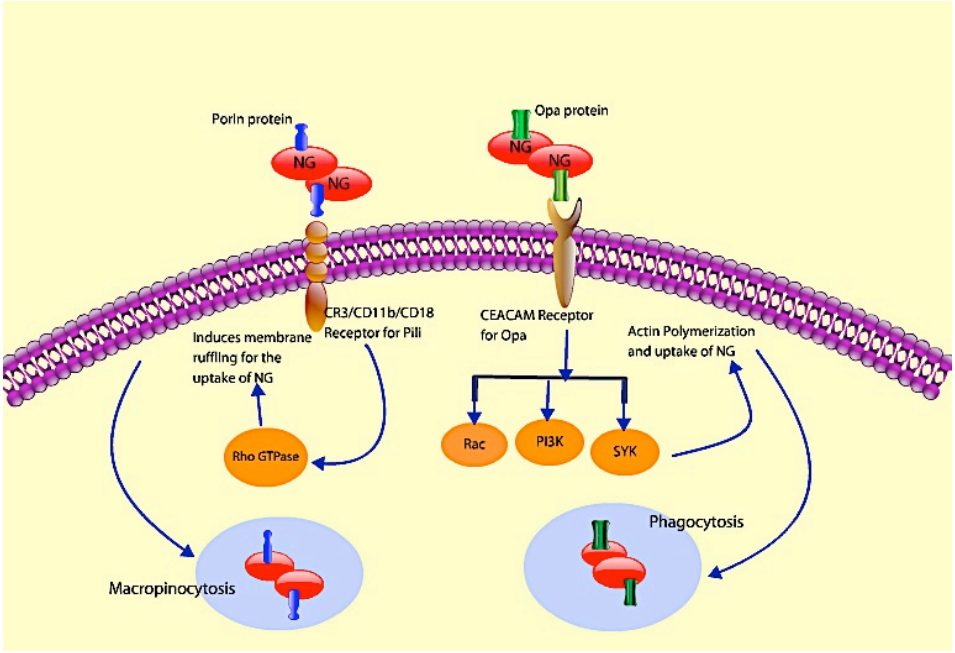


Figure 2: Adhesion and Internalization of NG into host cells

1.2.5 Multiplication

NG can undergo both phase and antigenic variation, which enables them to evade the host defense mechanisms and propagate within the host[33]. The Opa proteins can be switched on or

off randomly post entry thereby changing NG-NG and NG-host cell interactions[34]. It has been hypothesized that this ability of Opa surface molecules [35-37] makes it possible for NG to infect various anatomic locations of epithelium and generate different pathological conditions of the infection. Replication of NG begins with the adherence to non-ciliated cells resulting in death triggered by the LOS. They undergo parasite-directed endocytosis where they are taken up by the microvilli of the non-ciliated cells acting like phagocytes. Inside the cell, the NG replicate within vacuoles where they can avoid phagocytosis, antibodies, and anti-microbial agents. They are transported to the base of the non-ciliated cells in these vacuoles where they are exocytosed into sub-epithelial tissue creating inflammation or disseminating into the bloodstream.

1.2.6 Outer membrane constituents associated with NG virulence

Prior reports have demonstrated that pili protein play a critical role in forming an initial attachment with host cells, exhibit twitching motility and provides mechanism by which non-motile gonococci are able to colonize and to ascend mucosal[38-42]. CD46 (or membrane cofactor receptor), which is a complement regulatory protein, is a human-specific transmembrane protein expressed by all nucleated cells and has been reported to serve as a receptor for NG pilus[43, 44]. Pilus has also been demonstrated to play a role in host cell cytoskeletal rearrangements [45-47]. All these data together suggest that pili can modulate host cell signaling mechanisms to aid NG epithelial invasion.

Opa proteins are divided into two classes based on their ability to recognize host cell surface molecules and are thought to contribute to the cellular tropism exhibited by NG; Opa50 recognize host cell HSPG, and Opa52 recognize members of the CEACAM/CD66. A direct Opa_{50}-HSPG interaction has been demonstrated in conjunctiva epithelial cells. This interaction is

thought to trigger a signal transduction cascade resulting in the modulation of the cytoskeletal rearrangements required for endocytosis of the cell-associated gonococcus [48, 49]. Opa-expressing NG clinical isolates are able to bind CEACAM1, suggesting that an Opa-CEACAM association can occur in-vivo and such interaction has also been demonstrated on professional phagocytes [50-53].

LOS expressed by NG may modulate Opa and host cell interaction. NG lacks LPS that is prevalent among gram-negative bacteria. LOS on NG lacks the repeating O-antigen sugar that comprises the polysaccharide side chain of the LPS. LPS[54]. The oligosaccharide substitutions of LOS exhibit variability [55-57]. The spontaneous conversion of oligosaccharide determinants can change the manner in which the NG associates with host tissues. LOS oligosaccharide side chains terminate in epitopes those molecularly mimic sugar moieties of mammalian glycosphingolipids providing NG with a method of immune avoidance [54, 58-62].

Porin, the water-filled channel through which small molecules traverse the NG outer membrane is thought to play multiple roles in potentiating disease. A unique feature ascribed to NG porin is its ability to translocate into eukaryotic cell membranes [63, 64]where it forms a voltage-gated channel that is modulated by host cell ATP and GTP [65]. In recent studies, it has been observed that porin may be able to induce apoptosis in epithelial cells during mucosal infection *in-vivo* but such observation was not true for in-vitro epithelial cell studies [66].

1.2.7 Response to host defense

Once a host is infected with NG, it is one of the rare bacteria, which does not form an immune reinfection due to its capability of antigenic variation where it can rearrange proteins on its surface. Pili genes can be altered due to the high rate of genetic rearrangement producing new

variants making it possible to evade the host immune response. Opacity proteins are surface proteins that have a critical role in the adhesion to epithelial, endothelial cells and polymorphonuclear neutrophils [51]. It has been found that Opa proteins bind to CEACAM which is a receptor on the CD4+ T cells and this interaction inactivates the proliferation of the and hinders the memory of the immune system as well as increases susceptibility to opportunistic pathogens and co-infections with other STIs. There is also an IgA1 protease secreted by NG that cleaves the IgA1subunit of the IgA immuno-globin, which protects the host's mucous membranes and makes the mucous membrane more susceptible to infection.

1.2.8 Immune response against NG

The immune response generated to NG is broad. Tissue phagocytes are able to kill the bacteria in the phagolysosomes after entry and multiplication by producing reactive oxygen and nitrogen intermediates and proteolytic enzymes in case of mild infections. In addition, these cells are able to secrete pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8 and TNF-α, which initiates inflammatory response[67]. In early stages of infections, interactions between NG and epithelial mucosa triggers immune response with release of these cytokines, which serve to recruit and activate PMN to the site of infection, (Figure 3) induce their migration across the epithelium, where they bind and phagocytose the bacteria and promote their bactericidal activity limiting bacterial penetration into sub-mucosal tissues [68-70]

After exposure to pro- inflammatory cytokines and microbial products, immature DCs undergo maturation involving up-regulation of MHC molecules, co-stimulatory molecules, secretion of pro-inflammatory cytokines and migration to draining lymph node where mature DCs activate T cells [71]. In women with NG, the cervical secretions also contain PMNn [72]

and bacteria are attached to and within PMNn [73]. When bacteria crosses the layer of epithelial cells, they encounter macrophages and DCs[74]. Interaction of DCs with NG involves Pili and Opa on NG mediated by members of the CD66 family, CD46 and CR3 on host cells but the C-type lectins like MLG and DC-SIGN are the the main DCs expressed receptor for NG[75].

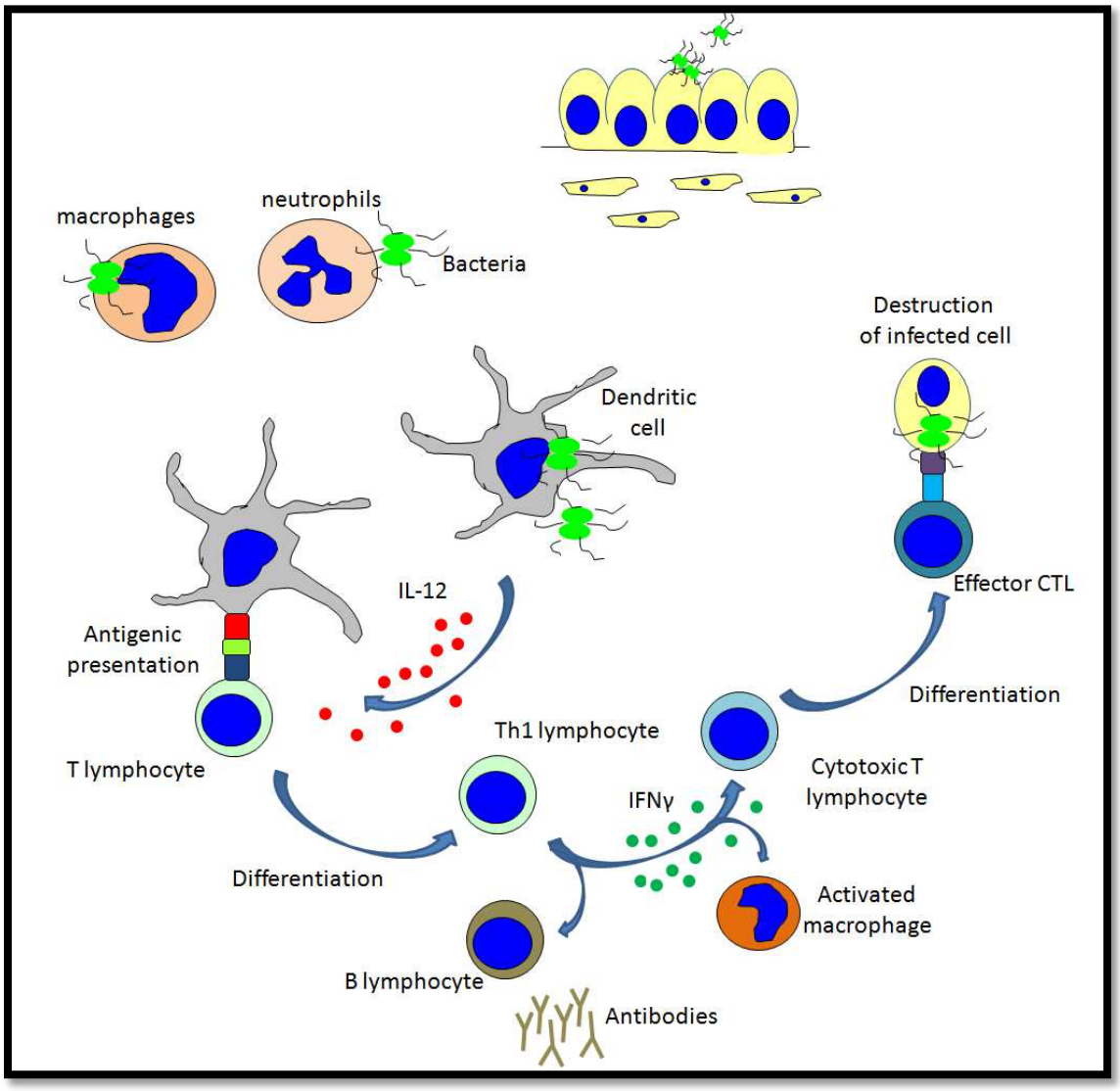


Figure 3: Representation of summary of immune response against NG

1.3 HIV-1 AND AIDS

1.3.1 Origin

Human Immunodeficiency Virus also referred to, as HIV-1 is the causative agent for one of the most alarming sexually transmitted infections discovered in the twentieth century, which came to be known as AIDS. In 1981, unusual clusters of *Pneumocystis carinii,* pneumonia and Kaposi's sarcoma were reported by the Centers for Disease Control and Prevention among gay men in parts of the US [76-78]. These were the first reported cases of Acquired Immune Deficiency Syndrome (AIDS). More than thirty years later, the global HIV/AIDS epidemic has killed millions of people and millions more are living with the infection Acquired Immuno Deficiency Syndrome (AIDS) [79]. This virus attacks the immune system of the body and decreases the body's natural ability to fight infections. The virus primarily infects and destroys the CD4+ T helper cells and even if untreated it takes about 10-15 years to develop AIDS. The decrease in the number of CD4 positive T helper cells after infection with HIV-1 weakens the immune system and makes it susceptible to secondary infections and if untreated the person infected with HIV-1 usually succumbs to these infections [80].

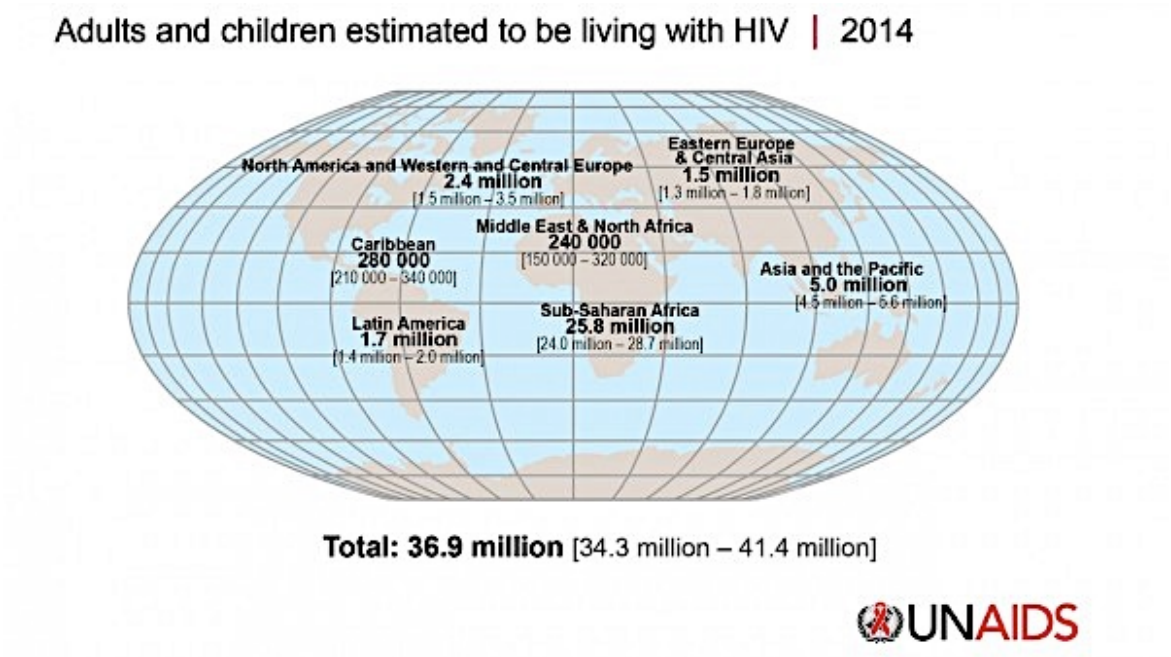
There are mainly two types of HIV-1 that causes AIDS among the human population [81]. The HIV-1 is more virulent, more easily transmitted and is the cause of the vast majority of HIV-1 infections globally also has a higher progression rate and the HIV-2 found mainly in the western part of Africa, which is less pathogenic, less transmissible with a slower progression rate leading to AIDS[82] [83]. HIV-1 is the most commonly found and is believed to have first originated from the Simian Immunodeficiency Virus (SIV) in a particular specie of Chimpanzee (*Pans tranglodytes tranglodytes*) [84] and in wild gorillas that got transmitted to the humans

through hunting and blood contamination[85]. These HIV-1 formed four phylogenetic groups with genetic differences after diverging from their descendants with the course of transmission [86]. These groups were M or main group [87]that originated from chimpanzees and group O also referred to as the outlier group[88] that formed from the gorillas from Cameroon. There are two other less prevalent groups of HIV-1 which have slower progression compared to M and O groups: the N group (the non M non O group) and the P (putative) group the origin of both these groups are still unknown [89]. Group M among the variants of HIV-1 is the most prevalent globally and had now expanded into several other subgroups A-D, F-H, J and K [90].

1.3.2 Epidemiology

The transmission of HIV-1 is still a widely discussed and concerning subject in this era and have a wide variety of modes of transmission. In spite of knowing all these modes of transmission, it is still not fully understood why the HIV-1 epidemic has spread so heterogeneously across the globe. There are many internal as well as external factors, which influence the transmission of HIV-1. Heterosexual transmission at mucosal surfaces is the most common route of HIV-1 infection, and women account for nearly half of HIV-1- infected individuals worldwide and more than 70% in sub- Saharan Africa[91, 92]The susceptibility of an individual to HIV-1 infection may also depend on predisposing genetic factors, as well as the presence of preexisting infections by other microbial agents which may be major factors driving the different HIV-1 epidemics. Of all the regions around the world, sub-Saharan Among south east Asia, Thailand is also hit by high explosive epidemics in the general population due to increase in injection drug use (IDU) and sex work which are conducive to the spread of HIV. There has also been an epidemic among IDU in the former Soviet Union that spread to the general population by sexual

activity. Some countries in Central America and the Caribbean have growing HIV-1 epidemics with adult prevalence second only to sub-Saharan Africa. More than 1.2 million people in the United States alone are living with this infection, and 12.8% of them are unaware of the infection in their body [93]. Recent reports also by the UNAIDS estimated that 36.9 million people were living with the virus, and that number continues to increase because 15 million of them now are taking lifesaving antiretroviral drugs. South Africa alone has 6.8 million HIV-infected people—more than any other country (Figure 4) [94] .



(open access journal)

Figure 4: UNAIDS global reports of new estimates of people living with HIV-1 in different regions of the world in 2014.

1.3.3 Structure

HIV-1 belongs to a group of retrovirus called the lentivirus. Each virus particle comprises a viral envelope that is associated with a matrix enclosing a conical capsid composed of p24 protein. This encloses two copies of its genome, made up of single stranded positive sense RNA. This RNA is 9.7 kb long and comprises of a 5' cap (Gppp), a 3' poly A tail which encloses several open reading frames. The structural viral proteins are typically produced from longer ORFs and the smaller ORFs contains transcripts which code for regulator proteins required in the viral life cycle starting from binding, fusion, attachment, entry, replication and assembly. This RNA is bound by p7 (nucleocapsid) and p6 (late assembly) proteins along with enzymes required for the production of virion which are the reverse transcriptase and integrase. The nucleocapsid associated with the genomic RNA protects the RNA from digestion. Virion particle also carries the accessory proteins Vif, Vpr, Nef and the viral proteases and the p17 protein surrounds the capsid to secure the virion. The entire virion is enveloped in a protein case derived from the host, which includes the gp120 and gp41 proteins (Figure 5).

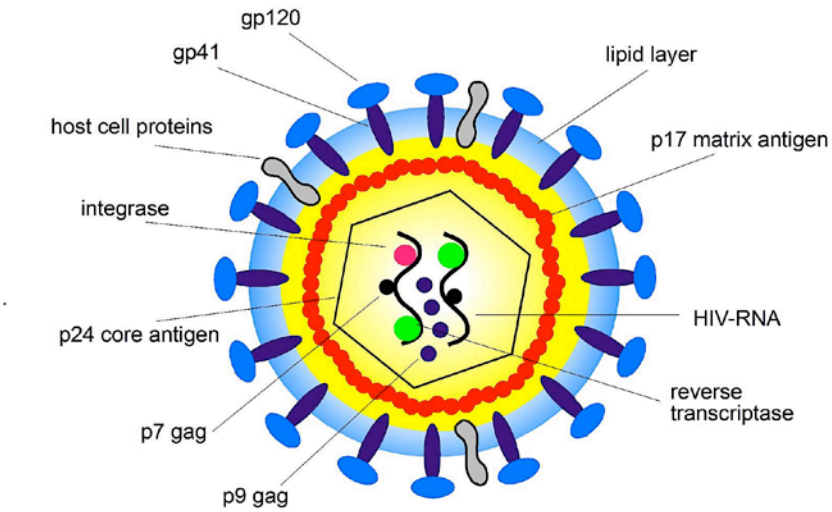


Figure 5: Structure of HIV-1 virion

1.3.4 HIV-1: LIFE CYCLE:

1.3.4.1 Un-coating of the virion 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, which enters the cell during the initial process. This is then released into the host cytoplasm and is dissociated from the capsid protein subunit after undergoing morphological changes. Host factors like Cyclophilin-A are required for uncoating which is a crucial step for the nuclear import of the virus [95, 96].

Following the process of fusion and un-coating of the virion, the two strands of HIV-1 RNA along with the protease, integrase and reverse transcriptase are released into the host cell cytoplasm which along with the host cell proteins like transportin-SR2 and importin 7, and viral proteins form the pre-integration complex and aided by Vpr gets translocated into the nucleus [97, 98]. The RT, which is the most important of the viral proteins, consists of many subunits. The first subunit converts ssRNA to ssDNA, second subunit aids in the conversion of ssDNA to dsDNA using polymerase domain and finally the RNA template is cleaved and degraded by the RNase H domain. The low proofreading ability of reverse transcriptase results in high mutation rates of HIV-1. IN protein catalyzes the integration of viral dsDNA by cleaving the 3’ end of the viral DNA into the sites of the host chromosome that are being actively transcribed at that moment [99]. This integrated retrovirus is the provirus, which contains two long terminal repeat (LTR), which plays very important roles in transcriptional regulation of the provirus (Figure 6).

1.3.4.2 Transcription and Translation

HIV-1 uses the host cell RNA pol II to transcribe the integrated provirus DNA along with the help of the viral transcription activator (Tat)[100], which is a unique sequence-specific

transcription activator for HIV-1. These Tat proteins produced from the initial viral transcripts are imported into the nucleus to stimulate transcription of proviral DNA. These viral RNA then undergo post-transcriptional modification by usual 5'cap and 3' poly A tail formation and then spliced. Tat and Rev proteins are produced from multiple spliced transcripts while some unspliced transcripts are packaged into virions or used for the synthesis of polyprotein precursors like Gag and Gag-pol. Rev protein transports into the nucleus, attaches itself to the Rev response element in the Env and transports the transcript back to the cytoplasm which are then translated into the viral proteins and packaged into virions [101].

1.3.4.3 HIV-1: LIFE CYCLE

Assembly of virion and Maturation: After synthesis of the viral RNA and its transport into the cytoplasm where the translation occurs using host cell machinery, the viral proteins and RNA are packaged to form the virion. These are then directed to the plasma membrane for assembly. The assembly of viral components takes place by the myristylation of the N- terminal of viral Gag polyprotein, which is then followed by the budding of assembled viral components from the plasma membrane and the cleavage of Gag and Gag-Pol polyproteins by viral proteases for the formation of matured virion[102, 103].

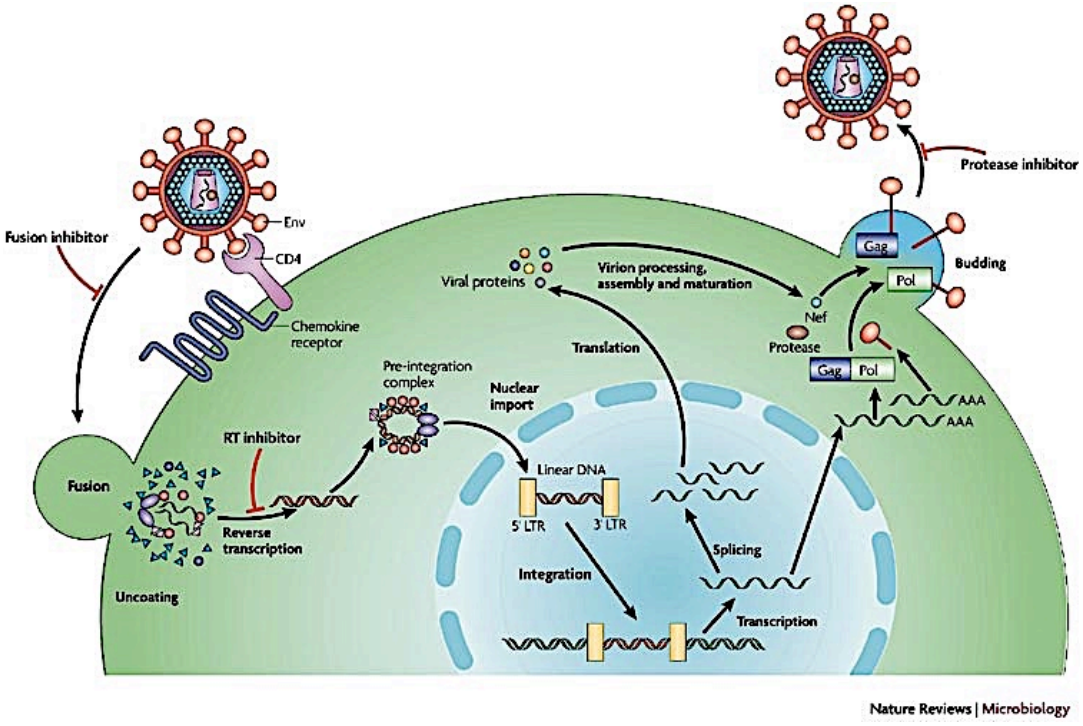


Figure 6: Schematic representation of the HIV-1 life cycle.

Figure taken from Yefei Han et, al. Nature Reviews Microbiology 5, 95-106 [101] Permission granted from rights holder, Nature Publishing Group.

1.3.5 HIV-1: Pathogenesis

HIV-1 causes disease in humans by infecting and disrupting the innate and adaptive immune systems. The course of the viral infection can be classified into broad stages namely the acute stage, the chronic stage and the AIDS stage. These infection states are mainly based on the patients CD4 counts, viral loads and the clinical presentation.

When the HIV-1 first infects the system it is characterized by an initial high peak of plasma viremia and associated depletion of CD4+ T cells and is known as the acute phase of infection. This onset and reduction of CD4+ T cells in the peripheral blood is followed by a prominent and drastic reduction of CD4+ T cells in the gut-associated lymphoid tissues due to

the HIV-1 targeting and infecting the CCR5+ CD4+ memory T cells [104, 105]. Numerous studies have pointed out that seropositive patients usually lose the majority of mucosal CD4+ T cells within the third week of infection, following a decline in the CD4+ T cell count in the first few days of infection [106-108]. This course of event is followed by production of neutralizing antibodies by the B cells but its function in the viral infection control is still not clearly understood.

This is then followed by development of adaptive immune response and HIV-1 specific CD8+ T cells, which leads to a decrease in the very high viremia, and this response is important for determining the plasma set-point of HIV-1 and the subsequent CD4+ T cell decline of the patient in the course of infection [109]. This Acute phase infection characterized by rapid increase in viral load and decrease in the T cell counts is followed by the chronic stage where there is reduced and stable viral load and CD4+ T cell numbers recover and stabilize[107, 108]. The chronic stage does have a high amount of immune activation with increase in cytokines and chemokine, which leads to further increase in CD4+ T cells which is also a target for HIV-1[105]. The reduction in the number of Th17 CD4+ regulatory T cells in the gut leads to increase in the intestinal permeability and microbial translocation across the gut membrane leading to an increase in the activation of immune cells [110, 111].

During the later stage of chronic infection, extreme immune activation causes loss of CD4+T cells, and apoptic loss of CD8+ T cells and B cells all of which are the main cause of HIV-1 pathogenesis [112-114]. The last stage of HIV-1 infection is AIDS that is characterized by dropping CD4+ T cell numbers of 200/mm^3 or lower[115, 116]. During this course, naive T cell population are depleted, memory T cells degraded and a further exploded immune activation leads to loss of B cell, which progresses the disease [108, 117]. The CD8+ T cells stop receiving

effective and appropriate co-stimulatory signals from CD4+ T cells which results in activation-induced cell death of CD8+ T cells upon re-stimulation with HIV-1 antigen[118, 119]. The resulting immunodeficiency leads to opportunistic infections such as kaposi's sarcoma, tuberculosis, pneumonia, cryptococcosis and finally to the body's immune system completely surrendering to the virus (figure 7) [108].

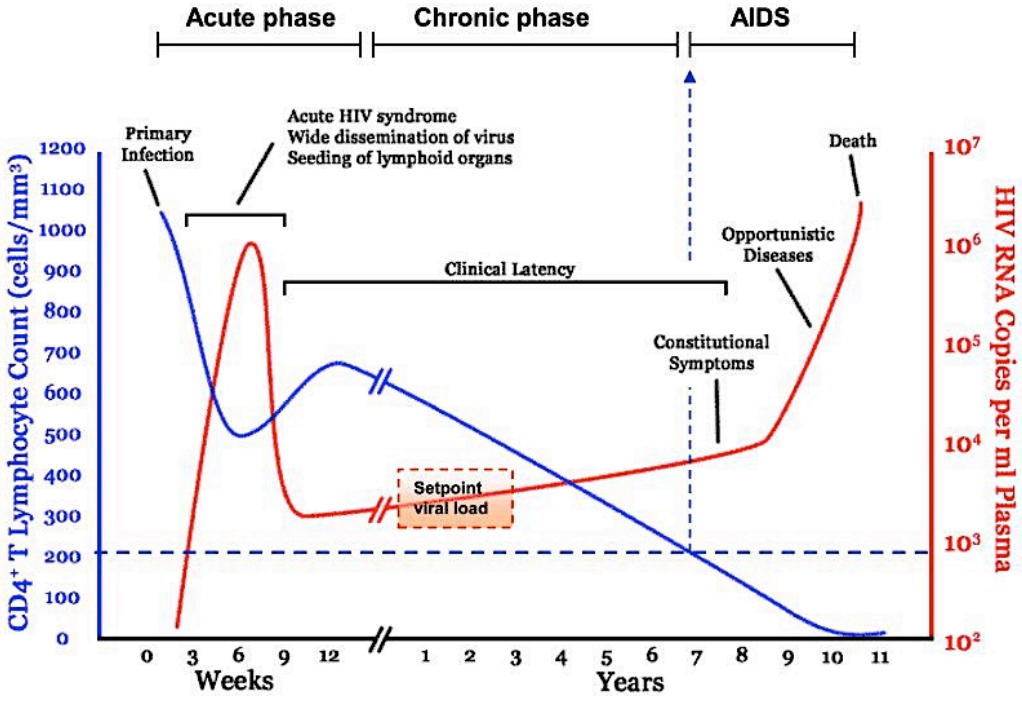


Figure 7: Course of HIV-1 infection and pathogenesis [120]

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

1.3.6 Epidemiology of HIV-1 transmission

HIV-1 transmission occurs mainly through usage of contaminated needle among IDUs or by blood transfusion, which are both referred to as parenteral transmission, or mother-child

transmission and primarily during sexual intercourse [121-124]. Some population is effected more than the general population by HIV-1. They are the drug users, sex workers, homosexual men, and transgender women and men. The spread of HIV-1 occurs primarily through the genital and rectal mucosa during sexual intercourse [125] which presently accounts for the largest demography of HIV-1 transmission in adults. The ecto-cervical and vaginal tissues have underlining HIV-1 target cell population like dendritic cells and spatially distributed CD4+T cells just beneath the epithelial layer, which makes it easier for the infection process to occur [126, 127]. Thus once the virus traverses the mucosal barrier, they can easily access and infect the underlying target cells.

1.3.7 Cellular and molecular mechanisms of HIV-1 transmission

Heterosexual transmission at mucosal surfaces is the most common route of HIV-1 infection, and women account for nearly half of HIV-1- infected individuals worldwide and more than 70% in sub- Saharan Africa [11, 91]. The relative contributions of vaginal, ecto-cervical and endo-cervical transmission of HIV-1 is not known, but due to the larger surface area and presence of relatively less mucus, the vagina and ecto-cervix are considered the primary sites for HIV-1 infection. Many different mechanisms for HIV-1 infection across vaginal/cervical mucosa have been suggested. Haldik et al demonstrated that superficial epithelial cells do not appear to be susceptible to endocytosis and viral particles are less likely to be transcytosed through the outer layers of squamous epithelium[126].

Breakage in mucosal epithelia during intercourse is one of the natural ways for HIV-1 to cross the epithelial layer, but HIV-1 could also cross the epithelial layer via other a-traumatic mechanisms but how HIV-1 crosses the cervical/vaginal epithelium layer under a-traumatic

condition are currently unknown. This might occur by increasing receptor expression, HIV-1-LTR activation, enhance HIV-1 infection in cells or bring more target cells in the vicinity of infection for HIV-1 to infect. The local proliferation of founder population of the HIV-1 takes place within the first week of infection, and produces viruses and virus-infected cells [128, 129]. This localised expansion of HIV-1 is important to cause further dissemination of the infection to the draining lymph nodes [129, 130]. During the second week of infection, virus spreads to the lymphatic tissue[131]. These lymph nodes have higher population of HIV-1-target cells, compared to the portal of entry and it is here that the virus replicates and spreads rapidly [129, 130]. This virus level in the blood peaks up at the end of the second week and gradually declines to a steady level by week four [126, 127]. The virus continues to replicate in these lymphatic organs and the proviruses gets integrated into the host genome and are hidden in latently infected cells [132]. These lymphatic regions are therefore reservoirs for viruses and during reactivation, it can infect the nearby CD4+T cells in these regions which then undergo depletion, eventually leading to disease progression [126, 127].

1.3.8 Types of HIV-1 transmission

Parenteral transmission can also occur by the transfusion of infected blood, so screening blood and reducing the unnecessary use of transfusions are necessary to minimize transmission by this route. Contaminated needles for injections and needle stick injuries among health professionals are another source of infection. Contaminated blood products and infected organs or semen have also been shown to transmit infection. IDU transferred HIV-1 is also known as another source of parenteral transmission of HIV-1.

Mother-to-child transmission (MTCT) of HIV-1 is another extremely significant route of transmission and millions of children worldwide has been infected with the virus since the onset of the epidemic. This is believed to be responsible for a large set of transmission from the early days of epidemic (UNAIDS). Two thirds of MTCT occurs in the uterus and during delivery and about one-third occurs during the process of breast-feeding. (Highly active anti-retroviral therapy) HAART have been shown to be highly effective in preventing MTCT to nearly 90 % but shorter, cheaper regimens have also been shown to reduce MTCT by 50% in infants who are not breast fed during the gestation process (UNAIDS)[133]

Sexual transmission is by far the most common mode of transmission globally. Although the spread of HIV-1 is not very highly efficient from seropositive men to sero-negative women, occurring in only about one of every 1,000 episodes of unprotected sexual intercourse, there are epidemiologic and clinical studies that strongly indicate that the presence of other ulcerative and non-ulcerative sexually transmitted infections (STIs) increase the likelihood of HIV-1 transmission along with many other factors[134]. Though male to female transmission rate is about 2 to 3 times more effective than female to male and there is some evidence that first sexual intercourse for females may be associated with particularly high transmission probabilities [135]. The rate of sexual transmission also depends on the infectiousness of the infected partner. Higher viral loads in the later stages of the disease are associated with increased probability of transmission but infectiousness has also been shown to be higher around the time of sero-conversion since it is the acute stage of the disease.

1.3.9 Mechanisms of sexual transmission

Intact vaginal and cervical epithelium provides a considerable barrier to HIV-1 infection [136] but it is not clear how wither cell-free or associated HIV-1 enters the intact mucosa to start an infection through the sexual route[137]. The rate of HIV-1 transmission in a single exposure from an infected male to an uninfected female is 3 in 1000 making this sexual route extremely inefficient [138-140]. This low transmission to women can be affected by factors including stages of menstrual cycle, vaginal pH, endocrine hormone levels and contraceptive use[138, 139][141]. HIV-1 virions and infected lymphocytes are inactivated by the low pH present in vaginal secretions making the process more inefficient. Vaginal pH can be raised to 7.0 by presence of blood and semen in the vagina either during the menstrual cycle or intercourse respectively. Inflammation and trauma to the vaginal epithelia, caused by other sexually transmitted diseases, and hormonal contraceptives can increase the risk of HIV-1 transmission, which may allow the immigration of the virus to susceptible cells in the sub-mucosa [142, 143].

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 DC-SIGN (same as for NG) can either confine the virus in the lumen[144], or become infected and transfer the virus to susceptible cells. It has also been demonstrated that gp340 on cervico-vaginal epithelium binds HIV-1 envelope and can enhance the entry of HIV-1 through the epithelium[145].

Several entry mechanisms have also been discussed. They are a) entry of cell-free virus into epithelial cell junction, captured internalized by intraepithelial Langerhans cells; b) fusion and productive infection of virus with the intraepithelial CD4+ T cells; c) migration of cell-free or cell-associated virus through un-breached vaginal epithelium into the mucosal stroma and then

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[146]. Regardless of all these mechanisms for HIV-1 transmission, virus crosses the mucosa and infects underlying susceptible cells. Heterosexual sex accounts for 80% of new infections[147] and the cervix and vagina are the main sites of HIV-1 acquisition during intercourse[148, 149].

1.3.10 Factors effecting sexual transmission of HIV-1

There are many viral and host factors that influence the transmission pattern of sexual transmission of HIV-1.

1.3.10.1 Mucosal integrity

The physical factors are very important in influencing the acquisition and sexual transmission of the HIV-1. Presence of genital ulceration infections plays a major role in HIV-1 transmission [135, 150], which has been proposed to be one of the main reasons for the rapid spread of HIV-1 in sub-Saharan Africa [151]. Male circumcision is also associated with protection from HIV-1 infection since there is a high frequency of dendritic cells in the mucosa of the foreskin that could provide an accessible pool of HIV-susceptible target cells for infection to take place [151, 152].

1.3.10.2 Hormonal factors

Hormones can also influence mucosal integrity. 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. The hormones, which peak during these different phases of

menstrual cycle, play a crucial role in antiviral defense in the female genital tract [153-155]. It has been shown in animal models that Macaques treated with progesterone were more susceptible to SIV infection through vaginal exposure[156] whereas animals administered with estrogen had a protective effect[157]. The influence of hormone on infection risk has not been well studied in humans, but the use of any forms of progesterone contraception is thought to increase the likelihood of infection [158].

1.3.10.3 Genetic factors

Genetic factors also play a role in susceptibility to HIV-1 transmission. The most common of these genetic factors is the deletion of the 32nd amino acid in the major CCR5 chemokine co-receptor for HIV-1 entry into the CD4+ T cells known as the CCR5Δ32 mutation, which lowers the transmission and infection chances in a cell [159, 160]. Homozygotes that have this deletion in the CCR5 receptors do not express the receptor at the CD4+ T cell-surface and can only be infected by CXCR4 using strains of HIV-1 and so there is always a chance of transmission[161-163]. It has also been demonstrated that cells from highly exposed persistently sero-negative (HEPS) donors are often less infective by HIV-1 strains even when there is no CCR5 mutation since these cells produce higher than average of the HIV-suppressing chemokines, MIP-1α MIP-1β and RANTES 56, which bind to the CCR5 receptors and lower infection rate[164]. HLA class I and class II types have been associated with susceptibility to HIV-1 infection and transmission. In a cohort of highly exposed Kenyan sex-workers, HIV-1 resistance was found to be associated with HLA-A2, A*6802, B18 and DR1, while the HLA-A23 is associated with increased HIV-1 infection [165].

1.3.10.4 Immunological Factors

There may be potential immune mechanisms, which might be associated with protection from HIV-1 infection in individuals with documented HIV-1 exposure who remain HEPS, and these individuals were found in some cases to have HIV-1 specific CD4+ T cells. A number of studies have shown that a significant proportion of HEPS donors have HIV-specific T-cells in their blood and genital mucosa which proliferated and secreted IL2 in response to the antigen [166-170] but were still not infected since they were also seen to be able to secrete HIV-1 suppressing chemokine [171, 172]. It has also been observed that lymphocytes from HEPS donors reconstituted into the immune system of mice with SCID leads to these mice becoming resistant to HIV-1 infection [173]. Many of these HEPS donors were also found to have HIV-1 specific cytotoxic T cells in the genital mucosa [174].

1.3.10.5 Presence of prior sexually transmitted infections

Due to shared routes of infection, HIV-infected persons are frequently co-infected with other STIs. The presence of an STI may increase both genital and plasma HIV-1 RNA levels, enhancing the transmissibility of HIV-1, with important public health implications.

Globally, HSV-2, a genital ulcerative disease (GUD) have been shown to have strong synergistic relationship with HIV-1 [175]. HSV-2 plays an important role in acquisition and spread of HIV-1 and a prior established HSV-2 infection increases the incidence of HIV-1[176, 177]. Mechanisms of increased HIV-1 acquisition include the presence of mucosal disruption and the influx of cells [178, 179]. Additionally, there is increased HIV-1 shedding in genital secretions [180] due to local inflammation and the interactions between HSV-2 proteins and the HIV-1 long terminal repeat (LTR) genes and Tat protein [181-183]. STIs have been shown to

increase HIV-1 shedding at mucosal sites during co-infection, and treatment decreases this viral load in the genital fluids [184-187].

Syphilis represents the second most common cause of GUD among HIV-1-infected people and is on a rise, especially in the MSM population [188-190]. Studies have pointed out that syphilis infection is associated with an increased risk of acquiring and transmitting HIV-1[191, 192] and these mechanisms include disruption of the mucosa and the influx of CCR5+ HIV-1 target cells to the site of infection making it easier for HIV-1 to infect.

Another set of non-ulcerative diseases occur in people, which are the topic of our study and is the Neisseria gonorrhea (NG) infection and Chlamydia trachomatis (CT). These do not cause the usual abrasions associated with GUD STIs but can also impact the acquisition of HIV-1. A study of seminal HIV-1 levels in HIV-1 patients with NG/CT found that the presence of NG or CT resulted in an increase in the HIV-1 RNA levels [185]. Like in HSV2 infections, clearance in NG/CT decreases the HIV-1. But another study said that HIV-1 infected people on ART did not have reactivate HIV-1 RNA levels detectable upon infection[186]. NG infection positively affects the acquisition of HIV-1 and has been demonstrated in many cell-based studies. The interaction of NG and HIV-1 and how NG influences the HIV-1 acquisition is discussed more in the following sections.

1.4 FEMALE LOWER GENITAL TRACT CELLULAR STRUCTURE

The female lower genital tract mucosal surface is complex yet an important part of the innate and adaptive immune defense acting as a perfect barrier against the external aseptic and internal sterile environment[193] [126]. The lower female genital tract consists of the vaginal mucosa,

the ecto-cervix and the endo-cervix [194]. The vaginal mucosa and the ecto-cervix in turn is made up of several layers of lined non-keratinizing stratified squamous epithelium that helps to form a natural barrier to block HIV-1 and other bacterial and viral infection. The ecto-cervix transitions to a single layer of mucin- secreting columnar epithelium called the endo-cervix near the transformation zone, which is a multilayered squamo-columnar junction[5]. This protects the underlying cells and system from the bacterial and viral infections and microbes, which can breach this structure or enter through receptors, can infect the FRT.

1.4.1 NG interaction with Epithelium and ecto-cervical mucosa

In FRT, epithelia are the interface that separates the host from the environment. It serves as the first line of defense against stimuli like mechanical damages, chemical stimulants, toxins, and microbial pathogens such as NG. NG can establish infection in the epithelium by three interrelated processes based on our current knowledge: 1) initial attachment and close association between the surfaces of the NG and the host epithelium, 2) invasion of NG into the host epithelium and survival of the bacteria within the cell, and 3) dissemination into deeper tissues [195].

Type IV pili is structure for the initial contact with host epithelial cells which retracts bringing the bacteria close to the host cell surface which in turn allows anchorage and micro-colony formation at sites of attachment [196]. Bacterial surface proteins Opa and LOS then interacts with the host cell receptors and induces the formation and elongation of microvilli which surrounds the bacteria and this process of ruffling helps in the engulfment of the bacteria. The Opa to interacts with different host cell receptors including Heparan Sulfate Proteoglycan receptors and CD66 family receptors [53, 197-200]. Different types of Opa interact with

different receptors and triggers a slightly different host cell response depending on the signaling pathway the interaction induces [195] [196, 201, 202]. The LOS is also capable of binding to host as a glycoprotein receptors and assists with uptake of the NG [203]. It has been demonstrated previously that LOS containing lacto-N-neotetrose structures is required for NG invasion [204]. The actin rearrangement leading to ruffling that occurs in the host epithelial cell as a response to NG and host interaction helps engulf the bacteria in vacuoles, which enables the NG to survive in host cell post entry. NG Porins help them survive in the epithelial cells by altering the host signaling[205]. Some studies show that NG also secrets IgA protease that enables NG to escape proteolytic degradation in the vacuoles[206], while other studies suggest NG remain within a vacuole once internalized by epithelial cells and in both cases they are capable of replication[41]. Therefore, the NG adherence and invasion are two critical processes for establishing infection.

Since female genital tract sheds epithelial cells during the menstrual cycle, adherence and invasion may not be sufficient for NG to survive and establish chronic infection. To escape this they use transmigration, which allows for penetration into deeper tissue and leads to persistence and dissemination of the disease. Using human fallopian tube organ-culture as a NG infection system, and the only organ culture used till date, the phenomenon of transmigration across epithelium was demonstrated and NG was found in both the epithelial layer and sub-epithelial connective tissue [207]. The NG crosses the epithelium using paracellular or intracellular pathways. The intracellular pathway involves the transcytosis of invading NG through the cytoplasm of epithelial cells and the escape of transcytosed bacteria from the basal surface near sub-epithelial connective tissue and this pathways is more supported, based on the ability of NG

to undergo receptor-mediated invasion[208]. The paracellular pathway involves NG passing through the space between epithelial cells to reach sub-epithelial tissues.

Previous studies also showed that different NG strains expressing various surface molecules induce transmigration in different ways[209, 210] implying that NG has developed diverse mechanisms to invade and survive within hosts, by manipulating existing host cellular apparatus[211]. Even though the adherence and invasion mechanisms utilized by NG have been extensively studied, the mechanisms by which NG actually works on the adherence and invasion process has not been studied in details due to lack of models to do so. Moreover, the NG-epithelium interaction studies conducted have not been able to represent the NG infection in-vivo due to the lack of a model for epithelium in the FRT.

1.4.2 HIV-1 Interaction and transmission through ecto-cervical mucosa

The HIV-1 must first cross the epithelial mucosal barrier region to establish successful transmission across the female ecto-cervical mucosa. It may be possible for the virus to transverse the epithelial layer by mechanical abrasions caused during intercourse on the mucosal surface and SIV studies on macaques have established that the infection may be established both by cell free and cell associated virus [7, 125, 126]. In spite of this primary known fact, mechanism of HIV-1 transmission through the ecto-cervical 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[212, 213], but whether these are enough to establish infection are not known since their contribution in the ecto-cervical epithelium is unknown. Like in the case of NG infection, HIV-1 transfer can also take place by transcytosis in primary genital

epithelial cells from the apical to the basal region and be released from the cells and infects susceptible target immune cells but this percentage of transcytosed viruse through epithelial cells is very small [6, 125]. Cell associated viruses appear to be more efficient in transcytosis compared to cell-free virus as shown in many studies. Conversely, cell free viruses are more efficient in the process of transmission than cell-associated virus. [125, 126, 214, 215]. Keeping all this in consideration, transmission seems to occur mostly via cell-free route. The common way of transmission for the majority of sexual transmission is thus not transcytosis. The ecto-cervical and vaginal tissues have target cell population like dendritic cells and spatially distributed CD4+T cells just beneath the epithelial layer [125, 126]. Thus once the virus crosses the mucosal barrier either by transytosis or by receptor mediated transmission, they can easily access and infect the underlying target cells(Figure 8) .

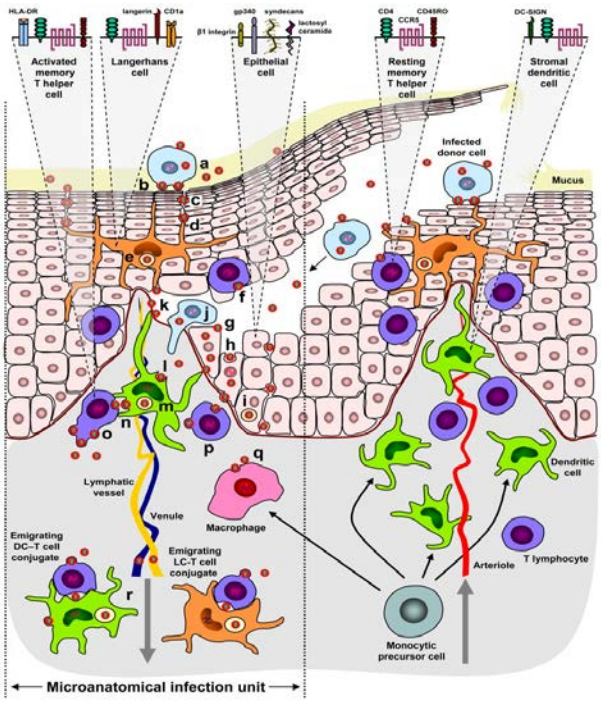


Figure 8: HIV-1 transmission across mucosal epithelium.

Figure taken from Hladik et,al Nat Rev Immunol 8(6): p. 447-57 June 2008 [125]
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1.5 MODELS FOR STUDYING SEXUAL TRANSMISSION OF NG INFECTION

It is difficult to study NG pathogenesis since pathogenicity solely occurs in the human host. The in depth study of NG pathogenesis is hindered by the lack of a suitable animal model that can closely mimic human disease and that can be efficiently used to study the pathology the infection. There are reports on several animal models, which have been studied for NG pathogenesis [216, 217], but no animal model exists that can closely mimic the full spectrum of disease. During the early study of the pathogenesis, human male volunteers were used, and a few but not useful tissue and organ cultures were developed, but most of the studies were mostly confined to immortalized or malignant tissue culture cell lines.

The first studies on NG pathogenesis were the male experimental challenge model [39] and the development of a primary human male urethral epithelial cell culture system [218] which gave insights into specific factors involved in early events that NG uses to colonize male urethral epithelium but these did not analyze any cellular or molecular events that occurred during real infection and these studies were not reflective of what was happening in the female reproductive tract. Since the severity of NG in women is adversely high it is unethical to have women voluntarily used as live models for studying early events in NG pathogenesis among women.

Primary cell and organ culture systems have also been developed to examine the gonococcal interaction within the upper female genital tracts; however, these systems are site specific and a global analysis of the female genital tract is not possible. As discussed, the female genital tract is composed of a heterogeneous mixture of epithelia, forming a complex microenvironment, which changes with the women's menstrual cycle and is not easily replicated in vitro. Therefore, primary cell cultures give an incomplete and biased picture of NG pathogenesis as it occurs in-vivo at any site within the female genital tract. The development of a

female mouse model of genital tract infection has served and will continue to serve as a valuable tool by which to study gonococcal pathogenesis in an immunologically defined environment (116, 132, 243). However, it is currently not clear whether data obtained from the mouse model of female genital tract infection is reflective of infection as it occurs within the human female genital tract, because mice fail to express several human-specific receptors (e.g., CR3, CD46, and CEACAM) that are believed to play critical roles in potentiating gonococcal disease. Additionally, high levels of estrogen are required to establish infection in mice (116, 243), and the contribution of increased estrogen to the data obtained is not known (15).

Researchers routinely rely on the use of immortal and malignant cell lines to study molecular factors involved in NG pathogenesis. These systems are advantageous in that they are readily available, easy to maintain, and amenable to manipulation. Cell lines are often altered in their protein and receptor expression patterns because of their immortal nature and continued laboratory maintenance and this makes it difficult to extrapolate data obtained from the use of immortal cell lines to NG infection at any specific site within the human host, and it keeps varying from one-cell lines to another. Therefore, the conclusions derived from these immortal cell lines studies cannot be representative of the in-vivo encounter of NG with the epithelium. However, the use of immortal cells has greatly contributed to the present day knowledge of both the human and the NG constituents that might play a role in potentiating disease in-vivo.

1.6 MODELS FOR STUDYING SEXUAL TRANSMISSION OF HIV-1 INFECTION

The study of HIV-1 disease process has been carried out in different cell lines, which included both primary cells a well as either engineered or immortalized cell lines. But these have not been

able to demonstrate the transmission and dissemination process occurring at sexual sites for HIV-1 acquisition. Different cell models have been used to study the infection process and to identify receptors for HIV-1, but there is still gaps in studying the exact mechanisms of sexual transmission (both in the cervix and the rectal tissues) during the exposure of HIV-1. In-vitro studies in primary or immortalized cells are still being used to elucidate the essential principles that play the major role in the interactions between HIV-1 and isolated target cells. However, there has been a lot of technical barriers in using such primary isolates, especially with the simple strategies that are involved with cell studies hampering the analysis of the real interaction of cells in-vivo. This has prevented all the information obtained from these studies to being efficiently translated to the more complex scenario of HIV-1 spread in the host in-vivo where different cells interact complexly with each other and has limited the understanding of the impact of host physiological parameters on the spread of HIV-1. Therefore, there was an urgent need to visualize HIV-1 spread and the adaptation to complex cell system approaches and then organotypic ex vivo models and animal models.

The closest models, which have been used for studying the HIV-1 transmission, are the transmission in SIV infected macaques. These monkey species are infected and transmit SIV (the animal virus counterpart of HIV-1) in nearly the same mechanisms as that of HIV-1. For studying HIV-1 transmission at the sexual epithelial surface level, some organ cultures have been used. The earliest models included were primate models for the study of simian immunodeficiency virus (SIV) and this was followed by the analyses of HIV-1 infected fetal and neonatal human thymus since these were sites of infection of HIV-1 target cells [219-221]. All these paved way to the establishment of the earliest human organ culture system which was the thymic organ culture model[222]. This was immediately followed by inoculation of tonsil

histoculture for the study of HIV-1 pathogenesis[223]. Macaque studies on SIV pathogenesis have suggested the importance of immune cell trafficking in the establishment of infection as well is in the process of viral dissemination post infection. However, these non-primate models don't permit the earliest stage of infection that is the HIV-1 transmission and dissemination studies. These studies though showed that SIV (the surrogate for HIV-1 infection) does enter genital mucosa at the level of cervix or vagina during sexual transmission. Data using human cervical explants and the nonhuman primate (NHP) model of simian immunodeficiency virus (SIV) infection suggest that HIV-1preferentially enters the genital mucosa at the level of the cervix or vagina[224].

There was a need to develop cervical and vaginal based models for the study of sexual HIV-1 transmission based on these data and this led to the development and improvement of humanized mouse models [225]with a strong human derived immune cell reconstitution of the female genital tract which then rendered these genetically engineered mice susceptible to intra-vaginal HIV-1 infection allowing for the study of the mechanisms involved in HIV-1 transmission and dissemination in-vivo. These mice are immune-deficient and transplanted with human hematopoietic stem cells (HSCs), lymphoid tissue or peripheral blood cells according to the design of the experiments. Nevertheless, the lack of human cells in the peripheral lymphoid organs and the inability to mount functional immune responses limited the applicability of these humanized mice models.

All these primate and mouse models are in general costly and labor intensive. Keeping all this in mind, the final development was that of the easiest and closest human cervical tissue based organ explant models which became an important tool in the understanding of specific mechanisms of sexual HIV-1transmission and this pointed out the importance of lower and upper

genital tract contributions for sexual transmission like that in the NHP models[226]. Defining the HIV-1 transmission pattern in-vivo is difficult, the use of mucosal tissue ex-vivo helped to identify key steps in HIV-1 entry and early dissemination [6, 227]. The use of mucosal tissue recapitulates the epithelium and immune cells that would be exposed in vivo to virus and drugs and can be extended to studying effects of other organisms on HIV-1 transmission like the one we are doing in this study.

1.7 NG AND HIV-1 INTERACTION

Unlike most sexually transmitted infections, NG infection is oftentimes quite symptomatic as discussed before and it is usually an ascending infection that can spread to the upper female genital tract and cause PID. Most NG infections are highly inflammatory, with adhesion proteins, including pili, opacity-associated (Opa) proteins, lactosyl lipooligosaccharide (LOS) and porin proteins causing cytokine/chemokine secretion [228] via TLR activation and related activities. CD4+ T cell targets are recruited to the endo-cervices of women infected with NG. During chronic HIV-1 infection, NG infection increased plasma viremia and also type 2 cytokines such as IL-4 and IL-10 in the plasma along with an enhanced activation of circulating CD4+ T cells and decrease in CD4+ T cell counts [229] and were observed in HIV-1 infected sex workers with NG infections[230]. Conflicting results were observed in studies evaluating the immune responses in women with mucosal NG co-infection during HIV-1 acquisition among high-risk HIV-1 sero-negative sex workers [231]. NG co-infection during HIV-1 acquisition has also been associated with increased breadth and magnitude of systemic HIV-1 specific CD8+ T cell

responses (IFN-γ and MIP-1β as cytokine biomarkers) and this association was only found for NG when studying different STIs co-infection with HIV-1 acquisition [232].

Most STIs can change the cervico-vaginal environment and act as important co-factors for the acquisition, transmission and progression of HIV-1 through mucosal surfaces in the FRT [233]. Likewise, NG has been shown to enhance HIV-1 acquisition [234, 235] and stimulate vaginal cytokine production [236-240], but its effect on HIV-1 infection of target cells (T cells, dendritic cells and macrophages) in-vitro has been conflicting. NG exerts cell-type dependent enhancing or inhibiting effects suggesting that these interactions may be better studied at the tissue level. NG infected women have more endo-cervical CD4+ T cells [241] providing more target cells for HIV-1 infection. NG can active the HIV-1long-terminal repeat (LTR) in a transformed Jurkat CD4+ T cell line as well as replication of HIV-1 in primary resting CD4+ T cells. It has also been shown that this effect is not dependent on outer membrane Opa and Pili[14, 242]. However, the bacterial LOS has been observed to promote HIV-1 infection in the primary CD4+ T cell after entry. It was also observed that this effect was strictly confined to the primary CD4+ T cells which are HIV-1 target cells and not on the other cell lines and the impact of NG on cell lines varied greatly[243, 244] and this was so restrictive that it was found to influence only the in activated CD4+ T cells and not activated CD4+ T cells[14, 242]. NG LOS from various strains has also been demonstrated to trigger TLR4 activation and thereby inhibit HIV-1 infection of MDM[245]. Defensins, human antimicrobial peptides are frequently induced by pro-inflammatory cytokines or TLR activation upon NG exposure [246, 247] and the defensins can also induce cytokines and chemokine in the host cells. Recently, heptose monophosphate (HMP) secreted from NG has been shown to activate the HIV-1 LTR in CD4+ T cells[248]. The presence of NG at human mucosal surfaces initiates the recruitment of abundant

polymorphonuclear leukocytes (neutrophils; PMN) to the site of infection as discussed and can increase chances of HIV-1 infection. NG has also been reported to promote the activation of MDDC and infection by HIV-1 but not in monocytes [249]. NG induced TNFα in vaginal biopsies and this TNFα was reported to increase the susceptibility of Langerhans cells to HIV-1 through activation of TLR2 [250]. Again contradicting to the increase in HIV-1 replication by NG in these above mentioned cells, NG exposure blocks HIV-1 production in CD8-depleted PBMCs from HIV-infected individuals as well as in-vitro HIV-infected PBMCs[251]. NG exposure has been reported to increase the expression of chemokines and IL-8 in macrophages and epithelial cells, respectively [252, 253]. Another form of defensin has also been shown to up-regulate IL-6, IL-8, IL-10, MCP-1, IL-1β, MIP-1β and RANTES in PBMC [254, 255]. IL-8 in one study has also been observed to enhances HIV-1infection in cervical tissues[256]. While defensins have strong anti-HIV-1 activity, their immune-modulator role to induce inflammation in the host leads to recruitment of HIV-1target cells to the site of NG infection and defensing production and facilitate HIV-1dissemination and amplification. Keeping all these observations in the picture, it appears that NG may facilitate HIV-1 acquisition through multiple mechanisms. NG infection results in inflammatory protein production by epithelial and immune cells, and recruits CD4+ T cells and other immune cells to the site of inflammation.

In spite of all these findings, it has not yet been completely understood as to how NG enhances HIV-1 acquisition process. All these observations have led to the speculation that NG may induce inflammatory cytokines and other cellular factors that are also enhanced during HIV-1 infection in cervix and may be responsible for increased HIV-1 acquisition in the FRT during sexual transmission. The relative contributions of vaginal, ecto-cervical and endo-cervical transmission of HIV-1 is not known, but due to the larger surface area and presence of relatively

less mucus, the vagina and ecto-cervix are considered the primary sites for HIV-1 infection. Regardless of how HIV-1 crosses the epithelium, virus exposure to the epithelial layer or epithelial cells has been shown to induce production of cytokines and chemokine including IL-6, IL-8, SDF-1, MIP-1α, MIP-1β, TNFα, GM-CSF, Type 1 IFNs and RANTES [4][5]. These signaling molecules may play an important role in HIV-1 transmission by attracting immune cells; inducing maturation of DCs; enhancing the function of T cells, B cells and macrophages; and inducing accumulation of plasmacytoid DCs (pDC) and CD4+ T cells. It is difficult to study the mechanism of NG mediated enhanced HIV-1 transmission in humans because of lack of suitable model that mimics the in-vivo situation. The role of NG in promoting cytokine production has been studied in primary cells and cell lines. However, they do not reflect in-vivo microenvironment of the female cervical- vaginal area. The mechanism by which NG influences the risk of HIV-1 acquisition in-vivo is not well-defined in part due to the lack of ideal animal models to study NG and HIV-1 co-infection as both NG and HIV-1 primarily infect humans and cervix of females. Rhesus macaque studies have been used for SIV transmission studies as a surrogate for HIV-1, and maybe used for NG pathogenesis studies to some degree in future but such studies have not been reported also because of the cost of study as well as the time. Though the results of studies on the effect of NG on HIV-1infection using specific immune cells may be important, it is important to gain insights into the impact of NG on HIV-1infection in a multi-cellular system such as tissue explants or in animal models. In this study we developed a cervical tissue based organ culture to study the effect of NG on HIV-1 transmission across the cervical mucosa [6].This cervical tissue based organ culture model designed to examine the effect of NG on HIV-1 acquisition and transmission at a tissue level will provide a unique opportunity to study the interaction of HIV-1 and these genital infection in a cervical tissue

matrix. Since this model uses primary, intact tissues rather than a monolayer of cells (primary or transformed), it provides the natural tissue architecture including epithelial cells, sub-mucosa, and immune cells, such as T cells, macrophages and Langerhans cells, thus mimicking the in-vivo situation. The long term aim of this dissertation project is therefore to use this developed cervical tissue based organ culture which had been earlier established for HIV-1 transmission as an extension to study the mechanisms as to how NG enhanced the acquisition of HIV-1 at this epithelial/mucosal surface keeping in mind the similarities of the infection process of the two microorganisms.

2.0 SPECIFIC AIM

Neisseria gonorrhea (NG) increases sexual transmission of HIV-1 in women [1-4]. However, molecular determinants of NG induced enhanced HIV acquisition in the female genital tract are still unknown due to lack of a suitable ex vivo model that mimics in vivo situation. In addition, we do not know the mechanism of HIV transmission through the epithelia of the cervical mucosa, especially when epithelia do not express CD4 and CCR5/CXCR4 [5]. It has been suggested that the pro-inflammatory cytokines often present in genital secretions of patients with NG [5, 257] [258, 259] are responsible for enhancement of HIV-1 transmission by some unknown mechanism. Regardless of how HIV-1 crosses the epithelium, virus exposure to the epithelial layer or epithelial cells has been shown to induce production of cytokines and chemokine including IL-6, IL-8, SDF-1, MIP-1α, MIP-1β, TNFα, GM-CSF, Type 1 IFNs and RANTES[260] [238]. These signaling molecules may play an important role in HIV-1 transmission by attracting target immune cells to fuel HIV infection in sub-mucosa and hence transmission. Therefore, our hypothesis is that NG induced pro-inflammatory cytokines interact with cellular and viral factors responsible for HIV-1 transmission and enhances HIV-1 transmission by increasing availability of target cells in sub-mucosa.

Overall goal of the study was to develop a cervical tissue derived ex vivo model to study NG and HIV interaction and elucidate the molecular mechanism of NG induced enhanced HIV transmission. To test our hypothesis, we have undertaken the following specific aims. These are:

Aim 1: To establish a cervical tissue based organ culture model to examine cellular responses induced by *Neisseria gonorrhoea* (NG)

i) An Ecto-cervical tissue based organ culture was established to evaluate inflammatory response to NG. Following NG exposure, microscopic visualization of Haematoxylin and Eosin (H&E) stained tissue, and adherence assays were carried out to determine viability. Scanning Electron microscopy (SEM) was performed on these tissues to evaluate ruffling of epithelial membrane, a characteristic feature of NG infection in vivo. Integrity of epithelial tight junction was evaluated by measuring expression of genes for tight junction proteins by RT-PCR.

ii) Pro-inflammatory cytokines were determined by measuring cytokine messages in NG exposed tissues using real time RT-PCR and secreted cytokine proteins in culture supernatant using a multi-panel MSD multiplex assays.

iii) NG strains with deletions in Pili and Opa genes were used to assess the role of outer membrane proteins on the induction of the inflammatory response by the NG on the cervical tissues

iv)Migration of CD3+ immune cells to the intraepithelial and sub-epithelial region was evaluated using immunohistochemistry.

Aim 2: To evaluate the role of NG on the replication and transmission of HIV-1.

i) Spent culture fluid from cervical issue exposed to NG was used to evaluate its effect on a) transcriptional activity of HIV by measuring HIV LTR expression in an β-Gal assay, b) Replication of HIV-1 in latently HIV-1 infected lymphocytic ACH2 cells and monocyte derived U1 cells, c) Transmission of HIV across cervical mucosa in a cervical tissue based organ culture model

Effect of microbicides on HIV transmission in the presence of NG was evaluated in a cervical tissue based organ culture model.

Aim 3: To identify cellular factors responsible for NG induced enhanced HIV-1 transmission.

i) Transcriptome analysis was performed on micro-dissected epithelial layer from NG and HIV exposed cervical tissues using next generation sequencing in an Ion Torrent technology platform. Differentially expressed genes from transcriptome analysis were confirmed using RT-PCR technique and bioinformatics performed to determine the interactive partners of the putative cellular proteins involved in HIV-1 transmission across cervical mucosa.

ii) Inhibition studies with specific inhibitor for putative cellular proteins were performed to verify the functional role of putative proteins.

3.0 MATERIALS AND METHODS

3.1 BACTERIAL CULTURES

Highly piliated (Pil^+) and opaque (Opa^+) *Neisseria gonorrhea* (NG) phenotype, a clinical isolate from the clinical lab at the Alleghany county hospital (gift from Dr. Timothy Meitzner, University of Pittsburgh) was used for the experiments. This NG strain were routinely grown in 5% CO_2 at 37°C on gonococcal medium base (GCB) (Difco) or in chocolate agar plates (Remel) for 18 to 24 hours. Strains of NG , which were locked out mutants in surface protein Pilli or Opacity proteins NG pil- and NG opa- were obtained from the labs of Dr Elizabeth A Stohl (North Western University) and Dr Allison Chris (University of Virginia) and cultured and maintained under similar conditions as the wild type NG. *Lactobacillus planetarium* (LB) was grown on Lactobacillus MRS media plates (Remel) in a 5% CO2 incubator at 37^0C for 24 hours. The working cultures of each bacteria was generated with two to three colonies from each culture types from the plate suspended in 10% RPMI with the absorbance adjusted to 0.01A corresponded to a concentration of 1x 10^7 cfu/ml for each experiments.

3.2 MTT AND ADHEARANCE ASSAY

To analyze the adhesion of the NG on the tissue epithelium, the infected cells were treated with 1% saponin (Sigma Chemical Co.) solution in PBS for 5 min at 37°C to lyse the cells from the epithelial surface and release the adherent and internalized bacteria. Appropriate dilutions of the cell lysate were plated on GC agar to determine the number of viable bacteria.

To analyse whether the tissues were viable after NG exposure the tissues were put in tubes and 200μL of **0.5**mg/mL MTT (3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide)solution. This was incubated at 37°C for 3 hr. The tissues were then taken out, and placed in 1mL of Methanol and incubated overnight in the dark. Next day the tubes were vortexed, tissues removed and absorbance measures at 595nm. Methanol was used as a blank. The tissues were dried overnight for weighing the next day.

3.3 PRIMARY CELLS

PBMCs were isolated from buffy coats obtained from HIV-1 negative donors by ficoll centrifugation at a speed of 2000 g for 20 minutes. Primary CD8-negative PBMCs were prepared by immune- magnetic depletion of CD8+ T cells from peripheral blood mononuclear cells (PBMCs) of these uninfected controls, as previously described [261]. CD8- negative PBMC were grown in the presence of phytohemaglutinnin (PHA) for 3 days to activate and expand the CD4+ T cell population and this activated CD8- PBMCs were primarily used for most of the studies.

3.4 VIRUS CULTURE

HIV-1 BAL strain obtained from the NIH AIDS reagent program (NIH AIDS reagent Catalog # 11445) was used in all experiments. They were grown in Phytohemagglutinin (PHA)- stimulated CD8 depleted peripheral blood mononuclear cells from control donors. These cells were maintained in PHA stimulated RPMI 1640 (Mediatech) containing 100 U/mL penicillin/0.1 mg/mL streptomycin, 10% fetal bovine serum (FBS) (ref). These CD8- PBMCs were first infected with the virus briefly for two to three hours and washed to remove excess virus and then transferred to a bigger culture with more cells for further infection running for 7 days. Virus was collected from the culture supernatant on day 7 after infection.

Another set of the same HIV-1 BAL was cultivated in PM1 cells, a T-cell line that expresses CD4, CXCR4 and CCR5. These 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. These were then infected with the virus for 3 hours followed by washing to remove excess virus on the cells and then cultured. The cell supernatant containing the virus was collected on day 7 post infection, and was filtered using an amicon ultra-15 filter device (Millipore, Billerica, US) to remove the soluble cytokines. The control culture supernatant was prepared in the same way but the cells were not infected with HIV-1. This control culture supernatant was used as control for some of the experimental designs in aim 3.

3.5 TITRATION OF VIRUS CULTURE

In both the types of virus produced, viral stocks were quantified using an HIV-p24 ELISA (Fredrick, MD), and virus titers (TCID50/mL) were determined by titration in PBMCs [262]. For the virus titers in both the viral stocks, briefly, CD8 depleted PBMCs were treated with PHA for thouree days to activate the CD4+ cells and other PBMCs to be infected by the virus. This activation was followed by centrifugation at a speed of 500xg for 10 minutes. These CD8 depleted PBMCs were then seeded in a 96-well plate and incubated with cell-free HIV-1 BAL from both the sets, which were serially diluted (1:5) in 10% RPMI media for 7 days at 37oC. These culture supernatants were collected at different time-points during the course of infection and HIV-1 p24 using ELISA ASSAY was analyzed for infectivity titer according to the manufacturer's protocol.

3.6 HUMAN ECTO-CERVICAL TISSUE CULTURE

Ecto-cervical 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 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 were given out. Ecto-cervical tissues were collected and processed within 2 hours of surgery. For processing these tissues, the cervix was

first 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 ecto-cervical 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. The different bacteria (NG, GV, AV or LB) at concentrations of 1 x 107 cells/ml ; cell-free HIV-1 Bal(TCID50 of 106) ; control media ; control supernatant; NG induced tissue supernatants (NGIS) ; microbicides; Recombinant cytokines or inhibitors for cytokines were added on the epithelial layer of the tissue depending on the experiments requirement in the upper chamber and complete 10%RPM1 [RPMI media, heat-inactivated fetal bovine serum (10%)] or IL2 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 different time intervals according to the demand of the experimental set up in a CO2 incubator. Post exposure to these, the tissues were harvested and processed differently for each experiment.

For the study of inflammatory response, the tissues post exposure to the different bacteria were washed and frozen down at -80O C and then homogenized and subjected to RNA extraction and RT-PCR Analysis. The supernatants from the bottom compartment of the organ culture was collected and subjected to MSD multiplex assay to measure the secreted cytokine protein from the tissues after exposure to the bacteria.

For the effect of NG on HIV-1 transmission studies, ecto-cervical tissues were harvested on the third day after inoculation with the cell-free HIV-1 BAL (TCID50 of 106). The biopsies were washed with PBS to remove excess virus, and frozen at at -80O C. HIV-1 p24 production was measured by ELISA (SAIC-Frederick) to monitor virus growth in the

supernatant. The supernatants were collected for a course of 21 days at intervals designed for the studies during the culture period.

For the ion torrent studies, the tissues post exposure to NG and HIV-1 BAL were washed and frozen at -80oC after being embedded in OCT (Thermo Fisher. USA), and then cryo-sectioned (7μm thickness). This cryo-sectioned tissues were then subjected to micro-dissection and RNA was extracted from the epithelium and was either used for a whole genome transcriptional profile analysis or RT-PCR to verify the significantly dys-regulated genes obtained from the ion torrent analysis. The culture supernatants from the bottom well was again collected and frozen and subjected to MSD multiplex assay to measure the secreted cytokine protein from the tissues after exposure to the bacteria as well as HIV-1.

3.7 CELL LINES USED:

TZM-BL cells, a HeLa cell line stably expressing CD4 and CCR5, and carrying luciferase and B-galactosidase genes under control of the HIV-1 promoter were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID from John C Kappes, Xiaoyun Wu and Transzyme Inc (Catalog number 8129).

ACH2 cells (Catalog number 349) are HIV-1 latent T cell clones with one integrated proviral copy from which productive highly infectious HIV-1 expression can be induced using phorbol myristate acetate (PMA). This was also obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID. This cell line constantly produces a low level of supernatant RT and p24.

U1 cells (Catalog number 165), again obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID is a pro-monocytic cell line which also shows a minimal constitutive expression of virus which is highly inducible using PMA. All these thouree cell lines were maintained in 10 % RPMI media which is RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine, 1% penicillin, and 10µg/ml streptomycin, at 37oC in a 5% C02 incubator.

E6/E7 cells, which are cervical/vaginal tissue derived HPV transformed epithelium cells (ATCC CRL-2616) were grown and maintained in Keratinocyte media with serum supplements, 1% penicillin, and 10µg/ml streptomycin (DIBCO).

3.8 HISTOLOGY AND IMAGE ANALYSIS

Ecto-cervical tissues were cryo-sectioned, and stained with hematoxylin and eosin (H&E) and examined by light microscopy to assess tissue morphology after exposure to NG and control media. After obtaining bright field images of H&E stained ecto-cervical 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.

3.9 IMMUNOHISTOCHEMISTRY AND ANALYSIS

Ecto-cervical tissues, which were exposed to NG for 24 hours were washed and fixed in formaldehyde and then frozen in OCT at - 80oC. These tissues were again cryo-sectioned in 7um

sections and then the slides with the tissue sections were used to stain for using CD3+ T cells in the sub epithelium of tissues exposed to NG as well as in the control media exposed tissues. The antibodies used for these analyses were commercially available, previously characterized murine monoclonal antibodies, rabbit polyclonal antibodies, and affinity-purified goat polyclonal antibodies. The Antibodies used were rabbit polyclonal-α-CD3 (A0452, Dako, Glostrup, Denmark), mouse polyclonal-α-CD68 (M0814, Dako, Glostrup, Denmark), mouse polyclonal-α-CD20 (M0755, Dako, Glostrup, Denmark), mouse polyclonal- α -DC-SIGN (551249, BD Technologies, NC, USA), and mouse polyclonal p55 (M3567, Dako, Glostrup, Denmark).

3.10 SCANNING ELECTRON MICROSCOPY

Human Ecto-cervical tissues were exposed to NG for 24 hours and fixed in 2.5 % glutaraldehyde for 1 hour at room temperature. The biopsies were washed with 0.1 M PBS (pH 7.4) a few times and then the tissues were in 1% OsO4 in 0.1 M PBS for 60 minutes. This was again washed thoroughly in 3 changes 0.1 M PBS for 15 minutes each. and processed using a protocol from the CBI imaging center from the University Of Pittsburgh as previously described [263]. SEM 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).

3.11 HOMOGENIZATION AND MICRO-DISSECTION OF EPITHELIAL LAYER AND RNA EXTRACTION

For the first Aim of the project, ecto-cervical tissues exposed to NG and Control media was washed and frozen at -80oC and then homogenized using tissue grinding techniques. The E6E7 cells were also frozen down suspended in RNA Bee till the extraction process was started. The frozen tissue was first chopped into small bits and then homogenized in RNA Bee buffer for extraction of the whole RNA from the tissues. For the third Aim of the project, where studies were focused on the epithelial layer of the tissues, the ecto-cervical tissues exposed to NG, HIV-1 BAL, media or control supernatant, were washed and frozen at -80oC in OCT and cryo-sectioned at -28oC in 7um sections on slides. This was followed by micro-dissection of the epithelial layer under microscope to minimize contamination from sub-mucosal layer. This was put in RNA Bee and RNA was extracted from the micro-dissected epithelial layer using chloroform extraction (TEL-TEST, INC, Friendswood, TX) method.

3.12 TAQMAN® REAL-TIME PCR

RNA was isolated from either the completely grinded tissues exposed to NG and Media control or the micro-dissected ecto-cervical epithelial mucosal layer exposed to NG, HIV-1 control media or control supernatant 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). For the first set of experiments in aim 1, GAP-DH was used as an indigenous housekeeping control

for the ecto-cervical tissues against which the other RNA transcripts were measured. The mRNA levels of cytokeratin 13 and cytokines of CXCL10, IL-6, IL-1β, IL8, TNFα and IFNγ were measured in the micro-dissected epithelial layers by real-time RT-PCR as described before [107]. Krt13 (endogenous control for epithelial cells), human CXCL10, CXCL11, IL-6, IL-1β, IL8, IL-10, TNFα, IFNγ primers and probes labeled with FAM / MGB were purchased from Life Technology (Table 1).

Table 1: Genes analyzed for aim 1 on pro-inflammatory cytokines induced by NG exposure on cervical tissues.

Gene	Primer and probe *
IL-1β	Hs99999029_m1
IL-6	Hs99999032_m1
IL-8	Hs99999034_m1
TNF-α	Hs99999043_m1
IFN-γ	Hs00989291_m1
CXCL10	Hs00171042_m1
GAPDH	Hs9999905_m1

Table 2: Genes analyzed for aim 3 for confirming the expression of genes differentially regulated upon NG and HIV-1 exposure on cervical tissues.

Gene	Primer and probe *
SAA1/SAA2	Hs00761940_s1
MUC1	Hs00159357_m1
IL36A	Hs00205367_m1
CXCL10	Hs00171042_m1
FMO2	Hs00155158_m1
IL8	Hs99999034_m1
WARS	Hs00188259_m1
CXCL3	Hs00171061_m1
CCL20	Hs01011368_m1
TNFAIP6	Hs01113602_m1
IL6	Hs99999032_m1
IL1B	Hs99999029_m1
LCE3A	Hs00820288_s1

*** 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 a (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 secs and 60 °C for 1 min.

Results were expressed as fold-change relative to control. Primers especially designed for HIV-Gag was used for the detection of HIV-1 mRNA in the tissues using the same RT-PCR techniques[264]. The main primers for these were:

Forward 5'-CCCATGTTTTCAGCATTATCAGAA-3',

Reverse 5'-CCACTGTGTTTAGCATGGTGTTTAA-3',

Probe 5'-FAM-AGCCACCCCACAAGA-MGB-3'

3.13 NEXT GENERATION SEQUENCING USING ION TORRENT TECHNOLOGY

3.13.1 RNA extraction and library construction

RNA was extracted from micro-dissected epithelial layer of the tissue as described earlier. The mRNAs from each sample were isolated from the total RNA with a commercially available kit (Dynabeads® mRNA DIRECTTM Micro Purification Kit, Life Technologies). The cDNA Library was then constructed using an Ion Torrent Total RNA-Seq Kit (Life Technologies) for whole transcriptome libraries and Barcodes 1 through 8 from an Ion Xpress 1-16 barcoding kit were used (Life Technologies) for each individual sample. cDNA libraries were quantified by qPCR using an Ion Library Quantitation Kit (Life Technologies) to determine a suitable template dilution factor for subsequent emulsion PCR and sequencing. Template preparation for sequencing was conducted using the OneTouch Ion™ Template Kit in the OneTouch machine (Life Technologies). Ion Torrent sequencing was conducted using the Ion Proton Sequencing Kit (Life Technologies) on an Ion Proton Machine (Life Technologies) using a P1(v2)-chip (Life Technologies). [265].

3.13.2 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 (Poured Quality score higher than 20). Quality trimming and adapter sequence clipping were performed prior to downstream analyses to narrow down unwanted data. The trimmed reads were then mapped to Homo sapiens (hg19) mRNA sequence. Bioconductor edge R 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.

3.13.3 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 for the HIV-1 induced genes and CXCL10, CXCL3, CXCL20, TNFA1P6, IL8, IL6 and IL-1B for the NG induced genes as described earlier.

3.13.4 IPA analysis

INGENUINITY IPA analysis software was used to determine the regulated pathways and disease progression pathways by the NG and HIV-1 exposure on cervical epithelium and also used to compare the two sets of data to locate common pathways.

3.14 STATISTICAL ANALYSES

Data are presented as mean ± standard deviation and was plotted using the PRISM software student's edition. All the analyses were also done using the same software. 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 NG exposed as well as HIV-1 exposed groups relative to controls. To determine significance ($p<0.05$) in cell numbers in NG and control exposed tissues and of TJ protein expression between NG exposed group and controls, nonparametric paired student t test was applied. For comparisons of mRNA cytokine expression levels in the ecto-cervical tissues treated with NG and HIV-1, T-Test Unequal Variance analysis was performed with significant level at $p<0.05$. To compare the susceptibility to HIV-1 infection, again nonparametric paired student t test was used. Fold change comparison were all done with one sample Students T test. To correct for tissue variability, paired tests were done within one tissue sets in control and exposed groups.

4.0 SPECIFIC AIM 1

TO ESTABLISH A CERVICAL TISSUE BASED ORGAN CULTURE MODEL TO EXAMINE THE CELLULAR RESPONSES INDUCED BY *Neisseria gonorrhea* (NG)

4.1 RATIONALE AND HYPOTHESIS

Heterosexual transmission is the most common route of HIV-1 infection in women, accounting for nearly half of HIV-1- infected individuals worldwide and more than 70% in sub- Saharan Africa. The relative contributions of vaginal, ecto-cervical and endo-cervical transmission of HIV-1 is not known, but due to the larger surface area and presence of relatively less mucus, the vagina and ecto-cervix are considered the primary sites for HIV-1 infection. A key co-factor in cervical transmission of HIV-1 is the prior existence of bacterial, viral, and parasitic microbes in cervix that can alter cervical environment and thereby influence HIV transmission. In that regard *Neisseria gonorrhea* (NG), a gram-negative diplococcic, is one of the most severe, recurring and common forms of STI [266, 267] that has been shown to increase HIV-1 acquisition [267]**.** Although currently the molecular mechanism of NG induced enhanced HIV transmission is unknown**,** pro-inflammatory cytokines often present in the vaginal fluid of NG-infected women led to the speculation that NG induced inflammatory cytokines either directly or indirectly through interaction with certain cellular factors could increase HIV transmission. Unlike many

other ulcerative STIs, infection with NG in the female genital tract is usually quite asymptomatic in the initial phase and since sexual NG infection is restricted strictly to cervical or urogenital cells of human origin, it gets difficult to study this infection process in any animal model. In addition, there is no suitable ex vivo tissue model of cervical NG infection that mimics in vivo situation. NG interaction has been mostly studied in in vitro cell culture. Using CD4+ PBMC and endometrial epithelial cells and immortalized cell lines [14, 268]. However these cell systems do not accurately reflect in vivo situation of NG and HIV-1 interaction that occur in human cervix/vaginal tissue. **Our hypothesis is that NG would induce pro-inflammatory response in cervical tissues and could induce the main physical properties in the cervical tissue based organ culture model**.

To prove this hypothesis we established a primary cervical tissue based organ culture model to study HIV-1 and NG interaction that provides the natural cervical tissue architecture observed in cervix of NG infected women. Using this organ culture we showed that NG exposure to cervical tissues induced inflammatory cytokine response reminiscent of in vivo situation. In addition we have extended our study to bacterial vaginiosis causing microorganisms, another STI commonly found in cervix of women.

4.2 RESULTS

4.2.1 Establishment of the cervical tissue based organ culture as a model to study NG induced responses *ex-vivo*.

Ecto-cervical tissues obtained from HIV-1 negative, premenopausal women with normal hysterectomy from the Magee's women hospital was used to set up ecto-cervical tissue based organ cultures as described in methods. Briefly, a polarized cervical tissue based organ culture with 6 mm circular biopsy was set up in a 12 well trans-well with epithelial layer of the cervical tissue orientated on top and the area surrounding the tissue sealed with 3% agarose. NG at a concentration of 3×10^6 was added onto the top of the tissue exposing epithelial layer in a trans-well as described in materials and methods. After incubation for 24 hours tissue were examined for viability and morphological characteristics (Figure 9).

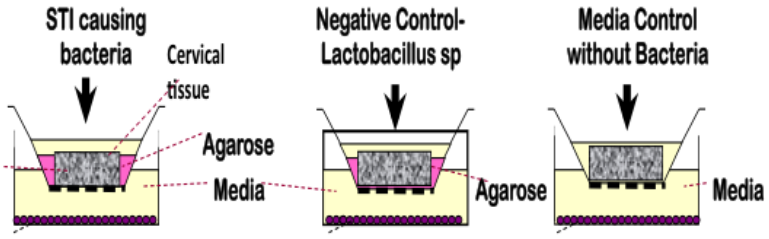


Figure 9: Ecto-cervical tissue based organ culture setup for assessing physical responses and induction of inflammatory cytokines from the cervical tissues in presence of NG and BV (STI) causing organisms.

Ecto-cervical 6 mm biopsies were sealed on trans-wells with agarose with the apical epithelium side exposed to the NG or other BV causing microorganisms (GV and AV) or control media. The bottom of the well was filled with 10%RPMI media to collect supernatant. The tissues were harvested post 24 hours, 48 hours or 7 days for RT-PCR analysis.

Tissues exposed to NG were then examined to determine the integrity of mucosal surface of the epithelial layer following incubation in the organ culture. The morphology of epithelial layer and the basal layer in the ecto-cervical tissues as determined by the hematoxylin and eosin (H&E) staining remained largely unchanged after exposure to NG for 24 hours compared to ecto-cervical tissues exposed to control 10% RPMI. Stained tissues showed retention of the epithelium and lamina-propria cell integrity after 24 hours in culture compared to the tissues subjected to control media, and was characterized by multilayered stratified squamous epithelial cells, basal layer and submucosa (Figure 10).

NG exposed tissue **10% RPMI exposed tissue**

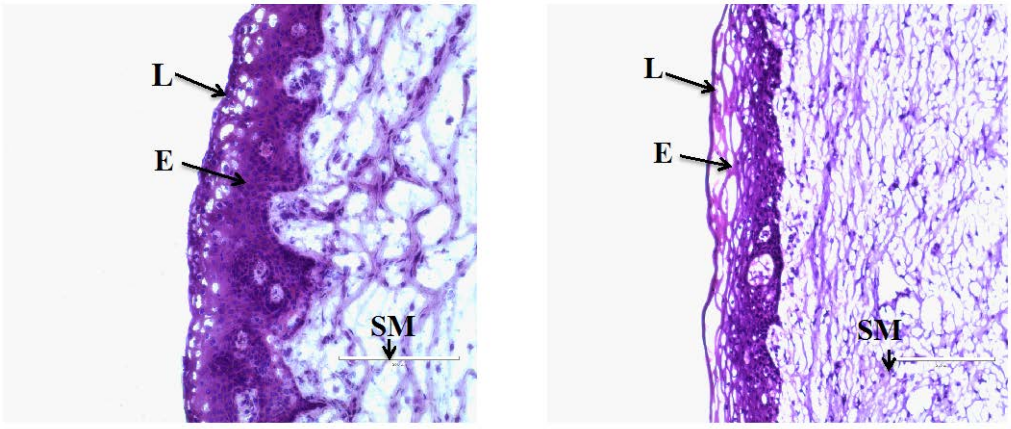


Figure 10: Effect of NG exposure on the integrity of the ecto-cervical mucosal epithelia.

H&E staining was performed on ecto-cervical tissues exposed to NG ($1X10^7$ cells/ml) or 10%RPMI as control in the organ culture for 24 hours after they were fixed in formalin. Images were obtained by bright field microscopy E: Epithelium; L: Lumen of ecto-cervix; SM: Sub-mucosa of the ecto-cervix. Magnification for viewing these ecto-cervical tissue sections was 40X for NG exposed and 20X for control exposed .

Viability of the same NG exposed tissues were examined by the MTT assay and found to be 97% compared to the control media exposed tissues**.** We also examined if there was an overgrowth of the bacterial culture on the tissues post 24 hours and also how much bacteria could adhere to the tissue surface by using a prior established method [204]. No overgrowth of NG was observed on the organ cultures. Moreover, We observed that 20 percent of the inoculated NG ($3x\ 10^6$) was found to be adherent on the tissues after 24 hours of exposure and was viable because they could be regrown on 1% NG agar plates

Another physical feature observed in cervical cell lines upon NG exposure infection process was ruffling that has also been observed in cervical tissue biopsies of hysterectomy patients with gonorrheal cervicitis. [269] Ruffling is the formation of a motile cell surface that contains a meshwork of newly polymerized actin filaments and is regarded as one of the earliest structural changes observed in the cell. It has also been demonstrated that NG Opacity (Opa) and Pili (Pil) proteins present on the cell surface interacts with host cells to form microvilli which helps in the ruffling process [45]. Scanning Electron microscopic analysis of the epithelial layer of tissues exposed to NG showed evidence of membrane ruffling (Figure 11), reminiscent of another in-vivo infection process of NG.

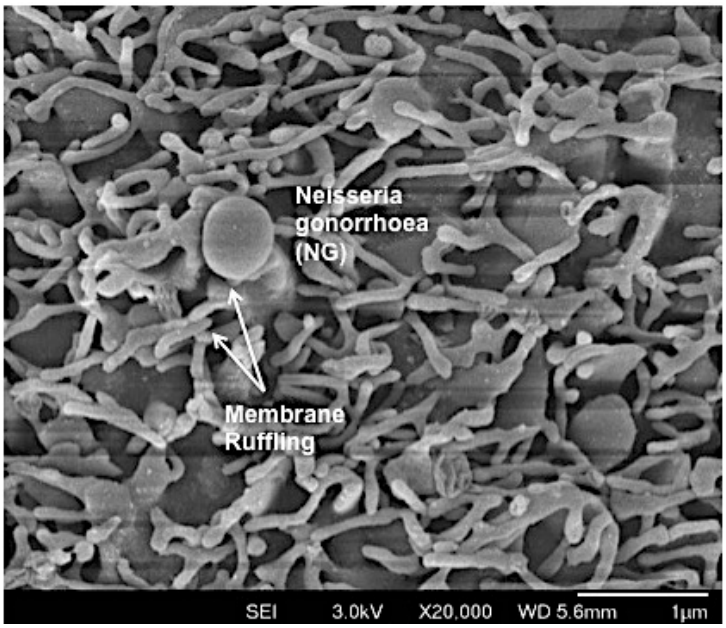


Figure 11: NG induced Membrane ruffling observed in cervical biopsy post 24 hours exposure under SEM

Membrane ruffling was observed using SEM with a magnification of 20,000. The microvilli projecting out which was a primary feature of NG infection process was clearly noted here.

4.2.2 Expression of tight junction protein on the ecto-cervical tissue epithelium upon NG exposure.

The epithelial layer in ecto-cervical tissues provides a robust barrier against microorganisms. This epithelium lining on the female reproductive tract (FRT) has a crucial role in reproduction and immune defense. The lower female genital tract including the vagina and ecto-cervix consists of non-polarized squamous epithelial cells, and the region from the endo-cervix to the fallopian tubes contains polarized columnar epithelial cells [270-272]. The apical junction sealing the space between adjacent epithelial cells consists of two major types of cell-cell junctions called the tight junctions and adherence junctions. The ecto-cervical epithelium of the lower female genital tract is the primary site of sexual NG infection, and it has been reported earlier that one of the ways by which NG could cross epithelium layer was by opening of the

intercellular junctions [273]. Our lab before has already demonstrated the presence of tight junction proteins in the cervical tissue epithelial surface and we examined the possibility of NG induced tight junction changes by examining the expression of two tight junction proteins, ZO1/TJP1 and Claudin1/CLD1 in the ecto-cervical epithelium by measuring their messages using RT-PCR. Results shown in Figure 12 indicate that there was no significant difference in the expression level of these tight junction mRNA in NG exposed tissues compared to control exposed tissues which was in line with observations noted earlier in cell lines that the NG does not show any change in expression of tight junction proteins [274].

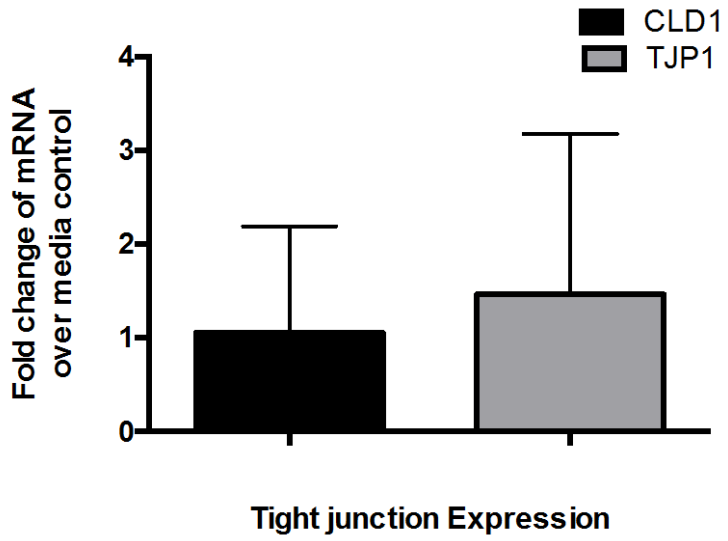


Figure 12: Tight junction protein mRNA from cervical tissues exposed to NG compared to control.

Epithelial layers micro-dissected from ecto-cervical tissue biopsies exposed to NG showed no significant changes in the expression level of tight junction proteins Claudin 1 (CLD1) and tight junction protein 1 (TJP1) / Z0-1 as compared to ecto-cervical biopsies exposed to control media. (n=4). P<0.05 was considered to be statistically significant compared to the control tissues analyzed by one sample student's T test.

4.2.3 Inflammatory cellular response in the cervical tissue based organ culture upon Neisseria gonorrhea (NG) exposure.

Cytokine analyses of culture supernatants from NG exposed PBMC compared to unexposed control have demonstrated the production of TNF-α, IL-6, IL-8 and IL-1β [242, 268]. It has also been observed in earlier studies that NG can interact with some epithelial cells and induce these inflammatory responses [275]. Lactobacillus species decrease those NG induced inflammatory responses[276]. Lavages taken from patients with NG infection also contained some of these cytokines. Following the setting up of cervical tissue based organ culture model and demonstrating that the model could replicate some of the physical characteristics of NG infection on cervical tissues, we used this model to examine the cellular responses that are elicited by NG on cervical tissues in-vivo. For this purpose the tissues were inoculated with 3 $X10^6$ NG and cultured for 24 hour (to simulate the acute infection cellular response in cervix) or 48 hours and 7 days (to simulate for a chronic state of infection). Following exposure to NG, cytokine induction was examined by measuring cytokine messages in the tissues using real time RT-PCR and secreted cytokines in culture supernatant using a multiplex assay in MSD platform. High levels of inflammatory cytokine mRNA induction were observed for IL-1β, IL-6, IL-8 and TNFα post 24 hours ranging from 3 fold to as high as 15 fold compared to tissues exposed to control media (Figure 13). When the 7-day tissues were analyzed for those particular cytokines, they were found to be consistently elevated at the mRNA level compared to the 7-day control exposed tissue with a higher increase in IL8 transcript production compared to IL-1β.

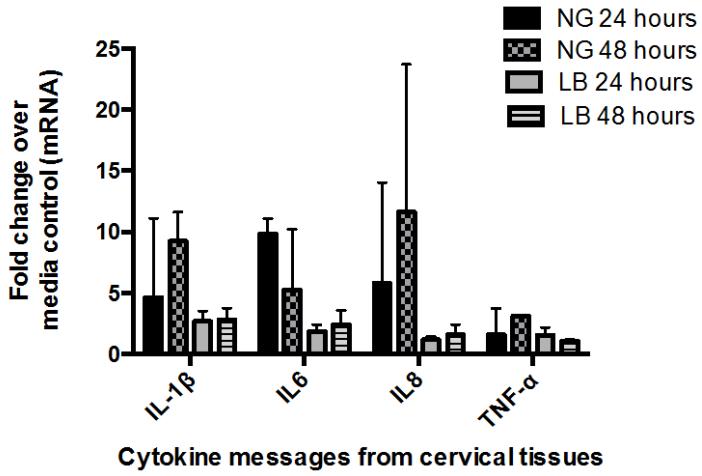


Figure 13: mRNA profile of inflammatory cytokines from cervical tissues exposed to NG and LB over media control.

Cervical tissue biopsies exposed to NG showed elevation in inflammatory cytokine mRNA production at 24 hours as well as 7 days whereas LB did not induce any inflammatory cytokine production in the tissues compared to the media exposed control post 24 hours. Experiments were carried out in triplicate with each experiments having two biopsies for times points for experimental and control (n=3). P<0.05 was considered as statistically significant compared to the control tissues analyzed using one sample student's T test

Analysis of culture supernatant showed high level of cytokine production for IL6, IL8, IL-1β and TNF-α at all time points after NG inoculation with IL-1β being highest compared to tissues exposed to media control (~2500pg/ml for IL-1-1β compared to 20 pg/ml in control media and ~500pg/ml for TNF-α compared to 10-17pg/ml in control media). We also found high production (10,000- 20,000 pg/ml compared to 8,000-12,000pg/ml in the control media) of IL8 and IL6 but the fold changes were lower compared to the IL-1β and TNF- α (Figure 14). In contrast, same number of *lactobacillus planaterum* (LB), commensal bacteria, did not induce cytokines upon their exposure to the cervical tissues.

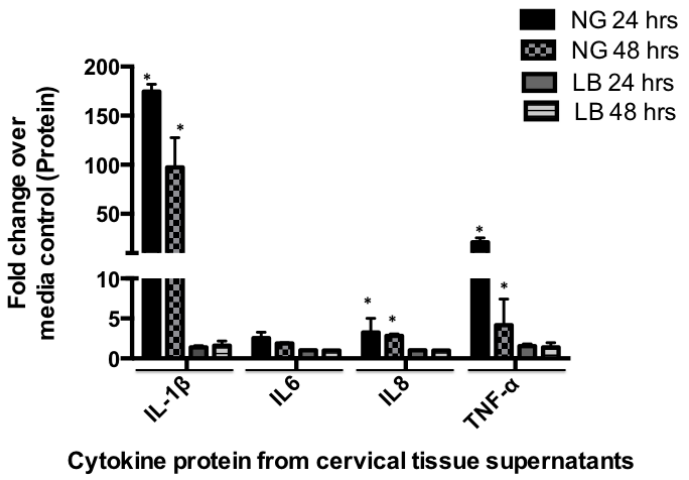


Figure 14: Secreted inflammatory cytokines from cervical tissues exposed to NG and LB over media control.

Cervical tissue biopsies exposed to NG showed elevation in secreted cytokines as measured in the supernatants suing MSD multiplex assay at 24 hours, 48 hours whereas LB did not induce any secreted inflammatory cytokine production in the tissues compared to the media exposed control at 24 hours or 48 hours post exposure. Experiments were carried out in triplicate with each experiments having two biopsies for each experimental and control time points (n=3). P<0.05 was considered to be statistically significant compared to the control tissues analyzed by student's T test.

4.2.4 Inflammatory responses upon NG exposure in tissues were similar to responses in epithelial cell lines.

Since epithelial layer exposed to NG induced the pro-inflammatory response, we sought to determine whether epithelia per se could induce pro-inflammatory cytokines when exposed to these bacteria. For this purpose, ecto-cervix derived epithelial cells E6/E7 were evaluated for their ability to induce inflammatory response upon exposure to NG. LB was used as a control organism (Figure 15). We observed that like in the cervical tissues, these epithelial cells upon exposure to NG induced an increase in the expression of intracellular IL-1β, TNFα, IL8 and IL6

cytokine messages and secreted cytokine proteins as compared to control media alone (figure 16) both at the mRNA as well as the secreted protein level.

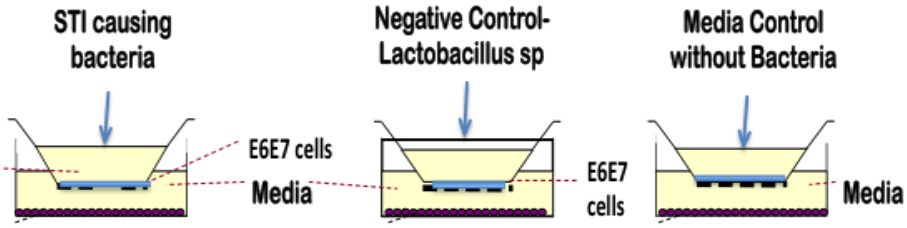
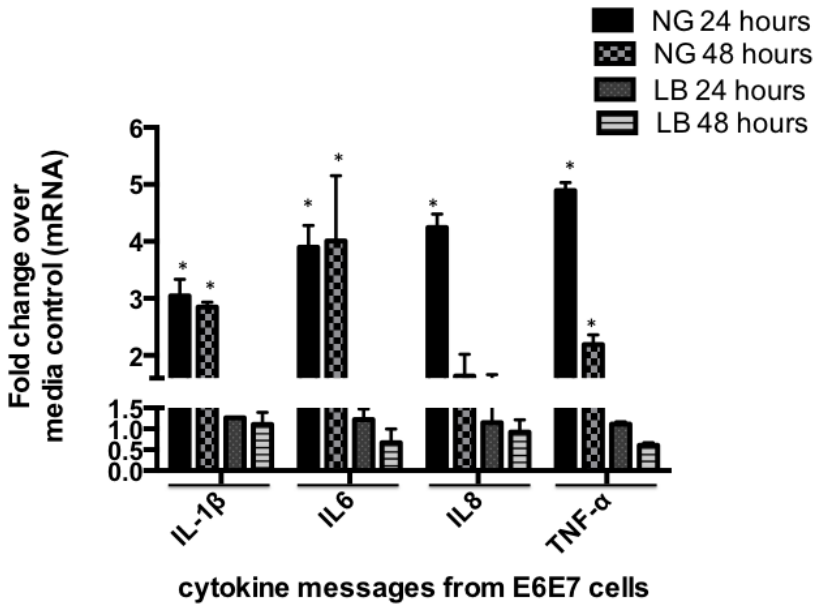


Figure 15: Ecto-cervical epithelium E6E7 cells for assessing inflammatory cytokines response induced by NG and BV causing (STI) organisms.

E6E7 cells (50,000 cells/ well) were seeded on transwells and exposed to the NG or LB for 24 hours and 48 hours. The bottom of the well was filled with keratinocyte media for keeping the cells viable and to collect supernatant. The cells were harvested post 24 hours and 48 hours for RT-PCR analysis.

a



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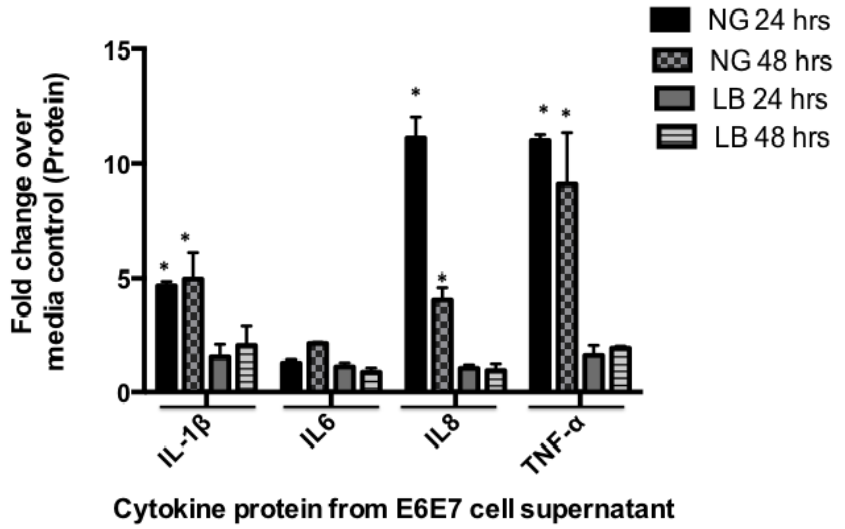


Figure 16: Inflammatory response in E6/E7 cells at both the mRNA and protein levels induced by NG and LB exposure compared to media control.

The (a) mRNA profile of the E6E7 Cells upon exposure to NG and LB and (b) the secreted cytokine profile of E6E7 cells upon exposure to NG and commensal LB showed a similar increase in cytokine responses. Experiments were carried out in triplicate. P<0.05 was considered to be statistically significant compared to the control tissues analyzed by one sample student's T test.

In contrast, similar to what was noted in the tissue model, Lactobacillus did not induce any significant level of inflammatory cytokines compared to media control. Peak cytokine m-RNA response by RT –PCR during 24 hour incubation showed a 3-5 fold increase of IL- 1β and TNFα expression in response to exposure to NG, compared to cytokine response in cells exposed to control medium. This cytokine profile observed in the cervical cells were in line with the observations in the cervical tissue based organ culture demonstrating that the cellular response from the cervical tissues induced upon exposure to these microorganisms was a response mainly from the cervical epithelial cells.

4.2.5 Effect of live and replicating NG on inflammatory responses from cervical tissues.

We next examined whether live replicating NG was required for induction of cytokine response. For this purpose, cervical tissues in the organ culture were exposed to live or heat (65 ^{0} C for 30 min) killed NG for 24 hour and 48 hour. Following incubation, the level of secreted inflammatory cytokines response was measured by MSD multiplex assay. No significant difference in the cytokine levels between the heat killed and live NG was observed (Figure 17).

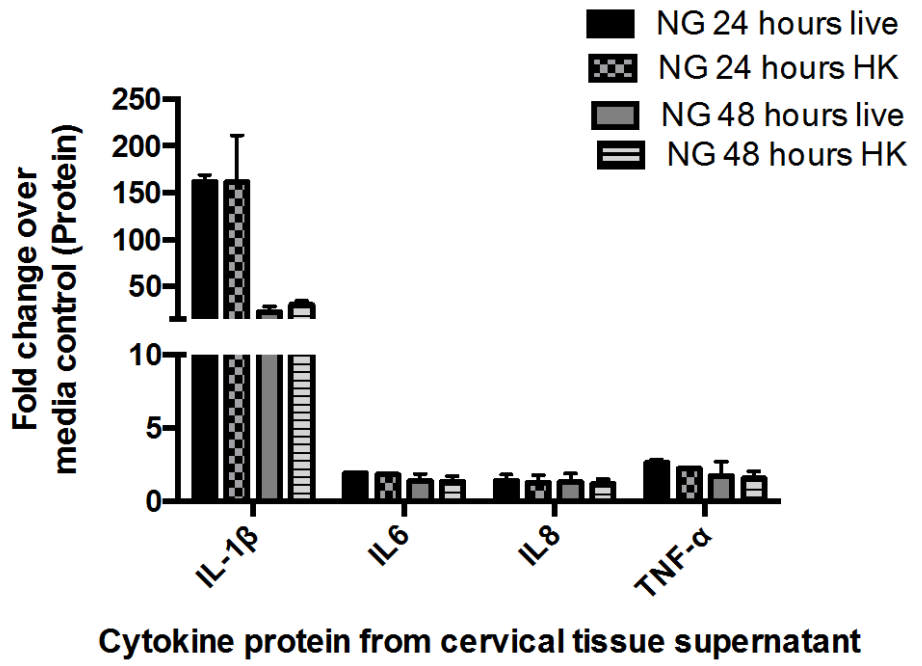


Figure 17: Secreted inflammatory cytokines from cervical tissues exposed to live or heat killed NG compared to media control.

Ecto-cervical tissue biopsies exposed to either live and replicating NG or heat killed NG showed no significant difference in the induction of inflammatory cytokines. Both the live and heat killed bacteria were able to induce similar responses showing that the outer membrane structure of the NG was enough to induce these responses. (n=2).

These results indicated that live NG was not important for the inflammatory cytokine response, implying that outer membrane structures of NG may be sufficient for inducing these inflammatory responses

4.2.6 Effect of outer membrane proteins Opa and Pili on the induction of inflammatory response.

Our heat-killed experiment has shown that outer membrane structures of NG may be sufficient for inducing inflammatory responses. Infection of the genital mucosa by NG involves attachment to and invasion of epithelial cells [16, 277]. NG infection of epithelial cells involves the phase-variable expression of several adhesins or outer membrane proteins like pili, opacity-associated (Opa) proteins, lactosyl lipooligosaccharide (LOS), and PorB protein. These outer membrane proteins have been observed to aid in the engulfment process of the bacteria by the epithelial cells[45] but has not been demonstrated to cause induction of inflammatory cellular responses. To determine the role of Pili and Opa proteins in inducing inflammatory response in cervical tissues, we examined the effect of pili and opa deletion mutants of NG on induction of inflammatory response in cervical tissues.

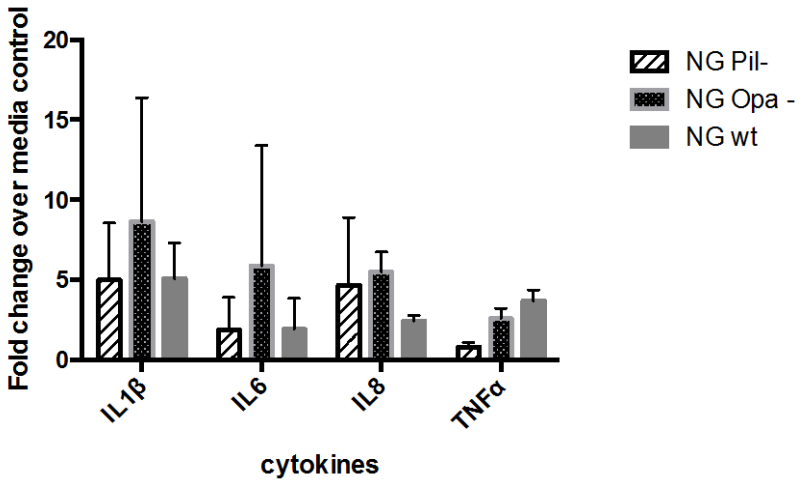


Figure 18: Deleting outer membrane proteins did not cause significant change in IL-1β or TNF-a.

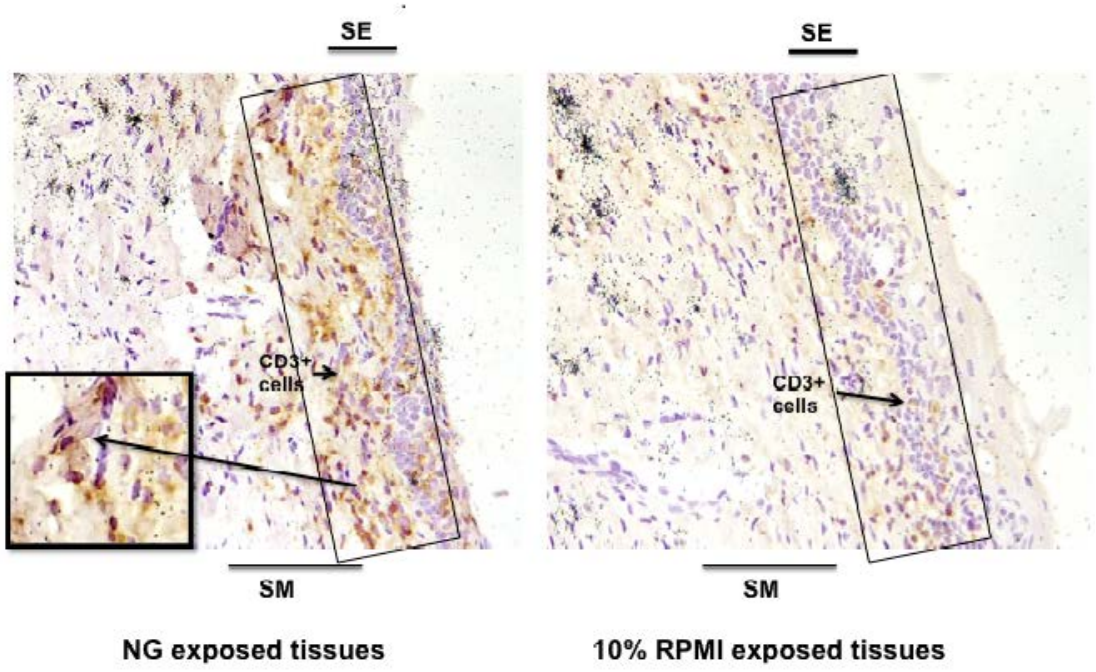
Deleting Pili or Opa proteins from the outer membrane of the NG did not cause any significant change in the inflammatory response that was observed in case of wildtype NG at the m RNA level. (n=2) significance was calculated using one sample Student paired t test.

For this purpose cervical tissues were exposed to wildtype NG (Pil+ Opa+), Opa- NG and Pil- NG in similar organ culture set up and induction of cytokines was determined by measuring cytokine messages. As shown in Figure 18, deletions in the Opacity and Pili proteins did not show any significant difference in the inflammatory cytokine induction of NG from the tissues compared to the wildtype NG even though they could cause infection. These finding are in line with earlier finding showing that Opa+ or Opa– NG did not have any differences in the cytokine profile induced from infection on primary cells [278]. This observation led us to conclude that the outer membrane proteins on the NG surface was more important for the process of attachment and infection as pointed out in earlier studies and are subject to phase variation even after the exposure to avoid host defense mechanisms and are not the primary components used by NG to induce inflammatory responses.

4.2.7 Effect of Immune cell recruitment towards epithelium upon NG exposure.

There have been reports that NG exposure recruits CD4+ T cell, which are targets for HIV-1 infection, to the endo-cervices of women infected with NG [241]. It has been shown that the pili present on the NG could also influence the activation of these CD4+ HIV-1 target cells [279]. Both of these findings taken together raise the possibility that NG infection may increase HIV-1 acquisition by the recruitment and activation of HIV-1 target cells near the epithelium. We tested this possibility whether the exposure of tissue to NG increases recruitment of immune cell recruitment at the intraepithelial region. For this purpose we carried out immunohistochemistry (IHC) staining for immune cells on the tissue exposed to NG as well as control media. The immune markers that we examined included: CD3 (T cells), CD20 (B cells), CD68 (monocytes/macrophages), CD209/DC-SIGN (DCs). As shown in Figure 20 we observed

significantly higher accumulation of the CD3+T cells in tissue exposed to NG towards intraepithelial layer, compared to tissues exposed to control media. This observation was in line with the previous findings in cervicitis patients (Figure 19). However, when stained with other cell markers, we could not detect any other immune cells. There was no change in intensity of stains for CD20 (B cells), CD68 (monocytes/macrophages) or CD209/DC-SIGN (DCs), in the tissue section or towards the periphery of the epithelium for NG exposed tissues or in the tissues exposed to control (Figure 20).



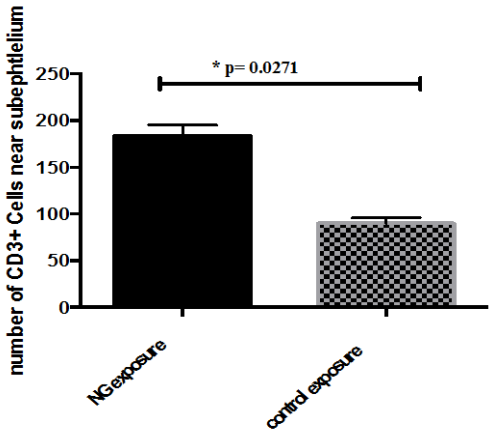


Figure 19: Migration of CD3+ cells were observed towards the sub-epithelium in NG exposed tissues over media exposed tissues.

Figure is a representative slide at 10X magnification. P=0.027 showed statistically significant increase in CD3+ T cells on NG exposed tissue compared to media exposed tissues. (n=2). Significance was calculated using nonparametric paired t test.

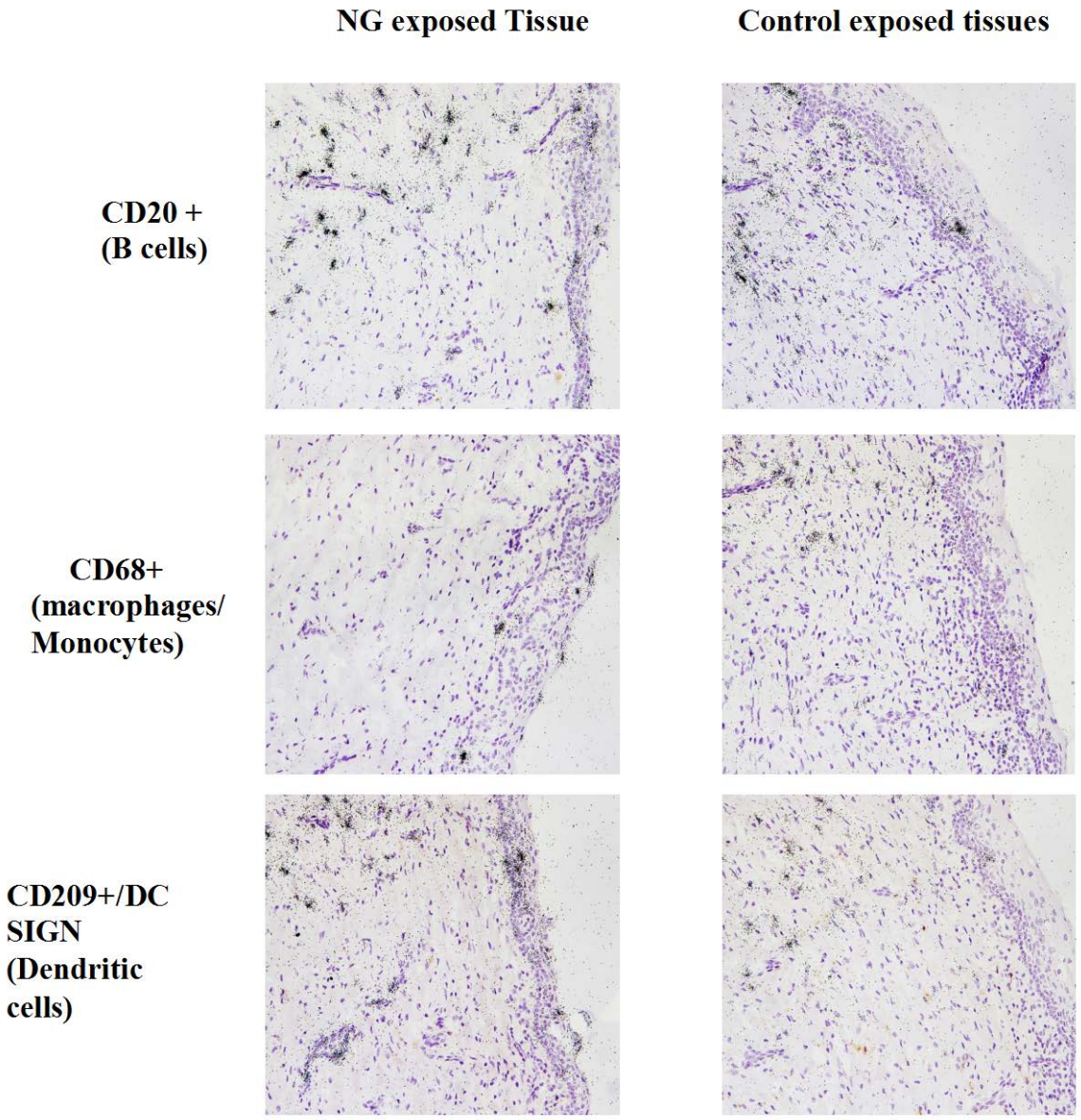


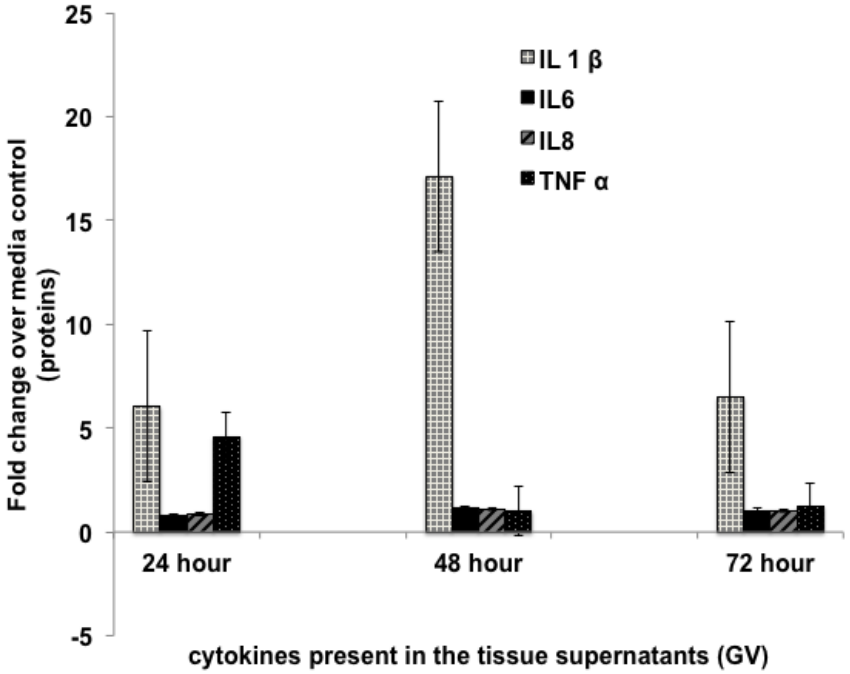
Figure 20: Representative Immunohistochemistry staining for CD20 (B cells), CD68 (monocytes/macrophages), CD209/DC-SIGN (DCs) at 20X magnification for Control media or NG exposed tissue sections.

No immune cells could be detected using markers for the stains for dendritic cells, B cells or marcrophages.

4.2.8 The organ culture model to study other bacterial vaginiosis causing organisms.

Following the inflammatory response results obtained from NG exposed cervical tissues we evaluated the cytokine response of two more bacterial vaginiosis (BV) causing microorganisms *Gardenerella vaginalis* (GV) and *Atopobium vaginae* (AV) on the cervical tissues for 24 hours, 48 hours and 72 hours by measuring their secreted cellular inflammatory response in supernatants using MSD multiplex assay. Both of these BV associated microbes GV and AV induced a very similar increase in inflammatory response compared to media control, but the extent of increase was not as large as observed by NG (Figure 21). IL-1β and TNF-α were still found to be the cytokines with highest fold changes compared to the control media.

a



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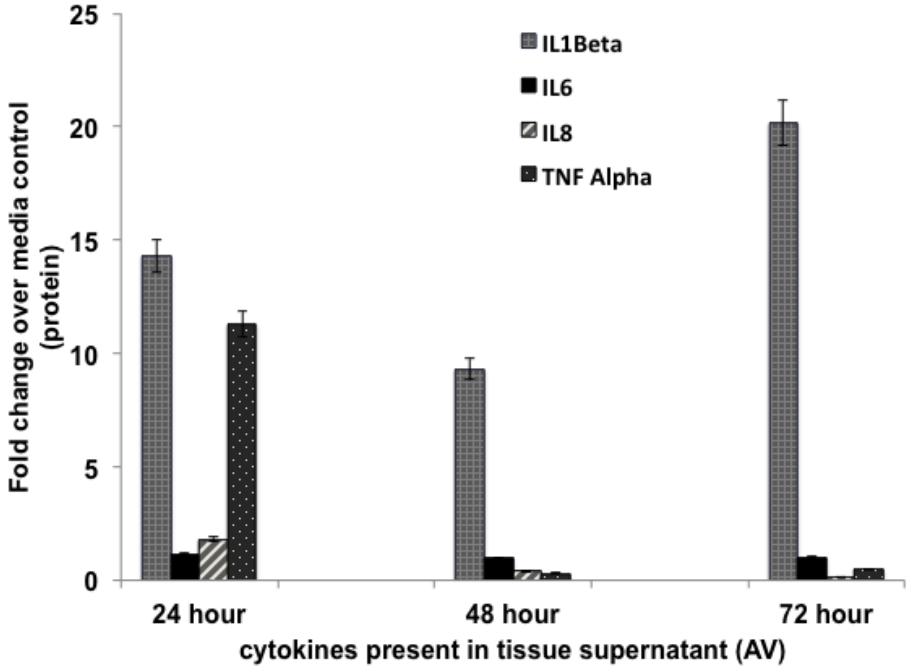


Figure 21: Secreted inflammatory cytokines from cervical tissues exposed to (a) *Gardenerella vaginalis* (GV) and (b) *Atopobium vaginae* (AV) compared to media control.

Cervical tissue biopsies exposed to (a) GV and (b) AV showed elevation in secreted cytokines as measured in the supernatants suing MSD multiplex assay at 24 hours, 48 hours and 72 hour compared to the media exposed control. Experiments were carried out in triplicate with each experiments having two biopsies for each experimental and control at times indicated (n=3). P<0.05 was considered as statistically significant compared to the control tissues analyzed by student's T test.

4.3 CONCLUSIONS

Our overall aim of this part of the project was to develop a cervical tissue based model to study NG that can be applied to study HIV-1 and NG interaction. For this purpose we modified the prior existing cervical tissue based organ culture model for HIV-1 transmission to evaluate the effect of STIs on the HIV-1 transmission. In this model we have shown that NG is non toxic to

the tissues, does not cause tight junction disruption, and causes epithelial membrane ruffling all of which are physical properties observed upon NG infection in-vivo.

In addition, NG exposure to cervical tissue induces an increase in synthesis of high level of cytokine production of IL-1β TNF-α, IL6 and IL8 with IL-1β and TNF-α being the highest fold change compared to control. In contrast, commensal bacteria, *Lactobacillus planeterum*, did not show any increase in cytokine response upon their exposure to the cervical tissues. NG mediated cytokine response does not require live bacteria, implying the outer membrane structure of NG is sufficient for inflammatory cytokine response. Deletion of two NG outer membrane proteins did not cause any changes in the cytokine profile implying that the LOS, which was present as an integral part of the membrane, may be largely responsible for induction of these cytokines from the tissues as described in studies earlier.

All of these observations together showed that this cervical tissue based organ culture model is an appropriate system to study NG infection in cervical tissues and could be used to study the effect of NG infection on the transmission of HIV-1.

5.0 SPECIFIC AIM 2

TO EVALUATE THE ROLE OF NG ON THE REPLICATION AND TRANSMISSION OF HIV-1 ACROSS CERVICAL MUCOSA

5.1 RATIONALE AND HYPOTHESIS

NG infection has been found to be associated with enhanced acquisition of HIV-1 in women. Epidemiological studies suggest that it is not the NG microbe per se, but NG induced cervical milieu that is responsible for increased HIV-1 acquisition/transmission in women. NG culture supernatants or some of its components have been shown to activate HIV LTR. NG has been reported to activate the HIV-1 long-terminal repeat (LTR) in a transformed Jurkat CD4+ T cell line[1, 2][3]. Heptose monophosphate (HMP) secreted from NG has been shown to activate HIV LTR in transformed $CD4^+$ T cells It has also been reported that NG induced human defensins (as induced on Hela cell lines) increased the transmission of HIV-1 in a cervical tissue based model.[4] Therefore we investigated whether culture supernatants from cervical tissues exposed to NG, (referred to as NG Induced supernatants, abbreviated as NGIS) had any effect on the HIV-1 expression and transmission across cervical mucosa.

Our hypothesis in this aim is that NGIS, reminiscent of NG induced cervical milieu and not NG *per se* enhances HIV-1 transmission across cervical mucosa by increasing HIV-1 expression and replication.

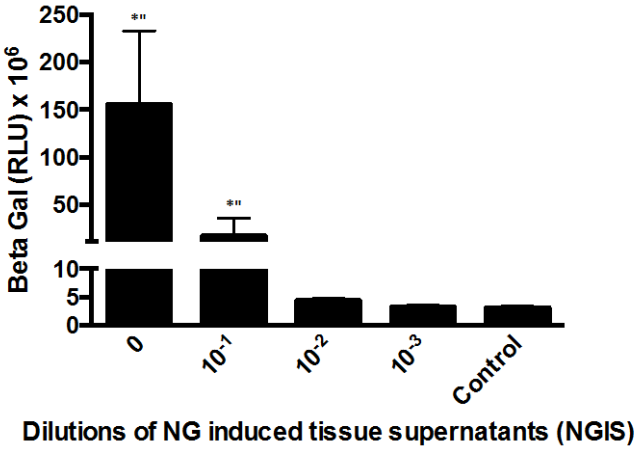
5.2 RESULTS

5.2.1 Effect of NG and NG induced tissue supernatant on HIV-1 transcription.

We investigated the effect of NGIS on HIV transcription by examining its effect on HIV LTR driven reporter gene expression in TZM-bl Cells. TZM-bl cells have integrated copies of the luciferase and ß-galactosidase genes under the control of the HIV-1 LTR. The TZM bl cells were incubated for 24 hour with NG bacteria alone, NGIS or control culture supernatants from cervical tissue exposed to media only (hereafter referred as control conditioned media (CM)) and then expression of β-galactosidase was measured .

NG bacteria alone did not show any significant activation of HIV-1 LTR in TZM-bl cells. However, a dose dependent stimulation of HIV-1 LTR activity was observed with a serial ten-fold dilution of the NGIS. No stimulation of HIV-1 LTR activity was observed with control CM (Figure 22).

a



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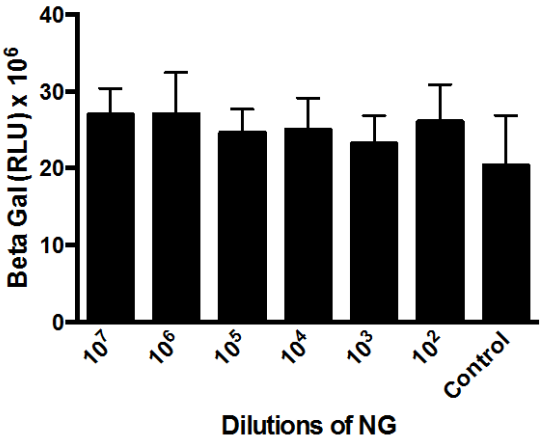


Figure 22: Effect of NG and NG induced tissue supernatants (NGIS) on the transcription of HIV-1 LTR in a TZM-bl cell based assay.

(a)NGIS showed a significant dose dependent increase in the beta galactosidase activity implying an increase in the transcription of HIV1- LTR in the TZM-bl cells compared to the control (CM). In contrast, (b)NG per se did not show any stimulation of the HIV-LTR over control media in TZM-bl cells. All experiments were run in triplicates P<0.05 was considered statistically significant compared to the control tissues analyzed by one sample students T test.

5.2.2 Effect of IL-1β and TNFα on the transcription of the HIV-1 LTR.

As mentioned earlier, NGIS has a number of pro-inflammatory cytokines which include IL-1β, TNF α, IL6 and IL8 with IL-1β and TNF α being the highest level compared to CM. We therefore examined whether these cytokines, primarily TNF-α and IL-1β, present in NGIS could

b

be responsible for this enhanced HIV-1-LTR expression in TZM-bl cells. For this purpose, purified cytokines IL-1β and TNF-α were evaluated for their ability to activate HIV-1expression in the TZM-bl cells

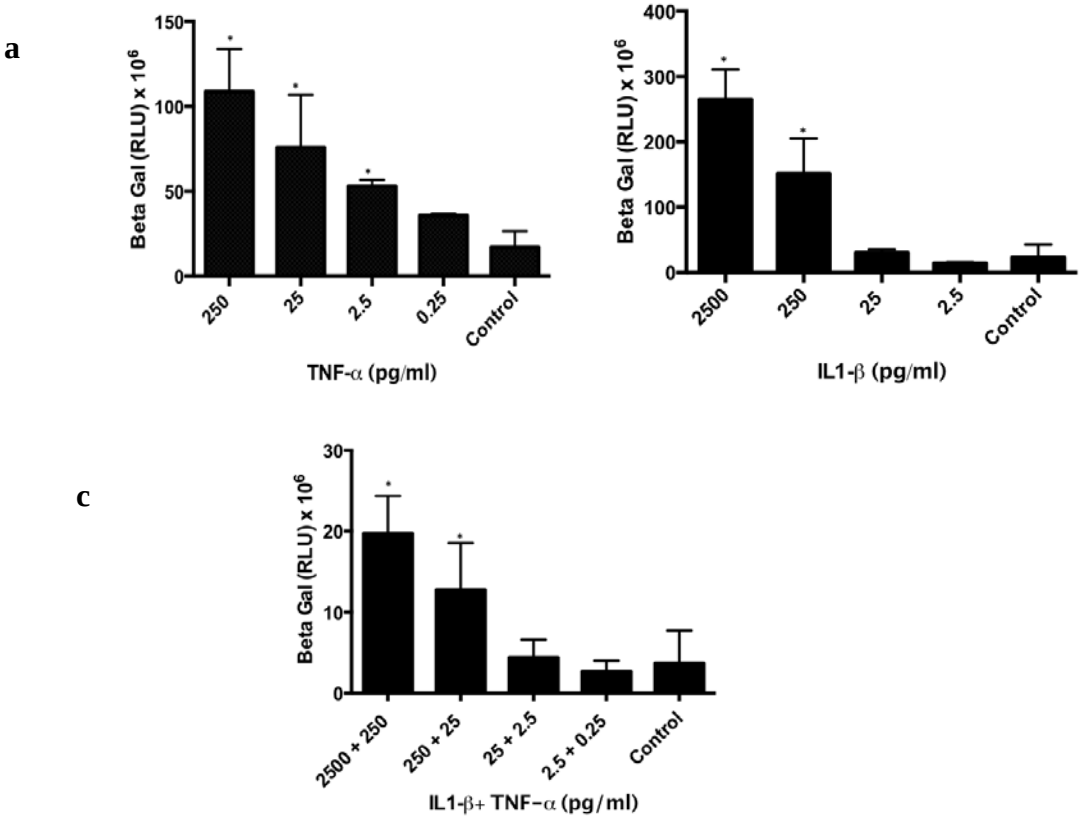


Figure 23: Effect of recombinant IL-1β and TNF-α at concentration detected in the NGIS on the expression of HIV-1 LTR in the TZM-bl cells.

(a)IL-1β and (b) TNF-α both showed a significant increase in the beta galasctosidase activity measured in RLU implying an enhancing effect on the HIV-1 LTR activity from the TZM-bl cells but failed (c) to show any additive effect on the transcription from the HIV-1 LTR. All experiments were run in triplicates and P<0.05 was considered to be statistically significant compared to the control tissues analyzed by one sample students T test.

As shown in Figure 23, both these cytokines at the concentrations (2500pg/ml for IL-1β and 250pg/ml for TNF-α) detected in the NGIS showed a significant dose dependent enhancement of the HIV-1 LTR activity. However, when the two cytokines were added together in the TZM-bl cells, it did not show an additive effect in the enhancement of HIV-1 LTR expression, which

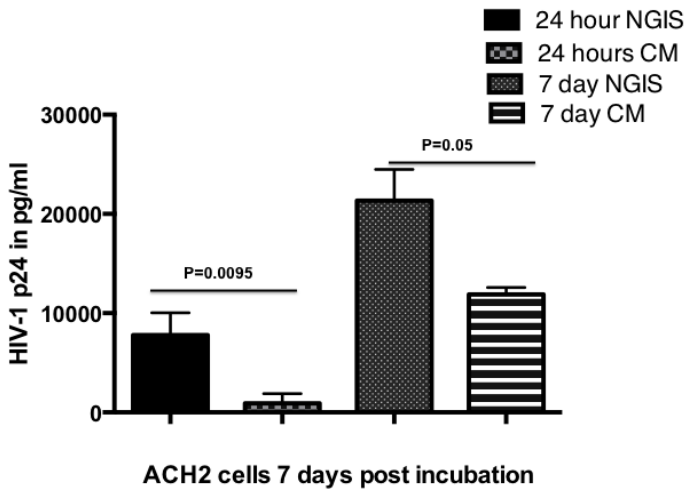
indicated that there could be more than just the two cytokines in the NGIS that was responsible for this increased effect on the HIV-1 transcription by the NGIS.

5.2.3 Effect of NG induced supernatant on HIV-1 replication and virus production from latently infected cells.

Having demonstrated enhancement of HIV-1 LTR activity by NGIS, we examined its effect on HIV replication. Studies have shown that supernatants from gonococcal-infected PBMCs stimulated HIV-1 replication in Jurkat cells.[1] We thus examined the effect of NGIS on reactivation of replication competent HIV-1 from latently infected cells ACH2 (T cell derived) and U1 (monocytic) cells.

For this purpose ACH2 and U1 cells were cultured in the presence of NGIS or control CM for 24 hour. In this part of the study, we examined the effect of both NGIS collected at 24 hours and 7 days post NG exposure to cervical tissues to evaluate if there was any change in the cellular protein content in NGIS during acute and chronic infection that would impact the replication and reactivation of HIV-1 from the latently infected cells. Following incubation, measuring HIV-1 p24 in the cell culture supernatant using ELISA monitored induction of HIV-1.

a



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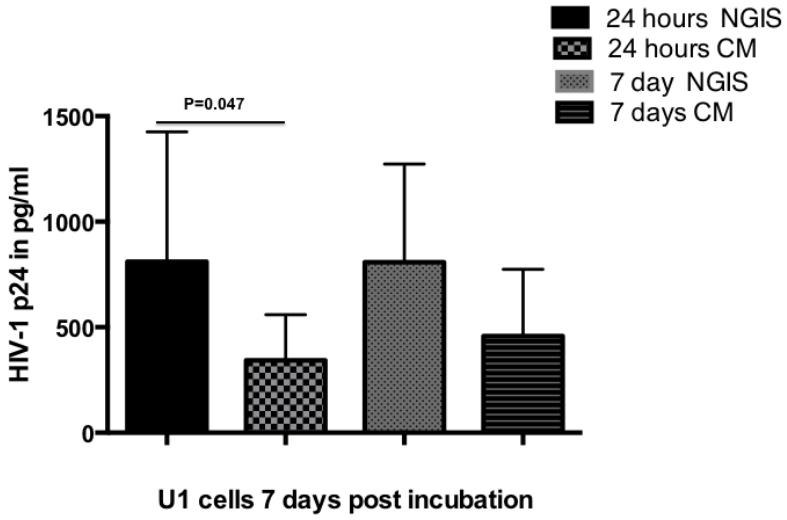


Figure 24: Effect of NGIS on HIV-1 replication and productive virus formation.

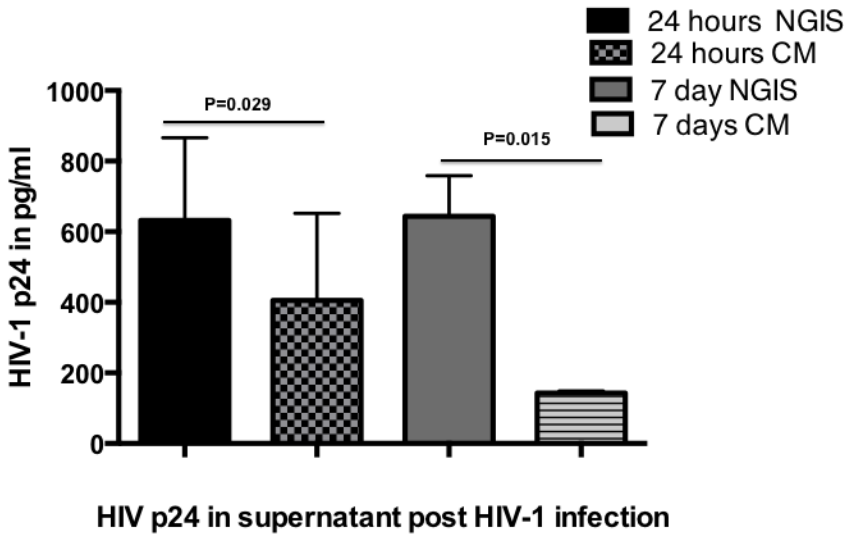
Both 24 hours and 7 days NGIS were able to significantly induce higher production of virus particles from (a) latently infected cell lines ACH2 compared to CM showing that they could induce the replication of HIV-1. (b) The NGIS could also induce higher virus production from U1 cells but this finding was not statistically significant at both time points. Experiment done in triplicates in three different sets (n=3). p <0.05 was considered as significant using the students T test.

NGIS from both 24 hours and 7 days supernatant significantly re-activated high levels of HIV-1 from both ACH2 and U1 cells (only 24 hours), compared to cells exposed to media alone or to control conditioned media (Figure 24).

5.2.4 Effect of NGIS on the HIV-1 transmission across cervical tissues in the organ culture system.

We had been able to show that NGIS increased HIV-1 transcription from the HIV-1 LTR as well as HIV-1 replication in latently infected cells. We next wanted to examine whether NGIS could increase the HIV-1 transmission across the cervical epithelium. For this purpose our standard organ culture for measuring HIV-1transmission was set up in a trans-well with CD8 depleted PBMC as indicator cells in bottom well. [5]. NG ($3x10^6$) or undiluted NGIS (24 hours or 7 days) were added onto the top of epithelial layer of the cervical tissue and pre-incubated for 24 hours after which HIV-1 Bal ($TCID_{50}$ $1x10^6$) was added onto the tissue. Viral growth in the bottom well was used to monitor HIV-1 transmission across cervical epithelium. After three days post HIV-1 exposure, the tissues were removed from trans-well and analyzed for HIV -1 transcription by measuring Gag-mRNA by RT-PCR in the tissues. The culture in bottom well was continued for 7 days to monitor transmitted HIV-1 across the cervical tissues using ELISA p24 assay. Results shown in Figure 25 indicate a 45% increase in the HIV-1 transmission across the cervical tissues with NGIS collected 24 hour after NG exposure and a 75% increase in HIV-1 transmission with NGIS collected 7 days after exposure to NG, compared to control CM (Figure 25 a). In contrast, NG by itself did not enhance HIV transmission (Figure 26). This was in line with our earlier finding, which showed that the NG by itself did not increase transcription from the HIV-1 LTR.

a



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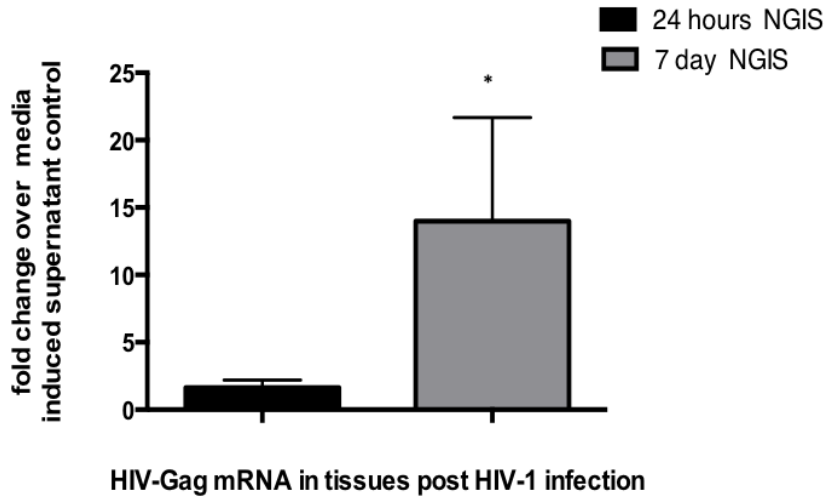


Figure 25: Effect of NGIS on HIV-1 transmission across cervical mucosa.

NGIS increased the transmission of HIV-1 across the cervical mucosa as (a) monitored by p24 in the transmitted supernatant. (b) An increase in HIV-1 Gag mRNA was monitored in the tissues which were exposed to NGIS pre infection with HIV-1. (n=3). p <0.05 was considered as significant using the students T test.

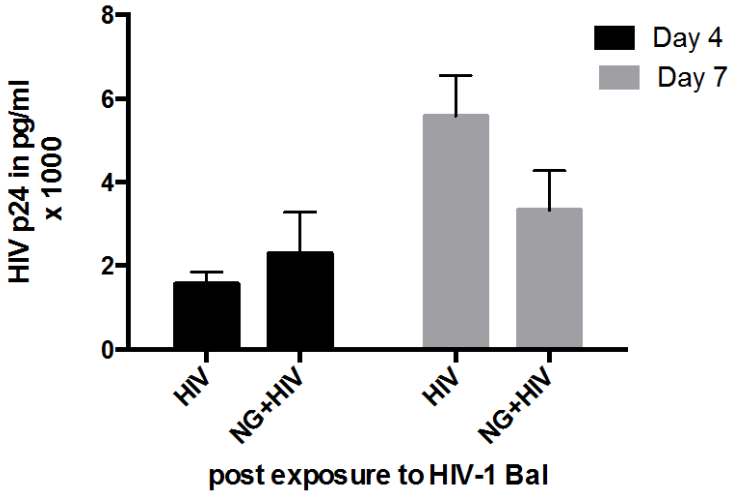


Figure 26: Effect of NG on HIV-1 transmission across cervical mucosa.

NG by itself when exposed to cervical tissues could show no significant (p=0.05 by students T test) impact on the transmission of HIV-1 across the mucosa as demonstrated in a cervical tissue based organ culture model. (n=3).

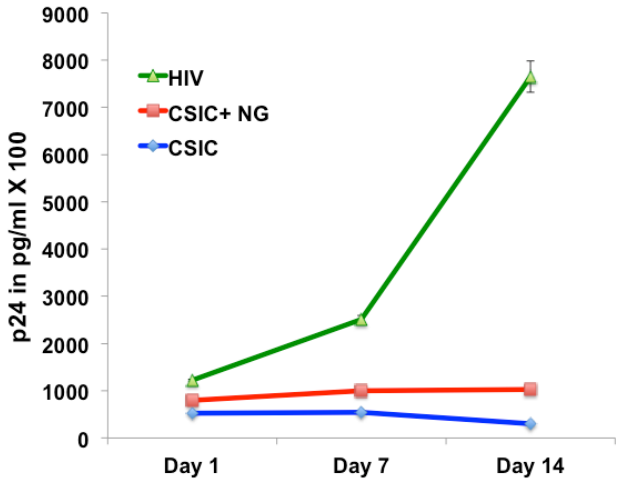
Results shown in Figure 25 b showed that there was a significant increase (4 fold increase) in HIV-1 transcription with 7 day as well as 24 hr NGIS (2 fold) compared to control CM. The increase in HIV-1 transcription with 24 hour NGIS was not statistically significant, although we observed statistically significant HIV-1 transmission as determined by HIV-1 p24 in bottom well of the organ culture set up.

5.2.5 Effect of NG on the action of microbicides against HIV-1.

In the absence of an effective vaccine, microbicides have become extremely important tools for HIV-1 prevention. Two large clinical trials have demonstrated that microbicides can significantly reduce HIV-1transmission: iPrEx with Truvada, an oral, once daily combination of emtracitabine and tenofovir disoproxil fumarate (TDF), and CAPRISA 004 with a pericoital 1% tenofovir (TFV) vaginal gel [6, 7]. An intracellular tenofovir-diphosphate (TFV-DP)

concentration of 16 fmol per million viable PBMCs was associated with upto 90% reduction in HIV-1 acquisition in high risk MSM groups relative to the placebo arm. In a separate study called STRAND, a direct dosing of TFV-DP concentrations when analyzed with this iPrEx model, corresponded with HIV-1 acquisition risk reduction of 76% for 2 doses per week, 96% for 4 doses per week and as high as 99% for 7 doses per week. In the CAPRISA study among high-risk females, Tenofovir gel reduced HIV-1 acquisition by an estimated 39% overall, and by 54% in women with high gel adherence statistically. A Retrocyclin (cyclic antimicrobial peptides) derivative entry inhibitor RC-101 that exhibits potent activity towards a broad range of HIV-1 in-vitro has been shown to be highly efficient against HIV-1 and could decrease the transmission across a cervical tissue [8, 9]. A reverse transcriptase inhibitor CSIC has also been demonstrated to be efficient in decreasing the transmission of HIV-1 across cervical mucosa in an organ culture[10]. However, a compounding problem in the development of microbicides is that they need to be active in the presence of STI-related microbes, which are often present in ecto-cervix of women in the developing world. Therefore, anti -viral activity of microbicides may be reduced in the presence of STIs. NG poses a great problem in this respect. Therefore, we evaluated antiviral activity of CSIC and RC101 in the presence of NG in an organ culture model.

a



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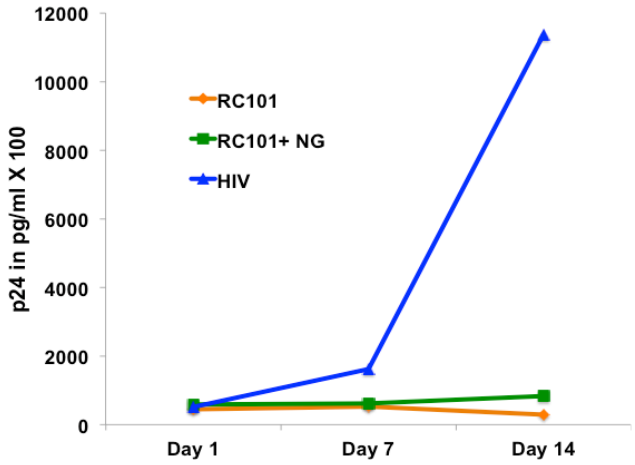

Figure 27: Presence of NG did not change the efficiency of CSIS or RC101 microbicides.

None of the microbicodes a) CSIC or b) RC101 lost their efficiency in decreasing the transmission of HIV-1 across the cervical mucosa as monitored in the organ culture setup.

For this purpose we pre-incubated the cervical tissues with RC-101(40ug/ml) or CSIC (100uM) with or without NG for 2 hour and then inoculated the tissue with HIV-1 Bal ($TCID_{50}$ $1x10^6$). As a control, tissues were incubated with media for 2 hour and inoculated with HIV-1 Bal. CD8-depleted cells in the bottom chamber were continued for an additional 7 days. We then

analyzed the culture supernatants in bottom well for HIV-1 p24 using ELISA to monitor the transmission of HIV-1 across the mucosa (Figure 27).

As shown in Figure 27, presence of NG did not have any effect on the antiviral activity of these two microbicides These results indicate that these microbicides could be used as potential preventative tools in developing countries where the population was at higher risk of acquiring HIV-1 as well as at risk for sexual transmission of NG, which possess a threat to public health.

5.3 CONCLUSIONS

Our overall aim of this part of the project was to investigate the effect of NG or NG induced tissue supernatant (NGIS) on the acquisition and transmission of HIV-1. In this aim we found that it is not NG, but 24 hour induced NGIS from tissues positively affected the activation of HIV-1LTR in TZM-bl cells. IL-1β and TNF-α at concentrations found in the 24 hour induced NGIS could also increase the HIV-1 LTR activity. Both 24 hours as well as 7-day NGIS also re-activated high levels of HIV-1 from both latently infected ACH2 and U1 cells. Most importantly both 24 hour and 7-day NGIS could also significantly enhance the transmission of HIV-1 across cervical mucosa with a concomitant increase in HIV-1 transcription in the cervical tissues. Finally, we showed that the effect of microbicides did not decrease in the presence of NG in the cervix.

All of these observations together showed that NGIS and not NG could increase the acquisition and transmission of HIV-1 across the cervix in our organ culture model. It also showed that the presence of NG do not compromise the antiviral activity of microbicides.

6.0 SPECIFIC AIM 3

TO IDENTIFY CELLULAR FACTORS RESPONSIBLE FOR NG INDUCED ENHANCED HIV-1 TRANSMISSION.

6.1 RATIONALE AND HYPOTHESIS

Currently molecular mechanisms by which NG enhances HIV-1 acquisition/transmission in female genital tract are uncertain. Part of this uncertainty arises from the fact that we do not know the mechanism of HIV-1 transmission through the epithelia of the cervical mucosa, especially when epithelia do not express CD4 and CCR5/CXCR4 [5]. Regardless of how HIV-1 crosses the epithelium, virus exposure to the epithelial layer of the cervix or epithelial cells has been shown to induce cytokines and chemokine including IL-6, IL-8, SDF-1, MIP-1α, MIP-1β, TNFα, GM-CSF, Type 1 IFNs and RANTES[260] [238]. These signaling molecules may play important roles in HIV-1 transmission by attracting target immune cells to fuel HIV-1 infection in the sub-mucosa and hence increase transmission. In addition, due to the multilayer nature of cervical epithelia, NG possibly does not enter the sub-mucosa right upon exposure on the onset of infection, and leads to an increased cytokine production at the epithelium surface from the epithelial cells as observed in aim 1. Therefore it is possible that that NG induced inflammatory cytokines through interaction with certain cellular factors that could increase HIV-1

transmission. Thus our hypothesis for this part of the study is that **the interaction of NG induced inflammatory cytokines and other epithelial cellular factors that are responsible for enhancing the HIV-1 transmission across the cervical mucosa**

To prove this we sought to identify a broader range of cellular factors that were expressed upon NG and HIV-1 exposure only to epithelial layers of the cervical tissues. Studies on SIV in non human primate (NHP) models indicates that virus crosses cervical and vaginal epithelial layers within hours of exposure leading to the accumulation of CD4+T cells, macrophage/monocytes and DC at the intraepithelial layer. It has been implicated that this is likely due to the cellular factors up-regulated during HIV-1 infection which drives the migration of these cells to the site of exposure[288]. Here we used next- generation sequencing technology [289], to analyze the differentially regulated cellular factors that are enhanced during the NG as well as HIV-1 exposure on cervical epithelium. This was further analyzed to evaluate whether NG exposure increases cellular signals that are responsible for HIV-1 transmission across the cervical mucosa and hence increase HIV-1 transmission.

6.2 RESULTS

6.2.1 Assessment of the global cellular response in cervical epithelia in response to NG.

To identify cellular factors in epithelia that may be expressed upon NG exposure on the epithelia, we performed a comprehensive transcriptome analysis using next generation sequencing technique on an Ion Torrent platform with the RNA extracted from epithelia of ecto-cervical tissues (n=6) exposed to NG or control supernatant. The mapped sequences, which were based

on the human genome mRNA database, were analyzed to compare the expression levels of individual gene by statistical software R and expression levels of about 52,000 genes were analyzed. The genes that had no changes in expression or with low read counts were excluded for further analysis from the study. Expression levels of the remaining genes were further analyzed based on false discover rate (fdr) <0.05 to examine if their expression levels were significantly changed after exposure to NG. The data showing differentially expressed genes were summarized in heat maps with red indicating low differential expression and green indicating high differential expression (Figure 28). 33 genes were found to be most differentially expressed (-3 to 8 fold) with high significance ((p<0.05) upon NG exposure compared to control media exposed to ecto-cervical epithelia (Table 3).

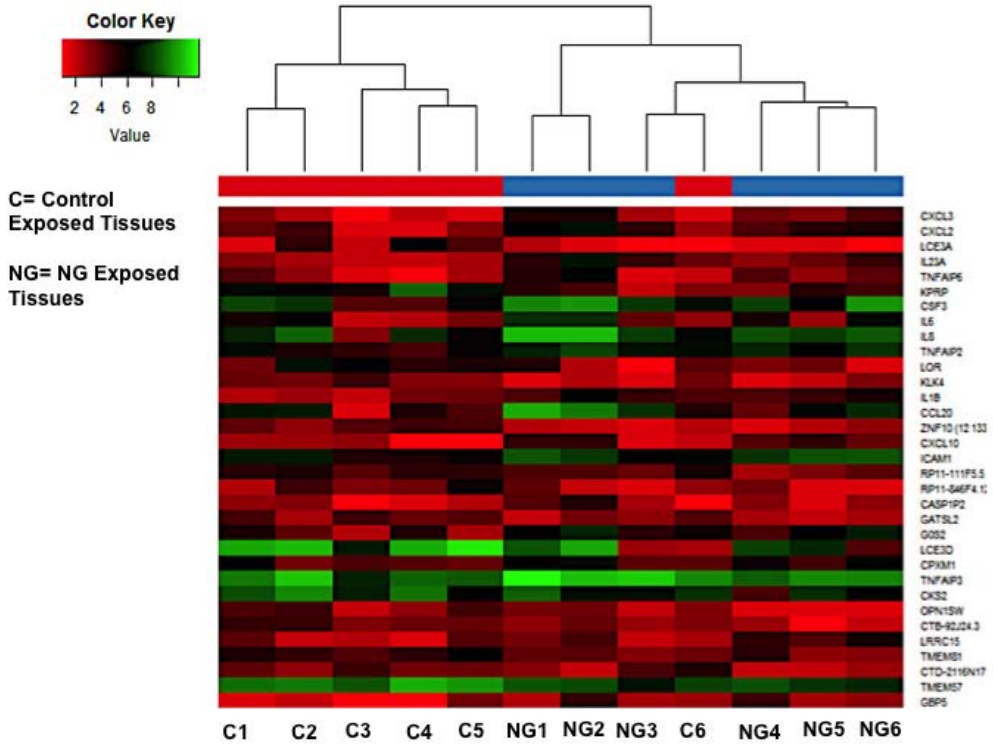

Figure 28: Heat map for NG induced gene expression on cervical epithelium.

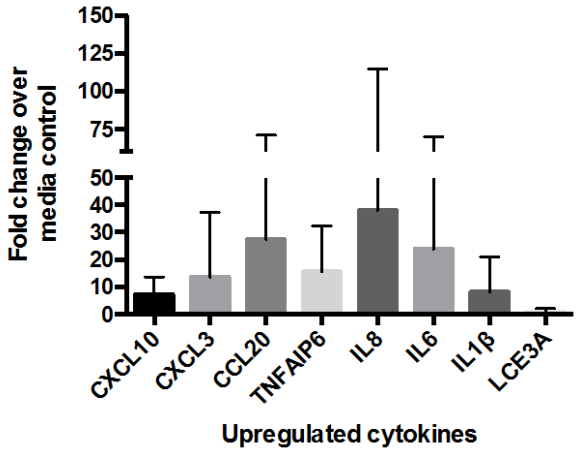

Figure 29: Epithelial transcriptome analysis by Ion torrent on six cervical tissues exposed to NG.

Seven most highly up regulated (more than five folds) and one most highly down-regulated gene selected from the differentially regulated genes upon NG exposure were quantified using RT-PCR separately for verification. Significance was set at $p< 0.05$.

The differentially expressed genes which were most significantly up-regulated and the one gene that was significantly down-regulated were verified using RT-PCR (Figure 29).

Table 3: Thirty-three Differentially expressed genes in NG exposed ecto-cervical epithelia compared to the controls as evaluated by next generation sequencing in an Ion Torrent platform

DEGs	Fold Change	P-Value	FDR	Gene Function
CXCL2	7.309515451	1.45E-10	0.000000294 *	antimicrobial gene from chemokine superfamily involved in immunoregulatory and inflammatory processes.
CXCL3	8.678235343	2.90E-10	0.000000294 *	codes for secreted growth factor and plays a role in inflammation and as a chemoattractant for neutrophils.
LCE3A	-13.11748544	6.58E-09	0.00000445*	Unknown
IL23A	5.941496535	3.13E-08	0.0000159 *	gene stimulate the production of IFNγ preferentially acts on memory CD4(+) T cells.
TNFAIP6	5.255028001	2.13E-07	0.0000864**	involved in extracellular matrix stability and cell migration.
CSF3	5.198292754	3.18E-07	0.000107 **	gene codes for cytokine that controls the production, differentiation, and function of granulocytes.
IL8	5.271190737	4.14E-07	0.000102 **	codes for major mediator of inflammatory response and secreted by several cell types. It functions as a chemoattractant
KPRP	-4.376721506	6.50E-07	0.000149 **	Unknown
IL6	4.840391408	6.61E-07	0.000149 **	involved in inflammation and the maturation of B cells. The protein is primarily produced at sites of acute and chronic inflammation.
CXCL10	6.360649828	1.34E-06	0.000272 **	involved in stimulation of monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression.
CCL20	5.563422645	1.64E-06	0.000302 **	involved in immunoregulatory and inflammatory processes. chemotactic activity for lymphocytes and can repress proliferation of myeloid progenitors.
LOR	-5.356203549	1.79E-06	0.000302 *	Unknown
IL1B	4.690655887	3.03E-06	0.000473 **	produced by activated macrophages and is an mportant mediator of the inflammatory response
TNFAIP2	3.143938559	1.24E-05	0.00180 *	This gene expressioncan be induced by TNF in the endothelial cells
KLK4	-4.32665441	2.51E-05	0.00340 *	diverse physiological functions but not directly related to inflamatory responses from the tisssues
ZNF10 (12 133707160..133736051)	-4.235329542	3.42E-05	0.00433 *	codes for protein that function as a transcriptional repressor.
ICAM1	3.358138783	5.08E-05	0.00606 *	codes for receptor for many bacteria and viruses and some reports say it is also a receptor for HIV-1

Table 3 Continued

DEGs	Fold Change	P-Value	FDR	Gene Function
LCE3D	-3.485271457	7.86E-05	0.00884 *	unknown but is one of the envelope proteins on HIV-1 that is derived from the host and may be inportant for the formation of virions after NG infection an HIV-1 coinfection
G0S2	4.064623803	8.28E-05	0.00884 *	Codes for genes required during growth of the cell. Other actions unknown
RP11-846F4.12	-3.406640896	1.58E-04	0.016 *	unknown
RP11-111F5.5	-2.591112673	2.08E-04	0.0198 *	unknown
CPXM1	2.969112701	2.15E-04	0.0198 *	This gene likely encodes a member of the carboxypeptidase family of proteins
CASP1P2	4.134089524	2.26E-04	0.0199 *	Unknown
LRRC15	3.346601799	2.68E-04	0.0223 *	unknown
TNFAIP3	2.363362563	2.74E-04	0.0233 *	This gene induced by TNF. The encoded protein, is involved in the cytokine-mediated immune and inflammatory responses.
GBP5	4.606825877	4.07E-04	0.0297 *	Unknown
SELE	6.069378117	4.10E-04	0.0297 *	responsible for accumulation of blood leukocytes at sites of inflammation
GATSL2	-2.950273312	4.11E-04	0.0297 *	Unknown
CTD-2116N17.1	-2.892427875	5.73E-04	0.0401 *	unknown
OPN1SW	-3.260377178	6.30E-04	0.0415 *	This gene belongs to the G-protein coupled receptor 1 family, opsin subfamily.
CTD-2292P10.4	-3.63591427	6.34E-04	0.0415 *	unknown
CTB-92J24.3	-2.890129738	6.71E-04	0.0425 *	unknown
FLG	-3.051920168	7.66E-04	0.0471 *	codes for protein filaments in mammalian epidermis.Maybe involved in ruffling process

The Ingenuity IPA software was used to construct pathway maps to evaluate how all these differentially expressed genes more than 1.5 folds differentially regulated interacted with one another and could be involved in different cellular functions. Such analysis predicted five different groups of cellular functions and molecular pathways to be significantly up regulated

upon NG exposure, which were broadly Cell-cell interaction and signaling, cellular movement, cellular development, cellular growth and proliferation and cellular function and maintenance. (Figure 30). The thirty three genes when analyzed for the canonical pathways they were involved in were found to be all mostly involved in the up regulation of canonical pathways, which comprised of T cell and monocyte migration, maturation and activation (Figure 31). These findings were in line with previous findings [279] [241] and our results in Aim 1 showing migration of CD3+ cells towards the epithelium upon NG exposure.

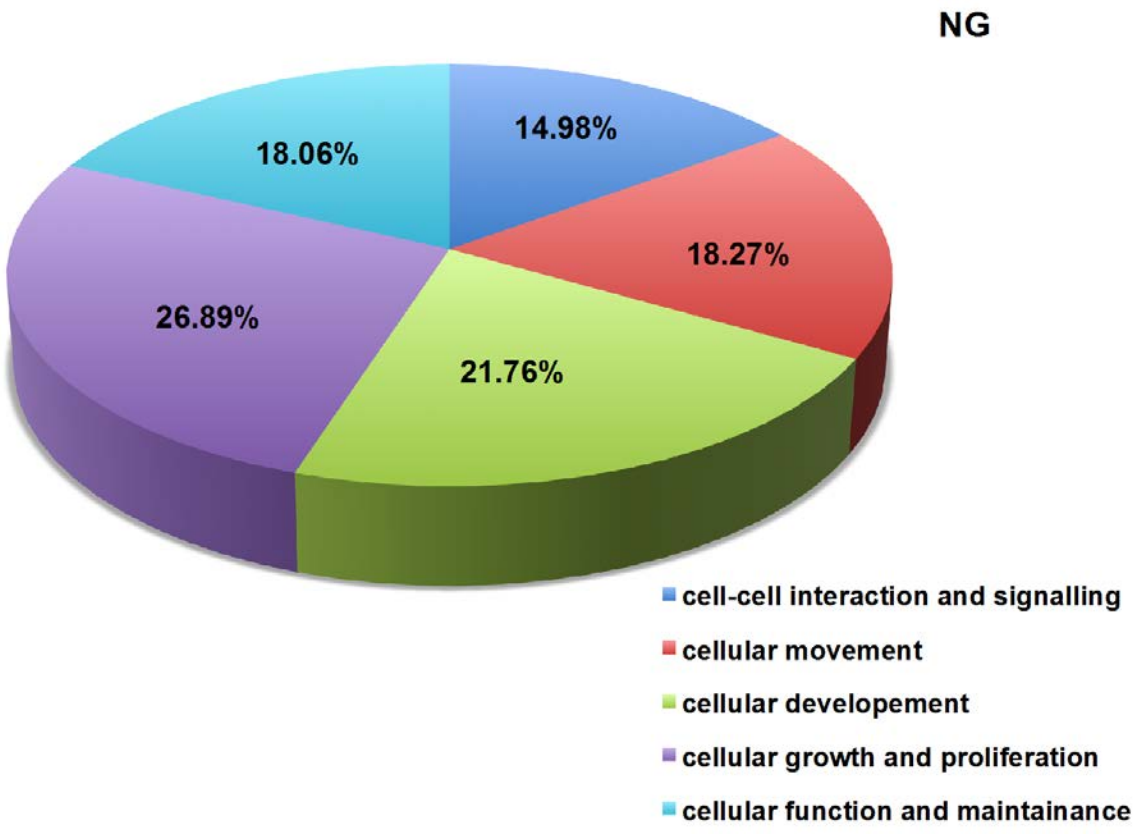

Figure 30: Pie chart with cellular function containing genes regulated upon NG exposure on cervical epithelium

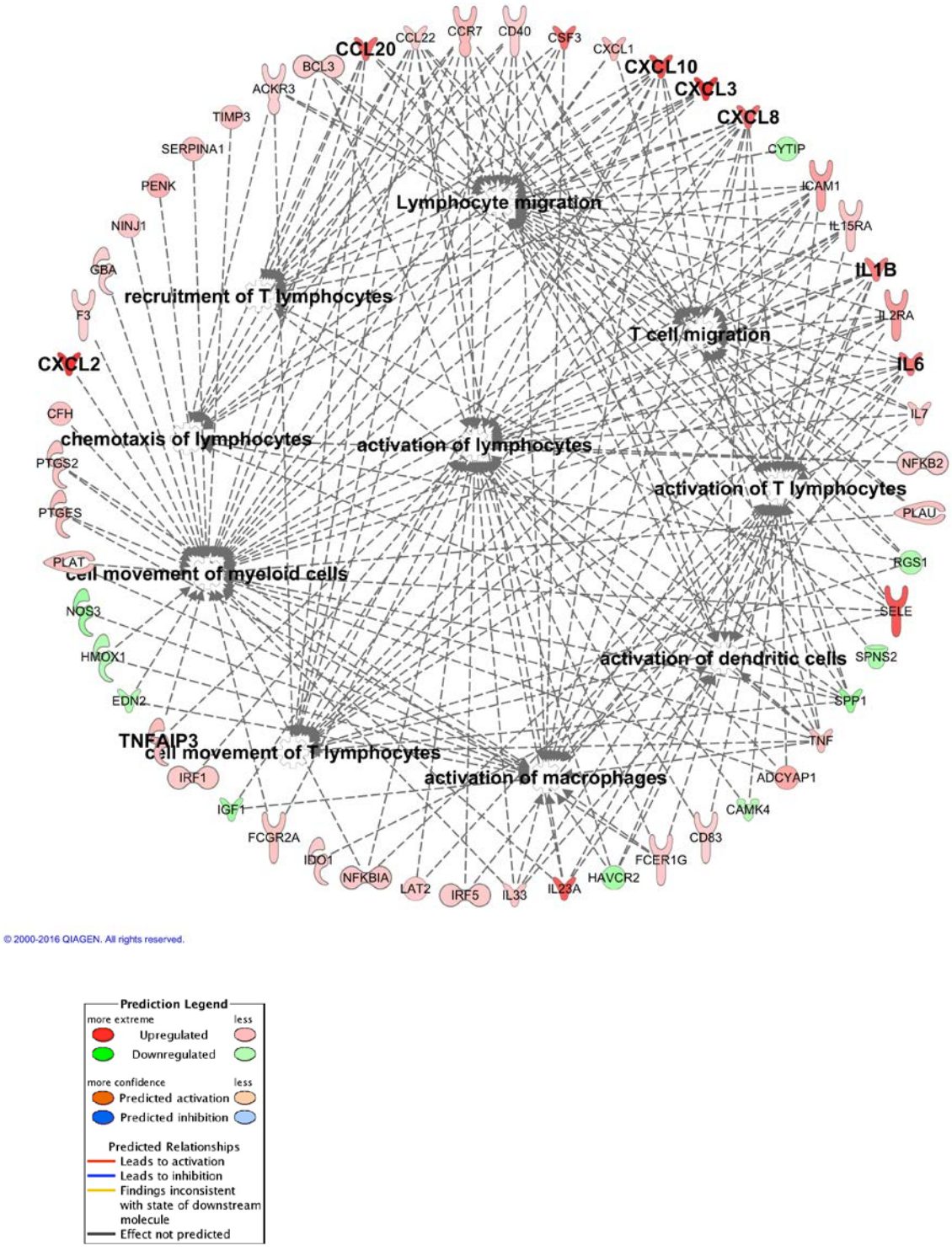

Figure 31: Cellular functions up-regulated upon NG exposure on cervical tissue epithelium along with interacting genes in those particular pathways.

6.2.2 Confirmation of genes differentially expressed upon NG exposure on cervical tissues using RT-PCR.

Out of the 33 significantly differentially expressed (< 5 fold) genes detected by the ion torrent analysis, expression of the 7 genes (CXCL10, CXCL3, CXCL20, TNFA1P6, IL8, IL6, IL-1β) which were the most highly up regulated with great significance were selected to be confirmed using the RT-PCR assay in 10 tissues exposed to NG or control supernatant. The LCE3A gene, which was not an up-regulated gene was included as a negative control (Figure 32). Results indicate that these seven genes but not LCE3A were highly elevated (9-29 fold)(Table 4)

Table 4: Confirmation of genes that were differentially up regulated upon NG exposure on cervical tissues using RT-PCR.

Sample Number	CXCL10	CXCL3	CCL20	TNFAIP6	IL8	IL6	IL1b	LCE3A
E-9202 (NG)	3.39	3.32	2.38	9.32	1.6	2.46	1.68	0.02
E-9235 (NG)	0.18	0.73	0.74	0.79	0.57	0.59	0.75	0.39
E-9249 (NG)	6.06	5.35	17.63	NA	NA	NA	NA	NA
E-9326 (NG)	5.82	61.82	113.77	44.63	174.85	106.15	30.91	3.23
E-9395 (NG)	7.26	5.82	26.72	8.63	8.51	5.24	4.29	0.57
E-9431 (NG)	19.56	4	2.79	14.12	4.66	4.29	2.89	0.68
E-9318 (NG)	26.17	3.41	4.89	7.46	7.67	4.32	8.4	4.53
E-9457 (NG)	13.83	5.94	10.41	3.07	42.22	3.48	8.17	3.16
E-9727 (NG)	4.29	15.78	6.59	2.91	6.06	10.85	22.94	0.86
E-9761 (NG)	3.81	3.12	34.54	3.39	15.24	5.13	6.96	0.68
Avg FC (n=10)	9.04 *	10.93 *	22.05 *	10.48 *	29.04 *	15.84 *	9.67 *	1.57 *
p value	0.0041	0.0039	0.0039	0.0078	0.0117	0.0078	0.0078	0.887

Seven genes out of which six were the most highly up-regulated genes and the seventh one not significantly up regulated was verified using RT-PCR on additional tissues making the n=10. A nonparametric-paired t test was used for calculating the statistical significance.

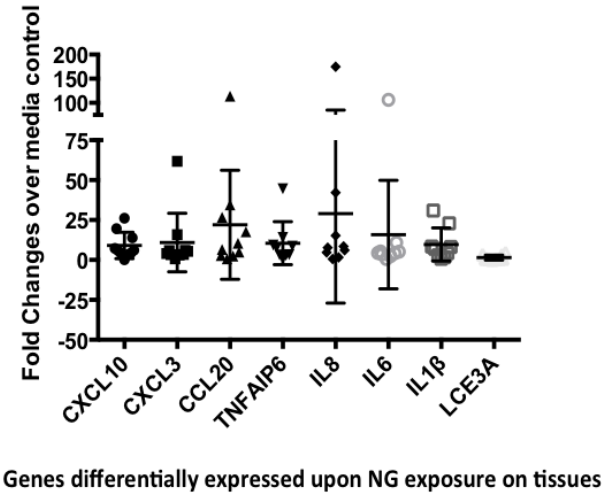

Figure 32: Confirmation of genes most significantly up regulated or down regulated upon NG exposure on cervical tissues.

6.2.3 Assessment of the global cellular response in cervical epithelia in response to HIV-1 and their confirmation using RT-PCR.

IL8, MIP1 Beta, MCP-1, TSLP, TNFα, IL 6 which play an important role in recruiting immune cells, have been shown to increase post exposure to HIV-1 on cervical and epithelial cells[213, 290, 291]. An increase in expression level of cytokines like MIP3α, MIP-1β has also been observed in studies on rhesus macaque's cervical epithelium which recruits immune cells like pDCs and CD4+T cells [290, 292]. These reports and conclusions are drawn from *in-vitro* human cell lines and primary cell studies and *in-vivo* monkey studies focusing on early events of SIV transmission. Studies on early events of HIV-1 transmission during the first few days of infection have not been performed in humans due to ethical and practical reasons. In view of all these observations, primary cervical epithelial cells in the tissue matrix may be of prime importance in

the transmission and infection process of HIV-1. These cells may induce local inflammatory responses and other cellular factors, which may facilitate HIV-1 transmission. To identify these epithelial proteins that may be involved in HIV-1 transmission across cervical epithelia, transcriptome analysis using second-generation sequencing was performed on cellular RNA isolated from micro-dissected epithelial layer of 6 tissues exposed to HIV-1 or control supernatant. As done with the tissues exposed to control and NG, the mapped sequences aligned to the human genome mRNA database were analyzed to compare the expression levels of individual gene and this was done using statistical software R. Here again, expression levels of about 52,000 genes were analyzed. The genes that had no changes in expression or with low read counts were excluded for further analysis from the study. Expression levels of the remaining genes were further analyzed based on false discover rate (fdr) <0.05 to examine if their expression levels were significantly changed after exposure to NG. Seven genes were found to be most significantly differentially expressed (2-7 fold) upon HIV-1 exposure compared to control media exposed to ecto-cervical epithelia (Figure 33)(Table 5).

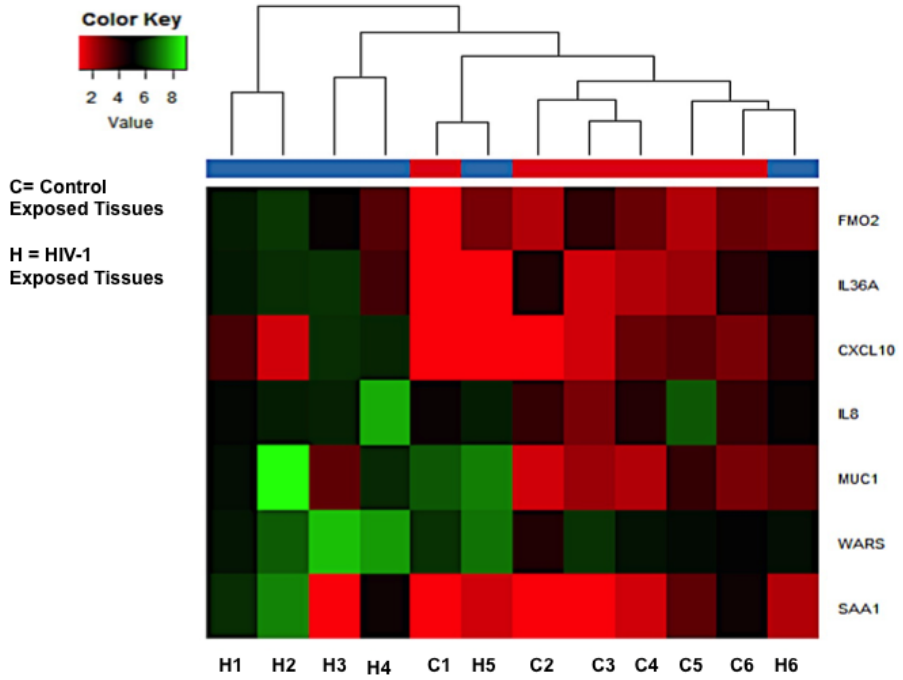

Figure 33: Heat map for HIV-1 induced gene expression on cervical epithelium

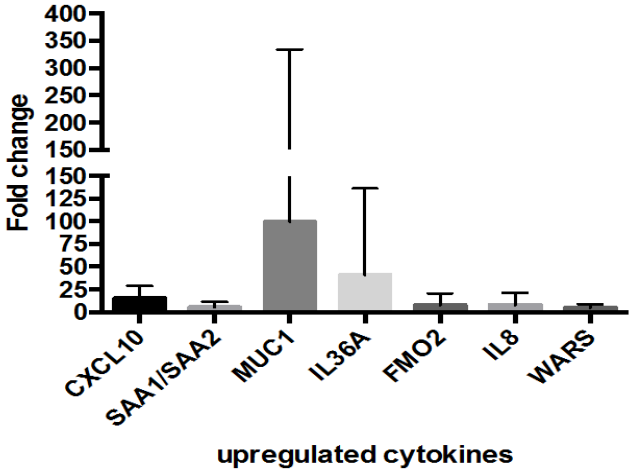

Figure 34: Epithelial transcriptome analysis by Ion torrent on six cervical tissues exposed to HIV-1.

Seven genes differentially regulated more than five folds upon HIV-1exposure was quantified using RT-PCR separately for verification. Significance was set at $p < 0.05$.

Table 5: Differentially expressed genes in HIV-1 exposed ecto-cervical epithelia compared to the controls as evaluated by next generation sequencing in an Ion Torrent platform

DEGs	Fold Change	P-Value	FDR	Gene function
IL36A	6.2376	1.77E-07	0.001541254 *	plays role in innate and adaptive immunity and functions to generate an inflammatory response
FMO2	5.2504	0.000000237	0.001541254 *	Unknown
CXCL10	5.4587	0.00000387	0.016779861 *	codes for protein that stimulate monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression.
MUC1	6.3156	0.00000955	0.027737094 *	protective mucous barrier on epithelial cells and plays role in intracellular signalling
SAA1	7.3825	0.0000107	0.027737094 *	acute phase protein that is highly expressed in response to inflammation and tissue injury.
IL8	2.6766	0.000000318	0.036655145 *	major mediator of the inflammatory response secreted by several cell types. It functions as a chemoattractant,
WARS	2.5519	0.000000414	0.095439132	major role in protein translation central role in linking amino acids with nucleotide.

Similar to our analysis of the NG exposed tissues, IPA analyses of these differentially expressed sets of genes which included most of the genes with more than a 1.5 fold change, predicted five different groups of cellular functions and pathways namely cell-cell interaction and signaling, cellular movement, cellular development, cellular growth and proliferation and cellular function and maintenance to be up regulated which were common to most of the functions up regulated upon NG exposure (Figure 35). When analyzed further, all these seven genes were predicted to be the genes either directly involved or were upstream regulators of other genes associated with T cell and monocyte migration, maturation and activation (Figure 36). All of these cells are known target cells for HIV-1 infection.

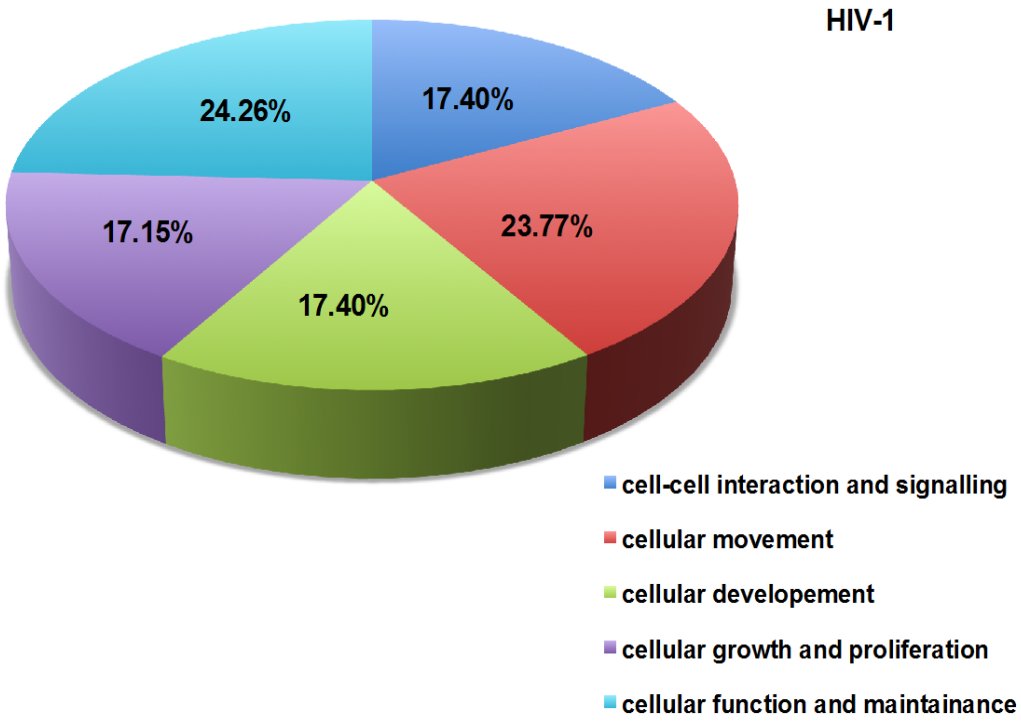

Figure 35: Pie chart with cellular function containing genes regulated upon HIV-1 exposure on cervical epithelium

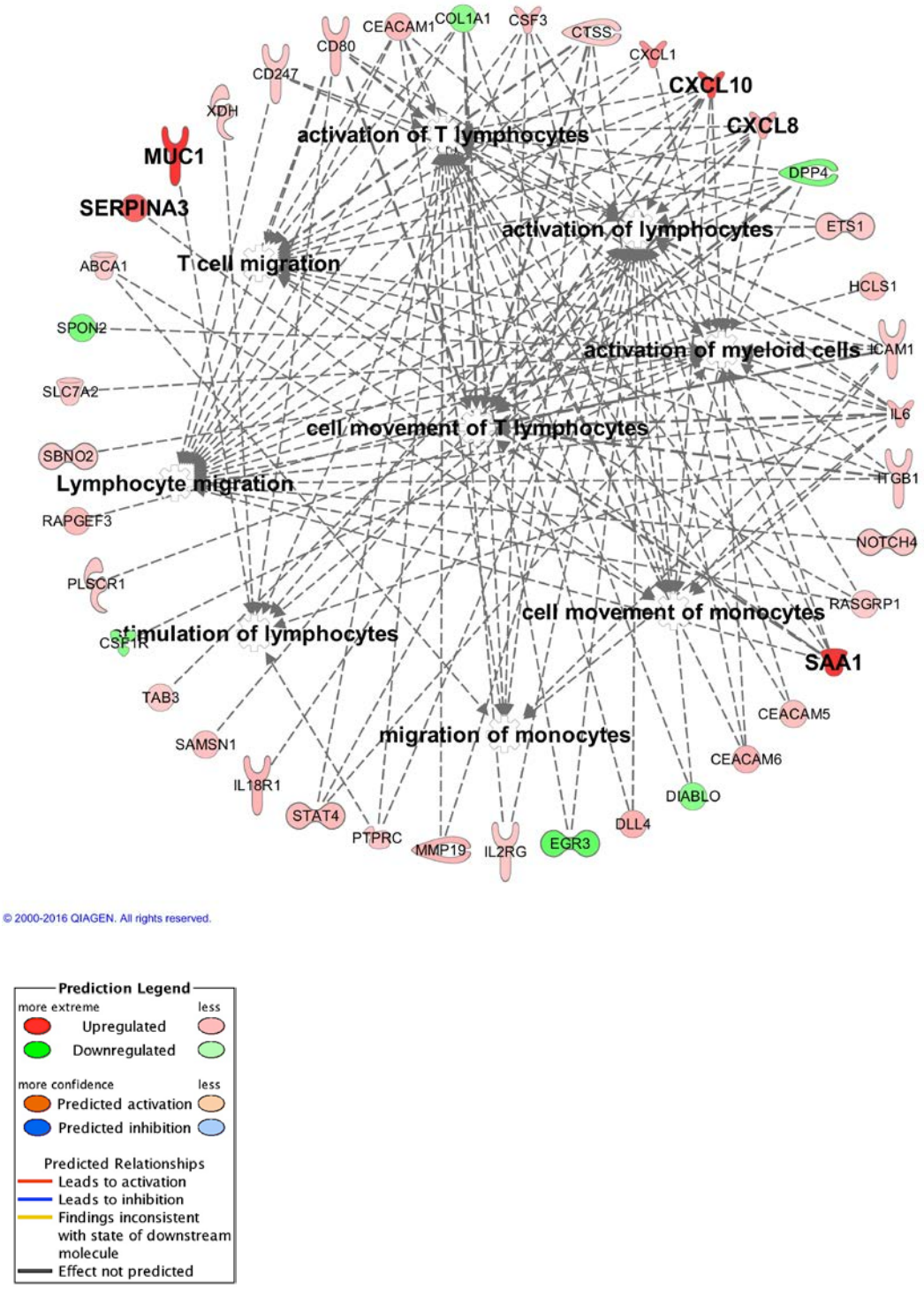

Figure 36: Cellular functions up-regulated upon HIV-1 exposure on cervical tissue epithelium along with interacting genes in those particular pathways.

The 7 differentially expressed genes detected using the sequencing analysis was confirmed using RT-PCR in 14 sets of tissues exposed to HIV or control supernatant. All 7 genes were found to be up regulated (3-21 fold) with statistical significance in all the tissue sets except for SAA1/SAA2, which was not statistically significant (Figure 37) (Table 6)

Table 6: Confirmation of DEGs (Differentially expressed genes) that were differentially regulated upon HIV-1 exposure on cervical tissues using RT-PCR.

Sample Number	CXCL10	SAA1 /SAA2	MUC1	IL36A	FMO2	IL8	WARS
E-8776 (HIV)	11.16	8.75	6.54	3.29	5.06	3.51	4
E-7688 (HIV)	40.22	2.22	2.83	0.99	2.6	1.56	9.45
E-8901 (HIV)	5.7	0.72	1.32	0.84	0.8	0.99	1.51
E-8916 (HIV)	19.7	16	580.04	235.57	34.06	34.78	5.31
E-9274 (HIV)	12.64	4.11	3.76	2.95	3.41	5.39	9.45
E-9301 (HIV)	1.41	0.26	0.37	1.41	0.79	2.16	1.03
E-9323 (HIV)	9.19	4.06	2.57	1.61	1.3	0.75	3.66
E-9387 (HIV)	15.67	1.57	0.79	0.58	1.09	1.42	1.69
E-9483 (HIV)	3.56	0.93	1.75	2.53	2.19	2.19	2.11
E-9493 (HIV)	2.51	1.2	0.68	1.57	1.59	1.12	1.69
E-9564 (HIV)	15.24	1.8	10.27	NA	9.65	3.16	10.34
E-9571 (HIV)	2.57	0.2	1.04	0.38	0.47	0.29	1.09
E-9769 (HIV)	7.26	0.74	0.18	2.04	0.8	0.26	0.33
E-9864 (HIV)	13.27	1.21	5.39	NA	8.51	1.79	11.16
Avg FC (n=14)	11.44 *	3.13	44.11 *	21.15 *	5.17 *	4.24 *	4.49 *
p value	0.0001	0.104	0.0245	0.05	0.0157	0.0245	0.0009

Seven genes out of which six were the most highly up-regulated genes and the seventh one not significantly upregualated was verified using RT-PCR on additional tissues making the n=14. A non-parametric paired t test was used for calculating the statistical significance.

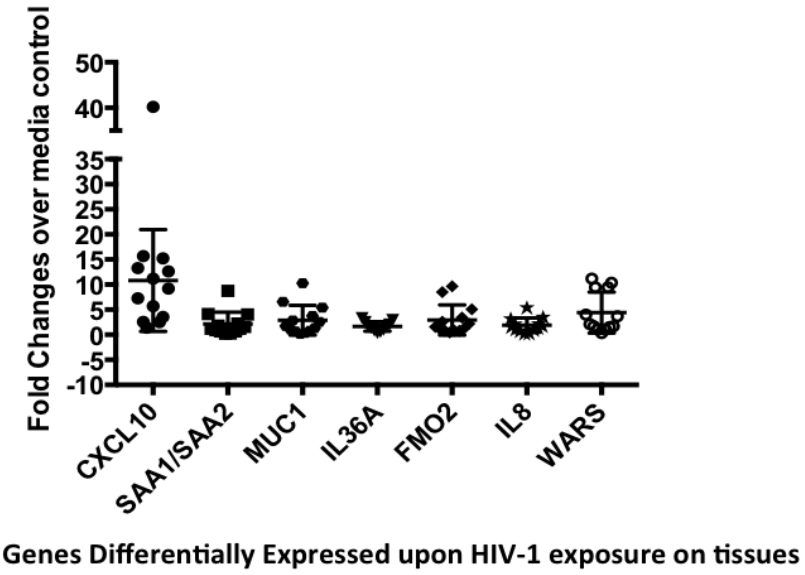

Figure 37: Confirmation of genes that were differentially up-regulated upon HIV-1 exposure on cervical tissues using RT-PCR.

6.2.4 Assessment of common cellular pathways and cellular factors up-regulated in cervical epithelium both upon NG as well as HIV-1 exposure.

After confirmation of these differentially regulated genes from both NG and HIV-1 exposed tissues, we compared up-regulated cellular pathways, in tissues exposed to HIV-1 and NG. This was done to evaluate whether there was any common cellular pathways that may be regulated in both sets of exposures (Figure 38). Comparison of both these exposures showed that they up regulated most of the migration, activation and stimulation pathways for the HIV-1 target cells upon exposure to the cervical epithelium, which may aid in the acquisition of HIV-1 by increasing target cells near the site of exposure. Furthermore, IPA analysis also suggested that cytokines signaling, TLR signaling, immune responses, NF-κB signaling, and communication between innate and adaptive immune cells were some of the canonical pathways that were up-regulated upon exposure for both the NG and HIV-1 on the tissues.

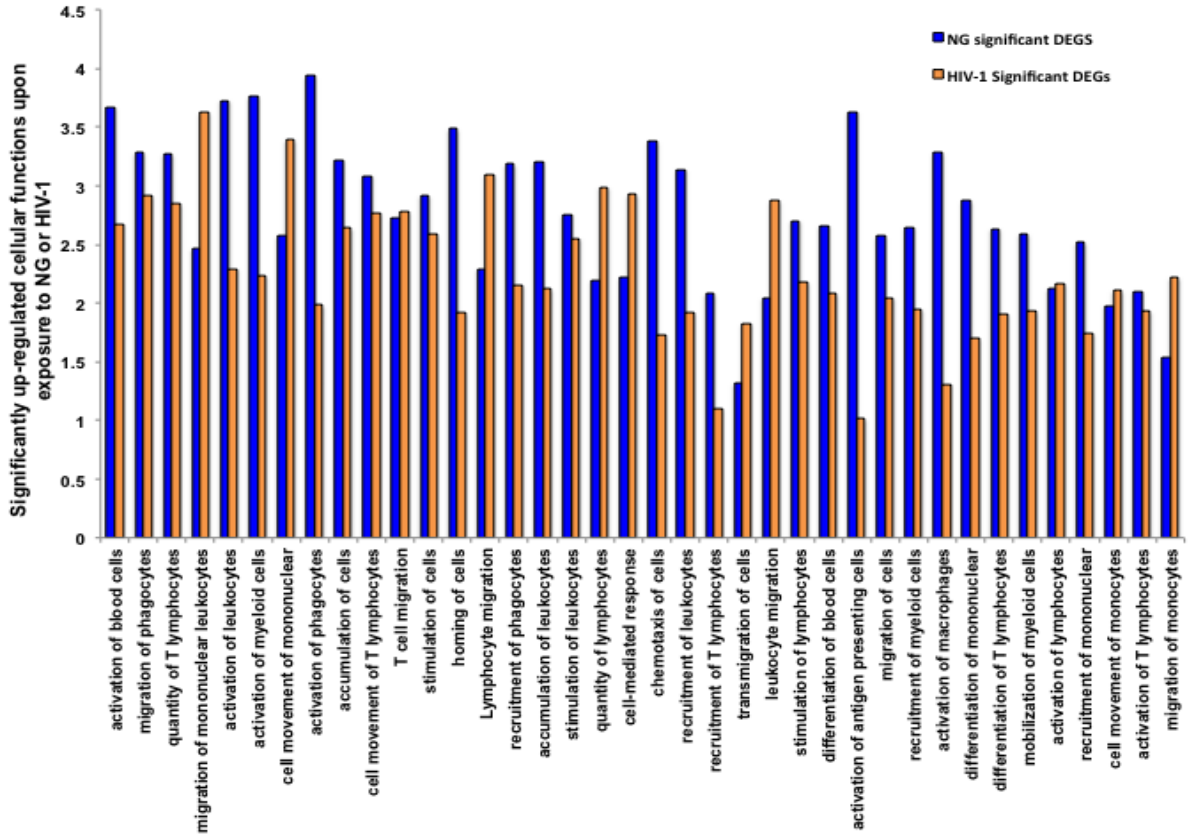

Figure 38: Graphs showing common cellular functions up-regulated upon both NG as well as HIV-1 exposure on cervical epithelium.

As mentioned earlier we hypothesized that NG induced cellular cytokines may help in increasing the epithelial cellular proteins and other factors that are involved in transmission of HIV-1 across epithelium. To prove this hypothesis, we compared differentially expressed genes between NG (n=10) and HIV-1 (n=14) exposed epithelia. Venn diagram analysis indicated that only two genes CXCL10 and IL8 were found to be common and up regulated between tissues exposed to HIV-1 and NG groups compared to control (Figure 39). A comparison of the RT-PCR data of the transcripts between the NG and HIV-1 exposed tissues also confirmed that CXCL10 and IL-8 were the two genes common between these two groups that were significantly

elevated (11.44 and 4.24 in HIV exposed tissues, respectively and 9 and 29 fold in NG exposed tissues, respectively).

IL-1β was found to be the most significantly up-regulated upstream modulator for most of the pathways which were activated upon exposure for both NG as well as HIV-1 and it was also one of the most significantly up-regulated gene in general for the NG exposure as evaluated using the IPA analysis.

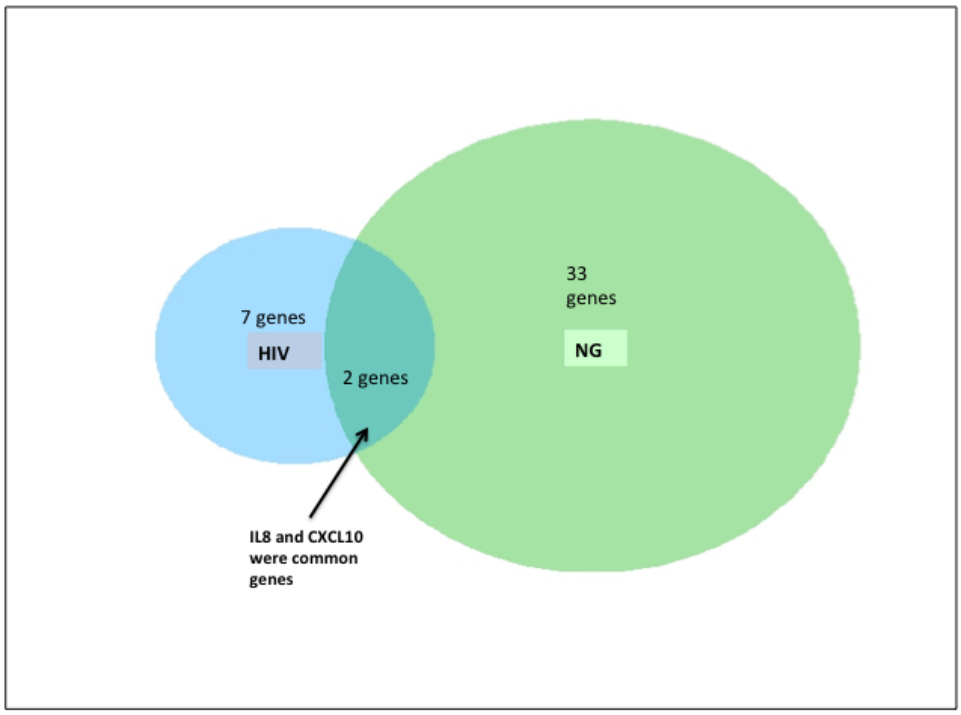

Figure 39: Ven diagram of the differentially expressed genes, which are commonly, up regulated both upon NG as well as HIV-1 exposure on the ecto-cervical tissue epithelium.

6.2.5 Effect of IL8 and CXCL10 on the HIV-1 transmission across the cervical mucosa.

IL8 and CXCL10 both have the ability to increase the migration and activation of T cells and macrophages. Prior literature and some of our preliminary findings showed that these proteins being chemokine could attract CD3+ T cells and possibly macrophages to the intraepithelial site

to act as target cells for HIV-1 infection and hence would increase HIV-1transmission. It has been shown earlier in cervical tissue explants that IL8 is able to increase the transmission of HIV-1 [256]. There have also been epidemiological reports that plasma cytokine and chemokine CXCL10 may increase the risk of HIV-1 transmission [293]. To determine their role directly in HIV-1 transmission across epithelia, tissues were incubated for 24 hour with IL8 and CXCL10 (10 ng/ml and 100 pg/ml respectively) at concentrations found in the NGIS and then exposed to HIV-1. It was observed that there was a significant increase (5-8 folds) in the transmission of HIV-1 across the cervical epithelium as determined by the transmitted virus measured by p24 in supernatants by ELISA (Figure 40a) and a concomitant increase in HIV-1 transcription as observed by an increase in the HIV-Gag-mRNA in the tissues (Figure 41 d).

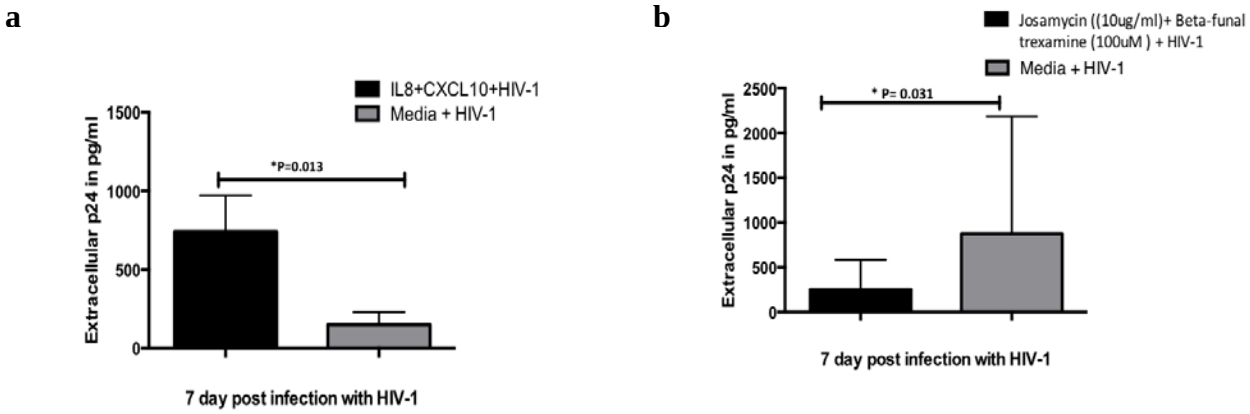

Figure 40: Role of CXCL10 and IL-8 in HIV-1transmission

a) Addition of IL8 and CXCL10 at concentrations present in the 24 hour NGIS increases the transmission of HIV-1 across cervical mucosa in the organ culture. b) Addition of anti IL8 drug Josamycin ((10ug/ml) and anti CXCL10 drug Beta-funal trexamine (100uM) decreased the transmission of HIV-1 across the mucosa in the same model. The experiments were run in triplicates and $p<0.05$ was considered statistically significant using a paired students T test.

To further confirm the role of CXCL10 and IL8 on HIV-1 transmission, we examined the effect of inhibitory drugs for IL8 and CXCL10 (10ug/ml Josamycin and 100uM Beta-funal trexamine) on HIV-1 transmission. The mechanism of action of macrolides such as Josamycin is via inhibition of IL8 biosynthesis by binding reversibly to the ribosome and inhibiting translocation of peptidyl tRNA. As shown in Figure 40 b, addition of IL-8 inhibitor Josamycin and CXCL10 inhibitor Beta-funal trexamine showed a significant decrease (56%) in the HIV-1 transmission. .

6.2.6 Effect of IL-1β on the HIV-1 transmission across the cervical mucosa in a cervical tissue based organ culture system.

Earlier studies showed that IL-1β might drive the production of IL8 [294] and CXCL10 [295]. We therefore investigated effect of IL-1β on the production of CXCL10 and IL-8 and on HIV-1 transmission in cervical tissues and its effect on HIV-1 transmission. We therefore exposed the cervical tissues to IL-1β (2500pg/ml) at concentration found in the NGIS for 24 hour and then infected the tissues with HIV-1. There was an increase in the HIV-1 transmission as monitored by p24 ELISA in the bottom wells as noted after exposure to IL-1β (Figure 41 a). This exposure to IL-1β also induced an increase in the expression of IL8 and CXCL10 in the tissues as monitored by RT-PCR (Figure 41 d). We further wanted to ascertain the role of IL-1β produced by NG exposure on tissues on the HIV-1 transmission by using 10ug/ml trans-chalcone, an inhibitor of IL-1β. Trans-chalcone acts by decreasing NF-κB-mediated inflammation and up-regulation of IL-1β thereby decreasing the production of this cytokine in the system. Treatment of tissues with trans-chalcone, decreased the transmission of HIV-1 across the cervical epithelium by 50% (Figure 41b)

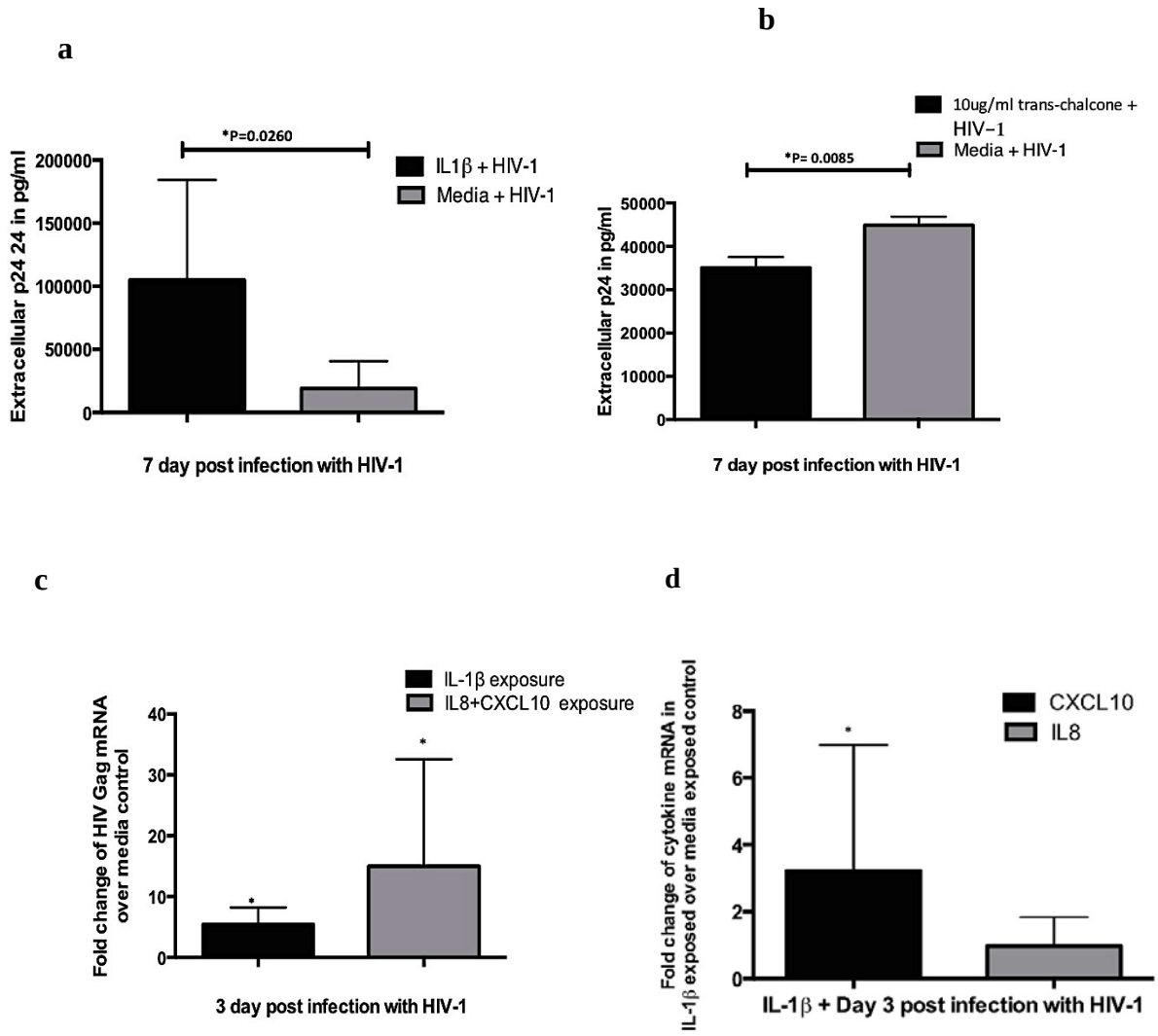

Figure 41: Role of IL-1β on HIV-1 transmission

a) IL-1β in concentrations present in the 24 hour NGIS increases the transmission of HIV-1 across cervical mucosa in the organ culture. b) Addition of 10ug/ml trans-chalcone used to inhibit IL-1β decreased the transmission of HIV-1 across the mucosa in the same model. c)Both IL-1β, CXCL10 and IL8 increased the HIV-1 transcription in the tissues compared to media exposed control. d) the IL-1β exposure on the cervical tissues increased the CXCL10 and IL8 in the tissues. The experiments were run in triplicates and $p<0.05$ was considered statistically significant using a paired students T test.

6.3 CONCLUSIONS

Our overall aim of this part of the project was to identify NG induced epithelial proteins that may be involved in increased HIV-1 transmission across cervical epithelia by evaluating common cellular factors and canonical pathways if any that was differentially regulated both upon NG as well as HIV-1 exposure on the cervix. Transcriptome analysis of micro-dissected epithelial layer of the tissues exposed to NG and HIV-1 identified with high statistical significance 33 differentially expressed genes in NG exposed and 7 differentially expressed genes in HIV-1 exposed tissues compared to same tissues exposed to control medium. Differential expression of these genes was subsequently confirmed by RT-PCR. A comparison of transcriptomes between NG and HIV-1 exposed epithelium showed IL8 and CXCL10 as the two cellular factors, which were significantly up regulated in both the NG as well as HIV-1 exposed tissues. Ingenuity Pathway Analysis (IPA) assigned the significantly up-regulated set of expressed genes in both tissue exposed sets to known canonical pathways and functional networks which were associated with inflammatory responses, acute cellular responses, and T cell and monocyte migration, activation, stimulation and differentiation of these cells. Addition of IL8 and CXCL10 at concentrations found in the cervical supernatant (NGIS) significantly increased the transmission of HIV-1 across cervical mucosa. Inhibiting these cytokines showed a significant decrease in the HIV-1 transmission confirming its role in HIV-1 transmission. Major upstream interacting factors for CXCL10 and IL-8 predicted by IPA for gene up regulation in both the sets of exposure included IL-1β and NFκ-B and ion torrent analysis also showed IL-1β to be one of the highest significantly up-regulated gene in case of NG exposure. Role of IL-1β in HIV-1 transmission has been demonstrated by enhanced HIV- transmission with concomitant increase of CXCL10 and IL8 by direct addition of IL-1β and its inhibition by an anti-IL-1β inhibitor.

Since IL-1β was one of the main cellular factors up-regulated by NG and its exposures also led to an increase in IL8 and CXCL10 expression in epithelia. All these data suggest that NG secreted IL-1β may be involved in enhancing HIV-1 transmission by up-regulating IL8 and CXCL10 in epithelia.

7.0 DISCUSSION

Mucosal surfaces in cervix and vagina are the primary sites for HIV-1 transmission during heterosexual intercourse Despite the importance of cervical transmission in the AIDS epidemic [306], the present knowledge regarding the mechanism of HIV-1 entry across mucosa is limited to in vitro studies. These studies involve primarily cervical cells and intestinal cell lines, and these cells do not reproduce completely the complexity and specificity of the cervical milieu where many different cells interact with one another in the in-vivo setting. Adding on to this HIV-1 transmission related research short coming is the problem of STIs like NG and other cervical pathogens which pose an increased threat of HIV-1 acquisition. Even though clinical findings show that NG increases the rate of HIV-1 transmission in-vivo, it has not been demonstrated in any ex-vivo setting because of lack of an in vitro model that can mimic in vivo situation of HIV-1 and NG interaction. Cell line based studies often give contradicting results where some cell types show that NG increased HIV acquisition and others show a decreased HIV acquisition by NG. NG infection in women has been difficult to study especially due to its asymptomatic nature. NG infects strictly the cervix via CR3 receptors present uniquely on the human cervical epithelium. NG though this interaction with CR3 induce ruffling of epithelial membrane which allows NG to enter the cells through macro-pinoctic mechanism. This interaction does not often cause any immediate inflammatory reactions making NG infection in women asymptomatic in the early stages. The inflammatory milieu of the cervical epithelial region changes with time and this may result in enhanced susceptibility to secondary infections.

Due to these issues of NG being asymptomatic at the onset of infection and being a strict human cervical pathogen, it is difficult to study NG induced enhanced in any animal model. Thus despite the worldwide problem of increased HIV-1 transmission by NG, molecular mechanism of NG induced enhanced HIV-1 transmission is largely unknown because of unavailability of a suitable *in-vitro* model.

The purpose of the current study was to understand the molecular mechanisms by which the presence of prior NG infection in the cervix enhances of HIV-1 transmission across the cervical mucosa. Here in this study, we describe a cervical tissue based organ culture model that mimics largely the *in-vivo* situation and could help us study the inflammatory changes that happen in the cervical milieu upon the onset of the NG infection and its effect on HIV-1 transmission. In this study, we used the previously developed ecto-cervical tissue-based organ culture model for HIV-1 transmission and extended it for studying NG and HIV-1 interaction is such cervical tissue setting [6]. Unlike working with a single kind of cell line, the ecto- cervical tissue based organ culture model is a more coherent system since it comprises the epithelial layer, sub-epithelial region that contains all the major immune cells like T cells, Langerhans cells and macrophages which are targets for the HIV-1 infection in-vivo and this structure allows evaluation of HIV-1 transmission across the mucosa [265].. NG did not show any toxicity in tissue after 24-hour incubation and the tissues were 97% viable compared to tissues exposed to media control. One of the most important characteristics of NG infection in vivo is epithelial membrane ruffling by which Opa and Pili proteins present on the cell surface interacts with host cells to form microvilli and engulfment of the bacteria, could also be observed in this explant system ex-vivo. [45]. There was no bacterial overgrowth. NG also did not cause any leakiness of the epithelial layer as evidenced by lack of transmission of blue dextran across cervical mucosa.

Moreover, there was no change in tight junction and adherence protein expression post 24-hours, which is in line with prior literature, indicating that NG does not cause any tight junction disruption in epithelial layer to enter the host system but some reports do mention that it could disrupt tight junction proteins [284]. In spite of all these promising results in the set-up of the culture system, there is certain weakness with this organ culture model for long-term experiments. Although tissues continue to secrete inflammatory cytokines at least up 7 days it is a technical challenge in maintaining this epithelium layer intact for more than two to three days in the organ culture. Our previous study indicates that although epithelial starts to become thinner, basal layer remain intact. Due to our inability to maintain these cultures for longer span of time, we could not simulate in-vivo cervical milieu condition during chronic infection of cervical milieu in our organ culture ex-vivo explant model. Since HIV-1 transmission probably occurs within 24-72 hour of inoculation, 3-day window allows sufficient time to study the effect of NG or induced culture supernatant (reminiscent of cervical milieu) on HIV-1 transmission. To the best of our knowledge this is the first reported system which could replicate most of the in-vivo NG cervical infection in a simple ex-vivo setup and can be utilized in further research and also preliminary data on other cervical pathogens showed that this modified version of our earlier established organ culture system for studying HIV-1 transmission could well be extended to the study of other microorganism's infection process and its effect on HIV-1 transmission.

After demonstrating that the organ culture model could replicate most of the physical characteristics of NG infection on cervical tissues, we used the model to replicate the cellular responses that are elicited by NG on cervical tissues. Most NG infections have been shown to be highly inflammatory in cell lines, and the outer membrane proteins have been reported to cause cytokine/chemokine secretion via TLR activation and related activities[228, 236-240, 273-275].

Most STIs along with NG can change the cervico-vaginal environment and act as important co-factors for the acquisition, transmission and progression of HIV-1 through cervical mucosal surfaces[233]. In response to NG exposure, genital epithelial cell lines have already been shown earlier to release cytokines [259, 274], some of which can strongly activates human myeloid DC, leading to robust induction of CD4+ T cell proliferation, promoting HIV-1 replication in these activated T cells [126]. These serve as targets for HIV-1 infection, and have also been observed to recruit to endo-cervices of women infected with NG[241]. Different cells are able to secrete pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8 and TNF-α as a response to NG which initiates inflammatory response[67]. In this study we showed that NG exposed to cervical tissue induced an increase in synthesis of high level of cytokine production for IL-1β and TNF-α compared to uninfected controls. In contrast, commensal bacteria, *Lactobacillus planeterum*, did not show any increase in cytokine response upon their exposure to the cervical tissues. Most data from in-vivo NG infected cervical lavage shows a cytokine profile high in IL8 and IL6 compared to our findings and this is mainly since the data collected clinically is after the NG has already established itself in the system, the infection is chronic and the upstream cytokines have produced more IL8 and IL6 in the system. This makes it difficult to correlate the immediate acute inflammatory milieu upon NG exposure obtained in the study to clinical reports from cervical lavages from chronic infections. In spite of this the findings were in line with earlier observations that which showed that there was an increase in pro-inflammatory cytokine production in the genital secretions in NG infected women. We also showed that such cellular response did not require live bacteria which demonstrated that outer membrane structure was enough to induce these responses. Deletion of outer membrane proteins Pili and Opa of NG did not cause any significant change in the cytokine profile induced by NG, which was in line with

prior literature on cell lines which showed that inflammatory responses was not dependent on outer membrane Opa and Pili [14, 242]. . On the other hand, the Lipo-oligosaccharide (NG lipopolysaccharide) has been shown earlier to cause the induction of the inflammatory response from *Neisseria meningitides* in animal models [307]. LOS, which interacts with TLR4[308] and has been shown to induce the secretion of TNF-α, IL-1β, IL-6, and IL-8 in primary urethral epithelial cells [309] [310]. Our results with findings in earlier literature implies that it is LOS in the outer membrane of NG which is the structure likely responsible for inflammatory responses from the tissues after the exposure and uptake of NG by the cervical tissues. Further studies are needed to assess the role of NG LOS in such cellular responses. NG-infected women have more endo-cervical CD4+ T cells providing more targets for HIV-1 [241]. We could show an increase in the CD3+ T cells towards the intra epithelium region upon NG exposure for 24 hour compared to the control exposed tissues. We did not observe migration of other immune cells, such as monocytes, dendritic cells into intra-epithelium region upon NG exposure. Part of the reason is that we could not evaluate the tissues for longer period of time in culture. Also, the tissue biopsies that we use in this setup are devoid of blood supply in the organ culture, which is the major source for immune cell migration and 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 NG exposure. Taken together, this cervical tissue based organ culture model was an appropriate system to study NG infection in female cervix and could be used to study the effect of NG infection on the transmission of HIV-1 which we demonstrated in the later part of the project.

It has been observed in earlier studies that NG can enhance the acquisition of HIV-1 [234, 235], activate the HIV-1 long-terminal repeat (LTR) in a transformed Jurkat CD4+ T cell line as well as increase replication of HIV-1 in primary resting CD4+ T cells [242, 278]. Previous research has shown that TNF-α treatment of ACH2 cells can lead to increase in steady-state levels of HIV-1 RNA and HIV-1 transcription indicating a positive effect on HIV-1 replication [292]. Recently, heptose monophosphate (HMP) secreted from NG has been shown to activate the HIV-1 LTR in CD4+ T cells[248]. Previous studies have also shown that HIV-1 replication was suppressed in Primary Human CD4+ T Cells by host factors. It has also been reported that supernatants from gonococcal-infected PBMCs stimulated HIV-1 replication in Jurkat cells while effectively inhibiting HIV-1 replication in primary CD4+ T cells [278, 293]. LOS been observed to promote HIV-1 infection in the primary CD4+ T cell after entry. The presence of NG at human mucosal surfaces initiates the recruitment of abundant polymorphonuclear leukocytes (neutrophils; PMNs) to the site of infection as discussed and can increase chances of HIV-1 infection. NG has also been reported to promote the activation of MDDCs and infection by HIV-1 but not in monocytes [249]. NG induced TNF-α in vaginal biopsies could increase the susceptibility of Langerhans cells to HIV-1 through activation of TLR2[250]. Since we and other groups already showed that there is no TJ disruption during the NG infection process and so the increase in HIV-1 transmission as a result of NG exposure does not occur through this route. In spite of these cell based studies, direct effect of NG on HIV-1 infection of target cells (T cells, dendritic cells and macrophages) *in-vitro has been conflicting* since it has been shown to exert cell-type dependent enhancing or inhibiting effects suggesting that these interactions may be better studied at the tissue level. In fact, we showed that NGIS increased HIV transcription by up regulating HIV-1 LTR and reactivated replication competent

HIV-1 from latently infected T cells (ACH2) and monocytes (U1) which are known to the target cells for HIV-1. We also successfully showed that NGIS caused an increase in transmission of HIV-1 across the cervical mucosa tissue in an organ culture model.

Epithelial cells in cervix are the first cells exposed to NG or HIV-1. Also due to multilayer nature of cervical epithelia, NG possibly does not enter the sub-mucosa and therefore it is the epithelial cells exposed to NG that induce the changes in cellular activity resulting in enhanced HIV-1 transmission. IL8, MIP1 Beta, MCP-1, TSLP, TNFα, IL 6 which play an important role in recruiting immune cells, have been shown to increase post exposure to HIV-1 on cervical and epithelial cells[213, 300, 301][300, 302]. In view of all these observations, epithelial cells may be of prime importance and be target cells for both NG and HIV-1 to induce local inflammatory responses and other cellular factors, and these may facilitate HIV-1 transmission. It has been observed that there is a range of inflammatory cytokines which increases upon NG interaction with the epithelium cells [277] and also been shown by is in this tissue culture model. NG exposure has been reported to increase the expression of IL-8 in macrophages and epithelial cells, respectively[252, 253]. This NG exposure also induces host defensins, which inspite of having strong anti-HIV-1 activity, are immune-modulator that induce inflammation in the host leading to recruitment of HIV-1 target cells to the site of NG infection and facilitate HIV-1dissemination and amplification. Keeping all these observations in the picture, it appears that NG may facilitate HIV-1 acquisition through multiple mechanisms and most importantly might up-regulate cellular pathways which are up-regulated in the case of HIV-1 infection which aids in HIV-1 transmission across the mucosa. We therefore used whole genome transcriptome analysis to further identify a broader range of cellular factors that were expressed upon NG and HIV-1 exposure separately only to epithelial layers of the cervical

tissues. Such analysis identified with high statistical significance 33 differentially expressed genes in NG exposed and 7 differentially expressed genes in HIV-1 exposed tissues compared to same tissues exposed to control medium. Differential expression of these genes in HIV-1 and NG exposed tissues were also confirmed by RT-PCR. A comparison of these two transcriptomes indicated that only two genes CXCL10 and IL8 were found to be common up regulated genes between tissues exposed to HIV-1 and NG groups. When the up-regulated genes in both the exposed groups were compared using the Ingenuity Pathway Analysis, we observed that NG as well as HIV-1 exposure up-regulated important cellular functions involved in immune regulatory and inflammatory processed in the tissues. These pathways heavily focused on migration, activation, stimulation and differentiation of different myeloid cells and T lymphocytes to the periphery of exposure which are known to be target cells for HIV-1 infection. This increase in target cell availability upon NG exposure might play an important role in establishing infection in the ecto-cervical region during viral transmission.

IL8 and CXCL10 are both strong chemo-attractant cytokines and have the ability to increase the migration and activation of T cells and macrophages along with neutrophils which are important HIV-1 target cells. It has been shown earlier in cervical tissue explant studies independent of NG, that IL8 at very high concentrations could increase the transmission of HIV-1 few folds[256]. There have also been epidemiological reports that CXCL10, may increase the risk of HIV-1 transmission[303]. Addition of recombinant IL8 and CXCL10 to our organ culture, in concentrations found in NGIS, significantly increased HIV-1 transmission across the cervical mucosa in. We further confirmed the importance of these cytokines in the transmission process by using inhibitory drugs against these cytokines, which significantly decreased the HIV-1 transmission in the system. Another major finding of our study was the consistent up-

regulation of IL-1β upon NG exposure found through simple RT-PCR, MSD protein analysis as well as the whole genome transcriptional profile assay. Earlier studies have showed that IL-1β may drive the production of IL8 [304] and CXCL10 [305], and we observed significant increase in HIV-1 transmission post exposure to IL-1β and IT induced the expression of IL8 and CXCL10 in the tissues. Inhibition of IL-1β production significantly decreased the transmission across the mucosa.

This study was important because it not only gave insights into major cellular factors up-regulated upon NG exposure on tissues which may aid in HIV-1 transmission but also looked into cellular factors up-regulated upon HIV-1 exposure on cervix opening a whole new area of work for investigating initial responses that occur with HIV-1 exposure on cervix which may influence the transmission process. Altogether these data are consistent with a model for NG induced enhanced HIV-1transmission (Figure 42): **NG infection secretes IL-1β which induces increased production of CXCL10 and IL-8 resulting in the migration of HIV-1target cells CD3+T cells and macrophages towards intraepithelial region which fuels HIV-1 infection and replication in sub-mucosa and consequently enhances HIV-1transmission.** Identification of IL-1β and its target cellular proteins in NG induced enhanced HIV-1transmission could be useful in development of drugs that impair HIV-1transmission. Further work in non-human primates or humanized mouse models could provide further confirmation of the role of CXCL10 and IL-8 in HIV-1 transmission and its modulation by NG secreted proteins like IL-1β in vivo.

8.0 WORKING MODEL

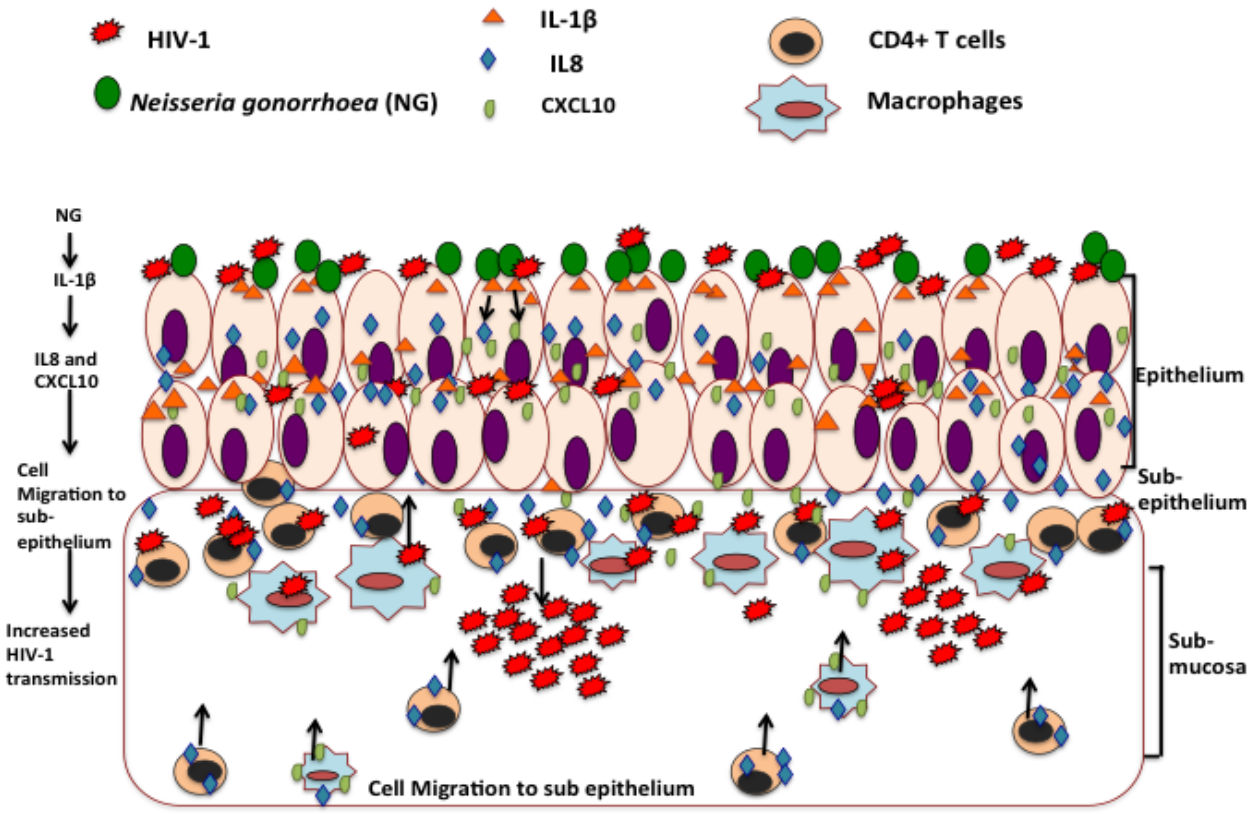

Figure 42: Schematic representation of NG induced enhanced HIV-1 transmission across the ecto-cervical tissues

The ecto-cervical epithelium is made of multilayered squamous epithelial cells with tight junctions between the epithelial cells. Acute exposure of NG on cervical tissues produces different inflammatory cytokines. The cytokine milieu produced by this exposure on epithelium increases the transcription, replication as well as the transmission of HIV-1 across the cervical mucosa. The cytokine that is significantly elevated in case of this exposure is primarily IL-1β, which further enhances the production of IL8 and CXCL10 in and across the cervical epithelium. This IL8 and

CXCL10 act as chemo-attractants aiding in the migration of immune cells to the periphery of the epithelium. In the presence of HIV-1 on the epithelial surface along with these cytokines produced by NG, more immune cells are recruited towards the epithelial layer thus increasing the target cells availability for HIV-1 infection. Cellular factors such as IL36A produced in the cervical epithelium upon HIV-1 exposure, activates the already migrated immune cells increasing the pool of target cells which can now be infected with HIV-1 and fuel the local expansion that is necessary for establishment of infection in the ecto-cervical tissues once they cross the epithelial barrier.

In summary, the current study provides evidence that although the squamous ecto-cervical epithelium act as a physical barrier against pathogen entry, NG can infect the cervix and elicit immune responses which can aid in the acquisition and transmission of HIV-1. However, NG exposure at the ecto-cervical epithelium results in up-regulation of cellular factors including inflammatory cytokine expression in the epithelial cells, as well as cellular pathways involved in cell migration, activation, stimulation and differentiation which is a rapid response and aids in the increase in HIV-1 acquisition. 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. Regardless, our results suggest that the **IL-1β** could be one of the most important genes in this entire process, which is elevated upon NG exposure and further downstream up regulated production of IL8 and CXCL10 through NFKB dependent signaling and recruits HIV-1 target cells to the periphery of exposure. Upon HIV-1 exposure, it becomes easier for the HIV-1 to infect a system already enrich in target cells thus enhancing the acquisition process. Our data also showed that NG infection causes migration of CD3+T cells towards the intra epithelium region, which was in line with the rest of the findings of the project.

9.0 PUBLIC HEALTH SIGNIFICANCE

HIV-1 infection is a global health concern and is an epidemic in most of the underdeveloped countries. Making the public health problem worse is the increasing rate of NG infection, which is another major problem in these nations. It is alarming since the presence of these STI like NG has been epidemiologically shown to increase the susceptibility to HIV-1. Such issues are difficult to address since it is still not clear as to how the presence of NG increases the chance of HIV-1 acquisition. Since NG infections are asymptomatic in the acute stage and could unknowingly increase the rate of HIV-1 acquisition among high-risk population, it becomes important to study the interaction between these two organisms. To develop a highly effective method to prevent HIV-1 transmission, it is crucial to gain a clear understanding on the mechanism of NG induced enhanced HIV- 1 transmission across ecto-cervical mucosa. Currently there is no suitable in viro model to study HIV-1 and NG interaction. In the current study, we described the use of a primary cervical tissue based organ culture model to study HIV-1 and NG interaction that provides the natural cervical tissue architecture observed in cervix of NG infected women.

Using this organ culture, we showed that NG exposure to cervical tissues induced inflammatory cytokine response reminiscent of in vivo situation. Furthermore, we have determined that NG secreted IL-1β induced cellular factors IL8 and CXCL10 that may be responsible for NG induced enhanced HIV-1 transmission across cervical mucosa. CXCl10 and

IL8 are chemo-attractants, which aid in the migration of HIV-1 target cells like T cells, DCs and macrophages from the sub epithelium to the epithelium which fuel HIV-1 to infect them and hence transmit across the mucosa.

Another important finding of the study is the acute cervical milieu that was detected upon NG infection. Due to NG infection being asymptomatic in the onset, the lavages from patients are usually collected after the infection becomes chronic resulting in a change in the cervical milieu. The increase in IL-1β and other cytokines that we detected in our study could be an interesting and crucial finding for the health organizations around the world.

First, as mentioned earlier, these differentially expressed cytokines and cellular factors present in the epithelium during acute NG exposure could be useful in designing preventive tools to act as biomarkers to identify acute NG infection. These could be used to monitor NG infections in high-risk population that could help decrease the rate of NG infection hence reduce the acquisition risk of HIV-1 among this population. Second, after confirming the relevance of these genes in acute NG infection as well as in HIV-1 transmission, drugs that block the pathways involving these genes can be used to prevent the acquisition and HIV-1 transmission. Thirdly, the presence of NG was found not to interfere with the antiviral activity of microbicides against HIV-1. This finding was important and implied that microbicides could still be used as an effective control regimen against HIV-1 in countries where STIs were a huge problem.

This work has profound public health significance because these findings will further enhance our understanding on the mechanism by which acute NG infection increases susceptibility of HIV-1 transmission, providing the basis to develop strategies to block HIV-1 transmission.

10.0 FUTURE DIRECTIONS

To the best of our knowledge this is the first reported tissue based system, which could replicate some major in-vivo NG cervical infection process and physical properties of this infection. . This system was an extension of earlier primary endo-cervical cell models obtained from cervical tissues. Here we showed that NG was non-toxic in cervical tissue, causes membrane ruffling, and induces pro-inflammatory cytokine response. This model thus helped up evaluate cellular factors that are important for NG mediated enhanced HIV-1 transmission.

In our work, we did not observe changes in the expression of tight junction proteins in the epithelial cells following NG exposure to the cervical tissues by using RT-PCR. However we did not check for the distribution pattern of tight junction protein following NG exposure using confocal microscopy. Therefore a study should be performed using confocal microscopy staining for the tight junction proteins to analyze if there is any redistribution of the tight junction proteins on cervical epithelium post NG exposure. If there is a redistribution of the proteins, this could open up a new mechanism for NG induced enhanced HIV-1 transmission.

Our preliminary findings in the study also demonstrated an increase in the CD3+ T cells towards the intra epithelium region upon NG exposure for 24 hour compared to the control exposed tissues presumably due to an increase in chemo attracting cytokines. However, we did not observe migration of other immune cells, such as monocytes, dendritic cells into intra-epithelium region upon NG exposure. Since our study was limited to only 24 hour NG exposure we should extend the study for at least up to 72 hour and stain with better antibodies to other

immune cells to follow their migration. In addition, it is possible that our failure to detect migration of other immune cells is due to the fact our organ culture is devoid of blood supply, which made it difficult to study the cell migration that usually would occur under *in vivo* conditions. To make our findings replicate in-vivo and to study the long term NG infected cellular responses and how they impact HIV-1 transmission at various stages, further experiments will be required using the humanized mouse models which could have a continuous blood supply and help study cell migration pattern. We also did not study which cellular responses were elicited from which cells types post examining the cytokine responses as a whole from these tissues upon NG exposure. We could carry out confocal microscopic studies and co-stain with the markers for different cell types as well as the cytokines detected in the supernatant to elucidate which cells were responsible for induction of these responses in particular and how these could impact the HIV-1 transmission process across the cervical epithelium.

We got evidence of induction of strong cellular immune responses at mucosal tissue with an increase in a number of cytokines, which played important roles in increase of HIV-1 transmission across the cervical epithelium. However, this system was geared towards studying the acute inflammatory or cellular functions elicited by the cervical tissue upon NG infection. Due to our inability to maintain cultures for longer period of time we could not study chronic NG infection in tissues and its effect on HIV-1 transmission. This needs to be extended to a model which can sustain long term infections by altering culture condition to give a better understanding of molecular events taking place in the cervical tissue at different stages of NG infection starting from acute to chronic infection.

We showed in this organ culture model that the outer membrane structures like the Opacity protein and the Pilin protein was not important for the inflammatory responses caused

by the bacteria since their deletion did not cause any change in the response from the wild type. However, it might be possible that the bacterial LOS was responsible for this inflammatory response, which is an integral membrane protein. Further studies using purified LOS from NG as well as deletion mutants of other accessory proteins like Porin should be studies to investigate their roles in this cellular response.

So far in this organ culture model, we have shown that IL8 and CXCL10 are important for HIV-1 transmission. Furthermore NG induced IL-1β has been shown to enhance production of these two cytokines and thus presumably increase HIV-1 transmission. By using the humanized mouse model, we could verify the role of IL8 and CXCL10 in HIV transmission and its modulation by IL-1β. In addition we could verify the effect of inhibitors of IL8 and CXCL10 on HIV-1 transmission. This could open up more avenues on development of drugs that could target cellular factors important for HIV-1 transmission and block HIV-1 transmission. We could also study the direct effect of CXCL10 and IL8 on the ability to induce cell migration by setting up chemotactic experiments.

We also detected a number of other cellular factors which were up-regulated both in the case of NG as well as HIV-1 in the cervical tissue epithelium. We could further investigate the role each of these cellular factors have on HIV-1 transmission by using similar experiments we designed for IL8 and CXCL10 effect on HIV-1 transmission. We also demonstrated that NGIS increased transmission across the cervix. We could use NG infected cervical lavages to evaluate this increase in transmission in our system as well as in humanized mouse models.

Based on the preliminary data that we generated in this study, this organ culture system could also be extended to the study of other cervical pathogens and their infection process and their effect on HIV-1 transmission.

APPENDIX A: LIST OF ABBREVIATIONS

NG: *Neisseria gonorrhea*

AV: *Atopobium vaginae*

GV: *Gardenerella vaginalis*

PID: pelvic inflammatory disease

CEACAM: carcinoembryonic antigen-related cellular adhesion molecule

HSPG: heparin sulfate proteoglycans

MGL: macrophage galactose-type lectin

AIDS: acquired immune deficiency syndrome

MDDCs: monocyte-derived dendritic cells

DCs: Dendritic cells

MDMs: monocyte-derived macrophages

HIV: Human immunodeficiency viruses

IL: Interleukin

KRT 13: Cytokeratin 13

LC: Langerhans cells

mRNA: Messenger ribonucleic acid

pDCs: Plasmacytoid dendritic cells

NGIS: *Neisseria gonorrhea* induced tissue supernatant

CM: Culture media induced supernatant

RNA: Ribonucleic acid

SD: Standard Deviation

SEM: Standard error of the mean

SIV: Simian immunodeficiency virus

SEM: Scanning electron microscopy

TJ: Tight junctions

TLR: Toll like receptor

Opa: Opacity protein

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