2015

Immune defense of the female lower reproductive tract and the use of monoclonal antibody-based topical microbicide films to protect against HIV infection

https://hdl.handle.net/2144/16278

Boston University
BOSTON UNIVERSITY
SCHOOL OF MEDICINE

Thesis

IMMUNE DEFENSE OF THE FEMALE LOWER REPRODUCTIVE TRACT AND
THE USE OF MONOCLONAL ANTIBODY-BASED TOPICAL MICROBICIDE
FILMS TO PROTECT AGAINST HIV INFECTION

by

COREY J. COSTANZO

B.S., Boston University, 2013

Submitted in partial fulfillment of the
requirements for the degree of

Master of Science

2015
Approved By

First Reader__________________________________________________________
Deborah Anderson, Ph. D
Professor of Obstetrics and Gynecology, Microbiology

Second Reader__________________________________________________________
Jeffrey Pudney, Ph. D
Research Associate Professor, Reproductive Biology
DEDICATION PAGE

For Pam, Steve, and Dan
ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. Deborah Anderson, Dr. Jeffrey Pudney, Dr. Ayesha Islam, and everyone else in the Anderson Reproductive Biology Lab at Boston University School of Medicine. Thank you for all of your help and instruction, and for allowing me to be a part of this research.
ABSTRACT

Research on the human-immunodeficiency virus (HIV) and HIV transmission prevention methods is extensive, yet a female-controlled prevention method still does not exist. In many cultures where HIV prevalence is highest there are many social and cultural barriers to the prevention options currently available, specifically around the use of condoms. Topical microbicides could potentially offer a viable solution.

In heterosexually transmitted HIV, the epithelium of the lower female genital tract is the first place of contact with invading pathogens. A topical microbicide that could protect against infection across this barrier, without causing inflammation, would be an ideal product to protect women against infection. In previous studies, several microbicides were shown to cause inflammation and increase the risk of HIV infection; therefore, it is vitally important that any new topical microbicide products developed do not have the same effect. Our laboratory is collaborating with Mapp Biopharmaceuticals in the development of a new vaginal microbicide product based on human monoclonal antibodies produced in plants. Our prototype microbicide, MB66, is a film containing two antibodies: VRC01 (anti-HIV) and HSV-8 (anti HSV-2).
hypothesize that the new MB66 topical microbicide film will provide protection against these sexually transmitted viruses without inducing inflammation in the vaginal epithelium, and will prove to be a viable topical microbicide product that does not cause inflammation and increase the risk of HIV infection.

MatTek vaginal epithelium models were used to test for any potential inflammation by the MB66 film. Active MB66 and placebo films were placed on the MatTek models. Vaginal tissue models grown in the presence of estradiol were also exposed to the active film. After being exposed to the active MB66 film, apical and basal supernatants were collected and analyzed for proinflammatory cytokines; IL-6, IL-8, MCP-1, and TNF-alpha. There were no significant increases in IL-6 or IL-8 expression in vaginal epithelium when exposed to the MB66 film. There was a significant decrease in the expression of TNF-alpha in the apical supernatant of the film-treated cultures at the 24 hour time point, and a very small (<1%) but statistically significant increase in the expression of MCP-1 in the basal supernatant of the film and the placebo after 24 hours. These results indicate that the active MB66 film did not induce a significant amount of inflammation in the vaginal epithelium.

Immunohistochemistry was used to ensure that the MatTek vaginal models are a valid representation of the native vaginal-ectocervical epithelium. Expression of toll-like receptors (TLRs) 2, 3, 4, 5, and 9 was compared in the MatTek models (partial and full thickness), as well as native vaginal and ectocervical tissue. Results show similar expression of all the TLR’s in the native
tissue and the partial and full thickness MatTek models. There was a greater degree of similarity between the native tissue and the full thickness MatTek models. Namely, there was a lack of expression with TLR 5 and TLR 9, and positive expression of TLRs 2, 4, and 9.

Overall, the TLR expression validated the use of these models in testing current and future topical microbicides in order to find an effective female-controlled prevention method. In addition, cytokine analysis provided evidence that the new Mapp MB66 topical microbicide films do not cause significant inflammation to the vaginal-ectocervical tissue. Our data indicate that there should be further development of the MB66 film as a vaginal topical microbicide.
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<td>CLR</td>
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<td>IPC</td>
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<td>NLR</td>
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<td>PMPA</td>
<td>phosphonomethoxypropyl adenine monohydrate</td>
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<td>PREP</td>
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<td>PRR</td>
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<td>SLPI</td>
<td>secretory leukocyte peptidase inhibitor</td>
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<td>TBST</td>
<td>tris-buffered saline tween</td>
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<td>TLR</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-β</td>
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INTRODUCTION

Human Immunodeficiency Virus Type 1

The human immunodeficiency virus type 1 (HIV-1) is a human retrovirus that recently evolved from closely related simian immunodeficiency viruses endemic in wild ape populations in West Central Africa (Sharp, P. M., & Hahn, B. H., 2011). The HIV-1 virion has a core that contains three components: capsid proteins, two copies of the viral transcriptome in the form of RNA, and three enzymes including reverse transcriptase, which synthesizes complementary DNA from viral RNA. In this way, the viral genome is incorporated into the host cell genome and successfully replicates. In addition to the core, the virus has an outer envelop which contains gp120 spikes, which enable the virus to infect target cells by interacting with the CD4 molecule present on the surface of CD4 T cells, macrophages and other cell types. CD4 molecules act as high affinity receptors for gp120, facilitating binding of the virus to the host cell. After HIV-1 binds to the CD4 receptors on the surface of target cells, a conformational change occurs in gp120, exposing gp41 which binds to HIV co-receptors CCR5 or CXCR4 leading to fusion of the viral envelop with the cell membrane allowing the virion to successfully insert its’ genetic components into the host cell (Huang, C., et al, 2007). HIV infection destroys the CD4 T cells in the body which leads to chronic inflammation resulting in an immunodeficiency state, termed the acquired immunodeficiency syndrome (AIDS). Since the start of the HIV/AIDS epidemic, over 78 million people have been infected with the virus and over 39 million
people have died. Currently there are 35 million people living with HIV infection worldwide, with about 2.1 million new infections per year (UNAIDS, 2014). Sub-Saharan Africa is the hardest hit region with over 70% of the HIV/AIDS cases (UNAIDS, 2014).

**HIV Prevention Challenges**

When addressing HIV globally, social and cultural norms play a large part in why HIV continues to be spread. In many cultures, there are social factors that contribute to condom rejection and in turn the spread of HIV. Lack of dialogue between partners regarding condom use and also unequal power between partners when it comes to decisions regarding sexual health are two social factors that contribute greatly (Mash, 2010). Many women do not want condoms to reduce sexual pleasure for their partner, and the idea that condoms indicate a lack of trust and intimacy also deters use. Other social factors that contribute are religious beliefs that forbid the use of condoms and fear that using condoms will negatively affect a woman’s reputation (Mash, 2010). Even with more access to condoms and education about their use, these social and cultural norms and beliefs still persist and contribute to the spread of HIV. This is a major reason why a female-controlled, discreet, and accessible HIV prevention method is essential in curbing the spread of this virus.

**HIV Prevention Methods**

Many HIV prevention strategies have been developed and tested. These strategies include condom use, abstinence, circumcision, drugs for pre-exposure
prophylaxis (PREP) or treatment as prevention, and topical microbicides. Multiple clinical trials, specifically ones conducted in Africa, have shown that circumcision is protective against HIV infection (Gray, R. H., et al, 2007). A randomized control trial conducted in Kisumu, Kenya showed that the protective effect of circumcision was 60% and suggests that wherever and whenever safely possible, voluntary circumcision services should be incorporated into HIV prevention interventions (Bailey, R. C., et al, 2007). Although circumcision has been shown to be effective, a major weakness of this service is the accessibility and expense of this service. In order to have this procedure, a patient must have access to health services that are able to conduct the procedure safely. This access is lacking in many locations, specifically sub-Saharan Africa, where HIV incidence rates are highest (White, R. G., et al, 2008). Abstinence and abstinence only programs are another HIV prevention method that have been explored. Although abstinence is completely effective in preventing sexually transmitted HIV infection, studies on abstinence only programs have shown that these types of programs do not impact delay in initiation of sexual intercourse, age at initiation of sex, number of sexual partners, or condom use; therefore, there is little hope that abstinence promotion would be effective at preventing the continued spread of HIV (Kirby, D. B., 2008).

Condom use is a method of prevention that has proved very effective. A review of condom effectiveness in reducing heterosexual transmission concluded that proper condom use reduced HIV transmission by approximately 80%
(Weller, S., & Davis, K., 2002). In addition, low cost and high accessibility are major strengths of this prevention method. Unfortunately, as previously discussed, social norms and cultural values are often barriers of condom use in the developing world. Treatment as prevention is another method that has been researched and tested by providing patients at risk of infection with prophylaxis drugs containing two medications, Tenofovir and Emtricitabine, that prevent the virus from establishing infection (CDC, 2015). Although pre-exposure prophylaxis has shown to be effective in those with high adherence, barriers including cost, accessibility, awareness, and low adherence are significant weaknesses of this HIV prevention method (Krakower, D. S., & Mayer, K. H., 2015).

In the past, topical microbicides have also been studied as a potential prevention method as they inactivate the pathogens that spread HIV. Specifically, Nonoxynol-9 is a microbicide that has been distributed and used widely for decades as a spermicide to kill sperm and potentially protect against the spread of HIV-1 virus (Fichorova, R. N., Tucker, L. D., & Anderson, D. J., 2001). Paradoxically, studies have shown that Nonoxynol-9 actually increases susceptibility to HIV and other sexually transmitted pathogens. A large-scale placebo controlled trial conducted with 892 female sex workers showed that vaginal gel containing Nonoxynol-9 did not protect against HIV and caused toxic effects that increased HIV infection (Van Damme, L., et al, 2002). In other studies, susceptibility to HIV increased with Nonoxynol-9 use because the microbicide increased inflammation in the vagina (Fichorova, R. N., Tucker, L. D.,
Vaginal inflammation attracts easily infected HIV host cells to the genital tract epithelium and activates transcription of the virus in infected cells. This occurrence causes the vaginal epithelium to be substantially more vulnerable to the virus. Due to similar results from numerous studies, Nonoxynol-9 is no longer considered an HIV prevention option.

Other nonspecific microbicide compounds have been developed for HIV prevention and tested in clinical trials. Compounds researched include Buffergel, Pro2000, Carragard, SAVVY vaginal gel, and cellulose sulphate. Results of these clinical trials provided evidence that none of these gels are significantly effective in preventing HIV infection (Abdool Karim, S. S., et al, 2011; McCormack, S., et al, 2010; Skoler-Karpoff, S., et al, 2008; Feldblum, P. J., et al, 2008; Van Damme, L., et al, 2008). These results indicate that more research is needed in order to find alternative microbicides that would allow people, specifically women, to successfully protect themselves against HIV transmission.

An additional class of microbicides being researched is topical microbicide gels containing antiretroviral medications that inhibit the viral reverse transcriptase enzyme. The reverse transcriptase enzyme converts viral RNA to DNA in an infected cell and is an essential component the virus needs to spread. One antiretroviral microbicide that has been studied is Tenofovir gel. The active antiretroviral component used in Tenofovir gel is 9-[(R)-2-phosphonomethoxy) propyl] adenine monohydrate (PMPA) (Karim, Q. A., et al, 2010). Tenofovir is a nucleotide reverse transcriptase inhibitor and was the first antiretroviral to be
tested in microbicides (Wilson, D. P., et al, 2008). Another antiretroviral being tested in microbicide gels is Dapivirine, which is a nonnucleoside reverse transcriptase inhibitor (NNRTI) (Wilson, D. P., et al, 2008). Tenofovir and Dapivirine are the two antiretroviral microbicides that have been tested most extensively.

In the Caprisa clinical trials, results showed that Tenofovir gel reduced HIV infection by approximately 39% and women who used the gel consistently were up to 54 percent less likely to contract HIV (Karim, Q. A., et al, 2010). In contrast, the results of the VOICE clinical trials, where Tenofovir tablets or Tenofovir gel were used, showed that neither of the products are effective in preventing HIV infection (Saag, M. S., 2015). In addition, results of the FACTS clinical trial, which tested the effectiveness of Tenofovir gel, showed that the gel showed a protective effect in women who used the gel consistently, but the adherence to the gel was too low to show the gel’s effectiveness (FACTS 001 Results, 2015). Overall, clinical trials of Tenofovir have had conflicted results, so more trials are needed to definitely determine the effectiveness of Tenofovir as an HIV prevention method.

**Topical Microbicides Containing Monoclonal Antibodies**

Additional research is currently being completed with a goal of finding a female-controlled HIV protection method. One promising avenue of research is the use of plantibodies to protect against infection. Plantibodies are plant-made human monoclonal antibodies that are obtained from plants genetically altered to
express specific antibody genes and in turn produce specific antibodies (Jaeger, G. D. et al, 2000). Antibodies bind to specific antigens on pathogens resulting in neutralization and destruction of the pathogen by immune cells (Overbaugh, J., & Morris, L., 2012). Once the pathogen is detected by an antibody, other white blood cells are recruited, including neutrophils, macrophages, and dendritic cells. These cells are often able to phagocytose the pathogen and neutralize it with enzymes. These antibodies can be targeted against specific antigens or pathogens, including HIV (Overbaugh, J., & Morris, L. 2012).

A cost effective way to manufacture human monoclonal antibodies is to produce them through genetic engineering in plants. By growing these types of plant antibodies, cost is reduced greatly as compared to mammalian produced antibodies (Stoger et al, 2002). In addition, these antibodies can be applied topically and work in the localized region of the vagina to provide protection. Studies have shown that these types of plant-derived antibodies can function effectively for hours when exposed to the human vaginal environment (Stoger et al, 2002). Overall, this plantibody technology has shown to be effective in protecting against HIV transmission in mammals and is a promising step in finding an effective topical microbicide.

Two antibodies, VRCO1 and HSV-8-N have been studied in depth and are effective in targeting HIV-1 and HSV-2, respectively. The first, VRCO1, is a broadly neutralizing monoclonal antibody that is able to target multiple different strains of HIV (Su, B et al, 2014). VRCO1 binds to the CD4 binding site of the
specific envelope glycoprotein subunit, gp 120, found on the surface of cell free HIV-1 (Pantophlet, R., & Burton, D. R, 2006). This antibody was originally found and obtained from a patient infected with HIV and has been shown to be effective at protecting against HIV infection in both nonhuman primates and mice (Su, B, et al, 2014). The second antibody, HSV-8-N, is also a monoclonal antibody. This antibody is able to target HSV-2 (herpes simplex virus 2). HSV-8-N antibody functions by binding to the viral envelope glycoprotein D subunit located on the surface of the HSV virus and interfering with infection by neutralizing the virus (Whaley, K., Hume, S., & Zeitlin, L., 2014). Studies have shown that HSV-8-N is a potent neutralizer of HSV-2 and can target multiple strains of the virus (De Logu, A., et al, 1998). HSV-8-N is used in combination with VRCO1 as the HSV antibody protects against HSV infection, a major risk factor promoting HIV infection (CDC, 2010). Studies have shown that HSV-2 infection increases HIV acquisition by six-fold and that HSV-2 induced ulcerations create a breach in the genital epithelium allowing HIV virus to cross this physical barrier (Kaul, R., et al, 2008). Overall, both of these antibodies, HSV-8-N and VRCO1, can be produced using plantibody technology making them extremely valuable in the search for a topical microbicide that will prevent the spread of HIV and HSV viruses.

**Innate Versus Adaptive Immunity Against HIV**

The immune system of the human body has two different components that work in conjunction to protect the body from pathogens, such as HIV. These two components are innate immunity and adaptive immunity. The adaptive response
is a longer-term response conducted against pathogens. It is delayed in onset and has highly specific receptors (Pudney, J., & Anderson, D., 2011). Major contributors to the adaptive immune response are lymphocytes and products of lymphocytes, and an important difference in the adaptive immune system is the presence of immunologic memory that offers the body a form of long lasting protection. There are two different adaptive immune responses. Humoral immunity, which involves antibodies produced by B cells, and cellular immunity, which involves T cell lymphocytes (Pudney, J., & Anderson, D., 2011). The most distinguishing feature of the adaptive immune system is its' highly specific response tailored to each specific pathogen (Alberts, 2002).

In the female genital tract, the adaptive immune system mounts specific immune responses towards different bacterial, viral, and fungal pathogens, but it can take days for the adaptive immune system at the mucosal surfaces to be activated and effective against these pathogens (Wira, C. R., et al, 2005). Antibodies specific to each pathogen are generated during this time period. In order to produce these specific antibodies, the mucosal surface and epithelial cells of the female genital tract utilize antigen-presenting cells (APCs), a type of dendritic cell that reacts when a pathogen is present. APCs internalize the foreign antigen, break them down to immunogenic fragments, and present them to T cells using class I or class II major histocompatibility complex molecules (Wira, C. R., et al, 2005). The activated cytotoxic T lymphocytes (CTLs) then travel around the body and when they encounter the same Class I molecule-
antigen complexes, they release two enzymes, perforin and granzulysin, that cause the cell to lyse (Stenger, S., et al, 1998).

B cell responses to the HIV-1 virus are different as they result in the development of antibodies against the virus. Once a matching antigen on a pathogen is detected, the B cells are triggered and T cells help them mature into plasma cells that produce antibodies against the specific antigen. Antibodies are then released into the blood and mark the cells that are infected for destruction and clearance by the liver and spleen. In the genital tract with HIV, this B cell response occurs for approximately one week and is followed by a phase where anti-gp1 antibodies circulate through the body. Anti-gp120 antibodies are also produced a few weeks later and target the V3 loop of gp120 on the HIV virion envelope (Overbaugh, J., & Morris, L., 2012). In addition, neutralization antibodies (NAbs) against the virus do appear, but not until months after the initial infection. Unfortunately, at this point in the infection there has been extensive spread of the virus and T cell counts are often extremely low.

**Innate Immunity**

The initial acting component of the human immune system is innate immunity. The innate immune response is the fast-acting immediate response that provides the body with a way to detect microbial pathogens promptly and control infection (Pudney, J., & Anderson, D., 2011). Cells that conduct this innate immune response are consistently synthesized and circulate throughout the body, providing a continuous defense against infection or pathogens. Major
contributors to this innate immune response include epithelial barriers of the skin, gastrointestinal tract, genital and respiratory tract (Kumar, V., Abbas, A., & Aster, J., 2013). In addition to physical barriers, various types of cells play a major part in the innate immune response. These cells include leukocytes (neutrophils and macrophages), natural killer cells, and various complement plasma proteins, which often have phagocytic, cytolytic, and antimicrobial properties that help provide immediate defense against pathogens (Woods, J. A., et al, 1999). An important feature of the innate immune system is its’ lack of specificity in targeting pathogens. The innate immune response is not specific to the particular pathogen, and this provides the body with a faster protection mechanism to defend against pathogens, as compared to the adaptive immune response.

The innate immune system in the female genital tract provides protection against sexually transmitted pathogens. Firstly, the epithelial cells form a continuous mucosal barrier between the lumen and internal environment and external pathogens. Epithelial cells in the genital tract have the unique ability to provide a physical barrier against pathogens while also allowing the passage of sperm or ovum (Wira, C. R., et al, 2005). This physical barrier is composed of stratified squamous epithelium in the vagina and ectocervix, and tight junctions between columnar epithelial cells in the endocervix, endometrium, and fallopian tubes (Wira, C. R., et al, 2005). Epithelial cells in the female genital tract also contain toll like receptors (TLRs) that detect foreign pathogens and activate the innate immune system through molecular pathways that enhance the secretion of
inflammatory cytokines and interferons. In addition, microbicidal substances secreted from the epithelium help protect the female genital tract against pathogen infiltration. These microbicidal substances include soluble factors, such as defensin, secretory leukocyte peptidase inhibitor (SLPI), lysozyme and lactoferrin enzymes, and tracheal anti-microbial peptide (Wira, C. R., et al, 2005). Studies have shown that secretions by the epithelial cells in the female genital tract have a chemotactic effect that results in an increase of pro-inflammatory cytokines, such as IL-6, IL-8, MCP-1, and TNF-alpha, in the area (Wira, C. R., et al, 2005). As such, epithelial cells of the female genital tract are an essential component of the innate immune system in protecting the lower female reproductive tract against pathogens.

**Harnessing the Innate Immune System For HIV Prevention**

The innate immune system is especially important when looking at the spread of HIV and how to prevent infection. In the 1980’s and 1990’s, the majority of the research concerning HIV and the spread of this virus was focused on the adaptive immune system. After no successful vaccine was developed, there was a shift towards focusing on the innate immune system and developing a way to utilize the innate immune response to fight HIV-1 virus. When attempting to harness the innate immune response for HIV prevention, there are unique features of this system that require attention. Firstly, unlike the adaptive immune system that recognizes specific antigenic sequences, the innate immune system recognizes microbial features and patterns on the surface of the pathogen (Levy,
The ability to discriminate foreign pathogens from its own cells is a vital function of the immune system and is carried out by the innate immune system via pattern recognition receptors (PRRs) (Pudney, J., & Anderson, D., 2011). Pattern recognition receptors detect pathogen-associated molecular patterns (PAMPs) and can identify a variety of foreign pathogens attempting to infiltrate the body (Kumar, V., Abbas, A., & Aster, J., 2013). PRRs are found on the cell membrane and also inside the cytoplasm. One major advantage of PRRs is their ability to detect a variety of pathogens, including fungi, bacteria, viruses, and protozoa (Kumar, V., Abbas, A., & Aster, J., 2013). Their ability to identify and detect a range of pathogens is a valuable and unique feature of the innate immune system and is important in the body's response to HIV.

A second important feature of the innate immune system and its ability to defend against HIV is the anti-HIV soluble components that contribute to innate response. These components include mannose-binding lectins (MBLs) and complement (Levy, 2001). Mannose-binding lectin is a liver-derived serum protein that has the ability to bind and lyse HIV directly. Also, MBLs are able to differentiate the body's normal cells from cells that are foreign, or the body's own cells that have been transformed or altered; therefore, it can detect cells that have been infected by the virus (Takahashi, K., & Ezekowitz, R. A. B., 2005). Studies have shown that individuals with lower than normal levels of mannose-binding lectin have an increased risk of HIV infection, and if they are infected with HIV there is rapid progression from infection to disease; therefore, mannose-
binding lectin protein is an influential anti-HIV soluble component to consider when studying HIV infection (Levy, 2001).

Another important anti-HIV soluble component of the innate immune system is complement. Complement is one of the many proteins in the complement cascade. The complement system is a major mediator of inflammation, and the system is composed of over 20 proteins that function in a cascade to induce inflammation when a pathogen is detected. In the innate immune response, when a pathogen is detected complement binds to the surface of the pathogen (Su, B. et al, 2014). When the first complement protein binds, there is a conformational change in the complex, which induces enzymatic activity leading to activation of the cascade and results in the lysis of the virus (Carroll, M. C., 2004). In addition, complement can act as an opsonin and trigger phagocytosis of the virus (Levy, 2001).

Another important component that allows the innate immune system to quickly and effectively protect against pathogens are antimicrobial peptides (AMPs). AMPs are small, cationic and amphipathic peptides that play a protective role in insects, invertebrates, and vertebrates (Reddy, K. V. R., Yedery, R. D., & Aranha, C., 2004). In humans, defensins were one of the first antimicrobial peptides to be discovered and studies have shown that defensin is secreted by the female genital tract epithelial cells in its' innate immune defense. Specifically, human α-defensin-1, -2, and -3 coordinate anti-HIV actions in the female genital tract by contributing to the anti-HIV-1 activity of CD8 antiviral
factor secreted by infected CD8 T cells (Reddy, K. V. R., Yedery, R. D., & Aranha, C., 2004). Other antimicrobial peptides such as cecropin and mellitin contribute by suppressing viral transcription, while polyphemusin prevents entry of HIV virus (Reddy, K. V. R., Yedery, R. D., & Aranha, C., 2004).

Anti-HIV cellular components also function in the innate immune system to decrease the risk of infection or spread of the virus. The first of these cellular components are Interferon-producing cells (IPCs) and Type I Interferon (IFNs). Interferons block HIV replication and activate other components of the innate immune system, such as Natural Killer (NK) cells or macrophages, to help counter the virus (Levy, 2001). Another influential anti-HIV cellular component is CD8+ noncytotoxic T cell. Studies have shown that these cells control HIV replication in cells that have already been infected through the CD8+ T cell noncytotoxic antiviral (CNAR) response or by fragmenting the genetic material of the virus and digesting it using macrophages (Levy, 2001). Overall, interferons and CD8+ noncytotoxic T cells are two other important cellular components of the innate immune response against HIV.

**Inflammation and HIV Expression**

In addition to the soluble and cellular components that contribute to the defense system of the female genital tract, it is also important to note the role of inflammation in transmission of HIV, specifically in heterosexual transmission to women. Inflammation can occur when a pathogen infiltrates the mucosal surfaces or there is epithelial damage. When this occurs, the innate immune
system is activated via pattern recognition receptors. The pattern recognition receptors up regulate the transcription of genes that encode various proinflammatory cytokines. A few of the most common proinflammatory cytokines produced include Tumor Necrosis Factor (TNF-alpha), interleukin (IL-1, IL-6, IL-8), and MCP-1. When these cytokines are synthesized, they are produced at the site of injury, and they recruit additional inflammatory cells to the area. The cytokines also function to regulate cell death, adjust vascular endothelial permeability, and recruit blood cells (Takeuchi, O., & Akira, S., 2010). Although, inflammation can help to repair injury to tissues, it has been found that in the female genital tract it actually makes the vaginal epithelium more prone to HIV infection. When inflammation occurs due to abrasion of the tissues or presence of sexually transmitted disease, the immune system is activated and inflammatory cytokines, specifically MCP-1, recruit CD4 cells to the site of tissue injury. Cells expressing CD4 are major HIV target cells and their presence increases HIV susceptibility (Deshmane, S. L. et al, 2009).

**Toll-Like Receptor Function**

The inflammatory response can be triggered by various toll-like receptors (TLRs). This major class of pattern-recognition receptors has an important function in detecting pathogens (Lancaster, G. et al, 2005). TLRs have been found to act as receptors for various types of ligands. These ligands include: bacteria, virus, fungi, and genomic DNA (Valanne, S., Wang, J.-H., & Rämet, M., 2011). When a ligand reacts with a Toll like receptor, various signaling pathways
are activated, and different genes are expressed in an immune response. Two main signaling pathways are activated when the TLR reacts with its’ respective ligand (Chapel, H., & Haeney, M., 2014). The first signaling pathway activated is common to all toll-like receptors and is regulated by myeloid differentiation marker, MyD88. This pathway results in the activation of NF-κB, a transcription factor that regulates various genes that encode for proteins controlling inflammation. Once NF-κB is activated, it turns on transcription of these genes and as such acts as a master switch for inflammation within the body (Chapel, H., & Haeney, M., 2014). The second signaling pathway is not dependent on MyD88. Instead, when the toll-like receptors are activated by their respective ligands, the signaling cascade is regulated by TRIF. TRIF is an adaptor protein and activation leads to the secretion of Type 1 interferons, which as discussed have the ability to block HIV replication and activate other components of the innate immune system to help counter the virus (Chapel, H., & Haeney, M., 2014).

When pathogen ligands react with toll-like receptors in mammals, they trigger different pathways depending on the specific toll like receptor. There are 10 different toll like receptors in the body, but for this study we chose to evaluate the expression of TLR2, TLR3, TLR4, TLR5, and TLR9. Studies have shown that TLR2 mediates HIV-LTR trans-activation and HIV replication. Also, TLR9 activation has also been shown to lead to HIV replication in transgenic mouse spleen cells.
TLR2 responds to a variety of ligands, including yeast, lipoproteins, and Gram-positive bacteria. A unique feature of TLR2 is that dimerization of the TLR2 cytoplasmic domain does not trigger cytokine production. On the contrary, when the cytoplasmic domain of TLR2 reacts with the domains of either TLR1 or TLR6, cytokine production does occur (Chapel, H., & Haeney, M., 2014).

Unlike TLR2, the ligand that interacts with TLR3 is double stranded RNA (Bell, J. K. et al, 2005). Double stranded RNA is a distinct characteristic of viruses. The TLR3 receptors are usually located intracellularly in order to detect any viruses that infect the cell (Akira, S., Takeda, K., & Kaisho, T., 2001).

TLR4 receptors interact and respond to lipopolysaccharides, which constitute an important part of the outer cell membranes of Gram-negative bacteria (Akira, S., Takeda, K., & Kaisho, T., 2001). Previous studies have shown that in the female reproductive tract, TLR4 expression is highest in the upper tissues including the fallopian tubes and the endometrium. The next highest expression of TLR4 is seen in the cervix followed by the ectocervix (Pioli, P. A., et al, 2004). On the contrary, in a different study TLR4 was expressed in the endocervix, endometrium, and uterine tubes, but was not present in the vagina and ectocervix (Fazeli, A., Bruce, C., & Anumba, D. O., 2005). In addition, TLR4 in the human intestine has been shown to be decreased and prevent pro-inflammatory responses against the body’s own bacteria in the gut (Pudney, J., & Anderson, D. J., 2011).
TLR5 is different than TLR4 as it recognizes and interacts with a bacterial component, flagellin. Flagellin is a protein present in the flagella of bacteria, which are finger-like projections located on the outer cell membrane of bacteria. Their primary function is locomotion as they help bacteria move and propel forward. TLR5 can identify flagellin in both Gram-positive and Gram-negative bacteria (Bell, J. K., et al, 2005).

The ligand that TLR9 reacts with is unmethylated CpG DNA. Unmethylated CpG DNA has been shown to stimulate human lymphocytes (Witkin, S. S., Linhares, I. M., & Giraldo, P, 2007). It also has the ability to directly stimulate many immune cells including: B cells, macrophages, and dendritic cells. These immune cells then secrete cytokines, which are major contributors and regulators of the body’s immune response to HIV (Akira, S., Takeda, K., & Kaisho, T., 2001).

Viral recognition by TLRs is mainly by TLR9 as it recognizes DNA and also by TLR3 that recognizes single and double stranded RNA (Mogensen, T. H., et al, 2010). Other pattern recognition receptors that recognize pathogen-associated molecular patterns and contribute to the innate immune system include C-type lectin-like receptors (CLRs), NOD-like receptors (NLRs), and RIG-like receptors (RLRs) (Takeuchi, O., & Akira, S., 2010). CLRs are a transmembrane protein, similar to TLRs, while NLRs and RLRs are located in the cytoplasm. Whenever these PRRs are activated by their ligands, they upregulate the transcription of genes that encode a variety of inflammatory components.
including: proinflammatory cytokines, type I interferons, chemokines, and antimicrobial peptides (Takeuchi, O., & Akira, S., 2010).

**Figure 1. Toll-like receptors and their respective ligands.** Toll like receptors all have specific ligands that they interact with. TLR2 with yeast, lipoproteins, and gram positive bacteria; TLR3 with double stranded DNA; TLR4 with lipopolysaccharides; TLR5 with bacterial flagellin; and TLR9 with CpG DNA.

When TLR recognizes the foreign pattern of surface molecules on the pathogen various molecular pathways are activated. TLR 3 and 9 are expressed in the cytoplasm, while TLRs 2, 4, and 5 are expressed on the cell membrane, and whether the response is NF-kB dependent or independent depends on which TLR was activated. With TLR2, TLR3, and TLR4, this cascade of events leads to NF-kB separating from phosphorylated IkB and translocating to the nucleus.
where it increase the transcription of genes encoding proinflammatory cytokines (Takeuchi, O., & Akira, S., 2010). The NF-kB independent pathway can also be activated by TLR4 and this cascade leads to IRF3 being phosphorylated and translocated into the nucleus, where it induces transcription of genes that produce proinflammatory cytokines and type I interferons. A similar molecular pathway is activated with TLR5 and NF-kB is translocated into the nucleus and upregulates the transcription of genes that code for proinflammatory cytokines. With TLR9, CpGDNA reacts with the TLR inside an endosome in the cytoplasm and leads to a MyD88 complex being recruited. The formation of this complex results in IRF7 translocating into the nucleus and upregulating the expression of type I interferon genes (Takeuchi, O., & Akira, S., 2010).

Some of the key cytokines that regulate inflammation include interleukin 6 (IL-6), interleukin 8 (IL-8), monocyte chemo attractant protein 1 (MCP-1), and tumor necrosis factor alpha (TNF-α). These are some of the most common proinflammatory cytokines induced when infected with HIV virus, which is why they were chosen for this study. IL-6 plays an important role in the inflammatory response as it stimulates the activation of T cells, the differentiation of B cells, and production of acute phase proteins by the liver (Jones, S. A., et al, 2001). The function of IL-8 is to recruit neutrophils to the site of injury during the inflammatory response (Fichorova, R. N. et al, 2001). Studies have shown that both IL-6 and IL-8 promote the spread of HIV infection, as IL-6 induces HIV-1 expression while IL-8 stimulates HIV-1 replication by recruiting susceptible cells
to the area of inflammation (Fichorova, R.N., et al, 2004). MCP-1 is another major chemo attractant during the inflammatory response that specifically recruits monocytes, memory T lymphocytes, and natural killer cells to the site of inflammation. Due to this recruitment, memory CD4+ T cells and monocytes are primary targets for HIV infection, and this contributes greatly to these types of cells spreading the virus (Deshmane, S. L. et al, 2009). TNF-α is also an inflammatory cytokine produced by macrophages and monocytes during the acute inflammatory response. TNF-α has many diverse functions and roles in the body and in the immune system as it activates neutrophils and platelets, enhances the killing abilities of macrophages and natural killer cells, and also mediates apoptosis by activating caspases that cleave proteins. In addition, TNF-α is able to alter the anion and cation channels of the cell membrane and in turn affect the cell volume, which can result in necrosis or apoptosis of the cell (Idriss, H. T., & Naismith, J. H., 2000). Specifically with HIV-1, studies have shown that TNF-alpha can increase epithelial tight junction permeability and increase the risk of HIV infection across the genital epithelial barrier (Nazli, A., et al, 2010).

**Genital Mucosal Barrier**

To date the most common paths of becoming infected with HIV are through sexual transmission or injection drug use (Shen, R., Richter, H. E., & Smith, P. D., 2014). Heterosexual contacts constitute approximately 34% of HIV infections (Kumar, V., Abbas, A., & Aster, J., 2013). In addition, women account for approximately 52% of HIV-1 infections (Women and HIV/AIDS, 2012). The
frequency of heterosexual transmission makes the genital mucosal barrier a particularly important aspect to consider when researching HIV-1 and sexually transmitted infections. For women, the site of primary heterosexual HIV-1 infection is the vaginal epithelium, and it is the first point of contact between sexually transmitted pathogens and the mucosal surface (Anderson, D. J., Marathe, J., & Pudney, J., 2014). The epithelium of the vaginal wall has different layers. The outermost layer of the epithelium is the vaginal stratum corneum, and it is a permeable layer composed of non-keratinizing stratified squamous cells (Anderson, D. J., Marathe, J., & Pudney, J., 2014). In this layer there exists an abundance of antimicrobial peptides and glycogen. This superficial layer is made up of flattened cells that have experienced cornification, a terminal cell differentiation where the nucleus and intercellular organelles are lost and DNA and RNA are broken down, inhibiting further production of new proteins. This cornification process is influential as it causes intercellular junctions to act less effectively and diminishes the ability of the epithelial cells to respond to microbial exposure (Anderson, D. J., Marathe, J., & Pudney, J., 2014)

**HIV Transmission Mechanisms**

There are multiple transmission routes to acquire the HIV virus. The heterosexual route of transmission is the most common transmission route worldwide. Mother to child, or vertical, transmission of HIV-1 can occur during birth or after birth of the child. The virus can be transmitted from mother to child during pregnancy, labor, delivery, or breast feeding. Another route to acquire HIV
is through needles or syringes, often associated with injection drug use. Lastly, blood transfusion or transfusion of blood products can spread the virus as well (CDC, 2014). These different transmission routes have different rates of HIV infection, and although heterosexual routes of transmission are the most common transmission routes, this route has the lowest infection rates with heterosexual activity resulting in HIV infection in only 1 per 1,500 sexual encounters. Vertical transmission has a much higher infection rate with 1 out of every 3 babies born to a mother with HIV being infected with the virus (Pier, G. B., Lyczak, J. B., & Wetzler, L. M., 2004).

When searching for a female-controlled HIV prevention method, heterosexual transmission is the most important route to research. Heterosexual transmission has been seen to occur rapidly within 30 to 60 minutes of initial exposure when HIV virus crosses the epithelial barrier of the female genital tract. Ex-vivo studies have also shown that both cell-free and cell-associated HIV-1 transmission can occur and that HIV infection can occur solely through invasion of the vaginal mucosa (Hladik, F., & McElrath, M. J., 2008). Cell free transmission occurs when HIV virions from infected seminal cells interact with the genital tract epithelial cells and cross the epithelium through transcytosis, endocytosis, subsequent exocytosis, or by moving through the spaces in between the epithelial cells (Hladik, F., & McElrath, M. J., 2008).

Some studies have also shown that HIV-1 virions can bind to and enter epithelial cells, be sequestered in endocytic compartments or cytosol, and then
be released and infect susceptible leukocytes. These results show that these cell-associated HIV virions are often more effective at transcytosing the epithelial barrier, as compared to cell-free virions. In addition, as suggested in mouse studies, HIV-1 can potentially infect by being transported by lymphocytes and macrophages through cervicovaginal epithelium (Hladik, F., & McElrath, M. J., 2008). The results of ex-vivo studies conducted in sheets of vaginal epithelium show that HIV-virions are located specifically in the basal and suprabasal epithelial cells, suggesting that HIV-1 most likely dispersed through the spaces between the epithelial cells versus entering and transversing through them. With this route, HIV virions can directly contact the susceptible Langerhans cells (LCs) and CD4+ T cells underneath (Figure 2). Cells located in the suprabasal or basal epithelium of the female genital tract often have distinguishing characteristics that cause them to be more prone to sequestering and trancytosing HIV-1 virus. Langerhans cells have a unique ability to extend their dendrites up through the epithelial spaces towards the surface of the epithelium and bind HIV (Hladik, F., & McElrath, M. J., 2008). Studies have shown that Langerhans cells can even take up HIV-1 virions into their cytoplasm and then exit the genital epithelium to transport the virus to different locations, effectively spreading the virus (Hladik, F., & McElrath, M. J., 2008). Interestingly, human explant studies have shown that HIV-1 is extremely efficient at targeting CD4+ T cells in the genital tract for infection and that initial infection predominantly occurs in CD4+ T cells, versus Langerhans cells (Hladik, F., & McElrath, M. J., 2008).
Cell surface components of the epithelial cells in the female genital tract are also very influential in the transmission of HIV-1. Surface proteins located on epithelial cells may support the attachment of HIV-1 and glycosphingolipids, such as lactosylceramide and galactosylceramide, have the ability to bind the gp120 on the HIV-1 viral envelope and induce transcytosis of HIV virions in the epithelial cell (Hladik, F., & McElrath, M. J., 2008). Recent studies have also shown that another glycoprotein, gp340, located on genital epithelial cells binds to the HIV viral envelope protein and fosters the movement of HIV through the epithelial barrier to the susceptible leukocytes in the basal layer (Stoddard, E., et al, 2007).

In addition to the characteristics of the epithelial barrier that affect infection, inflammation is a major risk factor in HIV transmission. Inflammation can occur due to epithelial tissue damage and cause a breach in the epithelial barrier. HIV virions can then cross the epithelial barrier with more ease and infect the inflammatory cells, such as CD4 cells, that are responding to the tissue injury. Specifically, small lesions of the mucosal epithelium caused by sexual intercourse can provide HIV-1 virions with direct access to the underlying dendritic cells, T cells, and macrophages that are easily infected (Figure 2). Certain topical microbicides or sexually transmitted infections can also cause these types of abrasions leading to increased HIV-1 susceptibility (Hladik, F., & McElrath, M. J., 2008).
Figure 2. HIV transmission across epithelial barrier. HIV virions are easily able to access susceptible dendritic cells (a), macrophages (b), and T cells (c) when physical abrasions or lesions result from sexual intercourse. Langerhans cells are able to reach up through the epithelial barrier and provide access to HIV virions as well. (Figure from Shattock, R. J., & Moore, J. P., 2003).

Goals of Current Research

The current research seeks to evaluate the safety of MB66 microbicide film through cytokine analysis and TLR expression analysis. As seen through past studies, many topical microbicides have been shown to induce inflammation and in turn increase HIV susceptibility; therefore, it is imperative to ensure that this effect is not seen with the new MB66 microbicide film before testing the film.
in human trials. By researching a new topical microbicide we are contributing to the efforts of creating a female-controlled HIV prevention method to curb the spread of sexually transmitted HIV infection.
SPECIFIC AIMS AND OBJECTIVES

Up until recently HIV prevention has been mainly focused on the use of condoms or trying to generate an HIV vaccine. The development of vaginal films containing microbicide could potentially provide a way for women to protect themselves against HIV transmission and other sexually transmitted infections, without the use of a condom. It is important to determine any adverse effects these films could potentially have before testing them in large scale human clinical trials; therefore, the goal of the present research is to test if any markers of inflammation are produced by these films on MatTek vaginal-ectocervical models. If a severe reaction is observed, this could potentially have a similar effect on vaginal-ectocervical tissue in women. Also, it is important to determine if the safety of the MB66 microbicidal film is affected in any way when exposed to high levels of estradiol. In addition, it is important to validate the use of the MatTek models in testing new topical microbicidal products and ensure that there is a similar immune response in both native tissue and the MatTek partial and full thickness vaginal-ectocervical tissue models. Specifically, the aims of this research were:

1) Determine IL-6, IL-8, MCP-1, and TNF-alpha cytokine expression changes in vaginal-ectocervical tissue epithelium when utilizing the MB66 and placebo films on MatTek models.
2) Determine IL-6, IL-8, MCP-1, and TNF-alpha cytokine expression changes in vaginal-ectocervical tissue when utilizing MB66 and placebo film in tissue is grown in the presence of estradiol.

3) Compare expression of TLR2, TLR3, TLR4, TLR5, and TLR9 by native vaginal and ectocervical tissue with partial and full thickness MatTek vaginal-ectocervical tissue models.

From these studies, I hope to learn if the MB66 microbicidal films cause any inflammation in the vaginal/ectocervical tissue. Also, I hope to validate the use of MatTek models for microbicide screening and discover any differences in TLR expression between the partial versus full thickness MatTek models and the native vaginal and ectocervical tissue.
METHODS

TLR Expression Experiment

Tissues

The tissues used for this research were obtained from human subjects; therefore, this research has been approved by the Institutional Review Boards of Boston University. The female reproductive tissues were obtained as discarded surgical specimens. They originated either from samples of the vaginal mucosa from women undergoing a vaginal repair procedure or ectocervical tissues from women undergoing a hysterectomy. Nine samples from three different women were obtained from women undergoing vaginal repair and five samples of the ectocervix from four different women undergoing a hysterectomy. The ectocervix is contiguous with the vaginal vault and histologically the epithelium is the same as that for the vaginal mucosa. The surgical tissue samples obtained were coded and patient identifiers were eliminated from all laboratory records to ensure patient confidentiality.

The MatTek tissue model is a three-dimensional organotypical vaginal-ectocervical model produced by the MatTek Corporation, located in Ashland, Massachusetts. They are made from human-derived vaginal-ectocervical epithelial cells. These tissue models were used in the cytokine analysis and the TLR expression experiments. For the TLR expression experiment, there were five MatTek organotypic vaginal/ectocervical partial thickness samples and five full thickness model samples. The partial thickness tissue is composed of only
epithelium, while the full thickness model is made up of the epithelium resting on a lamina propria derived from autologous fibroblasts. All tissue specimens were fixed in 10% unbuffered methanol-free formaldehyde and processed for embedding in wax.

**Immunohistochemistry**

5-µm-thick sections of tissue samples were collected on glass slides, de-waxed, and rehydrated in a graded series of ethanols. An antigen retrieval protocol was used to unmask reactive epitopes. The sections were immersed in citrate buffer pH6 and placed in pressure cooker which was then heated to a temperature of 125°C for 30 seconds. The slides were then cooled for ten minutes after which they were washed several times with distilled water and placed in Tris-buffered saline containing 0.1% Tween 20 (TBST).

Sections were incubated for 30 minutes with a serum-free protein blocking solution (Dako, Carpinteria, CA, USA), to block any non-specific binding of antibodies, and then drained from the tissue sections prior to the application of primary antibodies. Antibodies specific for TLRs 2, 4, and 9 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and the TLR 2 and 5 were supplied by Novus Biologicals, Littleton, CO, USA.

Rabbit or mouse primary antibodies were diluted in a proprietary antibody diluent (Dako) at the following concentrations: TLR2 1:90, TLR3 1:90, TLR4 1:80, TLR5 1:80, and TLR9 1:100. Tissue sections were incubated with the primary antibody for one hour at room temperature and then washed twice in TBST for
five minutes each. Primary antibodies were detected using proprietary secondary reagents (MACH4 Universal AP Polymer Kit Biocare, Concord, CA, USA). This system detects both mouse and rabbit primary antibodies. For mouse antibodies (TLR2 and 4) the sections were incubated with the MACH4 Universal AP Probe for 15 minutes. The sections were then washed twice in TBST for 5 minutes and then incubated with the MACH4 MR AP Polymer for a further 15 minutes. Finally the sections were washed twice in TBST buffer. For the rabbit antibodies (TLR3, 5 and 9) the sections were only incubated with the MACH4 universal AP Probe for 30 minutes. They were then washed twice in TBST buffer. The antibodies were visualized by incubating the sections with a substrate for alkaline phosphatase (Fast Red Dako) that stains positive cells and tissues a bright red. Development of the staining was monitored under a light microscope. After a final wash, the sections were counterstained in aqueous hematoxylin, mounted in a glycerin-based mounting medium and cover slipped. For negative controls that act as a check of non-specific binding of antibodies, primary antibodies were replaced by the antibody diluent only. The tissue sections were then treated exactly as those exposed to the primary antibodies.

**Protein Cytokine Analysis MB66 Film Experiment**

Two experiments were completed in order to evaluate the effect of Mab films on vaginal tissue. In the first experiment, placebo film and active film (MB66) with antibodies were added to MatTek vaginal-ectocervical tissue models and compared to the control. These films were manufactured by Kentucky
Bioprocessing, LLC and are meant to protect against the transmission of HIV-1 and herpes simplex virus (HSV-2). The antibodies used on the films were formed using plantibody technology. The active film is composed of polyvinyl alcohol (60%), maltitol (25%), histidine (0.1%), polysorbate 20 (0.01%), water (5%), HSV8-N monoclonal antibody (5%), and VRCO1-N monoclonal antibody (5%). The MB66 film contains 10 mg of VRCO1-N and 10 mg of HSV8-N. This film is 2 inches by 2 inches, with a thickness of 0.06 mm and a mass of 0.2 grams. The placebo film includes all of the same components as the active film, except for the HSV8-N and VRCO1-N monoclonal antibodies. Before being added to the tissues, a quarter of the film was dissolved in 1 mL of medium and 100 µL were placed on top of MatTek models. A Bio-Plex Pro Human Cytokine 4-plex Assay was used to determine if the films triggered immune response or inflammation among the cells in the vaginal epithelial model. This kit was used to detect four different human cytokines; IL-6, IL-8, MCP-1, and TNF-α. The tissues were then incubated at 37 degrees Celsius for four or twenty-four hours, and then harvested for basal and apical supernatants. The basal and apical supernatants were then tested for expression of the various cytokines.

In the second experiment, the films were added to the MatTek tissue models that were exposed to different environmental growth conditions. The tissues were grown in the presence of either basal medium or basal medium with 100 nM estradiol (E2) in order to mimic menstrual cycle peak. In addition, either active MB66 film or no film was placed directly onto the different vaginal tissue models.
and incubated at 37 degrees Celsius for 24 hours. In this case, the models without the film acted as the control. The basal supernatants were then harvested, since here the surface of the tissue was at the air interface, and tested for any expression of cytokines IL-6, IL-8, MCP-1, and TNF-alpha.

**Statistical Methods**

In experiment 1, log transformed data were analyzed by Kruskal-Wallis non-parametric analysis of variance (ANOVA). Significant (p<0.05) Kruskal-Wallis tests were followed by Fisher’s PLSD post-hoc tests for pairwise comparisons. No statistics were conducted for experiment two as only singleton or duplicate samples were obtained.
RESULTS

Cytokine Analysis Results

Experiment 1

The first cytokine analysis experiment added microbicide Mapp MB66 film to the vaginal tissue models to see if it induces inflammation. As seen in figures 3 and 4, there were no significant differences seen in IL-6 and IL-8 cytokine expression between the control, placebo, and MB66 film exposed tissue at 4 or 24 hours.

Figure 3. IL-6 expression in basal and apical supernatants at 4 and 24 hours in control, placebo film, and MB66 film exposed MatTek tissue models. Results show greater expression of IL-6 in basal supernatants as compared to apical supernatants in all models, yet the differences between the conditions (control, placebo, or MB66 film) were not statistically significant at either time point.
Figure 4. IL-8 expression in basal and apical supernatants at 4 and 24 hours in control, placebo film, and MB66 film exposed MatTek tissue models. Results show greater expression of IL-8 in apical supernatants as compared to basal supernatants in all models, yet the differences between the conditions (control, placebo, or MB66 film) were not significantly different at either time point.

In figure 5, the expression of TNF-alpha in the apical supernatant of the control after 24 hours was significantly greater than the expression in both the placebo and MB66 film at the same time point (both p<0.05). Less TNF-alpha expression suggests that a decrease in inflammation could be a positive side effect of the MB66 and placebo films. As seen in figure 6, the expression of MCP-1 in the basal supernatant after 24 hours was significantly greater in both the MB66 film and the placebo, as compared to the control (p<0.001). However, although the results are statistically significant (Figure 6), the difference in MCP-1 expression between the control, placebo, and MB66 film is quite small in magnitude.
Figure 5. TNF-alpha expression in basal and apical supernatants at 4 and 24 hours in control, placebo film, and MB66 film exposed MatTek tissue models. Results show greater expression of TNF-alpha in apical supernatants as compared to basal supernatants in the control and placebo models at the 4 hour time point. At the 24 hour time point, the expression of TNF-alpha was significantly reduced ($p<0.05$) in the apical supernatants of the placebo and the film.

Figure 6. MCP-1 expression in basal and apical supernatants at 4 and 24 hours in control, placebo film, and MB66 film exposed MaTek tissue models. Results show the expression of MCP-1 in the basal supernatant at the 24 hour time point was significantly greater ($p<0.001$) in both the film and the placebo as compared to the control.
Experiment 2

In the second experiment, the film was placed on top of the MatTek vaginal tissue grown in estradiol and compared to the control grown in medium. Only basal supernatants were analyzed, as the film was not dissolved in this experiment. In addition, duplicate or singleton samples were analyzed so no statistics were completed, although trends in the results were observed. In figure 7, there was an increase in the expression of IL-6 cytokine in the estradiol growth condition as compared to the control. There was a 1.8 fold increase in expression of IL-6 on the tissue exposed to estradiol and microbicide film, as compared to the control tissue exposed to estradiol and no film.

![Basal IL-6 24 Hour VEC-Full Thickness Tissue](image)

**Figure 7. IL-6 expression in basal supernatant of tissue exposed to medium or estradiol.** Results show that when the film was added to the MatTek tissue
model, there was an increase in the expression of IL-6. The model exposed to estradiol and film showed a greater increase in IL-6, as compared to its' media control.

In figure 8, the results do not show a great difference in the expression of IL-8 between the control and the MB66 film exposed tissue with the medium exposed tissue models. Under the estradiol growth condition, the expression of IL-8 in the tissue model increased the most when exposed to the microbicide film. A 1.7 fold increase in IL-8 was seen when the estradiol model was exposed to the film.

Figure 8. IL-8 expression in basal supernatant of tissue exposed to medium or estradiol. The results show that there was not a large difference in the expression of IL-8 between the controls and the film exposed tissue in the
medium model. There was a 1.7 fold increase in IL-8 in the estradiol model exposed to film, as compared to the estradiol control.

In figure 9, the results do not show a great difference in MCP-1 between the control and the MB66 film exposed tissues under the different growth conditions. A unique result seen is the slight decrease in MCP-1 when exposed to film, as compared to the control model exposed to only medium and no film. Although this change was very small, it was the only result that showed a decrease when MB66 film was added to the tissue model. In contrast, with the estradiol model, there was a slight increase in MCP-1 expression when film was added, as compared to the estradiol model with no film.

![Figure 9](image)

**Figure 9.** MCP-1 expression in basal supernatant of tissue exposed to medium or estradiol. Results do not show a great difference in the expression...
of MCP-1 in the MB66 film exposed tissue compared to the control under any of the growth conditions. There is a slight decrease in expression of MCP-1 with the film when exposed in the model grown in medium, as compared to the control with no film.

In figure 10, the results show that overall expression levels of TNF-alpha are very small compared to the other cytokines. There is hardly any expression of TNF-alpha in both the control and the MB66 film exposed models when grown with medium. A two-fold increase in expression of TNF-alpha in the MB66 film exposed tissue, as compared to the control, was seen with the models grown in the presence of estradiol.

Figure 10. TNF-alpha expression in basal supernatant of tissue exposed to medium or estradiol. Results show the least amounts of TNF-alpha expression under all growth conditions, as compared to the other cytokines analyzed. There
was almost no expression of TNF-alpha in the control and the film exposed tissues, with the tissue models grown in medium. A two fold increase in TNF-alpha expression was seen in the film exposed estradiol model, as compared to the estradiol control with no film.

**Detection of TLRs in Vaginal Tissue by Immunohistochemistry**

Immunohistochemistry was used to evaluate and compare TLR expression on the partial and full thickness MatTek tissue models, as compared to the native vaginal and ectocervical tissue samples. A bright red signal indicated positive staining for the specific toll like receptor.

**TLR 2**

A.  

B.  

C.
**Figure 11. TLR 2 expression in native tissue as compared to partial and full thickness MatTek tissue models.** Immunohistochemistry showed that TLR 2 expression was present in the epithelium of all native tissue samples. All of the MatTek tissue samples also stained positive for TLR 2. Panel A shows the native tissue, panel B shows the partial thickness MatTek model, and panel C shows the full thickness MatTek model.

The epithelium of all samples of the vaginal and ectocervical mucosa stained positive for TLR 2. This expression of TLR 2 was mostly present in basal layer of epithelial cells, as seen in Figure 11A. For a few of the tissues, staining for TLR 2 was localized along the length of the epithelium but for the majority of the epithelium tissue in the section, TLR 2 expression was seen. Blood vessels and cells, most likely white blood cells, were present in the lamina propria and also stained intensely positive for TLR 2.

All the MatTek tissue samples analyzed also stained positive for TLR 2. Similar to the native vaginal mucosa, the expression of TLR 2 by the partial thickness model was restricted to the basal layer of epithelial cells, as witnessed in Figure 11B. However, in the full thickness tissues, the expression of TLR2 was detected in several layers of the basal epithelium, as shown in Figure 11C. Fibroblasts present in the lamina propria like tissue of the full thickness model also stained intensely positive for TLR 2.
Figure 12. TLR 3 expression in native tissue as compared to partial thickness MatTek tissue models. Immunohistochemistry showed that the majority of the native vaginal and ectocervical tissue samples, as seen in panel A, did not show much TLR 3 expression. Panel B, also showed that there was limited expression of TLR 3 in the MatTek vaginal tissue models as well.

The epithelium of all the samples of vaginal and ectocervical tissue, except one section, stained negative for TLR 3. Although, as seen in Figure 12A, the one positive tissue sample had only a small localized portion of the basal layer of epithelial cells that expressed TLR 3. Blood vessels and cells in the lamina propria were only found to stain weakly positive for TLR 3. Similar to the vaginal mucosa, the epithelium and fibroblasts of all of the full thickness tissue models stained negative or showed very weak positive staining for the expression of TLR 3. In contrast, it was observed that the basal layer of the epithelium of the partial thickness MatTek model stained intensely positive for TLR 3, as seen in Figure 12B.
Figure 13. TLR 4 expression in native tissue as compared to partial and full thickness MatTek tissue models. Immunohistochemistry showed that the majority of the native tissue samples stained positive for TLR 4 expression, as seen in panel A. Also, as seen in panel B, all the samples of the MatTek full thickness model stained positive for TLR 4.

The epithelium of 11 out of the 14 native samples stained positive for TLR 4. This group was composed of 5 vaginal samples and 6 ectocervical samples. This expression of TLR 4 was detected primarily on the basal layer of epithelial
cells and was very extensive. As seen in Figure 13A, the staining often occurred along the entire length of the epithelium that was present in the tissue section. Blood vessels and cells in the lamina propria also often stained intensely positive for TLR 4.

In addition, all the samples of the MatTek full thickness model stained positive for TLR 4. As seen in Figure 13B, this expression was seen in the basal layers of the epithelium. Also, the fibroblasts present in the lamina propria like tissue stained intensely positive for TLR 4. One unique result was that no specific staining for TLR 4 was detected in the epithelium of any of the MatTek partial thickness tissue models. This was in contrast to the strong positive staining of the full thickness models.

**TLR 5**

When staining for TLR 5, no specific positive staining was detected in the epithelium of any of the vaginal or ectocervical tissue samples. Also, there was none or extremely weak positive staining of blood vessels or cells in the lamina propria. In addition, there was no expression of TLR 5 in the epithelium of either the full or partial thickness MatTek tissue models. Fibroblasts that were present in the lamina propria like tissue of the full thickness model also did not stain positive for TLR 5.
Figure 14. TLR 9 expression in native tissue as compared to MatTek tissue models. Immunohistochemistry showed positive staining and expression of TLR 9 in the native vaginal and ectocervical tissue samples, as seen in panel A. Similarly, in panel B, the MatTek full thickness models stained positive for TLR 9 as well. In addition, the MatTek partial thickness models showed many cells staining positive for TLR 9.

TLR 9 positive staining was seen in epithelial cells in all vaginal and ectocervical mucosa samples. Although, the amount of positive staining was
quite variable. The amount positive cells ranged from very few to extremely numerous in the different samples. As can be seen in Figure 14A, the cells that did stain positive were scattered within the supra-basal and apical layers along the entire length of the epithelium. In addition, there was no expression of TLR 9 seen in either the basal epithelial cells or by blood vessels and cells in the lamina propria.

Similar to the vaginal mucosa, TLR 9 positive epithelial cells were present in varying numbers in the suprabasal layers of the epithelium of the MatTek full thickness vaginal-ectocervical tissue model. This staining can be seen in Figure 14B. In addition, no positive staining for TLR 9 was detected for fibroblasts in the lamina propria like tissue of the full thickness tissue model. Finally, as seen in Figure 14C, numerous TLR 9 positive epithelial cells were detected in the partial thickness model.

**Negative Controls**

A. 

B. 
Figure 15. **Negative controls for immunohistochemistry.** This picture shows the negative controls for the native vaginal epithelium (panel A), the full thickness MatTek vaginal-ectocervical model (panel B), and the partial thickness MatTek vaginal-ectocervical model. Little to no background or non-specific staining was found on the negative controls.

There was no or very little background or non-specific staining that could be detected on the negative controls of the vaginal mucosa (Figure 15A) or either of the two MatTek models (Figure 15B and 15C).
DISCUSSION

Inflammation in the vaginal epithelium can cause more susceptibility and increase the risk of infection by HIV by depleting this physical barrier and recruiting more HIV susceptible cells to the site of tissue injury. Previous studies have shown that topical microbicides can induce inflammation in the vaginal epithelium and cause higher rates of HIV transmission. For my thesis research, I set out to prove that the new MB66 topical microbicide film produced by Mapp Pharmaceuticals, does not induce inflammation in the vaginal epithelium and is therefore a viable topical microbicide that could potentially be used and distributed as a safe female-controlled HIV prevention method. This subtle prevention method dissolves when in contact with liquid. Also, through immunohistochemistry I attempted to validate the use of MatTek vaginal-ectocervical models when testing products for vaginal inflammation through a comparison of TLR expression in partial and full thickness vaginal-ectocervical models and native vaginal and ectocervical tissue.

MB66 Films and Inflammation

Previous studies have shown that topical microbicides using Nonoxynol-9 have induced inflammation in the vaginal epithelium and increased the risk of HIV transmission; therefore, it is vital to ensure that any new topical microbicides do not cause the same effect before testing these products in healthy volunteers. The MB66 film experiments conducted did not show a significant or great increase in inflammation when the MB66 active film was added to the vaginal-
ectocervical tissue model. There was no significant increase in IL-6 and IL-8 cytokine expression in the vaginal epithelium when exposed to the MB66 films. IL-6 and IL-8 are two major cytokines that play a major role in the inflammatory response of the innate immune system; therefore, the lack of increased expression when exposed to the microbicidal film indicates that the film did not induce inflammation in these tissue models. Since inflammation increases susceptibility to HIV infection by potentially exposing leukocytes in the basal layer to HIV virions, the lack of IL-6 or IL-8 expression suggests that use of these microbicidal films will not increase the risk of HIV infection in initial human safety trials. In addition, the cytokine experiment analysis of TNF-alpha showed greater expression of TNF-alpha in the apical supernatant of the control as compared to the placebo film and the active MB66 film at the same time point. This suggests that the addition of the film to the tissue could have quieted the inflammatory immune response, leading to less inflammation and TNF-alpha cytokine production than the control tissue. More research should be done to determine the extent of this effect on vaginal-ectocervical tissue in women. Future research should include testing with a larger panel of cytokine analytes or through a safety study in health volunteers by testing cervico-vaginal lavages. Finally, when MCP-1 expression was analyzed results showed that expression of MCP-1 in the basal supernatant at the 24 hour time point was significantly greater in both the film and the placebo as compared to the control. As MCP-1 is a proinflammatory cytokine, formerly known as CCL2, this is an important result to consider.
Although this result indicates that both the active MB66 film and the placebo film could have induced migratory potential of macrophages and dendritic cells within the epithelium of the vaginal-ectocervical tissue, it should be noted that the expression of MCP-1 was statistically significant but was not vastly numerically different between the three conditions. This could be a statistical artifact. Also, there was no significant inflammation indicated in the apical supernatant. The MCP-1 expression was increased in detection only in the basal supernatant. Since the apical side of the epithelium is the first point of contact with HIV virus, it would be more concerning if the inflammation was induced at the host-pathogen interface. The basal layer lies underneath the apical layer; therefore, there is still some epithelial barrier left between the virus and this slightly inflamed area. Although unlikely, additional research should be done to explore the extent of this MCP-1 expression in the basal layer lamina propria to ensure that it does not increase the risk of HIV infection.

In the second cytokine experiment, the MB66 film was placed on vaginal-ectocervical models grown in the presence of medium and ones grown with high doses of estradiol. Estradiol levels in a female fluctuate throughout the menstrual cycle; therefore, it is important to know how the safety of the microbicidal film is affected by these hormonal changes, if at all. In addition, studies have suggested that high estrogen levels during ovulation could cause the mucosal tract to become less viscous and more alkaline, causing increased vulnerability to HIV-1 infection (Hladik, F., & McElrath, M. J., 2008). Results showed that there was an
increase in basal IL-6, IL-8, and TNF-alpha expression in the vaginal-ectocervical models grown in the presence of estradiol. Although statistics were not completed, as there were singleton or duplicate samples, these results could suggest that at certain points during the menstrual cycle when estradiol levels are at their greatest, the MB66 microbicidal films could potentially increase inflammation slightly in the epithelium and in turn increase risk of HIV infection. Although these results may suggest that these topical microbicides films may not be a perfect option for HIV prevention, it is important to note that the estradiol concentration, 100 nM, is significantly greater than the maximum estradiol present in the female human body at any point in the menstrual cycle; therefore, these results may not be representative of the actual inflammatory response that may occur in native tissue. Another unique result from this experiment was the decrease in basal MCP-1 expression when active film was added to the vaginal-ectocervical model exposed only to medium. This result suggests that in some cases, the MB66 microbicidal film could quiet the immune response, as the MCP-1 expression was decreased when the film was added. Although in the model grown only in medium, the difference in MCP-1 expression between the control (no film) and MB66 film exposed tissues was not a great difference, it is still a trend that should be considered and checked for when completing future research with these microbicidal products.
TLR Expression in Native Tissue and MatTek Models

The TLR expression experiment results were performed to validate the use of these vaginal-ectocervical models when testing new topical microbicidal products. We hypothesized that the native vaginal and ectocervical tissues would show similar TLR expression when compared to the partial and full thickness vaginal-ectocervical models. Immunohistochemistry results showed a great degree of similarity between the native tissue and the models. TLR 2 expression was positive in the native tissue as well as the partial and full thickness MatTek models. With TLR 3, there was a lack of expression in both the native and the full thickness models; in contrast, the basal layer of epithelium of the partial thickness MatTek model stained intensely positive for TLR 3. This difference between the native and the partial thickness tissue model could indicate that the full thickness model better represents the native environment of the lower female reproductive tract; therefore, the full thickness vaginal-ectocervical models should be used when testing new HIV prevention methods and products. With TLR 4, there was positive staining on the majority of the native samples and similar expression of TLR 4 was seen in all of the full thickness MatTek models. Surprisingly, no specific positive staining for the expression of TLR 4 was seen in the partial thickness MatTek models. Similar to the results with TLR 3, these results could indicate that the full thickness MatTek models are a better representation of the native vaginal-ectocervical environment.
A lack of TLR 5 expression was seen in native tissue as well as both the partial and full thickness models. There was no positive staining on the native vaginal or ectocervical tissue samples or any of the MatTek models. This result shows that the native tissue and the models have similar immune and inflammatory responses making both full and partial thickness MatTek models a good representation of the native environment. Finally, there was similar staining and expression of TLR 9 in all the native tissue samples, as well as the MatTek partial and full thickness models. Although there was variability in the staining itself and it was not localized to a specific area, as compared to the other TLRs, this same type of variable staining was seen in the all of the native and MatTek model samples. This result suggests that TLR 9 expression was very comparable in the native tissue and in the vaginal-ectocervical tissue models. In addition, throughout all of the samples TLR expression, when seen, was concentrated mainly in the basal layer. Since the basal layer lies deeper than the less apical layer, any inflammation occurring is not present at the host-pathogen interface, making the TLR expression here less concerning. Overall, these TLR expression results indicate that the MatTek vaginal-ectocervical models provide a valid and suitable method for testing out new microbicidal products and can successfully replicate the native environment of the lower female reproductive tract.
CONCLUSION

Even after more than 30 years of research, the scientific community has not yet discovered a female-controlled HIV prevention method. As HIV prevalence through heterosexual transmission is still quite high in the United States and even more so around the world, it is imperative that we continue to pursue research and find a discreet and accessible method for women. Topical microbicide products, such as the MB66 film, may be the method that could fill this void. Through these experiments, the MB66 films were not shown to increase inflammation in the vaginal epithelium, suggesting that this product will not increase the risk of HIV transmission. Future research should be conducted to determine the effectiveness of the MB66 microbicidal film at different points in the menstrual cycle to ensure that the fluctuating hormonal levels do not cause women to be at increased risk of HIV transmission. Overall, great strides are currently being taken in research to find a topical microbicide that can successfully curb the spread of HIV, and efforts in this area should continue until women around the world are afforded the ability to protect themselves against this incurable virus.
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Ayehunie, S., Cannon, C., Lamore, S., Kubilus, J., Anderson, D. J., Pudney, J., &


VITA

COREY J. COSTANZO

Address: 1 Homestead Boulevard Drive, #4224
Westborough, MA. 01581
508-887-1842

Email: costanzo@bu.edu

Year of Birth: 1991

Education:
Boston University
Bachelor of Science in Human Physiology, May 2013

Boston University School of Medicine, Boston, MA
Candidate for Master of Science in Medical Sciences, May 2015
Coursework: Biochemistry and Cell Biology, Advanced Human Physiology, Pathology, Biotechnology, Biostatistics

Boston University School of Public Health
Candidate for Master of Public Health, Concentration in Maternal and Child Health, May 2015
Coursework: Epidemiology, Reproductive Health, Health Law, Health Policy, Social and Behavioral Health, International Health

Rocky Vista College of Osteopathic Medicine
Candidate for Doctor of Osteopathic Medicine 2019

Professional Research Experience:

Center of Regenerative Medicine-Boston University School of Medicine
Boston, MA
Clinical Research/ IRB assistant—Kotton/Murphy Labs 8/13-Present
- Collect blood samples used for stem cell research
- Log stem cell line information into Freezerpro database
- Contribute to IRB protocols and will be completing IRB amendments as needed
- Enroll participants and complete blood draws for clinical trials when recruiting begins
**VDAART Study-Boston University School of Medicine**
Boston, MA
Clinical Research Assistant 4/10-Present
- Screen new patients and conduct regular study visits with participants and their children (ages newborn-4 years) in a clinical trial of vitamin D and pregnancy
- Safely complete patient blood draws and height/weight measurements, as well as biological specimen collections
- Complete visits in the Women’s Clinic at Boston Medical Center, and complete home visits to patient’s homes in various Boston neighborhoods, including Roxbury, Dorchester, East Boston, and South Boston
- Educate participants on the importance of prenatal vitamins during pregnancy
- Consult with doctors and nurses about potential study participants
- Update patient files in database and schedule patient appointments
- Accurately take inventory of supplies and restock any necessary equipment

**University of Massachusetts Medical School**
Worcester, MA
Research Assistant-Emergency Medicine 01/13-05/13
- Recruit patients waiting in the emergency room for the Patient Reported Outcomes study
- Thoroughly explain the study to the patient and answer the patient’s questions
- Administer survey to patients and clearly describe how to use iPad technology

**Slone Epidemiology Center-Boston University**
Boston, MA
Research Assistant 02/10-01/11
- Research drugs that women have reported to have taken while pregnant
- Organize and add new entries to the Slone Center’s Drug Dictionary

**OMNI Carb Study-Brigham and Women’s Hospital**
Boston, MA
Research Assistant 02/10-12/10
- Recruit new participants and thoroughly explain the study to them with clarity
- Accurately take participants blood pressure, height, and weight
- Schedule screening visits and construct files for new participants, perform data entry

**Leadership Experience:**
**Boston University Students for Sexual Health** 09/12-09/13
Position: Founder, President
- Provide resources and accurate sexual health information to Boston University students
- Encourage students to be informed and ask questions about their sexual health concerns
- Improve the sexual health of the BU community as a whole

**Sargent College-Boston University** 09/10-06/11
Position: Dean’s Host
- Introduce prospective students to Sargent College and our various programs
- Answer any questions prospective students’ have about Sargent College
- Offer tours of Sargent College and insight on BU student life

**Awards:**

**Scarlet Key Award-Scarlet Key Honor Society Boston University** 05/13
- The Scarlet Key Society is Boston University’s honor society for extracurricular achievement. The Scarlet Key Award is the highest honor awarded to Boston University student leaders who have exhibited exceptional leadership and excellence in University student activities and organizations, commitment to the individual’s school or college, and scholarship.

**Volunteer Experience:**

**Burton Chill Program** 12/13-Present
- Teach snowboarding to at risk and underserved youth and use snowboarding to increase self-esteem and teach life skills

**Boston Marathon Medical Tent Volunteer** 04/11-Present
- Boston University volunteer in Medical Records

**Horizons for the Homeless Volunteer Playspace Activity Leader** 10/12-05/13
- Volunteered weekly in a teen living homeless shelter in Roxbury, MA

**VIDA Medical Volunteer-Nicaragua and Costa Rica** 07/12-08/12
- Set up medical clinics and learned how to provide primary care in a rural Nicaragua and Costa Rica
- Gathered patient histories, vital signs, and developed a potential diagnosis and treatment plan for our patients