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17-beta estradiol alters the innate immune response to Neisseria gonorrhoeae

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17-BETA ESTRADIOL ALTERS THE INNATE IMMUNE RESPONSE TO
NEISSERIA GONORRHOEAE

by

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DEDICATION

I dedicate this work to my loving grandparents Thomas and Louise Ackerman!

From the time I was a toddler my nana instilled the importance of education in me letting me know I can be whoever and whatever I wanted to be. I thank my papa for my work ethic! I watched a man who never learned to read take apart a car and put it back together as a hobby, there was nothing that he couldn’t do and I appreciate every life lesson they gave me! I love and miss you more than words can say!
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me throughout this journey.
ABSTRACT

Data compiled by the Centers for Disease Control demonstrates that African American women, in particular young people between the ages of 14-25, have an increased incidence of infection with the sexually transmitted pathogen *Neisseria gonorrhoeae*. Estradiol is a key regulatory hormone in female reproductive function. It has been studied extensively in the cardiovascular field, and has been linked to breast and endometrial cancers in women. However, its impact on infectious diseases is largely unknown. Given what is known about the effect of estradiol on immunologic and inflammatory disorders in women, I hypothesize that estradiol alters the infectivity of *Neisseria gonorrhoeae* in the female reproductive tract by altering the host inflammatory immune response. This may explain a risk factor for increased rates of infection in some populations. I sought to develop a relevant in vitro model. After screening a number of candidate cell lines, I selected the human endometrial adenocarcinoma cell line, Ishikawa. These cells express specific estrogen receptors and respond to exogenous estrogen stimulation. Estrogen treatment of Ishikawa cells did not have an impact on the invasion of *N. gonorrhoeae*, nor did it impact bacterial growth. However, gonococcal induced chemokine secretion was reduced by estrogen, as measured by interleukin-8
secretion. I conclude that estrogen blunts the inflammatory response to *Neisseria gonorrhoeae* without altering bacterial infectivity.
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LIST OF ABBREVIATIONS

AF-1/AF-2 – Activation Function 1/2
AMP – Antimicrobial Peptide
BMI – Body Mass Index
CEACAM – Carcinoembryonic Antigen Cell Adhesion Molecules
CFU – Colony Forming Unit
DAPI – 4',6-diamidino-2-phenylindole
DGI – Disseminated Gonococcal Infection
DND – DNA Binding Domain
DMEM – Dulbecco’s Modified Eagle Medium
dPBS – Dulbecco’s phosphate-buffered saline
END E6/E7 cells – Endocervical Epithelial cells transformed with E6E7 gene
ELISA – Enzyme – Linked Immunosorbent Assay
ER α – Estrogen Receptor α
ER β – Estrogen Receptor β
ERE – Estrogen Response Element
FRT – Female Reproductive Tract
FSH – Follicle Stimulating Hormone
GC – Neisseria gonorrhoeae
GFP – Green Fluorescent Protein
GW – Graves Wavers
H – human
h – hour
IFN – Interferon
IK – Ishikawa Cells
IL – Interlukin
LBD – Ligand Binding Domain
LH – Luteinizing Hormone
LOS – Lipooligosaccharide
LPS – Lipopolysaccharide
MAPK/ERK – Mitogen Activated Protein Kinases/ Extracellular Signal Regulated Kinases
MCF-7 – human breast adenocarcinoma cell line
Min - Minutes
MOI – Multiplicity of Infection
MUC – Mucin
NF-κB – Nuclear factor – kappa B
NOD – Nucleotide-Binding Oligomerization Domain
OD – Optical Density
Opa – Opacity Associated Protein
PAMP – Pathogen Associated Molecular Pattern
PID – Pelvic Inflammatory Disease
PilE – Pilin E
PilS – Pilin S
PMN – Polymorphonuclear cells
PorB – Porin. 1B
PRAB – Progesterone Receptor AB
PRKCD – Protein Kinase C type Delta
PRR – Pattern Recognition Receptor
RLR – Rig-I-Like Receptor
RSK – Ribosome s6 Kinase
STI – Sexually Transmitted Infection
TIFA – TRAF-interacting protein with forkhead-associated domain
TNF – Tumor Necrosis Factor
TLR – Toll Like Receptor
CHAPTER ONE:

Introduction

Background and Significance:

*Neisseria gonorrhoeae* is a gram negative diplococci bacterium with humans being the only natural host. *Neisseria gonorrhoeae* infects the mucosal membrane including but not limited to the cervix, rectum and pharynx. Gonorrhea is transmitted via close sexual contact as well as passed vertically from mother to child causing conjunctivitis which if left untreated can lead to blindness in neonates. While the Centers for Disease Control (CDC) and the World Health Organization (WHO) have documented that the number of cases are slowly declining worldwide, there is an alarming disproportionate rate in infection within specific subsets of people when the numbers are teased apart according to age and ethnicity (Wira et al., 2010). Table 1a shows the ethnic breakdown by the CDC of gonococcal infection in the United States per 100,000 people, according to race and ethnicity (CDC). Here, we see African Americans have a greater incidence of infection compared to their ethnic counterparts, this disparity is echoed in the Boston area (Table 2).
Table 1. Incidence of Gonococcal Infection in the United States: A)
Gonococcal infection rates according to race and ethnicity B)
Currently, the reason behind this observation is poorly understood. According to the most recent statistics from the CDC, about 20 million new cases of sexually transmitted infections are reported yearly within the United States. Among this number, about 820,000 are *N. gonorrhoeae* infections. This epidemic disproportionally affects young people and minorities. Of the 820,000 cases of *N. gonorrhoeae* infection, about 71% of those infected are young people aged 15-24 years (Satterwhite *et al.*, 2013). It is important to note that this solely accounts for the reported cases; the actual numbers of gonococcal cases are thought to be at least double this number.

Disease signs and symptoms in males vs. females contributes to the vast difference in reported vs. actual gonococcal infections. When males are infected with gonorrhea, they often present with clear symptoms of infection characterized by painful and burning urination along with purulent discharge within a few days of infection. These clinical symptoms push males to seek treatment. However, females often present with subclinical symptoms that can be confused with normal menstrual cramps and discharge allowing the infection to be overlooked. Some strains, when unnoticed, can disseminate to other areas of the body, traveling up into the reproductive tract and causing complications such as severe pelvic pain, pelvic inflammatory disease, tubo-ovarian abscess, and more serious long-term complications such as tubal scarring, ectopic pregnancy, and ultimately infertility. Disseminated gonococcal infection can also cause arthritis and septicemia (Wira *et al.*, 2010).
Table 2. Incidence of Gonococcal Infection in the Boston Area:

Gonococcal infection rates according to race and ethnicity. Graph adapted from the Boston Public Health commission.

http://www.bphc.org/healthdata/other-reports/Documents/Gonorrhea2015_FINAL.pdf
In most first world countries, due to an overuse of antibiotics pathogenic bacteria such as *Neisseria gonorrhoeae* have evolved to become resistant to common antibiotics. Currently, gonorrhea is resistant to all antibiotics excluding a small group of cephalosporins (Kirkcaldy *et al.*, 2013). Completely resistant gonococcal strains with high resistance to cephalosporins were first isolated by a group in Japan and subsequently deemed the super bug (Tapsall, 2005). Since then, such strains have been identified in Europe, Canada, and Hawaii making this a very important bacteria to continue to study.

With the amount of antibiotic resistant gonococcal strains on the rise, there has been an increase exploration into vaccine development. Beginning as early as 1970, groups sought to use whole – cell or gonococcal outer membrane proteins as targets for vaccines, and found that protection against infection was not achieved (Greenberg *et al.*, 1974, Tramont, 1989, Boslego *et al.*, 1991). Variation in gonococcal membrane proteins has been attributed to the difficulties of vaccine development, therefore multi-target approaches are being explored.
**Microbiology of Neisseria gonorrhoeae:**

The hallmark of *Neisseria gonorrhoeae* is its evasion of the immune system and potential re-infection of the host. The bacterium has four distinct outer membrane moieties that aide in bacterial fitness, invasion, survival, and evasion within the host (Edwards & Apicella, 2004). These outer membrane proteins are pilin, opacity protein, porin, and the glycolipid lipooligosaccharide. By modifying these outer membrane antigens, Neisseria can evade immune cells like macrophages, neutrophils, and natural killer cells sent out to clear infection. Figure 1 outlines a simplistic view of gonococcal infection.

Type IV pili, whose major subunit is comprised of the PilE protein is responsible for initial host contact, motility, and virulence of the bacterium. These finger like appendages have been shown to undergo robust antigenic variation to evade the host immune response allowing for potential reinfection of the host (Forest & Tainer, 1997, Dehio *et al.*, 2000). Antigenic variation occurs when the major PilE subunit recombines with a silent PilS protein on the bacterium, turning pilin off and causing the bacterium to be overlooked by the immune system (Haas & Meyer, 1986, Swanson *et al.*, 1986, Koomey *et al.*, 1987). Seifert inoculated human volunteers with *Neisseria gonorrhoeae* intraurethrally and harvested bacteria via urethral swabs hourly to investigate pili expression. They found that overtime pilin expression changed and variation was based on combinations of the original inoculum (Seifert *et al.*, 1994). Pilin variation is thought to be RecA dependent, where RecA is a major DNA recombination repair gene. Our strain, FA1090b, has a total of 19 silent copies on the chromosome that codes for pilin,
making it very easy to change in the presence of medication or immune cells (van der Woude & Baumler, 2004, Jordan et al., 2005, Helm & Seifert, 2009).

Pilin adopt a grappling hook method adhering to host cells and neighboring bacteria pulling it close for invasion (Helm & Seifert, 2009). One way that this is achieved is through cytoskeletal rearrangement. As actin filaments shorten the host cell membrane begins to ruffle, allowing for the bacterium to enter (Edwards et al., 2000).

Opacity-associated adhesion protein (Opa), a highly conserved protein made up of an eight strand β-barrel, is another key outer membrane protein working alongside pili to initiate bacterial attachment and invasion (Swanson, 1978). Opa binds two distinct receptors within the host, one being carcinoembryonic antigen cell adhesion molecules (CEACAM) via Opa52 and heparin sulfate proteoglycan (HSPG) via Opa50 (Bos et al., 1997, Dehio et al., 1998).

Antigenic variation of the Opa protein occurs within the structural unit of Opa via frame shift mutations, triggering changes in pentameric repeats (Song et al., 2000). This variation causes formation of multiple Opa variants within one bacterium enabling immune evasion, potential reinfection, and difficulty in vaccine development (Murphy et al., 1989).

Pore forming proteins (Porins) represented by alleles porin 1A and 1B (P.1A, P.1B), also play a role in gonococcal pathogenesis. Porin is formed by trimeric β-barrels that transverse the outer membrane of gonococci. *N. gonorrhoeae* dominant allele PorB has been shown to translocate vertically into the membrane of host cells binding to ATP and GTP, creating voltage-gated channels that allow for the transfer of infectious
particles into uninfected host cells. As a result, porin is essential for gonococcal infection (Blake & Gotschlich, 1982, Gotschlich et al., 1987, Rudel et al., 1996). Along with pilin, porin has been shown to facilitate cytoskeletal rearrangement via actin nucleation within host cells (Edwards et al., 2000). Porin is also a PAMP recognized by TLR2 on the host membrane (Massari et al., 2006).

Finally, gram-negative bacterial pathogens like *N. gonorrhoeae* come equipped with an endotoxin lipopolysaccharide (LPS) on its cellular surface. *N. gonorrhoeae* LPS is referred to as lipooligosaccharide (LOS) as it is comprised of only three oligosaccharides attached to a highly conserved lipid A core, unlike conventional LPS. These specific oligosaccharides are prone to phase variation via insertion of guanine residues within glycosyltransferases producing varying LOS isoforms (Song et al., 2000). LOS can mimic host structures to blend in with surrounding environments. Antigen variation of LOS occurs via slippage during replication, causing a frameshift mutation (Danaher et al., 1995, Burch et al., 1997). It has been hypothesized that this variation can enhance immune evasion by allowing the bacterium to change phenotypes between invasive and serum-resistant, where serum resistance refers to the ability of serum to kill the bacterium (van Putten, 1993). This is often seen in gram negative bacteria that cause bacteremia and septicemia, two complications of untreated gonococcal infection. LOS is a PAMP that is recognized by pattern recognition receptor (PRR) Toll-like Receptor (TLR) 4, one of the innate immune system key activators.

These features all prove problematic when trying to develop a vaccine based on these outer membrane proteins. Along with both phase and antigenic variation, *N.*
*gonorrhoeae* has other ways in which it can evade the host adaptive immune system. *N. gonorrhoeae* can hijack this system to evade the host immune response by suppressing T lymphocyte activation and using T cell regulators – Tregs - to suppress activities of antigen presenting cells (Boulton & Gray-Owen, 2002).
**Figure 1. Gonococcal Infection Model:** Speculative model of how *Neisseria gonorrhoeae* invades human epithelial cells, along with induction of innate immune cells. Epithelial cell receptors CD46 and CEACAM binds pili and Opa, respectively.
Mucosal Immunity of the Female Reproductive Tract

The female reproductive tract (FRT) is divided into two sections: the non-sterile lower FRT composed of the vagina and ectocervix, and the sterile upper FRT composed of the uterus, endometrium, and fallopian tubes. The endocervix serves as the transition area between the two (Radtke et al., 2012). The non-sterile lower FRT is exposed to outside stress and commensal bacteria and is lined with stratified squamous epithelium that acts as the initial barrier against foreign particles. To protect against fungi, viruses, and bacteria, the FRT is equipped with barriers that separate the outside environment from the inside (Hickey et al., 2011). Along with the initial epithelial barrier, the reproductive tract also contains a rich microbiome and the ability to excrete mucus and alter pH to defend against impending pathogens (Sonnex, 1998). The vaginal microbiome is comprised of commensal bacteria largely made up of lactobacillus, which along with other members of the flora regulate vaginal pH (Doerflinger et al., 2013). The vagina normally has a very acidic pH of 4.5 not suitable for pathogen survival; however, when pathogens enter, the pH can change. As the pH changes, commensal bacteria will secrete lactic acid, bringing the pH back to its resting state (reviewed in (Hickey et al., 2011)). Interestingly, Ravel et al. explored and compared the microbiome of African American, Asian, Hispanic, and Caucasian women and found that African American and Hispanic women had a higher vaginal pH that creates an environment suitable for other bacteria to thrive. They further hypothesized that these bacteria could increase the chances of developing vaginal infections such as bacterial vaginosis that in turn could increase the
chances of contracting other infections due to disruptions in the epithelial barrier (Ravel et al., 2011).

The sterile upper FRT is lined with columnar epithelium held together by specialized tight junctions upholding the integrity of the epithelial wall (Godfrey, 1997). Along with hormonal regulation of tight junctions, several cytokines have been shown to regulate the integrity of these molecules. Capaldo et al reviewed this mechanism. In states of increased inflammation, pro-inflammatory cytokines were shown to remodel tight junctions between epithelial cells via actin rearrangement, allowing pathogens to pass through disrupted barriers. Cytokines such as TNF-α and IFN-γ have been shown to play key roles in actin remodeling that further disrupts junctional molecules (Blum et al., 1997, Capaldo & Nusrat, 2009). This area remains sterile to protect the semi-allogeneic fetus during fertilization and implantation. One way in which this barrier is maintained is by the production of mucus. Cervical crypts within the endocervix are the main source of mucins. Mucins are members of the MUC family and are O-linked glycosylated proteins that can be secreted, membrane bound or soluble. There are 19 known mucins documented, and they are known to be thick and sticky, trapping pathogens and blocking the infection process (Radtke et al., 2012).

Mucus is also known to be regulated by cyclically produced sex hormones during the menstrual cycle. Mucins are known to have either estrogenic or progestational characteristics, where estrogenic mucus is present more heavily during the proliferative phase leading up to ovulation. This mucus is thin and slippery in consistency, allowing for easy passage of sperm into the sterile upper FRT for fertilization. Progestational
mucus, on the other hand, has as its main role to block polyspermy. This is achieved by the production of thick viscous mucus increasing after ovulation during the secretory phase. This cervico-vaginal mucus plays a crucial role in blocking pathogens from reaching the sterile upper reproductive tract where disease can proliferate (Elstein, 1978, Gibbons, 1978, Gipson et al., 1997, Vigil et al., 2009). Along with protecting against pathogens, mucins also store and produce antimicrobial peptide (AMP). These AMP fight against viruses and bacteria by disrupting pathogen membranes and metabolic processes to kill them. Examples of AMP are α and β-defensins, SLPI, lactoferrin, and lysozyme (Ganz, 1999, O'Neil et al., 1999, Ganz, 2003, Radtke et al., 2012).

**Innate Immune System**

While the body is capable of fending off infection in the lower FRT via mucus lining the cellular surface, antimicrobial peptides (AMP), and low vaginal pH, this is not wholly sufficient. The body is equipped with a delayed, more effective response to clear pathogens called the adaptive immune response. The adaptive immune response is more long term and has the ability to recognize pathogens for clearance of future infections. The innate immune system, comprised of macrophages, dendritic cells, and epithelial cells, act as sentinels by presenting pathogenic antigens on its cell surface to ignite the adaptive immune system (Hickey et al., 2011). Macrophages and dendritic cells are most abundant within the endometrium right before menstruation, when the sex hormone estradiol is at its highest concentration during the menstrual cycle. During this time it has been speculated that pathogens have their greatest efficiency at entry. Interestingly,
macrophages that populate the vagina are not altered by changes in hormone concentrations (Starkey et al., 1991, Wira et al., 2005).

Polymorphonuclear cells (PMNs) also referred to as neutrophils, are the major phagocytic granulocyte that is abundant at high concentrations within the fallopian tubes early in the cycle but have a short half-life. Along with macrophages, PMNs are able to recognize a foreign pathogen and internalize it for degradation. These phagocytic cells are equipped with membrane bound pattern recognition receptors (PRRs), specifically toll like receptors (TLRs) that bind pathogen-associated molecular patterns (PAMPs) on the surface of bacteria, viruses, and fungi. Once contact occurs, pathogens are internalized into phagosomes. At this point, the internal environment of the phagosome changes, becoming more acidic to kill the pathogen. The phagosome then fuses with the lysosome for degradation. Dendritic cells also internalize pathogens, but unlike PMNs and macrophages, they present antigens on their surface, activating the more robust adaptive immune system to clear infection (Kis et al., 2004).

*N. gonorrhoeae* expresses a number of PAMPs on its outer membrane such as LOS, lipoproteins, and porin that are key activators of the innate immune system. These PAMPs are recognized by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) (reviewed in (Lien & Ingalls, 2002, Nasu & Narahara, 2010)). Other important receptors for innate immune activators are NOD-like receptors and Rig-I-like receptors (RLR) (reviewed in (Benko et al., 2008, Eisenacher & Krug, 2012)).

Toll-like receptors were originally discovered to play a significant role in *drosophila* development and were later found to have similar gene expression in humans.
There are approximately 13 TLRs expressed in the human genome. The endotoxin LOS is recognized by and binds TLR4 with the help of adaptor molecule MD-2, and outer membrane proteins PorB (Massari et al., 2006) and H8 (Fisette et al., 2003) are recognized by and bind TLR2.

Both TLRs bind adaptor protein myeloid differentiation primary response 88 (MyD88) which is recruited to the cellular surface by MyD88 adaptor like protein Mal. IL-1 receptor associated kinase 1 and 4 (IRAK 1, 4) are then recruited and activated. TNF receptor associated factor 6 (TRAF-6) is recruited and activated to recruit serine threonine kinase TGF-β activated kinase 1 (TAK 1). TAK 1 then phosphorylates IκB kinase (IKK) complex which then phosphorylates IκB to release nuclear factor kappa-light-chain-enhancer of activated B – NFκB for nuclear translocation to transcribe pro-inflammatory genes to elicit inflammation (reviewed in (Lien & Ingalls, 2002, Akira & Hemmi, 2003, Takeda & Akira, 2003)).

Activation of the innate immune activation results in production of several cytokines and chemokines. One such chemokine, human IL-8, is important during infections because once activated, it attracts and ushers in f neutrophils to the site of infection. This cell type is of particular importance because it has been shown to aid in immune evasion and pathogen survival. Once gonococcal infection is initiated, there is an influx of PMNs. Normally, these cells release redox species, oxidative bursts, and AMPs for pathogen clearance, but *N. gonorrhoeae* has evolved to evade the action of neutrophils. *N. gonorrhoeae* is able to hijack this system by producing factors that neutralize oxidative stress and fight against PMN uptake. These factors allow for the
bacterium to survive and replicate within PMNs, evading immune system clearance. We know that *N. gonorrhoeae* suppresses PMN killing because live gonococcal bacteria can be found within cultures from both infected males and females (Johnson & Criss, 2011). This survival can potentially lead to host re-infection.

Interestingly, studies have chronicled the differences in cytokine production between different ethnicities and have shown that African American women carry allele variants that elicit a more intense pro-inflammatory response measured by cytokine induction as compared to their Caucasian counterparts (Ness, 2004). This is important because it shows that this specific population is more prone to inflammation that could possibly intensify infections (Ness, 2004).

**Estrogen Receptor**

Estrogen signals through two distinct receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). These steroid receptors are members of the class III nuclear receptor superfamily binding palindromes on estrogen response elements as a homodimer (Mader *et al.*, 1993). Nuclear receptors consist of a N terminus A/B region, a highly conserved C region consisting of the specialized DNA binding domain, a D region that acts as a link between C and the highly conserved E region where the ligand binding domain is located, and finally an F region consisting of an unknown carboxyl terminus (Figure 2) (Mader *et al.*, 1993, Aranda & Pascual, 2001). Transcriptional activation function AF-1 located in the A/B region on the receptor interacts with ERα to enhance transcriptional activity in estrogen responsive genes, whereas estrogen receptor β has
been shown to inhibit AF-1 induced transcriptional activity in estrogen responsive genes (Paech et al., 1997).

Estrogen receptor is known to signal through a classical pathway. This pathway is ignited when the ovaries (or placenta) receive a signal from the brain to release estradiol. Intracellular estrogen receptor when unbound to estradiol is inactive. This inactive complex is bound to heat shock proteins and immunoglobulins. Once estradiol is sensed, the complex dissociates and estradiol binds estrogen receptor. This new complex then translocates into the nucleus, modifying chromatic structures to bind estrogen response elements in the promoter region of DNA to influence gene transcription (Figure 3). Recently there has been another pathway identified in estrogen signaling involving G–protein coupled estrogen receptors. This receptor was shown to be activated by estradiol, resulting in activation of classical MAPK/ERK signaling pathways leading to changes in gene transcription. This secondary pathway is still being characterized (Cabas et al., Sanchez et al., 2002, Weatherman, 2006).
**Figure 2. Schematic of Nuclear Receptor:** Estrogen receptors are members of the nuclear receptor family made up of functional fields which bind specific ERE regions on genes. A/B region binds (ligand independent) activation function 1; conserved C region binds DNA; Region D acts as a hinge point linking regions C and E; Conserved region E binds ligand binding domain and activation function 2 (ligand dependent); region F is still being explored. (Adapted from (Aranda & Pascual, 2001, Sanchez et al., 2002)).
Figure 3. Classical Estrogen Signaling: Estrogen receptors are bound in an inactive complex that dissociates only when estradiol binds. The new complex then translocates into the nucleus binding estrogen response elements on the target gene to alter transcription. Adapted from (Sanchez et al., 2002).
**Estradiol and the Female Reproductive Tract**

The human female reproductive tract (FRT) consists of a unique microenvironment suitable for housing a fetus along with fighting off pathogens. This system has been shown to be regulated by sex hormones in a cyclic manner. The main female sex hormones are estradiol and progesterone, where estradiol is the dominant hormone regulating the FRT. The role of these hormones is to promote female characteristics, regulate the menstrual cycle, and ultimately prepare the body for fertilization and implantation. Studies from Charles Wira (Wira *et al.*, 2010), Kenneth Beagley (Beagley & Gockel, 2003), Sabra Klein (Klein, 2012) and Elisabeth Garcia-Gomez (Garcia-Gomez *et al.*, 2013) all show that estrogen is a critical regulator of the FRT environment. The hypothalamus signals the pituitary gland at the base of the brain via gonadotropin releasing hormone to release follicle stimulating hormone (FSH) and luteinizing hormone (LH). LH and FSH stimulate 17 β-estradiol, the dominant circulating estrogen produced in the ovaries, the adrenal gland, and the placenta during pregnancy (Garcia-Gomez *et al.*, 2013).

The female menstrual cycle is a complex cycle with variations between cycles in a given woman, between different women, and in the daily amount of estrogen produced (Stricker *et al.*, 2006). It is important to note findings showing circulating serum levels of estradiol may be 5 times lower than that of local levels found within the FRT. According to Huhtinen, local estradiol levels may be 5-8 times greater than that of serum in the proliferative phase (Huhtinen *et al.*, 2012).
Early studies have shown a correlation between the menstrual cycle and gonococcal infection. Sweet (Sweet et al., 1986) looked at women who were hospitalized with salpingitis, searching for a connection between that diagnosis and gonococcal or chlamydial infection. They recovered gonorrhea from 40% of women who began to have symptoms within a week of the start of their menstrual cycle. They concluded that there was a connection between infection and the menstrual cycle.

Estrogen, as it functions within the menstrual cycle, is thought to be protective of the FRT (Salem et al., 2000). Along with protecting the FRT, estrogen also stimulates the production of pro-inflammatory cytokines like interleukin 1, 6 (IL-1, IL-6) and tumor necrosis factor alpha (TNF-α) and inhibits anti-inflammatory cytokines like IL-4 and 10 and transforming growth factor beta (TGF-β) (Salem et al., 2000, Straub, 2007). During the ovulatory phase of the menstrual cycle, that takes place approximately 14 days after the onset of menses, estrogen concentrations are at their highest. During this time, the body is preparing for fertilization and implantation, and the immune system is suppressed so as not to attack the allogenic fetus. Antimicrobial peptides and pro-inflammatory cytokines are also suppressed (Keller et al., 2007). Estrogen can also regulate the adaptive immune system by targeting the function of antigen presentation and phagocytic cells (Wira et al., 2010). This data proves that estrogen regulates both the innate and adaptive immune system of the FRT in a cyclic manner.

Estrogen also plays a role in pathogen invasion. Wira determined the integrity of epithelial cells by testing transepithelial resistance (TER). TER is a method to study barrier function by measuring electrical physical resistance. They saw that in human
uterine epithelial cells (ECC-1) treated with estrogen for 48 h, TER was significantly decreased (Grant-Tschudy & Wira, 2004, Wira et al., 2005). The upper reproductive tract lined with columnar epithelium is held together by tight junctions. Wira along with other groups showed that when estrogen is present at increasing concentrations, it has the ability to disturb these tight junctions, lowering their integrity and allowing for easier pathogen entry.

We know that estrogen levels vary in and between women based on data presented in other studies. Estrogen has also been shown to aid in the disruption of epithelial barriers at high concentrations, allowing pathogens to gain entry into the human host. Statistical differences in the incidence of infection among ethnic groups presented by the CDC and the WHO gave rise to the question of whether estrogen concentrations are also different among different ethnic groups. Two studies are relevant to the racial disparity noted by the CDC and WHO data. Pinheiro (Pinheiro et al., 2005) and Marsh (Marsh et al., 2011) both show that there is a significant difference in the amount of estrogen produced by women of different ethnicities. Pinheiro looked at premenopausal hormone levels in African American, Caucasian, and Asian women. They controlled for cycle progression and life style differences and showed that African American woman had an increase in circulating estrogen level. Marsh controlled for BMI, age, and life style and came to the same conclusion. They both found that African American women had more free and bound estradiol than their Caucasian or Asian matched controls.

This data is very interesting and suggests estrogen may play a role in infection propagation within the human host. Based on published data surrounding sex hormones
and their ability to cyclically regulate the female reproductive tract, we hypothesized that estradiol alters the infectivity of Neisseria gonorrhoeae in the female reproductive tract by altering the host inflammatory immune response. In order to prove our hypothesis, we propose the following aims:

**AIM1:** Determine the hormone responsiveness of known cell lines (compared to primary cells) that represent relevant tissues found in the female reproductive tract.

**AIM2:** Determine the effects of varying levels of sex hormone on innate immune responses to *N. gonorrhoeae* infection.
CHAPTER TWO:
MATERIALS AND METHODS

Cell Culture: The Ishikawa endometrial adenocarcinoma cell line was kindly provided by Dr. Wendy Kuohung (Boston University School of Medicine, Boston, MA). Cells were maintained in a 37°C 5% CO2 incubator in phenol red free Dulbecco’s Modified Eagle Medium (DMEM) (Gibco) supplemented with 5% (v/v) charcoal stripped fetal bovine serum (Gibco) and 2 mM Glutamine (Gibco). Complete DMEM (cDMEM) was changed every 3 days until 80% confluency was reached. Cells were trypsinized with 0.05% trypsin, 0.53mM EDTA, (Corning) for 75cm² for 5-8 minutes and inactivated with 10 mL of cDMEM. Cells were then centrifuged at 500 x g for 10 minutes and split 1:4 into new 75cm² flasks with fresh cDMEM.

Bacterial culture: Neisseria gonorrhoeae (GC) strain FA1090b is derived from the common lab strain FA1090 (Connell et al., 1988, Murphy et al., 1989), and engineered to express only OpaB (Cole et al., 2010). Bacteria from frozen stocks were inoculated onto chocolate agar and cultured overnight at 37°C with 5% CO2. For broth cultures, fresh colonies from the chocolate agar plate were used to inoculate Wade Gravers (GW) media and cultured at 37°C for 3 – 4 hrs. Growth curves were obtained by growing N. gonorrhoeae overnight on chocolate agar plates from frozen stocks in a 37°C 5% CO2 incubator. GW broth was inoculated the following morning at an optical density (OD) of 0.1, hourly samples were taken, and using the Eppendorf Biophotometer OD600 spectrophotometer, OD was obtained. Samples were then diluted and plated onto chocolate agar plates for overnight growth to determine colony formation unit (CFU).
GFP tagged FA1090b was kindly provided by Dr. Sanjay Ram (UMASS Medical School, Worcester, MA) and was grown in the same manner as FA1090b. Killed bacterial stocks were prepared from broth cultures by subjecting the stocks diluted to a specific concentration to a freeze-thaw cycle of -80°C for 30 min followed by thawing on ice. Confirmation of bacterial killing was performed by streaking onto chocolate agar.

**Invasion Assays and Quantitative Culture of Bacteria:** Ishikawa cells were seeded in a 6-well plate at a density of 1-5x10^5 cells/ml and grown to confluency as described above. FA1090B was grown as described above, and Ishikawa cells were inoculated at an MOI of 10 and 100:1 for 5 h (in separate plates). Every hour cells were washed 3 times with PBS and then treated with 200ug/ml gentamicin (Gibco) for 60 min. Wells were then washed 3 times with PBS to get rid of extracellular bacteria. Wells were scraped and diluted onto chocolate agar plates (Rimel, ThermoFisher Scientific) and placed in a 37°C 5% CO2 incubator overnight for colony formation.

**PCR Amplification of Eukaryotic Gene Expression:** Ishikawa cells were seeded in 6-well plates at a density of 5x10^5 cells/mL in the presence or absence of 75nM estradiol and grown to confluency. Cells were then inoculated with GC (grown as described above) at differing multiplicity of infection (MOI). FA1090b was grown in GW media until an optical density (OD) of 0.7 – 0.8 was obtained. Cultures were then diluted to an OD of 0.3 (which corresponds to 1x10^8 gc/mL) and was centrifuged at 1500 x g for 10 minutes and diluted to the appropriate MOI for Ishikawa inoculation. RNA was extracted from Ishikawa cells using the Qiagen RNeasy kit. cDNA was then made for semi-quantitative PCR. Thermoprofiles were as follows: ERα – 4 min. denaturing at 94°C
followed by 35 cycles as follows: 15 sec at 94°C, 15 sec annealing at 59°C, 30 sec extension at 72°C; PRAB – 1 min. denaturing at 94°C followed by 35 cycles as follows: 1 min at 94°C, 1 min annealing at 59°C, 2 min. extension at 72°C; RSK and PRKCD – 1 min. denaturing at 94°C followed by 40 cycles as follows: 5 sec at 95°C, 20 sec annealing at 60°C, 30 sec extension at 72°C. GAPDH – 5 min. denaturing at 94°C followed by 25 cycles as follows: 30 sec at 94°C, 30 sec annealing at 52°C, 45 sec extension at 72°C. Primers (Invitrogen: Chart Below). PCR primers and conditions for TLR expression were as previously described (Fichorova et al., 2002).
<table>
<thead>
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<th>Table 3: PCR Primers (Invitrogen)</th>
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<tr>
<td><strong>ERα</strong></td>
</tr>
<tr>
<td>F 5'-agacatgagagctgccaacc-3'</td>
</tr>
<tr>
<td>R 5'-gccaggcacattcttgaagg-3'</td>
</tr>
<tr>
<td><strong>RSK</strong></td>
</tr>
<tr>
<td>F 5'-caaggacctgtgcaagatg-3'</td>
</tr>
<tr>
<td>R 5'-agatgtggctctctgtgttt-3'</td>
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Flow Cytometry: Ishikawa (IK) cells growing in a 6-well dish were inoculated with FA1090b or FA1090b-GFP and incubated for 6 and 24 h. At the end of the incubation period, the wells were washed and scraped and washed again with dPBS to obtain Ishikawa cells with bound bacteria. For bacterial association analysis, cells were blocked for 30 minutes in 20% normal donkey serum, then incubated for 60 min using mAb 2C3 kindly provided by Dr. Sunita Gulati (University of Massachusetts Medical School) against outer membrane Neisseria lipoprotein, H8 (Cannon et al., 1984). For CEACAM1 analysis, cells were blocked for 30 min with 1% PBST, then incubated for 60 min with an anti-hCEACAM1/CD66a (R&D systems; Minneapolis, MN) antibody. Cells were then placed in a 1:5 solution of paraformaldehyde and were read on the BD FACScan and analyzed using FloJo v10.0.6 in the Flow cytometry core.

Immunohistochemistry: Ishikawa (IK) cells were seeded at a density of 1x10^5 cells/500uL in a 24 well dish onto tissue culture treated coverslips and grown until confluence was reached. Cells were then inoculated with GFP positive FA1090b at an MOI of 10 and 100. Cells were washed twice with Tris buffer solution, blocked for 30 min in 20% normal donkey serum, then incubated for 60 min using the mAb 2C3 antibody. Cells were washed again, then incubated with anti-mouse Cy3 (1:1000) for 30 min. After a final wash, coverslips were mounted onto slides using anti-fade DAPI and viewed under a fluorescent microscope.

Enzyme – linked immunosorbent assay (ELISA): Human interleukin-8 secretions from Ishikawa cells were measured by ELISA using a human IL-8 kit; samples were run and analyzed according to the manufacturer’s guidelines (R&D).
Western Blot: Ishikawa cells were seeded at a density of 1x10^6 cells/well in a 6-well plate in the presence or absence of varying concentrations of estradiol for 24 h. Cells were then washed with PBS, scraped off wells, and spun for 5 min at 5000 rpm twice. Cells were lysed with RIPA buffer (Boston BioProducts; Cambridge, MA), sonicated for 30 sec, and incubated on ice for 30 min. Cells were spun for 5 min at 5000 rpm, and whole cell lysates were collected.

Cell protein concentration was determined by bicinchoninic acid (BCA) assay (Thermo Scientific). A standard curve was prepared diluting BSA in RIPA lysis buffer solution. A working reagent was made by mixing BCA reagent A (consisting of sodium carbonate, sodium bicarbonate, bicinchoninic acid, and sodium tartrate in 0.1M sodium hydroxide) and BCA reagent B (consisting of 4% cupric sulfate) at a ratio of 1:8. 100 uL of working reagent was added to each well, along with 2 uL of protein sample, and the plate was mixed on a shaker for 30 sec. The plate was then incubated at 37°C for 30 min, and absorbance was read on spectrophotometer at or near 562nm.

For Western blot analysis, protein concentrations were diluted to appropriate concentrations using RIPA buffer solution. 1x loading dye was added, and samples were boiled for 5 min at 100°C to denature proteins. Proteins were separated on a 4–12% gel run at 120 volts in running buffer for 1 h. Gels were then transferred to nitrocellulose at 80 volts for 1.5 hr in transfer buffer (transfer buffer stock, methanol, dH2O). Nitrocellulose was then washed in TBST, blocked in 5% milk in TBST for 30 min, and probed overnight at 4°C in primary antibody (estrogen receptor α from Millipore and β from Upstate). Membranes were then washed twice the following morning in TBST for
10 min and were incubated in the dark with secondary antibody (horseradish peroxidase
goat anti rabbit IgG) for 1 h on a shaker. Membranes were then washed 3 times with
TBST. To develop the blots, a 1:1 chemiluminescent substrate solution was made and
placed on membranes for 1-2 min, and then membranes were exposed to film.

**Graves Wavers Media:**

1L normal strength M199 cell culture medium (with Earle’s salts without L-glutamine,
phenol red or sodium bicarbonate

500mL distilled water

Glucose (10g; 37mM)

Ammonium bicarbonate (2g; 17mM)

Sodium acetate trihydrate (1g; 4.9mM)

L-glutamine (0.75g; 3.4mM)

L-arginine (0.1g; 383mM)

Hypoxanthine (0.05g; 245mM)

Uracil (0.05g; 298mM)

Oxaloacetate (0.05g; 252mM)

Thiamine hydrochloride (0.05g; 99mM)

L-ornithine (0.01g; 39mM)

Nicotinamide adenine dinucleotide (0.01; 10mM)

Sodium DL-lactate (2.5mL of 60% w/w syrup; 13mM)

*Hypoxanthine and uracil were each dissolved in 2-3mL NaOH

*pH was adjusted to 6.8 with 1M HCL and was sterilized through a 0.22micron filter
**Statistics:** All data was analyzed using t tests. A P-value < 0.05 was considered statistically significant. All experiments were performed at least 3 times to allow for calculation of standard error and standard deviation.
CHAPTER THREE:
ISHIKAWA CELLS EXPRESS SPECIFIC HORMONE RECEPTORS WHILE RESPONDING TO EXOGENOUS ESTRADIOL TREATMENT

Rationale: One main drawback to studying gonococcal infection is that it’s only natural host is the human body. This creates an obstacle when studying and mapping out disease progression. Many groups have established and utilized the transgenic mouse model to carry out their bacterial experiments (Jerse, 1999, Packiam et al., 2010); however, to establish infection, hormone priming must take place that would interfere with our hypothesis. Along with transgenic mouse models, there are epithelial cell models that are used to study the general progression of gonococcal infection (Fichorova et al., 2001, Fichorova et al., 2002, Fisette et al., 2003), but it is vital for our work to identify a hormonally responsive human cellular model that can be used to investigate the effects of on the response of the human host to N. gonorrhoeae.

In the FRT, N. gonorrhoeae normally infects the endocervix, but primary hormonally responsive human endocervical tissue is not always available. We began with a well-known endocervical cell line, END E6/E7, immortalized with the human papillomavirus E6/E7 gene (Fichorova et al., 1997, Fichorova & Anderson, 1999). Using Western blot analysis, we identified protein expression of the dominant estrogen receptor ERα, but when challenged with exogenous estradiol to determine responsiveness, we do not see any change in expression (Figure 4a). This held true when estradiol concentrations were altered. We then sought to examine innate immune activation measured via IL-8 induction. When compared to media alone, estradiol stimulation did
not alter IL-8 induction (Figure 4b). We concluded that while the bacterium does
naturally infect the endocervix and the estrogen receptor is present, we did not see
changes in ER expression when challenged. Therefore, we did not continue with these
cells.
Figure 4. ERα Gene Expression along with IL-8 induction was not altered in the presence of estradiol in END E6/E7: A) ERα gene expression measured by western blot as outlined above. B) IL-8 induction. Experimental conditions were run in triplicate, and performed at least 3 times *p-value<0.05
We then examined a different endocervical cell line, SA2EN/A2EN. Because we were identifying the role of estradiol in endocervical cells, we utilized special growth medium and fetal bovine serum that was free of phenol red and charcoal stripped to remove estrogen like particles that could activate estrogen pathways and alter gene expression. While initial results showed that this cell line does express relevant receptors, when phenol red and other estrogen like components were depleted, the cells did not thrive and experiments could not be carried out in this system. For both these cell lines, we used a breast cancer cell line, MCF-7 cells, as a control for estrogen receptor expression and responsiveness. These data are summarized in Table 4.

Because of the limitations of the endocervical cell lines, we chose to move upward in the FRT to find tissue that is known to be infected by Neisseria and regulated by estradiol. Based on chronic complications caused by disseminated gonococcal infection we chose to explore the human endometrium, the tissue lining the uterus. When left untreated, *N. gonorrhoeae* has been shown to ascend to the upper FRT and infect the endometrium, causing complications that may lead to endometritis and pelvic inflammatory disease (PID). Because of this, we focused on the endometrium to understand the relationship between the bacterium and upper FRT complications. We explored two endometrial cell lines, Hec-1b and Ishikawa cells. Again we sought a cell line that would thrive under our estrogen free growth conditions; while hec-1b cells expressed our receptors they did not grow well in our system. Therefore, we pursued the Ishikawa cell line – a human endometrial adenocarcinoma cell line. This cell line was
isolated from a 39 – year old women with endometrial adenocarcinoma and was shown to express both estrogen and progesterone receptors (Nishida et al., 1985). These studies are detailed in the next section.
<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Source</th>
<th>ER Expression</th>
<th>ER responsive</th>
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<tbody>
<tr>
<td>END E6/E7</td>
<td>Immortalized endocervical cells</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A2EN</td>
<td>Immortalized endocervical cells</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Hec-1b</td>
<td>Immortalized endometrial cells</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Immortalized breast cancer cells (control)</td>
<td>+</td>
<td>+</td>
</tr>
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</table>
Estrogen Receptor Expression in Ishikawa Cells

Using semi-quantitative PCR and Western blot analysis, we sought to determine the gene and protein expression profile of Ishikawa cells when incubated with estradiol. Estradiol, a known regulatory hormone, has two distinct receptors present in the female reproductive tract: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). ERα is the dominant receptor found in the uterus, endometrium, and other areas of the FRT (Warner et al., 1999).

To characterize Ishikawa cells, we first had to confirm the presence of hormone receptors, specifically for estrogen and progesterone. Using semi-quantitative PCR, we first showed that Ishikawa cells express estrogen receptors at baseline. We observed a dose-dependent increase in ERα gene expression with increasing doses of 17-β estradiol as compared to media alone (Figure 5A). When evaluating the progesterone receptor, we also observed a dose-dependent increase in PR gene expression when incubated with increasing doses of 17-β estradiol as compared to media alone. Because progesterone has two different receptors that could be present in our cells, we chose a primer that covered both receptors for characterization (Figure 5B). It is important to note that estradiol concentrations vary greatly from woman to woman, day to day, and cycle to cycle; therefore we chose an array of concentrations to test expression.

Because ERα is the dominant receptor in this tissue, we chose to proceed solely with this receptor. To further prove the presence of ERα in our cell type, we chose to assess protein expression. We saw that ERα protein is present at baseline and is altered overtime when incubated with 75nM estradiol (Figure 6). Taken together, we can
conclude that Ishikawa cells express relevant hormonal receptors at both the level of gene and protein in response to stimulation with 17-β estradiol.
Figure 5. Ishikawa Cells Express Estrogen and Progesterone Receptors While Responding to Estradiol Treatment: Ishikawa cells were stimulated for 18 hr (overnight) with 17-β estradiol at increasing concentrations. RNA was extracted and cDNA was made for semi-quantitative PCR as described in the methods section. A) ERα B) PRAB expression patterns were identified. Conditions were run in duplicate, and experiments were performed at least 3 times. Graphs were normalized to GAPDH. *P-value <0.05.
Figure 6. Ishikawa Cells Express ERα Protein and is altered by Exogenous Estradiol Treatment Overtime: IK cells were stimulated with 17-β estradiol at 75nM along with media alone for 48hrs. Whole cell lysates were then harvested at 3, 7, 24 and 48hr for Western blot analysis as described in the materials and methods. Conditions were run in duplicate, and experiments were performed at least 3 times.
Expression of gonococcal receptors on Ishikawa cells

Because we wanted to examine the effect of estradiol on gonococcal binding and invasion in Ishikawa cells, we wanted to determine if these cells expressed known receptors for gonococcal outer membrane proteins. Studies show the Opa protein binds CEACAM on the surface of the epithelium, therefore, we investigated whether Ishikawa cells expressed CEACAM1 and if expression changed in our system. Ishikawa cells were stained using a CEACAM1/CD66a monoclonal antibody against CEACAM 1 and 6 at 6h and 24h. Compared to the isotype control no shift in expression occurred indicating Ishikawa cells do not express CEACAM receptors on its’ cell surface (Figure 7). We also incubated Ishikawa cells with 75 nM estradiol and FA1090b and CEACAM expression was not induced overtime. In contrast, Ishikawa cells have been reported to express CD46, the receptor for gonococcal pilin (Liu et al., 2014). Thus at least one of the known gonococcal receptors would be present on the surface of the Ishikawa cells.
Figure 7. Ishikawa Cells do not Express CEAMCAM1: Ishikawa cells were seeded and stained with CEACAM1 antibody at A) 6hr and B) 24hr. Compared to isotype control not significant shift in expression was observed. Histograms analyzed via FLOJO v10.0.6.
**Hormonally Responsive Gene Expression in Ishikawa Cells - Estrogen Responsive Genes**

Once we established that Ishikawa cells express ERα and respond to varying concentrations of estradiol, we wanted to compare our cell model with trends that occur *in vivo*. We chose specific genes that are known to be regulated by estradiol and assessed our cells for changes in expression. Ribosome s6 kinase (RSK) and protein kinase C type delta (PRKCD) are both serine/threonine kinases that are involved in growth and development. RSK is a member of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway known to be activated by epidermal growth factor (EGF) and is known to regulate the transcription of ERα within the FRT (Joel *et al.*, 1998). As an activating mitogen, RSK has been shown to be involved in processes such as cell growth and proliferation. In conditions where estradiol concentrations are at their highest, RSK is upregulated. To confirm that our cells are relevant and responsive, we used semi-quantitative PCR. Here we show that Ishikawa cells do express RSK and that its expression is significantly increased in the presence of estradiol (Figure 8A).

Next, we wanted to see if *N. gonorrhoeae* infection would alter RSK gene expression. The specialized gonococcal strain FA1090b locked in an OpaB confirmation to limit phase and antigenic variation as described above, was used to carry out our experiments. It is important to note that hormone incubation did not significantly alter bacterial growth of FA1090b (Figure 9, Figure 10). Ishikawa cells were incubated with or without estradiol for 24 h, then stimulated with FA1090b for an additional 24 h at a
multiplicity of infection (MOI) of 10:1 or 100:1 (10 or 100 times as many bacterium as cells). We also found that 75 nM estradiol intensifies RSK gene expression in Ishikawa cells in the presence of *N. gonorrhoeae* in a MOI-dependent manner (Figure 8A).

Next we wanted to explore genes that are down regulated in the presence of estradiol. We chose PRKCD, a member of the protein kinase B/AKT family that plays a role in apoptosis when activated by EGF (Ise et al., 2005). Ishikawa cells express PRKCD gene transcripts at baseline, and gene expression is down regulated in the presence of 75 nM estradiol (Figure 8B). This was an important finding, because during ovulation genes that promote apoptosis should be turned off in preparation for fertilization and implantation. Interestingly, we saw a significant MOI-dependent down regulation of PRKCD when Ishikawa cells were stimulated with *N. gonorrhoeae* for 24 h (Figure 8B). Identifying Ishikawa cell expression patterns for estrogen responsive genes further confirms that our *in vitro* model is adequate to study gonococcal infection and its effect on the immune response.
Figure 8. Estradiol Pretreatment Along With *N. gonorrhoeae* Stimulation Alters Expression of known estrogen responsive genes in Ishikawa Cells.

A) RSK and B) PRKCD gene expression profiles were identified via semi-quantitative PCR. Ishikawa cells were stimulated for 24 hrs with media alone or 75nM estradiol in conjunction with *Neisseria gonorrhoeae* as described in Materials and Methods. Conditions were run in duplicate, and experiments were performed at least 3 times. Graphs were normalized to GAPDH. *p*-value <0.05.
Figure 9. Estradiol Incubation does not Alter Bacterial Growth.

FA1090B was grown overnight as described in the Materials and Methods section and then inoculated in Graves Waver's broth in the presence of 75nM estradiol and grown to stationary phase. Optical Density was measured using a spectrophotometer.
Figure 10. Estradiol Incubation does not Alter CFU as Measured by Quantitative Culture. FA1090B was grown as outlined in Figure 9; bacteria was harvested and re-plated hourly onto chocolate agar for colony formation (CFU).
CHAPTER FOUR:

ESTRADIOL ALTERS THE INNATE IMMUNE RESPONSE TO GONOCOCCAL INFECTION

The Role of Estradiol in Bacterial Association and Invasion

It has been speculated that during times of heightened estradiol concentrations such as ovulation, epithelial barriers such as tight junctions may be compromised, allowing pathogens to invade the epithelium (Wira et al., 2005). The exact mechanism is still being unknown. We therefore wanted to explore if estradiol would affect association with and invasion of FA1090b to Ishikawa cells. Using flow cytometry, we determined the binding efficiency of FA1090b to Ishikawa cells over time. Ishikawa cells were stimulated for 6 and 24h with FA1090b expressing GFP. We showed that FA1090b binds sufficiently to Ishikawa cells after both 6 and 24h. Interestingly, there was no significant difference in bacterial association between cells treated with 75 nM estradiol and those not treated with estradiol (Figure 11). As a control we stimulated with WT FA1090b to account for auto-fluorescence. These data show that variation in invasion is not attributed to efficiency of bacterial association.

To further assess bacterial association, we targeted constitutively expressed outer membrane antigen H8 to detect extracellular bacteria via (Hitchcock et al., 1985). Ishikawa cells were incubated with 75 nM estradiol for 24 h on coverslips and then inoculated with FA1090b expressing GFP for 1 h, 2 h, and 3 h at MOI of 10 and 100. Association was then assessed via fluorescent microscopy as outlined in the Methods section. Bacteria that fluoresced green were considered internalized bacteria, while those
fluorescing red were considered external as they bound the Cy3 antibody targeting outer membrane protein H8. Here we show untreated Ishikawa cells stained with nuclear DAPI stain. The particles that fluoresce green designate bacteria that invaded the cell monolayer, while those particles that fluoresce red are non-invasive (extracellular) bacteria (Figure 12). While we did see binding and internalization of FA1090b to Ishikawa cells, we did not see a difference when comparing media and 75nM estradiol treatment. We also did not see an effect of estradiol on bacterial growth (Figure 9; Figure 10). These data further confirm that the difference in infection outcome is not solely based on how easily Neisseria gonorrhoeae invades the host. Instead, it is likely that estradiol is altering the host response to the pathogen.
**Figure 11: Bacterial Association is not Altered by Estradiol Pretreatment of Ishikawa Cells:** Ishikawa cells and FA1090b expressing GFP were grown and inoculated as outlined in the materials and methods section. Samples were then analyzed via flow cytometry. Conditions were ran in duplicate and experiments were performed at least 3 times. Graphs were normalized to mean fluorescence. *p*-value<0.05.
A) 75nM E2
Figure 12: Estradiol Does Not Alter Bacterial Association of Ishikawa Cells:

Ishikawa cells were inoculated with FA1090b expressing GFP, stained, and harvested for fluorescent microscopy as outlined in the Methods section. A) Representative fluorescent microscopy images. Grey cells indicate nuclear DAPI stain, green fluorescence indicates internal bacteria, red fluorescence indicates extracellular bacteria. (Representative graph 3hr MOI100 10X Magnification)

B) Quantitative graph of bacterial invasion. Conditions were run in triplicate. N=3

*p-value <0.05, **p-value <0.005
Because we do not see a difference in bacterial invasion when comparing estradiol to media treatment, we concluded that the bacterium must invade at the same rate regardless of the hormonal environment, causing the immune response to account for the difference. Although the literature shows disruption of the epithelial barrier in increased estrogen environments, our model did not utilize polarized cells. In our closed system, we stimulated our cells for 24-48 hrs. However, Holinka showed that changes in cell growth and expression were only observed after at least 10 days in estradiol treated culture (Holinka et al., 1986).

Next we chose to examine the host response to gonococcal infection in vitro. The innate immune system has evolved as the first line of defense against invading pathogens. At pathogen entry, the innate immune system is activated when pattern recognition receptors on the surface of immune cells recognize pathogen associated molecular patterns. When this system is activated, innate immune cells such as neutrophils and macrophages are exported to the site of infection for pathogen clearance. It has been speculated that during conditions where estradiol concentrations are increased, the immune system may be dampened, potentially creating a window for infection. Here we explored how estradiol interacts with the host during gonococcal infection.
TLR Expression and the Induction of IL-8 in Ishikawa Cells

Before examining the host response to gonococcal infection, we first wanted to confirm the expression of innate immune receptors in the Ishikawa cell line. Upon recognition, pathogen associated molecules, often referred to as pathogen-associated molecular patterns (PAMPs) activate pattern recognition receptors (PRRs), including the family of Toll like receptors (TLRs) that have been studied extensively (Fichorova et al., 2002). Expression of relevant TLRs would not only be important in identifying positive control treatments for the assays but also for predicting gonococcal ligands capable of activating inflammatory pathways. Young previously reported that Ishikawa cells weakly express TLR2 and TLR5, (Young et al., 2004). We conducted PCR analysis for TLRs 1-10 and came to a similar conclusion (Figure 13).

We next needed to identify a biological marker for assaying Ishikawa cell activation and chose Interleukin-8 (IL-8). IL-8 is a crucial chemokine known to recruit neutrophils to the site of infection, and it is one of the few cytokines synthesized by this cell line. Therefore, we examined IL-8 (IL-8) induction in response to defined TLR agonists in Ishikawa cells before examining IL-8 induction in response to gonococcal infection. Ishikawa cells were seeded and allowed to rest overnight and then were stimulated by key TLR and NOD agonists for 24 h. Ishikawa cell supernatants were then collected for IL-8 ELISA analysis. As predicted by the TLR expression profile, these agonists did not significantly induce IL-8 secretion (Figure 14). We did, however see an induction by TNF-α, which signals independent of TLRs (Figure 14).
Figure 13. TLR Gene Expression of Ishikawa Cells: Ishikawa cells were seeded and assessed for TLR gene expression as outlined in the materials and methods. Ishikawa cells weakly express only TLR 2 (169bp) and 5 (430bp). Specific bands are indicated by an arrow head.
Figure 14: Toll Like Receptors Do Not Induce IL-8 in Ishikawa Cells:

Ishikawa cells were incubated overnight with media alone and a range of TLR and NOD ligands. Cellular supernatants were harvested for IL-8 ELISA as previously described in the methods section. All conditions were done in triplicate and experiments were performed at least 3 times. BDL = below the level of detection for the assay.
**Neisseria gonorrhoeae upregulates IL-8 secretion by Ishikawa Cells**

While we did not see a significant induction of IL-8 in the presence of common TLR and NOD agonists, we did see an induction when Ishikawa cells were stimulated with *N. gonorrhoeae* (Figure 14). Based on previous data, we sought to explore if immune activation was based upon epithelial invasion. Ishikawa cells were seeded in the presence and absence of estradiol overnight and then stimulated with live FA1090b or freeze-thawed (dead) FA1090b at an MOI of 2, 5, and 10 for 24 h. Supernatants were collected the following morning for IL-8 ELISA. We show IL-8 is induced by both live and dead bacteria, suggesting that activation is not solely dependent upon the bacterial invading the epithelium (Figure 15).

We then wanted to see if estradiol altered IL-8 induction in our system. Ishikawa cells were seeded and incubated with or without 75 nM estradiol for 24 h; they were then inoculated with FA1090b at an MOI of 10 and 100. IL-8 induction was dampened in the presences of estradiol. Because we also see a modulation in IL-8 induction by TNF-α, we can postulate that estradiol may be acting upstream of IL-8, and the mechanism has yet to be established (Figure 16).
**Figure 15. IL-8 Induction is not Solely Dependent on Live Bacteria.** Ishikawa cells were seeded in A) media alone and B) 75 uM estradiol overnight. Cells were then stimulated with both live and freeze thawed dead bacteria for 24 h. Supernatants were harvested for IL-8 ELISA. Conditions were run in triplicate and performed at least 3 times. *p-value <0.05, **p-value<0.005.
Figure 16. Estradiol Dampens the Induction of IL-8 in Ishikawa Cells:
Ishikawa cells were incubated overnight in the presence or absence of 17-β estradiol then stimulated for 24 hrs with TNF-α (50ng/ml) and FA1090b as described above. Supernatants were harvested for IL-8 ELISA. Conditions were run in triplicate and performed at least 3 times. *p-value <0.05, **p-value<0.005.
CHAPTER FIVE:

DISCUSSION OF AIMS

Estradiol is the main hormone regulating the female reproductive tract and plays a major role in cell differentiation and promotion of female characteristics. This hormone is cyclically produced in the ovaries in response to signals received from the pituitary gland. Once estrogen binds estrogen receptor, it translocates to the nucleus, where it binds estrogen response elements present in DNA to alter gene transcription of estrogen responsive genes. Estrogen may play a role in acquisition of sexually transmitted infections. For example, it has been speculated that estrogen produced at high levels lead to disruption of the epithelial barrier, enabling pathogens such as *N. gonorrhoeae*, *Chlamydia trachomatis*, and Human Immunodeficiency Virus (HIV) to breach this barrier.

To explore the interaction between gonococcal infection and estradiol, we first had to identify a relevant cell model to carry out our experiments. Because humans are the only natural host for *N. gonorrhoeae*, we sought to develop an *in vitro* model using human cells that could be used to study innate immunity and bacterial invasion in the female reproductive tract. *N. gonorrhoeae* primarily infects the endocervix, so we began there. We found that while our endocervical cell model expressed specific estrogen receptors, it did not respond to exogenous estrogen treatment.

Next, we explored cell types that are affected after prolonged gonococcal infection. In the female host, some gonococcal strains are asymptomatic, leading to undiagnosed infection. This allows for amplified replication and antigenic variation that
increases immune evasion, which could lead to host reinfection and bacterial dissemination. Disseminated gonococcal infection (DGI) can cause arthritis, septicemia, and pelvic inflammatory disease (PID), that can lead to infertility (Sweet, 1986). We chose to study Ishikawa endometrial adenocarcinoma cells. We first confirmed hormonal receptor expression in our cells. We concluded that Ishikawa cells express both estrogen and progesterone receptor at the level of gene and protein. Next, we wanted to determine if gene expression in Ishikawa cells in culture reflect that which occurs in the body. To detect this, we measured the gene expression of known estrogen responsive genes. Based on our measurement of RSK and PRKCD gene expression in the presence of estrogen stimulation, we conclude that Ishikawa cells respond to stimulation with estradiol and are regulated by estrogen. To further characterize these cells, we sought to determine the effect of *N. gonorrhoeae* on gene expression, and we concluded that *N. gonorrhoeae*, in conjunction with estradiol, also alters gene expression in ways that favor bacterial infection.

Next, we questioned whether estradiol increased host-pathogen interaction in our system. To explore this, we looked at the binding efficiency of *N. gonorrhoeae* to Ishikawa cells in the presence and absence of estradiol using flow cytometry. While bacterial association increased over time in Ishikawa cells, there was no significant difference in binding when comparing cells pretreated with estradiol and those in media alone. This conclusion was confirmed by immunohistochemistry. This led us to conclude that the difference in bacterial infection efficiency may not be solely dependent upon invasion efficiency, but also upon the way in which the host responds to infection.
Because we did not see any change in invasion or association between *N. gonorrhoeae* strain FA1090b and Ishikawa cells, we postulated that differences in infection may be linked to the innate immune response. Our data suggests that the bacterium gains entry into the cellular host with similar efficiency when cells are pretreated with estradiol as compared to media alone. Therefore, we hypothesized that estradiol treatment somehow impeded the effects of the innate immune system upon gonococcal infection. To test this hypothesis, we explored induction of the primary chemokine (IL-8), due to its interaction with neutrophils. IL-8 has been shown to play an important role in uterine function, specifically during menses (Critchley *et al.*, 2001, Kayisli *et al.*, 2002). Other cytokines such as IL-6 were studied, but data suggests that IL-6 production is dampened by estradiol (Kurebayashi *et al.*, 1997).

It was noteworthy that we did not see induction of IL-8 by our TLR agonists, but did see induction by live and dead bacteria. This led us to conclude that in our Ishikawa model, FA1090b is not signaling through the typical TLR pathways. Recently, a group explored innate immune induction not activated by TLR agonists but by a TRAF-interacting protein with forkhead-associated domain (TIFA) dependent NF-κB pathway. This pathway is activated by the heptose biosynthetic pathway (HBP), a conserved intermediate of the LPS pathway present only in gram negative bacteria and not the human host. They isolated the byproduct, HldA, which is known to activate NF-κB. Using conditioned bacterial growth media to stimulate cells, they achieved innate immune activation without signaling through the TLR pathway. Based on this, they
concluded that instead of activating through classical MyD88, activation was spearheaded by NF-κB through this new TIFA dependent pathway (Gaudet et al.).

Taken together, these data suggest that as estradiol increases, chemokine recruitment decreases as measured by IL-8. Our model (Figure 17) proposes that as estradiol concentrations increase, *N. gonorrhoeae* binds CD46 on the surface of Ishikawa cells at a steady state. Under these conditions, tight junctions are loosened, allowing for bacterial invasion between the cellular monolayer. Once this occurs, the innate immune system is activated. However, the recruitment of pathogen clearing cells is not as robust as under conditions of moderate estradiol concentrations.

While we were able to characterize this cell type and efficiently induce an immune response following gonococcal infection, there were a few limitations to this study. One of the major drawbacks is the inability to verify these findings in primary human cells. The most appropriate cell type would be endocervical cells from a young person ages 14-29 with normal hormonal levels and physiology, compared to a high estradiol age-matched control. However, these cells were not available. Under optimal conditions, we would have utilized a cell type that expressed all of the major innate immune activator receptors including TLR/NLRs. In our experiments, our cell type does not express these receptors and could be signaling through an alternative pathway to elicit immune activation. Another limitation to our model is the lack of CEACAM receptors on the cell surface. Studies show that this is a major receptor that induces *N. gonorrhoeae* invasion by binding Opa on the bacterial surface. We speculate that *N. gonorrhoeae* is invading our cells by binding CD46 and traveling between cells under conditions of high
estradiol. Finally, our cells were not polarized, whereas apical and basal surface of polarized cells may secrete factors that can mimic the *in vivo* environment. We explored A2EN cells because they are able to be polarized, and this may provide more accurate results when testing invasion and innate immune activation.
Figure 17. New Gonococcal Infection Model: Speculative model of gonococcal invasion during times of increased estradiol and decreased induction of innate immune cells.
CHAPTER SIX:
SUMMARY AND NEXT STEPS

We hypothesized that estrogen alters the immune response to *N. gonorrhoeae*, causing a more intense and robust infection. However, when exploring invasion of and association with Ishikawa cells by *N. gonorrhoeae*, we did not see a significant difference in infection as we had anticipated. Numerous studies have chronicled the role of estradiol in cancer, but few have explored the connection between estradiol and gonococcal infection. Gonococcal infection is most prominent in African American women, who also have increased free and bound estradiol (Pinheiro *et al.*, 2005, Marsh *et al.*, 2011).

Studies have explored the link between estradiol and obesity in this same at risk population. They show that obese African American women have higher estradiol concentrations than their non-African American obese counterparts, independent of fat content. They also show that this same obese population had an increased risk of developing breast cancer (Stolzenberg-Solomon *et al.*, 2012). Raheem Paxton, et.al. highlighted this same trend when he concluded that premenopausal African American women with an increase in waist circumference/waist to hip ratio had higher concentrations of serum estradiol that correlated with an increased incidence of breast cancer (Paxton *et al.*, 2013).

There are other theories as to why African American women have higher estrogen levels. Many health and beauty products marketed to the African American community have been shown to contain estrogen or estrogen like particles referred to as endocrine disrupting chemicals. These endocrine disruptors are known to activate specific estrogen-
regulated pathways (James-Todd et al., 2011, Myers et al., 2015, James-Todd et al., 2016). These studies contend that hair products and skin moisturizers used by African American girls promote premature breast and pubic hair growth in children by triggering puberty (Yoon et al., 2014). These endocrine disruptors are introduced to the body through skin absorption and act on specific hormone receptors by mimicking estradiol (Darbre, 2006). Tiwary, et al., showed that with excess use of topical creams, children developed breast tissue and public hair. However, when the children reduced use of beauty products, breast tissue development and pubic hair regressed (Tiwary, 1998). These effects may also prove problematic later in life due to the link between chronically increased estradiol exposure and the risk of developing estrogen-dependent diseases such as breast and endometrial cancers.

The earlier onset of puberty in African American girls is well-documented in research on puberty, although the time of menarche is unchanged. A cross-sectional study exploring secondary sex characteristic development in prepubescent girls showed that there is a difference in age of onset of pubertal development between African American and Caucasian girls (Herman-Giddens et al., 1997). Young girls were followed until the age of 12. African American girls as young as 6 years old had a higher incidence of breast tissue and pubic hair development than Caucasian girls of the same age. This trend continued as the girls grew older. While African American girls presented with early secondary sexual characteristics, both groups experienced the onset of menses at approximately the same age. The study authors concluded that when evaluating
development of sexual characteristics, racial differences should be taken into consideration (Herman-Giddens et al., 1997).

With the constant use of products that can activate estrogen dominant pathways, dysregulation of estrogen regulated genes and proteins may result. This constant exposure can prime the body, keeping it in a pro-inflammatory state and enable the development of infection and disease. One of the drawbacks of our in vitro system is that we treat cells for 24 h, when in vivo affected tissues may be exposed to estrogen and estrogen like particles for years. If estrogen has the ability to disrupt barriers with increased concentrations, then it is likely that prolonged exposure will weaken cellular barriers, possibly accounting for differences in infection rates and disease propagation between ethnicities.

Overall, our data suggests that IL-8 induction by TNF-α is altered in the presence of estradiol. This is particularly interesting because there is conflicting data showing changes in TNF-α expression in the presence of estradiol based on age and stage of menstrual cycle (Bouman et al., 2001, Rogers & Eastell, 2001, Bouman et al., 2004). We want to explore this phenomenon further in the future, to study how estradiol changes IL-8 induction by TNF-α and to identify expression patterns of these cytokines and chemokines.

Because the female reproductive tract is not an isolated area, we know there are other microorganisms in the resident microbiome including commensal and pathogenic bacteria that may play a role in disease progression. Our future studies would include a 3D model co-culture system where we have stromal cells interacting with epithelial cells.
to better mimic the reproductive environment. This system can allow for introduction of other resident microorganisms and factors that may ultimately alter gonococcal infection. We would introduce commensal bacteria to our system to determine the role they play under our high estradiol conditions. These experiments can better recapitulate the ways by which N. gonorrhoeae interacts with epithelial cells in vivo.
**LIST OF ABBREVIATED JOURNAL TITLES**

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Subcell Biochem  
Trends Microbiol  

Sexually Transmitted Diseases  
Sexually Transmitted Infections  
Subcellular Biochemistry  
Trends in Microbiology
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AWARDS

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TEACHING EXPERIENCE

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TEACHING ASSISTANT – MATH FOR BIOTECH FALL 2015, 2016

- Led group discussions to reinforce mathematical equations
- Held weekly review sessions to solidify concepts
- Tutored students upon request
- Administered and proctored quizzes, midterm and final exams

TEACHING ASSISTANT-ADVANCED CELL CULTURE SUM 2014, 15, 16

- Prepared and led all cell culture labs, including growing and maintaining cultures and developing assays for students
- Tutored students upon request
- Prepared students for oral presentations held at the end of the semester

TEACHING ASSISTANT – GENERAL CELL CULTURE SPR 2013 – 2016
• Prepared and led all cell culture labs, including growing and maintaining cultures and developing assays for students
• Held weekly review session to reinforce assays ran in class
• Tutored students upon request
• Administered and proctored quizzes, lab practical’s, midterm and final exams

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• Assisted with teaching new genetics concepts during class and review sessions
• Served as a liaison between the students and instructor
• Facilitated topic discussions and peer learning

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• Identify an adequate cellular model to study gonococcal infection
• Characterize our cellular model and determine bacterial fitness under our conditions
• Determine bacterial association and invasion patterns in the presence of estradiol
• Determine IL-8 induction in our cellular model during bacterial infection

UNIVERSITY OF CHICAGO, CHICAGO, IL
Post-Baccalaureate Research Education Program (PREP) Scholar 2009 – 2010
• Used a novel technique to study asthma using human lung tissue
• Mimicked human asthma by inducing airway constriction in human lungs to simulate an asthma attack
• Imitated tidal breathing to overcome airway constriction in both human and animal models

PUBLICATIONS AND PAPERS
• “Estradiol Dampens the Innate Immune Response to Gonococcal Infection” Maston, Essence, D.; Ingalls, Robin. Abstract for Poster Presentation, New
England Science Symposium, Harvard Medical School, Boston, MA


- “Role of Estradiol in Regulating the Host Response to *Neisseria gonorrhoeae*” Maston, Essence, D; Ingalls, Robin. **Abstract for Poster Presentation**, Annual Sexually Transmitted Disease Conference, Boston, MA, 2014

- **“17-β ESTRADIOL, A KEY REPRODUCTIVE HORMONE, PLAYS AN ESSENTIAL ROLE IN PROPAGATING NEISSERIA GONORRHOEAE INFECTION WITHIN THE FEMALE HOST”** Maston, Essence D; Ingalls, Robin; Ayehunie, Seyoum. **Abstract for Poster Presentation**, EVANS Day Boston, MA. 2013