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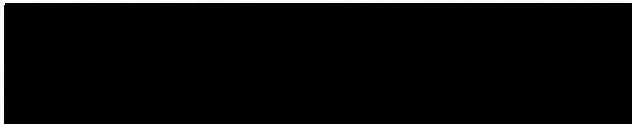
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Anita Marinelli

November 4, 2014

Examination Of The Role Of Secretory Leukocyte Protease Inhibitor (SLPI)
During Gonococcal Infection

by

Anita Marinelli

Dissertation submitted to the Faculty of the
Emerging Infectious Disease Graduate Program
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[Dissertation approval form inserted here]

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DEDICATION

To M. P., for unknowingly inspiring me to strive to become the best version of myself, every single day.

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Anita Marinelli

November 4, 2014

ABSTRACT

Title of Dissertation:

Examination of the role of Secretory Leukocyte Protease Inhibitor (SLPI) during gonococcal infection

Anita Marinelli, Doctor of Philosophy, 2014

Thesis directed by:

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Neisseria gonorrhoeae is a human-restricted pathogen that has evolved several mechanisms to evade host innate defenses. Secretory leukocyte protease inhibitor (SLPI) is an antimicrobial and anti-inflammatory peptide present in mucosal secretions. Here we show that wild-type *N. gonorrhoeae* strains are differentially susceptible to SLPI *in vitro*. The observed strain variability could not be explained by differences in the expression of the phase-variable opacity proteins as suggested by a recent study, or by differences in the expression of the MtrCDE active efflux pump, which expels other antimicrobial peptides. To further examine the interplay between *N. gonorrhoeae* and SLPI and to test the relevance of SLPI during gonococcal infection *in vivo*, we measured expression of SLPI in experimentally infected female mice via qRT-PCR and ELISA and studied the impact of SLPI on gonococcal colonization in SLPI-deficient mice. In BALB/c mice,

increased SLPI expression occurred on day 1 of infection, followed by down-regulation at time points that coincided with a neutrophil influx. Strikingly, infected C57BL/6 mice, which do not mount a pro-inflammatory response to *N. gonorrhoeae*, showed increased SLPI expression that was sustained over time. This differential expression pattern however, did not occur at the protein level, as the concentration of SLPI in vaginal lavages remained constant despite the presence of infection or inflammation. Furthermore, SLPI-deficient mice cleared infection similarly to wild-type mice, suggesting that, despite being susceptible to SLPI *in vitro*, *N. gonorrhoeae* has the ability to protect against SLPI bactericidal activity *in vivo*. The analysis of a pro-inflammatory cytokine panel in SLPI-deficient mice revealed no differences in the inflammatory response to gonococcal infection compared to wild-type mice. Taken together, the results of this dissertation work indicate that SLPI is bactericidal against the gonococcus *in vitro*, but its effects *in vivo* are impaired by the low concentrations present in the lower female reproductive tract and, or by unidentified gonococcal resistance mechanisms. In the approaching era of untreatable gonorrhea, topical administration of bactericidal amounts of SLPI or the use of agents that induce higher levels of expression could be envisioned as a valid therapeutic strategy against gonococcal infections.

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CHAPTER 1: INTRODUCTION

1.1 OVERVIEW OF GONORRHEA

Neisseria gonorrhoeae is a human-specific Gram-negative pathogen and the causative agent of the sexually transmitted disease gonorrhea. Neisser and Baumm identified the gonococcus as the definitive etiological agent of gonorrhea in the late 1800s, but reports on the disease manifestations date back to antiquity. Ancient Chinese documents as well as the Old Testament describe a sexually transmitted disease with signs and symptoms consistent with gonococcal urethritis. Hippocrates made the first scientific observations on gonococcal infections and Galen was the first to use the word ‘gonorrhea’ to describe the disease. For centuries after the emergence of syphilis there was a great deal of confusion as the two sexually transmitted infections were often found simultaneously in the same patient, leading to the belief that gonorrhea was merely one of the first symptoms of syphilis. It was only in the mid-1800s that Philippe Ricord systematically studied over 1,000 clinical cases and definitively proved that gonorrhea and syphilis were two distinct, unrelated diseases (131).

Despite the fact that therapy has made significant progress over the centuries, after moving away from rudimentary measures such as leeches and urethral ligation used in Hippocrates’ era to the advent of antibiotics, gonorrhea still remains a major public health problem and is threatening to become an untreatable disease due to the emergence of multi-drug resistant strains and the absence of an effective vaccine. Novel therapeutic agents that fail to induce the development of resistance and a more thorough

understanding of the host innate responses to gonococcal infection are therefore urgently needed.

Organism

Neisseria gonorrhoeae is a Gram-negative, non-motile, oxidase-positive diplococcus, identifiable in liquid culture as kidney-shaped cocci arranged in pairs. It belongs to the family *Neisseriaceae* that groups the genera *Neisseria*, *Moraxella*, *Acinetobacter*, and *Kingella*. The genus *Neisseria* comprises species isolated from humans and other animals; among those, 12 species and biovars were isolated from humans. *N. gonorrhoeae* is a human-specific pathogen and it colonizes the mucosal surfaces of the cervix, male and female urethra, rectum, pharynx and eye, causing symptomatic or asymptomatic infections (73). *N. meningitidis*, on the other hand, is the causative agent of meningococcal disease but can also be present as a commensal in the normal flora of the nasopharynx in healthy adults, and the prevalence of carriage varies geographically. Of the commensal *Neisseria* species, *N. lactamica* occurs more frequently in the nasopharynx of children than adults and has been reported to cause opportunistic infections (73).

Disease manifestations

Gonorrhea affects both men and women, but women are disproportionately burdened due to the frequent occurrence of complications that involve the upper reproductive tract and can lead to ectopic pregnancies, premature membrane rupture and infertility. *N. gonorrhoeae* can colonize the genital, rectal, oral and ocular mucosae and uncomplicated infections usually manifest as cervicitis and urethritis in women, and urethritis in men. While urethral infection in men most often results in acute symptoms

such as pain while urinating and the development of a purulent discharge, genital tract infection in women is often asymptomatic. Rectal and pharyngeal infections are usually asymptomatic and very common, and represent a major reservoir of infection because they are often undiagnosed (71; 161). Asymptomatic cervical infections are also likely to be left undiagnosed and therefore untreated, which increases the likelihood of ascending infection leading to upper reproductive tract complications. Ascending infections in men usually follow symptomatic infection and cause epididymitis, or more rarely prostatitis, which in some cases can result in infertility. Ascending infections in women result in pelvic inflammatory disease (PID), which manifests as any combination of endometritis, salpingitis, and pelvic peritonitis (156). PID has long-term consequences that have high impact on public health, and include chronic pelvic pain, ectopic pregnancies and, or infertility (30). PID occurs in 10-47% of women with gonorrhea and the risk of infertility increases exponentially with the number of PID episodes (30; 105; 157). A small percentage (0.5-3%) of untreated gonorrhea cases results in disseminated gonococcal infection (DGI). DGI is a result of gonococemia (gonococcal bacteremia) and manifests as hemorrhagic vesiculopustular skin rash and arthralgia or arthritis, rarely leading to gonococcal endocarditis (53). In addition to the reproductive tract, *N. gonorrhoeae* also colonizes the rectal, pharyngeal and ocular mucosae in both males and females (53). Gonorrhea during pregnancy can lead to premature membrane rupture, and infection can be transmitted perinatally from the mother to the neonate during passage through the birth canal. *N. gonorrhoeae* can infect the conjunctivae of the newborn causing ophthalmia neonatorum, which can result in blindness if not promptly treated (53).

Epidemiology

The World Health Organization estimates that 106 million cases of gonorrhea occur every year worldwide (163). In the United States, gonorrhea is the second most common reportable infectious disease, second only to chlamydia. The rates of gonorrhea in the U.S. underwent a dramatic decline from 1975 to 1997, and kept a slight negative slope until they reached a historical low in 2009. However, since 2009 rates have been rising steadily until 2012, when 107.5 cases per 100,000 people were reported, amounting to 334,826 total cases, which is a 4.1% increase compared to 2011 (108). However, given the high occurrence of asymptomatic infections, and the fact that sexually transmitted diseases have a tendency to be unreported, this figure is probably an underestimate and the number of real cases is closer to 700,000 (154). While sexual behavior can affect the rate at which gonorrhea is acquired, socio-economic status can also contribute to the prevalence of gonorrhea in particular communities. Gonorrhea rates are slightly higher in women than men; the highest rates are observed in the adolescent and young adult population (15-24 years old), in some ethnic groups and, or geographical locations (108). Gonorrhea represents a major public health problem due to the high occurrence of asymptomatic infections that are left untreated, the potential for long-term upper reproductive tract complications in women and the negative impact on neonatal health. Additionally, drug resistance is now a major concern due to the widespread circulation of multi-drug resistant strains. *N. gonorrhoeae* rose to the status of “superbug” in 2007, when the isolation of quinolone-resistant strains led the CDC to remove quinolones from the treatment protocol for gonococcal infections and associated syndromes such as PID (7). Cephalosporins are the only class of antibiotics effective against most *N. gonorrhoeae* strains. In 2012 however, due to the documented emergence of resistance to

cephalosporins, the CDC changed treatment guidelines from a single treatment with either oral (cefixime) or injectable (ceftriaxone) cephalosporins to dual therapy with injectable ceftriaxone and oral azithromycin or doxycycline (8; 162). The rapid acquisition of multi-drug resistance determinants observed over recent years, together with the lack of antimicrobial drug candidates in the pipeline, leaves us in desperate need for novel antimicrobial agents and, or an effective gonococcal vaccine to neutralize the threat of untreatable gonorrhea (4; 144).

1.2 PATHOGENESIS AND HOST IMMUNE RESPONSES

Gonococcal virulence factors

N. gonorrhoeae is a human-specific pathogen that is highly adapted to survive in the human genital tract. The first step in establishing colonization is ensuring firm attachment to the mucosal epithelium to resist the mechanical action of mucus shedding and urine flow. This first interaction between gonococci and epithelial cells is mediated by the gonococcal type IV pili, through binding to an as of yet unidentified eukaryotic receptor. After this first contact, lipooligosaccharide (LOS) and a class of outer membrane proteins called the opacity (Opa) proteins are responsible for establishing a tighter interaction with the host cell surface. Once colonization is established, it becomes crucial for the gonococcus to escape the surveillance of the host immune system and evade the innate and adaptive immune responses that are elicited during infection.

Some of the gonococcal membrane factors involved in establishing colonization are described below, followed by mechanisms by which the gonococcus evades host immune responses.

Pili

Pili are surface fibrils composed of thousands of identical small subunits of pilin that undergo both phase and antigenic variation at high frequency (90). Pilin is encoded by the *pilE* locus. The *N. gonorrhoeae* chromosome has at least one functional *pilE* allele and several partial copies called *pilS* (for “silent”). Antigenic variation occurs by a mechanism involving genetic recombination between *pilE* and *pilS* loci, giving rise to numerous distinct antigenic combinations (130). The main function of pili is to mediate initial attachment of the gonococcus to host tissues (103), but pili are multifunctional organelles that are also required for horizontal DNA exchange, interbacterial aggregation, twitching motility, and type IV secretion. Pili are also important in activating signaling pathways in host cells and may play a role in protecting the gonococcus from reactive oxygen species (ROS) and antimicrobial peptides (APs) (17; 135).

Porin

The most abundant protein on the gonococcal surface is porin (PorB or P.I), which trimerizes to form β -barrel pores that function as membrane channels that mediate the passive influx of nutrients and antimicrobial substances. Two classes of PorB exist, PorB1a and PorB1b, each of which is encoded by a different allele of the same locus. Gonococcal strains can be classified in two major serogroups according to the porin type, and into serovars within each serogroup. Importantly, PorB mediates serum resistance by binding the complement regulatory proteins factor H (fH) and C4 binding protein (C4BP), which in turn inhibit complement deposition on the gonococcal surface (111; 155). The porin type expressed by a gonococcal strain influences the degree of serum resistance, but both PorB1a and PorB1b can mediate serum resistance. Additionally,

PorB is found in close association with a membrane protein called reduction modifiable protein (Rmp or P.III), which is the target of non-complement-fixing-IgG antibodies that interfere with binding of complement-fixing IgM, therefore contributing to serum resistance (129).

Lipooligosaccharide (LOS)

The lipopolysaccharide (LPS) of the *Enterobacteriaceae* is composed of a lipid moiety core (lipid A) covalently attached to long chains of repeat oligosaccharide units (O-antigen). Lipid A connects to the O-antigen through 2-keto-3-deoxyoctulosonic acid (KDO) (107). The glycolipid component of the *N. gonorrhoeae* cell wall is significantly different from the LPS of other Gram-negative bacteria because it lacks the higher molecular weight long chain polysaccharides (O-antigen), and for this reason it is referred to as lipooligosaccharide (LOS). Gonococcal LOS is heterogeneous among different strains and subject to antigenic variation within the same strain and its individual, antigenically distinct components can be visualized on an SDS-PAGE gel (82). The mechanism of LOS antigenic variation depends on frame shift mutation of the genes involved in the biosynthetic pathway of the oligosaccharide components (107). The oligosaccharide side chains can mimic the host glycosphingolipids present on the membrane of the erythrocytes (83), resulting in immune evasion. Moreover, the presence of such lipids allows for deposition of host-derived sialic acid, which confers resistance to serum and AP-dependent killing (see section 1.4).

Opacity (Opa) proteins

The opacity (Opa) proteins belong to a family of 24-30 kDa membrane proteins that are responsible for inter-gonococcal adhesion, which determines the degree of photo-

opacity of a colony when observed under a stereomicroscope (136). The transition rate between transparent and opaque state *in vitro* has been determined to be 10^{-4} - 10^{-3} per cell per generation (85). Each gonococcal strain carries several unlinked *opa* loci on the chromosome. For example, 11 distinct *opa* loci have been identified in both strains FA1090 (13) and MS11 (3). The structure of each *opa* gene is highly conserved (Figure 1A). The signal peptide-coding region contains a pentameric repeated sequence element (CTCTT) that is subject to slipped-strand mispairing during DNA replication, resulting in the downstream sequence being in or out of frame during transcription and the Opa protein expression being “on” or “off” as a consequence (94; 134). The transcription of the *opa* genes is constitutive, but according to the number of repeats in the signal peptide region, the transcripts can result in either full-length or truncated Opa proteins (134). Since each *opa* gene is under control of its own constitutive promoter, and phase variation occurs randomly, each gonococcus can express one Opa protein, none, or any combination of Opa proteins on its surface at any given time (137). In the remainder of the structural sequence of the gene there is a high degree of identity among all *opa* genes. The bulk of divergence is localized on two short regions denoted hypervariable (HV) region 1 and 2. There is a limited repertoire of HV sequences shared among the *opa* genes of a particular strain. For example, strain MS11 has seven distinct HV₁ and seven HV₂, while strain FA1090 has six different HV₁ and five different HV₂, occurring in nine distinct combinations. There is some homology between HV sequences of different strains, but overall the HV sequences are not conserved across strains (3; 13) and antibodies raised against the HV regions of one Opa protein do not cross-react with Opa proteins from the same strain or other strains (2; 138). Some minor variability is also

found at the N-terminal of the mature Opa protein, in the so-called semi-variable (SV) region (13). Structural studies predict eight membrane-spanning β -strands, likely organized as a β -barrel in the outer membrane, and connected by four external loops: the semi-variable (SV), the hypervariable 1 (HV₁) and 2 (HV₂), and the fourth conserved (4L) loop (Figure 1B) (3).

Opa proteins cooperate with pili in the interaction with host cells; after the initial contact mediated by pili, Opa establish more intimate contact by binding eukaryotic cell surface receptors (74). Among the eukaryotic receptors identified, are heparan-sulfate proteoglycans (HSPG), which are ubiquitously expressed (22), and carcinoembryonic antigen cell adhesion molecules (CEACAM), expressed on a variety of cell types including epithelial, endothelial, and professional phagocytic cells. The type(s) of Opa present on the gonococcal surface dictate the specificity of binding, and Opa proteins can be classified based on their affinity for HSPG and, or particular CEACAMs (113).

Opa-positive gonococci are strongly selected for during infection of the human male urethra, without a bias for particular Opa proteins over others (61; 139). The isolation of Opa-positive gonococci is cyclical during infection of women and female mice, and it appears to be affected by factors under hormonal control that remain as of yet unidentified (12; 57; 60; 123). The high frequency of Opa phase variation and selection can be instrumental for evading the host immune responses, including innate factors such as serum proteases, and, or allowing colonization in niches where a particular type of receptor is expressed.

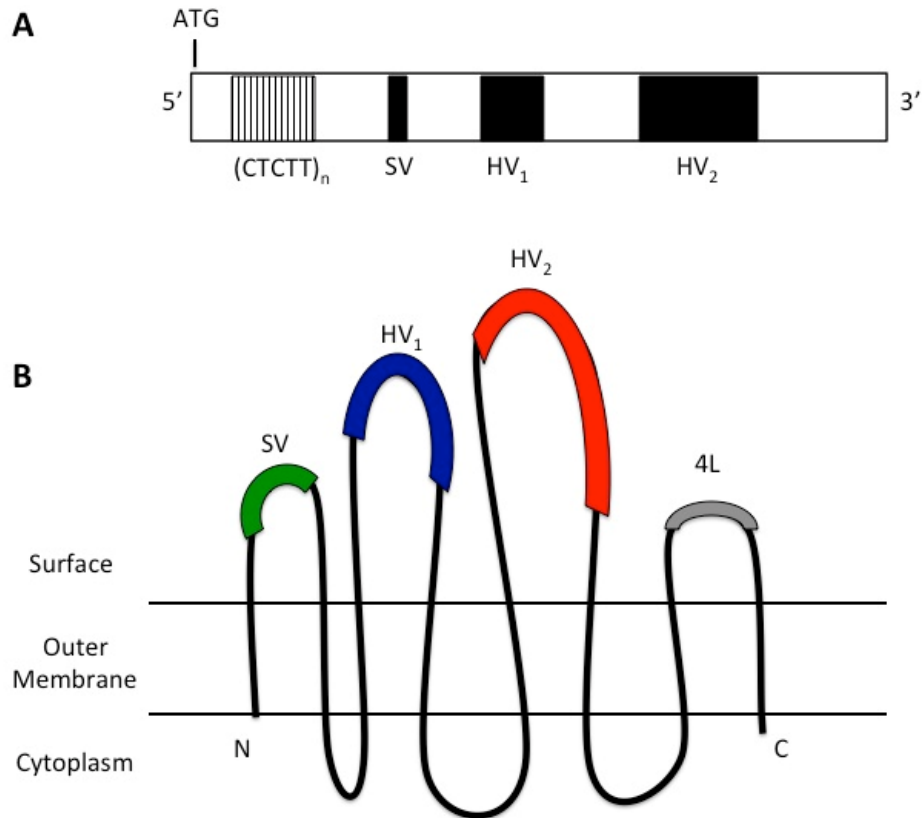


Figure 1. Structure of the *opa* gene and Opa protein.

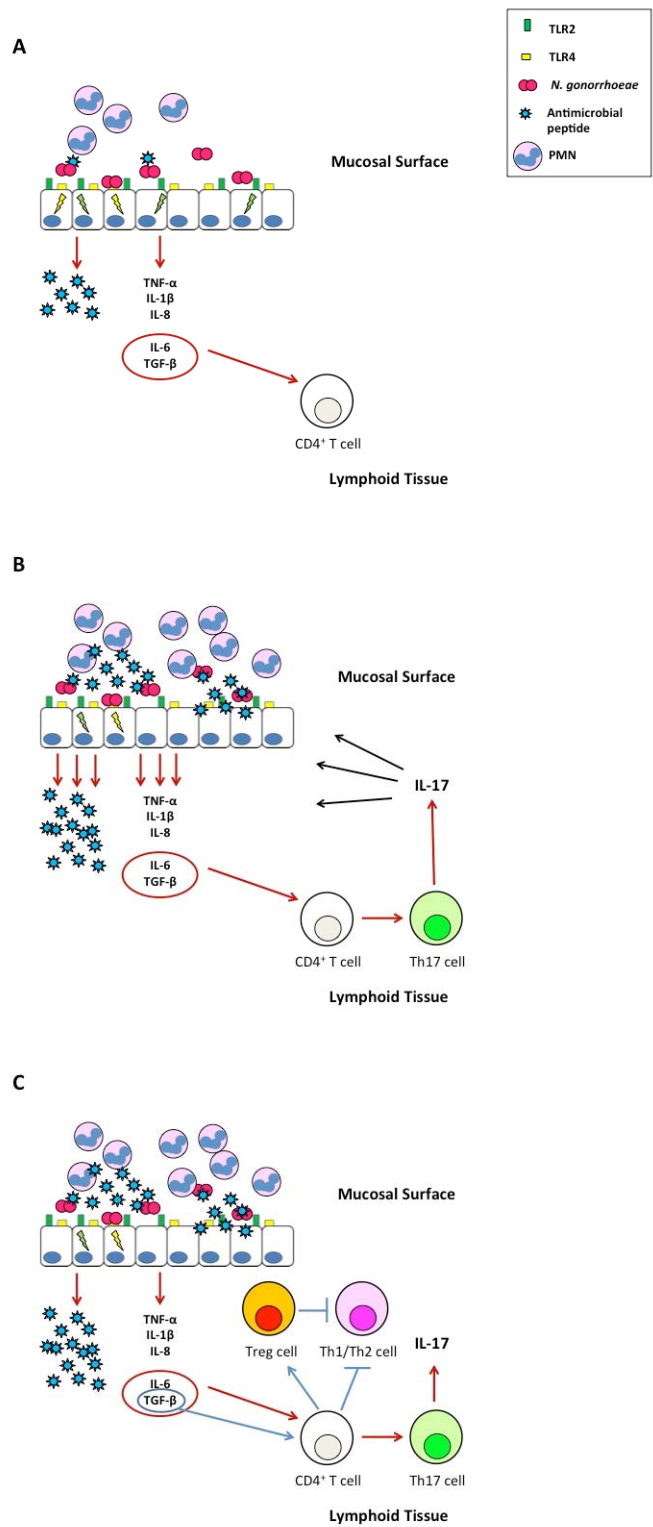
(A) Schematic representation of the structure of the *opa* gene, showing the pentameric repeat (CTCTT), the semivariable (SV) and hypervariable regions 1 (HV₁) and 2 (HV₂). (B) Linear sequence of the Opa protein showing topology of insertion into the outer membrane. Highlighted in color are the external loops corresponding to the variable regions in the sequence.

Host immune responses

It has been known for decades that repeat infections with *N. gonorrhoeae*, even with the same strain as the primary infection, are extremely common. Over the years investigators have focused on examining the antibody response to gonococcal infection and discovered that, while an antibody response occurs in most infected individuals, the serum titers are low and wane rapidly, therefore allowing for repeat infections to occur. It was not until the years 2000s, however, that the development of the mouse model granted the ability to definitively identify receptors and signaling pathways involved in the immune response to gonococcal infection and elucidate the mechanisms of immunosuppression triggered by the gonococcus. In general, symptomatic infections result in recognition of bacterial components by the mucosal innate immune system, which responds with a prominent inflammatory response, which manifests with a purulent exudate containing high numbers of polymorphonuclear leukocytes, or PMNs. While a powerful innate immune response is initiated upon gonococcal infection, the adaptive immune response is weak and inadequate at preventing repeat infections. Although an antibody response is elicited, the serum titers are low and the humoral response is usually short-lived, leading to a high incidence of repeat infections, often with the same serotype. A more detailed description of the innate and adaptive responses to *N. gonorrhoeae* is given below and is summarized in Figure 2.

Figure 2. Immune responses to *N. gonorrhoeae* infection.

Cartoon representation of the immune events that follow *N. gonorrhoeae* infection of mucosal sites. (A) Stimulation of TLR2 and 4 expressed on mucosal epithelial cells by gonococcal PAMPs leads to production of pro-inflammatory cytokines and APs and recruitment of PMNs. (B) IL-6 and TGF- β produced during the inflammatory response lead to maturation of CD4⁺ T cells into Th17 cells, which produce IL-17 that amplifies the inflammatory response by engaging IL-17R receptors on epithelial cells. (C) TGF- β also inhibits development of Th1/Th2 responses directly and indirectly, by inducing maturation of Treg cells.



Innate responses

The female reproductive tract can be divided in three sections that differ with regards to the degree of tolerance for commensal organisms and expression of innate immune receptors and effectors: the non-sterile vagina and ectocervix, the transiently sterile endocervix, and the sterile upper reproductive tract (uterus, Fallopian tubes and ovaries). The response to infection starts at the mucosal surface, where epithelial cells represent the first line of defense. Mucosal epithelial cells express innate immune receptors such as Toll-like receptors (TLRs), NOD and RIG, which recognize pathogen-associated molecular patterns (PAMPs) and in response trigger a signaling cascade that results in production of pro-inflammatory cytokines. TLRs1-6 are constitutively expressed throughout the female reproductive tract, with differences in expression levels of TLR2 and TLR4 in the upper versus lower tract. Specifically, TLR2 is present at higher levels in the vaginal and ectocervical epithelium, whereas TLR4 is more abundant in the endometrium and Fallopian tubes (34; 48; 104). TLR2 forms heterodimers with TLR1 and TLR6 and this directs the specificity of binding towards lipoproteins, peptidoglycan and lipoteichoic acid (LTA). TLR2 is also involved in the recognition of atypical LPS, such as *N. gonorrhoeae* LOS (95). TLR4, in association with the co-receptors CD14 and MD-2, binds to and recognizes the lipid A component of LPS and gonococcal LOS. Engagement of the TLR4 complex triggers an intracellular signaling cascade that results in I κ B degradation and nuclear translocation of NF- κ B, which ultimately results in production of pro-inflammatory cytokines (95). TLR4-dependent responses are protective in the female mouse model of gonococcal infection, as mice deficient in TLR4 signaling have higher bacterial burden compared to wild-type mice and TLR4-deficient PMNs have decreased activity against gonococci *ex vivo* (100).

Gonococcal products such as LOS, peptidoglycan (PGN) and outer membrane proteins stimulate signaling from TLR2 and TLR4 leading to secretion of the cytokines IL-1 β , IL-6, IL-8, TNF- α and TGF- β , as well as production of APs that will be described in detail in section 1.3. The pro-inflammatory cytokines IL-1 β , IL-6, IL-8, and TNF- α released in the genital tract during *N. gonorrhoeae* infection are potent chemotactic factors, which lead to the recruitment of high numbers of PMNs.

A prominent influx of PMNs is considered the hallmark of symptomatic gonorrhea, occurring in the majority of infected men and about 50% of infected women. Overall, the function of PMNs in gonococcal infections remains controversial. On the one hand, they successfully employ a variety of systems to kill gonococci. On the other hand however, they fail at clearing infection and cause significant tissue damage during the inflammatory process, possibly facilitating gonococcal migration to secondary sites of infection and, or transmission (20). PMNs are professional phagocytes that can kill bacteria via both oxidative and non-oxidative mechanisms. The cytoplasm of PMNs contains three sets of granules that are released either in the extracellular milieu or in the phagolysosome in a specific order: first, tertiary gelatinase granules are released to degrade the extracellular matrix and facilitate migration; next, secondary and primary granules containing APs and other toxic factors are released to kill the pathogens. PMNs can engulf the gonococcus via opsonic and non-opsonic mechanisms. Non-opsonic uptake is mediated by the interaction between gonococcal Opa proteins and CEACAM1, CEACAM3 and CEACAM6 on PMNs (113). Upon internalization and fusion of the phagosome with the lysosome, the granule content is released in the lumen and the NADPH oxidase complex assembles on the membrane producing toxic reactive oxygen

species (ROS) in a process known as “oxidative burst”. In spite of this impressive arsenal, however, gonococci cannot only survive but can also replicate within PMNs and viable gonococci are routinely recovered from gonorrheal exudates. *N. gonorrhoeae* has evolved several mechanisms to protect against PMNs (67). First, gonococcal porin and sialylated LOS bind the complement regulatory factors C4BP and factor H (fH), inhibiting C3 deposition and therefore avoiding opsonophagocytosis. Additionally, *N. gonorrhoeae* produces several enzymes that detoxify ROS, such as superoxide dismutase (SOD), catalase (KatA), and a variety of peroxidases. The manganese transporter MntABC provides a non-enzymatic system against oxidative damage by importing Mn^{2+} ions to scavenge ROS (164). Furthermore, purified porin and Opa-negative gonococci have been shown to inhibit the PMN oxidative burst *in vitro*, although the exact mechanism and *in vivo* relevance remain to be determined (19). However, studies have shown that the mechanisms of resistance to oxidative killing listed above have limited importance in affording survival against PMNs *in vitro* and *in vivo* (18), thus non-oxidative killing via the release of granules and APs likely represents the main challenge for the gonococcus. To protect against non-oxidative defenses, especially APs, the gonococcus has evolved mechanisms of resistance that will be described in detail in section 1.4.

Adaptive responses

Despite the prominent inflammatory response induced during infection, the adaptive immune responses to *N. gonorrhoeae* are weak, short-lived and ineffective at clearing infection. Anti-gonococcal antibodies are detected in gonorrhea patients, but the levels are remarkably low. Notably, a history of previous infection does not alter the

antibody levels in response to secondary infection, and as a direct consequence, repeated infection with *N. gonorrhoeae*, even with the same strain, is common (44). These findings have been effectively recapitulated in the mouse model, as infection in mice does not result in a prominent antibody response, and primary infection does not affect antibody titers or susceptibility to secondary infections (128). It has become clear over the years that *N. gonorrhoeae* inhibits the development of effective humoral responses to infection. Studies performed on the mouse model have demonstrated that rather than inducing a proper adaptive immune response with development of Th1 and Th2 responses, *N. gonorrhoeae* induces a Th17 response. The Th17 axis of the immune response functions as a bridge between the innate and adaptive immune systems. CD4⁺ Th17 cells differentiate from naïve CD4⁺ lymphocytes under the influence of the cytokines TGF- β , and either IL-6 or IL-1. The female reproductive tract is naturally rich in the anti-inflammatory cytokine TGF- β to maintain tolerance against the allogeneic sperm and fetus, and it has been shown that *N. gonorrhoeae* induces IL-6 and IL-1 β production from mucosal epithelial cells. Mature Th17 cells produce signature cytokines such as IL-17, IL-22 and IL-23. The IL-17 receptor IL-17RA is expressed on mucosal epithelial cells and IL-17-dependent signaling events lead to production of pro-inflammatory cytokines and APs, and recruitment of PMNs. The importance of Th17 responses during gonococcal infection has been demonstrated in the mouse model (35) and corroborated by the clinical finding of elevated serum levels for IL-17 and IL-23 in humans with gonorrhea (38). While inducing Th17 responses that include production of APs and influx of PMNs, both of which *N. gonorrhoeae* can survive, the cytokine milieu produced during gonococcal infection also contributes to the inhibition of Th1 and Th2

responses, which would be important for the development of immunological memory and could result in protective immunity. Inhibition of Th1/Th2 adaptive responses occurs both indirectly, via induction of regulatory T cells (Treg) (55), as well as directly via inhibition of maturation of Th1 and Th2 cells mediated by TGF- β . The direct interaction of the gonococcus with CD4⁺ T cells as well as B cells has been shown to inhibit their proliferation, with a mechanism dependent on the expression of CEACAM1-specific Opa proteins (6; 102). Additionally, it has been demonstrated that the uptake of gonococci by memory B cells and subsequent stimulation of the intracellular receptor TLR9 leads to increased polyclonal B cell proliferation and secretion of broadly reactive polyclonal IgM antibodies, that are not specific for gonococcal antigens (125). In addition to causing immunosuppression, *N. gonorrhoeae* is also extremely well adapted at evading specific antibodies via several mechanisms, which include phase and antigenic variation of surface antigens, molecular mimicry of host glycolipids, production of an IgA1-specific protease, and intracellular survival.

1.3 ANTIMICROBIAL PEPTIDES IN THE FEMALE REPRODUCTIVE TRACT

APs are small (≤ 100 kDa) peptides with broad-spectrum activity against a wide variety of pathogenic bacteria, fungi and viruses. The mechanisms of antimicrobial activity range from enzymatic or mechanical disruption of essential structures, to sequestration of essential nutrients (or “nutritional immunity”). Many APs also have important immunomodulatory functions.

Alexander Fleming in 1922 first described the antimicrobial properties of mucosal tissues and identified lysozyme in human nasal secretions. Numerous other human mucosal APs have been identified in body secretions and epithelia since, and in this

section I will focus exclusively on the APs present in the cervicovaginal mucus and their contribution to the innate defenses of the female lower reproductive tract.

Calprotectin

Calprotectin is a heterodimer formed by two small peptides called S100A8 (8 kDa) and S100A9 (14 kDa), which is abundantly present in the cytoplasm of neutrophils and can also be produced by epithelial cells and keratinocytes in response to inflammatory stimuli. Calprotectin is present in vaginal fluids at a concentration of micrograms per milliliter and can be found at even higher concentrations in inflammatory fluids (11). Calprotectin has bactericidal activity against *Candida* spp. and *Cryptococcus neoformans* among the fungi (132) and the bacteria *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Klebsiella* spp., *Salmonella typhimurium*, and *Acinetobacter baumannii* (15; 52; 79; 132). The mode of action of calprotectin consists of chelation of manganese and, or zinc, which represent essential nutrients for many bacterial pathogens and are usually present in limited amounts in inflamed tissues (15). Interestingly, calprotectin also has pro-inflammatory activity and acts as a potent chemoattractant for neutrophils (165).

Defensins

Defensins are small (3-4 kDa), cysteine-rich, cationic peptides characterized by a structure rich in β -strands, stabilized by intra-molecular disulfide bonds. There are three classes of human defensins, α , β , and θ defensins, which are classified according to the number and position of disulfide bridges. Expression of θ defensins, however, has not been detected in human tissues, probably due to a nonsense mutation identified in the signal sequence that results in a premature stop codon (58). Leukocytes and epithelial

cells are the main sources of defensins at different body sites. Among the six human α -defensins identified to date, α -defensin-1, -2, and -3 are primarily found within neutrophil granules and are therefore designated human neutrophil peptides, referred to as HNP-1, -2, and -3, respectively, but they are also present in the placenta and cervical mucus plug during pregnancy; α -defensin-5 and -6 (HD-5 and -6) can be induced upon infection in epithelial cells, HD-6 however is not found in the female reproductive tract; α -defensin-4 (HNP-4) is present in neutrophils but its exact tissue distribution has not been determined yet (72). Increased concentrations of HD-5 have been detected in secretions from men with gonococcal and chlamydial urethritis (106).

The human β -defensins (HBD) are predominantly expressed by epithelial cells. Epithelial cells produce six types of HBD (HBD-1 through -6), and expression can be constitutive or induced by microbial products and pro-inflammatory cytokines (72). Expression of HBD-1 is detected at the mRNA level in epithelial tissue extracts from various sites of the female reproductive tract, including the ectocervix, endocervix, vagina, fallopian tubes and endometrium, but not the uterine stroma and myometrium (146). While the concentration of HBD-1 in healthy vaginal fluid might be too low to result in antimicrobial activity, production might be induced by infection or damage, and high concentrations have been demonstrated in the cervical mucus plug that forms during pregnancy (45; 146).

The antimicrobial activity of defensins has been demonstrated *in vitro* against a variety of bacteria, viruses, and fungi. It is worth noting, however, that the concentrations required for antimicrobial activity are higher than the physiological range, and the actual contribution of defensins to infection clearance might be less than indicated by *in vitro*

studies (118). Perhaps more interesting than their antimicrobial activity is the immunomodulatory function of defensins. It has been shown that defensins can bind to innate immune receptors such as TLR2 and TLR4, resulting in either increased or decreased signaling depending on the peptide concentration, the pathogen and the cytokine environment (118).

Lysozyme and lactoferrin

Lysozyme is a 14.6 kDa protein with both enzymatic and non-enzymatic antimicrobial activity. The enzymatic activity results in cleavage of the bond between N-acetyl muramic acid and N-acetyl-D-glucosamine in peptidoglycan, whereas the non-enzymatic activity depends on its highly cationic charge (11). Lysozyme is present at low concentrations in vaginal fluids (145), but is abundant in the cervical mucus plug during pregnancy (45). Lysozyme alone is inactive against many pathogens because in some cases it cannot reach the peptidoglycan layer and some pathogens can modify the structure of peptidoglycan resulting in resistance to lysozyme. For these reasons, the activity of lysozyme is enhanced by synergy with other mucosal APs, particularly lactoferrin (29; 124). Lactoferrin is a homolog of transferrin, and as such is able to bind iron. The bactericidal activity of lactoferrin is two-fold: via iron sequestration ('nutritional immunity'), and via membrane disruption. The iron-independent activity of lactoferrin supports the non-enzymatic activity of lysozyme against Gram-negative pathogens (11; 29).

Cathelicidin (LL-37)

The human cathelicidin hCAP-18 was identified in 1995 and subsequently, antimicrobial activity was attributed to a highly cationic, 37 amino acid-long C-terminal

peptide within its sequence that was named LL-37. Upon secretion in the extracellular milieu, hCAP-18 is processed by tissue proteases to release the active peptide LL-37. Cathelicidin is constitutively expressed or induced in most epithelia, and it is stored as precursor hCAP-18 in the intracellular granules of leukocytes. Neutrophils in particular contain high amounts of cathelicidin in tertiary granules, and secretion is stimulated by infection or damage, resulting in transiently high tissue concentrations (147). The antimicrobial activity of LL-37 and its mouse homolog CRAMP (Cathelicidin-Related Antimicrobial Peptide) has been demonstrated against a broad range of viruses, fungi, and bacteria, including *N. gonorrhoeae* (119; 147). There are several models for the mechanism of action of LL-37 against bacterial pathogens, and they all describe some degree of disruption of surface structures. Studies in Gram-negative bacteria have shown that the mode of action of LL-37 likely involves an initial interaction with the outer membrane, followed by penetration in the periplasmic space, disruption of the inner membrane, and potentially damage to intracellular structures, particularly nucleic acids (147). It also appears that LL-37 interferes with cell division by localizing to the division septum and preventing separation of the daughter cells (127).

Like many other APs, cathelicidin is also capable of modulating the immune response by acting as a chemotactic stimulus and activating innate immune cells to release pro-inflammatory cytokines (147).

Cathelicidin is present as both LL-37 and hCAP-18 in vaginal fluid. Following intercourse, hCAP-18 is processed into LL-37 by a protease present in seminal plasma, which becomes active due to the acidic pH of vaginal fluid, as a mechanism to prevent

infection (11). The concentration of LL-37 in vaginal fluid in physiological conditions is in the micromolar range (145).

Secretory Leukocyte Protease Inhibitor (SLPI)

Secretory Leukocyte Protease Inhibitor (SLPI) is the main focus of this dissertation and as such its structure, functions, and expression dynamics will be described in detail in section 1.5.

1.4 GONOCOCCAL MECHANISMS OF RESISTANCE TO ANTIMICROBIAL PEPTIDES

APs have broad spectrum of action because they target essential structures shared by a variety of different pathogens, and for this reason, resistance is not developed as rapidly as it is for conventional antibiotic drugs. Nevertheless, pathogens have co-evolved with their hosts and acquired systems that confer protection against APs (Figure 3).

For most APs the first step in targeting pathogens involves binding to the surface. The most common mechanism of resistance to APs, therefore, is modification of outer surface structures to inhibit binding by APs. The negatively charged LPS makes Gram-negative bacteria particularly susceptible to cationic APs, hence most bacteria (including *N. gonorrhoeae*, see below) have evolved enzymes that introduce LPS modifications that result in a decrease of the overall negative charge and therefore decreased AP binding. Capsule production and biofilm formation can also result in decreased susceptibility to APs (42). Some pathogens, including *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus pyogenes*, and the protozoan *Trichomonas vaginalis*, actively prevent binding of APs by releasing proteases or trapping factors that sequester and neutralize the peptides (27; 42). One of the major

mechanisms by which pathogens protect against APs and antibiotics is through the expression of active efflux pumps that actively expel antimicrobial substances from the bacterial cell. *N. gonorrhoeae* utilizes the MtrCDE active efflux system to protect against structurally diverse APs in addition to macrolide antibiotics, nonionic detergents, bile salts, and progesterone.

The importance of LOS modifications and the MtrCDE active efflux pump in the context of gonococcal resistance to APs will be described in more detail below.

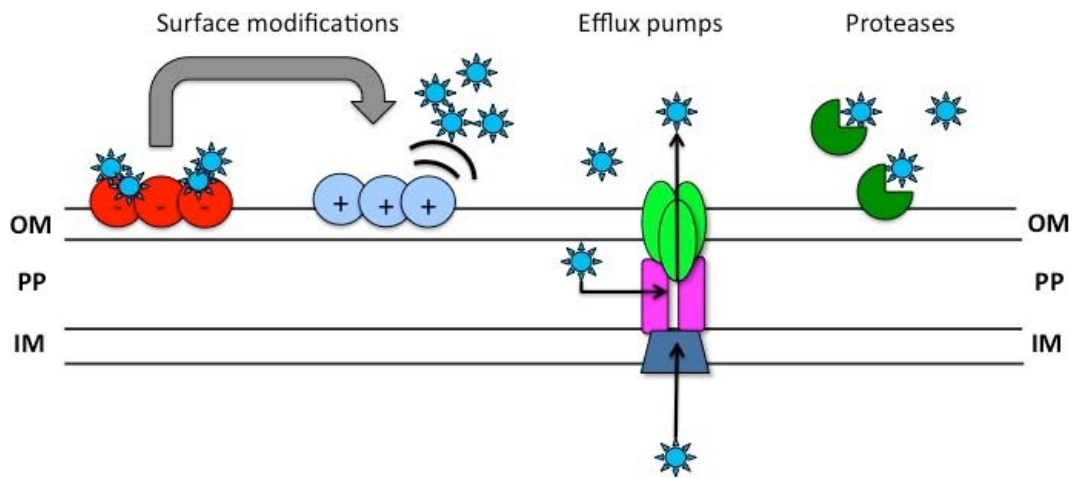


Figure 3. Bacterial mechanisms of resistance to antimicrobial peptides. Cartoon depiction of the three main modes of resistance to APs evolved by pathogenic bacteria: modification of cell wall components to reduce binding, expression of antimicrobial efflux pumps, and secretion of proteases or other inhibitory factors that prevent APs from reaching the bacterial surface (OM: outer membrane; PP: periplasm; IM: inner membrane).

LOS modifications

In addition to exhibiting heterogeneity due to variable length of the glycan chains, gonococcal LOS can also be modified by addition of sialic acid (84) or phosphoethanolamine (PEA) moieties (76; 101). Gonococcal sialyltransferase, which is localized to the outer membrane and encoded by the *lstA* locus, catalyzes the addition of sialic acid from the donor molecule CMP-NANA onto the terminal lacto-N-neotetraose residue of LOS. Gonococci cannot produce 5'-cytidine monophospho-N-acetylneuraminic acid (CMP-NANA) and therefore have to scavenge it from the host. Our laboratory has shown that *lstA* mutants exhibit reduced binding and susceptibility to the APs LL-37, its mouse homolog CRAMP, and bactericidal permeability increasing protein (BPI) *in vitro*. Furthermore, these mutants are attenuated compared to wild-type gonococci in competitive infections of wild-type female BALB/c mice but not CRAMP-deficient mice, indicating that LOS sialylation is a fundamental mechanism by which gonococci survive CRAMP killing *in vivo* (Wu and Jerse, unpublished data).

The enzyme PEA transferase, encoded by the *lptA* gene, is responsible for the addition of PEA to the position 4' of gonococcal lipid A. This modification makes the gonococcus highly resistant to complement and APs such as polymyxin B, LL-37 and CRAMP, as shown by the increased susceptibility of *lptA* mutants to the bactericidal activity of normal human serum and AP killing (76). Importantly, *lptA* mutants are also attenuated during experimental infection of female mice and human male volunteers (50; 101), which underscores the importance of this LOS modification for gonococcal adaptation to and survival in the genital tract.

MtrCDE active efflux pump

The Mtr (multiple transferable resistance) system is a tripartite efflux pump that was first identified during the characterization of clinical isolates with increased resistance to hydrophobic compounds such as certain antibiotics, nonionic detergents, bile salts and progesterone (120). Importantly, the MtrCDE active efflux pump is effective at protecting *N. gonorrhoeae* against APs such as protegrin-1 (PG-1) and LL-37 (119). This efflux system belongs to the RND (resistance/nodulation/division) family, which also contains the homologous systems AcrA-AcrB-TolC and MexA-MexB-OprM of *Escherichia coli* and *Pseudomonas aeruginosa*, respectively (120). RND pumps are active efflux systems, in that they utilize the proton motive force to actively pump out toxic substances. The MtrCDE system in *N. gonorrhoeae* has three structural components: the inner membrane protein MtrD, the periplasmic adapter MtrC, and the outer membrane channel MtrE (Figure 4A). Each of these components is essential to the function of the pump. The *mtr* locus consists of the *mtrCDE* operon and the *mtrR* gene, which is divergently transcribed upstream of *mtrCDE* and encodes MtrR, a repressor of *mtrCDE* transcription. Expression of *mtrCDE* is also regulated by the transcriptional activator MtrA, the gene for which, *mtrA*, is located elsewhere on the chromosome (120). MtrA-mediated activation of *mtrCDE* is induced by the presence of sub-lethal concentrations of hydrophobic agents, but the details of the activation process are unclear. MtrR is a 24.5-kDa TetR-like repressor protein that forms homo-multimers and has an N-terminal helix-turn-helix (HTH) domain that mediates binding to DNA. The binding site of MtrR has been mapped to the *mtrCDE* promoter region; however, a microarray study revealed that, in addition to *mtrCDE*, MtrR regulates the expression of over 60 gonococcal genes (36).

These transcriptional modulators of *mtrCDE* expression have a profound impact on gonococcal survival in the mouse model. Mutants lacking the MtrE outer membrane channel, and therefore lacking a functional efflux system, are highly attenuated during infection of female mice (63), as are mutants lacking the MtrA activator (152). In contrast, *mtrR* mutants, which overexpress the pump, have a significant fitness advantage over wild-type gonococci in a competitive infection system (152). Several mutations have been described that affect both the structural gene *mtrR* and the promoter (153), and mutations that result in loss of repression by MtrR are relatively common among resistant outbreak isolates. The widely used laboratory strain MS11, originally isolated in the 1960s from a case of uncomplicated cervicitis, has two mutations that abrogate MtrR function, including a structural mutation in the sequence coding for the HTH domain and a single base change in the *mtrCDE* promoter that creates a stronger, MtrR-independent promoter (98; 153) (Figure 4B). The mouse infection data and the presence of *mtr* mutations in strain MS11, which was isolated prior to the introduction of macrolide antibiotics as gonorrhea treatment, indicate that the MtrCDE active efflux pump plays a fundamental role in protecting gonococci against toxic host factors encountered in the genital tract, such as APs.

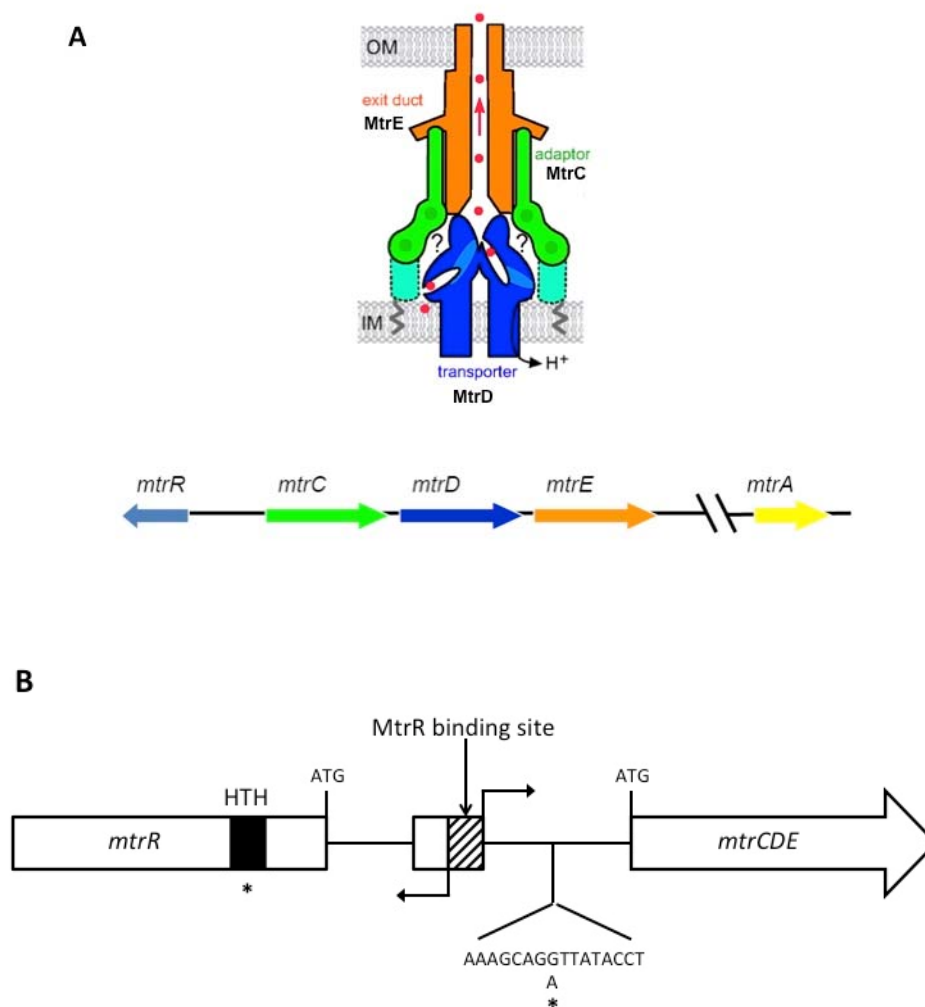


Figure 4. MtrCDE active efflux pump and *mtr* locus mutations.

(A) Diagram of the tripartite MtrCDE active efflux pump adapted from (141). The three components are indicated in different colors. The same colors are maintained in the diagram of the *mtrCDE* operon showing the divergently transcribed *mtrR* gene and the unlinked *mtrA* activator gene. (B) Diagram of the intergenic region between the *mtrR* and the *mtrCDE* loci displaying the relative promoters and MtrR binding site. The asterisks correspond to the position of the mutations occurring in the HTH region of the *mtrR* structural gene and in the intergenic region in strain MS11, that result in overexpression of the MtrCDE system (adapted from (153)).

1.5 SECRETORY LEUKOCYTE PROTEASE INHIBITOR (SLPI)

Secretory Leukocyte Protease Inhibitor (SLPI) is an 11.7 kDa, 107 amino acid residue, single-chain cationic peptide that belongs to the family of Whey Acidic Proteins (WAP). Members of the WAP family are identified by the presence of the WAP domain that contains a four-disulfide core. SLPI has two highly homologous WAP domains, an N-terminal and a C-terminal one. The crystal structure of SLPI has been resolved and shows the two independently folded and spirally arranged WAP domains each containing an internal β -strand region, in a structure described as “boomerang-like” (41). The cysteine content is fairly high for a peptide this size: a total of sixteen cysteine residues are engaged to form 8 intra-molecular disulfide bonds, four in each WAP domain. The abundance of lysine and arginine residues confers a high cationic charge to the protein (net charge: +11), which is a common feature for many APs. Although its structure is exceptionally stable (97), SLPI can be degraded by host and bacterial proteases such as cathepsin B, L, and S (114), proteinase-3 (112), *Trichomonas vaginalis* secreted cysteine proteases (27), and a *Pseudomonas aeruginosa*-derived metallo-elastase (114).

SLPI can be found at many mucosal surfaces and in body fluids, and it is present at high concentrations in saliva, respiratory secretions and cervicovaginal fluid. Its expression is mostly constitutive in mucosal epithelial cells and keratinocytes, while it is induced by inflammatory stimuli in immune cells such as mast cells, neutrophils, and macrophages.

Functions of SLPI

SLPI was first identified for its anti-protease activity, which is specifically targeted against neutrophil proteases and particularly potent against neutrophil elastase

(NE). In fact, SLPI was classified as “alarmin”, a term that defines a class of proteins which are produced by the host in response to primary inflammatory stimuli, including bacterial products and pro-inflammatory cytokines such as interleukin-1 beta (IL-1 β) and TNF- α . The role of the alarmins is to counteract the negative effect of inflammation on tissues in terms of damage caused by phagocytes, and to promote resolution of inflammation and return to homeostasis. While SLPI does an excellent job as an alarmin due to its high binding affinity to NE, it would be reductive to limit its description to the protease inhibitory activity. Since it was first identified, SLPI has in fact also been characterized as a mediator of innate immunity with anti-inflammatory activity, and as an antimicrobial peptide that is effective against a variety of pathogens.

SLPI as protease inhibitor

SLPI was first identified in human respiratory secretions and characterized as a potent NE inhibitor. Due to its high local concentration and binding affinity, SLPI is considered the main physiological elastase inhibitor in human airway secretions. It was lately shown that SLPI also binds to and inhibits cathepsin G, trypsin and chymotrypsin. The binding site within SLPI for all the target proteases is the C-terminal WAP domain and the active residue is a leucine in position 72. The high affinity for elastase results in a 1:1 binding stoichiometry, whereas binding to the other proteases occurs with a 2:1 stoichiometry (5). Innate immune effectors such as neutrophils deployed during inflammatory responses have considerable potential to cause harm and damage the host. Alarm antiproteases like SLPI are crucial to limit the damage caused by proteases and ensure prompt resolution of inflammation and return to homeostasis.

SLPI as immune mediator

Several studies have suggested that, in addition to its anti-protease activity, SLPI may play a direct role in the regulation of innate immunity and inflammation. SLPI can reduce tissue damage in models of lung fibrosis and ischemic brain injury, and this activity cannot be attributed to its anti-elastase role (92; 151). Furthermore, it has been shown that SLPI secretion is reduced in patients with *Helicobacter pylori*-induced gastritis (158) and serum levels of SLPI increase in response to sepsis (40), suggesting a potential anti-inflammatory role. In response to these observations, the focus shifted towards identifying possible mechanisms by which SLPI exerts its anti-inflammatory activity. For example, SLPI has anti-chemotactic activity, and inhibits migration of eosinophils and neutrophils in models of eye and joint inflammation. In the same models, SLPI also seems to interfere directly with the activity of eosinophils and neutrophils by inhibiting degranulation and oxidative burst, respectively (93; 117). SLPI has the capacity to limit inflammation indirectly by inducing production of the anti-inflammatory cytokines IL-10 and TGF- β in macrophages (115). Interestingly, the expression of SLPI itself is negatively regulated by TGF- β as shown *in vitro* in human bronchial epithelial cells (59), uncovering the existence of a potential negative feedback regulatory loop that could be instrumental for the gradual resolution of inflammation. On a molecular level, the interaction between SLPI and bacterial LPS has been characterized, stemming from the observation that SLPI knock-out mice are more susceptible to endotoxin shock (116). Jin *et al.* demonstrated that SLPI inhibits LPS-induced NF- κ B activation in macrophages (66) and that SLPI expression is induced in response to direct LPS stimulation as well as in response to the LPS-dependent cytokines IL-6 and IL-10 (65). Several mechanisms have been proposed to explain the role of SLPI in inhibiting NF- κ B signaling in response

to bacterial products. It has been shown that SLPI interferes with the recognition of LPS by TLR4 by inhibiting binding of LPS to the TLR4 co-receptor CD14 on the surface of macrophages (25), leading to decreased uptake and therefore signaling. Given its affinity for lipid membranes, it has also been proposed that SLPI has intracellular activity, since overexpression of a non-secreted SLPI isoform in endothelial cells and macrophages leads to inhibition of LPS-, oxLDL- (oxidized LDL) and TNF- α -dependent NF- κ B activation (47; 66). It has been then shown that SLPI binds to the NF- κ B inhibitor I κ B and prevents its degradation via the proteasome-dependent and independent pathways. As a consequence, I κ B keeps NF- κ B sequestered in the cytoplasm, precluding access to the nucleus and activation of inflammatory genes (75; 143). Furthermore, there is evidence that SLPI can directly inhibit NF- κ B activity by competing with the NF- κ B subunit p65 for binding to promoters that contain the NF- κ B binding site, in particular the TNF- α and IL-8 promoters, leading to a decreased production of these cytokines upon stimulation with LPS (142).

SLPI as an antimicrobial peptide

The cationic nature of SLPI and its similarity to an equine neutrophil peptide with bactericidal activity (16) spurred investigations on the role of SLPI as antimicrobial peptide. Hiemstra *et al.* were the first to show that SLPI has *in vitro* activity against *Escherichia coli* and *Staphylococcus aureus* and that the bactericidal activity resides in the N-terminal WAP domain (49). SLPI has since been found to be inhibitory against other pathogens including *Mycobacterium tuberculosis* (96), HIV (88; 89; 109), and more recently *Neisseria gonorrhoeae* (14). On a molar basis the activity reported for SLPI against *E. coli* and *S. aureus* was lower than that of other two classes of APs, lysozyme

and defensins, and was ablated by high salt concentrations (49). In a recent study the activity against *N. gonorrhoeae* appeared to be much higher (14), with the caveat that none of these studies were performed *in vivo*, and the levels of activity detected might not correspond to those occurring in physiological conditions. However, SLPI was effective against *M. tuberculosis* at relatively low concentrations and SLPI-deficient mice were more susceptible to infection compared to wild-type controls (96).

Although the cationic nature of SLPI suggests a mode of action that consists of membrane binding and disruption, like many other cationic peptides, the exact mechanism of action of SLPI has never been elucidated.

Regulation of SLPI expression

Expression of SLPI is constitutive in human keratinocytes (160) and in PMNs, where it is stored in secondary granules together with lactoferrin (56). However, expression of SLPI is inducible in other cell types and tissues upon stimulation with pathogen-associated molecular patterns (PAMPs), cytokines, or corticosteroids. In mouse-derived macrophages SLPI expression is induced *in vitro* by treatment with bacterial LPS, lipoteichoic acid (LTA), IL-6 and IL-10, and inhibited by exposure to IFN- γ (65; 66). A *Mycobacterium tuberculosis* study showed that expression of SLPI in response to live or heat-killed bacteria depends on TLR2-dependent signaling but not on MyD88 activity (26). SLPI is present in intestinal lavage fluids recovered from human subjects and *in vitro* it is constitutively expressed by intestinal epithelial cells, but its secretion is significantly enhanced by the pro-inflammatory cytokines IL-1 β and TNF- α (122). Conversely, SLPI expression is strongly inhibited by TGF- β , as shown *in vitro* in human bronchial epithelial cell lines (59) and *in vivo* in a rat model of chronic obstructive

pulmonary disorder (COPD) (81). The expression of SLPI is higher in dendritic cells (DCs) isolated from mucosal lymph nodes compared to DCs from peripheral lymph nodes, and it is dependent on stimulation of the cells with bacterial products and other TLR agonists (150).

There is also evidence that SLPI expression is hormonally regulated. It has been shown that SLPI expression was increased in the presence of progesterone in a breast epithelial cell line and in cervical explants due to direct activation of the SLPI promoter that contains a progesterone response element (PRE) (23; 69). Estrogen also appears to induce overexpression of SLPI, as demonstrated *in vitro* in human uterine epithelial cells treated with physiological concentrations of estrogen (33), and *ex vivo* in cervicovaginal secretions and endocervical tissue explants from menopausal women that had received treatment with vaginal estrogen (121).

There are also indications that infections lead to substantial alteration of SLPI expression. Reduced levels of SLPI were found in tissue biopsies from patients with *Helicobacter pylori* infection (159) and in cervicovaginal lavages from women with lower reproductive tract infections (28). However, incubation of gastric cell lines with *H. pylori* lead to increased transcription of the SLPI gene, but did not affect protein levels, indicating that regulation of SLPI expression might occur at the post-translational level (159).

Expression of SLPI in the female genital tract

SLPI is expressed throughout the female genital tract, and during pregnancy it is present at exceptionally high concentration (ranging from 150 to 10,000 µg/ml) in the cervical mucus plug, which represents a key barrier to protect the upper reproductive tract

from infections (45; 46; 54). The concentration is much lower in cervical and vaginal fluids from non-pregnant women, but there are some discrepancies among reports, probably due to differences in sample collection. For example, Moriyama *et al.* reported that the concentration of SLPI in cervical fluid samples from non-pregnant women varies widely during the menstrual cycle, averaging 188 ng/ml in the follicular phase, 110 ng/ml in the luteal phase, and 880 ng/ml in the ovulatory phase (91). On the other hand, Valore and colleagues found that there was little variation in SLPI concentration among samples collected in different stages of the menstrual cycle, but the concentration in all cases was lower than 1 µg/ml, consistent with previous reports (145). The expression of SLPI in the Fallopian tubes is reportedly maintained at constant levels throughout the menstrual cycle, but is increased in ectopic pregnancy, which could indicate that SLPI plays an important role in the innate immune responses of the upper reproductive tract in a diseased state, but could also be a mere consequence of the increase in progesterone that occurs during pregnancy (70). SLPI has also been detected in the nucleus of myocytes in human myometrial explants, and its expression increases at the onset of labor, perhaps to control the excessive inflammation and tissue damage that occur during labor (77).

Interestingly, Draper *et al.* (28) reported that decreased amounts of SLPI are found in cervicovaginal secretions of women with lower genital tract infections, including gonorrhea, compared to healthy controls, suggesting that pathogens might have evolved ways to cause degradation of this peptide or down-regulation of its expression in order to resist killing. In a previous study, in fact, Draper *et al.* had also shown that *Trichomonas vaginalis* secretes cysteine proteases that degrade SLPI (27). An alternative explanation, not suggested by these investigators, is that host genetic polymorphisms that

result in decreased expression of SLPI might be a factor in determining susceptibility to reproductive tract infections. In both cases, this study suggests that SLPI might play an important role in the innate defenses of the female reproductive tract.

1.6 OVERALL GOAL AND SPECIFIC AIMS OF THE DISSERTATION

The goal of this dissertation was to examine the role of SLPI during *N. gonorrhoeae* infection of the female genital tract using the mouse model of gonococcal infection. SLPI is present throughout the reproductive tract, and its antimicrobial activity has been demonstrated *in vitro* against a variety of pathogens, including *N. gonorrhoeae*. However, the gonococcus is extremely well adapted to survive in the genital tract and it is not known whether SLPI challenges *N. gonorrhoeae* during infection. It is likely that some of the resistance mechanisms that the gonococcus has evolved against antimicrobial compounds (e.g. efflux pumps) might effectively protect against SLPI. Furthermore, it is not known how, or if, *N. gonorrhoeae* infection modulates the expression of SLPI in the female genital tract. Draper et al. showed that genital tract infections cause a decrease in the amount of SLPI measured in cervicovaginal secretions, but it is not clear whether this downregulation was caused by the presence of the pathogens or whether host genetic polymorphisms that result in lower amounts of SLPI being expressed increase susceptibility to infections.

We hypothesized that SLPI challenges the gonococcus during infection, but that *N. gonorrhoeae* has likely evolved mechanisms to resist killing. We also hypothesized that the expression of SLPI in the reproductive tract is affected by gonococcal colonization. In order to test these hypotheses, we designed two specific aims. The results of the work outlined in the specific aims are presented in chapter 3.

Specific aim 1

The first aim was **to measure the *in vitro* bactericidal activity of SLPI against wild-type strains of *N. gonorrhoeae* and to determine whether resistance mechanisms are in place to evade killing**. We developed an *in vitro* bactericidal assay to measure SLPI activity and tested mutants in the MtrCDE active efflux pump system to determine whether SLPI was a substrate of the pump. We also tested the hypothesis recently advanced by others that Opa phenotype contributes to SLPI susceptibility.

Specific aim 2

The second aim was **to determine whether *N. gonorrhoeae* infection and, or the host inflammatory response alter the expression pattern of SLPI in the female genital tract and whether SLPI challenges the gonococcus during infection**. We measured SLPI expression at the RNA and protein level over the course of infection in female mice that do and do not develop an inflammatory response to *N. gonorrhoeae* and tested the impact of SLPI expression on gonococcal colonization by infecting SLPI-deficient female mice.

CHAPTER 2: MATERIALS AND METHODS

GONOCOCCAL STRAINS AND CULTURE CONDITIONS

Neisseria gonorrhoeae strain FA1090 (PorB1b, serum-resistant) was originally isolated from a female patient with disseminated gonococcal infection (DGI) and has been characterized extensively in male volunteer studies (51) and the mouse model (64). Strain FA19 Sm^R (PorB1a, serum-resistant) is a streptomycin (Sm) resistant mutant of strain FA19, which was also isolated from a case of DGI (63). Strain MS11 (PorB1b, serum-intermediate) was isolated from a case of uncomplicated cervicitis (139; 140), and strain F62 Sm^R (PorB1b, serum-sensitive) is a Sm-resistant mutant of strain F62, which was originally isolated from an uncomplicated urogenital infection (31). Strains RD1 and JF1 are an *mtrE* insertional mutant and an *mtrR* deletion mutant, respectively, of strain FA19 and were described previously (152). Strain JF6 was derived from strain JF1 by complementation with a copy of *mtrR* under control of its own promoter (36). All *N. gonorrhoeae* strains were cultured at 36°C in the presence of 7% CO₂ on GC agar with Kellogg's supplement I and 12 M Fe(NO₃)₃. GC agar supplemented with vancomycin, colistin, nystatin, trimethoprim sulfate, and streptomycin (GC-VCNTS) was used to isolate *N. gonorrhoeae* from mouse vaginal swabs as described previously (60).

SLPI BACTERICIDAL ASSAYS

The bactericidal activity of recombinant human SLPI (R&D systems) was tested in a microtiter plate assay similarly as described by Hiemstra et al. (49). *N. gonorrhoeae* strains were cultured to mid-logarithmic phase (OD₆₀₀ 0.5) in GC broth (GCB) with Kellogg's supplement I, 12 M Fe(NO₃)₃ and 5 mM NaHCO₃ at 37°C under agitation at

150 rpm. Forty μl (4×10^3 CFU) of mid-logarithmic phase bacteria were then directly added to an equal volume of SLPI (31.25, 62.5, 125 $\mu\text{g/ml}$) prepared in a 1:1 solution of 10 mM NAPB (pH 7.4) containing 500 mM glycine and supplemented GCB in a polypropylene microtiter plate. Microtiter plates were incubated at 36°C , and the number of CFU was determined at the start of incubation and 3 hours later by culturing serial dilutions on GC agar in duplicate. The percent survival was calculated by the formula $CFU_{3h}/CFU_{untreated} \times 100$, in which CFU_{3h} or $CFU_{untreated}$ is the number of bacteria obtained after the 3h incubation in the presence or absence of SLPI, respectively. The results of the bactericidal assays are expressed as the average CFU percent of the untreated control as the mean $\pm$ standard error of the mean (SEM). Results from three independent bactericidal assays were used to construct point-to-point curves from which the concentration of peptide that caused a 50% decrease in CFU (50% inhibitory concentration) was calculated.

FLOW CYTOMETRY

For detection of SLPI binding via flow cytometry, equal volumes of mid-logarithmic phase gonococci at a concentration of $2 \times 10^5/\text{ml}$ in GCB and 62.5 $\mu\text{g/ml}$ SLPI prepared as described above for the bactericidal assays were incubated for 30 minutes at 36°C in the presence of 7% CO_2 . Unbound SLPI was removed by centrifugation at $9,279 \times g$ for 5 minutes and the bacterial pellet washed once with GCB containing 50 mM NAPB. The pellet was suspended in 100 μl of anti-SLPI biotinylated antibody (3 $\mu\text{g/ml}$, R&D systems) diluted in 1% BSA or 100 μl of 1% BSA and incubated for 1 hour at room temperature. Bacterial pellets were washed once with 1% BSA, resuspended in 100 μl of PE-labeled streptavidin (0.6 $\mu\text{g/ml}$, eBioscience) diluted in 1% BSA or in 100 μl of 1%

BSA and incubated for 30 minutes at room temperature. Pellets were first washed once with 1% BSA, then once with HBSS prior to being suspended in 4% paraformaldehyde and analyzed. For each sample, a total of 10,000 events were acquired. SLPI-positive gonococci were gated based on the fluorescence intensity of secondary-only controls. Sample analysis was performed in the USUHS Biomedical Instrumentation Center Flow Cytometry Core using the BD LSRII Flow Cytometer. Data were collected and analyzed using FACS Diva and FlowJo software.

SLPI DEGRADATION ASSAY

A protocol similar to the one used for bactericidal assays was used to detect degradation of SLPI over the course of a 3-hour incubation with gonococci. *N. gonorrhoeae* strains FA1090 and MS11 were grown to mid-logarithmic phase and 4×10^3 CFU were added to 62.5 $\mu\text{g/ml}$ SLPI in wells of a polypropylene microtiter plate in a final volume of 80 μl . Control wells contained either bacteria or SLPI alone. After a 3-hour incubation, 50 μl of 2x Laemmli sample buffer (Sigma) containing sodium dodecyl sulfate (SDS) and β -mercaptoethanol and supplemented with 10 mM urea (27) were added to 50 μl of each sample. The lysates were boiled for 10 minutes and stored at -20°C until ready for analysis. For SLPI detection the samples were separated via SDS-PAGE on 4-15% precast polyacrylamide gels (Bio-Rad) and transferred onto a nitrocellulose membrane. After blocking for 4 hours at room temperature in Pierce[®] protein-free T20 blocking reagent (Thermo scientific), membranes were incubated with anti-human SLPI goat polyclonal antibody (1:500, R&D systems) overnight at 4°C , followed by HRP-conjugated rabbit anti-goat IgG (1:25,000, Bethyl laboratories) for 30 minutes at room temperature. Antibodies were diluted in blocking reagent and the

membranes were extensively washed with 0.1% Tween 20 in TBS after each incubation. Signal was detected with ECL Western Blotting Reagent (Amersham).

OPA PROTEIN ANALYSIS

Opa-positive variants of strain MS11 were isolated by serial passage of a primarily Opa-negative stock of MS11 on solid agar and inspection of isolated colonies under a stereomicroscope to detect differences in photo-opacity. Colonies with increased photo-opacity were sub-cultured and suspended to an OD₆₀₀ of 0.1. Bacteria within the suspension were lysed in Laemmli buffer (Sigma) containing sodium dodecyl sulfate (SDS) and β -mercaptoethanol and boiled for 10 minutes to denature proteins. Samples were separated on 12% SDS polyacrylamide gels and transferred onto a nitrocellulose membrane. After blocking overnight in 3% non-fat milk diluted in 0.1% Tween 20, membranes were incubated with monoclonal antibody 4B12 (1:1,000) (kindly provided by Dr. Alison Criss, University of Virginia) for 2 hours at room temperature followed by HRP-conjugated goat anti-mouse IgG (1:10,000, Sigma) for 30 minutes at room temperature. Antibodies were diluted in 2% milk containing 0.1% Tween 20 and the membranes were extensively washed with 0.1% Tween 20 after each incubation. ECL Western Blotting Reagent (Amersham) was used for signal detection.

EXPERIMENTAL MURINE INFECTION

Female BALB/c or C57BL/6 mice (four to six weeks old; National Cancer Institute) and female wild-type or *Slpi*^{<Tm1Smw>} (SLPI-deficient) B6.129SF2/J mice (four to seven weeks old; Jackson Laboratories) were allowed to acclimate to the animal facility for 7-10 days before each experiment. Vaginal smears were stained with Hema-3 to determine the stage of the estrous cycle (21) and mice in the diestrus or anestrus stage

received 0.5 mg of water-soluble 17 β -estradiol (Sigma) subcutaneously two days prior to, the day of and two days after intravaginal inoculation with 10^6 CFU of *N. gonorrhoeae* strain FA1090 in 20 μ l of phosphate-buffered saline (PBS) or PBS as described (128). The inoculum was quantitatively cultured by serial dilution to confirm the bacterial dose for each experiment. To suppress the overgrowth of commensal flora that occurs under the influence of estrogen, mice were given antibiotics (streptomycin sulfate, vancomycin hydrochloride, trimethoprim sulfate) as described (64). Colonization load and the vaginal PMN response were assessed as follows. Vaginal mucus was collected with a PBS-soaked polyester swab and suspended in 1 ml of GCB with 0.05% saponin to disperse cell aggregates. A portion of each sample was used to inoculate HIA agar to monitor the presence of facultative anaerobic commensal flora and to prepare glass slides for enumerating the percentage of PMNs among 100 vaginal cells as described (60). Undiluted and 1:10-diluted vaginal swab suspensions were cultured onto GC-VCNTS agar using the Autoplate 4000 (Spiral Biotech). After 48 hr incubation, the number of CFU recovered was determined using the Spiral Biotech Q-Counter Software. The lower limit of detection was 20 CFU per plate.

ASSESSMENT OF ASCENDING INFECTION

On day 10 post-inoculation with bacteria, SLPI-deficient and -sufficient mice were anesthetized with ketamine and xylazine and euthanized by cervical dislocation. The peritoneal cavity was surgically exposed, and the whole genital tract excised. Tissues were placed in sterile tubes containing 500 μ l of GCB with 0.05% saponin, and homogenized with a hand-held homogenizer. The entire volume was cultured onto GC-

VCNTS agar and the colonies enumerated after a 48-hour incubation at 36°C in the presence of 7% CO₂.

IMMUNOHISTOCHEMISTRY

Prior to conducting immunohistochemical staining with test samples, the optimal concentration of goat polyclonal anti-SLPI antibody (R&D systems) required to minimize background staining and maximize signal was determined by testing increasing dilutions of the antibody on whole genital tract sections excised from an uninfected female BALB/c mouse. Tissues were fixed in 10% formalin for 24 hours, and stored in 70% ethanol prior to embedding in paraffin and sectioning onto slides for immunohistochemical staining. To determine the dynamics of SLPI expression during gonococcal infection, whole genital tracts were harvested from 4 uninfected and 4 infected BALB/c mice on days 1 and 5 post-inoculation with bacteria, and tissues were fixed as done for the antibody titration prior to staining with goat polyclonal anti-SLPI antibody (2 µg/ml). The titration and the experimental staining were both performed by Histoserv, Inc. (Germantown, MD).

QUANTITATIVE REVERSE TRANSCRIPTION REAL-TIME PCR (QRT-PCR)

Vaginal material was collected on days 1, 3, 5, and 7 post-inoculation with bacteria or PBS using a PBS-soaked polyester swab from infected and uninfected mice 2 hours prior to culture and suspended in 500 µl RLT buffer (Qiagen). Samples were stored at -80°C until use. Total RNA was extracted with the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The RNA concentration was estimated using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Inc.) and a minimum of 10 ng of RNA per sample was reverse-transcribed to cDNA using the QuantiTect

Reverse Transcription Kit (Qiagen). The cDNA reaction mixtures (final volume 20 μ l) were diluted to a volume of 100 μ l in nuclease-free water and 5 μ l were used as template for the qRT-PCR reactions. Each 25 μ l reaction contained 12.5 μ l of SYBR Green Master Mix (AB) and primers at a final concentration of 0.4 μ M. The PCR amplification was carried out on the ABI 7500 Sequence Detector as follows: 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The cycle threshold (C_t) value was determined by using Sequence Detector v.1.7a software (ABI). The data were analyzed with Microsoft Excel using the comparative C_t method ($\Delta\Delta C_t$) for relative quantification of gene expression using β -actin as the normalizer, as described previously (149). The experiment was performed twice with four to six mice per group. The oligonucleotide sequences used were described previously (149).

MEASUREMENT OF SLPI CONCENTRATION AND CYTOKINE ANALYSIS

Two hours prior to collecting swabs for RNA extraction, vaginal washes were collected from mice on days 1, 3, 5, and 7 post-inoculation with bacteria or PBS. Washes were performed by pipetting 50 μ l of endotoxin-free PBS (10 times in and out); three washes were pooled from each mouse at each timepoint, and centrifuged at 15,682 $\times$ g for 5 minutes at 4°C to remove cellular debris. Supernatants were stored at -80°C until use. The concentration of SLPI in vaginal lavages was measured using a commercially available Enzyme-Linked Immunosorbent Assay (ELISA) kit specific for mouse SLPI (MyBioSource) following the manufacturer's instructions. The concentration of pro-inflammatory cytokines was measured with the 20-plex mouse pro-inflammatory cytokine and chemokine panel (Invitrogen Life Sciences). The cytokine analysis was performed at the Duke University Human Vaccine Institute (Durham, NC).

DATA ANALYSIS

Differences in susceptibility of FA19 *mtr* mutants to SLPI were analyzed using two-way ANOVA with Bonferroni correction. Gonococcal burden (CFU/ml) and number of PMNs per 100 vaginal cells are reported as mean $\pm$ standard error of the mean (SEM). Differences in PMN influx between infected BALB/c and C57BL/6 mice were assessed using repeated-measures ANOVA with Bonferroni correction for multiple comparisons. All differences were considered statistically significant at *P* values less than 0.05.

CHAPTER 3: RESULTS

SPECIFIC AIM 1

SLPI has bactericidal activity against *N. gonorrhoeae* *in vitro* and susceptibility differs between strains

The bactericidal activity of SLPI has been demonstrated against several pathogenic bacteria (49; 96) and recently Cooper *et al.* reported that SLPI is also bactericidal against *N. gonorrhoeae* (14). As a first step towards examining the role of SLPI in gonococcal genital tract infection, we tested the activity of SLPI against four laboratory strains: FA1090, FA19, MS11 and F62. These strains differ in terms of type of infection from which they were originally isolated, susceptibility to normal human serum, expression levels of the active efflux system MtrCDE, and major porin type (Table 1). Consistent with Cooper's findings, we found that strains FA1090, FA19 and F62 were all susceptible to SLPI killing to varying extent, whereas strain MS11 was fully resistant to 125 µg/ml, the highest concentration tested. The effective dose necessary to kill 50% of bacteria in the inoculum (ED₅₀) for FA1090, FA19 and F62 was calculated as 76.3 µg/ml, 87 µg/ml and 125 µg/ml, respectively. Flow cytometry analysis revealed no substantial difference in the degree of SLPI binding to the bacterial surface between MS11 and the susceptible strain FA19, although binding of SLPI to FA1090 seemed to occur to a greater extent compared to the other strains (Figure 5).

Susceptibility to SLPI killing is not affected by differential Opa protein expression

Cooper *et al.* showed that SLPI killing is dependent on binding of SLPI to Opa₅₂ on the gonococcal surface.

TABLE 1. Features of wild-type *N. gonorrhoeae* strains used in this study

Strain	Origin	Serum susceptibility	MtrCDE expression	Major porin type
FA1090	Disseminated gonococcal infection	Resistant	+	PorB1b
FA19	Disseminated gonococcal infection	Resistant	++	PorB1a
MS11	Uncomplicated cervicitis	Intermediate	++++	PorB1b
F62	Urogenital infection	Sensitive	+	PorB1b

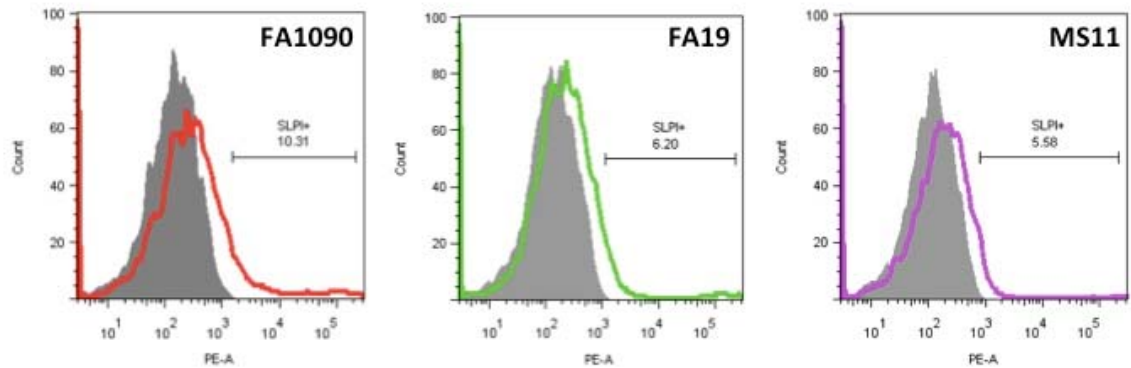


Figure 5. Flow cytometry analysis of SLPI binding

Histograms showing the percentage of gonococci binding SLPI over the course of a 30-minute incubation. The gray histograms represent background signal obtained from unstained bacteria. A shift of the peak to the right compared to background indicates SLPI binding. The percent values listed were obtained by subtracting nonspecific signal from bacteria incubated with the secondary antibody only.

To test the possibility that differences in Opa phenotype may be responsible for the strain-specific differences we observed in susceptibility to SLPI among these four strains, we performed Western blot analysis of whole cell lysates obtained from primary subcultures of the bacterial stocks used in the SLPI killing assay. For Opa protein detection, we used the monoclonal antibody 4B12 that recognizes most Opa types (1). At least one predominant Opa protein was detected in lanes loaded with lysates from strains FA1090, FA19, and F62, with the Opa proteins exhibiting different migration patterns on the gel (Fig 6). Interestingly, no protein was recognized in the MS11 bacterial lysate. We conclude that the stocks of FA1090, FA19, and F62 used in the susceptibility assays were predominantly Opa-positive while that of strain MS11 was Opa-negative. This conclusion seems consistent with SLPI killing being dependent on Opa expression.

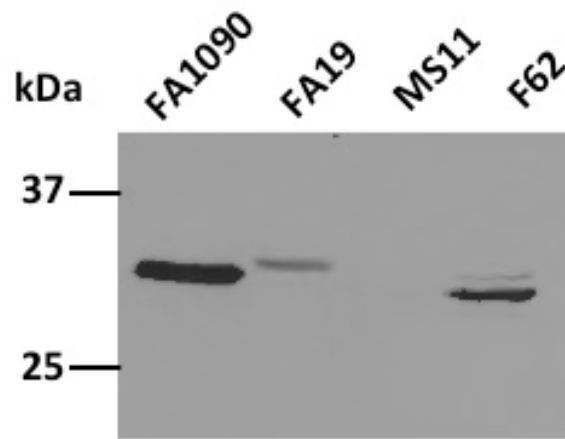


Figure 6. Opa phenotype of FA1090, FA19, MS11, and F62.
4B12 Western blot of whole cell lysates from wild-type *N. gonorrhoeae* strains showing expression of at least one Opa type in each strain with the exception of MS11.

To verify that the resistance of MS11 to SLPI was in fact due to the lack of expression of Opa proteins, we isolated Opa-positive variants from the original Opa-negative MS11 stock (Figure 7A) and tested one of the Opa-positive MS11 variants in the SLPI bactericidal assay. MS11 bacteria that were sub-cultured from the original Opa-negative stock were tested in parallel. No difference in the susceptibility to SLPI was observed (Figure 7B). It could be argued that, if SLPI preferentially binds and kills Opa-positive gonococci, then over the course of the incubation with SLPI Opa-negative gonococci would have a survival advantage and therefore be selected over Opa-positive bacteria. Western blot analysis of lysates from gonococci that survived the challenge with SLPI shows a slight decrease in the intensity of the Opa band for the Opa-positive gonococci incubated with the highest concentration of SLPI (Figure 8). While this result could be indicative of selective killing of Opa-positive gonococci, a Western blot is not the most sensitive method to detect differences in Opa expression at the single cell level. A better approach would be colony blot, since it would allow calculating the exact number of Opa-positive and Opa-negative colony-forming units surviving SLPI challenge. However, the colony blot approach is unsuitable in our case because a pan-Opa antibody reactive against Opa proteins in their native conformation on the gonococcal surface is currently not available.

Finally, we tested the activity of SLPI against naturally occurring Opa variants of strain FA1090: A21.3, an Opa-negative variant, A30, an OpaB-positive variant and A35, an OpaI-positive variant. No difference in susceptibility to SLPI killing was detected among these three strains (Figure 9). We conclude that the Opa phenotype is not a factor

in determining the differential susceptibility to SLPI that we observed, and that a different factor must be responsible for the resistance to killing exhibited by strain MS11.

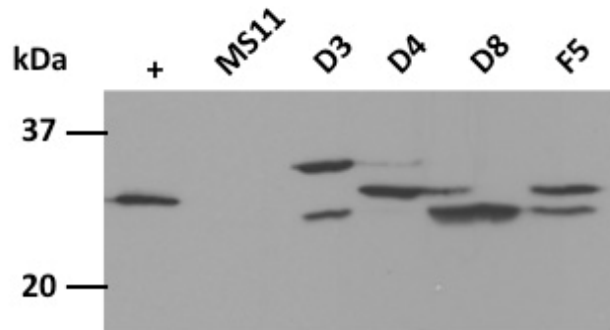
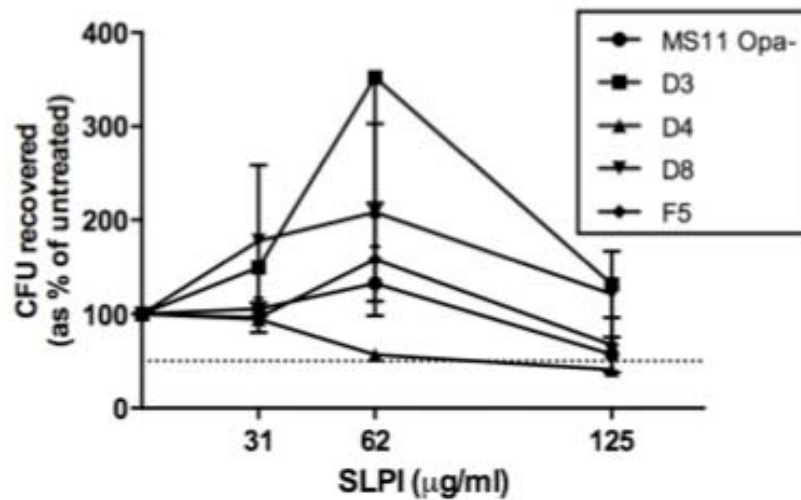
A**B**

Figure 7. Opa-positive variants of strain MS11 are not susceptible to SLPI
 (A) 4B12 Western blot of whole cell lysates from Opa-positive colony variants isolated from strain MS11 and arbitrarily named D3, D4, D8, and F5. The positive control (+) is a lysate obtained from a predominantly OpaI-positive FA1090 strain. (B) Bactericidal activity of SLPI against Opa-negative and Opa-positive variants of MS11. The number of CFU recovered is expressed as percent of the untreated control. The dashed line indicates 50% killing. Data are presented as mean $\pm$ SEM of three independent experiments. The differences between the curves are not statistically significant.

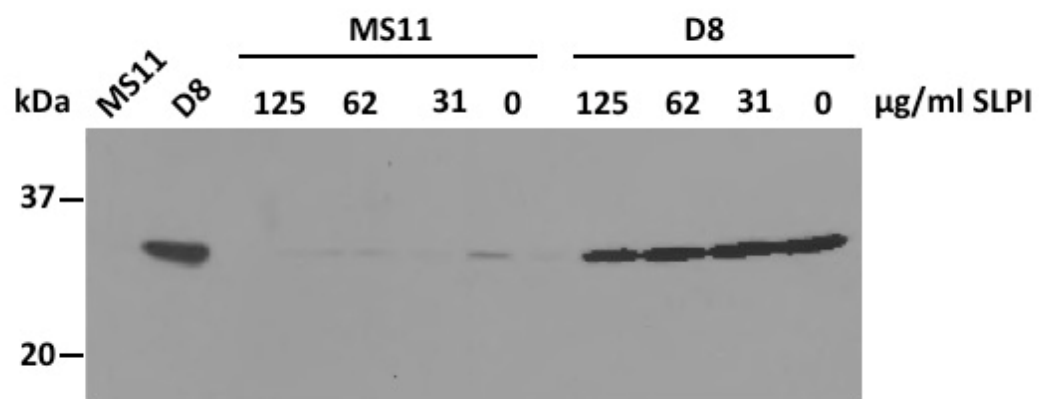


Figure 8. SLPI does not selectively kill Opa-positive bacteria.
 4B12 Western blot of whole cell lysates obtained from Opa-negative MS11 and Opa-positive D8 before and after a 3-hour incubation with decreasing concentrations of SLPI.

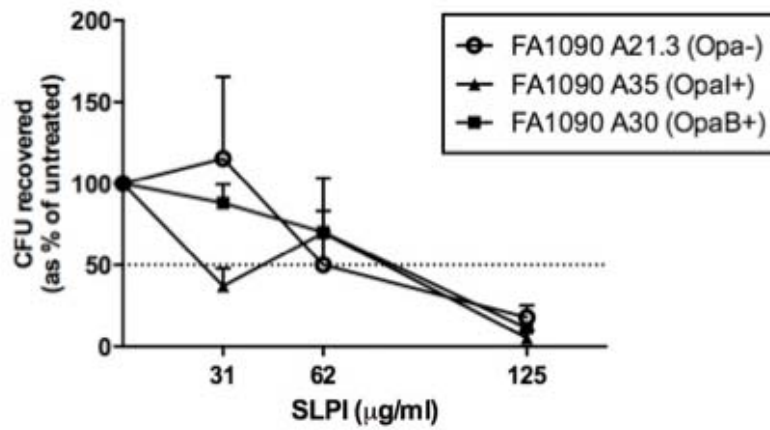


Figure 9. Opa phenotype does not affect the susceptibility of strain FA1090 to SLPI. Bactericidal activity of SLPI against three different Opa variants of FA1090: A21.3 (Opa-negative, open circles), A30 (OpaB-dominant, closed squares), and A35 (OpaI-dominant, closed triangles). The number of CFU recovered is expressed as percent of the untreated control. The dashed line indicates 50% killing. Data are presented as mean $\pm$ SEM of three independent experiments.

Resistance to SLPI is not due to degradation by a gonococcal protease

Draper et al. (27) showed that *Trichomonas vaginalis* secretes a cysteine protease that causes degradation of SLPI, which results in the release of fragments detectable as a series of low molecular weight products on a SLPI Western blot. We hypothesized that strain MS11, but not strains FA1090, FA19 and F62, might similarly secrete a protease that degrades SLPI, effectively avoiding killing. This hypothesis was reasonable because strain MS11 is one of few *N. gonorrhoeae* strains that possess a genetic pathogenicity island (24). Among the products of the island is a putative collagenase (43). Collagenases are a class of proteases that act on substrates containing a polyproline-folding motif, which is typical of collagen. Since SLPI is rich in proline (12 residues out of a total of 107 amino acids), and it has been shown to assume a polyproline-like conformation (66), we hypothesized that production of such protease or overexpression of the sIgA1 protease or others by strain MS11, but not susceptible strains, could be responsible for protection against SLPI killing. To test this hypothesis, we incubated mid-logarithmic phase MS11 and FA1090 with 62.5 µg/ml of SLPI for 3 hours, and verified whether SLPI degradation had occurred by separating whole lysates obtained from each experimental sample on an SDS-PAGE gel and probing with a SLPI-specific antibody by Western blot. Incubation with FA1090 did not result in cleavage of SLPI, as expected, and SLPI was also not degraded over the course of the incubation with MS11 (Figure 10), indicating that resistance to SLPI killing by this strain is not due to the secretion of a protease. A caveat to the interpretation of these results is that a monoclonal antibody was used here to detect SLPI; therefore we would not have been able to detect degradation products by Western blot in the event that the epitope recognized by the antibody was disrupted by the action of a bacterial protease.

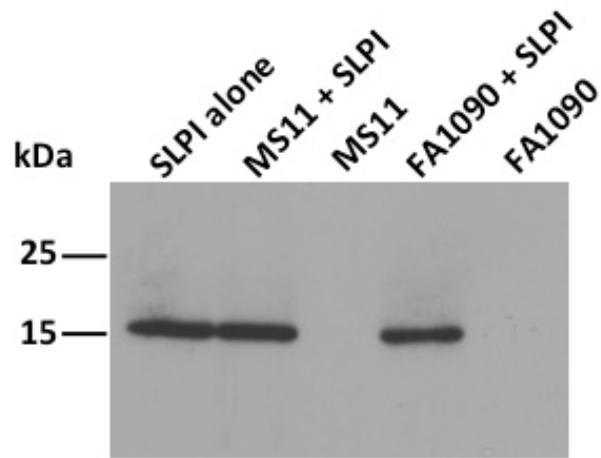


Figure 10. *N. gonorrhoeae* does not degrade SLPI

SLPI Western blot indicating that there is no degradation of recombinant human SLPI (~15 kDa) during a 3-hour incubation with either the SLPI-resistant strain MS11 or the SLPI-susceptible strain FA1090. As a positive control, recombinant human SLPI was incubated for 3 hours in the same buffer used for the assay, and as negative controls the two strains of *N. gonorrhoeae* were also incubated in the same conditions.

The MtrCDE active efflux system does not protect gonococci against the bactericidal activity of SLPI

MS11 overexpresses the MtrCDE active efflux pump as the result of a mutation in the region of the *mtrR* gene encoding the helix-turn-helix and a single base pair mutation upstream of *mtrCDE* that creates an MtrR-independent promoter for the *mtrCDE* operon (98; 153). To test the hypothesis that overproduction of the MtrCDE active efflux pump increases resistance to SLPI, we measured the bactericidal activity of SLPI against *mtr* mutants in the SLPI-susceptible FA19 background. JF1 is an *mtrR* mutant that expresses higher than wild-type levels of MtrCDE, similarly to MS11; RD1 is an *mtrE* mutant that lacks the exit duct of the efflux system and is therefore more susceptible to antimicrobial substances than the wild-type strain. We hypothesized that if SLPI is a substrate of MtrCDE, JF1 should be more resistant than wild-type FA19, whereas RD1 should be more susceptible to SLPI than wild-type bacteria. Our data show no difference in susceptibility among the different mutants, indicating that the factor protecting against SLPI is not the MtrCDE active efflux pump, nor a factor regulated by MtrR (Figure 11).

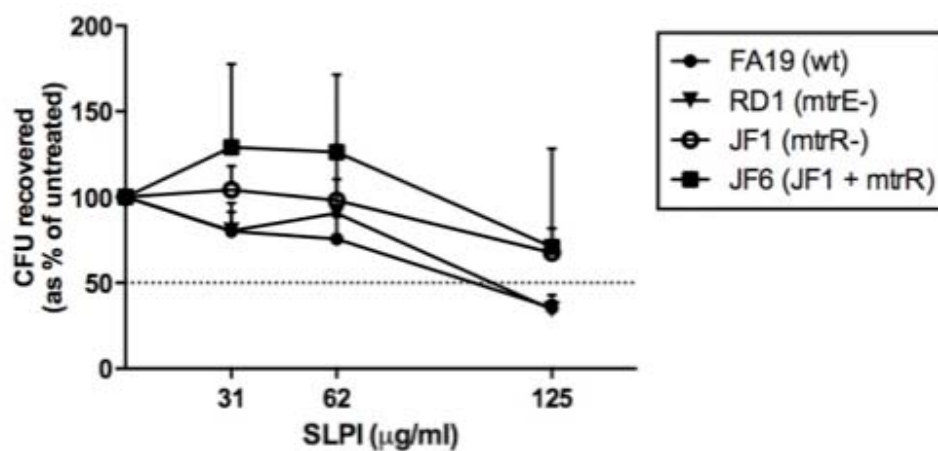


Figure 11. SLPI is not a substrate of the MtrCDE active efflux pump. Bactericidal activity of SLPI against wild-type FA19 (closed circles), RD1 (*mtrE* mutant, closed triangles), JF1 (*mtrR* mutant, open circles), and JF6 (complemented JF1, closed squares). The number of CFU recovered is expressed as percent of the untreated control. The dashed line indicates 50% killing. Data are presented as mean $\pm$ SEM of at least three independent experiments.

SPECIFIC AIM 2

Expression of SLPI during experimental murine infection is inversely correlated with the PMN response at the RNA but not the protein level

We next determined whether the expression of SLPI in the genital tract of female mice is affected by gonococcal infection. We hypothesized that infection would increase SLPI expression since it has been shown that SLPI is induced in tissue culture after stimulation with bacterial LPS and pro-inflammatory cytokines (65). However, it has also been reported that SLPI is present in lower amounts in vaginal fluids from women with gonococcal cervicitis compared to healthy controls (28). It is therefore possible that down-regulation of SLPI may occur during infection. It has been shown that SLPI has anti-inflammatory activity that is due to preventing LPS binding to the soluble TLR4 co-receptor CD14 and therefore uptake and detection by TLR4 (25). SLPI also inhibits binding of the p65 subunit of NF- κ B to NF- κ B-dependent promoters (142). An important consequence is the ability of SLPI to decrease IL-8 production, as shown in a study of cystic fibrosis patients who received aerosolized SLPI (87). Since IL-8 is a potent PMN chemoattractant, we also hypothesized that the expression levels of SLPI inversely correlate with the inflammatory response and the influx of PMNs in the mouse model of gonococcal infection.

To test these hypotheses, we conducted experiments in BALB/c and C57BL/6 mice, which do and do not produce an inflammatory response to *N. gonorrhoeae* infection (99). Both strains of mice were colonized for an average of 10 days, with a bacterial load ranging from 10^3 to 10^6 CFU/ml. Infection of female BALB/c mice resulted in a robust inflammatory response beginning on day 4 post-inoculation as measured by

the number of PMNs present in vaginal smears. As expected, infection of female C57BL/6 mice in parallel failed to induce an inflammatory infiltrate, despite similar numbers of *N. gonorrhoeae* recovered ((99) and Figure 12). Increased expression of the SLPI gene was detected in infected mice in both the symptomatic and asymptomatic models of infection on day 1 post-inoculation with bacteria compared to uninfected controls (Figure 13). This result is consistent with studies that linked induction of SLPI with detection of bacterial products and TLR signaling (26; 65; 150). Interestingly, while expression remained upregulated throughout infection in C57BL/6 mice, levels of SLPI transcript decreased at time points that preceded the influx of PMNs in infected BALB/c mice (Figures 12 and 13), and a statistically significant difference in SLPI expression was detected between BALB/c and C57BL/6 mice on day 3 post-inoculation. Since SLPI has been described as an anti-inflammatory molecule (39; 87; 142), its down-regulation could be instrumental to the induction of a PMN response at the infection site.

To confirm that SLPI expression is reduced later in infection in BALB/c but not C57BL/6 mice, we repeated these experiments and measured the concentration of SLPI in vaginal lavages from uninfected and infected mice on days 1, 3, 5 and 7 post-bacterial inoculation by ELISA. Surprisingly, there was no difference in the concentration of SLPI between infected and uninfected BALB/c and C57BL/6 mice, as well as between infected BALB/c and C57BL/6 at any time point (Figure 14). Interestingly, we observed a temporal pattern in the fluctuation of SLPI concentrations in both infected and uninfected animals, with decreased amounts measured on days 3 and 5 post-inoculation compared to days 1 and 7, which suggests that SLPI levels in vaginal fluids may be under hormonal regulation. Immunohistochemical staining of reproductive tract tissue from uninfected

and infected BALB/c mice with SLPI-specific antibodies showed high levels of expression in vaginal epithelial cells on day 1 post-inoculation, and lower levels on day 5, and consistent with the ELISA data, no differences were apparent between infected and uninfected mice at these time points (Figure 15). Overall, our data indicate that, although the expression of SLPI might be affected at the transcript level by gonococcal infection and, or the resultant inflammatory response, post-transcriptional or post-translational mechanisms might be in effect to maintain the concentration relatively unchanged in the female genital tract.

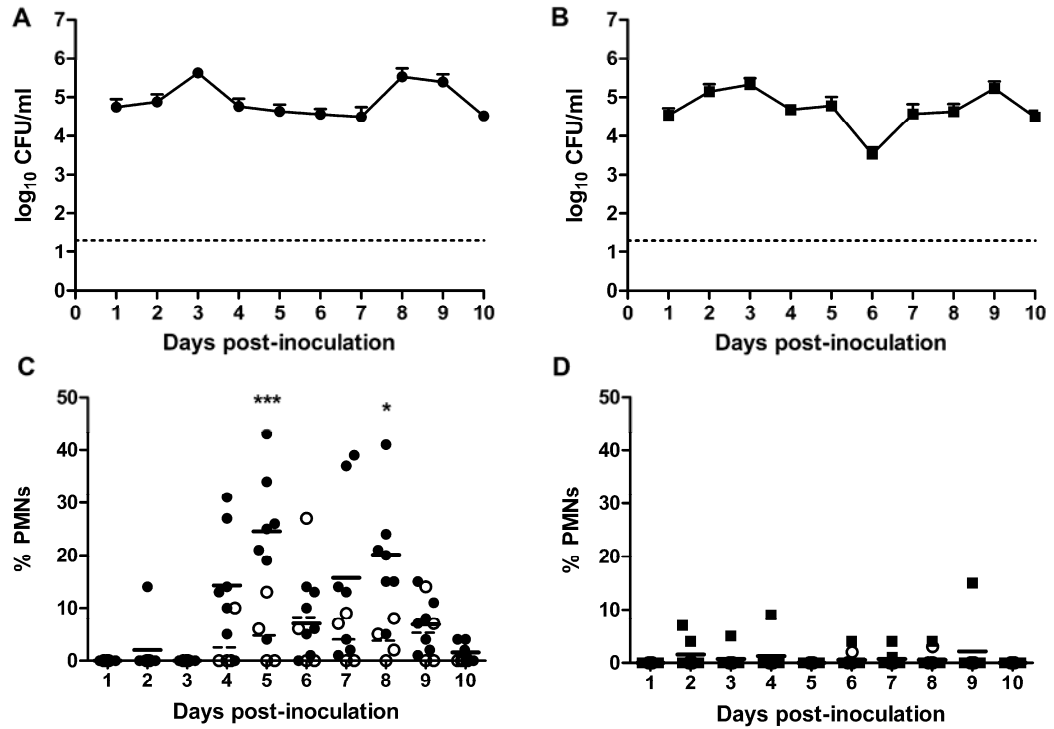


Figure 12. Gonococcal colonization and PMN influx in female BALB/c and C57BL/6 mice.

The colonization level observed in BALB/c (A) and C57BL/6 (B) mice was similar. Gonococcal burden (CFU/ml) and number of PMNs per 100 vaginal cells are reported as mean $\pm$ standard error of the mean (SEM). The dashed line indicates the lower limit of detection. A prominent PMN infiltrate occurred in infected BALB/c mice (C, closed circles) compared to uninfected mice (C, open circles), whereas no inflammatory response was detected in infected C57BL/6 mice (D, closed squares) similarly to the uninfected controls (D, open squares). The percent PMNs indicates the number of PMNs occurring per 100 cells counted on vaginal smears. *P* values lower than 0.05 were considered significant (*, *P* < 0.05; ***, *P* < 0.001). Representative data from three independent experiments (*n* = 5 to 7 mice per group) are shown.

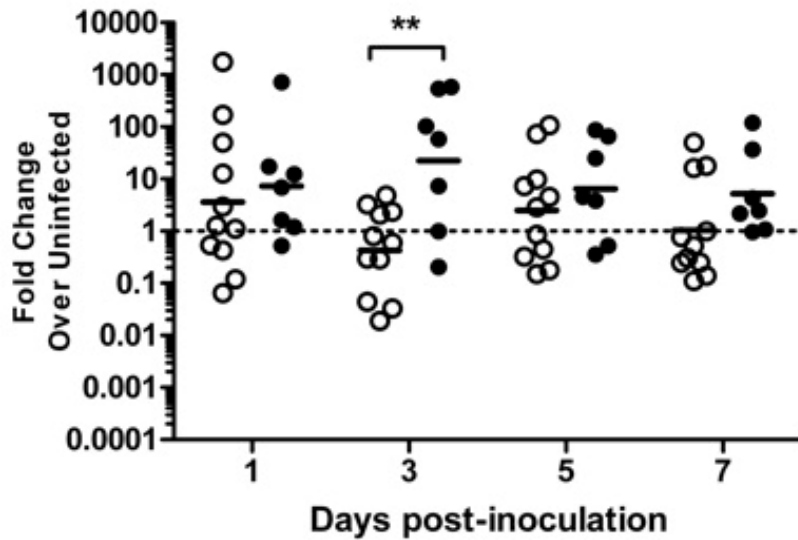


Figure 13. Fold-change in expression of the SLPI gene during *N. gonorrhoeae* infection of female BALB/c and C57BL/6 mice.

SLPI expression was measured via qRT-PCR in vaginal material collected from BALB/c (open circles) and C57BL/6 (closed circles) mice during *N. gonorrhoeae* infection. Results are expressed as fold-change relative to uninfected controls and expression levels of β -actin is used as an internal control. Each circle represents the fold-change in expression for an individual infected mouse relative to the average of the uninfected controls for the same time point. The solid lines correspond to the geometric mean values and the dashed line indicates a geometric mean of one, which corresponds to no difference in expression levels between infected mice and uninfected controls.

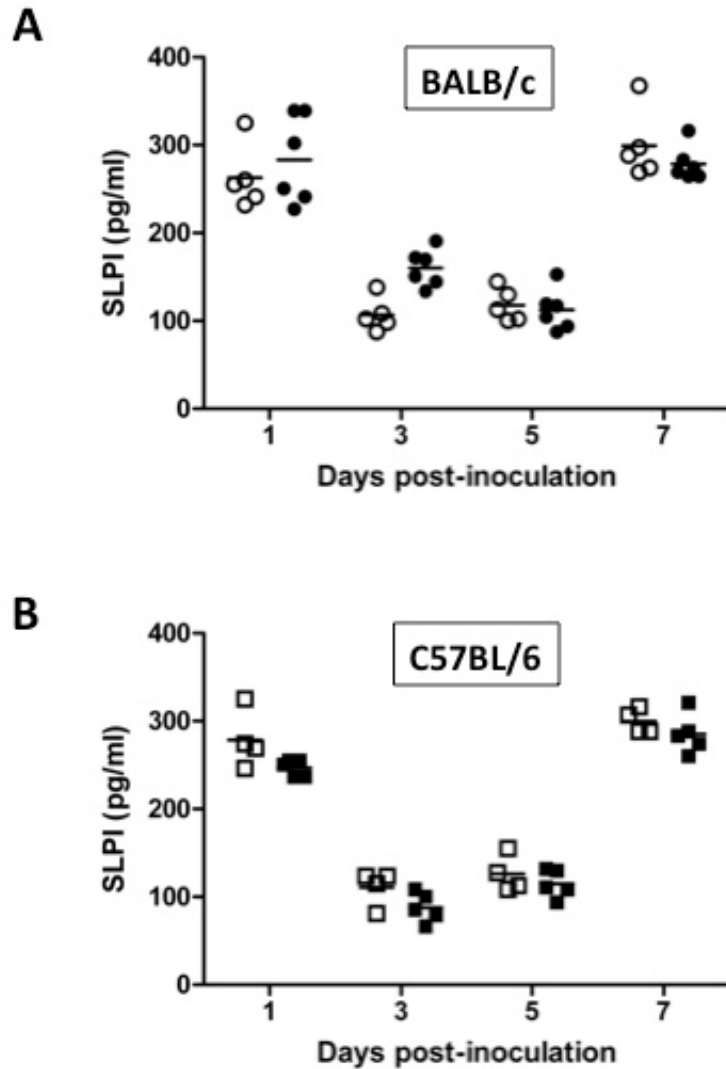


Figure 14. Concentration of SLPI in vaginal lavages from BALB/c and C57BL/6 mice during *N. gonorrhoeae* infection.

The concentration of SLPI in vaginal lavages was measured via ELISA in infected (closed symbols) and uninfected (open symbols) BALB/c (A) and C57BL/6 (B) mice throughout the duration of a *N. gonorrhoeae* infection.

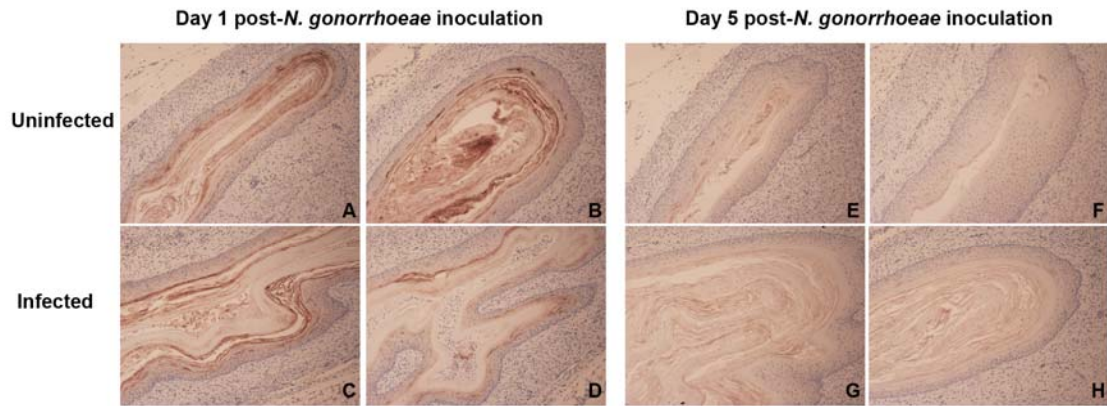


Figure 15. Immunohistochemical staining of genital tract tissue from female BALB/c mice with SLPI antibody.

The pictures show the presence of SLPI (brown staining) in vaginal tissue sections from uninfected and infected BALB/c mice on days 1 and 5 after inoculation with *N. gonorrhoeae*. Images from two representative mice per group are shown at each time point at 100x magnification. Panels A-D show intense SLPI staining localized to squamous epithelial cells in the vaginal lumen on day 1. Positive staining can also be observed on nucleated cells in the stratified epithelium. Panels E-H show weaker staining on day 5 in both uninfected and infected mice.

SLPI does not affect the gonococcal load or the host inflammatory response during experimental infection of female mice

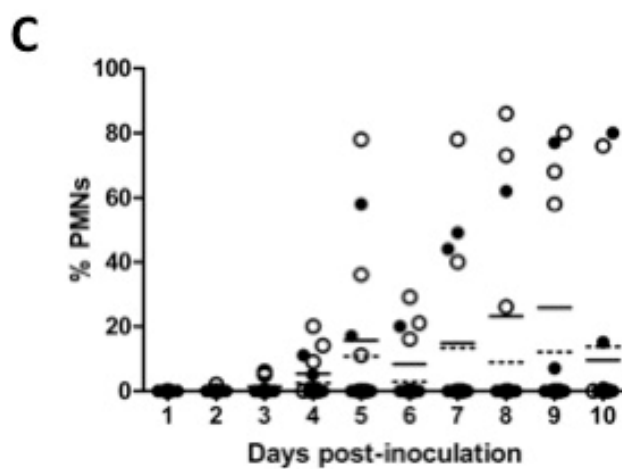
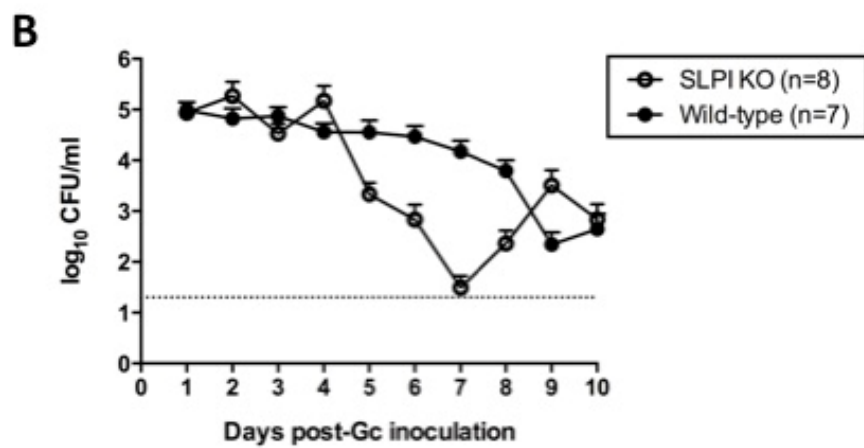
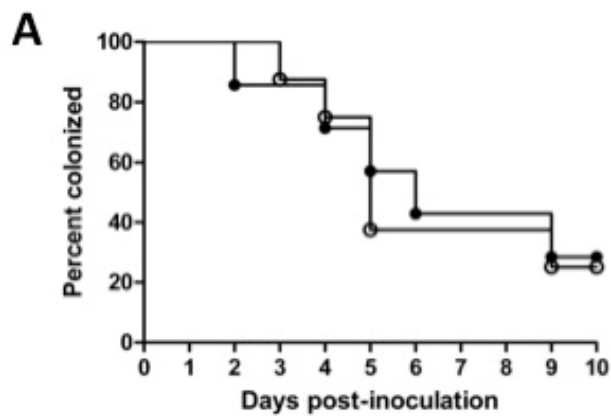
To determine whether SLPI challenges *N. gonorrhoeae* in the female genital tract, we compared the gonococcal colonization in SLPI-deficient and -sufficient B6.129SF2/J mice inoculated with strain FA1090, which is a strain that showed susceptibility to SLPI *in vitro*. We hypothesized that if SLPI were effective against *N. gonorrhoeae in vivo*, we would observe a higher bacterial load or a longer duration of colonization in SLPI-deficient mice compared to wild-type controls. Additionally, given the anti-inflammatory activity of SLPI, we were expecting to see an exacerbated inflammatory response with a more pronounced PMN influx and cytokine production in SLPI-deficient mice. However, we found no significant difference in clearance of infection or number of gonococci recovered between the two mouse strains (Figure 16A-B). Since studies have shown that the expression of SLPI is high in the upper reproductive tract (45; 46; 54), we hypothesized that SLPI-deficient mice would be subject to a higher rate of ascending infection compared to wild-type mice. However, we failed to detect viable gonococci from whole reproductive tract cultures regardless of the mouse background. We also found no statistically significant differences between SLPI-deficient and wild-type mice in either the percent PMN detected on vaginal smears (Figure 16C) or the amount of IL-1 α , TNF- α , MIP-1 and VEGF measured in vaginal lavages on days 1, 3, 5, and 7 (Figure 17).

These results, together with the *in vitro* bactericidal ED₅₀ and the SLPI ELISA data, lead us to conclude that SLPI might not be present at high enough concentration in the genital tract of female mice to result in a significant challenge for the gonococcus.

Alternatively, *N. gonorrhoeae* might utilize as of yet undefined mechanisms of resistance to protect against SLPI during infection. Furthermore, the PMN and cytokine data suggest that SLPI does not play a significant anti-inflammatory role during gonococcal infection of the female genital tract.

Figure 16. Outcome of infection with *N. gonorrhoeae* in SLPI-deficient and wild-type mice

(A) Kaplan-Meier curves showing the percentage of SLPI-deficient (open circles) and wild-type (closed circles) mice colonized with *N. gonorrhoeae* at each time point. There was no difference in infection clearance between the two groups. (B) Gonococcal burden (CFU/ml) in SLPI-deficient (open circles) and wild-type (closed circles) mice. The differences observed on days 6-8 were not statistically significant. (C) PMN influx in response to *N. gonorrhoeae* infection in SLPI-deficient (open circles, solid lines) and wild-type (closed circles, dashed lines) mice, indicated as number of PMNs occurring in 100 vaginal cells. The lines correspond to the geometric means. There is a trend towards a higher PMN influx in SLPI-deficient mice compared to wild-type mice, but it is not statistically significant as determined by two-way ANOVA with Bonferroni correction.



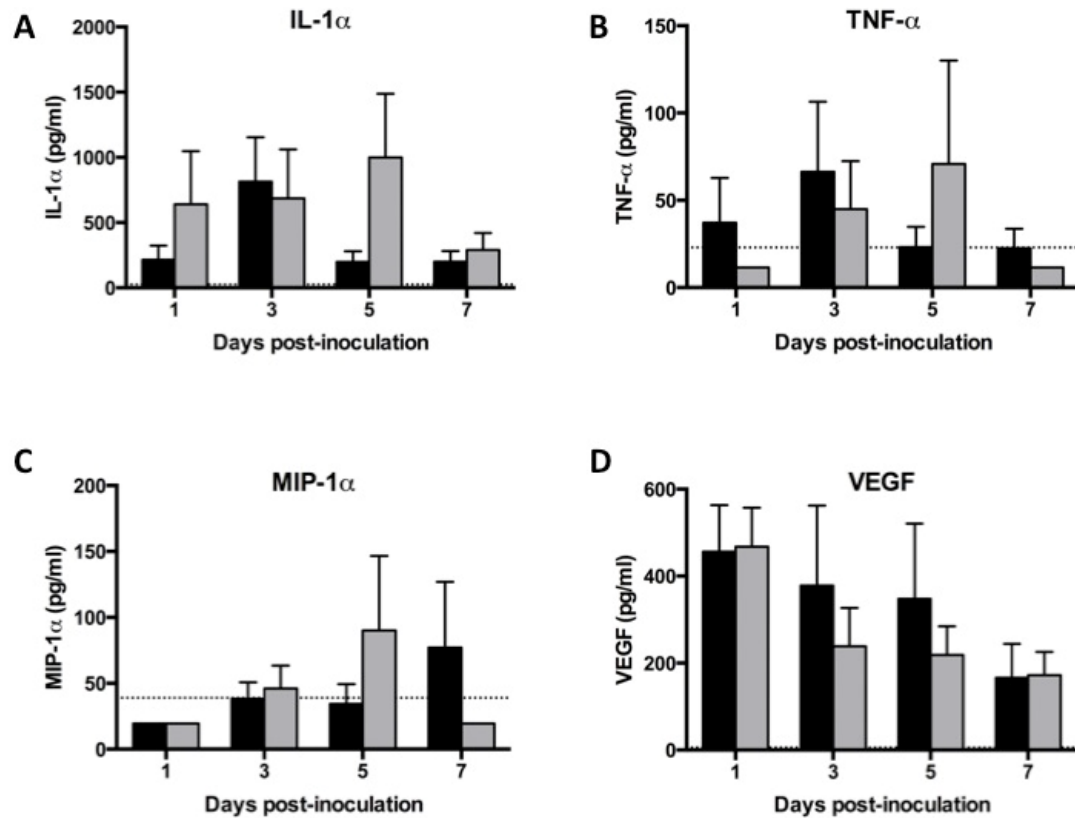


Figure 17. The absence of SLPI does not alter the pro-inflammatory cytokine profile of *N. gonorrhoeae* infected mice.

Vaginal levels of pro-inflammatory cytokines in infected SLPI-deficient (black bars) and wild-type (gray bars) mice. Concentrations (pg/ml) over time from a single experiment are shown as mean $\pm$ SEM (n = 7 to 8 mice per group). The dashed lines correspond to the lower limit of detection in each graph.

CHAPTER 4: DISCUSSION

SUMMARY OF FINDINGS

The main findings of this dissertation work address the goals and specific aims presented in the introduction. We focused on the bactericidal activity of the mucosal antimicrobial peptide SLPI and its ability to target *N. gonorrhoeae* *in vitro* and *in vivo*, using a murine model as a surrogate model for gonococcal genital tract infection in women. We also addressed the expression dynamics of this peptide at the transcript and protein level in the presence or absence of infection, and during either symptomatic or asymptomatic infection.

In Specific Aim 1, we found that SLPI is bactericidal against wild-type strains of *N. gonorrhoeae* and that susceptibility varies among different strains. We identified strain MS11 as resistant to the highest concentration tested, and we determined that resistance is not due to the lack of Opa protein expression on the gonococcal surface as suggested by a recent study (14), and is not mediated by the MtrCDE active efflux pump.

In Specific Aim 2, we found that the expression of SLPI at the transcript level is differentially regulated during gonococcal infection in BALB/c mice, which respond to infection with a prominent cytokine production and PMN influx (99). Following inoculation of BALB/c mice with *N. gonorrhoeae* SLPI was immediately upregulated, but downregulated at a time point that preceded the influx of PMNs in the genital tract. Conversely, in C57BL/6 mice that do not mount an inflammatory response to infection, while initial upregulation of SLPI transcription was also seen, levels stayed elevated compared to uninfected mice throughout the course of infection. While this differential downregulation might have indicated an important role for SLPI in controlling

inflammation, it was not recapitulated at the protein level, as the concentration of SLPI measured in vaginal fluids remained constant despite the presence of infection or inflammation. Finally, we tested the impact of SLPI on gonococcal colonization and inflammatory response in SLPI-deficient mice and found that, despite its *in vitro* bactericidal activity, SLPI does not represent a significant challenge for the gonococcus *in vivo* and it does not have an impact on the inflammatory response to infection.

BACTERICIDAL ACTIVITY OF SLPI AGAINST *N. GONORRHOEAE*

We show here that SLPI is bactericidal against wild-type strains of *N. gonorrhoeae* at supra-physiological concentrations, with ED₅₀ values ranging from 76.3 to 125 µg/ml. While these values are comparable to those obtained by Hiemstra et al., who were the first to report that SLPI has bactericidal activity (49), these levels are higher than the concentrations measured in vaginal fluids from healthy women (91; 145). It is therefore unclear how accurately the *in vitro* ED₅₀ values represent the actual activity of SLPI under physiological conditions. Detecting AP activity *in vitro* can be particularly challenging, however, as activity is usually dependent on ionic charge and factors like salt concentration and pH need to be carefully optimized (133). Even the type of materials used can have a substantial impact on the outcome of a SLPI bactericidal assay. In our experience we could not detect SLPI activity in tissue-culture-treated polystyrene plates, likely due to the negative charge on the surface residues, which might bind and sequester SLPI, reducing the actual concentration of free peptide in the wells. The simple switch to polypropylene plates was sufficient to overcome the loss in activity we had initially observed in our *in vitro* assays. Therefore, even if the conditions of our *in vitro* assay were designed to maximize activity, we cannot exclude that SLPI could be active at

lower ED₅₀ in a physiological environment. It is also possible that SLPI secretion is focalized at the apical surface of epithelial cells, which could result in the formation of discrete areas of high, potentially bactericidal, concentrations. Additionally, the commercially available recombinant human SLPI used for *in vitro* bactericidal assays might be less active than naturally occurring SLPI. Recombinant human SLPI is produced in *E. coli*, and while the details of the purification process are proprietary, we can hypothesize that purification requires denaturing and re-folding steps, which could potentially lead to peptide mis-folding and loss of activity. The manufacturer (R & D systems) tests each protein lot to guarantee anti-protease activity, but does not address the issue of bactericidal activity. Since the anti-protease and bactericidal activities reside on two separate SLPI domains and are independent from each other (37; 49), we cannot exclude the possibility that the bactericidal activity of recombinant human SLPI might be compromised during the purification process.

While in this study we confirmed the bactericidal activity of SLPI against *N. gonorrhoeae* reported earlier by Cooper et al. (14), our results had some substantial differences. First, the ED₅₀ values obtained in the Cooper study are significantly lower than what we observed and, while the authors reported not being able to achieve 100% killing even at concentrations in the range of 100 µg/ml, we were able to reach nearly complete killing of strains FA1090 and FA19 with 125 µg/ml SLPI. Cooper and colleagues measured killing after only 30 minutes of incubation with SLPI; however, we were unable to detect any bactericidal effect at 30, 60 or 120 minutes of incubation (data not shown), and consistently observed killing of susceptible strains after a 3-hour incubation. The major discordance in our methodology is that in our assays we tested

mid-logarithmic phase gonococci, whereas Cooper et al. harvested bacteria from overnight growth on agar plates, which are mostly in stationary phase. Although the mode of action of SLPI has not been characterized yet, the longer incubation time needed in our protocol to achieve killing could indicate that SLPI is only active during bacterial division and therefore has a mode of action similar to that of another mucosal antimicrobial peptide, the cathelicidin LL-37 (127). Our experimental conditions and results are also consistent with those reported for *Escherichia coli* and *Staphylococcus aureus* (49) and we believe the experimental conditions of our assays best approximate what may happen *in vivo* on mucosal surfaces, where gonococci are actively proliferating (62).

Controversial role of Opa proteins in determining susceptibility to SLPI

A second major difference between our work and that of Cooper and colleagues pertains to the role of the phase-variable surface proteins called Opa proteins in determining susceptibility to SLPI. They found that SLPI binds directly to one Opa variant (Opa₅₂) and gonococci that do not express any Opa are protected against SLPI killing. We found that a predominantly Opa-negative isolate of strain MS11 was indeed resistant to SLPI, but so were Opa-positive variants isolated by serial passage and colony purification from the same strain. There was no selective killing of Opa-positive gonococci during incubation with SLPI as indicated by Western blot analysis of the bacteria that survived SLPI challenge. Similarly, the FA1090 strain variants A21.3, A30 and A35 (Opa-negative, OpaB-positive and OpaI-positive, respectively) were equally susceptible to the bactericidal activity of SLPI. Therefore we concluded that the Opa phenotype is not a factor in determining susceptibility to SLPI. As described in the

introduction, Opa types are determined mostly by sequence differences occurring in the hypervariable (HV) regions 1 and 2, however different strains possess a different repertoire of HV₁ and HV₂ sequences. Opa₅₂ was identified in strain MS11, and while there can be some degree of homology among HV sequences across different strains, it is not possible to identify a true homolog of Opa₅₂ in other strains. Sequence alignment shows that the HV₁ region of MS11 Opa₅₂ is 100% identical to the HV₁ sequence found in FA1090 OpaD and OpaI; the HV₂ region of Opa₅₂ is identical to the HV₂ sequence found in FA1090 OpaI. Since Cooper *et al.* did not map the exact binding site of SLPI on Opa₅₂, we can speculate that in FA1090 the Opa protein most similar to Opa₅₂ would probably be OpaI. However, our studies clearly show that the expression of OpaI in FA1090 does not increase susceptibility to SLPI, as the lack of expression of Opa proteins in the same strain does not confer resistance.

Other aspects of Opa protein biology bring into question the likelihood that Opa phenotype is an important factor in dictating gonococcal susceptibility to SLPI. The typical mode of action of APs such as SLPI is to target structures and, or processes that are shared by a variety of pathogens, which is what makes them such an effective component of the first line of defense against infections. For this reason, it would be unrealistic to suggest that susceptibility to SLPI in gonococci is conditional to the expression of a specific variant of a surface protein (Opa₅₂) which is subjected to high frequency phase variation, not found in other organisms, not conserved among gonococcal strains and whose expression is selected for over the course of infection in the genital tract where SLPI is normally present.

The demonstration that selection for Opa-positive gonococci occurs in the human male urethra (61) and the lower genital tract of female mice without a bias for any particular Opa protein (12; 60; 123) further diminishes the likely importance of one particular Opa protein in increasing gonococcal susceptibility to SLPI.

Potential mechanisms for gonococcal resistance to SLPI

We found that susceptibility to SLPI varies among gonococcal strains. Among the four strains tested, strain MS11 was resistant to SLPI, and since we found that the Opa phenotype was not a determinant of susceptibility, we sought to investigate what other resistance mechanisms could potentially be in effect.

Draper *et al.* showed that *Trichomonas vaginalis* secretes a cysteine protease that is responsible for *in vitro* degradation of SLPI (27). *N. gonorrhoeae* has a well-characterized secretory IgA1 (sIgA1) protease that cleaves within the hinge region of sIgA1 antibodies. Interestingly, this protease also cleaves and inactivates the lysosomal membrane protein LAMP1, and thus contributes to the intracellular survival of the gonococcus (78). The hinge region of sIgA1 is rich in proline, similarly to SLPI, and we asked whether SLPI could be an additional substrate for the gonococcal sIgA1 protease or for other, uncharacterized proteolytic enzymes produced by *N. gonorrhoeae*. Particularly attractive was the hypothesis that a putative collagenase encoded in the gonococcal pathogenicity island present in strain MS11 but not in the other strains we tested (24) might be responsible for degrading SLPI by targeting its polyproline-like folding. We found however, that SLPI was not degraded by the SLPI-susceptible strain FA1090 or the resistant strain MS11 over the course of a bactericidal assay (Figure 10), ruling out the protease hypothesis.

Strain MS11 is naturally de-repressed for *mtrCDE* expression and thus more resistant to substrates of the MtrCDE active efflux pump, which include APs such as the cathelicidins LL-37 and its mouse homolog cathelicidin-related antimicrobial peptide (CRAMP) (153). Therefore, we tested whether the level of SLPI resistance could be determined by MtrCDE expression. We found that this was not the case. A deletion mutant of the *mtrE* gene, which encodes the exit duct of the system, was not more susceptible to SLPI than the wild-type strain. Similarly, a deletion mutant of the *mtrR* gene that encodes the transcriptional repressor MtrR was not resistant to SLPI. We conclude that SLPI is not a substrate of the MtrCDE efflux pump and resistance to SLPI is probably not mediated by any product of the 69 genes under control of MtrR (36).

It is not clear at this point why MS11 is resistant to SLPI. Strain MS11 is more infectious than strain FA1090 in both the mouse and the human volunteer challenge models, but the reasons behind this difference are not clear (51). The mechanism of action of SLPI has not been described yet, making it difficult to speculate on the potential basis for resistance. If we postulate that SLPI acts similarly to LL-37, by interfering with cell envelope formation during cell division (127), then it is reasonable to hypothesize that resistance in MS11 could be mediated by a cell envelope component that is present in higher amounts in MS11 compared to other strains, or conversely, that susceptibility to SLPI is due to the presence of a membrane component that is expressed in most of the strains we tested but absent in MS11. Zielke et al. have recently completed a comprehensive quantitative proteomic analysis of different common laboratory strains of *N. gonorrhoeae*, including MS11, highlighting the presence of factors that are present in higher abundance in the cell wall of MS11 compared to other strains (166). Most of the

proteins identified are as yet uncharacterized and, or have been attributed putative functions. Therefore, further studies are needed to examine the impact of cell wall composition on SLPI resistance in *N. gonorrhoeae*.

ROLE OF SLPI DURING GONOCOCCAL INFECTION

We showed here for the first time how SLPI expression in the genital tract of female mice is affected by gonococcal infection. Recent reports in the literature have highlighted the potent bactericidal activity of SLPI *in vitro* against *N. gonorrhoeae* and the interesting downregulation of SLPI in vaginal fluids from women with gonorrhea, which both suggest an important role for this peptide in the innate response to gonorrhea, and we were able to directly test the impact of SLPI on gonococcal colonization and the host inflammatory response here by studying the outcome of *N. gonorrhoeae* infection in SLPI-deficient mice. Contrary to the existing literature on SLPI, our results show in fact that vaginal SLPI concentrations are not significantly affected by gonococcal infection, and do not seem to have a correlation with the host inflammatory response. Furthermore, the modest bactericidal activity that we detected *in vitro* was completely lost *in vivo*, indicating that the concentrations found in the genital tract are not high enough to result in gonococcal killing, and, or that some of the mechanisms that protect the gonococcus against components of the innate immune system, or as yet uncharacterized ones are effective at protecting against SLPI killing *in vivo*.

A more detailed discussion on our *in vivo* findings can be found in the following sections.

Regulation of SLPI expression during *N. gonorrhoeae* infection

Expression of SLPI by mucosal epithelial cells of the female reproductive tract has been detected in both primary and immortalized cell lines, as well as tissue biopsies (32; 68; 70; 126). Interestingly, Draper *et al.* (28) showed that decreased amounts of SLPI are found in women with gonorrhea, suggesting that downregulation of SLPI occurs as a consequence of infection, or that host genetic polymorphisms that result in decreased levels of SLPI lead to increased susceptibility to infection. With the second specific aim of this dissertation we sought to determine whether the mouse model of gonococcal infection recapitulates this finding and if so, identify the molecular basis of such down-regulation.

BALB/c mice respond to infection by mounting a prominent inflammatory response, which manifests in the production of pro-inflammatory cytokines and ultimately in the influx of PMNs in the vaginal lumen, effectively modeling symptomatic infections. Conversely, C57BL/6 mice do not mount an inflammatory response to *N. gonorrhoeae*, and thus mimic asymptomatic infections (99). Given the demonstrated anti-inflammatory activity of SLPI, we expected to see a downregulation of SLPI expression in BALB/c mice, possibly to allow for the inflammatory response to occur. Conversely, we predicted that SLPI would be upregulated in infected C57BL/6 mice, which could have explained the enigmatic lack of inflammation following infection in this mouse genetic background. We found that transcription of SLPI was upregulated in vaginal epithelial cells early on in response to *N. gonorrhoeae* infection in both symptomatic and asymptomatic mouse models. This was not surprising, as several studies have shown that SLPI expression is induced by bacterial products and other PAMPs (9; 65; 66; 126). Strikingly, while expression remained upregulated throughout infection in C57BL/6

mice, the levels of SLPI transcript decreased in infected BALB/c mice compared to uninfected controls on day 3 of infection, which corresponds to the time point that immediately precedes the PMN influx. Interestingly, it has been shown that TGF- β is a potent inhibitor of SLPI expression both *in vitro* and *in vivo* (59; 81). The female genital tract is naturally rich in TGF- β and *N. gonorrhoeae* infection further increases the concentration of TGF- β in the vagina (80). It is therefore possible that the downregulation in SLPI expression observed in BALB/c but not C57BL/6 mice on day 3 might be due to higher levels of TGF- β produced in response to *N. gonorrhoeae* infection in BALB/c compared to C57BL/6 mice. This hypothesis is consistent with the increased production of pro-inflammatory cytokines in BALB/c but not C57BL/6 in response to *N. gonorrhoeae* infection (99). Future studies should focus on identifying differences in TGF- β levels during gonococcal infection in BALB/c and C57BL/6, and how they relate to differences in the expression of the SLPI gene.

At the protein level, however, the concentration of SLPI in vaginal washes was not affected by the presence of infection or inflammation. SLPI ranged in concentration between 81.2 and 288 pg/ml of vaginal fluid; the lowest concentrations were detected on days 3 and 5 post-inoculation in both infected and uninfected animals. Steroid hormones affect the expression of SLPI, and both 17 β -estradiol and progesterone increase SLPI secretion (10; 69). In order to establish infection with *N. gonorrhoeae*, which is a human-specific pathogen, mice are treated with water-soluble 17 β -estradiol two days before, the day of, and two days after inoculation with *N. gonorrhoeae*. Serum estradiol levels peak on the day of inoculation (day 0) and by day 3 return to physiological levels, below the threshold that corresponds to proestrus levels (64). It is possible that the initial higher

concentration of SLPI that we measured in vaginal fluids could be a direct consequence of estradiol treatment, since both uninfected and infected mice are treated. The levels then drop on days 3 and 5, mirroring the decrease in serum estradiol, before the subsequent increase in concentration that might be a result of mice re-entering the estrous cycle. Further studies are needed to address the effect of serum estradiol on SLPI expression in the context of gonococcal infection in female mice.

The fact that the differential regulation observed at the gene level was not found at the protein level might indicate that the concentration of SLPI in vaginal fluids is tightly regulated by post-transcriptional and, or post-translational mechanisms, which contribute to maintaining constant levels of protein despite the presence of infection and, or inflammation. SLPI has pleiotropic functions, and it is reasonable to hypothesize that the host needs to keep its levels in check at all times to ensure the maintenance of homeostasis in case of damage caused by infectious agents or the endogenous inflammatory response.

In vivo* activity of SLPI against *N. gonorrhoeae

Our study shows that clearance of gonococcal infection by strain FA1090 in SLPI-deficient mice is not impaired compared to wild-type mice. This observation is consistent with the relatively high SLPI ED₅₀ values determined *in vitro* against *N. gonorrhoeae* and the low concentrations detected in vaginal fluids of wild-type mice by ELISA. *N. gonorrhoeae* is exceptionally well adapted to survive in the female genital tract, which is a very complex and dynamic environment, so it is not entirely surprising that a single antimicrobial peptide by itself does not represent a significant challenge for the gonococcus. In addition to the low concentrations of SLPI found in vaginal fluids, the

lack of activity of SLPI against the gonococcus *in vivo* could be due to gonococcal mechanisms of resistance that have not yet been characterized with respect to SLPI or to entirely novel, undiscovered factors. Of the known mechanisms of resistance that could be responsible for protecting the gonococcus against SLPI *in vivo*, LOS sialylation is perhaps the most likely. Sialylation can occur *in vivo* because *N. gonorrhoeae* can scavenge the substrate that provides the sialic acid moiety, CMP-NANA, from the host. We did not explore the possibility that sialylation might protect the gonococcus against SLPI, but future studies can address the susceptibility to SLPI of wild-type and sialyltransferase-deficient gonococci grown in the presence or absence of CMP-NANA. Sialylation however cannot be the only mechanism mediating resistance against SLPI, since our *in vitro* data show that strain MS11 is not susceptible to SLPI even in the absence of CMP-NANA.

Another hypothesis that would explain the lack of SLPI activity *in vivo* could be that human SLPI, which was used in the *in vitro* assays, is perhaps more active against *N. gonorrhoeae* compared to its mouse homolog. Mouse and human SLPI share only 52% sequence identity at the amino acid level, but they are very similar on the structural level (66). Nevertheless, it is reasonable to hypothesize that small differences could result in a significantly different biological activity, especially considering that *N. gonorrhoeae* is a human specific pathogen and human SLPI may have co-evolved with it, as opposed to its mouse homolog. Future studies could focus on comparing the *in vitro* activity of recombinant human and mouse SLPI on the gonococcus and, or on establishing a treatment model whereby the outcome of a gonococcal infection in female mice is analyzed in the presence or absence of treatment with human recombinant SLPI.

SLPI does not seem to have a significant impact on the inflammatory response to *N. gonorrhoeae*, as shown by the comparison of pro-inflammatory cytokine levels and the PMN response between SLPI-deficient and wild-type mice. The levels of IL-1 α , TNF- α , MIP-1 α and VEGF during infection were not affected by the lack of SLPI. SLPI has been reported to inhibit IL-8 production both *in vitro* and *in vivo* (87), however in our assay we were not able to detect the mouse IL-8 analog KC in either mouse background and therefore, we cannot make conclusions on this particular aspect of SLPI biology. We also failed to detect the pro-inflammatory cytokines IFN- γ , IL-1 β , IL-6, IL-12, IL-17, and MCP-1 in the mice used in this experiment. It should be noted that the genetic background of the SLPI-deficient mice is a mix resulting from the cross of the C57BL/6 and 129S1/SvImJ backgrounds, and to our knowledge this strain had never been utilized for gonococcal infections. A limitation of this system with respect to the study of SLPI anti-inflammatory activity is the fact that these mice have a similar genetic makeup to C57BL/6 mice, which do not mount an inflammatory response to *N. gonorrhoeae* infection (99). The similarity to C57BL/6 mice could therefore explain the overall low levels of pro-inflammatory cytokines that we detected in our study. A SLPI-deficient mouse strain in the BALB/c genetic background would probably represent a better biological system for elucidating the function of SLPI in the context of the inflammatory response to *N. gonorrhoeae*.

In addition to its very intriguing bactericidal and anti-inflammatory functions, SLPI has also the perhaps less “glamorous”, but not any less important role of neutrophil protease inhibitor, with the purpose of controlling the damage caused by the host inflammatory response. It is therefore possible that the main function of SLPI in the

female genital tract is to prevent excessive tissue damage caused by the PMN proteases during the processes of extravasation and de-granulation. This hypothesis is in contrast to what has been shown in other body sites, such as the respiratory system, where SLPI is fundamental for controlling inflammation and clearing *Mycobacterium tuberculosis* infection (87; 96; 148). Unfortunately, the mouse model of gonococcal infection does not fully recapitulate the symptomatology of gonorrhea in terms of tissue damage in the upper reproductive tract, and therefore it was not possible to determine whether the lack of SLPI activity in SLPI-deficient mice could be associated with a worse infection outcome in terms of pathology compared to wild-type mice.

SIGNIFICANCE OF THIS WORK

Neisseria gonorrhoeae is a human-specific pathogen that is extremely well adapted to survive within the host. The female reproductive tract is a complex and dynamic environment, characterized by the presence of innate immune receptors and effectors that are subject to hormonal influence. To evade the host innate and adaptive immune systems *N. gonorrhoeae* has evolved several sophisticated mechanisms, which include: expression of efflux systems for fatty acids and APs, surface antigen modification via phase and antigenic variation, inhibition of complement deposition and resistance to phagocytosis. *N. gonorrhoeae* infection skews the host immune responses towards a Th17-dominant type, which is pro-inflammatory in nature and results in production of inflammatory cytokines and APs while simultaneously inhibiting adaptive Th1 and Th2 immunity and the development of immunological memory. In the absence of effective adaptive immune responses, innate defenses, particularly APs produced by mucosal epithelial cells, represent the main line of protection for the genital tract.

However *N. gonorrhoeae* is well equipped to survive these innate immune measures, and the elicitation of a Th17 response could ultimately be beneficial to the gonococcus compared to Th1 and Th2 responses which have been linked to the development of sterile immunity (80).

Among the many antimicrobial factors present in the female reproductive tract, the human cathelicidin LL-37 and its mouse homolog CRAMP are the only ones that have been proven effective against *N. gonorrhoeae* (119; 153). This dissertation work focused on the activity of SLPI, an antimicrobial peptide abundantly present throughout the reproductive tract, with anti-inflammatory and anti-protease activities in addition to bactericidal activity. Our interest in this molecule was spurred by a well-cited report by Draper *et al.* that SLPI is present in lower amounts in cervicovaginal secretions of women with gonorrhea compared to healthy volunteers (28), and by our observation that mice infected with *Chlamydia muridarum* have decreased levels of SLPI transcript compared to uninfected mice and are more susceptible to *N. gonorrhoeae* (149). Both these studies suggest that SLPI could represent a significant challenge for the gonococcus *in vivo*. Therefore we sought to characterize the activity of SLPI against *N. gonorrhoeae* both *in vitro* and *in vivo*, hypothesizing that it would be a potent bactericidal factor both *in vitro* and *in vivo*, and potentially, an immunomodulatory agent with respect to the inflammatory response to infection.

The results of this study suggest however that in physiological conditions the role of SLPI in the innate immune response to gonorrhea may not be as striking as recent studies indicated. Nevertheless, our data represent a significant contribution to the understanding of the host innate defenses against gonorrhea. We were able to

characterize the *in vitro* activity of SLPI against wild-type *N. gonorrhoeae* strains and show that resistance is not mediated by some of the most common systems that protect the gonococcus against APs. Furthermore, to our knowledge this was the first report of the dynamics of SLPI expression in the genital tract of female mice, under physiological conditions and during gonococcal infection. Finally, we showed that SLPI has no impact on gonococcal colonization *in vivo* by utilizing SLPI-deficient mice, which have never been used to model *N. gonorrhoeae* infections previously.

Despite the lack of activity we observed *in vivo*, the possibility still exists that, at supra-physiological, bactericidal concentrations, recombinant SLPI could be developed as a therapeutic agent to treat gonorrhea. Since *N. gonorrhoeae* has rapidly developed resistance to every single class of antibiotics introduced as a treatment and there is no vaccine currently available, gonorrhea is likely to become an untreatable disease in the near future and there is a desperate need for novel therapeutic agents. Recombinant SLPI has been shown to be safe and to decrease IL-8 production when administered by aerosol to cystic fibrosis patients (86; 87). We suggest that SLPI could represent a valid candidate treatment for *N. gonorrhoeae*, since resistance to APs is not easily developed. If supplied in adequate concentrations, SLPI would likely produce the dual benefit of abating gonococcal burden and reducing inflammation, which is the main cause of morbidity associated with gonococcal infections. The best way to ensure high concentrations at the epithelial barrier would likely be to design a recombinant SLPI formulation for topical use, such as a vaginal/rectal microbicide gel. Antimicrobial peptides are attractive candidates for topical microbicide development because they would be recognized as “self” by the immune system and therefore they would be less

likely to cause irritation and induce an immune response, like synthetic products often do (110). The potential use of SLPI as treatment for gonorrhea would also benefit from its immunomodulatory properties and be particularly advantageous if used in conjunction with antibiotics. This treatment strategy would likely limit the inflammation elicited in response to infection and lead to a decrease in the morbidity associated with gonococcal infections. Future studies should focus on evaluating different formulation of recombinant SLPI in the context of *N. gonorrhoeae* infection in female BALB/c mice.

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