Role of Secretory Leukocyte Protease Inhibitor in Urinary Tract Infection

Anne Rosen
Washington University in St. Louis

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Role of Secretory Leukocyte Protease Inhibitor in Urinary Tract Infection
by
Anne L. Rosen

A thesis presented to
The Graduate School
of Washington University in
partial fulfillment of the
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Anne L. Rosen

Washington University, May 2021
Chapter 1

Introduction
1.1 Urinary Tract Infections

Urinary tract infections (UTIs) affect approximately 150 million people per year worldwide and account for more than 10 million outpatient and emergency room visits in the United States alone\textsuperscript{1,2}. More than 3 billion dollars are spent annually in the U.S. for sick leave and treatment of these patients, the majority of whom are women\textsuperscript{1,3,4}. The anatomy of women puts them at higher risk of developing a urinary tract infection than men for two reasons. The female urethra is positioned closer to the vagina and anus where bacteria are in high abundance which facilitates the translocation of bacteria to the periurethral region\textsuperscript{5,6}. Additionally, the female urethra is shorter than male, which aids bacteria entry into the bladder\textsuperscript{6}. More than 50\% of women will experience a UTI in their lifetime and of those who do, 25\% are likely to suffer from a second infection within 6 months\textsuperscript{7-9}.

Recurrent UTIs (rUTIs) following initial infections are likely attributed to a weak host adaptive immune response to the uropathogen causing infection although research has demonstrated that a strong response can be formed\textsuperscript{9,10}. Weak responses may result from early childhood exposure to a uropathogen or maternal history of UTI\textsuperscript{9,11}. While most acute urinary tract infections can be resolved with antibiotics, increased antibiotic resistance to uropathogens has made treatment of rUTI challenging\textsuperscript{9}. Recurrent infections risk developing into a chronic infection that can persist for months despite treatment\textsuperscript{9}.

UTIs are classified into two categories: uncomplicated and complicated. An uncomplicated UTI occurs in an otherwise healthy individual and is effectively treated with a course of antibiotics. A complicated UTI often occurs in patients with catheters, urinary abnormalities, or immunological disorders\textsuperscript{2}. Other risk factors for contracting a urinary tract
infection include prior UTIs, sexual activity, vaginal infection, diabetes, obesity, and genetic predisposition\textsuperscript{12,13}.

1.2 Uropathogenic \textit{Escherichia coli}

While UTIs can be caused by many types of bacteria and fungi, the primary culprit is uropathogenic \textit{Escherichia coli} (UPEC), a gram negative bacteria responsible for more than 75\% of community-acquired and 50\% of nosocomial infections\textsuperscript{1,14}. Microbiome studies measuring UPEC in healthy stool show that these strains are often commensal to the gut, but once shed, can migrate from fecal material to colonize the vaginal and periurethral regions\textsuperscript{15}. In the urethra, UPEC becomes pathogenic and upregulates production of multiple virulence factors like toxins, adhesins and proteases to ascend into the bladder\textsuperscript{16}. These factors help bacteria bind and invade bladder epithelial cells within the first few hours of arrival as shown in mouse models of UTI\textsuperscript{17}. Invading these cells allows UPEC to hide from the host immune system while replicating\textsuperscript{8}. Severe infections can further ascend into the kidneys where they become complicated to treat. Untreated, pyelonephritis can be fatal as bacteria may escape into the bloodstream causing bacteremia and sepsis\textsuperscript{4}.

One way UPEC increases its virulence is by upregulating expression of surface adhesion molecules like type 1 pili, which is essential for invasion of the luminal facet epithelial cells of the bladder\textsuperscript{18}. In fact, one study showed type 1 pili to be expressed in 95\% of isolates from urinary tract infections\textsuperscript{19}. Once inside the cell, UPEC replicates within the cytoplasm to form intracellular bacterial communities (IBCs) that make a biofilm-like structure and can be visualized by microscopy\textsuperscript{20}. IBC formation is an important part of UPEC pathogenesis as studies have shown mutant UPEC strains that do not form these structures are quickly cleared from the
bladder. After 12-16 hours of infection, these formations take on a filamentous structure to flux out of epithelial cells into the lumen of the bladder. This new arrangement can deposit bacteria on neighboring bladder cells to advance the infection and is too large to be engulfed by neutrophils. In an attempt to clear the infection, compromised superficial epithelial cells undergo a process similar to apoptosis and are exfoliated into the urine to be excreted.

Rather than incorporating into the filamentous structure, some UPEC escape to endocytic vesicles located within transitional cells deeper in the urothelium where they form quiescent intracellular reservoirs (QIRs). Containing up to 15 inactive bacteria, these reservoirs can exist for months without shedding bacteria into the urine, making them undetectable to the host immune system. Additionally, QIRs are resistant to 3 and 10 day courses of antibiotics, making them difficult to destroy. Though normally dormant, QIRs can revert to an active state during epithelial cell turnover to cause a recurrent infection. Their resiliency increases the difficulty of identifying and resolving UTIs.

1.3 Diagnosis and Treatment of UTI

1.3.1 Diagnosis

A UTI is suspected when a patient presents with symptoms of burning, urgency, and frequency of urination. These symptoms may be accompanied by fever, chills, hematuria, bladder spasms, and pelvic or back pain; the latter suggests pyelonephritis.

Diagnosis of a UTI involves culturing bacteria from a clean-catch urine specimen. The result of the culture is used to calculate the colony forming units per ml (CFU/ml) and defines a positive result as $10^5$ CFU/ml or higher. This technique, first developed in the 1950’s, has been adopted as the gold standard for UTI diagnosis; however, it has been criticized by multiple
research groups for its limitations in culturing slow-growing microbes, lack of selectivity, and inability to detect less than $10^3$ CFU/ml$^{5,26,27}$. A more sensitive method, expanded quantitative urine culture (EQUC), uses larger sample input, anaerobic conditions, and multiple media types to grow a variety of bacteria$^{26}$. Upon comparison, EQUC has exposed the current method to have a 90% false negative rate$^{5,26}$. Some caveats to this method are its use of more specialized and expensive equipment, along with slower results due to longer incubations.

Up to 20% of women who have physical symptoms of a UTI do not meet the diagnostic cutoff by culture, so alternate methods can help identify whether an infection is present$^{28}$. Other common diagnostic methods include the dipstick test which measures nitrite, protein, leukocyte esterase, and blood in the urine. While this information is somewhat helpful, results from a dipstick test alone tend to have a high false positive rate and can lead to inappropriate antibiotic usage$^{25}$. Microscopy and immersion culture media can also be used, but these methods only slightly improve diagnostic accuracy$^{25}$. The lack of reliable diagnostic testing leaves room for the development of other biomarkers of urinary tract infections to help in cases that do not meet the current diagnostic standards.

1.3.2 Treatment

Most urinary tract infections are treated with a single course of antibiotics$^{25}$. Appropriate antibiotic selection has become increasingly important not only because of the marked increase in antibiotic resistant organisms, but also due to the development of allergies in repeatedly exposed patients, a majority of whom are women$^{28,29}$. Therefore, alternative treatments are currently under development. One study shows promising results for the oral administration of methenamine hippurate as it metabolizes into formaldehyde, a toxin that kills bacteria, in the
Another treatment currently in clinical trials involves vaccination against UPEC adhesins that are essential for infection. The vaccination is designed to use the body’s immune system to help remove UPEC from the reservoir in the gut thereby reducing the likelihood of a UTI\textsuperscript{30,31}.

Treatment becomes more challenging when a patient experiences frequent and recurrent UTIs (rUTI). Often a long-term, prophylactic antibiotic treatment is employed, and although effective, can result in pathogen resistance and allergic reactions in the host\textsuperscript{32–34}. In extreme cases, surgical removal of the bladder is performed to break the cycle of recurrence\textsuperscript{35}.

Other conditions may be misdiagnosed as a UTI and lead to unnecessary antibiotic treatment. This occurs in part due to the overlap in symptomatology as sexually transmitted infections (STIs) can also cause dysuria, frequency and urgency\textsuperscript{36}. Studies in the emergency department have shown more than 50% of diagnosed UTIs return a negative culture of which almost 20% test positive for an STI\textsuperscript{36,37}. Overactive bladder syndrome along with myofascial pain of the pelvic floor can also be misinterpreted as UTIs\textsuperscript{38,39}. Possibility of STI and other urologic conditions should be considered during the patient examination, especially in cases returning negative cultures and failure of prior antibiotic treatment.

1.4 Asymptomatic bacteriuria

While some urine samples do not meet the diagnostic cutoff, patients with asymptomatic bacteriuria (ABU) often surpass this threshold. ABU is diagnosed by two consecutive, positive clean-catch urine cultures in the absence of signs or symptoms of infection\textsuperscript{40,41}. While the incidence rate of ABU increases with age, a higher proportion of cases continue to be in females\textsuperscript{40}. This rate is 1-5% in healthy premenopausal women and increases to 40% in female residents of long term care facilities over 80 years old\textsuperscript{35}. Although the cause of ABU is not well
understood, some evidence suggests the host response is weaker in ABU patients possibly reflecting genetic differences in innate immune mediators. A urine proteome analysis revealed significantly increased levels of immune markers including neutrophils, IL-8 and GRO-α, a neutrophil attractant molecule, compared to urine from healthy controls. This finding demonstrates that ABU patients may still have elevated immune marker levels typical of UTIs without experiencing any symptoms of infection. Increased immune markers along with a positive culture result could easily be misclassified as a urinary tract infection.

As in UTIs, more than one species of bacteria can contribute to ABU, but E. coli is most frequently cultured from patient urine samples. A urine culture analysis of acute pyelonephritis samples showed a higher occurrence of type 1 pili expressing E. coli serotypes compared to samples from patients with ABU. This data suggests that presence of bacteria in the urine may not be problematic in some cases. In fact, the presence of bacteria in ABU patients is thought to be protective against colonization of pathogenic bacteria that may cause infections. Since this discovery, clinicians have been advised not to treat a majority of these cases. Use of antibiotics in ABU patients has not only led to multidrug resistant bacteria, but also increased rates of urinary tract infections. Distinguishing ABU and UTI cases has been challenging and development of other markers of infection is needed to aid in this effort.

1.5 Urinary microbiome

The central dogma of UTI research postulates that healthy urine is sterile. However, discovery of bacterial DNA and live bacteria in the urine of women lacking UTI symptoms suggests the existence of a urinary microbiome. Using expanded quantitative urine culture (EQUC), one study found less than $10^4$ CFU/ml of bacteria in the urine on average, compared to
the colon which is inhabited by approximately $10^{11}$ CFU/g\textsuperscript{44}. The urinary microbiome is also less diverse than that of the gut and more on par with the microbiome of the eye\textsuperscript{44}. Similar to microbiomes found in other regions of the body, the microbial community found in the bladder may protect against invading pathogens\textsuperscript{44,45}. Across multiple studies of the urinary microbiome, \textit{Lactobacillus} species tend to be higher in healthy control groups\textsuperscript{27,44,45}. This genera has been well established as a healthy commensal in the neighboring vaginal microbiome\textsuperscript{45,46}.

The difficulty in exploring the urinary microbiome exists in the methodology. As discussed previously, current culturing methods are limited in sensitivity and selectivity. The development of more sensitive techniques like 16S rRNA gene sequencing and advanced culturing methods allowed detection of low abundance microbes, and provided compelling evidence that a urinary microbiome exists\textsuperscript{26}. Pearce \textit{et al.} performed a urinary microbiome study on women with and without urgency urinary incontinence (UUI)\textsuperscript{44}. Urine samples were collected by transurethral catheterization and analyzed using 16S rRNA sequencing and EQUC. Sequencing showed the cohorts had equal amounts of bacterial DNA, and were largely composed of one or two genera, usually \textit{Lactobacillus} or \textit{Gardnerella}. Additionally, compared to EQUC, they show the standard culturing method has a 90\% false negative rate across both cohorts. Results from EQUC and 16S rRNA sequencing revealed that both methods classify more than half of all samples positive for bacteria. To deconvolute the study, subjects with a history or current UTI were excluded, as these conditions are likely to disrupt the microbial community.

A major source of speculation in these studies is the likelihood of contamination by vaginal or gut flora during urine collection\textsuperscript{27}. To determine the method with minimal contamination, urine was collected from patients by three methods: voiding, transurethral
catheterization and suprapubic aspiration. Overall, bacterial composition was similar among the three techniques, with the most taxonomic overlap in catheterization and aspiration. Voided urine tends to have the most vaginal contamination, although catheterization can also introduce microbes into the bladder. The collection method chosen will need to be carefully considered when evaluating a study and improvement in technology will help distinguish contaminants, bladder commensals, and uropathogens.

1.6 Host immune response to UTI

The host immune system has a multifaceted response to a UTI. First, the innate immune response takes nonspecific action against pathogens through the use of pattern recognition receptors (PRRs). These receptors are expressed on the surface of epithelial and immune cells to recognize specific pathogen-associated molecular patterns (PAMPs) or danger/damage associated molecular patterns (DAMPs). Once a signal is detected, the cell creates a multiprotein complex, the inflammasome, to organize production and secretion of inflammatory molecules into the extracellular environment. This will also signal defensins, alarmins, and other antimicrobial proteins to be upregulated.

Antimicrobial peptides (AMPs) are one of the first host defenses to take action against a urinary tract infection. They are usually small, positively charged proteins produced by epithelial cells and neutrophils that can be constitutively expressed or induced by bacterial stimuli. Close association with bacterial membranes is believed to be one way in which they exhibit bactericidal effects. Bacteria express a variety of negatively charged proteins on the cell surface that may bind cationic AMPs. Some AMPs use special structures to bore into bacteria and polymerize to lyse bacterial cells. They can also create pores to release bacterial
contents and eventually kill the pathogen. Defensins are the most well studied AMPs in the urinary tract and are increased during infection. Neutrophils produce 4 types of α-defensins that employ non-oxidative antimicrobial activity and were increased in patients with chronic pyelonephritis. Cathelicidin is another AMP expressed by epithelial cells that, in vitro, increases in mRNA transcripts within 5-15 minutes of contact with bacteria. Since their discovery, these molecules have been explored as therapeutics in UTI, however they have a short lifespan and cannot compete with antibiotics.

One of the PRRs involved in activating the innate immune response against UPEC is Toll-like receptor 4 (TLR4). TLR4 expressed on macrophages recognizes lipopolysaccharide (LPS) found on gram negative bacteria including UPEC. Once LPS binds TLR4, a signaling cascade will activate the NFκB pathway to upregulate transcription of inflammatory cytokines including IL-1β, IL-6, and TNFα in addition to chemotactic molecules IL-8 (MIP-2 murine homolog) and G-CSF which attracts and stimulates expansion of granulocytes. This increase in cytokines occurs within 1 hour of bladder inoculation and peaks at 24 hours post infection. Accumulation of proinflammatory cytokines creates the environment needed to activate and recruit neutrophils to the site of infection. Upon exiting circulation, neutrophils migrate through the urothelium, arrive in the lumen of the bladder within 2 hours and peak in concentration at 24 hpi. Neutrophils kill bacteria by engulfment, degranulation and release of extracellular traps (NETs). These cells are thought to be the pinnacle innate cell type to combat bacterial colonization in the bladder. However, neutralization of G-CSF reduces the number of neutrophils in circulation and therefore recruitment, which surprisingly leads to decreased bacterial burden. G-CSF is specific to neutrophils but not their monocyte precursor; this implies monocytes may also play an important role in the innate immune response.
Monocytes may also work in conjunction with resident macrophages to increase expression of enzymatic matrix metalloproteinase 9 (MMP-9) production in neutrophils. MMP-9 breaks down collagen to aid neutrophil migration to the lumen of the bladder. Some evidence suggests that mast cell production of IL-10, an anti-inflammatory cytokine, also plays a crucial role in balancing the inflammatory environment in UTI. Additional in vitro studies found mast cell products, including TNFα, induce activation of NFκB in bladder epithelial cells. Mice deficient in mast cell production had higher UPEC titers in the bladder following infection.

Another facet of the innate immune response of the bladder involves γδ T cells which kill infected epithelial cells through secretion of granzymes. γδ T cell knockout mice have higher bacterial burden suggesting that although small in number, these cells play a significant role in fighting UTI. In fact, elevated levels of these cells in the lamina propria of naive mice improve host defense against UTI. They are also a major source of IL-17A in the infected bladder, suggesting they may activate recruited T cells at the site of infection.

Although most studies have focused on the innate immune system response to UTI, several groups have investigated the role of the adaptive immune system. Importantly, since the high rate of recurrence in UTI is thought to be because of a weakened adaptive immune response, it deserves further inquiry. Two cytokines associated with recruitment and activation of T cells, RANTES and IL-12 p40, are increased in mice 48 hpi. Furthermore, transfer of T cells from infected mice to naive ones diminishes UPEC colonization upon challenge. One group discovered that macrophages play a major role in the beginning of adaptive immunity by taking up bacteria soon after inoculation of the bladder. Depletion of resident macrophages in infected mice leads to improved adaptive immune responses. While initially a puzzling result, it revealed that early sequestration of bacteria for antigen presentation weakened the adaptive
immune response\textsuperscript{61}. Depleting macrophages allowed a 2 fold increase in antigen presented by dendritic cells and led to a more robust adaptive immune response\textsuperscript{61}.

1.7 Secretory leukocyte protease inhibitor

Secretory leukocyte protease inhibitor (SLPI) is an innate antimicrobial peptide expressed at mucosal surfaces including the bladder, but little is known about its effect on urinary tract infection\textsuperscript{57,66,67} (Figure 1.1). SLPI is one of 18 proteins in the whey acidic protein (WAP) family, only a few of which have been characterized past the gene level\textsuperscript{68}. A defining characteristic of this family is the prevalence of intramolecular disulfide bonds. SLPI, a 12 kD cationic protein with 107 amino acids, contains 8 of these bonds connecting its two domains: C and N\textsuperscript{68}. The C domain functions as an inhibitor of serine proteases while the N domain is associated with antimicrobial activity\textsuperscript{68}. Interestingly, these functions appear to be independent as truncated SLPI to include only the C or N terminal allows it to retain antiprotease or antimicrobial function respectively\textsuperscript{69–71}. Although, comparatively, intact SLPI is more efficient\textsuperscript{69}. Furthermore, even when covalently bound to the extracellular matrix physically blocking it from damage, SLPI still preserves its antiprotease activity\textsuperscript{72}.

1.7.1 Functions of SLPI

Antiprotease function of SLPI against serine proteases has been well established. At the site of inflammation, recruited neutrophils and activated mast cells release serine proteases: neutrophil elastase (NE), cathepsin G, chymase and tryptase, to increase local inflammation\textsuperscript{50}. SLPI also shows activity against digestive enzymes trypsin and chymotrypsin constitutively
expressed in the gut. Crystallization of SLPI complexed to NE has allowed further examination of this interaction. This complex increases in inflamed states of the lung and skin.

Originally discovered for its antiprotease activity, SLPI was found to also function as an antimicrobial demonstrating activity against gram positive and negative bacteria, fungi, protozoa and viruses. Not only does SLPI kill bacteria in culture as shown with *Escherichia coli* and *Staphylococcus aureus*, but a cell free system also suggests that it can bind bacterial mRNA and DNA to prevent protein synthesis. SLPI is also shown to directly bind *Neisseria gonorrhoeae* Opa protein expressed on the surface of bacteria. In addition to bacteria, cell culture assays show SLPI to associate with *Candida albicans*, a major cause of vaginal yeast infections, to prevent invasion of vaginal epithelial cells. Moreover, infection of Slpi−/− mice with *Leishmania major* showed increased parasite burden in Slpi−/− both at the site of injection and distal organs showing the infection becomes systemic without SLPI onboard. A large amount of research has investigated the role of SLPI in HIV infection and found it reduces perinatal transmission by competitively binding annexin A2 (AnxA2) to block viral invasion of macrophages. This cell surface receptor, AnxA2, is also expressed on bladder epithelium and may prevent UPEC expressing Yad fimbriae from invading the urothelium.

Some microbes have developed mechanisms to combat the bactericidal effects of SLPI. *Pseudomonas aeruginosa* can secrete pseudolysin, a specialized protease that cleaves SLPI. Additionally, a gastrointestinal cell line infected with *Helicobacter pylori* also demonstrates the ability to cleave SLPI. This could explain why biopsies from patients with *H. pylori*-induced gastritis have low levels of SLPI secretion in the antrum.

SLPI is also known to modulate inflammation and there are multiple hypotheses on the mechanism for this. One proposed hypothesis is the downregulation of immune cell recruitment
as shown in a guinea pig model of allergic conjunctivitis in which topically applied SLPI reduced eosinophil recruitment in infected animals\textsuperscript{86}. Other data supports the hypothesis that SLPI can modulate activity of activated immune cells. One study showed SLPI can prevent secretion of histamine from IgE-activated mast cells in the lung\textsuperscript{87}. Inside monocytes, SLPI can inhibit synthesis of MMP9, a protease that aids neutrophil migration through tissue\textsuperscript{88}. Furthermore, neutrophil phagocytosis, oxidative burst and NETosis can also be inhibited by SLPI\textsuperscript{80,89}. The third hypothesis is also the one most studied: SLPI inhibits the NFκB pathway to reduce inflammation. This direct inhibition has been demonstrated \textit{in vitro} to show SLPI can be taken up by monocytes and activated macrophages\textsuperscript{90}. Once inside the cell, SLPI translocates to the nucleus to bind NFκB promoter regions of inflammatory cytokines thus preventing their transcription to decrease inflammation\textsuperscript{90}. Macrophages and monocytes are known to produce and secrete SLPI, but whether or not it can be taken up \textit{in vivo} is still unclear. SLPI may inhibit NFκB indirectly by preventing degradation of NFκB inhibitors IkBα and IkBβ\textsuperscript{90}.

1.7.2 Expression and regulation of SLPI

SLPI is constitutively expressed in mucosal tissue of the respiratory, gut and urogenital tracts and is secreted by epithelial cells, neutrophils and macrophages into mucosal fluids including saliva, nasal mucus, vaginal secretions and urine\textsuperscript{50,66}. SLPI production is stimulated by microbial products LPS and viral RNA\textsuperscript{91,92}. Colonization of germ-free mice confirmed SLPI is upregulated in gut epithelial cells upon contact with bacteria, implying exposure to microbes and their products can induce production of SLPI\textsuperscript{93}. In addition to PAMPs upregulating SLPI, a variety of proinflammatory cytokines have also been reported to do this including INFγ, TNFα,
IL-1β, IL6 and TSLP\textsuperscript{73,91,94–96}. In return, SLPI can induce macrophages to secrete TGFB and IL-10 to reduce inflammation\textsuperscript{97}.

Estrogen increases production of AMPs including SLPI\textsuperscript{98–101}. In women, SLPI and estrogen levels are highest during the ovulatory stage\textsuperscript{98}. Indeed, cell culture assays confirm incubation of vaginal epithelial cells with estrogen increases SLPI production\textsuperscript{99}. Post-menopausal women have low levels of estrogen which can increase risk of developing rUTI\textsuperscript{102}. Vaginal application of oestrogen has reduced frequency rUTI\textsuperscript{22}. Estrogen also reduces epithelial cell exfoliation\textsuperscript{100}. Taken together, the reduction in urothelium cell turnover and increased concentrations of AMPs may act to prevent QIR activation and therefore their contribution to rUTIs\textsuperscript{100,101,103}.

1.7.3 SLPI in mouse models of disease

SLPI has been studied in a variety of diseases. The similarity in structure of human and murine slpi has made mouse models a promising technique for the study of SLPI in disease states relevant to humans\textsuperscript{104}. Development of a Slpi\textsuperscript{-/-} mouse has aided understanding of the specific role this protein has \textit{in vivo}\textsuperscript{96}.

Three models in which SLPI has been studied include wound healing, airway inflammation and lower reproductive tract infections. Since the discovery of SLPI in saliva in the 1980’s, a large amount of research has been dedicated to the study of SLPI in airway conditions\textsuperscript{105}. In the lung, SLPI is found in high abundance in part due to its sensitivity to oxidation from having multiple methionine residues\textsuperscript{50,82}. Meaning, a large amount of SLPI may be required to maintain homeostatic levels. Being highly expressed in the lung led to research on the role of SLPI in cystic fibrosis and asthma\textsuperscript{105}. Cystic fibrosis patients have high levels of NE
that worsen disease. Tested as a possible therapeutic, SLPI was aerosolized into the lungs of these patients and reduced NE and CXCL8, a neutrophil chemoattractant\textsuperscript{106}. However, it was unable to reach more diseased areas of the lung and has since been abandoned\textsuperscript{106}. In COPD and asthma, studies have found lower levels of SLPI in diseased patients compared to controls\textsuperscript{107,108}.

An ovalbumin mouse model of allergic airway inflammation (AAI), reported Slpi\textsuperscript{-/-} mice have increased eosinophil infiltration, goblet cells hyperplasia and worsened lung function\textsuperscript{91}. Using this model, another study showed SLPI to modulate airway microbiota\textsuperscript{108}.

SLPI is upregulated in human cutaneous wounds and improves healing by inhibiting host proteases and regulating inflammation\textsuperscript{76}. A group studying wound healing introduced a Slpi\textsuperscript{-/-} mouse line to study the specific effects SLPI has on chronic wounds\textsuperscript{96}. Wounded Slpi\textsuperscript{-/-} mice have reduced rates of clotting and healing as well as increased levels of TNF\textgreek{a}, neutrophils and NE\textsuperscript{96}. Activated neutrophils secrete NE which, in excess, can be detrimental to host tissue\textsuperscript{50,61}.

SLPI inhibiting NE improves wound healing in two ways: reduces host tissue damage and prevents cleavage of proepthilin, a growth factor\textsuperscript{96,109}. Inhibiting NE from digesting proepthilin to epithilin will increase cell growth, reduce both inflammation and neutrophil recruitment\textsuperscript{109}.

Lastly, SLPI is believed to have a protective role in lower reproductive tract infections. Lower levels of SLPI in the reproductive tract is thought to increase susceptibility to infection\textsuperscript{110}. One study found decreased SLPI levels in vaginal secretions of women with lower reproductive tract infections\textsuperscript{110}. SLPI may also be decreased in pregnant women with asymptomatic trichomoniasis\textsuperscript{9}. As previously mentioned, SLPI demonstrates antimicrobial activity against \textit{Candida albicans}, \textit{Neisseria gonorrhoeae} and HIV to prevent infection\textsuperscript{50}.

Seeing that SLPI plays a role in lower reproductive tract infection, we questioned whether it was also part of the host response to urinary tract infection and found this to be a gap
in knowledge. To investigate this we use a 129;BL/6 SLPI\textsuperscript{tm1Ssw/J} (Slpi\textsuperscript{-/-}) mice and a well
established model of UTI that strongly parallels disease in humans\textsuperscript{111}. C57BL/6 mice robustly
exfoliate bladder epithelial cells in response to UTI and can therefore clear bacteriuria within 7-
10 days\textsuperscript{9,24,112}. More than half of these mice will clear bacteria in the urine within 48 hpi,
however, they are still susceptible to rUTI possibly through QIR formation\textsuperscript{8,9,112}. To combat
rapid clearance of UPEC, a higher inoculum of 10\textsuperscript{7}-10\textsuperscript{8} CFU is often used to infect this mouse
strain\textsuperscript{56}.
Figure 1.3 Proposed mechanisms of SLPI in UTI

A. The antiprotease function of SLPI could inhibit serine proteases secreted by activated neutrophils including neutrophil elastase (NE) and cathepsin G. SLPI may also be found decorated on NETs following NETosis. Alternatively, SLPI may act against serine proteases secreted by UPEC to inhibit invasion of host epithelial cells.
B. As an antimicrobial protein, SLPI may exhibit bactericidal effects on invading UPEC though binding of surface molecules to inhibit adherence to the epithelium. SLPI may act against molecular molecules secreted by UPEC. Additionally, SLPI may prevent translation of proteins through direct binding of DNA and mRNA.

C. SLPI also demonstrated anti-inflammatory effects. This may be achieved through inhibition of the NFκB pathway which promotes inflammation through transcription of pro-inflammatory cytokines. Some evidence suggests it may translocate to the nucleus and directly bind promoter sites for NFκB genes. SLPI may also inhibit recruitment of immune cells to the bladder.
Chapter 2

Role of secretory leukocyte protease inhibitor in urinary tract infection
2.1 Introduction

Urinary tract infections (UTIs) affect approximately 150 million people per year worldwide\textsuperscript{1,2}. One year in the United States alone sees 10 million outpatient and emergency room visits with over 3 billion dollars spent toward sick leave and treatment of these patients, the majority of whom are women\textsuperscript{1–4}. Women are at higher risk of developing a UTI due to length and proximity of the urethra to other orifices where bacteria are abundant\textsuperscript{5,6}. More than 50% of women will experience a UTI in their lifetime, of which 25% are likely to suffer from a second infection within 6 months and risk causing future recurrent infections\textsuperscript{7–9}.

Uropathogenic \textit{Escherichia coli} (UPEC) causes more than 75% of community-acquired and 50% of nosocomial infections\textsuperscript{1,14}. Although commensal to the gut microbiome, these species become pathogenic in the urinary tract\textsuperscript{16}. In a mouse model of UTI, UPEC upregulates production of proteases and cellular adhesion molecules, including type I pili, to aid epithelial cell invasion\textsuperscript{16,17}. While protected from host defenses inside the cell, bacteria rapidly expand replication to form intracellular bacterial communities (IBCs). Some infected cells undergo exfoliation and apoptosis, resulting in cell contents being spilled into the urine\textsuperscript{9,23}.

Due to the increasing number of multidrug resistance genes found in UPEC strains, accurate diagnosis of a UTI is crucial\textsuperscript{2,113}. Currently, clinical diagnosis of a UTI uses an antiquated method of culturing bacteria from clean-catch urine specimens and applies a cutoff of $10^5$ colony forming units per milliliter (CFU/ml) to label a culture as positive\textsuperscript{103}. However, in cases of asymptomatic bacteriuria, patients return a positive culture in the absence of UTI symptoms\textsuperscript{40}. Furthermore, \textit{E. coli} is the species most frequently isolated from these patients and therefore significantly complicates distinguishing a UTI from other conditions\textsuperscript{40,41}. Recently, use of a more sensitive culturing technique called expanded quantitative urine culture (EQUC) has
provided evidence of a urinary microbiome in healthy people, challenging the belief that urine is sterile\textsuperscript{27,44}. In addition to culturing, urinalysis can help diagnose infection, but has a high false positive rate\textsuperscript{25}. Development of other biomarkers of UTI may assist in distinguishing a current infection from other conditions.

In response to a UTI, the host increases production of antimicrobial peptides (AMPs) as part of the innate immune response\textsuperscript{49}. These molecules, often small, cationic proteins, serve to fight invading pathogens. One AMP, secretory leukocyte protease inhibitor (SLPI), is known to be expressed by mucosal tissues, namely epithelial cells, and secreted into fluids\textsuperscript{50,66}. Some evidence shows SLPI is expressed in the bladder and excreted in urine, but has not been well characterized for its role in urinary tract infection\textsuperscript{57,66,67}. SLPI responds to LPS and demonstrates specific bactericidal activity against \textit{E. coli in vitro}\textsuperscript{69,92}. Additionally, reduced levels of SLPI in vaginal secretions of women with lower reproductive infections supports the idea that it is protective in this region\textsuperscript{110}. Indeed, SLPI can bind \textit{Neisseria gonorrhoeae} and \textit{Candida albicans} to prevent invasion of host epithelial cells\textsuperscript{77,78}. Along with antimicrobial activity, SLPI also possesses antiprotease and anti-inflammatory functions the latter of which are attributed to inhibition of the NFκB pathway from transcribing pro-inflammatory cytokines including TNFα\textsuperscript{90}.

In this study, we inoculated wild-type (WT) and \textit{slpi} knock out (\textit{Slpi}\textsuperscript{-/-}) mice with UPEC to investigate the role of SLPI in urinary tract infection. To determine whether it may be a useful biomarker for the diagnosis of UTI, we characterized the production of SLPI during infection and also examined the potential for SLPI to exhibit antiprotease and anti-inflammatory functions.
2.2 Results

2.2.1 Mouse model of UTI with UTI89-Kan\(^R\)

To study the effect of SLPI \textit{in vivo}, we used 129;BL/6 SLPI\(^{tm1Smw}\)/J mice which contain a nucleotide insertion that terminates SLPI gene expression\(^{96}\). Mice were backcrossed to C57BL/6J mice for 9 generations and homozygous breeding was used to produce WT and Slpi\(^{-/-}\) mice for experiments. Homozygous breeding was used to optimize experimental groups since Slpi\(^{-/-}\) mice tend to have infrequent litters with fewer pups surviving to weaning age compared to littermate WT mice (data not shown). Originally, the Slpi\(^{-/-}\) model was developed to characterize the roles of this protein in wound healing, but have also been used to explore the role of SLPI in immune tolerance and parasitic infection\(^{79,96,114}\).

We used an established model of mouse urinary tract infection to investigate the role of SLPI in the urinary tract (Figure 2.1). Briefly, we grew UTI89-Kan\(^R\), originally a UPEC clinical isolate, under static conditions to induce expression of type I pili on the bacterial surface\(^{18}\). This structure binds mannosides expressed on bladder epithelial cells and is crucial for efficient colonization of the bladder and kidney\(^{42,115}\). This strain has been modified to contain a kanamycin resistance gene on the bacterial chromosome\(^{56}\). Prior to inoculation, UTI89-Kan\(^R\) was resuspended in sterile 1X PBS at a concentration sufficient to deliver \(10^7\)-\(10^8\) CFU to the bladder\(^9\). A higher concentration inoculum is often used in C57BL/6 mice due to their robust exfoliation response and therefore fast clearance of bacteriuria compared to C3H/HeN mice, which are also commonly used to study UTI\(^9\). Urine was collected before transurethral inoculation of female mice and also at 3, 7, 12, 24 and 48 hours post infection (hpi). As part of the innate immune response, SLPI is upregulated early on during \textit{in vitro} models of bacterial
invasion. To investigate the roles of SLPI in the early response to UPEC, we included multiple time points for urine collection within a few hours of infection. Submandibular bleed was also performed on a subset of mice in which urine was collected prior to bleeding and also before bladder inoculation. At 12, 24 or 48 hpi, mice were euthanized by isoflurane overdose and bilateral thoracotomy before harvesting blood, bladder and kidney.

2.2.2. Increased bacterial burden in urinary tracts of S1pi−/− mice

Previous groups show SLPI has direct antimicrobial properties with specific bactericidal activity against *E. coli in vitro*. To test whether this was the case *in vivo*, we infected mice as described and plated sample dilution series on LB supplemented with kanamycin to select for UTI89-KanR. Plates were incubated overnight and CFU/ml or g were calculated the following day. In urine, we found S1pi−/− mice tended to have higher UTI89-KanR titers than WT overall with the 24 hpi timepoint showing a 10-fold higher titer in S1pi−/− mice compared to WT (p=0.002; Figure 2.2A). Although S1pi−/− mice tended to have higher titers overall, there were no significant differences found at other time points in the urine. Notably, two WT mice had urine counts below the limit of detection (LOD) at 48 hpi. We also saw that S1pi−/− mice had a trend towards higher UTI89-KanR titers in bladder tissue at 24 (p=0.06) and 48 hpi (p=0.08, Figure 2.2B). At 24 hpi, S1pi−/− mice also had higher UTI89-KanR titers than WT in the kidneys although a majority of samples from each genotype were below the LOD (p=0.02, Figure 2.2C). These data show S1pi−/− mice tend to have higher amounts of bacteria in the urinary tract thereby supporting our hypothesis that SLPI plays a role in urinary tract infection.
2.2.3 UTI leads to prolonged elevation of SLPI in urine of WT mice

Previous data showing upregulation of the slpi gene in the bladder suggested that SLPI protein may be increased as well as a result of UTI. However, given that UTI9-KanR titers in the urine were increased in Slpi−/− mice and knowing SLPI is normally a secreted protein, we thought to first examine urine for changes in SLPI concentration throughout infection. To investigate this, we measured SLPI in urine samples collected before and after infection in our WT mice (Figure 2.3). We found that prior to infection, all mice had low basal levels of SLPI in their urine. Strikingly, starting as early as 3 hpi, we observed a marked increase in urine SLPI in mice infected with UTI9-KanR. Urine SLPI levels peaked at 3 hpi then gradually decreased over the course of infection out to 48 hours, with all timepoints remaining above baseline on average. This result demonstrates that a UTI results in rapid upregulation of SLPI which remains elevated up to two days following infection.

2.2.4 Bacterial load and SLPI are positively correlated in urine past 12 hpi

SLPI is upregulated in the presence of bacteria and bacterial products like LPS. In this study, we have demonstrated that bacterial titers and SLPI concentration change in the urine during a UTI. We asked whether this increase in SLPI was related to the amount of bacteria present in the urine. Spearman’s rank correlation coefficient was used to test these two variables and showed no correlation at time points before 12 hpi (p = 0.61, rho = -0.14) (Figure 2.4A). However, 12, 24 and 48 hpi, we found that the concentration of SLPI and UTI9-KanR titers in the urine are positively correlated (p = 1.76x10^-8, rho = 0.73) (Figure 2.4B). These data suggest that after 12 hours, the amount of SLPI found in urine is closely associated with the amount of bacteria present, however earlier time points do not show this trend.
2.2.5 SLPI concentration predicts infection in WT urine

Upon discovering that SLPI concentration is linked to the amount of bacteria present in the urine, we asked whether SLPI concentration could be used to predict if a WT mouse has an infection. To test this idea, we generated a receiver operating characteristic (ROC) curve which tests different classification thresholds to distinguish a true positive (TP) from a false positive (FP). The rate of TP and FP is plotted as a line and the area under the curve (AUC) is calculated as a range from 0-1. The AUC represents the performance of all thresholds with values closer to 1 meaning the variable being tested is more likely to be a “perfect” classifier. Using this method, we show that SLPI concentrations successfully distinguish infected from uninfected urine samples (AUC = 0.91) (Figure 2.5). Additionally, the optimal threshold can be extracted from the ROC curve analysis to visualize how samples were classified. In this case, our analysis found this value to be 1.07 ng/ml of SLPI (Figure 2.4 dashed line). Using this threshold, more than 80% of samples were classified correctly as infected or uninfected (uninfected = 25, infected = 44; Figure 2.5 inset). Taken together, these data show that SLPI concentration can be used to distinguish infected versus uninfected urine samples from WT mice.

2.2.6 Recombinant human SLPI inhibits UTI89-KanR secreted proteases

SLPI has been extensively studied for its inhibitory effects on serine proteases produced by host cells including neutrophil elastase (NE), cathepsin G, and chymase. Secretion of NE from recruited neutrophils is increased in the lung during airway inflammation and is inhibited by SLPI. During an infection, bacteria like UPEC are also known to secrete proteases. UPEC strains produce and secrete serine proteases like SPATE and Pic/PicU to aid in tissue colonization. However, the effect of SLPI on UPEC proteases has not yet been explored. We
investigate whether recombinant human SLPI (rSLPI) has the capability to inhibit UPEC proteases by incubating rSLPI with supernatant from UTI89-KanR culture in presence of casein, a substrate for proteases. Protease activity of UTI89-KanR supernatant samples were measured with the addition of 5, 0.5 and 0.05 ug/ml of rSLPI (Figure 2.6). Results showed a dose-dependent decrease in protease activity within 15 minutes of incubation. This data provides evidence for the first time that rSLPI may also inhibit bacterial proteases during a urinary tract infection.

2.2.7 TNFα trends higher in infected Slpi−/− bladder

Previous studies that have investigated the role of SLPI in modulating inflammation in mouse models of asthma and colitis have shown increased levels of TNFα in Slpi−/− mice91,95. This finding is attributed to the ability of SLPI to inhibit activation of the NFκB pathway to reduce local inflammation90,91,95. An in vitro study found mast cells secrete TNFα that can then activate NFκB in urothelial cells62. This pathway is also activated during a urinary tract infection in response to LPS to upregulate expression of inflammatory cytokines including TNFα58,118. We tested whether this finding would hold true in our mouse model of UTI by measuring the amount of TNFα in homogenized bladder tissue from Slpi−/− and WT mice (Figure 2.7). We saw Slpi−/− mice tended to have higher levels of TNFα compared to WT. In congruence with previous findings, this data shows that Slpi−/− mice have increased inflammation during UTI perhaps due to the inability to block transcription of NFκB related genes.
2.3 Discussion

SLPI is known to be expressed on mucosal surfaces including the respiratory, intestinal and urogenital tracts. The role of SLPI in airway inflammation and wound healing is well studied, but only one group has addressed the regulation of SLPI in response to UTI using a spinal cord injury model in rats. After 24 hours of UPEC infection, sham rats had higher amounts of slpi transcripts in bladder tissue compared to injured rats. This data suggests that SLPI may be involved in innate immune response to bacterial insult, but the specific role of SLPI in urinary tract infection has not yet been described.

To fill this gap in knowledge, we used a Slpi⁻/⁻ mouse model to better understand the role SLPI plays in the host response to UTI. First, we inoculated mice with UTI89-Kan and found that Slpi⁻/⁻ mice tended to have increased bacterial titers in the urine, bladder and kidney compared to WT. This trend held true for most of the collected time points, however we observed the strongest difference at 24 hpi. This timepoint seems to be important in other human, mouse and cell culture models examining the role of SLPI as well. This may be attributed to a rapid increase in SLPI early on that declines as other members of the immune system come into play.

In WT mice, high SLPI concentrations in early time points supports our hypothesis that there is a rapid increase in SLPI. In fact, we saw an approximate 8 fold increase just 3 hours after bladder inoculation that gradually declined over time. As part of the innate immune system, it is not surprising that SLPI levels increased quickly. A similar finding has been shown for cathelicidin, another AMP found in urine. In vitro infection of bladder epithelial cells with UTI89 revealed increased cathelicidin transcripts that peaked just 5 minutes after infection and returned to control levels after 2 hours. Like other antimicrobials produced in response to
innate immune signaling, our data suggests that SLPI is quickly produced or secreted in large quantities to respond to bacterial insult. We hypothesize that transcription of SLPI decreases as other host defenses are activated to take control of the infection. Previous research shows that inflammatory cytokines that signal recruitment of immune cells are upregulated within 1 hour of infection in the urinary tract\textsuperscript{48,58}. Within 2 hpi, neutrophils arrive in the lumen of the infected bladder and peak in abundance at 6 hpi\textsuperscript{59,60}. Furthermore, we show UTI89-Kan\textsuperscript{R} titers and SLPI concentration are correlated in the urine at 12, 24 and 48 hpi, but not at earlier time points (Figure 2.4). Taken together, our data suggests that the concentration of SLPI before 12 hours is not proportional to the severity of infection. It’s possible that interactions taking place between SLPI, pro-inflammatory cytokines and immune cells within the first few hours of infection will still affect the immune response after 12 hpi and may in part explain the difference in bacterial burden at 24 hpi.

SLPI is known to have antimicrobial, antiprotease and anti-inflammatory properties and could therefore contribute to fighting UTI in multiple ways\textsuperscript{50}. \textit{In vitro} assays demonstrate SLPI to have bactericidal effects against gram negative bacteria including \textit{E. coli}\textsuperscript{69}. Additionally, SLPI binds LPS and CD14 thereby preventing LPS-CD14 complexes from forming and being taken up by macrophages in cell culture\textsuperscript{118}. This implies SLPI may also directly bind bacteria. Or, like other cationic AMPs, SLPI could be killing UTI89 by destabilizing its membrane\textsuperscript{49,55}. Yet another way SLPI may inhibit bacterial function is through its interaction with nucleic acids. SLPI has the potential to block protein synthesis by binding negatively charged mRNA and DNA in a cell free system\textsuperscript{14}. Such a mechanism would be dependent on the ability of SLPI to enter bacterial cells which has not yet been shown. However, an observation we made while attempting to express rSLPI (without a signal peptide) in \textit{E. coli} revealed reduced growth in
cultures even before IPTG induction (data not shown). This implies that SLPI expressed inside a bacterial cell can still carry out bactericidal activity. We have since adapted our protocol to include a plasmid containing GST-fusion to dampen the antimicrobial activity of SLPI which appears to have resolved the growth inhibition we observed when expressing SLPI alone (data not shown). A future direction we plan to pursue is to create a transposon library of mutant UTI89 and culture it with and without rSLPI to identify specific bacterial genes targeted by SLPI antimicrobial activity.

Another mechanism SLPI may employ to defend the host is through inhibition of proteases. Many groups have studied the ability of SLPI to directly inhibit neutrophil elastase (NE) in lung inflammation. Once recruited to the site of inflammation, neutrophils release non-specific proteases like NE that increase inflammation. However, NE can also attack host tissue to cause damage and in excess, actually slows the healing rate of tissue as shown in a mouse model of chronic wounds. To combat this detrimental effect on the host, neutrophils synthesize and release SLPI at the same time. This restores balance to the inflammatory environment and keeps host tissue damage under control. Therefore, Slpi−/− mice may have more inflammation in part due to reduced inhibition of host proteases like NE. In addition to its effects on endogenous proteases, we show that SLPI may also inhibit bacterial proteases (Figure 2.6). UPEC strains secrete serine proteases that weaken host tissue. Some of these proteases are important for colonization and invasion of epithelial cells. In this study, we show that rSLPI inhibits bacterial proteases secreted by UTI89-KanR in a dose dependent manner. This evidence suggests that SLPI may also act directly against bacterial proteases, which would suggest another mechanism SLPI may use to combat bacterial invasion.
In addition to these functions, SLPI also possesses anti-inflammatory properties that may be involved in fighting urinary tract infection. SLPI modulates inflammation by reducing LPS activation of TLR4 and therefore downregulating transcription of NFκB pro-inflammatory genes including TNFα\textsuperscript{90,118}. This may be achieved by preventing activation of IkBβ, the molecule that translocates to the nucleus to transcribe genes, or by competitive binding of SLPI to DNA in the nucleus to prevent transcription\textsuperscript{90}. We show Slpi\textsuperscript{-/-} mice tend to have increased levels of TNFα in bladder tissue at 24 hpi, although this effect may be due to the anti-inflammatory effects of SLPI or worsened UTI severity (Figure 2.7). While this is consistent with previous findings, further investigation of other inflammatory markers is needed to better understand if and how SLPI modulates inflammation in the urinary tract.

In our mouse model of UTI, increased SLPI in the urine is positively correlated to the amount of bacteria present starting at 12 hpi. We questioned whether SLPI could be used as a biomarker of UTI as current methods of diagnosis are under scrutiny in the scientific community\textsuperscript{25,27}. Not only is the gold standard culturing technique limited in selectivity, but ABU and evidence of a urinary microbiome can lead to false positive results and subsequent unnecessary antibiotic treatment. To investigate if SLPI would be a good candidate, we used a receiver operating characteristic (ROC) curve to show that SLPI measured in urine can distinguish infected and uninfected samples with a high level of accuracy with the model correctly classifying 80% of samples (Figure 2.5 inset). This model performs better at early time points of UTI likely due to the flood of SLPI produced within the first few hours. After 12 hpi, bacteria begin to clear and SLPI levels decrease. At 48 hpi, two mice had bacterial titers below the LOD which is expected in C57BL/6 mice as a result of their rapid clearance of bacteriuria\textsuperscript{8,9}. Although SLPI levels between baseline and 48 hpi are significantly different, the model struggles
to classify the mice that have titers below the LOD even though they still have bacteria in the bladder (Figure 2.5). However, the bacteria in the bladders of these mice could be mostly intracellular at this time period. More experiments including a long term UTI would help to clarify this observation.

One question we are still addressing is where SLPI comes from in a mouse model of UTI. SLPI is synthesized and secreted by multiple cell types including epithelial cells and macrophages in response to cytokine and LPS stimuli respectively\(^{92,108}\). Neutrophils are also a major source of SLPI and release it into the extracellular environment when activated\(^8\). Our findings imply that SLPI is likely produced by epithelial cells since a large quantity of SLPI is already present in the urine at 3 hpi and is unlikely to originate solely from recruited cells. However, this does not completely rule out the possibility that resident macrophages could be significantly contributing to this production and although neutrophils are shown to be most abundant at 6 hpi, it’s possible they may contribute to urine SLPI levels very early in UTI.

One way in which we attempted to answer this question was to compare serum levels of SLPI before UTI and 24 hpi. We took submandibular bleeds before infecting mice and collected urine before each manipulation. Serum levels of SLPI in these mice did not seem to change significantly over the 24 hour infection (data not shown), however we discovered all time points after submandibular bleed had increased levels of SLPI in the urine (Supplemental Figure 2.1). We saw increased levels of SLPI in urine collected right before bladder inoculation (0 hpi), compared to urine before the submandibular bleed (-2 hpi) which closely resembled baseline from other experiments. While this increase in SLPI following submandibular bleed was unexpected, we hypothesize this excess SLPI is produced at the mandible to aid in wound healing and excess amounts are filtered from the blood to be excreted in the urine. Intravenous
injection of SLPI in rats, dogs and humans has resulted in high levels of SLPI in the urine\textsuperscript{67,123}. Serum SLPI levels also increase during infection\textsuperscript{67}. Taken together, these findings suggest that exceeding the homeostatic level of SLPI in the blood leads to excretion of SLPI in the urine. In our model, a better way to investigate where SLPI is coming from is to perform flow cytometry on infected bladder tissue. This will inform us what cells are recruited to the site of infection at specific timepoints. Based on those experiments, we plan to use a mouse cre/LoxP system to knock out SLPI in one cell type to reveal its contribution during a UTI. Currently, we plan to examine this by breeding albumin-cre, to diminish SLPI from circulating immune cells, and uroplakin-cre, to knock out SLPI in the bladder epithelium.

One limitation of this current study is that the mice we used are incompletely backcrossed. Mice arrived to us as a mixed background 129 and C57BL/6J and we are in the process of fully backcrossing them to a BL/6 background. Mice with mixed backgrounds can have phenotypic differences from fully backcrossed versions and be a source of variability\textsuperscript{124}. Traditionally, a fully backcrossed mouse is one that has been bred to the desired background for a minimum of 10 generations (N10)\textsuperscript{125}. For these experiments, we used N5 generation mice with the intention of confirming our findings with N9 and N10 mice at a later date. So far, we have not seen any noticeable differences between the two generations, but experiments with fully backcrossed mice will be done to confirm our findings. We also hope to use heterozygous breeding with WT littermate controls in future experiments as well, however the viability of Slpi\textsuperscript{+-} mice we observed will make this challenging.

Another limitation of the current study is the lack of translation to humans. Thus far, we have successfully shown SLPI to be important in a mouse model of UTI, but this has yet to be investigated in humans. One connection we make in this study is to show recombinant human
SLPI inhibits the activity of proteases secreted by UTI89-KanR (Figure 2.6). Additionally, ongoing experiments using 5637 cells, an immortalized human bladder cell line, provide evidence that SLPI is secreted by bladder epithelial cells in response to infection with UTI89-KanR (data not shown). To unambiguously show that SLPI is upregulated during urinary tract infection in humans, we plan to measure and compare SLPI levels in urine samples from patients with and without UTI. These experiments will be used to guide future directions with the goal of developing a biomarker of urinary tract infection to reduce misdiagnosis and prevent further evolution of multidrug-resistant bacteria.

In conclusion, we demonstrate in this study that SLPI plays a protective role in urinary tract infection to reduce bacterial burden. Urine SLPI levels increase rapidly within the first few hours of infection and remain elevated up to 48 hours afterward. In addition to antimicrobial properties, this protein may also utilize antiprotease and anti-inflammatory properties to control infection and reduce inflammation in the bladder. We propose that SLPI levels can also be used to predict infection in a mouse model and may prove to be a biomarker of UTI in humans. Further investigation into human urinary tract infection is needed to evaluate the translation of these findings.
2.4 Materials and Methods

Mice

All animal procedures were reviewed by the Washington University Institutional Animal Care and Use Committee (Protocol#: 20180286). Cryo-recovered 129;BL/6 SLPI<sup>tm1Smw</sup>/J mice (Jackson Laboratories Bar Harbor, ME) were backcrossed to WT BL/6 mice for 9 generations. Mice used in this study were from the 5th and 9th generations.

Bacterial Strains and Cultivation

A kanamycin-resistant derivative of a human cystitis isolate, UTI89: UTI89-Kan<sup>R</sup> provided by Scott J Hultgren was used to infect mouse bladders.

Mouse Infection

Bacterial cultures were started from a single colony in 20 ml of Luria-Bertani broth (LB) and grown statically at 37C overnight, subcultured 1:1000 into 20 ml of sterile LB and grown statically at 37C for 18-24 hours. Bacteria were spun down (3,000rpm for 15 min) and resuspended in sterile 1X PBS. Inoculum was diluted to a final concentration of 1-2x10<sup>9</sup> colony forming units (cfu)/ml (1:10 OD<sub>600</sub>=0.25-0.28) and 50 ul (~0.5-1x10<sup>8</sup>cfu) was used to inoculate the bladders of 6-7 week old female mice by transurethral catheterization under 2.5% isoflurane.

Urine Collection and Bacterial Titering

Urine was collected at 0, 3, 7, 12, 24 and 48 hours post infection by applying suprapubic pressure and collecting the urine stream into a sterile 1.5 ml Eppendorf tube. Urine samples were
serially diluted 1:10 in sterile PBS and 5 ul of each dilution was spotted onto LB agar supplemented with 50 ug/ml kanamycin (LB/Kan50) five times. Colony counts were averaged for the highest dilution containing colonies in 4 or more replicates and CFU/ml was calculated. Remaining urine sample was frozen at -80C.

Tissue Bacterial Titering

Bladder and kidneys were aseptically harvested into pre-weighed tubes containing 1 ml of sterile PBS. Final weights were measured then samples were homogenized. Samples were serially diluted 1:100 in sterile PBS and titered as described above to calculated CFU/g of tissue.

Protein Quantification by ELISA

Urine samples were thawed and diluted 1:10 (pre-infected) or 1:20 (infected) in 1XPBS + 1% BSA. SLPI was measured using Mouse SLPI DuoSet ELISA (R&D Systems DY1735-05) according to manufacturer’s instructions.

Homogenized bladder tissue was spun down (10,000rpm, 5 min) and supernatant was transferred to a 0.22 um SpinX column (Costar CS8160) to remove bacteria. Filtered samples were saved at -20C until use. TNFα was measured by ELISA (BioLegend 430901) on undiluted samples following manufacturer’s instructions.

Protease activity

UTI89-KanR was grown under conditions previously explained. Culture was spun down (3,000rpm, 15min) and supernatant was filtered through 0.22 um SpinX columns to remove
residual bacteria. Protease activity was measured on supernatant samples using the Pierce Fluorescent Protease Assay Kit (ThermoFisher 23266) following manufacturer’s instructions.
Figure 2.1: Schematic of UTI mouse model
Transurethral inoculation of 6-7 week old female WT and Slpi−/− mice with 10^7-10^8 CFU of UTI89-Kan^R. Mice were infected for 12-48 hours. Optional submandibular bleed 2 hours before infection.
Figure 2.2: Increased bacterial burden in urinary tract of Slpi⁻/⁻ mice

A. Urine was collected at 3, 7, 12, 24 and 48 hpi and titers determined.
B. Mice were sacrificed and harvested. Bladder and kidney were homogenized and plated for titers at 12, 24 and 48 hpi (B and C).

Shape denotes number of backcrosses to C57BL/6 background. Dashed line represents the limit of detection for that specimen.
Statistical significance: one-tailed Wilcoxon rank-sum test, boxes indicate 25th and 75th percentiles and wiskers are 1.5 x interquartile range, *p<0.05, **p<0.01, ***p<0.001.
Figure 2.3: UTI leads to prolonged elevation of SLPI in urine of WT mice
ELISA quantification of SLPI in urine from before and after infection of WT mice.
Dashed line indicates optimal threshold (1.07 ng/ml) determined in Fig2.5.
Statistical significance: two-tailed Wilcoxon rank-sum test, boxes indicate 25th and 75th percentiles and whiskers are 1.5 x interquartile range, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 2.4: Bacterial load and SLPI are correlated after 12 hpi

A. Correlation between SLPI and UTI89-Kan^R present in the urine at 3 and 7 hpi.

B. Correlation between SLPI and UTI89-Kan^R present in the urine at 12, 24 and 48 hpi.

Dashed line indicates limit of detection for that specimen.
Statistical significance: Spearman’s rank-order correlation.
Figure 2.5: Urine SLPI concentration predicts infection in WT mice
Receiver operating characteristic (ROC) curve analysis of SLPI levels in urine to test to classify infected and uninfected urine. Classification of samples at optimal threshold.
Figure 2.6: Recombinant human SLPI inhibits UTI89-Kan\textsuperscript{R} secreted proteases

UTI89 Kan\textsuperscript{R} grown for 2 x 24 hours under type I inducing conditions was centrifuged. The supernatant was filtered and incubated in presence of 5, 0.5, 0.05 µg/ml rSLPI for 15 minutes. A FRET-based assay was used to determine protease activity.
Figure 2.7: Increased levels of TNFα in Slpi<sup>−/−</sup> mice with UTI
ELISA analysis of TNFα concentration in homogenized bladder tissue from WT and Slpi<sup>−/−</sup> mice. Statistical significance: one-tailed Wilcoxon rank-sum test, boxes indicate 25<sup>th</sup> and 75<sup>th</sup> percentiles and whiskers are 1.5 x interquartile range.
Supplemental Figure 2.1: Submandibular bleed increases SLPI levels in urine of WT mice
Submandibular bleed of mice was taken 2 hours prior to transurethral inoculation of the bladder. Urine was collected before each manipulation as well as at 3 and 24 hpi.
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