Abstract

WOOD, MICHAEL WILLIAM. The Role of Interleukin-6 During the Acute Phase Response to Urinary Tract Infection. (Under the direction of Drs. Edward Breitschwerdt and Gregg Dean.)

Urinary tract infections (UTI) are the second most commonly reported infection in humans however one should not confuse the presence of bacteriuria with the diagnosis of a pathogenic urinary tract infection. This differentiation between UTI and nonpathogenic bacteriuria is of clinical and microbiological importance for patient management, and has become increasingly important because of the emergence of antimicrobial resistance among many common uropathogens. A common characteristic of UTI is the presence of a mucosal response to the infecting organism. Therefore identifying and understanding the sequella of the mucosal response to UTI may provide a means to recognize subclinical and/or unresolved infectious in patients as well as promote the development of new therapeutic interventions to improve patient outcomes. The production of the cytokine interleukin-6 (IL-6) is recognized as a primary mucosal response following exposure of the urothelium to bacteria. Concentrations of IL-6 increase within minutes to hours after infection. Despite its presence during the acute phase response little is known about the role of IL-6 during UTI. The purpose of this body of work was to characterize clinically and experimentally the IL-6 response during infection. This includes identifying the origin, magnitude, and targets of IL-6 as well as pinpointing specific genes that are modulated by IL-6. To achieve this goal a canine specific IL-6 ELISA for use with urine and a Ussing chamber model of UTI were
developed during the course of this work. In clinical samples IL-6 was successfully measured, however urine IL-6 proved to be of little utility when examining cases of asymptomatic bacteriuria and chronic infections possibly due to the down regulation of the IL-6 response after the acute phase of infection. In contrast, during the first 5 hours of experimental infection uropathogenic *E. coli* did stimulate the synthesis of IL-6 by the 3 urothelial cell layers. This secretion of IL-6 was polarized towards the lamina propria. Urothelial cells were also the local site expressing IL-6 receptors on their cellular surfaces suggesting an autocrine/paracrine effect of IL-6. In urothelial cells IL-6 activation mediated the transcription of numerous genes within the urothelium including genes responsible for the cytokine/chemokine/growth factor response, cell adhesion and ECM remodeling, GAG production, cytoskeletal rearrangement, and cellular transport. More specifically IL-6 up regulated genes responsible for the production of heparan sulfate, syndecan 2, MMP-2, and IL-1β all of which help promote a pro-inflammatory state but may also be integral to tissue remodeling and bladder defense during infection.
The Role of Interleukin-6 During the Acute Phase Response to Urinary Tract Infection

by
Michael William Wood

A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

Immunology

Raleigh, NC

2010

APPROVED BY:

Jody Gookin  Sushila Nordone

Paul Orndorff  Shelly Vaden

Edward Breitschwerdt  Gregg Dean
Co-chair of Advisory Committee  Co-chair of Advisory Committee
BIOGRAPHY

May, 1997  University of Pennsylvania, Philadelphia, PA

B.A. in Biological Basis of Behavior, concentration - neuroscience

May, 2002  Tufts Univ. School of Veterinary Medicine, North Grafton, MA

Doctor of Veterinary Medicine

2002-2003  Veterinary Referral and Emergency Center, Norwalk, CT

Intern – Medicine and Surgery

2003-2006  North Carolina State Univ. College of Veterinary Medicine,
Raleigh, NC

Resident – Internal Medicine, ACVIM certified 2007

2007-2010  North Carolina State Univ. College of Veterinary Medicine,
Raleigh, NC

Center for Comparative Medicine and Translational Research
Trainee – Clinician Scientist, NIH Grant # T32 RR024394
ACKNOWLEDGEMENTS

Michael Wood is supported by the Ruth L. Kirschstein National Research Service Award T32 RR024394 as part of North Carolina State University's Comparative Medicine and Translational Research (CCMTR) Training Program. Additional support was provided by CCMTR Pilot Research Grants.

Laboratory resources were provided by the Vector Borne Disease Diagnostic Laboratory (Breitschwerdt, Birkenheuer, Levy), the laboratory of Gregg Dean, the laboratory of Jody Gookin, and the laboratory of Paul Orndorff all located at the North Carolina State University College of Veterinary Medicine.

Technical expertise was provided by Henry Marr, Stephen Stauffer, Maria Stone, and Mitsu Suyemoto.

Committee support provided by Edward Breitschwerdt, Gregg Dean, Jody Gookin, Sushila Nordone, Paul Orndorff, and Shelly Vaden.

Graduate school representation provided by Paul Siciliano.

Finally, I would like to acknowledge Edward Breitschwerdt and Shelly Vaden for their assistance in developing the concept of this body of work, Sushila Nordone for her help establishing the ELISA studies, and Jody Gookin for her help in the development and execution of the ex vivo work.
TABLE OF CONTENTS

LIST OF TABLES........................................................................................................ vi
LIST OF FIGURES........................................................................................................ vii
LITERATURE REVIEW.............................................................................................. 1
   Bacteriuria, Urinary Tract Infections, Asymptomatic Bacteriuria.......................... 1
   Urinary tract infection – Incidence................................................................. 3
   Urinary tract infection – Host defenses......................................................... 3
   Bacterial Interactions with the Urothelium.................................................. 16
   Clinical Application....................................................................................... 24
   Techniques.................................................................................................... 28

ACCOUNTING FOR URINE MATRIX EFFECTS ALLOWS FOR THE APPLICATION OF A CANINE SPECIFIC INTERLEUKIN-6 ELISA TO URINE, BUT FAILS TO DETECT MEASURABLE CYTOKINE CONCENTRATIONS IN PATIENTS WITH ASYMPTOMATIC BACTERIURIA.................................................... 35
   Introduction................................................................................................. 38
   Materials and Methods................................................................................. 41
   Results......................................................................................................... 45
   Discussion.................................................................................................... 50

UROPATHOGENIC E. COLI INDUCE UROTHELIAL AUTOCRINE IL-6 SIGNALING AND LOSS OF BARRIER FUNCTION IN AN EX VIVO MODEL OF ACUTE URINARY TRACT INFECTION................................................................. 58
   Introduction................................................................................................. 60
   Materials and Methods................................................................................. 61
   Results......................................................................................................... 66
   Discussion.................................................................................................... 70
LIST OF TABLES

Table 1   Forward and reverse primer pairs for the 10 target genes…………… 100
LIST OF FIGURES

LITERATURE REVIEW

Figure 1. Urothelial structure…………………………………………...... 34

ACCOUNTING FOR URINE MATRIX EFFECTS ALLOWS FOR THE
APPLICATION OF A CANINE SPECIFIC INTERLEUKIN-6 ELISA TO URINE,
BUT FAILS TO DETECT MEASURABLE CYTOKINE CONCENTRATIONS IN
PATIENTS WITH ASYMPTOMATIC BACTERIURIA

Figure 1. The urine IL-6 ELISA was optimized by adjusting 6
different components of the immunoassay………………. 56

UROPATHOGENIC E. COLI INDUCE UROTHELIAL AUTOCRINE IL-6
SIGNALING AND LOSS OF BARRIER FUNCTION IN AN EX VIVO MODEL
OF ACUTE URINARY TRACT INFECTION

Figure 1. Maintenance of transepithelial electrical resistance
(TER)(panel A) and secretion of IL-6 by urothelium
(panel B) in response to normal Ringer’s solution
(NR) or lumen infection with uropathogenic
E. coli J96 (1 × 10^8 cfu/ml)…………………………………….. 75

Figure 2. Immunofluorescence localization of IL-6 and IL-6
receptors in E. coli treated canine urothelium……….. 76

Figure 3. Light and transmission electron microscopic
appearance of canine bladder mucosa after incubation
in the Ussing chamber in the absence (A, C, and E) or
presence (B, D, and F) of 1 × 10^8 cfu/ml E. coli J96
on the lumen side of the urothelium……………………… 77
Figure 4. Immunofluorescence localization of ZO-1 and uroplakin-III in canine bladder mucosa treated with normal Ringer’s solution alone or infected with uropathogenic *E. coli* J96 (1 × 10⁸ cfu/ml) for a period of 5 hours in Ussing chambers……………….. 79

Figure 5. Infection of canine urothelium with *E. coli* promotes phosphorylation of STAT3…………………………... 80

Figure 6. Maintenance of transepithelial electrical resistance (TER) by canine bladder mucosa treated in Ussing chambers with exogenous recombinant canine IL-6 (20 ng/ml; submucosal reservoir) or normal Ringer’s solution (NR) alone………………………… 82

UROTHELIAL RESPONSE TO INTERLEUKIN-6 STIMULATION INCLUDES THE ACTIVATION OF GENES RESPONSIBLE FOR PRO-INFLAMMATORY EFFECTS AND TISSUE REMODELING

Figure 1. Exposure of canine urothelium to IL-6 fails to increase phosphorylation of STAT3 at 5h……………….. 101

Figure 2. Exposure of canine urothelium to IL-6 induces SOCS3 production………………………………………... 103
Chapter 1 — Literature Review

I. Bacteriuria, Urinary Tract Infections, Asymptomatic Bacteriuria

Based upon recent observations in human medicine, microbiological documentation of bacteriuria is not equivalent to a diagnosis of a urinary tract infection (UTI). Bacteriuria is the presence of bacteria in the urine. Traditional definitions of UTI require that the invading organism adhere to the urothelium, multiply, and persist within the urinary bladder. In the past 15 years there has been a continuing evolution of how bacteriuria and UTI are assessed in clinical patients. Regardless of urinary bladder colonization, all bacteria will not activate a host response or ultimately cause disease. This subset of bacteriuric individuals are diagnosed as having “asymptomatic bacteriuria” (ASB)[1].

This differentiation between UTI and ASB is of clinical and microbiological importance to patient management. In many humans untreated bacterial strains persist within the urinary bladder for years without deleterious effects. Greater than 17% of elderly women and 5% of adult women are carrying bacteria within their urinary tract without symptoms of a UTI[1]. Importantly, these bacteria are hypothesized to convey a degree of protection against the urinary tract colonization with more virulent bacteria. Evidence includes studies where treatment of patients with ASB has resulted in the subsequent development of pathogenic infections of the lower urinary tract[1].
In veterinary medicine, bacteriuria is routinely diagnosed particularly in immunocompromised individuals[2]. Many dogs present to their veterinarian with subtle to no overt clinical signs. Whether to treat these patients is a complicated decision. Currently, most veterinarians will treat any dog with urinalysis or urine culture evidence of bacteriuria because the possible ramifications of not treating (pyelonephritis and renal failure) can be grave. However, it is possible that such therapies may be a disservice to our patients as more virulent and resistant infections may then be allowed to colonize.

The first warning sign of a possible urinary tract infection is frequently the report of clinical signs such as stranguria, hematuria, and pollakiuria. Unfortunately, clinical signs vary widely depending on the characteristics of the pathogen and the response of the infected individual[2]. As a result, clinical signs are not always a reliable indicator of the presence of infection. Urine culture and microscopy are currently the gold standard for diagnosing UTIs[2]. These tests generally are specific, but they do not specify the extent of the urinary tract afflicted and their sensitivity varies. These diagnostic shortcomings can make it difficult to diagnose and treat certain urinary tract infections effectively. Hence, there has been a push in recent years to better understand the mucosal response to infection, to identify whether bacteriuria has activated a host immune response, and to develop additional diagnostic tests that focus on the mediators of the early host response to infection.
II. Urinary tract infection - Incidence

Urinary tract infections (UTIs) are the second most commonly reported infection in humans. Over 60% of women will develop a UTI during their lifetime and 25% of those people will have another infection within 6 months of clearing the first[3, 4]. By comparison, 14% of dogs are estimated to develop UTIs during their lives and <3% of cats presenting with lower urinary tract signs have been reported to have UTIs[2, 5, 6]. Regardless of the incidence, failure to detect and treat a UTI in a timely manner can be devastating for an individual particularly if the infection should ascend the urinary tract and involve the kidneys. Such infections can instigate and perpetuate irreversible renal damage[7]. Given the morbidity associated with these infections, costs pertaining to UTIs in humans are estimated to exceed 1 billion dollars annually[8].

III. Urinary tract infection – Host Defenses

A. Overview

The origin of most UTIs can be traced to the fecal flora. After vaginal colonization and ascent through the urethra, bacteria are able to infect the bladder [9]. To maintain a sterile environment, the urinary tract employs numerous mechanisms to prevent infection. Two dedicated defenses include the urine itself and the urinary tract anatomy. Characteristics of the urine including osmolality, pH, urea concentration, and flow all
inhibit UTI formation[10, 11]. Anatomically, urethral high pressure zones, secretions, and peristalsis also inhibit bacterial ascent through the urethra[9, 12]. There are also numerous mucosal defenses. These are primarily mediated by characteristics of the urinary tract epithelium. Using tight junctions, the cells of the urothelium create a passive, physical barrier to prevent invasion of organisms into the underlying tissues[13]. The urothelium also plays an active role in the defense of infections. Invading bacteria activate the urothelium promoting urothelial exfoliation and/or eliciting the release of cytokines that propagate a cellular inflammatory response.

B. Mucosal Defenses – Barrier Function

i. Overview

Of the mucosal defenses to UTI one of the most essential is maintaining the barrier function of the bladder. During disease processes, such as infections, an increase in the permeability of epithelia can occur[14, 15]. Destruction of the barrier allows for bacterial invasion as well as exposure of underlying tissues to potassium, urea, and ammonia that are present in high concentrations within the urine. The leakage of these molecules across the urothelial barrier, in particular potassium, have been demonstrated to produce tissue damage and induce clinical signs consistent with cystitis[16].

The ability of the bladder to remain largely unaffected by most substances found within the urine or blood is enabled by a complex urothelial microenvironment. Barrier
function is maintained by the combined characteristics of the glycosaminoglycan rich mucus layer overlying the apical cell surfaces, the asymmetric unit membrane constructing the umbrella cell apical surface, and tight junctions adjoining umbrella cells in the apical portion of the intercellular spaces. Together these cellular components mediate low permeability and high resistance in normal urothelium[17]. Depending on the bacteria and the organ system the mechanism by which the permeability defect occurs during infection can vary from effects on ion channel and tight junctional complexes, to an increase in epithelial membrane permeability, to an up or down regulation of a second messenger systems[14].

**ii. Epithelial Resistance**

Transepithelial electrical resistance reflects both paracellular and transcellular flow of ions across the urothelium and is considered the most sensitive measure of epithelial barrier function. Paracellular resistance is largely regulated by intercellular tight junctions. Transcellular resistance is conferred by apical and basolateral cell membranes that are inherently resistant to ion permeation except, in some urothelia, for movement of sodium through amiloride-sensitive sodium channels and apical leak of potassium and sodium ions[18]. Increases in ion flow are therefore associated with decreased transepithelial resistance. In tissue model systems it is possible to calculate this resistance (R) by measuring voltage (V) and current (I) changes across a membrane and applying Ohm’s Law (V/I=R)
Changes in permeability are not limited to the movement of ions. In the urine non-charged molecules such as urea and ammonia are present in high concentrations. Depending on the barrier defect present these molecules can move into the urothelium independent of ion shifts[19]. To combat the movement of all urine solutes the bladder is constructed to create one of the most impermeable barriers in the human body. The framework of the barrier begins with the urothelium.

iii. Urothelium construction

The barrier function of the bladder is maintained by a stratified urothelium constructed of basal, intermediate, and umbrella cells (Figure 1). Numerous intercellular attachments including hemidesmosomes connecting basal cells to the basement membrane, desmosomes connecting the urothelial cells, and terminal bars that adjoin umbrella cells exist. Of these only the terminal bars and their associated tight junctions are integral in preventing the paracellular movement of solutes and water[20, 21].

In normal individuals the turnover cycle for urothelial cells is quite slow taking approximately 200 days[22]. When the epithelium is damaged the luminal layer of cells can be repopulated and the barrier function repaired in as few as 5 days. The replacement occurs with underlying cells maturing as they migrate from the basement membrane to the lumen of the bladder. This progression creates a 3 tiered epithelium with the umbrella cells being the largest, most differentiated cellular layer. It is through the maturation process that the outermost umbrella cells attain the ability to create a tight
epithelium, impermeable to water and most solutes. This is also the urothelial layer that interacts with invading bacteria.

iv. Glycosaminoglycan Layer

In addition to interacting with *E. coli*, umbrella cells are of interest because they have numerous unique characteristics that help create the urothelial barrier. First, umbrella cells secrete a mucus layer rich in glycosaminoglycans (GAGs) that lines the luminal surface of the bladder[23]. This anionic layer is hydrophilic and traps water creating a wall of water between the urothelial cells and the urine which contains cationic solutes and potentially bacteria[16]. Prior research has demonstrated that disruption of the GAGs using protamine sulfate will increase epithelial permeability. This defect is reversible by the addition of GAG replacers such as heparin or pentosan polysulfate sodium[24]. One flaw of this work is that protamine sulfate can also disrupt the cells of the epithelium creating alternate permeability defects[25]. Other GAG disrupters, such as hyaluronidase, have failed to induce a similar changes in permeability suggesting that the role of protamine sulfate is multifactorial[26]. One isolated report suggested that soluble factors released by *E. coli* have a similar effect as protamine sulfate disrupting the GAG layer and decreasing the resistance across the urothelium. This study failed to distinguish the GAG defect and left the possibility of a primary urothelial defect[15]. While it remains possible that the increase in permeability observed in *E. coli* urinary tract infection may be related to a disruption in the GAG, the current data is incomplete at
best. Despite these variable observations, treatment with GAG replacers such as pentosan polysulfate sodium has shown promise in relieving pain and discomfort associated with interstitial cystitis as well as preventing bacterial adherence during urinary tract infections[16].

v. Asymmetric Unit Membrane

A second characteristic of the umbrella cells helping to create the urothelial barrier is their apical surface constructed of scalloped shaped uroplakin plaques and lipid rafts that together create the asymmetric unit membrane[17, 27]. Uroplakins are evolutionary conserved proteins that exist as four distinct units, Ia, Ib, II, and IIIa, with each possessing unique characteristics. Of these, Uroplakin II and particularly IIIa appear to be integral in maintaining the barrier function of the bladder. Urothelium deficient in uroplakin IIIa has an increased permeability to radiolabeled urea and water[19]. It is hypothesized that the structure provided by uroplakins prevents free lipid movement providing structure to a transcellular urothelial barrier. The importance of these lipids is demonstrated by the observation that disruption of the lipid rafts via nystatin treatment will decrease transepithelial resistance allowing ion fluxes across the membrane[28]. It is only when both uroplakin and lipid raft integrity is maintained that the urothelial barrier function is intact.
vi. Tight Junctions

A third characteristic of umbrella cells is that they are all joined at the apical membrane interface by tight junctions. Nowhere else in the urothelium are these cellular connections present. These junctions act to prevent the paracellular movement of charged an uncharged solutes and water by containing pores that limit the passage of molecules based on both charge and size. These selection criteria are independent of one another[29].

Claudins are transmembrane proteins associated with tight junctions[30]. Responsible for charge selectivity, up and down regulation of these structures has been associated with changes in transepithelial electrical resistance[29]. In umbrella cells the expression of claudins has been variably reported. However claudin-4 has been consistently associated with urothelial tight junctions[31, 32]. Claudin 4 increases epithelial resistance by excluding cations from passing across the tight junctions[30].

C. Mucosal Defenses - Inflammatory Response

i. Overview

There are three mechanisms in which bacteria may activate the cellular inflammatory response. The first is by direct interaction of microbial surface ligands with epithelial cell receptors. One of the more potent ligands is bacterial fimbiae. These interactions
induce a transmembrane signaling pathway that ultimately results in cellular transcription and the production of inflammatory cytokines. The second pathway involves the internalization of bacterial components into the epithelial cells directly resulting in the activation of inflammatory mediators. The third mechanism requires the activation of nonepithelial cells. In this instance bacteria cross the epithelial layer, where they are engulfed by phagocytotic immune cells such as mucosal macrophages. These cells then are capable of releasing mediators of inflammation that activate the surrounding epithelial cells to release cytokines[33].

**ii. Urothelial cytokine response**

The ability of bacteria to stimulate a mucosal cytokine response by epithelial cells throughout the body has been well established[34]. Despite the local release into the adjacent mucosal compartment, the epithelial derived cytokines can have both systemic and local effects. Systemically, cytokine driven responses may affect body temperature, blood flow and blood pressure. Locally, these same cytokines are integral in activating other epithelial cells, increasing vascular permeability, and promoting the recruitment and activation of inflammatory cells[33]. Some of these effects result in the clinical signs attributable to the disease state.

In urinary tissue the production of certain cytokines is disease state dependent with IL-1α, IL-1β, IL-4, IL-6, IL-8, INF-γ, TNFα, PDGF, and TGFβ all being produced in response to different pathological environments[35, 36]. Variable urothelial cytokine
production has also been induced by changing the type of bacteria and the adhesins expressed on the bacterial surface[37-39]. A more limited repertoire of cytokines including IL-6, IL-8, PDGF, and TNF-α were consistently produced by the cultured urothelial cells when exposed to 3 strains of *Escherichia coli* and *Citrobacter spp*[37]. Of these, it is the cytokines IL-6 and IL-8 that have been found to be constitutively produced by human kidney and bladder cell lines[40].

iii. IL-6 overview

Cytokines are small soluble proteins or glycoproteins that are frequently <30kDa. These molecules share a number of characteristics. The first is pleiotropy meaning that they exhibit more than one action frequently in numerous locations throughout the body. Despite these broad actions production is typically transient with a small radius of action. The high potency of cytokines allows for even limited cytokine production to exert effects after binding to cell surface receptors. Most of these actions are generated by altering gene expression within a cell and there is a great deal of redundancy between different cytokines. Together groups of cytokines released in serial or parallel produce a cytokine network promoting or inhibiting the actions of one another.

Cytokines are grouped in families based on their receptor structure. Interleukin-6 signals through a haematopoietin receptor and shares a similar receptor structure to IL-12. All cytokines within this family utilize gp130 in their intracellular signaling
pathways. Interleukin-6 utilizes the Jak-Stat, Ras-MAPK pathways in cellular signaling[41].

Synthesized by T-cells, B-cells, macrophages, fibroblasts, endothelial cells, and epithelial cells, IL-6 is stable within the bloodstream. This attribute allows IL-6 to be an effective pro-inflammatory and immunoregulatory mediator both locally and systemically[42]. Perhaps the most well documented role of IL-6 is in evoking the systemic acute phase response and modulating thermoregulation in the hypothalamus resulting in clinically apparent fever[13, 42]. Immunologically IL-6 is important for immunoglobulin production as well as leukocyte proliferation particularly the growth and differentiation of T and B cells[7]. Interleukin-6 and -3 act synergistically to promote the proliferation of pluripotent hematopoietic progenitor cells.

Locally, IL-6 has numerous effects. The cytokine has been recognized for its role in bone remodeling, obesity related insulin resistance, endothelial permeability, response to myocardial infarction, and IL-6 is a prominent cytokine in tumor biology[43-47]. At the epithelial cell layer IL-6 is specifically thought to promote the transition of B cells to IgA producing plasma cells in the mucosal compartment[33, 48]. The binding of IgA to bacteria is thought to help prevent bacterial adherence to the epithelium[13]. Other effects of IL-6 on epithelia are conflicting with the cytokine having both barrier protective and destructive effects. Exposure of the endothelium to IL-6 for has been shown to alter cellular shape and increase the permeability of endothelial monolayers by
affecting the arrangement of cytoskeletal actin filaments and ZO-1 proteins within tight junctions[45, 49]. In the intestine, models of septic shock have shown a correlation with IL-6 concentrations and ZO-1 alterations[50]. In contrast, other enteric studies have argued for a protective effect of IL-6 on epithelial barrier function and maintenance of cellular architecture. In a colitis mouse model, IL-6 treatment increased keratin expression and reduced permeability to Dextran when compared to IL-6 KO mice [51]. Given these findings, at best the role of IL-6 in epithelial cells can be described as unsettled.

iv. Urinary IL-6

In the normal individual levels of IL-6 are not detectable in the urine[42]. Even patients with a strong serum IL-6 response to systemic disease fail to obtain detectable levels of IL-6 in the urine[52]. However, within 30 minutes of instilling E. coli or fimbriae into the bladder an IL-6 response is measurable in the urine[13]. Experimentally, this cytokine release is purely local as IL-6 levels within the serum remain undetectable[34]. Clinically, the presence of bacteria limited to the lower urinary tract results in marked urine IL-6 elevations, while serum IL-6 approximated healthy controls[52]. As the disease state increases in severity the cytokine concentrations as well as the scope of the effects increase. Looking at a population of symptomatic, febrile patients with a urinary tract infection both urinary and serum IL-6 levels are elevated. Here bacteremic individuals have a strong serum IL-6 response, but the presence of blood
borne bacteria is not necessary for the serum IL-6 increase. Individuals may also be febrile without bacteremia. This indicates that as the magnitude of inflammation increases locally in the urinary tract, the local production of IL-6 can produce a systemic response. Hence a febrile UTI patient should be a suspect for either a bacterial infection of high virulence or considerable urinary tract pathology[42, 53].

v. Kinetics of urothelial IL-6 release

Exposure of the urothelium to gram negative bacterial products such as fimbriae and lipopolysaccharide has been shown to increase IL-6 cytokine production above constitutive levels within minutes to hours[40, 54]. In human bladder colonization experiments IL-6 was found to be released in a series of peaks and not continuously. Maximal concentrations of the cytokine were measured at means of 4, 17, and 33h. There was no significant difference between the concentrations of IL-6 measured at any of the three time points. The cytokine response does appear to be contingent on bacterial concentrations with $1 \times 10^5$ cfu/ml necessary to initiate the IL-6 response. Recolonization immediately after bacterial clearance does not affect the magnitude of the IL-6 response[34], however chronic UTI sufferers have lower IL-6 concentrations when re-infected than new infections[42]. Of note, continued bacteriuria does not result in a persistent IL-6 response. After 6 days of bacterial urothelial stimulation the IL-6 response nears baseline concentrations[34]. Treatment of UTI with antibiotics hastens the reduction with declines in urinary IL-6 concentrations recorded within 24h[42].
vi. IL-6 signaling pathway

Interleukin-6 binds to membrane bound or soluble IL-6 receptors (gp80) to recruit 2 membrane associated gp130 molecules. This complex recruits cytoplasmic auto-phosphorylating JAK kinases that phosphorylate gp130 and in turn the transcription factors STAT1 and STAT3[55]. Once phosphorylated two STAT3 molecules or 1 STAT3 and 1 STAT1 molecule dimerize within the cytoplasm and travel to the nucleus to induce cellular transcription. Once IL-6 binds to its receptor it takes about 10m before phosphorylated STAT can be identified within the cytoplasm. Maximal nuclear concentrations of STAT dimers are reached in about 1h after which time phosphorylated STAT concentrations begin to decrease. After 5h a new steady state is achieved[41].

The membrane associated complex IL-6-IL6R-gp130-JAK also activates the RAS/MAPK pathway by recruiting the tyrosine phosphatase SHP2. This pathway ultimately results in the translocation of the phosphorylated transcription factor ERK to the nucleus. SHP2 serves an additional role as it also dephosphorylates JAK kinases thereby reducing STAT phosphorylation[41].

Other inhibitors of STAT phosphorylation include nuclear phosphatase PP2 and protein inhibitors of activated STAT both of which directly dephosphorylate STAT within the nucleus and cytosol. A final group of STAT inhibitors are considered feedback inhibitors as STAT activation of transcription results in their production. These molecules titled suppressors of cytokine signaling (SOCS) reduce STAT phosphorylation
by inhibiting JAK and gp130 phosphorylation[56]. After IL-6 binds to its receptor it takes 30m for SOCS3 to be detectable in the cytoplasm. Maximal concentrations are reached after 2.5h and return to steady state occurs after 15h[41].

IV. Bacterial Interactions with the Urothelium

A. Overview

Urinary tract infections are caused by numerous etiological agents including *Escherichia coli*, *Staphlococcus saprophyticus*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Enterococcus* spp [57]. Of these, uropathogenic strains of the gram negative bacteria *E. coli* (UPEC) account for over 80% of UTIs [58, 59].

A defining feature of many UPEC strains and other gram negative uropathogens is the presence of fimbriae[60]. Expressed on the surface of the bacteria, fimbriae are integral components for adhesion, internalization, and intracellular colonization of bacteria within urothelial cells. A uropathogenic strain of bacteria may contain numerous gene clusters for encoding fimbriae[61] however, typically a bacterium will only express one type of fimbriae at a time. The organisms do have the ability to phase switch depending on the bacterial environment[62].

There are two main fimbrial units that play prominent roles in the development of UTI, type 1 and P-fimbriae. Both fimbriae when exposed to renal and urinary bladder
biopsies have been shown to elicit a stronger IL-6 response than nonfimbriated *E. coli* [63]. P-fimbriated bacteria are typically associated with the development of acute pyelonephritis, while type 1 fimbriated bacteria are associated with cystitis and lower urinary tract infections. This does not mean that P-fimbriated bacteria cannot be cultured from patients with cystitis however P fimbriae bind glycosphingolipid receptors, specifically α-D-Gal-(134)-β-D-Gal to activate the ceramide signaling pathway. These glycolipids are predominantly found in kidney tissue [64]. In contrast type 1 fimbriae recognize mannosylated glycoprotein receptors that are found in abundance along the urothelium [65]. The presence of type 1 fimbriated *E. coli* has been specifically shown to be important during the first 24h of urinary bladder colonization [66].

**B. Type 1 Fimbriae Attachment**

**i. *fim* Gene Cluster**

Two genera of bacteria, UPECs and at least one strain of *K. pneumoniae* [67] are known to invade urothelial cells in an in vivo mouse UTI model. A unifying characteristic of the two genera of bacteria is the presence of the *fim* operon coding for the expression of type 1 fimbriae. Clinically the presence of type 1 fimbriae is associated with an increased severity of infection [60] and they are the primary fimbriae found on the surface of UPECs during initial colonization of the urinary bladder [68]. Given their importance in establishing UTIs and intracellular colonization, knowledge of fimbriae
structural subunits is essential to understanding recurrent UTIs and potentially using the fimbriae structure as a target for UTI prophylactic therapies.

Type 1 fimbriae are constructed from numerous elements all encoded within the \textit{fim} gene cluster. Translated proteins include those of fimbrial structure as well as chaperone and usher proteins that through the chaperone-usher pathway promote the proper construction of fimbriae. The structural subunits consist of the main subunit, FimA, which creates 95% of the shaft of the fimbriae, minor subunits FimF and FimG, which support the fimbrial adhesin, and FimH, the minor subunit that is the adhesin located at tip of the fimbriae [60, 69]. Of these proteins, differences in both FimA and FimH have been proposed to be integral in affecting the ability of bacterial strains to colonize and invade urothelial cells [70, 71].

The main chaperone protein is FimC. Located within the periplasm, FimC binds to structural subunits of the fimbriae and performs three main tasks. First, it captures structural proteins within the periplasm preventing premature polymerization with each other. Second, FimC accelerates the folding of structural subunits increasing folding speed by more than 100 times [72]. Finally, once the structural subunit is properly prepared, FimC serves as a chaperone to traffic the subunits to the transmembrane usher protein, FimD, which serves to anchor the fimbriae to the bacterial outer membrane.

Type 1 fimbriae mediate bacterial attachment to the urothelium by the binding of FimH to mannosylated glycoproteins. Bacteria lacking an active \textit{fimH} gene lose the
capacity to bind D-mannose moieties even though the remainder of the fimbrial shaft remains intact [65]. Specificity of FimH adherence is achieved via \textit{fimH} allelic variation [73]. Although all FimH proteins bind tri-mannose receptors [74], within the urinary tract an increased FimH affinity for the mono-mannose receptors of urothelial glycoproteins is associated with increased virulence [75].

Variability of bacterial adherence has also been associated with the FimA protein configuration. FimA is quite heterogeneous across bacterial species. Bacterial chimeras containing one species’ FimH and another’s FimA will have a binding specificity consistent with the bacterial origin of the FimA protein. Changes in FimA structure may sterically restrict or promote the movement of the FimH tertiary structure [70]. Since FimH constructs lacking a FimA derived shaft have been commonly used to elucidate bacterial interactions with the bladder surface, the ability of FimA to alter bacterial binding to the urothelium is noteworthy.

Given their importance in fimbriae formation and function, FimC, FimH, and FimA all may be a viable therapeutic target when seeking to prevent bacterial colonization of the urothelium. In particular, FimH-specific antibodies have been shown to protect individuals from cystitis [71], making FimH inhibition an attractive therapeutic target to prevent recurrent UTIs.
ii. FimH-Urothelial Interactions

The ability of FimH to promote adherence and invasion of the bladder wall is generally accepted [59]. The FimH adhesin is known to bind numerous structures associated with the luminal surface of the urothelium[76]. However, multiple binding sites on the urothelium promoting bacterial internalization have been proposed.

Uroplakins (UP) are protein subunits that form heterodimers and subdomains to construct the urothelial plaques that comprise 90% of the bladder surface membrane [22]. Of these proteins only uroplakin Ia contains mannose residues accessible for and capable of binding type 1 fimbriae [77, 78]. Uroplakin Ia consists of four transmembrane domains with two hydrophilic loops projecting into the bladder lumen but with little domain in the cytoplasm [79]. The lack of a defined intracellular signaling domain suggests that uroplakin Ia itself may be an anchoring point for UPECs while intracellular signaling is associated with accessory molecules.

Additional signaling properties of uroplakin Ia can be hypothesized based on the protein structure. Uroplakin Ia is in the tetraspanin family of proteins [80]. This diverse group of proteins is known to associate with integrins forming tetraspanin-integrin complexes [81]. These complexes are important mediators of cell motility, proliferation, differentiation, and in some instances cell signaling. In other tissues cell signaling and potentially actin cytoskeleton rearrangement has been associated with tetraspanin/α3β1 integrin complexes [82]. While a similar link between UPIa and αβ1 integrins on
urothelial cells has not been identified, actin rearrangement has been associated with UPEC internalization [59] suggesting that a UPIa and αβ1 integrin interaction may serve as an alternate link between the tetraspanin UPIa and UPEC internalization.

Of the uroplakins only uroplakin IIIa has an intracytoplasmic domain that is capable of cellular signaling via C-terminal phosphorylation [22]. While FimH cannot directly bind to UPIIIa, a FimH construct and UPEC exposure to cultured human epithelial cells has been shown to induce UPIIIa intracytoplasmic phosphorylation [83] suggesting a signaling link between UPIa and UPIIIa. Although these two uroplakins do not dimerize and lack a means of direct interaction, the uroplakin dimers of UPIa/UPII and UPIb/UPIII do become co-localized via an exomembranous bridge connecting UPII and UPIII [80].

Besides complexing with tetraspanins, integrins are a diverse family of α and β adhesion receptors themselves. Heterodimers of an α and β subunit combine to form a unit that traverses the plasma membrane and provides a means for the cell interior to react to changes in the extracellular environment and visa versa. These responses can vary from initiating cell signaling to direct interactions with the actin cytoskeleton [84].

In the urinary bladder the α3, αV, and β1 integrins have been identified in vivo on urothelial cells lining the luminal surface [85] and UPECs have been shown to become associated with α3β1 heterodimers in vitro. This association appears to be mediated by bacterial FimH interaction with high mannose oligosaccharides on the surface of α3 and
β1 integrins [76]. Blocking of these α3 or β1 receptor sites is associated with a decrease in the ability of UPECs to move intracellularly[76].

The current urothelial integrin data provides evidence of the importance of α3β1 in binding and internalization of uropathogens as well as signaling pathways that the integrins modulates. Since the presence and density of cell surface molecules varies in vitro and in vivo, confirmation of the role of integrins in vivo is necessary. Overall, given the suggested role of UPIIIa and α3β1 integrin in the establishment of intracellular bacterial colonies it seems reasonable that uroplakins and integrins may co localize linking the currently independent mechanisms of UPEC adhesion and signaling. This hypothesis has yet to be proven. To date prophylactic therapies preventing bacterial adherence and internalization have centered on altering bacterial binding characteristics not modulating the urothelial expression of binding sites.

C. *E. coli J96* Bacterial Characteristics

*E. coli J96* is a uropathogenic strain isolated from a human case of pyelonephritis. This bacteria produces four different adherence factors including the aforementioned type 1 fimbriae, and two types (*pap* and *prs*) of P-fimbriae. Additional virulence factors include 2 hemolysins and cytotoxic necrotizing factor type 1(CNF-1)[86].

Hemolysins are exotoxins capable of destroying red blood cells in vitro, but are noteworthy during in vivo infections for their ability to induce leukocytes to release
inflammatory mediators [87]. In the urinary bladder hemolysins are important mediators of umbrella cell destruction and bladder hemorrhage[88].

Cytotoxic necrotizing factor type 1 is a common virulence factor in uropathogenic strains of *E. coli* that constitutively activates Rho family GTP-binding proteins in mammalian cells resulting in changes in the cell cycle, actin cytoskeleton, as well as gene signaling pathways[89]. During UTI CNF-1 induces submucosal edema[88] and is hypothesized to induce the acute production of cytokines by the urothelium. These cytokines include IL-6[90].

There are at least two pathogenicity islands within *E. coli* J96. Pathogenicity islands are sections of DNA at least 30kp in length that code for at least 1 virulence factor and can be deleted from the bacterial genome. PAI IJ96 is greater than170 kb long includes genes coding hemolysins as well as the pap P-fimbriae. PAI IIJ96 is 110 kb long and codes for hemolysins, the prs P-fimbriae, and CNF-1[91]. Since these genes can be deleted from the bacterial DNA en bloc it is possible for *E. coli* J96 to change its bacterial phenotype including fimbriae expression readily[87].

An equally important virulence factor expressed by *E. coli* J96 is lipopolysaccharide (LPS) and the toxin Lipid A that adhere LPS to the bacterial outer membrane. In the urinary tract LPS is known to bind to Toll-like receptor 4 that is expressed only on the apical surface of the umbrella cells [92]. LPS binding to its receptors is known to initiate the urothelial NF-κB and cAMP/CREB mediated pathway [93] ultimately resulting in the initiation of the innate immune response. This response may be responsible for many of
the urothelial changes associated with UTI including the loss of bladder permeability. Given that *E. coli* J96 is known to both adhere to and activate umbrella cells these bacteria are an ideal organism for studying the urothelial response to infection.

V. Clinical application

A. Urinary Tract Infection Diagnosis

Urinary tract infections pose a challenge to the clinician as treatment should be paired with an identification or understanding of the cause of the infection. If the primary reason for bacterial colonization is not identified and addressed the patient may be at risk for developing another UTI. Unfortunately in 25-30% of both human and dog patients the defect allowing re-colonization is not identified resulting in a frustrating cycle of repeat infections and treatment [94, 95].

Traditionally, treatment of recurrent infections has relied on characterizing the UTI as a re-infection or a persistent infection. Re-infections occur weeks to months after the initial UTI and are commonly treated as uncomplicated infections. In contrast, persistent infections are typically identified within 2 weeks of the initial infection. These cases are managed with a modified treatment plan to account for bacterial resistance or urinary tract pathology that may be causing the failure in treatment. The ability of bacteria to be harbored in a protected intracellular environment [96] only to re-emerge weeks to months
later means that some infections characterized as re-infections may actually be persistent infections and hence are treated inappropriately. The presence of intracellular colonies also provides an attractive explanation as to why in over a quarter of cases the defect allowing for recurrent infection is unidentified.

The internalization of bacteria into urothelial cells is rapid with invasion occurring within 6 hours of bladder inoculation [58]. Clinically this timeframe is important since it indicates that by the time a UTI is diagnosed bacteria may already be protected within the urothelium shielded from traditional antibiotic treatments. Misinterpreting and treating a persistent infection of the bladder wall as an uncomplicated re-infection will ultimately result in treatment failure and has been associated with the development of resistant UTIs [97]. These facts indicate that patients at risk for developing recurrent UTIs may be best served by a treatment plan that focuses not on treating urothelial bacterial colonies but instead on prophylactically blocking the organism’s ability to adhere and enter the urothelial cells.

**B. Urinary Tract Infection Treatment – Focus on Prevention**

The use of antibiotics is the accepted treatment for uncomplicated UTIs. However, in recent years an increase in multidrug resistant uropathogenic bacterial strains has led to treatment failure [98]. If persistent colonies are present within the urothelium treatment with antibiotics will increase the risk of developing a resistant infection over time [97]. Alternate therapies that keep the organism viable while disabling bacterial virulence
factors are being explored. Since these drugs are not bactericidal selective pressures yielding resistant strains should be minimal and the normal flora within the body would remain viable. Prophylactic use of these pharmaceuticals may effectively prevent infection in at risk patients by blocking the steps of adherence and internalization before colonies ever have a chance to form.

Since certain components of the chaperone-usher system are conserved across many bacterial species [99, 100], blocking the action of chaperone proteins such as FimC or PapD, necessary for type 1 and P fimbriae respectively, can prevent proper formation of fimbriae across a broad spectrum making this class of drug a desirable alternative to antibiotics. Experimentally, inhibition of both type 1 and P fimbriae formation by UPECs has been achieved using a bi-cyclic 2-pyridone class of pillicides. In vitro this pharmaceutical is efficacious, able to decrease bacterial adherence to cultured urothelial cells and biofilm formation by up to 90% [101]. Although this class of drugs is still in development, given their effectiveness and potential for a broad spectrum of activity, pillicides do hold promise for clinical utility in the coming years as an alternative or adjunctive therapy to antibiotics. The basis of this therapy is that pillicides compete with fimbrial structural subunits for FimC binding sites. Without FimC stabilization of structural subunits within the periplasm they are rapidly degraded [102]. More recent work argues that the binding of pillicides to chaperone proteins also alters the ability of
chaperone proteins to dock with the usher proteins on the bacterial surface thereby blocking structural subunit assembly[103].

The second therapeutic strategy aims to disrupt adhesion by blocking the ability of lectins on the tips of fimbriae to interact with carbohydrate moieties located on urothelial cells. This diverse class of drugs attempts to optimize the avidity in which bacterial lectins are blocked by using synthesized multivalent glycoconjugates and glycodendrimers[104]. In vitro experimentation has proven that these molecules specifically bind to the conserved lectin, FimH, located on the tips of bacterial fimbriae and are effective at blocking bacterial adherence to cultured urothelial cells [105]. In a mouse model, heptyl α-D-mannose incubation of bacteria reduced both urine and intracellular bacteria concentrations after bacterial inoculation of the urinary bladder [106]. While promising to date, this class of drugs still lacks clinical evidence of effectiveness.

Another anti-adherence therapeutic that has received considerable attention in recent years is the consumption of cranberries and cranberry extract. It has been demonstrated that proanthocyanidins (PACs) with type A-linkages isolated from cranberries can prevent P-fimbriated UPECs from binding to uroepithelial cells [107]. It is thought that like glycomimetics, PACs alter the lectin-mannose binding necessary for *E. coli* adherence. Unfortunately, these molecules do not appear to have a similar effect on type 1 fimbriated UPECs or other uropathogenic bacteria limiting their efficacy. There is data
suggesting that the consumption of cranberry juice can alter the urine composition such that the adherence of type 1 fimbriated UPECs to urothelial cells is inhibited [108]. The mechanism by which consumption of the juice acts differently than the PACs is not known.

Additional therapies that bear mentioning but fall beyond the scope of simple anti-adherence therapy include the development of anti-bacterial antibodies using various vaccination strategies [109, 110] and bacterial interference where the introduction of an asymptomatic competing bacterial strain into the bladder prevents colonization with a more pathogenic strain [111]. Both of these treatments have achieved modest success in clinical trials and may provide additional prophylactic treatment options for preventing bacterial adherence and colonization of the urothelium.

VI. Techniques

A. IL-6 Detection and Quantification

There are numerous methods for identifying and quantifying the production of IL-6 in tissues as well as in body fluids. Many previous studies have relied on measuring mRNA transcripts to estimate IL-6 production by different cell types[40, 112]. This is a potentially flawed method since the number of mRNA transcripts within a cell does not necessarily correlate with cytokine (protein) production.
Within body fluids cytokine measurements are typically done by immunoassay (specifically ELISA) or bioassay. Of the assays, the bioassay is more physiologically relevant since it only measures active cytokine, however it is not as specific as an ELISA and cytokine inhibitors may interfere with the measurement of the actual concentration of cytokine present.

The typical cytokine bioassay utilizes a target cell line that either has an absolute requirement for a cytokine or the presence of the cytokine results in cell death. Cytokine concentration is indirectly determined by assessing the response of the cell line. Cell line proliferation or cell death are two common standards used to quantify cytokine concentrations, however, assessing downstream cell functions induced by the cytokine such as phosphorylation of signaling pathways (ie STAT) or changes in protein production (ie SOCS3), can also be used.

A common alternate to bioassays are the use of direct and indirect ELISAs. Direct ELISAs utilize the direct binding of cytokine within body fluids to plate plastics and hence plate variations can have profound effects on cytokine measurements. Indirect or sandwich ELISAs utilize a capture antibody. The binding characteristics of the plates can be such that Fc receptors of the antibody preferentially bind to the plastic. Using such a technique may increase yield when performing an assay for cytokines that do not adhere to the ELISA plate plastics well. The use of ELISAs in measuring cytokine concentrations is advantageous because the modality is rapid and high volume and hence
is great when examining multiple samples. Pitfalls include the detection of inactive fragments and decreased sensitivity when compared to bioassays. A third problem caused by “matrix effects” is a common complication of performing an ELISA using complex biological fluids where the nonspecific binding of proteins is prevalent.

B. Current UTI Models

Despite efforts over the past two decades there remain large knowledge gaps regarding both urothelial barrier function and urothelial mucosal immunity. One reason for the paucity of data is that traditional methods for studying the bladder response to infection are either too complex to assess specific cellular changes or the model construction is unable to mimic real life physiology consistently.

i. In vivo models

In vivo research of urinary tract infections has performed controlled experiments utilizing animals varying from mice to humans. These models are quite effective at examining large scale responses such as the release of inflammatory mediators and the destruction of epithelial architecture, or for contrasting the responses of an animal after knock-out of a gene of interest[34, 113]. Responses that can be assessed using in vivo studies typically focus on the tissue or systemic response as a whole. Data is gleaned from examining ultrastructural changes, measuring secreted products, assessing the movement of cells and solutes, and generally examining the response of the tissue. In
vivo models are limiting when trying to identify the response of individual cells since it is difficult to provide a directed treatment to certain cells and prevent the influx of other cells and mediators. To provide this information cell culture models and epithelial monolayers have been used with varied success.

**ii. In vitro models**

In vitro models provide a mechanism to assess more specifically bacterial interactions, cell signaling activation, and secreted products of specific UTI effects on the urothelium. The main drawback of these models is that properties or urothelia that are vital for proper function in vivo have historically been absent from urothelial cell cultures[17]. Six requirements of cultured urothelium include high transepithelial resistances, an amiloride-sensitive transport system, active movement of umbrella cell cytoplasmic vesicles to the apical membrane, low permeability, the presence of differentiated umbrella cells expressing uroplakins, the presence of tight junctions between umbrella cells, and three differentiated cell layers[17]. It is not uncommon for cultured tissue sheets to meet a few of these requirements, but not all. This deficiency has resulted in work with unknown correlation to in vivo urothelial activity. It also makes comparing studies difficult when urothelial cell lines are grown under different conditions. Only recently has a well differentiated urothelium been grown in culture from normal porcine urothelial cells that met most of these criteria (all the criteria were not assessed)[114]. While the authors admit that it is unknown whether the model may mimic in vivo
function, this advancement may allow for cell culture experiments that can be compared directly and may have greater resemblance to in vivo physiology. Regardless of the in vitro model, a cell culture system will always have some drawbacks since urothelial interactions with the lamina propria and basement membrane cannot be mimicked. Traditional cellular interactions within the urothelium are also lacking such as the presence of nonepithelial cells.

iii. Ex vivo model

An optimal ex vivo model takes the benefits of the in vivo and in vitro models and hybridizes them. In the described research of this thesis a Ussing chamber model of UTI was designed to achieve this goal. The Ussing chamber model retains the physiological complexity of interactions between the lamina propria and native urothelium while enabling a mechanistic assessment of urothelial function during the acute phase response to UTI. Since intact canine urothelial tissue stripped of the muscularis layer is used in these experiments, the native architecture and biological responses of the urothelium and underlying lamina propria can be viably maintained for short term studies ex vivo. By providing physically separate saline reservoirs to warm, nourish and oxygenate the subepithelial and lumen side of the mucosa independently, the Ussing chamber allows for polarized quantitative measurement of epithelial barrier function, transport, signaling and secretory processes[115]. An additional benefit of this system is that since the tissue is kept viable independent of a blood supply, tissue responses are not affected by the influx
of inflammatory cells. Without these confounding effects, this system allows for an assessment of urothelial permeability and cellular signaling specifically by the resident cells of the urothelium.

There are drawbacks to using a Ussing chamber system. The experiments are time consuming in both maintenance and experimental time. The use of live tissue within the chambers also provides a degree of variability that can make it difficult to assess subtle physiologic changes within the tissue. Even small variations in tissue preparation and Ussing chamber parameters can have a profound effect on experimental outcomes. A third factor that must be considered before considering a Ussing chamber model is that there must be a source of tissue available for experimentation.
Figure 1 – Urothelial structure

Chapter 2

Accounting for urine matrix effects allows for the application of a canine specific interleukin-6 ELISA to urine, but fails to detect measurable cytokine concentrations in patients with asymptomatic bacteriuria.

Michael W. Wood DVM, Sushila K. Nordone PhD, Shelly L. Vaden DVM, PhD, Edward B. Breitschwerdt DVM

Department of Population Health and Pathobiology, North Carolina State University, Raleigh, NC 27606. (Wood, Nordone); Department of Clinical Sciences, North Carolina State University, Raleigh, NC 27606. (Vaden, Breitschwerdt); Center for Comparative Medicine and Translational Research, College of Veterinary Medicine North Carolina State University, Raleigh, NC. (Nordone, Vaden, Breitschwerdt)

Supported by a grant from the North Carolina State University Center for Comparative Medicine and Translational Research

Address correspondence to Michael Wood (michael_wood@ncsu.edu)

Acknowledgements

Michael Wood is supported by the Ruth L. Kirschstein National Research Service Award T32 RR024394 as part of North Carolina State University's Comparative Medicine and Translational Research Training Program.
**Objective**—To optimize a canine specific ELISA to facilitate accurate measurement of IL-6 concentrations in the urine of dogs and to apply the ELISA to dog urine samples to determine whether IL-6 is a viable biomarker of bacterial infection.

**Sample Population**—The urine of 50 dogs collected from patients that required urine culture at the Veterinary Teaching Hospital at North Carolina State University. Control urine samples were collected from 25 dogs without clinical or microbiological evidence of urinary tract disease.

**Procedures**—A chemiluminescent ELISA for detecting IL-6 in the urine of dogs was optimized using capture goat anti-canine polyclonal IL-6 and detecting biotinylated goat anti-canine IL-6 antibodies. The effects of urine matrix, urine specific gravity (USG), and pH on the ELISA were assessed.

**Results**—Urine matrix and variations in USG contributed to poor IL-6 detection via ELISA. Using matrix inhibitors, the IL-6 quantitative recoveries improved to 80-95% after a 1:8 dilution. Clinically, only 1 of 50 dogs presented with clinical signs of UTI. Thirteen of the 50 had documented bacteriuria. Only 4 of 50 had detectable concentrations of IL-6 within the urine. None of these 4 dogs had clinical signs consistent with a UTI and only one had bacteriuria.

**Conclusions and Clinical Relevance**—Urine matrix and USG must be accounted for accurate measurement of IL-6 in dog urine via ELISA. In this study, there was no
clinically relevant association between bacteriuria and urine IL-6 concentrations. IL-6 measurements appear to be a poor diagnostic aid in identifying pathologic bacteria in patients presenting without signs of UTI.
Introduction

A urinary tract infection (UTI) occurs when there is an anatomical or functional break in the host defense system allowing for the adherence, multiplication, and persistence of bacteria in part of the urinary tract. Depending on the site of infection, there is potential for serious pathology, hence identifying the bacterial species, the antimicrobial susceptibility, the anatomic location and preventing UTI recurrence are essential for ensuring optimal patient outcomes. Although not universally accepted, microbiological documentation of bacteriuria does not equate to an automatic diagnosis of a pathogenic UTI. Depending on bacterial characteristics such as expressed virulence factors, all bacteria will not activate a host response or ultimately cause disease. In human medicine, this subset of bacteriuric individuals are diagnosed as having “asymptomatic bacteriuria” (ASB).[116]

This differentiation between UTI and ASB is of clinical and microbiological importance for patient management, but has also become increasingly important because of the emergence of antimicrobial resistance among many common uropathogens. Antibiotics are not administered to human patients with ASB. In many humans, untreated bacterial strains persist in asymptomatic patients for years without deleterious effects. Greater than 17% of elderly women and 5% of adult women carry bacteria within their urinary tract without symptoms of a UTI.[116] Importantly, these nonpathogenic bacteria, which have presumably co-evolved within the human urogenital
tract, are hypothesized to convey a degree of protection against urinary tract colonization with more virulent bacteria. Antibiotic administration to patients with ABU can result in the subsequent development of pathogenic infections of the lower urinary tract.

In veterinary medicine, bacteriuria is routinely diagnosed and is most often considered to be indicative of UTI.[2] However, many bacteriuric dogs present to their veterinarian with subtle to no overt clinical signs indicative of a UTI. Whether to routinely treat dogs with non-clinical bacteriuria is perhaps a more complicated decision than is currently appreciated. Many veterinarians will treat any dog with urine culture evidence of bacteriuria because the possible ramifications of not treating (pyelonephritis and renal failure) can be life-threatening. However, it is possible that unnecessary antibiotic therapies are a disservice to our patients as bacterial resistance could be induced in resident bacterial flora or more virulent or resistant bacteria may be able to colonize. Ideally, the clinician would be able to differentiate diagnostically whether bacteriuria has activated a host immune response, which would be indicative on ongoing pathology and therefore necessitate treatment.

The essential early step that determines whether a host response is elicited occurs when invading bacteria bind to and activate cell signaling pathways in the urothelium eliciting the release of cytokines that propagate a cellular inflammatory response.[11] Interleukin (IL)-1β, IL-4, IL-6, IL-8, interferon gamma, and transforming growth factor beta have all been found to be produced by human urinary bladder and renal tissues in
response to different pathological conditions. However, only a few of these cytokines are made on a constant basis and available for immediate release by cells. One pro-inflammatory cytokine, IL-6, will increase in concentration within minutes to hours following exposure of urothelium to gram negative bacterial products such as fimbriae and lipopolysaccharide. These results suggest that urinary concentrations of IL-6 are not only important to better understand the initial inflammatory response in urothelium, but also may be a useful diagnostic marker of urinary tract inflammation and disease pathology.

Commercially available kits can be purchased for measuring IL-6 in the serum or plasma of dogs via ELISA. However, a similar assay for the measurement of IL-6 in the urine of dogs has not been described. Complicating the development of a reliable diagnostic urinary IL6 assay is the inherent variability in the biological nature of urine, which can readily change in composition in healthy individuals. For example, urine pH and specific gravity (USG) can vary substantially at different time points among sick or healthy animals. Other contributing variables include differing concentrations of urinary protein and carbohydrate moieties that create a matrix capable of hindering immunologic testing assays. These multiple confounding variables do not allow for a simple translation of a serum assay to a urinary assay.

The purpose of this study was to optimize a canine specific IL-6 ELISA to facilitate accurate measurement of IL-6 concentrations in urine. To accomplish this objective,
efforts were made to neutralize protein and carbohydrate matrix effects that are inherent to urine and to determine the influence of pH and urine specific gravity on IL-6 measurements. Subsequently, the ELISA was used to screen urine samples from dogs with and without documented UTIs for the presence of IL-6 and to determine whether IL-6 is a viable biomarker of urinary tract inflammation and bacterial infection in dogs.

Materials and Methods

Urine Sample Collection and Preparation—A convenience sample of 50 canine patient urine samples were collected over a 3 year period from the Clinical Pathology Laboratory at the Veterinary Teaching Hospital of North Carolina State University (VTH-NCSU). Criterion for inclusion in the study included that the urine was collected aseptically, urinalysis was performed by the Clinical Pathology Laboratory and that an aerobic urine culture was performed in the Clinical Microbiology Laboratory at the VTH-NCSU. Urine was refrigerated immediately after collection, with 500µL to 1000µL urine aliquots frozen at -80°C within 24h of collection to prevent cytokine degradation. Urine samples were centrifuged at 1800rpm for 10m to remove gross sediment before freezing.

A total of 25 control urine samples were obtained from a colony of purpose bred beagles housed at the College of Veterinary Medicine at North Carolina State University and from employee pets that lacked a history of clinical signs compatible with lower
urinary tract disease. All patient and control urine samples were collected aseptically by cystocentesis and had urinalysis by chemical examination\(^{a}\) performed as well as refractometer\(^{b}\) measurement of urine specific gravity (USG). Urine (1ml) was cultured on a 3% sheep blood agar plate\(^{c}\) for 14d to assess sterility. Urine samples were centrifuged at 1800rpm for 10m to remove gross sediment before freezing at -80°C in 500µL to 1000µL aliquots. Urine collection was approved by the North Carolina State University Institutional Animal Care and Use Committee, IACUC ID# 06-091-O.

**ELISA Optimization**—An indirect chemiluminescent sandwich ELISA was optimized for detecting IL-6 concentrations within the urine of dogs (Figure 1). Capture antibody concentrations, goat anti-canine polyclonal IL-6\(^{d}\), were tested in a range from 200ng/ml to 1000ng/ml in 200ng increments. The coating antibody was assessed for IL-6 detection using three different coating buffers, 1X phosphate buffered saline (PBS), 8.6 pH carbonate buffer (15mM Na\(_2\)CO\(_3\), 35mM NaHCO\(_3\)) (CBC), and 9.5 pH CBC. Blockers assessed included 3% W/V instant nonfat dry milk\(^{e}\) and 0.1% V/V Kathon\(^{f}\) in 9.5 pH CBC and 2% milk 1% bovine serum albumin\(^{g}\) (BSA) in CBC 9.5 pH. Detection antibody concentrations, biotinylated goat anti-canine IL-6\(^{h}\), were tested in 2 fold dilutions from 100ng/ml to 12.5ng/ml. Reagent diluents assessed included 1% BSA in 1x PBS and 1% BSA, 0.5% Tween 20\(^{i}\) in 1x PBS.
Optimal plate coating utilized 100µl per well of 800ng/ml capture antibody diluted in
9.5 pH CBC incubated for 12h at 4°C in commercially available ELISA plates¹. Blocking was performed for 2h at room temperature with 205µl per well of 3% W/V instant nonfat dry milk and 0.1% V/V Kathon in 9.5 pH CBC. Background was reduced by incubating 100µl of 25ng/ml detection antibody in the reagent diluent of 1% BSA, and 0.05% V/V Tween 20, in 1x PBS solution for 2h at room temperature. Streptavidin-HRP⁰ in a 1:200 dilution with reagent diluent was incubated for 20m. Manual washes between each step were performed using a wash solution consisting of 0.05% V/V Tween 20 in 1x PBS. After the addition of 100µL of luminol/enhancer stable peroxide solutions¹ chemiluminescence was measured using a plate readerᵐ at 1m and 5m. Results were analyzed using commercially available softwareⁿ to perform a curve fit analysis.

**Establishment of a Standard Curve**—Control urine samples from a single dog or proprietary sample diluents⁰ (SD1, SD2, or SD3) were spiked with 1000pg/ml of canine recombinant IL-6⁰. Two fold dilutions in the various sample diluents were made to 15.6pg/ml. 100µl of each sample titration was then added to an equal volume of proprietary assay diluents⁴ (AD1, AD2, AD3, or AD4) within the ELISA plate. Numerous permutations of assay and sample diluents were tested. Resultant standard curves and urine curves were compared to each other using quantitative recoveries of spiked samples.
Based upon the above described optimization, the diluent pair of AD3/SD2 was subsequently used in following experiments to create standard curves and as the urine diluents. Sample diluent SD2 was added 1:1 with sample and standard to perform dilutions (125µl each). From this sample 100µl was removed and mixed with 100µl of assay diluent (AD3) in the ELISA plate. The combination of AD3/SD2 was also exclusively used as the sample and assay diluent for the measurement of IL-6 in the clinical samples. Standard and sample incubations were performed at 37°C for 2h.

**pH**—Urine pH was measured using pH 5.0-10.0 pH-indicator strips in accordance with the manufacturer’s recommendations. A single urine sample was divided into 5 aliquots. Urine pH was adjusted by adding 1N HCl or 1N NaOH to establish a pH range from 5.5 to 9.5 increasing in increments of 1 pH unit. Resultant urine was spiked with 4000pg/ml of canine recombinant IL-6 and using two fold dilutions in AD2/SD3 a curve was created to compare the effects on urine pH on the ELISA.

**Urine Specific Gravity**—USG was determined for all urine samples by refractometry. To determine the effects of USG on the ELISA urine samples ranging from 1.014 to >1.040 were spiked with 4000pg/ml of canine recombinant IL-6. Two fold dilutions were performed in AD2/SD3 and curves were compared to the standard to calculate quantitative recoveries.
Clinical Urine Sample ELISA—For each sample a neat, 2x, 4x, and 8x measurement of IL-6 was determined using the optimized ELISA protocol in duplicate. Using a curve fit analysis IL-6 concentrations were determined by comparing chemiluminescence of sample wells to that of the standard curve.

Results

Urine Standard Establishment—IL-6 standard curves were established using reagent diluent spiked with 4000pg/ml of canine recombinant IL-6 decreasing in 2 fold dilutions to 3.91pg/ml. The limit of detection of the assay fell between 32.25pg/ml and 15.62pg/ml of IL-6. The intra-assay coefficient of variation averaged 2.9% (n=3) for the standard curve while the inter-assay coefficient of variation was higher with a 7.7% (n=3) average. In contrast, urine samples spiked with 4000pg/ml of canine recombinant IL-6 created curves that were highly variable with the coefficient of variation between the samples reaching 19.6% (n=5). In addition, the quantitative recovery of urine IL-6 compared to the standard curve was only 28.6% for neat undiluted samples. Even after dilutions of 1:8 (the number of dilutions used in the clinical study) the spike recovery of IL-6 was only 54.6%. The large variation of IL-6 chemiluminescence between urine samples as well as the low spike recovery indicated that a component of the urine was decreasing the sensitivity of the ELISA.

The interference of matrix proteins and carbohydrates on the immunologic assay was hypothesized to contribute to the poor quantitative recoveries found in the spike recovery
experiments. The large variability in chemiluminescence seen for different urine samples was hypothesized to be secondary to a variable characteristic of urine such as pH or USG.

**Matrix Effect Neutralization**—To neutralize the effects of urine carbohydrates and proteins on the ELISA and improve the ability of the ELISA to accurately measure quantities of IL-6 the use of proprietary sample and assay diluents were utilized. Standard curves were created using every combination of sample and assay diluents previously described in the Materials and Methods section. The combination of AD3 and SD2 provided the best quantitative recovery. Using neat urine samples compared to the respective standard curve the spiked recovery increased to 67.8% and reached 89.1% at a dilution of 1:8 (n=19). While neutralizing the matrix effects did improve the accuracy of IL-6 detection, an identified compromise was an increase in the lower limit of detection from between 32.5pg/ml and 15.62pg/ml to between 125pg/ml and 62.5pg/ml of IL-6. This higher limit of detection was attributed to a substantial increase in the background chemiluminescence. Following the addition of 3% goat serum to block the nonspecific binding of the diluents to the polyclonal goat capture antibody background fluorescence did not improve substantially. Despite the narrowing of the dynamic range of the ELISA, the assay’s IL-6 detection range still fell within the reported range of clinical IL-6 concentrations found in the urine of humans with urinary tract infections.

After neutralizing the matrix effects, spike recovery of IL-6 in numerous different dog urine samples remained variable with quantitative recoveries for neat samples ranging
from 45.4% to 87.4%. These data indicate that another urine variable in addition to the
matrix effect was affecting the detection of IL-6 by the ELISA.

**Urine pH and USG Effects on ELISA on assay variability**—Variation in urine pH
provided a plausible explanation of why different urine samples spiked similarly failed to
be quantified identically by this ELISA. Dog urine normally has a pH within the range of
6.0-7.5, but can fall outside of this range during various pathologic conditions. Identical
urine samples with a titrated range of pH from 5.5-9.5 were used in a spike recovery
experiment using 4000pg/ml of canine recombinant IL-6. Chemiluminescence was
compared to a standard curve using AD3 and SD2 to perform sample dilutions.
Quantitative recoveries of IL-6 were nearly identical varying by only 5% when
comparing a urine sample modified to represent the range of physiologic urine pH. The
coefficient of variation between the different pH samples also remained close to the
standard intra-assay variation at 4.9% (compared to 2.9%) indicating that variation in
urine pH does not explain the variation in quantitative recoveries when comparing two
different urine samples.

Urine specific gravity is affected by total solute concentration as well the molecular
size and weight of the constituent solutes. The differences in solutes within the urine
may interfere with an immunoassay and contribute to the variation in measured IL-6
between urine samples. Seven urine samples with USG ranging from 1.014 to >1.040
were spiked with 4000pg/ml of canine recombinant IL-6. After two fold dilutions using
AD3 and SD2 the resultant curves were compared to a standard as well as to each other. As the urine specific gravity increases and hence solutes increase within the urine, the spike recovery of the ELISA improved. At a urine specific gravity of 1.014 the quantitative recovery for neat urine was 65.3% and increased to 80.0% after a 1:8 dilution. In contrast urine with a specific gravity of 1.032 had a neat quantitative recovery of 77.3% and increased to 94.7% after a 1:8 dilution. Therefore, variability in USG between urine samples is a major factor affecting IL-6 detection by ELISA within urine. Although all analyses underestimated IL-6 concentrations within each sample, there was greater discrepancy in IL-6 results at lower USGs. Ideally a correction factor could be used to calculate an approximate IL-6 concentration across a range of USG using the same diluents. To assess the feasibility of establishing a correction factor, urine samples with a USG of 1.015 (n=4) and 1.028 (n=4) were spiked with 4000pg/ml of IL-6. Variation in recoveries was assessed for neat, 2x, 4x, and 8x dilutions. Different dog urine samples with the same specific gravity had an average variation in recovery of 12.9% for a USG of 1.015 and 8.8% for a USG of 1.028. This data indicates that even for defined urine specific gravity there is enough variation in IL6 recovery that a correction factor would remain an estimate.
Clinical Application—The urine from 50 dogs (28 females and 22 males) collected from patients from which urine was cultured for bacterial growth at the Veterinary Teaching Hospital at North Carolina State University was used to assess the clinical utility of the urine IL-6 ELISA as optimized for this study. The dogs had a median age of 7 years (range, 0.6-17 years). Twenty-nine breeds were represented with Golden Retrievers (n=8), Labrador Retrievers (n=4), and Maltese (n=4) being the three most common breeds. The median USG was 1.019 (range, 1.005-greater than 1.040). The median pH was 7 (range, 5-9). Only 1 dog had clinical signs consistent with a urinary tract infection at the time of presentation. The most common clinical diagnoses were recurrent UTIs (n=6), followed by hyperadrenocorticism (n=4), hypoadrenocorticism (n=3), chronic kidney disease (n=3), cystoliths (n=3), and pyelonephritis (n=3). Thirteen of the 50 dogs had bacteria cultured from their urine with *E. coli* being the most prevalent (n=6) isolate, followed by coagulase positive *Staphylococcus* sp. (n=2). The control urine (n=25) predominantly came from 1 year old male intact purpose bred Beagles (n=17) although 7 other breeds were represented within an age range of 0.4-9 years. The total control population consisted of 20 males and 5 females. The median USG was 1.035 (range, 1.014-greater than 1.040) and the median pH was 7.3 (range, 6-8).

Of the 50 clinical samples, only 4 had detectable concentrations of IL-6 within the urine. None of these four dogs had clinical signs consistent with a urinary tract infection and only one had bacteria cultured from the urine. The two urine samples containing the
highest IL-6 concentrations, 593pg/ml and 447pg/ml respectively, were obtained from two dogs presenting with histiocytic sarcoma. The former presented with disseminated intravascular coagulation and the latter and presented in shock with bacteriuria. The other two samples with lower IL-6 concentrations, 72pg/ml and 63pg/ml respectively, were found in dogs that presented for fever and neck pain respectively. The latter dog also had a cystolith. None of the control dogs had detectable concentrations of IL-6 within the urine. In this study, there was no clinically relevant association between bacteriuria and urine IL-6 concentrations. Of note, a fifth dog had an IL-6 concentration that fell just below the assay limit of detection of 62.5pg/ml of IL-6. This dog had a USG of 1.017. Because low USG underestimates IL-6 concentration, the dog likely did have IL-6 secretion into the bladder. This dog had growth of *Proteus mirabilis* on urine culture and had been historically diagnosed with recurrent urinary tract infections.

**Discussion**

In this study, urinary concentrations of IL-6 were detectable in 4 of 50 dogs with suspected urinary tract infection or inflammation, of which only one dog had a documented urinary tract infection. The finding of increased IL-6 concentrations in non-bacteriuric samples is not unique, as this cytokine can be secreted independent of UTI and has been associated with the presence of urothelial irritants, such as urinary catheters.[117] More puzzling is that our ELISA results are not consistent with similar
studies performed in people. In healthy individuals, IL-6 is not detectable in the urine. However, within 30 minutes of instilling *E. coli* or P-fimbriae into the human urinary bladder, an IL-6 response is measurable. In 86% of women with bacteriuria, IL-6 concentrations are measurable by an ELISA bioassay. In this study, only 7% (1 of 13) of dogs with bacteriuria had measurable concentrations of IL-6, therefore it is possible that urinary IL-6 concentrations in dogs with UTI do not mirror trends found in human patients. Unfortunately, comparative data from other studies measuring the concentration of IL-6 using ELISA in the urine of dogs are not available.

The lack of IL-6 detected in the patient population receiving urine cultures at the NCSU-VTH was potentially associated with the fact that IL-6 measurements tend to decrease to non-diagnostic levels after the acute phase of urinary tract infection in human patients. By 6 days after infection the IL-6 response in humans is in decline. One plausible explanation is that IL-6 peaks occur for only a limited time after infection in dogs despite persistent bacteriuria. Only one of the 50 dogs evaluated had current signs of UTI. The rest either had histories of recurrent infections or presented for another medical problem, unrelated to the urinary tract. Even during the acute phase response to a urinary tract infection, IL-6 is secreted in a series of peaks. It is possible that if multiple urine samples were obtained from each patient the likelihood of obtaining a sample during an IL-6 peak would have increased. Since a majority of the dogs that were ultimately diagnosed with UTI were asymptomatic at the time of presentation it is also
likely that they may have been harboring the bacteria for greater than 6 days, making IL-6 detection improbable (assuming that the kinetics of urinary IL6 in dogs is analogous to humans). Regardless of the reason, these results would argue that using urinary IL-6 measured by ELISA as a diagnostic aid in deciding whether a patient presenting for bacteriuria should be treated with antibiotics is not feasible based on this study.

Although the optimized ELISA in this study would have to be used with caution when measuring IL-6 in dogs with asymptomatic bacteriuria, our findings outline several important considerations when applying commercially available kits for use with cell supernatants and serum samples to urine samples. Urine composition is dependent on numerous factors including the blood plasma composition, renal function, and the addition of materials as the glomerular filtrate passes from the kidneys through the lower urinary tract. This final factor may be most important when designing a urine-based ELISA because substances secreted into the urine may inhibit detection of the immunologic target. For example, Tamm-Horsfall mucoprotein (THP) has been shown to bind cytokines such as IL-2,[120] and in vitro THP will bind to plastics particularly in the presence of extracellular matrix.[121] This nonspecific binding of THP may trap IL-6, thereby effectively preventing the binding of IL-6 to capture antibody. After numerous washes this trapped IL-6 would be removed, reducing the detection of IL-6 in a sandwich ELISA below what was actually present in the sample.
In this study matrix effects did play a large role in decreasing the recovery of spiked IL-6 in urine. The use of sample and assay diluents increased the quantitative recovery of IL-6 in neat urine samples by over 100%. These proprietary diluents are designed to reduce nonspecific binding of the matrix and to promote an ionic strength and pH optimal for antigen/antibody interactions. The use of diluents did have the deleterious effect of increasing background chemiluminescence by almost three fold. Based on deduction, this increase is likely a result of the diluents themselves interacting with the goat polyclonal detection antibody. Unfortunately, the addition of 3% goat serum to the diluents had a minimal effect on decreasing the background noise. The increased background would contribute to the chance of generating false negative diagnostic results in samples with low IL-6 concentrations. However in children with UTI, IL-6 measured by ELISA had a median concentration of 397pg/ml,[118] which is well above this assay’s threshold.

A second factor identified as affecting the urine IL-6 ELISA performance was the variability in urine specific gravity. Unlike the matrix effects that universally decreased spike recovery of IL-6 in all urine samples, USG had a less predictable effect, although higher USG generally allowed for a better recovery of IL-6. This unpredictability was expected since measurement of USG is affected by both total solute concentration as well as the molecular size and weight of various solutes. Hence, urine samples with the same urine specific gravity will have variable numbers of solutes to interfere with ELISA.
Puzzling was the observation that as USG increased IL-6 recovery improved. This observation conflicts with the premise that the solutes themselves are interfering with the ELISA by binding IL-6 since solutes appear to have a protective effect on IL-6 yield. A hypothesis to explain this observation is that the urine solutes act as a blocking solution, thereby preventing the nonspecific binding of IL-6 to the ELISA plate and storage container plastics. Therefore, the putative blocking effect of the urine solutes would promote IL-6 binding to the capture antibody hence increase ELISA yield.

The primary urinary solutes are urea, sodium, potassium, and ammonium salts. The concentrations of these solutes vary for each urine specimen. This unpredictability of the total solute concentration makes creating an accurate correction factor to account for differences in USG difficult. Published human studies have used an IL-6:creatinine ratio to normalize the variable nature of urine.[1] Our laboratory contends that such a correction is inappropriate. Creatinine is an estimate of the glomerular filtration rate (GFR) and by dilution can affect the concentration of urinary solutes. However, IL-6 is not freely filtered in dogs. Even patients with a strong serum IL-6 response due to a systemic disease process fail to develop detectable levels of IL-6 in the urine.[52] Instead urinary concentrations of IL-6 are produced by renal epithelial cells and the cells of the urothelium.[40] This secretion is not constant. Interleukin-6 is released in a series of peaks during the first 48 hours after infection.[34] Since the rate of IL-6 secretion is variable and independent of GFR, using the IL-6:creatinine ratio as a correction factor
would do little to correct for the effects of urine solutes on the ELISA. An alternative solution to using either USG or creatinine would be to measure urine osmolality. Since osmolality only measures the concentration of solutes and is not affected by solute size or weight it may be used to more accurately create an ELISA correction factor. Given these observations at this time we can say with confidence that the described optimized ELISA is an effective tool for identifying the presence of IL-6 within the urine, but caution should be used when comparing the IL-6 concentrations of two clinical urine samples due to the effects of urinary solutes. A third factor, urine pH, did not appear to have an effect on the ELISA.

This study applies a canine specific IL-6 ELISA to urine. It identifies the urine matrix as a major contributing factor to poor ELISA performance. The research identifies urine specific gravity as a complicating factor in measuring urine IL-6. These factors alter the immunoassay performance allowing for the detection of IL-6, but prevent the direct comparison of IL-6 concentrations between patients. Interleukin-6 measurements also appear to be a poor diagnostic aid in identifying pathologic bacteria in patients presenting without signs of UTI. Alternatively additional cytokines or immunoregulatory molecules could be assessed such as IL-8, PDGF, or TNF-α. All three have experimentally been shown to increase after urothelial cell challenge with Escherichia coli and Citrobacter spp[122] and therefore these molecules may be attractive targets for examining the role of urinary cytokines in dogs.
Figure legends

**Figure 1**—The urine IL-6 ELISA was optimized by adjusting 6 different components of the immunoassay. Conditions that expanded the dynamic range of the ELISA and/or improved quantitative spike/recovery are listed in bold type and were ultimately used in the clinical study.

a. Multistix reagent strips, Bayer, Elkhart, IN

b. Schuco clinical refractometer, Erma, Tokyo, JA

c. 3% sheep blood agar plates, BD, Franklin Lakes, NJ
d. Goat anti-canine polyclonal IL-6 antibody, R & D Systems, Minneapolis, MN

e. Nonfat dry milk, Carnation, Solon, OH

f. Kathon, Supelco, Bellefonte, PA

g. Bovine serum albumin, Equitech-Bio Inc, Kerrville, TX

h. Biotinylated goat anti-canine IL-6 antibody, R & D Systems, Minneapolis, MN

i. Tween 20, Acros, Morris Plains, NJ

j. Chromalux 96 well HB plates, Dynex, Chantilly, VA

k. Streptavidin-HRP, R & D Systems, Minneapolis, MN

l. Femto Luminol/Enhancer and Femto Stable Peroxide solutions, Pierce, Woburn, MA

m. Wallac Victor³ reader, Perkin Elmer, Waltham, MA

n. Prism 4 software, GraphPad Software, La Jolla, CA

o. Sample diluents SD1, SD2, SD3, Immunochemistry Technologies, Bloomington, MN

p. Canine recombinant IL-6, R & D Systems, Minneapolis, MN

q. Assay diluents AD1, AD2, AD3, AD4, Immunochemistry Technologies, Bloomington, MN

r. Colorphast pH 5.0-10.0 pH-indicator strips, EMD Chemicals Inc, Gibbstown, NJ
Chapter 3

Uropathogenic E. coli induce urothelial autocrine IL-6 signaling and loss of barrier function in an ex vivo model of acute urinary tract infection.

Michael W. Wood, Edward E. Breitschwerdt, and Jody L. Gookin*

Department of Clinical Sciences, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, North Carolina 27606

Running title – IL-6 and barrier function in uropathogenic E. coli

Corresponding author:

Jody L. Gookin, DVM, Ph.D.
Department of Clinical Sciences
College of Veterinary Medicine
North Carolina State University
4700 Hillsborough Street
Raleigh, NC 27606
Phone (919) 513-6295
Fax (919) 513-6336
Jody_Gookin@ncsu.edu
Abstract

The urinary bladder is one of the most common sites of bacterial infection in the human body with 70-90% of cases attributed to uropathogenic *E. coli*. The loss of urothelial barrier function and secretion of interleukin-6 (IL-6) are known sequelae of these urinary tract infections (UTI). However, our knowledge of the native-tissue origin, polarity of secretion, target cell type(s), and effect on urothelial barrier function of IL-6 produced during acute uropathogenic *E. coli* infections is limited by the extremes of complexity and over-simplicity of current in vivo and cell culture models. In the present study, we modeled acute uropathogenic *E. coli* infection ex vivo using intact canine bladder mucosa mounted in Ussing chambers to sustain viability, physically separate submucosal and lumen influences, and quantitatively measure transepithelial electrical resistance (TER) as a measure of urothelial barrier function. Using this model, we demonstrated that uropathogenic *E. coli* infection results in loss of TER in association with failure of umbrella cell tight junction formation and stimulates synthesis of IL-6 by all cell layers of the urothelium with selective secretion into the lamina propria. Further, the urothelium was identified as the only site of unequivocal IL-6 receptor expression within the resident bladder mucosa and autocrine effects of IL-6 were supported by activation of urothelial STAT-3 signaling. IL-6 was not identified as mediating the acute effect of *E. coli* on failure of urothelial tight junctions. This novel model is likely to provide a powerful and relevant tool for identifying both the mechanisms of *E. coli* urothelial pathogenesis and role of IL-6 in mediating or ameliorating these effects.
INTRODUCTION

The urinary bladder is one of the most common sites of bacterial infection in the human body with 70-90% of cases attributed to *E. coli* [123]. Nearly 11% of women ≥ 18 years of age experience at least one urinary tract infection (UTI) per year, resulting in annual medical costs exceeding 1 billion dollars [8]. Given the prevalence and cost associated with UTI our understanding of the host response to bladder infection remains surprisingly limited, perhaps due to the extremes of complexity and over-simplicity of the in vivo and cell culture models [17, 124] currently used to study *E. coli* pathogenesis.

The urinary bladder is lined by a complex multicellular urothelial barrier that restricts translocation of ions, solutes and bacteria into the body despite tremendous changes in the volume, tonicity and composition of urine. Barrier function of the urothelium is primarily attributed to the umbrella cells, whose expression of apical membrane uroplakins [19, 28] and intercellular tight junction connections [20, 21] contribute to high transcellular and paracellular resistance [20, 125]. Damage to the urothelial barrier is a common consequence of bacterial infection [126] where leakage of urine constituents into the underlying tissue is believed to contribute to the genesis of pain and discomfort [16].

In addition to serving as a mechanical barrier, the urothelium is a biologically active sentinel of UTI, a notable response of which is the synthesis and secretion of interleukin-6 (IL-6)[34, 37, 40, 127]. While synthesis of IL-6 is a salient response of the urinary
bladder to bacterial infection, the target cell type(s) and functional effects of IL-6 still remain unknown. Studies in endothelia [128] and intestinal epithelium [129-131] have demonstrated significant effects of IL-6 on tight junction and barrier function that vary in outcome from deleterious [128, 129, 131] to beneficial [130].

In the present study, we modeled acute uropathogenic *E. coli* infection ex vivo using intact canine bladder mucosa mounted in Ussing chambers to sustain viability, physically separate sub-urothelial and lumen influences, and quantitatively measure transepithelial electrical resistance as a measure of urothelial barrier function. Using this model, these studies identify the native-tissue origin, polarity of secretion, target cell type and the effect on urothelial barrier function of IL-6 induced by acute uropathogenic *E. coli* infection.

**MATERIALS AND METHODS**

**Animals.** Intact urinary bladders were obtained from beagle dogs (aged 6-mos to 1 year; Covance Laboratories) immediately after euthanasia using sodium pentobarbital given intravenously. Urine sterility of each animal was confirmed by aerobic culture (10% blood agar for 14 days at 37°C) of urine samples obtained by aspiration from the bladder. All studies were approved by the Institutional Animal Care and Use Committee.
**Barrier function studies.** Urinary bladders were bathed in an oxygenated Ringer’s solution (mM; 154.1 Na⁺, 6.3 K⁺, 1.2 Ca²⁺, 0.7 Mg²⁺, 137.3 Cl⁻, 24 HCO₃⁻, 1.65 HPO₄²⁻). Sterile Ringer’s solutions were filtered (0.22 µm) and treated with antibiotics (streptomycin, 50 µg/ml; penicillin, 50 IU/ml). The urinary bladder was bisected longitudinally and the seromuscular and submucosal layers removed by sharp dissection. The resulting mucosal sheets (urothelium and lamina propria) were mounted in 3.14cm² aperture Ussing chambers and bathed on both surfaces with Ringer’s solution containing glucose (10 mM submucosal) and mannitol (10 mM lumen). Solutions were oxygenated (95% O₂, 5% CO₂) and circulated by gas lift and maintained at 37°C by water-jacketed reservoirs. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. If the spontaneous PD was between -1.0 and 1.0mV, tissues were current clamped at 100µA for 5-sec and the PD was recorded. Transepithelial electrical resistance (TER; Ω·cm²) was calculated from the spontaneous PD and short-circuit current which were recorded at 30-min intervals.
**Bacteria.** Uropathogenic *E. coli* J96 (kindly supplied by Dr. Paul Orndorff, North Carolina State University) were grown to log-phase at 37°C in Luria-Bertani broth and washed three times in sterile Ringer’s solution prior to addition to the lumen reservoir of Ussing-chambered bladder mucosa to achieve a final concentration of $1 \times 10^8$ cfu/ml.

**Light and transmission electron microscopy.** Bladder mucosa specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (4 μm) and stained with hematoxylin and eosin for light microscopic examination. For transmission electron microscopy, specimens were fixed in Trump’s 4F:1G at 4°C, processed using standard techniques [132] and visualized using a FEI/Philips EM 208S transmission electron microscope.

**Immunofluorescence microscopy.** After removal from the Ussing chamber, mucosae were embedded in optimal temperature cutting media and frozen-sectioned at 4-μm thickness. Sections were fixed in 100% ethanol and blocked with 1% BSA (v/w) and 2% goat serum (v/v) in PBS+ (1× PBS, 0.12% of 1M CaCl₂, pH 7.4) for 1-hr at 4C prior to incubation with primary antibodies. Primary antibodies (1:50 in blocking buffer) were applied for 1h at room temperature and included biotinylated polyclonal goat anti-canine IL-6 and biotinylated polyclonal goat anti-human soluble IL-6 receptor (R&D Systems,
Minneapolis, MN), polyclonal rabbit anti-human uroplakin-III (Abcam, Cambridge, MA), and polyclonal rabbit anti-canine ZO-1 (Invitrogen, Eugene, OR). Fluorescence labeling was performed using streptavidin-conjugated Alexa Fluor 488 (1:100; Invitrogen, Eugene, OR), Cy3-labeled goat anti-rabbit (1:100; Jackson Immunoresearch, West Grove, PA), and FITC-labeled control mouse IgG1 (1:500; BD Pharmingen, Franklin Lakes, NJ) for 30-min at room temperature. Sections were counterstained with DAPI and visualized using an epifluorescence microscope. For demonstration of cytokine synthesis, bladder mucosae were treated with the golgi protein transport inhibitor monensin (1 µl/ml of 1.5× solution; BioLegend, San Diego, CA) applied to both the lumen and submucosal reservoir of the Ussing chamber.

**IL-6 ELISA.** A canine-specific IL-6 chemiluminescence ELISA (R&D Systems, Minneapolis, MN) was used to assay IL-6 in duplicate samples obtained from the lumen and submucosal reservoirs bathing the bladder mucosa. Components of the ELISA included capture (800 ng/ml) and detection (25 ng/ml) goat anti-canine polyclonal IL-6 antibodies, streptavidin-HRP (1:200 in Femto Luminol/Enhancer), and Femto Stable Peroxide substrate (Pierce, Woburn, MA). Recombinant canine IL-6 (R & D Systems, Minneapolis, MN) was used to create the standard curve and results reported as pg IL-6 per ml Ringer’s solution.
Western analysis. Soluble protein was extracted from liquid nitrogen-frozen samples of bladder mucosa by homogenization (Mini-Beadbeater; BioSpec Products, Bartlesville, OK) with stainless steel beads (3.2 mm) in RIPA buffer (25mM Tris-HCl, 150mM NaCl, 1% Nonidet P-40, 1% Sodium Deoxycholate, 0.1% SDS, 1% EDTA, pH 7.6) containing antiproteases (1% each of Halt anti-protease cocktail (Pierce, Woburn, MA), and anti-phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich, St. Louis, MO). Equal protein concentrations, measured by BCA assay, were electrophoretically separated in 4-12% Bis:Tris gradient gels (Invitrogen, Carlsbad, CA), transferred to nitrocellulose, and sequentially immunoblotted using anti-pSTAT3 [Ser727] (0.5µg/ml; Assay Designs, Ann Arbor, MI) and anti-total STAT3 (1:2500; BD Biosciences, San Jose, CA) monoclonal antibodies. Secondary reagents included anti-mouse IgG-HRP (1:10,000; Santa Cruz, Santa Cruz, CA) and the chemiluminescence substrate (Supersignal West Pico; Pierce, Woburn, MA). Pervanadate-treated HepG2 cell lysate was included as a positive control for pSTAT3 and equal protein loading was confirmed by immunoblotting cell lysates with polyclonal anti-goat GAPDH (200µg/ml) followed by secondary anti-goat IgG-HRP antibodies (400µg/ml; Santa Cruz, Santa Cruz, CA). Densitometry was performed using SigmaScan software (Systat, San Jose, CA) and reported as pSTAT3 normalized to total STAT for each sample.
**Statistical analysis.** Statistical analyses were performed using commercially available software (SigmaStat; San Jose, CA). Values are reported as mean ± standard error. Data were tested for normality (Kolmogorov-Smirnov) and variance (Levene median) and analyzed using parametric or non-parametric tests where appropriate. Parametric data were analyzed using a Student’s t-test. Non-parametric data were analyzed using a Mann-Whitney rank sum test. In all cases n = number of dogs.

**RESULTS**

**Uropathogenic *E. coli* mediates loss of urothelial barrier function.** To determine the acute effect of uropathogenic *E. coli* on urothelial barrier function, *E. coli* J96 (1 × 10⁸ cfu/ml) were inoculated into the lumen reservoir of Ussing-chambered bladder mucosa and the effect on transepithelial electrical resistance (TER) was measured over a 5 hour period. In the absence of *E. coli*, TER of the urothelium characteristically increased steadily over time [18], achieving measurements in excess of 1,300 Ω.cm². In the presence of *E. coli* however, recovery or urothelial barrier function was significantly impaired (Fig. 1A).

**Uropathogenic *E. coli* stimulates basolateral secretion of interleukin-6 by the urothelium.** Urothelial cells in culture will secrete IL-6 in response to infection with *E. coli* [37, 40, 127] and bacterial urinary tract infection in vivo results in increases in
urinary IL-6 concentrations [34]. Using intact bladder mucosa, we sought to determine if *E. coli*-induced synthesis of IL-6 is confined to the urothelium and whether the primary secretion is selectively directed to either the lumen or lamina propria of the bladder. As early as 3 hours after inoculation of *E. coli* J96 (1 × 10⁸ cfu/ml) into the lumen reservoir of Ussing chambered bladder mucosa, significant increases in IL-6 were measured in the submucosal reservoir. Significant concentrations of IL-6 were not detected in the lumen reservoir 10h after infection (Fig. 1B). Identification of IL-6 secreting cells by means of immunofluorescence demonstrated that IL-6 synthesis was restricted to urothelial cells with no apparent contribution by cells residing in the lamina propria. Further, all layers of the urothelium were identified as participating in the synthesis of IL-6 (Fig. 2A).

**Uropathogenic *E. coli* infection results in failure of urothelial tight junction formation.** To determine the basis for *E. coli*-mediated loss of urothelial barrier function, light and transmission electron microscopic examination of the urothelium was performed immediately following isolation of the mucosa and after infection by *E. coli* in the Ussing chamber. At a light microscopic level, intact urothelium were identified and not significantly altered in appearance by the presence of *E. coli* infection for periods up to 10 hours (Fig. 3). In contrast, we identified distinct ultrastructural differences in tight junction formation between treatment groups that closely corresponded to concurrent measures of transepithelial resistance. In uninfected urothelium, dissection and acute stretch were accompanied by an initial lack of defined tight junctions between umbrella
cells. After mounting in the Ussing chamber, increases in measured transepithelial resistance were accompanied by reformation of these tight junctions. Moreover, differences in recovery of barrier function between individual tissues corresponded in magnitude to maturation of tight junctions, with tight junctions readily apparent in urothelium exceeding \( \sim 850 \, \Omega \cdot \text{cm}^2 \) and poorly defined in urothelium below \( \sim 450 \, \Omega \cdot \text{cm}^2 \). These observations agree with experimental evidence that transepithelial resistances in excess of \( 500 \, \Omega \cdot \text{cm}^2 \) are compatible with tight junction formation [17, 133]. In contrast to uninfected mucosa, failure of the \textit{E. coli} infected urothelium to similarly restore barrier function was accompanied by apparent lack of tight junction reformation and isolated evidence of umbrella cell detachment (Fig. 3).

To further establish whether \textit{E. coli}-induced failure to maintain transurothelial resistance was due to tight junction loss and not umbrella cell detachment or altered expression of uroplakin, we examined the expression of ZO-1 and uroplakin III in situ by means of immunofluorescence. In the uninfected bladder mucosa uroplakin-III was expressed intensely by all layers of the urothelium with ZO-1 discretely expressed at the intercellular junctions of the umbrella cells. In response to \textit{E. coli} infection, uroplakin-III expression was unaltered, while the expression of ZO-1 was conspicuously absent (Fig. 4).
The urothelium is an autocrine recipient of IL-6 signals in response to uropathogenic *E. coli* infection. While synthesis of IL-6 is a salient response of the urinary bladder to bacterial infection, the target cell type(s) and functional effects of IL-6 still remain unknown. Studies in endothelia [128] and intestinal epithelium [129-131] have demonstrated significant effects of IL-6 on tight junction and barrier function that vary in outcome from deleterious [128, 129, 131] to beneficial [130]. We are unaware of any studies examining the effect of IL-6 on urothelial barrier function. To determine if IL-6 mediates the acute effect of *E. coli* on barrier function of the urothelium, we first identified the cellular target(s) of IL-6 by locating IL-6 receptor-expressing cells using immunofluorescence. In both uninfected and *E. coli* infected bladder mucosa, expression of IL-6 receptors was confined to the urothelial cells. Little evidence for IL-6 receptor expression by cells residing within the submucosa was observed (Fig. 2B). Because IL-6 receptors were constitutively expressed by the urothelial cells, we further examined if IL-6-mediated signals were generated within the urothelium in response to *E. coli* infection. Accordingly, uninfected and *E. coli*-infected urothelia were removed from the Ussing chamber after 5 hours and the cellular lysates immunoblotted for total and phosphorylated STAT3. Significant phosphorylational activation of STAT3 was observed in the urothelium infected with *E. coli* but not uninfected urothelium (Fig. 5).
IL-6 does not mediate the acute effect of uropathogenic E. coli on urothelial barrier function. To determine if IL-6 mediates the loss of urothelial barrier function in response to acute uropathogenic E. coli infection, we added canine recombinant IL-6 (20 ng/ml) to the submucosal reservoir of Ussing-chambered bladder mucosa in quantities sufficient to yield an IL-6 concentration approximately 2 times that induced by E. coli. Despite incubation periods of over 5 hours, there was no significant effect of IL-6 on transepithelial resistance (Fig 6). Accordingly, IL-6 produced in the acute phase response to E. coli infection is not sufficient to mediate the observed early decrease in urothelial barrier function.

DISCUSSION

A key biological response of the bladder to urinary tract infection is the secretion of IL-6 [34]. While not normally present in the urine [52], IL-6 is common in urine of patients with cystitis or pyelonephritis [52, 134] and appears in human urine within 4-hrs of experimental infection with E. coli [34]. Using cell culture models, studies have shown that urothelial cells secrete IL-6 in response to infection with E. coli [37, 40, 127]. Whether IL-6 synthesis is confined to the urothelium, is secreted directly into the urine, or translocates into urine secondary to an increase in bladder permeability are unknown. By clarifying the source and polarity of IL-6 secretion in the context of the entire bladder mucosa, the present studies have provided insight into the cellular targets and function of
this cytokine. Using an ex vivo preparation of canine bladder mucosa, these studies demonstrate that IL-6 is synthesized by all layers of the urothelium and is secreted exclusively into the underlying lamina propria and not into urine during the acute phase of *E. coli* infection. Whereas elevations in urine IL-6 were measured within hours of experimental urinary tract infection in humans [34], such elevations were not detected in the same timeframe of our ex vivo studies. A key difference between the two models is the absence of recruited inflammatory cells ex vivo. These observations suggest that IL-6 may secondarily leak into the urine in association with influx and transmigration of neutrophils [135]. This may further explain why urine IL-6 concentrations appear to correlate with clinical disease severity [134]. Based on some known actions of IL-6, it is reasonable to speculate that primary secretion of IL-6 into the lamina propria plays a role in both recruiting [136] and enhancing the microbicidal activity of these infiltrating neutrophils [130].

While IL-6 is a predominant cytokine secreted by the urothelium in response to urinary tract infection, the cellular target and biological responses mediated by IL-6 in this microenvironment have remained poorly understood. The present studies are the first to identify the urothelium as the only site of unequivocal IL-6 receptor expression within the resident bladder mucosa. As such, urothelial cells appear to be a key autocrine recipient of acute IL-6 signals; an assertion which is supported by our demonstration of increased urothelial STAT-3 signaling. A direct effect of *E. coli* on STAT3 activation
however cannot be ruled out. The functional response of the urothelium to stimulation by IL-6 remains unknown. However, because studies in endothelia [128] and intestinal epithelium [129-131] have demonstrated significant effects of IL-6 on tight junction and barrier function [128-131], we explored the possibility that IL-6 was responsible for the loss of urothelial barrier function characteristic of \textit{E. coli} infection. Direct submucosal application of recombinant canine IL-6 at concentrations that recapitulate \textit{E. coli}-induced IL-6 secretion did not result in loss of barrier function. Whether or not IL-6 is capable of inducing changes in urothelial permeability after more prolonged exposure was not examined due to the time limitations of our ex vivo studies.

Independent of IL-6, uropathogenic \textit{E. coli} mediated loss of urothelial barrier function as sensitively measured by failure of the urothelium to maintain transepithelial electrical resistance. Transepithelial resistance reflects the ability of the urothelium to resist paracellular and transcellular permeation of ions. Paracellular resistance is largely conferred by intercellular tight junctions that adjoin the umbrella cells while transcellular resistance is conferred by apical and basolateral cell membranes that are inherently resistant to ion permeation. Observations in the present study provide evidence that \textit{E. coli} infection perturbs tight junction control of paracellular permeability rather than transcellular integrity of the urothelium. First, the act of mounting bladder mucosa within the Ussing chamber gave rise to an initially low resistance that was rapidly restored to physiological levels in association with reformation of tight junctions between the
umbrella cells. Second, in response to *E. coli* infection, the urothelium remained largely intact however reformation of tight junctions between umbrella cells was not observed. Third, we found no direct evidence for disrupted membrane uroplakin expression as postulated in a prior study but not directly examined [15]. Neither did we observe changes in electrical current indicative of the flow of ions through transmembrane channels [18]. Finally, the size of the permeability defect resulting from *E. coli* infection is consistent with that reported for selective loss of tight junction function. For example, in patients with interstitial cystitis failure of tight junctions is associated with leakage of irritating sodium and urea (hydrodynamic radius of 2.6 and 2.3 Å, respectively) but not albumin (35.5 Å) into the suburothelium [137]. Similar size selectively was shown in the present study by the inability of basolaterally-secreted IL-6 (30.5-35.5 Å)[138] to leak from the lamina propria back into the bladder. This further supports our notion that a more dramatic loss of barrier function, such as that resulting from the inflammatory response, is required for IL-6 to translocate into the urine. Although the mechanisms by which uropathogenic *E. coli* disrupt urothelial tight junctions remain undetermined, enteropathogenic *E. coli* elaborate virulence factors and proteases directly cleave tight junctional proteins, modify the actin cytoskeleton, activate cellular signal transduction, or instigate transmigration of neutrophils to disrupt epithelial barrier function in the intestine [139]. It seems plausible that similar mechanisms may be active in UTI.
By isolating the native urothelial microenvironment from recruited inflammatory cells, the present studies demonstrate that uropathogenic *E. coli* infection results in synthesis of IL-6 by all cell layers of the urothelium with selective secretion into the lamina propria. Further, the urothelium was identified as the only site of unequivocal IL-6 receptor expression within the resident bladder mucosa and autocrine effects of IL-6 were supported by activation of urothelial STAT-3 signaling. While IL-6 was not identified as mediating the acute effect of *E. coli* on failure of urothelial tight junctions, the unclarified effects of IL-6 on the urothelium remain nonetheless intriguing. This novel model is likely to provide a powerful and relevant tool for identifying both the mechanisms of *E. coli* urothelial pathogenesis and for establishing the role of IL-6 in mediating or ameliorating these effects.

**ACKNOWLEDGEMENTS**

Michael Wood is supported by the Ruth L. Kirschstein National Research Service Award T32 RR024394 as part of North Carolina State University's Comparative Medicine and Translational Research Training Program. We would like to thank Maria Stone, Stephen Stauffer, Mitsu Suyemoto, and the Laboratory for Advanced Electron and Light Optical Methods for their valuable technical assistance.
Figure 1  Maintenance of transepithelial electrical resistance (TER)(panel A) and secretion of IL-6 by urothelium (panel B) in response to normal Ringer’s solution (NR) or lumen infection with uropathogenic *E. coli* J96 (1 × 10^8 cfu/ml). IL-6 concentrations were measured in the submucosal reservoir of the Ussing chamber after 5 hours of infection. IL-6 was below the limit of detection in the lumen reservoir. * p<0.05, ** p<0.01; n = 6 dogs for each treatment condition.
Figure 2  Immunofluorescence localization of IL-6 and IL-6 receptors in *E. coli* treated canine urothelium. Urothelium mounted in Ussing chambers were exposed to $1 \times 10^8$ cfu/ml of *E. coli* J96. Tissue was simultaneously exposed to monensin. Both IL-6 (A) and IL-6 receptors (IL-6R) (B) were concentrated within the urothelial cells. Fluorescence was not observed in sections incubated with mouse IgG$_1$ control antibodies (C).
Figure 3 Light and transmission electron microscopic appearance of canine bladder mucosa after incubation in the Ussing chamber in the absence (A, C, and E) or presence (B, D, and F) of $1 \times 10^8$ cfu/ml *E. coli* J96 on the lumen side of the urothelium. Umbrella cells (UC), intermediate cells (IC), and basal cells (BC) can be ultrastructurally identified under each treatment condition. After mounting in Ussing chambers, well-developed tight junctions (tj) between umbrella cells can be identified within a period of 5-hrs (C, E).
After a 5-hr exposure to *E. coli* J96, umbrella cells are rarely observed to detach (D) and those remaining lack defined intercellular junctions (ij)(F).
Figure 4  Immunofluorescence localization of ZO-1 and uroplakin-III in canine bladder mucosa treated with normal Ringer’s solution alone or infected with uropathogenic *E. coli* J96 (1 × 10^8 cfu/ml) for a period of 5 hours in Ussing chambers. Localization of ZO-1 to the apical-lateral junctions of umbrella cells was well-defined in mucosa treated with normal Ringer’s and absent in mucosa infected with *E. coli*. There were no differences in uroplakin-III expression by the urothelium between treatment groups.
Figure 5  Infection of canine urothelium with *E. coli* promotes phosphorylation of STAT3. Canine bladder mucosa immediately after removal of the seromuscular layers (NC; non-chambered), and after 5 hour treatment in Ussing chambers with normal Ringer’s solution (NR) or uropathogenic *E. coli* J96 (1 × 10⁸ cfu/ml) were immunoblotted for phosphorylated STAT3 (pSTAT3), total STAT3, and GAPDH (protein loading control). *E. coli* significantly increased phosphorylation of STAT3
compared to un-chambered (NC) and uninfected tissue (NR). Densitometric data represent relative fold increase in pSTAT3 to total STAT3. *** p<0.001, n = 4 dogs each.
Figure 6  Maintenance of transepithelial electrical resistance (TER) by canine bladder mucosa treated in Ussing chambers with exogenous recombinant canine IL-6 (20 ng/ml; submucosal reservoir) or normal Ringer’s solution (NR) alone. n = 6 dogs each.
Chapter 4

Urothelial response to interleukin-6 stimulation includes the activation of genes responsible for pro-inflammatory effects and tissue remodeling.

Michael W. Wood, Edward E. Breitschwerdt, and Jody L. Gookin
Abstract

The urinary bladder is one of the most common sites of bacterial infection in the human body with 70-90% of cases attributed to uropathogenic *E. coli*. One of the first responses of bacterial stimulated urothelia is the secretion of interleukin-6 (IL-6). This IL-6 acts locally in a paracrine fashion modulating gene transcription within the urothelial cells. The specific genes regulated in response to IL-6 activation and the role of these products in urinary tract infections is unknown. In the present study, we modeled the acute IL-6 response ex vivo using intact canine bladder mucosa mounted in Ussing chambers. Using this model, we demonstrated that exogenously added IL-6 does activate the IL-6/STAT3 pathway and regulates the transcription and translation of genes as demonstrated by increased concentrations of SOCS3 protein within the cytoplasm of urothelial cells. Further, 49 common genes were identified via microarray as being regulated by IL-6 interaction with the urothelium including genes responsible for the cytokine/chemokine/growth factor response, cell adhesion and ECM remodeling, GAG production, cytoskeletal rearrangement, and cellular transport. Finally, four genes, heparan sulfate, syndecan 2, MMP-2 and IL-1β, were verified via q-RT-PCR as being integral in promoting a pro-inflammatory state and tissue remodeling during infection.
Introduction

Urinary tract infections (UTIs) are the second most common infection of the human body. Nearly 11% of women 18 years or older experience at least one UTI per year with medical costs exceeding 1 billion dollars[8]. For a single episode of uncomplicated UTI patients experience on average 6.1 days with UTI symptoms and 2.4 days of restricted activity[140]. Given the prevalence, costs, and morbidity associated with these infections there has been an ongoing emphasis placed on understanding the mucosal response to bacterial bladder colonization and how it can be modified to improve clinical outcomes.

In the urinary bladder, the urothelium provides the first line of defense against pathogens. Bacteria adhering to and internalizing into urothelial cells[76] as well as the release of bacterial virulence factors such as LPS[93] initiate a local response that includes the release of cytokines, growth factors, and other pro-inflammatory molecules[33, 36]. These mediators of the acute phase response to UTI induce many local changes in the urothelial microenvironment including the recruitment of inflammatory cells to help clear the infection[33]. These changes can serve two functions including the destruction of the invading pathogens as well as secondary tissue damage that may augment the clinical signs of infection. Given the potential benefits and harm that each of the factors may initiate, understanding which factors promote which effects is crucial to better managing cases of UTI.
Experimentally, interleukin-6 (IL-6) has been used as a marker of bacterial activation of urothelial cells[141]. Constitutively produced, IL-6 is one of the first cytokines released in response to infection[13]. Recently we demonstrated using an ex vivo Ussing chamber model of UTIs that within 5h of initial contact with uropathogenic *E. coli* urothelial cells produce and respond to the cytokine IL-6 in an autocrine/paracrine manner. These data suggest a local role for IL-6 within the epithelium.

Throughout the body the local effects of IL-6 are numerous. The cytokine has been recognized for its role in bone remodeling, obesity related insulin resistance, endothelial permeability, epithelial B cell maturation, response to myocardial infarction, and is a prominent cytokine in tumor biology[43-47]. However within the urothelium, the specific genes regulated in response to IL-6 activation and the role of these products in urinary tract infections is unknown. The central hypothesis of the described work contends that IL-6, produced during the acute phase response of urinary tract infection, binds to urothelial membrane-associated IL-6 receptors and alters gene expression to promote tissue remodeling.

This research utilizes our recently described Ussing chamber model of UTIs to isolate and characterize the acute transcriptional effects of IL-6. This model maintains viable tissue in the absence of a blood supply and hence is able to specifically isolate the action of IL-6 on the urothelium by eliminating the confounding effects of recruited inflammatory cells. Given these features this work may best isolate changes in gene
expression particularly related to tissue remodeling using microarray and q-RT-PCR techniques.

**Materials and Methods**

**Animals**

Intact urinary bladders were obtained from beagle dogs (aged 6-mos to 1 year; Covance Laboratories) immediately after euthanasia using sodium pentobarbital given intravenously. Urine sterility of each animal was confirmed by aerobic culture (10% blood agar for 14 days at 37°C) of urine samples obtained by aspiration from the bladder. All studies were approved by the Institutional Animal Care and Use Committee.

**Barrier function studies**

Urinary bladders were bathed in an oxygenated Ringer’s solution (mM; 154.1 Na\(^+\), 6.3 K\(^+\), 1.2 Ca\(^{2+}\), 0.7 Mg\(^{2+}\), 137.3 Cl\(^-\), 24 HCO\(_3\)\(^-\), 1.65 HPO\(_4\)\(^{2-}\)). Sterile Ringer’s solutions were filtered (0.22 µm) and treated with antibiotics (streptomycin, 50 µg/ml; penicillin, 50 IU/ml). The urinary bladder was bisected longitudinally and the seromuscular and submucosal layers removed by sharp dissection. The resulting mucosal sheets (urothelium and lamina propria) were mounted in 3.14cm\(^2\) aperture Ussing chambers and
bathed on both surfaces with Ringer’s solution containing glucose (10 mM submucosal) and mannitol (10 mM lumen). Solutions were oxygenated (95% O₂, 5% CO₂) and circulated by gas lift and maintained at 37°C by water-jacketed reservoirs. The spontaneous potential difference (PD) was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short-circuited through Ag-AgCl electrodes using a voltage clamp that corrected for fluid resistance. If the spontaneous PD was between -1.0 and 1.0mV, tissues were current clamped at 100µA for 5-sec and the PD was recorded. Transepithelial electrical resistance (TER; Ω·cm²) was calculated from the spontaneous PD and short-circuit current which were recorded at 30-min intervals. Tissue treatments included 20ng/ml of canine recombinant IL-6 (R&D Systems, Minneapolis, MN) added to the submucosal reservoir or normal Ringer’s (NR) as a control. Total treatment contact time with the urothelium was 5h.

**Western analysis**

Soluble protein was extracted from liquid nitrogen-frozen samples of bladder mucosa by homogenization (Mini-Beadbeater; BioSpec Products, Bartlesville, OK) with stainless steel beads (3.2 mm) in RIPA buffer (25mM Tris-HCl, 150mM NaCl, 1% Nonidet P-40, 1% Sodium Deoxycholate, 0.1% SDS, 1% EDTA, pH 7.6) containing antiproteases (1% each of Halt anti-protease cocktail (Pierce, Woburn, MA), and anti-phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich, St. Louis, MO). Equal protein concentrations, measured
by BCA assay, were electrophoretically separated in 4-12% Bis:Tris gradient gels (Invitrogen, Carlsbad, CA), transferred to nitrocellulose, and sequentially immunoblotted using anti-pSTAT3 [Ser727] (0.5µg/ml; Assay Designs, Ann Arbor, MI) and anti-total STAT3 (1:2500; BD Biosciences, San Jose, CA) monoclonal antibodies or goat anti-human polyclonal anti-SOCS3 (1µg/ml; Lifespan Biosciences, Seattle, WA). Secondary reagents included anti-mouse IgG-HRP (1:10,000; Santa Cruz, Santa Cruz, CA) or donkey anti-goat IgG-HRP (1:10,000; Santa Cruz, Santa Cruz, CA) respectively and the chemiluminescence substrate (Supersignal West Pico; Pierce, Woburn, MA). Pervanadate-treated HepG2 cell lysate (Axxora, San Diego, CA) was included as a positive control for pSTAT3 and MOLT-4 cell lysate (Santa Cruz, Santa Cruz, CA) was used as a positive control for SOCS3. For the STAT blots protein loading was normalized by calculating a pSTAT3 to STAT3 ratio. For the SOCS3 blots equal protein loading was confirmed by immunoblotting cell lysates with monoclonal anti-mouse actin (1:2000; Chevicon, Billerica, MA) followed by secondary anti-mouse IgG HRP antibodies (1:5000; Santa Cruz, Santa Cruz, CA). Densitometry was performed using SigmaScan software (Systat, San Jose, CA) and reported as pSTAT3 normalized to total STAT3 or simply SOCS3.
RNA Extraction

Chamber tissues were placed in 600μl aliquots of RLT lysis buffer (Qiagen, Valencia, CA) and flash frozen in liquid nitrogen for storage at -20°C prior to extraction. Tissues were homogenized using two 3.2mm stainless steel beads (BioSpec Products, Bartlesville, OK) agitated with a Mini-Beadbeater (BioSpec Products, Bartlesville, OK). RNA was extracted from the resultant suspension using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) using the manufacturers recommendations and stored at -80°C. RNA purity and quality was assessed using an Agilent Bioanalyzer 2100 (Hewlett Packard, Corvallis, OR). RIN numbers for all samples met or exceeded 8.80. Resultant RNA was collected from a total of four dogs. Each dog bladder was divided such that tissue from all dogs received both NR and IL-6 treatments producing 4 paired RNA samples where the NR treated tissue served as the internal control.

Microarray

The paired RNA samples were amplified using Affymetrix GeneChip® One-Cycle cDNA Synthesis Kit and labeled using Affymetrix GeneChip® IVT Labeling Kit (Affymetrix, Santa Clara, CA). The cRNA was hybridized to GeneChip Canine Genome 2.0 Arrays (Affymetrix, Santa Clara, CA) containing probes for >20,000 genes. The
images were captured using Affymetrix Genechip Scanner 3000 7G (Affymetrix, Santa Clara, CA).

The data was analyzed via Principal Components Analysis. During analysis it was revealed that Dog 1 clustered by itself on both the 1st principal component and 2nd principal component with respect to the other three dogs. Based on this the specimen was removed from the analysis as it was considered an outlier and would potentially dilute the sensitivity. Important latent factors unrelated to the experiment of interest were estimated via Principal Components Analysis (PCA). We identified the first component at which the specimens are organized with respect to their biological groups. Since RNA profiles of specimens were paired as in a repeated measures analysis and differences were computed for each transcript for each paired subject (IL-6 treated versus control NR treated) Repeated Measures Permutation Analysis of Differential Expression (RM-PADE) was utilized in combination with an Overlap Analysis to identify target genes. Genes were required to have a minimum of 2 fold increase in transcripts over control, a p-value less than 0.05, and concordance in gene expression for all three dogs to be considered for further analysis.

**q-RT-PCR**

Ten genes were further characterized via quantitative reverse-transcriptase PCR (q-RT-PCR). To reduce confounding variables the paired NR and IL-6 RNA samples
extracted from 4 dogs for use in the microarray experiments were utilized for q-RT-PCR. Reverse transcription was performed using the Superscript II RT enzyme kit (Invitrogen, Carlsbad, CA) according to the manufacturers recommendations. 100ng of converted cDNA was amplified using SYBR Green PCR master mix (Applied Biosciences, Framingham, MA) in a BioRad ICycler. Primer pairs (Table 1) were designed using the canine predicted base sequences for the genes of interest as described in the NCBI database. Primers were validated by PCR and gel electrophoresis to ensure that only a single band of desired size was present. PCR products were verified by sequencing. Relative copy numbers were determined by comparing resultant amplification to the hypoxanthinephophoribosyltransferase (HPRT) canine housekeeping gene. To determine up regulation or down regulation gene expression paired transcript numbers were compared between the IL-6 treated tissue and the NR treated tissue using a paired Students t-test.

**Statistical analysis**

Statistical analyses were performed using commercially available software (SigmaStat software, Systat, San Jose, CA). Values are reported as means +/- standard error. Data were tested for normality and analyzed using parametric or non-parametric tests where appropriate. Parametric data were analyzed using the Student’s T-test. Non-parametric
data were analyzed using a Mann-Whitney rank sum test. Data were paired when appropriate. In all cases n = # of dogs.

Results

Exogenous IL-6 activates urothelial gene transcription

In previous work we have demonstrated that uropathogenic *E. coli* will induce the secretion of IL-6 and the activation of the IL-6 signaling pathway, STAT3, within urothelial cells. For the purpose of this study we are asking the question, what specific effects do IL-6 have on the urothelium? To determine whether exogenously added IL-6 will activate urothelium mounted within Ussing chambers, tissue was exposed to exogenous canine recombinant IL-6 at polarity and concentrations that recapitulate *E. coli* induced IL-6 secretion. Normal Ringer’s (NR) treatment was used as control. Nonchambered tissue was collected to rule out any chambering effects. After 5h of treatment, protein lysates created from the harvested urothelium were immunoblotted for total STAT-3 and phospho-STAT-3. Unexpectedly there was no statistical difference between the IL-6 and NR treated tissue (p=0.12) (*Figure 1*).

Although exogenously added IL-6 did not result in significant amounts of pSTAT3 when compared to control, it remained possible that negative regulatory factors such as SOCS3 may have already reduced pSTAT3 concentrations in the urothelial cells by 5h. Since the transcription factor pSTAT3 induces SOCS3 production, finding increased
concentrations of SOCS3 would have the dual effect of validating that exogenously added IL-6 activates the urothelium and demonstrate that during our experimental time frame transcription does occur. To determine if the lack of pSTAT3 seen in IL-6 treated tissues may be related to SOCS3 inhibition, protein lysates of the urothelium treated with IL-6, NR, and NC were immunoblotted for SOCS3. IL-6 treated urothelium had significantly more SOCS3 protein within its cytoplasm than the other treatments (p<0.05) (Figure 2). These results provide evidence that exogenously added IL-6 does activate STAT3 signaling, and it also induces the urothelial cells to begin transcription during our 5h experimental timeframe. The results also suggest that when exogenously added IL-6 is used to treat the tissue, the time course is predictably faster than in E. coli treated tissue since in the latter IL-6 must first be secreted by urothelial cells. Ultimately, these results indicate that exogenously added IL-6 to the Ussing chamber model system can be used for identifying urothelial genes regulated by IL-6.

**Differential urothelial gene expression induced by IL-6**

Given the autocrine/paracrine IL-6 urothelial activation pathway during UTI, we next sought to determine the pattern of IL-6 induced gene expression both globally within the urothelium and more specifically for genes associated with tissue remodeling. Sheets of canine urothelium were placed within Ussing chambers and treated with exogenously added canine recombinant IL-6 or NR control for a period of 5h after which the tissue was harvested and RNA extracted. Comparing gene lists from PCA, Overlap, and
Random Measure statistical analyses a total of 49 common genes had \( \geq 2 \) fold increases or decreases in expression, concordant results for all dogs, and a p value <0.05. Ten target genes important in tissue remodeling were selected for verification via q-RT-PCR. These included heparan sulfate D-glucosaminyl 3-O-sulfotransferase 3A1 (HS3ST3A1), syndecan 1, 2, 3, 4, metalloproteinase 1, metallopeptidase 2 (MMP-2), metallopeptidase 3, tumor necrosis factor \( \alpha \) (TNF\( \alpha \)), and interleukin 1\( \beta \) (IL-1\( \beta \)). Gene up regulation was statistically significant for HS3ST3A1 (4.40±1.15; \( p=0.03 \)), syndecan 2 (2.07±0.30; \( p=0.02 \)), MMP-2 (2.79±0.41; \( p=0.01 \)) and IL-1\( \beta \) (3.20±0.85; 0.04) when performing a paired comparison of fold change gene increase of IL-6 versus NR treated tissue (fold change±s.e.; p value).

**Discussion**

Interleukin-6 is a cytokine that epitomizes pleiotropy. Secreted by both lymphoid and nonlymphoid tissues IL-6 is widely known for its regulatory effects on lymphocytes, promotion of hematopoiesis, and as an acute phase reactant inducing the production of fibrinogen, serum amyloid A, and C-reactive protein[142-144]. Although diverse as described, these systemic actions constitute only a small subset of IL-6 duties. It can be argued that more important are the local effects of IL-6 within epithelia and other tissues throughout the body. Many of these actions remain unknown.

Despite being a frequent topic of research, the precise role of IL-6 in epithelia remains elusive. Given that IL-6 initiates gene transcription and protein production within
epithelial cells, identifying the genes transcribed after exposure of urothelial cells to IL-6 may predict IL-6’s actions. From the array results and q-RT-PCR four selected genes were identified as being consistently up regulated. These genes potential relatedness in urothelial tissue remodeling may underlie the ability of the mucosa to ready itself to respond to infection.

The first of these genes encodes heparan sulfate D-glucosaminyl 3-O-sulfotransferase 3A1 which is an enzyme responsible for the synthesis of the heparan sulfate. Heparan sulfate is a glycosaminoglycan carbohydrate that interacts with proteins in the extracellular matrix to regulate numerous responses throughout the body. It has been shown to be integral in maintaining the glomerular filtration barrier in the kidney and the intestinal epithelial barrier[145, 146]. Heparin sulfate expressed on the surface of epithelial cells in the lung and small intestine has been shown to have antimicrobial effects[147]. During inflammatory processes heparan sulfate interacts with heparan binding proteins to play integral roles in leukocyte extravasation and chemotaxis as well as promote the release of the pro-inflammatory cytokines[148].

In the urinary bladder heparan sulfate has a prominent role as it is a major component of the mucus rich glycosaminoglycan (GAG) that lines the luminal surface of the bladder and is produced by the urothelium[23]. This anionic layer is hydrophilic and traps water creating a wall of water between the urothelial cells and the urine which contains cationic solutes and potentially bacteria[16]. Prior research has demonstrated that disruption of the GAGs will increase epithelial permeability[24]. The actions of IL-6 increasing
heparan sulfate production may be an effort to repair a damaged GAG layer. There is precedence for this hypothesis as experimentally produced GAG defects are corrected by the exogenous addition of heparin[24, 149].

A second gene, syndecan 2, has heparan sulfate as its major constituent. Syndecans are transmembrane proteoglycans recognized as important in the recruitment of leukocytes to areas of inflammation, in the formation of cytokine gradients, chemokine and growth factor signaling, as well as tissue repair. Interestingly, of the four mammalian syndecans (1-4), only syndecan 2 was up regulated in our study. Both syndecan 1 and 4 are expressed on epithelial cells, while syndecan 2 is primarily found on endothelial cells and fibroblasts [148]. While syndecan 2 expression has been associated with IL-1α, IL-1β, and TGFβ production, it has previously not been associated with the IL-6 transcription pathway. Given that syndecan 2 is thought to be associated with increases in vascular permeability during inflammation, its production by urothelial cells during UTI is in line with a role for IL-6 in inflammatory cell recruitment to the area of infection.

Syndecans located on cell surfaces also connect epithelial cells to the extracellular matrix. The cleavage of syndecans from the cell surfaces by metalloproteinases increases cellular mobility and hence may be involved in bladder restitution[150]. A third gene, MMP-2, was found to be up regulated by both microarray and q-RT-PCR. The metallopeptidases have broad roles including the cleavage of extracellular matrix-cell contacts, the cleavage of cell-cell contacts, and the cleavage of proteins within the
extracellular matrix to their active form including growth factors, cytokines, and the aforementioned cell adhesion molecules[151]. Together these processes promote wound healing via inflammation and re-epithelialization. They also may affect epithelial permeability by breaking cell adhesions and promoting cell mobility.

Metalloproteinase 2 has also been identified as an important protein for activating certain cytokines. One of these cytokines’ genes, interleukin-1β (IL-1β), was also upregulated in our microarray and q-RT-PCR. Specifically MMP-2 cleaves the IL-1β precursor to its active form. IL-1β is known as a pro-inflammatory cytokine with important functions during the acute phase response including the production of cyclooxygenase-2, type 2 phospholipase A and inducible nitric oxide synthase. Perhaps of greater interest with regard to the urothelium is that IL-1β promotes the migration of inflammatory cells extravascularly and increases IL-6 production. These roles would appear critical in propagating the inflammatory response induced within the urothelium during UTI.

Together these 4 genes loosely demonstrate a compelling role of IL-6 during the acute phase response to urinary tract infection. Not unexpected, one role is to promote a pro-inflammatory state. This may occur via numerous mechanisms including the increase in vascular permeability and inflammatory cell extravasation via the actions of syndecans and IL-1β, the heparan sulfate activation of inflammatory cells, and the facilitation of cell mobility by degrading cell adhesions and the ECM by MMPs.
Of these genes, the finding that IL-6 promotes heparin sulfate production is particularly intriguing. Uropathogenic *E.coli* have been associated with damage to the GAG layer and an increase in urothelial permeability[15]. If the increased production of heparan sulfate can be linked to GAG layer repair it would seem probable that one function of IL-6 may be improved barrier function. Given that treatment with GAG replacers such as pentosan polysulfate sodium has shown promise in relieving pain and discomfort associated with interstitial cystitis as well as preventing bacterial adherence during urinary tract infections[16], a potential role for IL-6 in increasing heparan sulfate production during infection is an important observation. Given that current non-antibiotic UTI treatment strategies focus on altering bacteria adherence, IL-6/heparan sulfate may be a valuable pathway to pursue as an alternate to antibiotic therapy.
Table 1 - Forward and reverse primer pairs for the 10 target genes. Sequences were designed using canine predicted base sequences as described in the NCBI database. Primers were validated by PCR and gel electrophoresis to ensure that only a single band of desired size was present.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT (housekeeping)</td>
<td>CGC TGA GGA TTT GGA AAA AG</td>
</tr>
<tr>
<td></td>
<td>AAT CCA GCA GGT CAG CAA AG</td>
</tr>
<tr>
<td>TNF</td>
<td>TGC CTG CTG CAC TTT GG</td>
</tr>
<tr>
<td></td>
<td>GCT ACT GGC TTG TCA CTT GG</td>
</tr>
<tr>
<td>MMP1</td>
<td>CGC GTA AAT CCC TTC TAT CC</td>
</tr>
<tr>
<td></td>
<td>CAT CCT GAC CCT GAA CAA CC</td>
</tr>
<tr>
<td>MMP2</td>
<td>TGG AGC AAG AAG AAG ACC</td>
</tr>
<tr>
<td></td>
<td>CCC TTG AAG AAG TAG CTA TGA CC</td>
</tr>
<tr>
<td>MMP3</td>
<td>ACA CCA GCT GCA TGT GAC C</td>
</tr>
<tr>
<td></td>
<td>GAA CCC AGG TTC AAG TGT CC</td>
</tr>
<tr>
<td>HS3ST3A1</td>
<td>TCA TCG GCC TGA AGA AGG</td>
</tr>
<tr>
<td></td>
<td>GCG GGT GAC AAA GTA ACT GG</td>
</tr>
<tr>
<td>Syndecan1</td>
<td>GAA GAC CAA GAT GGC TCT GG</td>
</tr>
<tr>
<td></td>
<td>GCT GTG ACA AGG TGA TGT CC</td>
</tr>
<tr>
<td>Syndecan2</td>
<td>GGA GAA GCA CTC GGA CAA CC</td>
</tr>
<tr>
<td></td>
<td>TTC TTC CTC ATG CGA TAC ACC</td>
</tr>
<tr>
<td>Syndecan3</td>
<td>CGA TGA TGA ACT GGA TGA CC</td>
</tr>
<tr>
<td></td>
<td>GTC TCA ATG CCC GAC TCC</td>
</tr>
<tr>
<td>Syndecan4</td>
<td>TTG AGC TGT CTG GCT CTG G</td>
</tr>
<tr>
<td></td>
<td>CTC CAA TTC CTT GGG TTC G</td>
</tr>
<tr>
<td>IL-1β</td>
<td>CAG GAC ATA AGC CAC AAA TAC C</td>
</tr>
<tr>
<td></td>
<td>CAA AGC TCA TGT GGA ACA CC</td>
</tr>
</tbody>
</table>
Figure 1 – Exposure of canine urothelium to IL-6 fails to increase phosphorylation of STAT3 at 5h. Canine bladder mucosa immediately after removal of the seromuscular layers (NC; non-chambered), and after 5 hour treatment in Ussing chambers with exogenous IL-6 or normal Ringer’s solution (NR) were immunoblotted for phosphorylated STAT3 (pSTAT3) and total STAT3. Exogenous IL-6 failed to significantly increase phosphorylation of STAT3 compared to NR and NC tissue.
Densitometric data represent relative fold increase in pSTAT3 to total STAT3. HepG2 = Pervanadate-treated HepG2 cell lysate positive control; n = 4 dogs each.
Figure 2 – Exposure of canine urothelium to IL-6 induces SOCS3 production. Canine bladder mucosa immediately after removal of the seromuscular layers (NC; non-chambered), and after 5 hour treatment in Ussing chambers with exogenous IL-6 or normal Ringer’s solution (NR) were immunoblotted for SOCS3 with actin acting as a loading control. Exogenous IL-6 significantly increased SOCS3 production compared to NR, and NC tissue. MOLT-4 = positive control; ** p<0.05, n = 4 dogs each.
Chapter 5 — Future Directions

This thesis describes research that utilized two tools developed during the course of my graduate program, the canine specific IL-6 ELISA for use with urine and the Ussing chamber model of UTI. Both the ELISA and chamber model are new scientific contributions that other researchers may utilize in their work. During my program these tools were used to establish the following…

- The application of an ELISA developed for use with cell supernatants or plasma to other bodily fluids is unacceptable without understanding the variables present in the different fluids and how they may affect the immunoassay.

- Matrix effects must be neutralized when measuring IL-6 within the urine of dogs. The tremendous variability in solute composition within urine makes direct comparison of IL-6 measurements difficult between patients even with the same USG.

- Urine IL-6 can be measured in dogs, just not as an absolute value. IL-6 concentrations can be described as greatly increased, moderately increased, slightly increased and baseline.

- Urine IL-6 is not a useful tool when examining cases of asymptomatic bacteriuria and chronic infections.

- Uropathogenic *E. coli* interacting with urothelium alters barrier function by preventing tight junction formation in tissue mounted within Ussing chambers.
• Uropathogenic *E. coli* stimulates the synthesis of IL-6 by all urothelial layers. This IL-6 is secreted basally into the lamina propria.

• Urothelial cells themselves are the predominant local site of IL-6 receptors suggesting an autocrine/paracrine effect of IL-6.

• IL-6/STAT signaling does not mediate the acute effect of *E. coli* demonstrated by the failure of tight junction formation.

• IL-6 does mediate the transcription of numerous genes within the urothelium including the cytokine/chemokine/growth factor response, cell adhesion and ECM remodeling, GAG production, cytoskeletal rearrangement, and cellular transport.

• More specifically IL-6 up regulates the production of heparan sulfate, syndecan 2, MMP-2 and IL-1β all of which help promote a pro-inflammatory state and may be integral in tissue remodeling during infection.

This work is to be continued as follows…

1st – While the urine IL-6 ELISA fails to detect measurable cytokine in chronic and/or asymptomatic cases of UTI, there may still be a use in the acute phase of infection and in cases of upper urinary tract infection as a marker of disease severity, progression, and or resolution. In humans IL-6 is known to be released in the acute phase of UTI and successful treatment reduces IL-6 concentrations[42]. It is predicted that IL-6 concentrations can therefore be utilized to gauge the effectiveness of treatment and progression of disease in acute clinical diseases such as pyelonephritis. Comparable
human studies have proved successful using IL-6 as a biomarker in other diseases such as pancreatitis[152].

2nd – In addition, despite the failure of IL-6 as a predictive marker of asymptomatic and chronic infections, other cytokines and immunoregulatory molecules can be similarly assessed for their utility in predicting UTI versus sterile inflammatory states. For example, IL-8, PDGF, and TNF-α all have experimentally been shown to increase after urothelial cell challenge with *Escherichia coli* and *Citrobacter* spp[122] and therefore these molecules may be attractive targets for examining the role of urinary cytokines in dogs.

3rd – The effects of uropathogenic *E. coli* on barrier function remains unexplained. Why does *E. coli* infection alter the formation of tight junctions within the urothelium? What effect does *E. coli* have on the production of essential tight junction structure such as claudins and ZO? To answer these questions chambering of *E. coli* treated tissue and subsequent array analysis is underway. The goal is to compare IL-6 treated tissue, *E. coli* treated tissue, and NR treated tissue isolate gene regulation specific to *E. coli*. It is known that *E. coli* and its virulence factors interacting with the urothelium activate numerous cellular pathways and some of these participate in altering the cellular cytostructure[59, 82]. It is predicted that *E. coli* interaction with the urothelium, in particular through uroplakin binding will initiate the production and release of factors that destroy ECM, such as the MMPs, while down regulating genes specific to tight junction formation.
4th – The bulk of the planned work will focus on completing IL-6 array analysis and the
effects IL-6 has on the urothelium. Identifying presence of transcripts after IL-6
stimulation of the urothelium is not equivalent to knowing the concentrations of a
produced protein and whether that protein is active. Zymography is planned to assess
more specifically whether MMP-2 as well as other MMPs are enzymatically active in the
ECM after IL-6 exposure. The importance of MMPs in carcinomas of the urinary
bladder have been studied extensively, however, the role of MMPs in UTI is a relatively
unexplored field. Knowing that epithelial shedding is a key component of the mucosal
response to infection, the idea that MMPs may be important to urothelial restitution is
intriguing and has not been previously described. Given the increase in cytokine and
growth factor production during IL-6 stimulation and the importance of MMPs in
activating these molecules a more complete picture describing the roles of MMPs in the
urothelium is essential.

5th - As discussed previously, heparan sulfate D-glucosaminyl 3-O-sulfotransferase 3A1
is an important enzyme for the production of heparan sulfate and heparan sulfate is an
important factor in the pro-inflammatory response as well as in the formation of the
glycosaminoglycan (GAG) layer lining the apical surface of the urothelium. Given the
importance of the GAG layer as a barrier to infection, and the circumstantial clinical
evidence demonstrating that GAG repair helps to alleviate clinical signs of cystitis[16],
examining more closely the link between IL-6 and GAG production is an attractive
option linking the current work and the development of future therapeutics.
References


144. Ohsugi, Y., *Recent advances in immunopathophysiology of interleukin-6: An innovative therapeutic drug, tocilizumab (recombinant humanized anti-human interleukin-6 receptor antibody), unveils the mysterious etiology of immune-


