INCREASED BACTERIAL ADHERENCE AND DECREASED BACTERIAL CLEARANCE IN URINARY TRACT INFECTIONS WITH DIABETES MELLITUS

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Dedication

To my beloved family; mom and dad, Munevver & Ertugrul Ozer, my brother Yusuf, my sister Arife, my cousin Esra. I am very thankful to them for their endless support. I feel very lucky to have such a great family. I thank my friends, for all their support, help and friendship. Life is an easier path to walk together with you.
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List of Abbreviations

ABTS – 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid
AGE – advanced glycation end products
APC – antigen presenting cell
ASB – asymptomatic bacteriuria
AUC – area under curve
BSA – bovine serum albumin
cAMP – cyclic adenosine monophosphate
CCL – (C-C) chemokine ligand
CD – cluster of differentiation
cDNA – complementary deoxyribonucleic acid
CEL – 3, 4-N-ơ(carboxyethyl) lysine
CML – 3, 4-N-ơ(carboxymethyl) lysine
CFU – colony-forming units
ConA – concanavalin A
CXCL – (C-X-C) chemokine ligand
CUP – chaperone usher pathway
DC – dendritic cell
DM – diabetes mellitus
DSA – Dantura stramonium
DTPA – diethylenetriaminepentaacetate
EDTA – ethylenediaminetetraacetic acid
ELISA – enzyme-linked immunosorbent assay
FBS – fetal bovine serum
G-CSF – granulocyte-colony stimulating factor
GNA – Galanthus nivalis
GSL – glycosphingolipid
h – hour
HDAC – histone deacetylase
H&E – hematoxylin and eosin
HLA – human leukocyte antigen
HPA – Helix pomatia agglutinin
hpi – hours post infection
HRP – horseradish peroxidase
IBC – intracellular bacterial community
Ig – immunoglobulin
IHC – immunohistochemistry
IL – interleukin
IM – inflammatory monocyte
IACUC – Institutional Animal Care and Use Committees
IRF - Interferon regulatory transcription factor
i.p. – intraperitoneal
i.v. – intravenous
kD – kilodalton
KC – keratinocyte chemokine
LB – Luria Bertani
LCH – lentil lectin
LPS – lipopolysaccharide
MAA – Maackia amurensis lectin
MCP-1 – monocyte chemoattractant protein-1
MIP-2 – macrophage inflammatory protein 2-alpha (CXCL-2)
MPO – myeloperoxidase
mRNA – messenger RNA
MyD88 – myeloid differentiation primary response gene 88
NF-κB – nuclear factor kappa B (light polypeptide gene enhancer in B- cells)
NK – natural killer
NO – nitric oxide
PBS – phosphate-buffered saline
PCR – polymerase chain reaction
PNA – peanut agglutinin
PRR – pattern recognition receptor
QIR – quiescent intracellular reservoir
qPCR – quantitative real time PCR
RCA – Ricinus communis agglutinin
RT – reverse transcriptase
rt – room temperature
sec – second
SEM – standard error of the mean
STZ – streptozotocin
Th – T helper
THP – Tamm-Horsfall protein
TLR – Toll-like receptor
TIR – Toll/IL-1 receptor
TNF – tumor necrosis factor
Treg – T regulatory cell
TRAM – TRIF-related adaptor molecule
TRIF – TIR domain-containing adaptor inducing
IFN – interferon
TU – transurethral
UPEC – uropathogenic Escherichia coli
UTI – urinary tract infection
Wt – weight
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Increased Bacterial Adherence and Decreased Bacterial Clearance
in Urinary Tract Infections with Diabetes Mellitus

Abstract

by

AHMET OZER

Diabetic individuals are more susceptible to infections, and urinary tract infections (UTI) are the most common infection type in women with diabetes mellitus (DM). Bacteria colonize tissues predominantly by binding to cell surface carbohydrates, which may change in DM and thereby alter susceptibility to infections. Also, innate immune responses are impaired in DM, which may affect the outcome of infection. Complete knowledge of bacterial adherence and clearance effectiveness in UTI in diabetics is lacking.

The objectives of the studies comprising this thesis are to investigate two major events that occur in the course of UTI and impact the final outcome of this painful ailment: bacterial adherence, with its possible facilitation by advanced glycation end products (AGEs), and bacterial clearance efficiency, with an emphasis on neutrophil recruitment. Using a murine model of type 1 DM in vivo, we determined if AGE accumulation on uroepithelial surfaces contributes to increased adherence of uropathogenic Escherichia coli (UPEC) in UTI in DM mice relative to non-DM mice. Then we evaluated bacterial clearance efficiency and neutrophil recruitment to the
bladder in relation to the expression of local cytokines MIP-2, KC, MCP-1, and IL-6 during UPEC-induced UTI.

Significantly higher levels of UPEC adherence and AGEs were detected in bladders of diabetic mice compared with controls, and AGEs were shown to bind to isolated type 1 fimbriae in vitro. Diabetic mice showed prolonged bacterial clearance during UTI. Neutrophil recruitment was delayed and diminished early after UPEC installation in both bladder tissue and urine of diabetic mice compared with controls. Expression of the chemokines at the transcriptional and protein levels in response to UTI was markedly impaired in diabetic mice.

Our results show that AGEs accumulate on bladder urothelium during DM and may serve as bacterial adherence facilitators in UPEC-induced UTI. This new functional concept of AGEs can be subjected to an extended array of studies addressing host-pathogen interactions and new therapeutic targets in UTI in DM. Impaired bacterial clearance in diabetic mice may be partly due to the lower expression of cytokines in response to UTI, resulting in lower and postponed recruitment of neutrophils to the bladder.
CHAPTER 1

INTRODUCTION

Urinary Tract Infections: An Overview

The urinary tract is normally a sterile environment. Urinary tract infection (UTI) is reported as one of the most common infectious diseases in humans and, as expected, the most common urological disease by far [1, 2]. Bacteria have the ability to survive in urine and colonize all parts of the urinary tract including the urethra, ureters, bladder, and kidney. There are characteristic differences between the bacteria in fecal matter, the main source, and in the urinary tract, as bacteria need a set of virulence factors to colonize a challenging environment like the urinary tract [3]. Once infection or colonization of the urethra is acquired, bacteria must ascend the urethra against the flow of urine in order to reach the bladder, where colonization results in the most prevalent presentation of UTI called cystitis [4, 5]. The term, ‘lower urinary tract infection’ (LUTI), mainly describes this condition. If cystitis is not treated accordingly, bacteria further colonize the ureters and eventually cause kidney infection called pyelonephritis. Acute pyelonephritis is the most severe form of UTI, causing the main complications of the disease including renal scarring, kidney dysfunction, transmission of bacteria into the bloodstream (bacteremia),
and occasionally urosepsis [6, 7]. Generally, definite localization of the source of the bacteria in UTI is practically impossible, thus the diagnosis is dependent on the clinical symptoms. UTIs are divided into two main categories: symptomatic, with the hallmark symptoms being dysuria and malodorous urine with variable suprapubic pain, and asymptomatic. Another categorization in the clinic is made based on pre-existing urinary conditions. Complicated UTI occurs in patients with underlying anatomic, functional, or pharmacologic-associated urologic abnormalities that predispose an individual to infection, such as spinal cord injury and diabetic bladder dysfunction. Often these infections are caused by organisms other than *E. coli*, are resistant to many antibiotics, and spread to other parts of the body. Uncomplicated UTI occurs in individuals who lack any urologic abnormalities, and accounts for most occurrences of UTI [8, 9].

**Burden of UTI: Why UTI is a Problem**

Women are more susceptible to UTI than men for anatomical and physiological reasons including a short urethra, menstrual cycle-dependent pH alterations, and hygienic situations [10]. It is estimated that roughly 50% of women will experience a UTI in their lifetime, and at least 15 million women in the U.S. will suffer from this painful disease each year, with a recurrence rate reaching 30-40% within 4 months of the first episode in this group [10, 11]. The economic burden of UTI is also significant. LUTI itself accounts for more than 8 million physicians’ office visits, 1.5 million emergency room visits, and 300,000 hospital admissions annually in the U.S., with a total annual cost of more than $3 billion [12]. Recurrent UTI and chronic UTI are of special concern, especially in
vulnerable populations such as the elderly, diabetics, and infants [10, 13]. The importance of recurrent and chronic UTI is illustrated by the observation that up to 30% of all bacteremia and sepsis cases reported are due to pyelonephritis [14].

Risk Factors for UTI

Women are at higher risk than men for both community-acquired, uncomplicated UTI and nosocomial (mostly catheter-related) UTI [15]. There is an association between frequent vaginal sexual intercourse and UTI [16-18]. Exposure to spermicides or antibiotics may disrupt the normal vaginal flora and thereby facilitate colonization by more resistant UPEC strains [16, 19]. Certain phenotypes for blood-group antigens, especially non-secretor and recessive groups, are also considered to be risk factors for UTI [20]. An association of childhood UTI with a history of UTI in the mother suggests that genetic factors may play a role in a subset of UTI [16].

Additional groups that are considered to be more susceptible to UTI are diabetic individuals, patients with spinal cord injury or similar neurogenic disorders, patients who have undergone urethral catheterization, and individuals with urologic functional or anatomic abnormalities [10, 21]. Recurrent UTI in postmenopausal women is most strongly associated with urinary incontinence and a history of premenopausal UTI; long-term prophylactic antibiotic treatment has been recommended for these women [22]. Besides humans, other mammals with high susceptibility to UTI include cattle, horses, swine, and some sub-breeds of dogs [23, 24].
UTI and Its Clinical Presentations

Major symptoms of LUTI include increased urination frequency, a burning sensation or pain during urination (dysuria), suprapubic pain and/or lower abdominal discomfort, and cloudy and/or bloody urine with a bad odor. Pyelonephritis shows itself with flank pain and fever, and is diagnosed by the presence in urine of bacteria (bacteriuria) and neutrophils (pyuria). The urothelial inflammatory response against bacterial adherence and invasion is the source of those symptoms. Pain in the pelvic region is the most distressing symptom of UTI-caused cystitis and has been the subject of extended studies. In a recent study, Rudick et al. determined that lipopolysaccharide (LPS), an endotoxin in the outer cell wall of bacteria, can contribute to the complex pain response in a murine model of UPEC-induced UTI [25]. The most common presentation of UTI is asymptomatic bacteriuria (ASB), described as having high levels of bacteriuria without the classical symptoms of UTI. The main groups of people that ASB commonly occurs in are the elderly, pregnant women, school-age girls, and patients with diabetes [26-29]. Antibiotic treatment of ASB is recommended only for certain groups: In young women, ASB is a strong predictor of future symptomatic UTI, and thus treatment is recommended [30]. Pregnant women with ASB are within the indication of aggressive antibiotherapy, since risks exist for both the mother and child [31]. No benefits of treatment of ASB in the elderly have been reported, and antibiotic treatment is not indicated for this group [32].
Urothelium and Dynamism within Its Unique Biology

The bladder mucosa is a transitional epithelium with large, highly differentiated, binuclear, superficial cells called facet or umbrella cells lining the luminal surface (13) (Figure 1). The apical side of umbrella cells consists of a specialized intact membrane that maintains an impermeable barrier between urine and blood. The membrane is covered almost entirely with plaques consisting of a family of urothelium-specific integral membrane proteins called uroplakins (UPKs), which contribute to the barrier function of the uroepithelium [33]. UPKs also act as receptors for the adhesin FimH, a virulence protein on the tip of UPEC type 1 fimbriae that is responsible for the initial attachment of bacteria to the urothelium [34]. The basement membrane of the transitional epithelium lies on the lamina propria with its rich content of fibroblasts and blood vessels. In addition to functioning as an intact impermeable barrier, the bladder epithelium performs important sensory and signaling functions, and must undergo cyclical expansion and contraction along with the rest of the bladder wall to store and excrete urine [33]. Those dynamic structural changes rely on dynamic membrane changes. In order to achieve those changes, there are urothelial cell-specific fusiform vesicles that are considered to be actively endocytosed to reduce the membrane surface area during bladder contraction, and then exocytosed to increase membrane surface area during bladder expansion [33].
(A&B) H&E stained transverse section through the wall of the urinary bladder, showing the urothelium (red bracket) in the full-thickness of the bladder (A) and at high magnification (B). The urothelium, also called transitional epithelium, shows pseudostratified variation; the dome cells lining the luminal surface of the bladder are binucleated umbrella cells (arrow in B) (courtesy of Alvar W Gustafson, Ph.D., Tufts.edu). (C) Schematic representation of bladder mucosa (courtesy of Paramount Digital, Welmount).

Figure 1: H&E transverse section of the urinary bladder
Diabetes Mellitus and UTI

Diabetes mellitus (DM) is the most common endocrine disease worldwide, caused by physical or functional lack of the hormone insulin. A continuous rise in the prevalence of DM in recent years has been associated with an increased incidence of LUTI, which can occur at a rate 4 times higher in DM compared with non-DM individuals [35, 36]. Furthermore, several reports have suggested that diabetic women in general are more vulnerable to UTI than diabetic men (reviewed in [37-39]). Also, bacterial clearance is slower in diabetic than in non-diabetic subjects [40].

Pyelonephritis has also been found to be more common in diabetics. In a survey of 242 non-pregnant, 18 to 49 year-old women with acute pyelonephritis compared with a similar control population with no pyelonephritis diagnosis in the previous 5 years, DM was identified as a strong independent risk factor for pyelonephritis (odds ratio 4.1) [41]. In a population-based study in Canada, Nicolle et al. used health insurance claims to estimate the prevalence of diagnosed DM in the population and found that diabetic women 25 to 64 years of age were found to be almost 20 times more likely to be hospitalized for acute pyelonephritis than non-diabetic women [42, 43]. However, a U.S. nationwide study using the 1997 Health Care Cost and Utilization Project Nationwide Inpatient Sample and prevalence estimates from the 1997 U.S. Census did not reveal significant differences in rates of hospitalization for acute pyelonephritis in diabetic vs. non-diabetic women [15]. DM, especially uncontrolled DM, is associated with up to 95% of cases of life-threatening emphysematous pyelonephritis, with a prevalence ratio in women/men of 6:1.
Rosen et al., showed in two different studies that type 1 diabetic mice were considerably more susceptible than non-diabetic mice to UTIs caused by UPEC and also other less frequent uropathogenic bacteria including *Klebsiella pneumoniae* (TOP52 1721) and *Enterococcus faecalis* (0852) [44, 45]. Competition experiments demonstrated that *K. pneumoniae* was outcompeted by *E. coli* in the bladders of healthy mice, but not in diabetic bladders. Taken together, the majority of the studies have shown that both the prevalence and severity of LUTI increase with DM, making diabetic individuals more prone to several UTI-related complications [46-49].

**DM and Asymptomatic Bacteriuria**

Asymptomatic bacteriuria (ASB) is defined by the Infectious Diseases Society of America as “two consecutive (women) or a single (men) clean-catch voided urine specimen(s) with isolation of the same bacterial strain in quantitative counts of ≥10^5 colony forming units (cfu)/mL”, in the absence of classical symptoms of UTI [26]. The majority of studies have shown that DM patients have a higher prevalence of ASB and other infections compared with people without DM, although in most studies, the correlation with type of DM (type 1 or 2) was not reported clearly [29, 49-51]. A recent meta-analysis study reported that 12.2% of patients with DM had ASB compared with 4.5% of healthy control subjects [29]. In that study, ASB was observed more frequently in DM patients regardless of subgroup (type 1 and 2 DM, women, men, children and adolescents) compared with controls. Another study reported rates of ASB more than four times higher in all DM patients (26%) and 3.5 times higher in type 1 DM patients.
(21%) compared with non-DM patients (6%) [52]. Higher rates of ASB have also been observed in pregnant women with pregestational DM compared with non-DM pregnant women [53].

Uropathogenic *E. coli*: The Most Common Causative Microorganism

Uropathogenic *E. coli* (UPEC) belongs to the extraintestinal pathogenic *E. coli* family of strains and is one of the most common ‘pathotypes’ in this group [54]. UPEC is by far the most prevalent causative agent for community-acquired UTI and one of the most common in nosocomial UTI, accounting for >85% and 25%, respectively [55-57]. Staphylococcus strains come in the second place. Even though antibiotics have been considered very beneficial, and in many situations are necessary to completely resolve the infection, increasing antibiotic resistance is raising concerns worldwide [58]. An increasing prevalence of multidrug resistance strains of UTI, the lack of response to common first-line antibiotics used empirically, and severe complications of more potent antibiotics make recurrent and chronic UTI a more complex problem [59-62].

UPEC Virulence Factors Contributing to Development of UTI

UPEC are well-equipped for colonizing the urinary tract and persisting in the infected area against a group of very effective elements of the host defense system. UPEC produce a general class of virulence factors, including both cell surface and secreted factors, that are known or potentially important contributors to the establishment
of UTI in the host [63, 64]. Virulence factors that contribute to UPEC colonization of and persistence in the urinary tract include fimbrial adhesins, afimbrial Afa adhesins, flagellin (the active element of flagella), secreted toxins (mainly LPS, also secreted autotransporter toxin and cytotoxic necrotizing factor 1), the polysaccharide capsule, iron acquisition systems, tRNAs, and specific metabolic pathways ([65-67], reviewed in [64]) (Figure 2). A diverse array of UPEC virulence factors has evolved, due in part to the clustering of many genes encoding virulence proteins on pathogenicity islands, which are large (generally >10 kb) chromosomal or extrachromosomal DNA segments that also contain mobile genetic elements and are generally flanked by repeat sequences. Multiple pathogenicity islands are found in UPEC, but not non-pathogenic E. coli strains, and appear to have enabled en bloc horizontal transfer of virulence factor genes [68-70]. Among the virulence factors encoded on pathogenicity islands that are known to play important roles in UTI pathogenesis are: type 1 fimbriae, fimbrial Dr adhesins, flagella, TonB, hemolysin (HlyA), cytotoxic necrotizing factor 1, K2 capsule, PhoU, DegS, DegP, FliC, RfaH, pckA, dppA, and OmpR {[71-78] and reviewed in [64].

Single or combinatorial mutations hitting any of the genes responsible for the expression of the virulence factors listed above cause defects in adherence and/or colonization of the mucosa, the essential first steps in the establishment of infection [68]. The main challenges for bacterial infection in the host environment are osmotic stress, shear stress generated by urine flow, nutrient restrictions and metabolic needs, iron sequestration, and host immune factors that exist in the healthy urinary tract [79, 80]. Type 1 fimbriated UPEC appear to overcome the force of urine flow, intuitively presumed to wash bacteria off of the urothelial surface, through the formation of ‘catch
bonds’ between the fimbrial tip adhesin FimH and mannose, the key component of urothelial receptors for FimH discussed in the following section. Instead of being weakened by shear stress, catch bonds between FimH and mannose actually gain strength with increasing shear stress [81].

In short, various virulence factors collectively enable UPEC to overcome the obstacles in the host environment and colonize the bladder successfully. In this dissertation, I have emphasized the critical role of type 1 fimbriae in UPEC adherence, and also have used type 1 fimbriated UPEC to evaluate the innate immune response in diabetic mice during UTI.
Figure 2: UPEC virulence and fitness factors that contribute to bacterial pathogenesis during UTI.

[82]
UPEC Adherence with Type 1 Fimbriae (Pili) and FimH

After entry into the lower urinary tract, adherence to mucosal surfaces is the very first and crucial step for ascending UPEC to establish infection [68, 83]. As discussed above, UPEC strains have a rich variety of adherence factors that enable attachment to the uroepithelium. Fimbriae are filamentous organelles that allow bacterial cells to adhere to a number of different tissue surfaces or cell types, mainly through the adhesive properties of adhesin proteins in the fimbria tip. UPEC strains are defined mainly by the specific adhesive organelles expressed on the surface. Currently, a number of fimbriae and adhesins are known to be expressed by UPEC strains, including type 1 (fim), P (pap), S (sfa), F1C (foc), and Dr, and afimbrial Afa adhesins [64]. In addition to their direct role in adhesion, fimbriae can contribute to bacterial virulence in other ways such as stimulating host signal transduction pathways involved in internalization of UPEC and facilitating delivery of bacterial products such as LPS to host cells [84].

Regulation of UPEC fimbria expression is a complex process. Each type of fimbria is encoded by at least one cluster of genes, the products of which include structural proteins of the fimbria as well as proteins involved in regulating the synthesis of the structural proteins [85]. Fimbria expression is known to be phase-variable, meaning expression of fimbria genes can be switched on or off depending on environmental or culturing conditions [86]. Expressed proteins of many fimbriae are assembled on the outer bacterial membrane by a process called the chaperone-usher-pathway (CUP) [87]. Each UPEC strain encodes several CUP and other fimbria operons;
expression of the different fimbria operons must be coordinated with respect to each other and the environment [86, 88].

Many UPEC strains express the type 1 CUP fimbria as the major adhesive fiber for attachment to urothelium. It has been thought that it Since the expression of type 1 fimbriae is common to both pathogenic and resident bacterial strains in human environments, it has been difficult to address the functional importance of type 1 fimbriae in human UTI [89, 90]. Therefore, to date, virulence of the type 1 fimbria has been demonstrated mainly in murine models of UTI, where it has been shown to play a critical role in the development of cystitis [71, 91-94]. Specifically, studies in the mouse models have shown that type 1 fimbriae initiate mucosal inflammation, increase the survival of UPEC in the infection site, and enhance bacterial invasion and production of biofilm structures, which are very important for chronic and recurrent infections [71, 95-98].

Type 1 and other CUP fimbriae effect attachment and adherence of UPEC to urothelium by means of adhesins in their tips that recognize specific receptors on the urothelial surface. FimH, the type 1 fimbria tip adhesin, has been shown to bind mannosylated uroplakins [34, 99] as well as N-linked oligosaccharides on alpha-1 and beta-3 integrins and the pattern recognition receptor Toll like receptor 4 (TLR4) [discussed below under “Innate Immune Response in UPEC-induced UTI”], which are expressed on the luminal surface of human and murine bladders [100]. These FimH-mediated interactions facilitate bacterial colonization and invasion [4, 101]. FimH binding to uroplakin complexes on urothelial cells in culture induced phosphorylation of the cytoplasmic signaling domain of uroplakin IIIa on a specific threonine residue by casein kinase II, resulting in an increase in the intracellular calcium level [99]. Inhibition
of those events leading to calcium-mediated signaling attenuated bacterial invasion and apoptosis of urothelial cells in culture and in a murine UTI model [102]. Thus, FimH-induced uroplakin IIIa signaling is thought to be essential for the invasion of UPEC into bladder epithelial cells. Moreover, in a study with human bladder urothelial cells, purified FimH coupled to inert latex beads, in the absence of any other bacterial factors, can mediate internalization into the cultured cells [99, 103].

Possible Outcomes of UTI: Intracellular Bacterial Colonies (IBCs), Quiescent Intracellular Reservoirs (QIR), Recurrent and Chronic Cystitis

The UTI cascade includes a series of consecutive events that increase the complexity of the process in various ways (Figure 3). Once bacteria adhere to receptors in a FimH-dependent way and initiate signaling through uroplakin IIIa and other proteins, invasion events follow. Upon internalization into fusiform vesicles, UPEC can be exocytosed in a TLR-4 dependent process, but some bacteria can escape into the host cell cytoplasm where they can subvert innate defenses and replicate into biofilm-like intracellular bacterial communities (IBCs) of $10^4$-$10^5$ bacteria in a single host cell [55, 104, 105]. Subsequently, UPEC can disperse from the IBC, escape into the bladder lumen, and re-initiate the IBC cycle by binding to and invading neighboring uninfected urothelial cells [106]. The murine model has revealed two common outcomes of these acute events: i) resolution of the acute infection with the establishment of quiescent intracellular reservoirs (QIR), which are intracellular vesicles in transitional urothelial
cells containing a few latent bacteria that remain undetected by the host’s immune system and can serve as seeds for recurrence, or (ii) establishment of chronic bladder infection (chronic cystitis) [107] (Figure 3). Chronic cystitis is characterized by persistent, high titer bacteriuria ($>10^4$ CFU/ml) and bacterial bladder burdens $\geqslant 2$ weeks post-infection, and evidence of chronic bladder inflammation [106]. After antibiotic therapy that resolved infection, mice with a history of chronic cystitis were significantly more predisposed to chronic cystitis upon rechallenge with isogenic UPEC compared with naive controls and mice with a history of resolving UTI [55]. The ability of UPEC to form IBCs appears to be a critical determinant of disease outcome, as a previous study suggests that a high number of IBCs formed during the acute stages of infection precedes the development of chronic cystitis [108]. In silico analysis of the $fimH$ gene from hundreds of UPEC strains revealed two conserved amino acids that were shown to be required for IBC formation, without affecting phase variation, fimbria assembly, or mannose binding [109]. Thus, different selection pressures and bacterial population dynamics, as well as niche residence and population bottlenecks (e.g., ability to form IBCs) determine UPEC’s ability to survive and seed an acute infection and/or persist long-term in the urinary tract as a chronic extracellular infection [106]. Significantly, evidence for exfoliated IBCs was found in the urine of women with UPEC-UTI, but not in the urine of women with asymptomatic bacteriuria [110-112].
Attachment of type 1 fimbriated UPEC to binuclear umbrella cells that form the luminal urothelial lining of the bladder wall is the first step of UTI (a). Upon successful attachment, bacterial invasion of umbrella cells (b) and intracellular replication (c) follow, leading to exfoliation and removal of infected cells (c) or the establishment of intracellular bacterial communities (IBCs) (d). Upon reaching unsustainable numbers in IBCs, UPEC disperse from the host cells (e) and then attach to other umbrella cells (a) to continue the infectious cycle. Upon resolution of the acute infection over the course of multiple IBC cycles, a few non-replicating, intravesical bacteria may remain in umbrella cells as quiescent intracellular reservoirs (QIRs) (f), which may emerge later to seed a new infection. Alternatively, chronic cystitis may develop (g), where urothelial cells can no longer support IBC formation, but UPEC can replicate in extracellular clusters for long periods.
**Repertoire of Host Defenses Against UPEC-induced UTI**

A healthy urinary tract is considered to be a mostly sterile mucosal lining, and the sterility is maintained by a number of well-equipped host defense mechanisms. As emphasized above, the UPEC armamentarium includes an impressive repertoire of virulence factors and mechanisms for attachment and adherence to uroepithelium, as the first and foremost requirement for establishment of UTI [68], as well as for invasion, replication, and reinfection [64, 106, 112-118]. The host defenses against those bacterial elements can be divided into two categories: a) primary bladder defenses, namely preexisting mechanisms for preventing bacterial adherence to the urothelium; and b) secondary defenses, including host immune and other responses to successful bacterial adherence.

**Primary Bladder Defenses:** As discussed earlier, the shear stress generated by urine flow is intuitively considered to be a preventive factor among the host defense mechanisms against many microbial attacks, but type 1 fimbriae overcome that force through the formation of ‘catch bonds’ between FimH and mannose that are strengthened by shear stress, thereby potentiating the adherence of UPEC [81]. Apart from that adaptation, the bulk flow of urine during micturition still is an effective mechanism for clearing nonattached and poorly attached UPEC from the bladder [119]. Another line of defense is provided by the mucin layer that normally covers the luminal surface of the bladder, which is comprised of anionic, highly sulfated mucopolysaccharides, commonly called glycosaminoglycans [120], that are secreted by urothelial cells. The mucin layer, in
addition to aiding in the establishment of bladder wall impermeability, is considered to possess anti-bacterial adherence properties [120-123]. Other important preventive defense mechanisms in the urinary tract are the composition of the urine, which includes salts, organic acids, urea, and a steadily maintained low pH [64]; competition for UPEC binding by soluble Tamm-Horsfall glycoprotein (THP) in the urine [124] (see below), and the presence of highly cationic antimicrobial proteins called defensins, which are secreted into the urine in response to bacterial exposure and play a role in bacterial killing by disrupting the membrane integrity of capsulated bacteria [125].

- **Tamm-Horsfall Protein (THP) in UTI:** THP is a high molecular weight glycoprotein that was long known to be present in human urine [126] before its ability to bind *E. coli* type 1 fimbriae and decrease bacterial burdens in UTI was recognized [127-129]. The main source of THP seems to be kidney [130-132]. Studies revealed that soluble THP from both mouse and human urine was able to bind type 1 fimbriae by virtue of its mannose moieties, inhibiting fimbria interactions with uroplakin Ia and Ib receptors [132]. Infections could be established in THP-/- mice with inoculum numbers too low to infect wild type mice [133]. On the other hand, THP depletion did not enhance infection by UPEC expressing P fimbriae [133, 134]. Those studies suggest that molecules with mannose moieties may be useful for the resolution of UPEC-induced UTI.

**Secondary Host Response Related Defenses:** When UPEC manages to successfully adhere to the bladder mucosa, the host responds by activating a second line
of defense systems. These are grouped mainly as the exfoliation and regeneration of urothelial cells, and the innate immune/inflammatory response in the bladder.

-Urothelial Cell Exfoliation

Type 1 fimbria-mediated bacterial interactions with the bladder mucosa initiate a cascade of events that directly influences the pathogenesis and outcome of disease. The mammalian bladder urothelium is comprised of a 3-4 cell deep pseudo-stratified transitional epithelium that have high self-renewal capacity. The luminal layer of the urothelium facing bladder cavity is comprised of terminally differentiated, binucleated umbrella cells. Under physiological conditions, the urothelium has a slow rate of turnover; undamaged urothelium requires 40 weeks for renewal in mice [135]. However, acute injury caused by infection or toxins lead within hours to the onset of rapid self-renewal of the epithelium [136]. Studies using a mouse cystitis model demonstrated that urothelial umbrella cells exfoliate in response to infection by type 1-fimbriated E. coli strains via a rapid apoptosis-like mechanism involving host DNA fragmentation and the activation of caspases [4]. The exfoliation apoptosis-like process is promoted by FimH [4, 137, 138] and is dependent on expression of the FimH receptor uroplakin IIIa [99, 136]. The exfoliation of infected bladder cells is proposed to act as a host defense mechanism, as clearance of exfoliated cells from the bladder is a very effective mechanism for removing adherent UPEC [4, 139-143]. Inhibition of exfoliation by a general caspase inhibitor significantly reduced the rate of bacterial clearance from the bladder [4]. However, UPEC that persist within the bladder lumen following the
exfoliation response and in the face of additional host defense mechanisms may invade transiently exposed underlying transitional urothelial cells, where the bacteria are capable of forming QIRs [4, 140].

-Urothelial Regeneration in Response to UPEC Infection

Maintaining the impermeability of the urothelial barrier is vital for protection of the tissue from toxic substances in the urine. Exfoliation of the superficial umbrella cell layer during UPEC infection causes the disruption of the impermeability barrier. Thus, removal of infected urothelium must be accompanied by rapid regeneration of it. In a series of studies carried out by Mysorekar et al., the dynamics of urothelium regeneration in UPEC-induced UTI, crucial molecular signaling events, and some of the key players involving those processes have been delineated [101, 136, 140, 144]. Microarray analysis revealed that the expression of many genes involved in different important pathways, including cell differentiation, apoptosis, intercellular contacts, and the stress response machinery, increased in a time-dependent manner in response to UPEC infection, as compared with control groups receiving mock infection or non-inflammatory chemical exfoliation treatments [136]. Importantly, the levels of proteins in the inflammatory cascade composed of transcription factors, inflammatory cytokines and chemokines, and proteins in related signaling processes were significantly increased and may also contribute to urothelial regeneration [101]. Regeneration itself appears to be a function of uroepithelial stem cells present in the basal and mesenchymal layers of the urothelium, and depends on downstream signaling mediated by down-regulation of bone
morphogenetic protein 4 (Bmp4) through the Bmp4 receptor 1a [136]. Additionally, Pull et al. have shown in injured intestinal epithelium that epithelium-associated macrophages transmit MyD88-dependent proliferative signals to progenitor epithelial cells of the colon via direct cell-cell contact [145]. There is no evidence yet showing that macrophages play a role in UTI.

Innate Immune Response in UPEC-induced UTI

- TLR-Dependent Acute Response in UPEC-induced UTI

In addition to leading to exfoliation, successful adherence of type 1 fimbriated UPEC to the bladder urothelium triggers a rapid and robust immune response mediated primarily by Toll-like receptor (TLR) signaling [105, 146]. TLRs recognize specific components of pathogens and stimulate urothelial cells to secrete critical cytokines and chemokines, causing a vigorous neutrophil response and bacterial clearance [103, 147] (Figure 4). Although an adaptive immune response is considered to be a part of the secondary host defense against UPEC infection, the most effective and key event in bacterial clearance is innate immunity delivered largely by TLR activation leading to eventual chemokine release and neutrophil recruitment [147, 148]. Establishment of a prompt local tissue response and initiation of local inflammation relies on the production and secretion of many proinflammatory factors, including important cytokines and chemokines secreted by urinary tract epithelium [149-152]. It is also very important to
note that the antibacterial functions of the immune response must be tightly regulated in order to remain beneficial for the host, since an uncontrolled immune/inflammatory response could lead to detrimental local tissue damage.

As one of the core points in the scope of this dissertation, neutrophil recruitment to the infected bladder is critical for the successful eradication of bacteria from urinary tract, and the successful recruitment of neutrophils to the infection site requires locally secreted chemokines such as IL-6, IL-8 (in human), and MIP-2 and KC (in mice) [64, 151]. Signaling events upstream of the cytokine/chemokine response and eventual neutrophil recruitment are crucial for understanding the bacterial clearance process, thus discussions of the roles of TLR-4 signaling and Ca\(^{2+}\)-dependent signaling in the induction of cytokine and chemokine expression follow.
Figure 4: Schematic Illustration of TLR-4 Dependent Innate Immune Response in UPEC-induced UTI. Adapted with some modifications [153].
Local activation of the innate immune response in the bladder mucosa against adherent and invading bacteria relies largely on the recognition of bacterial components/products by TLRs [154-156]. To date, three TLRs, TLR4, TLR5, and TLR11, have been found to be clearly associated with the immune activation in the bladder upon UPEC infection [155, 157-161]. Among those, TLR4 has long been known to be effective in innate immunity against gram negative bacterial infection in multiple tissues including bladder. TLR4 is expressed ubiquitously in urinary tract epithelium and is necessary to evoke a proper and strong immune response in UPEC-induced UTI [157, 162]. The lipopolysaccharide (LPS) endotoxin on the UPEC cell wall is a major activator of TLR4 and is required for optimal TLR4 signaling in response to type 1-fimbriated UPEC [163]. Recognition of LPS by TLR4 requires co-expression of the adaptor molecule CD14, which is not expressed in uninfected urothelium [149]. However, UPEC infection of mice was shown to rapidly induce expression of CD14 mRNA in the bladder [164], suggesting a possible route for cooperative activation of TLR4 by type 1 fimbriae and LPS [149, 150, 165]. Importantly, two recent studies have shown that the type 1 fimbria tip adhesin FimH also has the ability to stimulate TLR4 signaling independently of LPS [166, 167]. Other studies have demonstrated LPS-independent activation of TLR4 by P fimbriae [168-170]. Studies in mice have shown that TLR5 plays a role in the response to UTI by mediating bacterial flagellin-dependent activation of inflammation [159, 171]. TLR5 null mice have increased susceptibility to UTI with increased bacterial burden upon UPEC infection [159]. TLR11, expressed in mice, but not in humans, also
has a role in murine UTI, as TLR11 null mice are more susceptible to UTI compared with their healthy counterparts [160]. A recent study have shown that diabetic state didn’t alter the expression levels of TLRs in alveolar macrophages in type 1 diabetic mice induced by STZ [172].

TLR signaling in response to UPEC infection can utilize NF-κB-dependent and -independent pathways leading to cytokine and chemokine expression [155, 173]. NF-κB is a crucial transcription factor, and its activation by translocation to the nucleus through TLR signaling is a key factor in the expression machinery of many proinflammatory cytokines and chemokines such as IL-6 and IL-8. Another transcription factor, interleukin regulatory factor 3 (IRF3), is also described as an important regulatory protein in the antibacterial response downstream of the TLRs [174, 175]. IRF3 has a remarkable role in distinguishing pathogens from endogenous flora and development of a proper immune response [176].

**Calcium-Dependent Signaling in UPEC-induced UTI**

A growing body of studies has shown that Ca$^{2+}$-dependent signaling is involved in the innate immune response against infections, and other signaling pathways that result in the expression of proinflammatory cytokines may intersect with the Ca$^{2+}$-dependent signaling [177]. The expressional control of some chemokines such as IL-8 is also indirectly affected by intracellular Ca$^{2+}$ level oscillations that affect NF-κB expression. Various bacterial components can affect Ca$^{2+}$ levels in the early infection period and initiate immune response pathways [178, 179]. Song et al., using *in vitro* infection of
human urothelial cells, showed that TLR4 triggered sequential Ca\(^{2+}\) and cyclic AMP signaling more rapidly than the classical NF-κB-mediated pathway, resulting in production of one of the key regulatory cytokines, IL-6 [105, 173].

The adaptation of multiple host response pathways initiating the expression of key cytokines and chemokines during UTI is presumably advantageous for the host, since an inherent or UTI-related deficiency in one of those signaling pathways could be overcome by an alternative path. In the next section, the importance of neutrophil recruitment and four crucial chemokines for bacterial clearance are discussed.

**Cytokines and Chemokines**

- **General Characteristics**

  UPEC adherence and invasion initiate rapid production of many proinflammatory mediators, among which cytokines and chemokines are crucial members [149-151].

  Cytokines, including chemotactic cytokines (chemokines), are small proteins secreted by a variety of cell types, such as epithelial cells and inflammatory immune cells like neutrophils and macrophages [180, 181]. They mediate nearly every biological process within the body including growth, differentiation, and activation functions that regulate and determine the nature of immune responses, via binding to their specific receptors on target cells [182]. The expression and release of cytokines is regulated temporally and geographically within firm ranges due to their high activities in even small amounts in tissues [182]. Chemokines, small chemotactic proteins of 8-12 kD,
mainly control immune cell trafficking, such as neutrophil recruitment through infected
tissues, and modulate the cellular distribution, maturation, and arrangement of immune
cells within organs [181, 183]. Chemokines can be classified functionally according to
their participation in inflammatory or homeostatic processes. Structurally, chemokines
are classified into four groups according to the positions of the first two of typically four
conserved cysteine residues in the sequence from the N-terminus. The four groups are C,
C-C, C-X-C, and C-X3-C chemokines [183, 184]. Several C-X-C chemokines contain a
glutamic acid-leucine-arginine (ELR) motif before the first cysteine residue; members of
this subgroup are very important for mucosal inflammation due to their ability to direct
neutrophil migration [183, 185, 186]. Included among the ELR chemokines are IL-8
(CXCL8) and murine IL-8 homologs KC (CXCL1) and MIP-2 (CXCL2) [180, 183].
Those two murine chemokines have been considered to be the closest relatives to human
GRO proteins, and studies have shown their important roles in murine UTI [187]. Two
other ELR chemokines, dendritic cell inflammatory protein-1 (DCIP-1, CXCL3) and
lipopolysaccharide-induced CXC chemokine (LIX, CXCL5), also have the ability to
recruit neutrophils in response to inflammatory signals [184, 188, 189], but in our study,
we have focused MIP-2 and KC among ELR chemokines due to their extensive
documentation during UTI. In addition to those, IL-17A has recently been reported that is
likely playing an important role in macrophage and neutrophil recruitment in UTI, thus
important for innate immune response during UTI [190]. Another crucial mediator, a C-C
chemokine called monocyte chemoattractant protein-1 (MCP-1, CCL2) which is known
mainly as a chemoattractant for monocytes, also plays a direct role in neutrophil
recruitment by enhancing the adherence and transmigration of neutrophils [191-193].
Neutrophil Recruitment Relies On Chemokines During UTI: Important Roles of MIP-2, KC, MCP-1 and IL-6

UPEC-induced UTI stimulates a robust chemokine response through various TLR4-dependent signaling pathways in the urothelium, as discussed earlier [156, 169], leading to recruitment of neutrophils, the main components of innate immunity directing effective bacterial clearance from the urinary tract (Figure 5). UPEC induces a mucosal inflammation upon adherence to the urothelium, and C-X-C and C-C chemokines have been detected in patients with urosepsis [194-196]. In humans, Interleukin-8 (IL-8), a member of the CXC chemokine family, is the major chemoattractant for neutrophils [180, 183-185]. Since IL-8 is the first described and broadly studied chemokine in neutrophil recruitment, there are a number of studies confirmed that IL-8 levels increase in blood and urine of symptomatic UTI patients, in some studies, along with IL-6 [152, 197-201]. A marked increase in the level of IL-8 was found in the urine of women with a history of recurrent UTI after instillation of UPEC into the bladder [197]. CXCR-1, the main receptor of IL-8, has a pivotal role in neutrophil recruitment [202, 203]. Furthermore, neutrophils of IL-8 receptor null mice show a diminished ability to migrate across the uroepithelium, leading to increased levels of bacteria in the urinary tract after UPEC infection [55, 161].

In mice, chemokines macrophage-inflammatory protein 2 (MIP-2) and keratinocyte chemokine (KC) are functional equivalents of IL-8 [180, 183, 197]. Those two have been considered as the closest relatives to human GRO proteins, and studies have shown their important roles in murine UTI [187]. Other ELR chemokines, dentritic
cell inflammatory protein-1 (DCIP-1, CXCL3) and LIX also have ability to recruit neutrophils in response to inflammatory signals [184, 188, 189]. MIP-2 is an effective neutrophil attractant in the urinary tract in mice, orchestrating neutrophils to pass epithelial barriers [204], and KC has been shown to be an important leukocyte attractant in in vivo models [205]. In a mouse pneumonia model, intratracheal infection triggered increased levels of MIP-2, KC, and LIX), the three functional murine homologues of IL-8 [206], in the lung tissues. UPEC installation into the bladder in mice induced MIP-2 expression and neutrophil accumulation in the urine [207], and blocking MIP-2 with a neutralizing antibody resulted in impaired neutrophil recruitment [204].

Monocyte chemoattractant protein-1 (MCP-1) has pleiotropic functions and activates multiple inflammatory pathways in both gram-negative and gram-positive bacterial infections [208]. MCP-1 promotes mainly the recruitment of monocytes to the infection area, but also contributes to the neutrophil migration during the course of severe infections [191, 209]. IL-6 has pleiotropic effects and is primarily considered to be a cytokine, however it also plays an important role in neutrophil trafficking as a chemokine [210, 211].

IL-6 is secreted primarily by mononuclear phagocytic cells, however, T and B lymphocytes, fibroblasts, endothelial cells, keratinocytes, hepatocytes, and bone marrow cells also produce IL-6 [212]. By interaction with IL-6, B lymphocytes differentiate into plasma cells and secrete immunoglobulins. IL-6 mediates T-cell activation, growth, and differentiation[185]. Like IL-1, IL-6 is an important inducer of pyrexia and participates in the synthesis of acute phase proteins [212]. IL-6 mediates several anti-inflammatory effects besides its proinflammatory effects. IL-6 terminates this upregulatory
inflammatory cascade and inhibits IL-1 and TNF synthesis. Furthermore, IL-6 stimulates synthesis of IL-1ra [185, 213]. In UTI, IL-6 is secreted early after infection, with primarily mucosal origin, and plays an important role in mediating inflammation and indirectly activating local immunity, cytokine expression and neutrophil trafficking [214]. Thus, it is considered that the primary innate immunity mediator secreted in response to UPEC infection is IL-6, which leads to neutrophil recruitment and the induction of local immunity [210]. In humans and mice, the IL-6 concentration in urine increases with the severity of UTI [210, 215].
Figure 5: Model for Signaling Processes in Urothelium upon UPEC-induced UTI

Adapted and modified from [64]. Activation of TLR4 gives rise to various signaling cascades and triggers the immune response. The process includes step-wise activation of adaptor molecules and kinases and eventual activation and nuclear translocation of NF-κB (red arrows, main pathway) and/or activation of alternative transcription factors through CREB and IRF3 (dotted arrows). A Ca²⁺-dependent pathway also contributes to the cytokine/chemokine expression and pairs up with TLR-4 signaling (dotted-dashed arrows). Chemokine expression and neutrophil recruitment are critical for successful killing and clearance of bacteria from the urinary tract.
Neutrophils: The First Line Soldiers of the Host Against UTI

Neutrophils are critical components of innate immunity, executing effective bacterial clearance from the urinary tract after pathogen-specific recognition and activation of the inflammatory mechanism by TLRs and downstream cytokines [64, 216]. Although the presence of neutrophils in healthy urothelium has not been shown [217, 218], possibly because of the relatively short life span of these cells [219], the vast majority of the inflammatory cells present in urine in the early phases of UTI are neutrophils [220, 221]. As the main performers of bacterial clearance from the urinary tract after pathogen-specific recognition, neutrophils exhibit at least three potential bacterial killing mechanisms. Those include phagocytosis, release of microbicidal molecules by degranulation or exocytosis, and NETosis, a distinct form of active cell death characterized by extrusion of chromatin fibers covered with antimicrobial proteins [222]. Based on those characteristics, neutrophils are considered to be non-resident immune cells that are very well-equipped to provide a vigorous innate immune defense against invading bacteria upon recruitment to the site of infection [223]. As a hallmark of bacterial infection, the detection of neutrophils in urine is used for diagnosis of UTI. Also, counting neutrophils in urine is used as a tool for evaluating the innate immune response in infected mice [220]. During UTI, murine bladders show characteristic changes of inflammation including urothelial wall thickening, significant accumulation of neutrophils and then other inflammatory cells, and edema in the lamina propria [3, 55]. UPEC activate mainly a rapid mucosal inflammation through pathogen recognition by TLRs, and the resulting chemokine response directs neutrophils to the infection site as the
first line of defense, since the neutrophil accumulation is the earliest and strongest response to the infected bladder [3, 197, 224]. Depending on the chemokine expression gradient, neutrophils migrate rapidly in the early hours of UPEC infection to the bladder tissue and urine, where they dominate the acute inflammatory response [147, 216, 225]. A number of studies have confirmed that neutrophil-dependent innate immunity has the major role in the defense against UTI, and thus neutrophils are the key elements for maintaining the sterility of the urinary tract [147, 148]. The components of adaptive immunity, including T lymphocytes and immunoglobulin-producing B lymphocytes, appear to be less effective in the immune defense against UTI, as suggested by experimental studies on nude, xid, SCID, TCRα/β, TCRγ/δ, and RAG knockout murine models [226-228]. On the other hand, depletion of neutrophils with antibodies significantly increases the bacterial burden and impairs clearance of bacteria from the bladder in mice with UPEC-induced UTI, revealing the pivotal role of neutrophils recruitment [147]. Additionally, inherited defects in the neutrophil response cause impaired bacterial clearance and leave the mice more susceptible to UTI. TLR4 deficient C3H/HeJ mice have increased susceptibility to UPEC-induced UTI and show an impaired neutrophil response against UPEC colonization in the bladder [220, 229].

Efficient migration of neutrophils from blood vessels through infected tissues relies on chemical gradients of chemokines, and also requires expression of intracellular adhesion molecule-1 by epithelial cells and integrin Mac-1 (CD11b/CD18) by neutrophils [218, 230, 231]. Recently, involvement of granulocyte-colony stimulating factor in the immune defense against UTI was described, found to be required for emigration of neutrophils from bone marrow upon stimulation [231]. Two distinct signaling steps have been
proposed for neutrophil recruitment and mucosal inflammation during UPEC-UTI; first step of inflammatory response is initiated upon UPEC adherence to urothelium by pathogen recognition and TLR activation (described above), and the second step is activated upon cytokine/chemokine expression and release from the infected urothelium [216]. Chemokine expression is crucial for the complete resolution of UTI with successful bacterial clearance from urinary tract, since this process ensures vigorous neutrophil recruitment to the infected bladder and urine. Many cytokines and chemokines have been found to be expressed upon bacterial colonization, but we majorly focused to four chemokines that are responsible for direct or indirect neutrophil recruitment in murine models of UTI.

**Advanced Glycation End Products (AGEs)**

Maillard is credited with discovering the glycation reaction in 1912 while examining foods [232]. He observed that sugars react with a multitude of proteins by nonenzymatic glycosylation (glycation) to form stable intermediates called Amadori products or fructosamines [233], which subsequently underwent further reactions to produce the familiar brown pigments in cooked foods. More recently, nonenzymatic glycation reactions of lipids and nucleic acids with sugars to form Amadori products have been identified [234]. Through a series of rearrangements and oxidative reactions of Amadori products, the second stage of glycation occurs, producing advanced glycation end products (AGEs) [235-238]. AGEs were originally identified by their yellow-brown
fluorescent color and their potential to form cross-links among proteins/amino acids [238].

The nonenzymatic Maillard reaction consists of the reaction between a reducing sugar such as glucose and an amino acid group on a protein, lipid, or nucleic acid to produce an unstable Schiff base that is a secondary aldimine. Further isomerization of the aldimine leads to formation of a stable, though reversible Amadori adduct (ketosamine or fructosamine). Ultimately, Amadori products undergo further chemical rearrangement through a series of dehydration, β-elimination, and condensation reactions to form irreversible adducts and cross-linked proteins (AGEs) [233, 237, 239-241]. Similarly, the glycation reaction of glyceraldehydes with protein establishes protein cross-linking to form α-hydroxyaldehydes [242, 243]. The large family of AGEs and their active intermediate products include some specific molecules that do not display color or fluorescence, or lead to cross-linking of proteins, such as 3,4-N-ε (carboxymethyl) lysine (CML) and pyrraline [244-246] (Figure 6).

The synthesis of AGEs in vitro and in vivo is contingent upon the turnover rate of the chemically transformed target, time, and the sugar concentration [247]. Interestingly, glucose has the slowest glycation rate of all sugars, whereas intracellular sugars such as fructose, glucose-6-phosphate, and glyceraldehyde-3-phosphate generate AGEs at a more accelerated rate [248-250]. Once formed, AGEs are considered practically irreversible [251]. Excessive production of AGEs in vivo can lead to tissue damage directly through extensive modification of proteins, lipids, and/or nucleic acids, by cross-linking proteins, and by binding to the receptor for AGEs (RAGE). Binding of AGEs or certain other ligands to RAGE activates inflammatory and pro-fibrotic signaling pathways [252].
A)

(A) Diagram showing AGE product generation through a set of chemical reactions. A) The first reaction between a reducing sugar and an amino group gives rise to an unstable Schiff base. B) The Schiff base slowly rearranges to form the Amadori product. C) Degradation of the Amadori product. D) Formation of reactive carbonyl and dicarbonyl compounds. E) Formation of Strecker aldehydes of amino acids and aminoketones. F) Aldol condensation of furfurals, reductones, and aldehydes produced in steps C, D, and E without intervention of amino compounds. G) Reaction of furfurals, reductones, and aldehydes produced in steps C, D, and E with amino compounds to form melanoidins. H) Free radical-mediated formation of carbonyl fission products from the reducing sugar.

(B) Maillard reaction active intermediate products.

(C) Some AGE products and chemical structures are depicted from [244].

Figure 6: Advanced glycation end products; chemical reactions and intermediate products
B

\[ \text{H}_2\text{N}{\text{Protein}} + \text{CHO} \rightarrow \text{CHO} \]
\[ \text{CHOH} \]
\[ \text{CHOH} \]
\[ \text{CHOH} \]
\[ \text{CHOH} \]

D-glucose

\[ \text{Schiff's base} \]

\[ \text{Amadori product} \]

reactive intermediates

glycolaldehyde

glyoxal

methylglyoxal diimine

\[ \text{H}_2\text{N}{\text{Protein}} \]

\[ \text{AGE} \]

C

\[ \text{NH} \cdot \text{CH} \cdot \text{CO} \]
\[ \text{NH} \cdot \text{CH} \cdot \text{CO} \]
\[ \text{NH} \cdot \text{CH} \cdot \text{CO} \]
\[ \text{NH} \cdot \text{CH} \cdot \text{CO} \]
\[ \text{NH} \cdot \text{CH} \cdot \text{CO} \]
\[ \text{NH} \cdot \text{CH} \cdot \text{CO} \]

\[ \text{Pyrraline} \]

\[ \text{Pentosidine} \]

\[ \text{Glucosepane} \]

\[ \text{Crosslines} \]

\[ \text{Fluorolink} \]
Importance of AGEs in Diabetic Complications

Studies conducted in diabetic adults and children revealed that hyperglycemia is the main factor that initiates and exacerbates diabetic complications, especially microvascular complications associated with both type 1 and type 2 DM [253, 254]. Both acute and chronically increased levels of glucose affect all levels of glycation, from early through advanced glycation events [253, 255]. Calorimetric and immunologic methods have revealed increased levels of several AGEs in serum and tissues of diabetic patients [249, 256, 257]. Schmidt et al. also reviewed that increased AGE accumulation is observed during hyperglycemic state and causes chronic vasculopathy [251]. AGEs also increase in saliva and are associated with dental complications in diabetic patients [258]. Some studies have found associations between exacerbated periodontal infections and AGE accumulation in the tissue in patients with DM, though a causative role for AGes remains to be delineated [259]. Diabetic vascular complications attributed to AGE accumulation include atherosclerosis [260], diabetic nephropathy [261, 262] and diabetic retinopathy [263-265] (also reviewed in [266] & [249]). Makita et al. have shown an association between the severity of diabetic nephropathy and the level of serum and tissue AGEs in patients [267, 268]. In the same study [267], an increased level of CML in skin was found to be associated with diabetic nephropathy. Elevated levels of CML and total fluorescence were also detected in skin collogens of patients with diabetic retinopathy, and found as independently related with this diabetic complication [269, 270]. It has also been shown that accumulation of AGEs on lipids exacerbates the renal complications in diabetic patients [271].
As a result of high blood glucose levels, diabetic patients tend to accumulate glycated proteins over time much more than non-diabetic individuals. Levels of glycated proteins in serum or plasma can be determined, as a measure of glycemic control, by the fructosamine assay, which measures the abilities of glycated proteins to reduce nitroblue tetrazolium under alkaline conditions [237, 272]. AGE formation and accumulation may perturb various cell functions and structures, and AGEs may modify cell surface proteins by cross-linking [reviewed in [234]]. Despite those well studied molecular and cellular perturbations, little information is available about a possible mechanistic role of AGEs in host-pathogen interactions. Bacteria colonize cell surfaces predominantly by binding to carbohydrate moieties on glycosylated proteins and glycolipids, therefore changes in cell surface carbohydrates due to AGE accumulation may directly result in an altered susceptibility to microbial infection. As mentioned in the earlier section on UPEC adherence, many UPEC strains express type 1 fimbriae, which mediate attachment to the urothelium through the type 1 fimbria tip adhesin FimH [18, 71]. FimH has been shown to bind mannosylated uroplakins [34] on the luminal surface of human and murine bladders [100]. The ability of type 1 fimbriae to bind to aberrant carbohydrates that are potentially on the luminal surface of the urothelium in DM, such as AGEs, has not been reported. The second chapter of this dissertation presents the results of our investigation into the role of AGEs accumulated in the urothelium of diabetic mice on UPEC adherence in the UTI model.
CHAPTER 2

ADVANCED GLYCATION END PRODUCTS (AGES) ARE BACTERIAL ADHERENCE FACILITATORS IN URINARY TRACT INFECTIONS
ABSTRACT

Despite well studied functional perturbations of advanced glycation end products (AGEs) in intracellular signaling pathways, a mechanical role in host-pathogen interactions is currently unknown. Bacteria colonize cell surfaces predominantly by binding carbohydrates. AGE accumulation greatly increases in tissues through diabetes mellitus (DM). Changes in cell surface carbohydrates and/or AGE accumulation may enhance the susceptibility to microbial infection by contributing to adherence of bacteria. In order to evaluate bacterial adherence in DM, streptozotocin (STZ)-induced female diabetic C57BL/6J mice and aged-matched controls were infected with uropathogenic E. coli (UPEC) to establish an acute urinary tract infection (UTI), and bacterial titers in bladder homogenates were determined. The AGE content of the superficial urothelium in DM and control mice, obtained by tryptic digestion, was measured by ELISA. Type 1 fimbria-AGE binding experiments were conducted in vitro by ELISA using various AGE-modified BSA species (CML-BSA, CEL-BSA, glucose-AGE-BSA, glyceraldehyde-AGE-BSA, and BSA alone) in the presence and absence of competition from soluble AGE-BSA or D-mannose, a known competitor of type 1 fimbria binding to urothelium. In addition, the effects of preincubation of UPEC with mannose or AGE-BSA products on bacterial adherence to the bladder were measured. Significant increases in UPEC adherence and AGE content were detected in the bladders of diabetic mice compared with controls. Type 1 fimbriae bound to AGEs in a manner inhibitable by mannose or the respective AGE. Furthermore, UPEC adherence decreased to a similar
extent when the bacteria were preincubated with AGEs or with D-mannose. Together, these data suggest that AGEs serve to mediate bacterial adherence and thereby exacerbate the pathogenesis of UPEC-induced UTI in DM. This novel functional concept of AGEs can lead to a better understanding of host-pathogen interactions in diabetic patients and novel therapeutic agents.

1. INTRODUCTION

Urinary tract infections (UTIs) are the most common type of infection reported to the National Healthcare Safety Network in the U.S. and among the most common worldwide, with huge economic and social burdens [273]. Infection of the bladder, referred to as lower UTI (LUTI) or acute cystitis, accounts for 95% of all UTIs [85]. Women are more susceptible to LUTI than men, especially during their reproductive years, but the risk becomes more similar between men and women with advancing age. It has been estimated that roughly 50% of women will experience a UTI in their lifetime, and millions of women in the U.S. will suffer from this painful disease each year, along with a recurrence rate reaching 30-40% [10, 11]. The majority of the published epidemiological studies of LUTI have shown that the prevalence and severity of LUTI increases with diabetes mellitus (DM), making diabetic individuals more prone to UTI-related complications [46-49].

DM is the most common endocrine disease worldwide, with various complications including UTI. The clinical and social ramifications of LUTI in this population are significant and lead to frequent hospitalizations and long-term antibiotic
therapy with no long-term benefit, but with increased antibiotic resistance [58, 274]. Delineation of the mechanism causing increased LUTI susceptibility with DM may lead to new therapeutic targets and more effective preventive strategies.

Uropathogenic *Escherichia coli* (UPEC) are the most frequently isolated uropathogens, responsible for approximately 80% of community-acquired LUTI and a large portion of nosocomial UTIs [55-57]. At present, the detailed pathogenesis of UPEC-induced UTI is yet to be completed, but many studies conducted to date demonstrate tremendous complexity in the pathogenesis of this disease. UPEC is thought to ascend the urethra from the perineum to colonize the bladder. The pathogenesis of LUTI in diabetic patients appears more complex and multifactorial than in non-diabetic individuals, owing to diverse factors such as conformational changes in the bladder epithelium and alterations of the host immune response. After entering the bladder, bacterial adherence to inner mucosal surfaces is the very first and crucial step for ascending UPEC to establish infection [83]. The type 1 fimbria is the major adhesion appendage on many UPEC strains that enables them to attach to bladder epithelium, and it plays a critical role in the development of cystitis [18, 71]. The luminal lining of the bladder consists of stratified epithelium called urothelium, with large umbrella cells comprising the superficial layer. A strong impermeable barrier is established on the apical surface of the umbrella cells, consisting of plaques composed of unique urothelial membrane glycoproteins, the uroplakins [275]. During a UPEC infection, uroplakins Ia and IIIa serve as the main receptors for the lectin-like adhesion protein (adhesin) FimH, expressed on the tip of type 1 fimbriae [4, 34, 95, 99]. Each type 1 fimbria contains a single FimH subunit on its tip that can recognize terminal oligomannose residues on
glycoproteins. UPEC pathogenesis in LUTI relies on FimH-uroplakin adherence, since type 1 fimbria-mediated interactions facilitate bacterial colonization and invasion as the first and the most critical step before the downstream events including invasion, replication, and exfoliation of urothelial cells [4].

The ability of bacteria to adhere to bladder mucosa might be altered in DM due to changes in the carbohydrate content of the luminal urothelial surface. In a small in-vitro study, urothelial cells harvested from diabetic patients were found to have increased binding capacity for type 1 fimbriated UPEC strains compared with cells from non-diabetic individuals, but the etiology behind the enhanced binding remains unclear [46]. Since binding of UPEC to host urothelial cells relies on FimH adherence to carbohydrates, normally mannose, on glycoproteins, changes in the carbohydrate moieties on the urothelial surface in DM may alter the binding ability of UPEC during UTI.

Over the course of disease progression in diabetic patients, there is increased accumulation of small carbohydrate moieties called advanced glycation end products (AGEs) generated by hyperglycemia and exacerbated in cases of poor glucose control. AGEs are implicated as mediators of several recognized diabetic complications [234, 237, 270, 276]. AGEs are generated in vivo and in vitro by a series of non-enzymatic chemical reactions between reducing sugars like glucose and macromolecules including proteins, nucleic acids, and lipids [277]. The initial product is a Schiff base, which is spontaneously converted to a more stable Amadori product, of which hemoglobin-A1c, a clinical parameter used to monitor mid-range glucose control in diabetic patients, is a representative [277]. AGE formation and accumulation may perturb various cell
functions and structures, and AGEs may modify cell surface proteins by cross-linking (reviewed in [234]). Despite well studied functional perturbations of molecular signaling pathways by AGEs within cells, little information is available about a possible mechanistic role in host-pathogen interactions. What is known is that microbes colonize cell surfaces predominantly by binding to carbohydrate moieties on glycoproteins and glycolipids, therefore changes in cell surface carbohydrates due to AGE accumulation could directly result in an altered susceptibility to microbial infection.

The aim of this study was to investigate the possible role of AGEs accumulated on bladder urothelium in DM as alternative binding receptors for type 1 fimbriated UPEC, which could be a mechanism for increased susceptibility to LUTI in DM. We confirmed increased adherence of UPEC to the urothelium in diabetic mice, measured the accumulation of carbohydrates, and AGEs in particular, in urothelium of diabetic and control mice, and determined if AGEs directly bind to type 1 fimbriae of UPEC both in vitro and in vivo, potentially impacting bacterial adherence to the urothelium.

2. ANIMALS, MATERIALS AND METHODS

- **Bacterial strain propagation and characterization of type 1-fimbriated UPEC**

  Growth of type 1-fimbriated UPEC and characterization of the fimbriae were conducted as described with minor modifications [278]. For each experiment involving bacterial instillation, UPEC strain 53498 was freshly streaked from a frozen glycerol stock onto a Luria agar plate and grown at 37 °C overnight. A single colony was inoculated into Luria-Bertani (LB) broth, incubated at 37°C overnight without shaking.
(static conditions), and then the bacterial suspension was subcultured at 1:1,000 into 2 ml of fresh LB and incubated again at 37°C overnight without shaking to aggravate expression of type 1 fimbriae. Bacteria were washed and concentrated to $10^9$/ml in 1xPBS, and type 1 fimbria expression was confirmed by mannose-sensitive agglutination of a 2% solution of guinea pig erythrocytes. Bacteria were maintained on ice (maximum 2 h) until installation.

➢ **Type 1 DM induction in mice:**

Female C57BL/6J mice 8 weeks-old (Jackson Laboratory, Bar Harbor, ME) received one or two intraperitoneal injections of high-dose STZ (150 mg/kg) to induce DM, or sodium citrate vehicle, as described [44]. Mice with blood glucose levels $>$250 mg/dl two days after STZ injection were considered diabetic. Mice were then monitored weekly until UPEC inoculation 6 weeks after STZ or vehicle injection, and only STZ-injected mice that maintained glucose levels $>$300 mg/dl were used in experiments. All protocols were pre-approved by the IACUC of Case Western Reserve University in compliance with the Public Health Service policy on humane care and use of laboratory animals.

➢ **Experimental UTI in mice and bacterial adherence assay:**

We followed established inoculation and adherence assay protocols [4, 279] with some modifications. Six weeks after injection of STZ or vehicle, 14-week-old female diabetic or control C57BL/6 mice were anesthetized with isoflurane and inoculated via transurethral catheterization with 50 μl of UPEC suspension ($1.6 \times 10^9$ CFU ml$^{-1}$) in 1xPBS, using a 24 GA 0.75 IN 0.7x19 mm shielded I.V. catheter (BD Angiocath™
Autoguard™). After 10 seconds of catheterization, catheters were removed and mice were returned to their cages. At 15 minutes after UPEC inoculation, mice were euthanized by cervical dislocation under anesthesia and their bladders were aseptically removed, weighed, and incised vertically from fundus to urethral trigone. The bladder parts were rinsed 5 times in 5 different 200mL sterile 1xPBS flasks and homogenized in 1 ml of 0.025% Triton X-100 in 1xPBS. To test for any pre-existing infection, urine was collected aseptically from mice before inoculation of bacteria. Bacterial titers were determined by plating serial dilutions of homogenates or urine onto LB agar plates. For the in vivo bacterial adherence assay with competitive binding, UPEC were preincubated for 20 minutes at room temperature with AGE-modified BSA (glucose-AGE-BSA, glyceraldehyde-AGE-BSA, Nε-(carboxymethyl) lysine [CML]-AGE-BSA, or Nε-(carboxyethyl) lysine [CEL]-AGE-BSA at 10ug/ml in 1xPBS), the FimH competitor mannose (5%), or 1xPBS alone prior to transurethral inoculation.

➢ AGE Productions: Production of AGE-modified proteins:

Collagen type 1 for production of AGE-modified collagen was obtained as a generous gift from Vincent M. Monnier, PhD (Department of Pathology, Case Western Reserve University). We followed the AGE-collagen preparation method described by Makita et al. [239]. Briefly, 5ul, 10ul, 50ul, and 100ul aliquots of collagen stock (5 mg/ml) were added to different wells in a 96-well plate, along with 135ul, 130ul, 90ul, and 40ul, respectively, of 100 mM NaPO4 to neutralize the collagen and allow gel formation. After shaking the plate for 10 min, the plate was wrapped tightly with parafilm and incubated at 4°C O/N. After O/N incubation, each well was washed twice with 0.1 M sodium
phosphate buffer, pH 7.4 to remove acetic acid. This reduced collagen was incubated with glucose, glucosepane, or methylglyoxal under the conditions described in sections 2.4.1-2.4.3 below to prepare AGE-modified collagen. For all of the AGE-collagen and AGE-BSA production protocols below, unincorporated sugars or dicarbonyl compounds were removed following incubation with the protein by repeated dialysis (3 × 18 h at 4°C) against PBS. Products were then separated into aliquots and stored at 20 °C before use. Condition-matched non-glycated (sham-modified) collagen and BSA controls were also prepared, by incubating the proteins under the same conditions, but without sugar or dicarbonyl compound. Levels of AGEs were tested by ELISA using a well-characterized monoclonal antibody against CML (Circulex, MBL International, Woburn, MA), and a polyclonal rabbit anti-AGE antibody generously provided by Dr. Vincent Monnier.

- **Preparation of glucose-AGE-collagen:**

  Each well of a 96-well plate containing reduced collagen was treated with 200ul of filter-sterilized 50mM glucose in 100mM NaPO4 buffer, pH 7.4 under sterile conditions. The samples were wrapped tightly with parafilm and incubated at 4°C for 21 days to allow formation of AGE-modified collagen (glucose-AGE-collagen).

- **Preparation of glucosepane-AGE-collagen:**

  Reduced collagen-containing wells were treated with 200ul of 50mM glucose in 100mM NaPO4 Buffer with 1 mM DTPA(Cu^{2+}/Fe^{+}) per well. The parafilm-wrapped samples were incubated at 4°C for 14 days to allow formation of glucosepane-collagen.

- **Preparation of methylglyoxal-AGE-collagen:**

  For the preparation of collagen modified by methylglyoxal, wells containing reduced collagen were incubated with 200ul of 1mM methylglyoxal in 100mM NaPO4.
Buffer with 1 mM DTPA (Cu^{2+}/Fe^{+}) per well. The parafilm-wrapped samples were incubated at 4°C for 14 days.

- **Preparation of glucose- and glyceraldehyde-AGE-BSA:**

  Glucose-AGE BSA and glyceraldehyde-AGE-BSA were produced as described with minor modifications [280, 281]. Briefly, to prepare glucose-AGE-BSA, low lipopolysaccharide and fatty acid-free BSA (50 mg/ml, Sigma-Aldrich, St. Louis, MO) was incubated with 0.5 M glucose in 0.2 M sodium phosphate buffer (pH 7.4) at 37°C for 8 weeks under sterile conditions. For glyceraldehyde-AGE-BSA, the BSA was incubated at 10 mg/ml with 0.1 M D-glyceraldehyde in 0.2 M NaPO₄ buffer (pH 7.4) at 37°C for 7 days.

- **Lectin binding activity of urothelium:**

  A combination of methods reported in the literature was employed to detect urothelial carbohydrate content using enzyme-linked lectin assays (ELLA) [282, 283]. Mouse urothelium was detached from the underlying basement membrane and suspended at 10 μg/ml in 1×PBS containing 0.1% Triton X-100 and 5 dissolved tablets of protease inhibitor (Complete Protease Inhibitor cocktail, Roche). The tissue was homogenized on ice for 2-3 min with a Power Gen 125 homogenizer at full speed. Aliquots of 100μl of homogenate were added to wells of a 96-well plate and incubated for 2 hours at 37°C to allow nonspecific adsorption of glycoproteins. The wells were then washed twice with 0.1% Tween 1×PBS, and non-specific lectin binding was blocked by adding 1% BSA (Sigma-Aldrich, St Louis, MO) to the wells and incubating for 1 hour at 37°C. After washing twice with 0.1% Tween in 1×PBS, 100 μl of a biotin-conjugated lectin (5 μg/ml)
was added per well and incubated at 37°C for 1 hour. The biotinylated lectins used were Helix pomatia agglutinin [HPA; specific for N-acetyl-alpha-D-galactosamine (α-D-GalNAc)], Maackia amurensis agglutinin [MAA; specific for (Siaα(2,3)Gal/GalNAc)], Concanavalin A [ConA; specific for GlcNAc/branched mannose], Lentil lectin [LCH; specific for α-Mannose>α-Glucose-GlcNAc], Ricinus communis agglutinin I [RCA-I; specific for 6-D-Gal], Phytohemagglutinin [PHA-L ;α-mannose residues, galactose, β(1,4)GlcNAc], Lens culinaris Agglutinin [LCA; specific for α-D-Man], Galanthus nivalis agglutinin [GNA; specific for mannose residues], Sambucus nigra lectin [SNA-I; specific for Siaα(2,6) Gal/GalNAC], and Dantura stramonium lectin [DSA; specific for β(1,4)GlcNAc] (EY Laboratories, San Mateo, CA). Following incubation, the wells were washed five times with 0.05% Tween 20 in 1×PBS. For detection, 100 µl of horseradish peroxidase (HRP)-conjugated streptavidin solution (Sigma-Aldrich) was added to each well and incubated for 30 min. The wells were washed 5 times with 0.05% Tween 20, and 100 µl of ABTS (2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) with hydrogen peroxide was added to each well. After 10-30 min, depending on color development, 100 ul of N, N dimethylformamide solution was added to each well to stop the reaction. The adherence of biotinylated lectins was detected by measuring the absorbance at 405 nm.

- Lectin binding activity of AGEs:

Lectin binding abilities of AGEs were assessed by ELLA, using AGE-modified-collagen and -BSA products generated as described above, as well as commercially available glucose-AGE-BSA, glyceraldehyde-AGE-BSA, CML-BSA, and CEL-BSA.
(Circulex, MBL International, Woburn, MA). AGE-collagens were homogenized on ice for 10 min with a Power Gen 125 homogenizer at top speed prior to aliquoting into 96-well plates. Aliquots of 100 μl of AGE-protein solutions (10 μg/ml) were added to wells of a 96-well plate and incubated 2 hours at 37°C for nonspecific adsorption. After incubation, the wells were washed with 1x PBS and blocked by adding 1% BSA (Sigma-Aldrich, St Louis, MO) and incubating at 37°C for 1 hour, to prevent non-specific lectin binding. After washing the wells, incubation with biotinylated lectins and detection with HRP-streptavidin and ABTS proceeded as described above for ELLA of urothelium.

➢ Type 1 fimbriae isolation:

Purification of type 1 fimbriae was conducted as reported by Horst et al. and Karch et al. with some modifications [284, 285]. A single colony of UPEC strain 53498, was expanded and cultivated under static conditions to enhance expression of type 1 fimbriae as described above. After confirming type 1 fimbriae expression, fimbriated bacteria were centrifuged for 20 min at 6,000 g, and the pellet was resuspended in 20 ml of 10mM Tris-HCl, pH 7.2. The fimbriae were detached from the bacteria by homogenization on ice for 10 min with a Power Gen 125 homogenizer. The bacterial debris was removed by centrifugation at 8000 g for 20 min three times. The supernatant was exposed to 24% ammonium sulfate at 4°C for 1 hour to precipitate the type 1 fimbriae, and then centrifuged at 15,000 g for 30 min. Then, the supernatant was separated and centrifuged at 27,000 g for 20 min. The pellet was resuspended in 20 ml of 10 mM Tris-HCL, pH 7.8, containing 0.02% sodium azide. After serial washing steps with 30K centrifugal filter
units (Amicon™ Ultra, Millipore), the type 1 fimbriae were dissolved in sterile water, and the protein concentration was measured using a Bio Rad Protein Assay Kit.

- **Biotinylation of type 1 fimbriae:**

  The purified type 1 fimbriae were biotinylated using a BiotinTag™ Micro Biotinylation Kit (Sigma-Aldrich), following the provided protocol with some modifications. A solution of type 1 fimbriae with a protein concentration of 10 mg/mL was prepared in 0.1 M sodium phosphate buffer, pH 7.2. The biotinylation reagent, BAC-SulfoNHS, was dissolved in 30 µl of DMSO, and then 0.1 M sodium phosphate buffer, pH 7.2 was added to a final volume of 1 ml to yield a BAC-SulfoNHS concentration of 5 mg/ml. A 10 µl aliquot of the freshly prepared BAC-SulfoNHS solution was added to 100 µl of the type 1 fimbriae protein solution with gentle stirring, and the mixture was incubated with mild shaking at 4 °C for 2 hours. The mixture was applied to a 10K Centricon centrifugal filter unit (Amicon, Millipore) and washed serially with sterile water to remove unreacted BAC-SulfoNHS reagent. The biotinylated type 1 fimbriae protein was concentrated and resuspended in sterile water, and the protein concentration was measured using a Bio Rad Protein Assay Kit.

- **Type 1 fimbriae - AGEs binding assays:**

  Binding of biotinylated type 1 fimbriae to AGE products was performed using a direct ELISA procedure with some modifications. AGE-modified proteins CML-BSA, CEL-BSA, methylglyoxal-AGE-BSA, glucose-AGE-BSA, and glyceraldehyde-AGE-BSA (Circulex, MBL International, Woburn, MA), as well as sham-modified BSA were
diluted in 1×PBS to a concentration of 10 µg/ml and adsorbed to wells of 96 well plates as described above. Biotinylated type 1 fimbriae (10 µg/ml) were preincubated with 5% mannose in 1×PBS, with a competitive AGE-modified protein solution (10 µg/ml) in 1×PBS, or with 1×PBS alone at room temperature for 20 min. Then, 100µl of preincubated, biotinylated type 1 fimbriae solution (final concentration of fimbria protein 10µg/ml) or BSA (10µg/ml) alone were each added to quadruplicate wells and incubated at 37°C for 1 hour. Mannose inhibition of the FimH subunit of type 1 fimbriae is well-known, since FimH binding to urothelium is mannose-dependent [286].

➢ Trypsin-assisted AGE harvesting and AGE ELISA:

Proteins of the superficial urothelium were harvested with a modified method of trypsin (Sigma-Aldrich) treatment of ‘inverted bladder balls’, a method that was originally developed to collect urothelium cells for primary cell cultures [287]. Briefly, bladders harvested from diabetic and control mice were inverted by pushing the dome downward through the bladder neck with a blunt 18 gauge needle. A suture was placed around the bladder neck and tightened with a half knot. The inverted bladder was inflated with 1×PBS through the needle and the suture was tightened while recruiting the catheter, producing a distended ball with only the urothelial surface exposed. Then, bladders were placed in Eppendorf tubes containing 1 ml of trypsin (0.05%) / EDTA (0.53 mM) without phenol red, and incubated at 37°C on an oscillating platform for 5 min, removing the tube every minute for brief vigorous shaking. Then, the bladders were removed and paraffin-embedded for H&E staining to ensure that the digest was effective, while removing no more than one or two cell layers of urothelium. The remaining trypsin digest mixture was
heat inactivated and centrifuged to spin down intact cells, and the supernatant was used for ELISAs after measurement of total protein concentration and dilution to 10 µg/ml in 1×PBS. ELISAs of CML and CEL were performed using specific anti-CML and anti-CEL antibodies (Abnova Corp., Jhongli, Taiwan). ELISA of CML was also performed on 5% samples collected aseptically from mice 6 weeks after DM induction, and diluted 20 times with 1×PBS before use.

➢ **Statistical Analysis:**

Data are expressed as means with SEM. Comparisons of measures in adherence assays and *in vitro* and *in vivo* binding assays were performed with the Student’s t test and one way ANOVA between groups. Differences were considered significant for p<0.05.
3. RESULTS

➢ Type 1 DM induction in female C57BL/6J mice.

At the time of euthanasia, 6 weeks after injection of STZ or vehicle, the mean blood glucose level in diabetic mice was about 5 times higher than in controls (p<0.001, Table 1). There were statistically significant differences between the two groups. The mean body weight and bladder weight was lower and higher, respectively in diabetic vs. control mice. Although those differences were not significant, combing the two measures revealed a significantly higher mean bladder weight/body weight ratio in the diabetic mice (Table 1), suggestive of bladder hypertrophy, as we have shown in STZ-diabetic male rodents [288].

Table 1. General characteristics of age-matched DM and control mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/dl)</th>
<th>Body wt (g)</th>
<th>Bladder wt (mg)</th>
<th>Bladder wt / body wt (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>111.2±8.5</td>
<td>22.4±2.2</td>
<td>27.4±3.1</td>
<td>1.22</td>
</tr>
<tr>
<td>DM</td>
<td>562.4±32.1</td>
<td>18.7±2.1</td>
<td>36.4±2.5</td>
<td>1.94</td>
</tr>
</tbody>
</table>

Values are means ± SEM, except for the ratio of mean bladder weight (wt) to mean body wt. n = 5 mice/group.
Increased Bacterial Adherence and Altered Carbohydrate Structure in urothelium with DM.

Consistent with studies showing a higher prevalence of UTI in women with DM compared to women without DM [1], our data revealed about 5 fold greater adherence of type 1-fimbriated UPEC to the bladder urothelium of mice with type 1 DM (6 weeks after injection of STZ) compared with control mice (Fig. 7). Since the carbohydrate composition of urothelial surface is critical for host-pathogen interactions, the increased adherence may be due to increased and/or altered carbohydrate levels in the urothelium of diabetic mice. Thus, we determined if the levels of different carbohydrates of urothelium differ between diabetic and healthy mice by taking advantage of the carbohydrate specificity of lectins. ELLA is an ELISA-based technique that utilizes small carbohydrate-binding proteins called lectins to detect and evaluate surface carbohydrate structures [289]. Each lectin has different ligand specificity as listed in Table 1, enabling detection of different alterations in the carbohydrate composition of tissues such as urothelium. Using ELLA, we observed significantly increased levels of mannose-competitive binding by several lectins to urothelium from diabetic mice compared with control mice, suggesting increased numbers of mannose binding sites for the FimH adhesion of many UPEC strains (Fig. 8). We also observed increased binding of lectins RCA-I and SNA-I, specific for 6-D-Gal Siaα(2,6) Gal/GalNAC, respectively, in diabetic vs. control mice.
Figure 7: Increased bacterial adherence in urothelium of diabetic mice
**Figure 7:** Figure shows increased colonization of UPEC to urothelium in type 1 diabetic mice. Mice were anesthetized and sterilized in the pelvic area, the bladder was emptied by manual massaging and washing, and bacteria (UPEC strain 53498, 1.6 x 10^9 CFU/ml) were instilled via intravesical route. Twenty minutes later, the bladder was washed serially with 1x PBS, then the mice were sacrificed and the bladder was excised and homogenized. Serial dilutions of homogenates were cultured in LB agar, and the colonies were counted and recorded as the number of adherent *E. coli*. The Student’s t test was used to compare diabetic mice with controls; *indicates statistical significance, p=0.0168.
Figure 8: Altered carbohydrate content in urothelium of diabetic mice
**Figure 8:** Urothelium samples from diabetic and control mice were homogenized, and duplicate aliquots of homogenates were adsorbed to wells of 96-well plates according to the protocol. Biotin conjugated lectins were added to microwells and incubated at 37 °C for 1 h. For each specific lectin, preincubation with competitive and non-competitive sugars were allowed to ensure specificity. Mean optical density values + SEM from two independent experiments are displayed, Student’s t test *p<0.05,  < 0.01 comparing the indicated diabetic to its corresponding control group.
Endogenous AGE product Levels on Urothelium.

Next, we measured the endogenous levels of common AGEs in urothelium and urine of diabetic and control mice. Using urothelial surface proteins released by trypsin as described in Materials and Methods, direct ELISAs of CML and CEL were performed. CML and CEL levels were significantly increased, by about 5- and 8-fold, respectively, on the urothelium of diabetic mice compared with control mice (Fig. 9A&B). CML is the most common AGE species that has been found in human biological specimens, along with glucosepane [234, 240]. Urine CML levels also were significantly higher in diabetic mice compared with controls (Fig. 9C).
Figure 9A: AGEs are elevated on urothelium in diabetic mice; CML on urothelium

Figure 9B: AGEs are elevated on urothelium in diabetic mice; CML and CEL on urothelium
Figure 9C: AGEs are elevated on urothelium in diabetic mice; CML in urine
Figure 9. AGEs are elevated on urothelium in diabetic mice. Urothelial surface proteins, released by trypsin as described in Materials and Methods, or urine samples from diabetic and control mice were adsorbed to wells of 96-well plates, and direct ELISAs were performed using HRP-conjugated anti-AGE monoclonal antibodies. (A) Urothelial proteins with anti-CML antibody. (B) Urothelial proteins with anti-CEL antibody. Results of (A) and (B) were normalized to controls in which the trypsinization procedure was carried out without bladder tissue. C. Urine with anti-CML antibody. Each sample was assayed in triplicate, and the mean absorbance value for each mouse, as well as overall means ±SEM for each group are displayed in the graphs. The Student’s t test was used to compare AGE levels in DM vs. control groups; statistical significance; ** p<0.01 and § p<0.001) was indicated.
Lectins and Type 1 Fimbriae Preferentially Bind to AGE products.

Binding of type 1-fimbriated UPEC to host receptors is mediated by the FimH adhesin located on the tips of fimbriae [99]. FimH is a lectin-like structure possessing an N-terminal carbohydrate-binding pocket capable of binding to mannosylated glycoprotein residues on cell surface proteins such as uroplakins, enabling it to mediate both bacterial adherence to and invasion of target host cells [84]. We determined the binding abilities of various lectins to two different AGE products in order to provide suggestive evidence about whether lectin-like structures can bind to AGE products. Using an in vitro ELLA for lectin-AGE product interactions, we observed that different lectins preferentially bind the AGE products glucose-AGE-collagen and glyceraldehyde-AGE-BSA compared with sham-modified collagen and BSA, respectively (Fig. 10A&B). Next, we determined if common AGEs reported in human specimens such as CML and CEL along with a methylglyoxal-AGE can bind to type 1 fimbriae of UPEC [240]. We determined that type 1 fimbriae bound to those AGE-modified BSA products significantly than to sham-modified BSA, and that the binding was partially inhibited by the FimH competitor sugar mannose (Fig. 10C).
Figure 10A: Various lectins bind to Glyceraldehyde-AGE
Figure 10B: Lectins Can Bind to Glyceraldehyde-AGE
Figure 10C: Type 1 Fimbriae (pili) of UPEC bind to CML and CEL AGEs, and Methylglyoxal-AGE
Figure 10. AGE products bind to lectins as well as UPEC Type 1 Fimbriae. AGE products were produced in the laboratory conditions as described and incubated in 96 well plates with various biotinylated lectins. Type 1 fimbriae were isolated as described, biotinylated and incubated in 96 well plates coated with various AGE products ± mannose competitor: (A) Glucose-AGE Collagen and unbound collagen as a control (B) Glyceraldehyde-AGE BSA and unbound BSA as a control. (C) the two most common AGEs, CML-BSA and CEL-BSA (MBL, Woburn, MA) and, Methylglyoxal-AGE BSA and BSA negative control, Biotinylated fimbriae were quantified by measuring absorbance at 405 nm. The Student’s t test (for graphs A, B and within each group for C) and two-way ANOVA with Bonferroni’s multiple comparison test (for C) were used to compare lectin or fimbriae binding; statistical significance *p<0.05, **p<0.01, §p<0.001 compared to controls.
Determination of the binding ability of AGEs via functional adherence assays.

FimH-mediated UPEC adherence can be inhibited by D-mannose and similar synthetic mannosides [290]. Prevention of the host receptor-FimH interaction has been shown to inhibit UPEC adherence and infection [291]. In a direct *in vitro* ELISA experiment, we found that D-mannose inhibition could block the binding of purified type 1 fimbriae to glucose-AGE-BSA or glyceraldehyde-AGE-BSA significantly, but complete blockage was not reached (Figure 4A). Preincubation of type 1 fimbriae with glucose-AGE-BSA or glyceraldehyde-AGE-BSA before probing an ELISA plate containing the same AGE-BSA species yielded a similar degree of inhibition as preincubation with mannose (Figure 4A), suggesting that AGEs also bind to FimH, or that binding of AGEs to another site on type 1 fimbriae is inhibited by mannose bound to FimH. To further investigate the possible role of AGEs in UPEC adherence, we coupled competitive inhibition of viable UPEC with the established *in vivo* adherence assay in a pilot study. UPEC were preincubated with different ligands including mannose, glucose-AGE-BSA, glyceraldehyde-AGE-BSA, CML-BSA, or CEL-BSA before instillation into the bladder in diabetic and control mice, and adherence assays were performed (Figure 4B). Although statistical analyses of the results were not performed due to the small number of mice per group (3-4), a trend toward attenuation of the increased adherence in diabetic mice was observed after preincubation UPEC, not only with mannose, but with any of the AGE-modified BSA species, supporting the *in vitro* assay results. These
results suggest that endogenous AGEs bind to type 1 fimbria by a specific receptor-mediated process that likely involves FimH, and that accumulated AGEs on urothelium can facilitate bacterial adherence, and thus UPEC pathogenesis, during experimental mouse UTI.
Figure 11: Type 1 Fimbriae of UPEC Bind to AGE products; *in vitro* functional adherence assay
Figure 11. Type 1 Fimbriae Bind AGES in a specific manner in vitro

AGE-modified or sham-modified BSA was adsorbed to wells of 96-well plates and incubated in the presence or absence of biotinylated type 1 fimbriae that had been preincubated with a) the competitive FimH blocker mannose, b) the respective adsorbed AGE-modified or sham-modified BSA, or c) PBS alone. In the BSA group, the glucose-AGE-BSA group, and the glyceraldehyde-AGE-BSA group, ‘AGE preincubation’ of type 1 fimbriae was with BSA, glucose-AGE-BSA, and glyceraldehyde-AGE-BSA, respectively. Each sample was loaded in quadruplicate, and absorbance at 405 nm was plotted as the mean plus SEM of the three independent experiments. One-way ANOVA was used to compare type 1 fimbria-AGE binding among the preincubation groups; $p<0.01$, and **$p<0.05$ compared with type 1 fimbriae binding in the absence of competitor.
Figure 12: Type 1 Fimbriae Bind to AGE products; in vivo functional adherence assay
Figure 12. Type 1 Fimbriae Bind AGEs in a specific manner; \textit{in vivo} functional assay

To delineate if the \textit{in vitro} binding of type 1 fimbriae to AGEs is reflective of \textit{in vivo} behavior of UPEC, we performed bacterial adherence assays in diabetic and control mice using type 1-fimbriated UPEC that were preincubated with different competing compounds. UPEC were preincubated with mannose (5%), with BSA modified with different AGEs (glucose-AGE-BSA, glyceraldehyde-AGE-BSA, CML-BSA, or CEL-BSA), or with BSA as a negative control prior to transurethral installation in 3-4 mice/group. The result from each mouse is shown in the graph; statistical analysis was not applied due to the low number per group, but the data, showing increased UPEC adherence in diabetic mice that was partially prevented by preincubation with mannose or any of the AGE-modified BSA species, provide preliminary support for the \textit{in vitro} assay results.
4. DISCUSSION

In the experimental UTI model, type 1 fimbriae mediate adherence of bacteria to the urothelium and initiate downstream events including invasion of urothelial cells, replication within the invaded cells, and emergence and dispersion of bacteria from the dying urothelial cells [93]. In this study, we have shown that, in addition to the well-known mannose residue receptors of UPEC type 1 fimbriae, AGEs may play an important role in bacterial adherence and enhance bacterial pathogenesis during UTI in DM. We have shown that increased UPEC adherence in diabetic mice (Figure 7) is associated with increased AGE content on the urothelial surface (Figure 8), and that isolated type 1 fimbriae bind to AGEs in a specific manner that can be inhibited by preincubation with either mannose or the respective soluble AGE-BSA species (Figure 10C). Furthermore, we have shown that binding of type 1-fimbriated UPEC to the urothelium in diabetic mice can be inhibited by preincubation with mannose or several AGE-BSA products (Figure 11&12). Together, those data strongly suggest that urothelial surface AGE modifications in DM mediate adherence of type 1-fimbriated UPEC, thereby augmenting the normal binding of UPEC to mannosylated uroplakins and exacerbating bacterial colonization of the bladder.

AGE accumulation has been shown in various other tissues in DM, including basement membranes of extracellular matrix in vascular structures, skin, eye, lung, kidney glomeruli, intestinal epithelium, heart, intervertebral cartilage discs, and atherosclerotic plaques [276, 292]. Matsumoto et al. detected the common AGEs CML and pentosidine within the extracellular matrix between bladder smooth muscle layers in
cystectomy specimens from cancer patients, but did not include diabetic individuals in their study [293].

AGEs form through a series of modifications of amino groups on proteins, lipids, and nucleic acids, initiated by non-enzymatic reactions of the amino groups with reducing sugars, and once formed, AGEs are essentially at an irreversible stage. Accordingly, hyperglycemia and/or oxidative stress accelerate AGE production, and there is evidence that accumulation of AGES is responsible for many complications of DM and aging [251, 276]. Various effects of AGEs have been studied and discussed for decades, but previous evidence supporting a role for AGEs in host-pathogen interactions has been reported. Our study is the first to show evidence for a direct effect of AGEs on infectious disease pathogenesis, by specifically binding to type 1 fimbriae of UPEC.

AGEs are known to cause tissue damage and functional alterations through three main mechanisms, which are: disruption of the functions of proteins, including regulatory and structural proteins, by direct modification or via cross linking of proteins; disruption of signaling between extracellular matrix and cells by modification of matrix molecules; and binding to specific membrane-bound receptors for AGEs, which triggers cellular responses [294-296]. Recently, gingival expression and accumulation of AGEs were identified as significantly increased in type 1 DM patients and were associated with chronic periodontitis [297]. In that study, AGE accumulation was detected in epithelium, vasculature, and fibroblasts. In another study, deterioration of periodontitis correlated with the level of serum AGEs, but not with the levels of other potential serum markers [298]. Levels of AGEs in serum and urine do not necessarily correlate with tissue levels, suggesting that AGE accumulation can vary among different tissues [299]. However, our
measurements of CML showed similar increases in both urine and the urothelial surface from diabetic mice compared with controls, suggesting that the urothelium is the major source of urinary AGEs.

Adhesins located at the distal end of UPEC fimbriae mediate various functions including tissue tropism, receptor interaction, and biofilm formation [96]. Fimbrial adhesins consist of two major domains, the N-terminal lectin-like receptor binding domain and the C-terminal pilin domain that functions mainly to anchor the adhesin to the fimbria [300] UPEC elaborate type 1 fimbriae on their surface, and the oligo-mannose-binding adhesin FimH at the type 1 fimbria tip is critical for multiple virulence functions in the course of UTI. Those include attachment and adherence of UPEC to urothelium, invasion of urothelial cells, and the distinct process of intracellular bacterial colony (IBC) formation [4, 44, 93, 301]. Chen et al. have shown that FimH is under positive selection in UPEC isolates from UTI patients [301]. The results of our in vitro studies provide evidence that UPEC type 1 fimbriae bind to various AGEs and thus endogenous AGEs that accumulate on the urothelial surface in diabetic mice may enhance type 1 fimbria-mediated binding to the bladder. Mannose did not completely abolish binding of type 1 fimbriae to AGEs, and non-mannosylated AGEs also exhibited specific, presumably receptor-mediated binding to type 1 fimbriae. The in vitro results were supported by our pilot in vivo UPEC adherence assay, in which preincubation of type 1-fimbriated UPEC with either mannose or various AGE-modified BSA species markedly decreased adherence of UPEC in the bladder. These findings further suggest that FimH and/or other as yet undefined adhesion molecules of type 1 fimbriae are capable of binding to AGEs. Such adhesin-AGE interactions could result in increased
UPEC colonization of the bladder in humans as well as mice. Future studies are necessary to determine if AGE accumulation increase in the bladder mucosa in diabetic and elderly people.

Recent attempts to find more effective treatment modalities for LUTI have focused on improving antibiotic regimens, reducing morbidity, limiting health care costs, and combating increasingly antibiotic resistant bacterial strains [38, 302]. As an alternative or complementary approach to antibiotics, a family of synthetic mannose derivatives called mannosides has been developed as orally bioactive drugs to serve as FimH competitors. Certain mannosides have been shown to be effective in preventing and treating UPEC infection and chronic cystitis in a murine model, either alone or in conjunction with an antibiotic, including sensitizing an antibiotic resistant UPEC strain to the antibiotic [302]. Additionally, other synthetic FimH blockers prevent bacteria from binding host receptors [303]. We have shown that binding of type 1 fimbriae to AGES can be inhibited partially by either mannose or the respective AGE. A more complete delineation of the AGE-type 1 fimbria binding mechanism using dose-response studies and combinations of inhibitors will be required to assess the potential efficacy of mannosides in treating UTI in DM patients.

In addition, AGE inhibitors have been found to be potentially helpful in modulating some diabetic complications [304-308]. The availability of non-toxic anti-AGE drugs for clinical use, such as alagebrium and the vitamin B₆ derivative pyridoxamine, has enabled the transition within the last decade from animal studies to clinical trials for treatment of diabetic and aging-related complications [309]. Alagebrium is an advanced glycation cross-link breaker, and pyridoxamine prevents the formation of
AGEs from Amadori products by scavenging reactive carbonyl species [276]. A more detailed characterization of the mechanism of AGE-type 1 fimbria binding may produce new contributions to the repertoire of treatments for UTI in diabetic patients, such as combining an AGE inhibitor with mannoside or antibiotic treatment.
CHAPTER 3

IMPAIRED CYTOKINE EXPRESSION, NEUTROPHIL INFILTRATION, AND BACTERIAL CLEARANCE IN RESPONSE TO URINARY TRACT INFECTION IN DIABETIC MICE
ABSTRACT

Diabetic patients have increased susceptibility to infections, and urinary tract infections (UTI) are the most common type in women with diabetes mellitus. Knowledge of bacterial clearance effectiveness following UTI in diabetics is sparse. In this study, the effects of diabetes in a murine model on bacterial clearance efficiency and components of the innate immune system in response to UTI were investigated. Streptozotocin-induced diabetic and control female C57BL/6J mice were infected with uropathogenic E. coli, and bacterial load, expression of chemokines, and neutrophil infiltration in the bladder over time were investigated. Expression levels of histone deacetylases were also measured to address a potential mechanism underlying the phenotype. Bacterial clearance during UTI was significantly prolonged in diabetic mice relative to controls. Neutrophil infiltration in bladder tissue and urine, and both mRNA and protein expression of chemokines MIP-2, KC, MCP-1, and IL-6 in bladder tissue were diminished and delayed at early time points after infection in diabetic mice relative to controls. In addition, mRNA levels of histone deacetylases 1-5 were increased in diabetic mice. This is the first study to show an association of impaired bacterial clearance in diabetic mice with suppression of UTI-induced chemokine expression and neutrophil infiltration in the bladder.
1. **INTRODUCTION**

Urinary tract infections (UTI) by uropathogenic bacteria are the most common urological disorders, and lower UTI involving bladder infections (or cystitis) specifically affect millions of individuals each year, carrying an extensive annual cost in the U.S. [1]. Women are more susceptible than men [38]. Individuals with diabetes mellitus (DM) have a higher prevalence of asymptomatic bacteriuria and of lower UTI and other infections compared with people without DM [46, 47]. The documented increasing prevalence of DM suggests an increasing prevalence of lower UTI [179]. The mechanistic basis of this association is still unclear; well-known corollaries of DM that may be contributing factors include glucosuria, which enhances growth of bacteria [44], bladder abnormalities, and impaired innate immunity [46].

UTI is a complex process, and its fate is determined within the first 24 hours in certain mice strains through dynamic host-pathogen interactions with various checkpoints and population bottlenecks [106]. Host resistance to UTI is dependent on a strong innate immune response, in which toll-like receptor 4 (TLR4), which recognizes LPS on Gram negative bacteria, plays a major role [105, 173, 310]. Uropathogenic E. coli (UPEC) is the predominant uropathogen, responsible for 80-85% of UTIs [55]. Bladder epithelial cells that have been invaded by type 1-fimbriated bacteria may exfoliate [140], or they may expel UPEC via a TLR4-dependent exocytic pathway regulated by cyclic AMP. TLR4 also activates NF-κB-dependent and -independent signaling pathways that lead to production of cytokines such as IL-6 and IL-8, and recruitment of neutrophils to the bladder [105, 106]. Neutrophils are essential for clearance of UPEC from the urinary tract.
TLR-defective C3H/HeJ mice are deficient in the neutrophil response to UTI and in bacterial clearance compared with wild type C3H/HeN mice [224]. Rosen et al. showed that UPEC infection of female C3H/HeN mice with streptozotocin (STZ)-induced type 1 DM yielded bladder titers 10,000 times higher than in non-diabetic C3H/HeN mice, whereas the difference in titers between UPEC-infected diabetic and non-diabetic C3H/HeJ mice was 100 fold, suggesting deficiencies in both TLR4-dependent and independent responses to UPEC in diabetic mice [44]. However, the neutrophil and related cytokine/chemokine responses to UTI in animal models of DM have not been examined.

Neutrophils are the main components of innate immunity directing effective bacterial clearance from the urinary tract after pathogen-specific recognition. In humans, IL-8 is the major attractant for neutrophils, and chemokines MIP-2 and KC are functional homologues of IL-8 in mice [197]. MIP-2 is an effective neutrophil attractant in the urinary tract in mice, orchestrating neutrophils to pass epithelial barriers [204], and KC has been shown to be an important leukocyte attractant in in vivo models [205]. MCP-1 has pleiotropic functions in both gram-negative and gram-positive bacterial infections, and activates multiple inflammatory pathways [208]. MCP-1 promotes mainly the recruitment of monocytes to the infection area, but also contributes to the neutrophil migration during the course of severe infections [209]. IL-6 is an early cytokine of mucosal origin that plays an important role in mediating inflammation and indirectly activating local immunity, cytokine expression and neutrophil trafficking [210]. Any defect in the circuitry of pathogen recognition, local cytokine/chemokine secretion, or
early neutrophil infiltration into the infection area may cause stalled and ineffective bacterial clearance, resulting in severe complications.

Diabetic alterations in the expression of many proinflammatory genes have been shown to be maintained even after the reinstitution of good glycemic control, suggesting epigenetic modifications. STZ-diabetic rats had increased levels of certain histone deacetylases (HDACs) and a reduced level of acetylated histone 3, which persisted after restoration of good glycemic control [311].

In the current study, we aimed to evaluate the efficiency of neutrophil infiltration into the bladder during acute UTI in diabetic mice, with respect to the expression profiles of regulatory chemokines and HDACs, the latter as a potential targetable mechanism underlying altered expression of the chemokines.
2. MATERIALS AND METHODS

- Propagation and characterization of type 1-fimbriated E. coli

Growth of type 1-fimbriated UPEC and characterization of the fimbriae were conducted as described with minor modifications [278]. UPEC strain 53498 were grown in static Luria-Bertani broth at 37 °C for 2 days to induced expression of type 1 fimbriae, and fimbriae expression was verified by mannose-sensitive agglutination of a 2% solution of guinea pig erythrocytes in 1xPBS.

- Type 1 DM induction in mice

C57BL/6J female mice 8 weeks-old received one or two intraperitoneal injections of high-dose STZ (150 mg/kg mouse) to induce DM, or sodium citrate vehicle, as described [44]. Blood glucose levels of mice were monitored weekly until UPEC inoculation 4 weeks after STZ or vehicle injection, and only STZ-injected mice that maintained glucose levels >300 mg/dl were used in experiments. All protocols were pre-approved by the IACUC of Case Western Reserve University in compliance with the Public Health Service policy on humane care and use of laboratory animals.

- Experimental UTI in mice and bacterial clearance assay

We followed the inoculation and clearance assay protocols of Mulvey et al [140] with some modifications. Twelve week-old female diabetic or control C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with isoflurane and inoculated via transurethral catheterization with 50 μl of UPEC suspension (1.6 x 10^9 CFU ml^-1) in 1xPBS. At the indicated times after UPEC inoculation, mice were euthanized by cervical
dislocation under anesthesia and their bladders were aseptically removed, weighed, and homogenized in 1 ml of 0.025% Triton X-100 in 1xPBS. Urine samples were collected aseptically.

- **Neutrophil counts in urine**

  At different times after UPEC inoculation of mice, beginning 4 weeks after STZ or vehicle injection, urine was collected aseptically by massaging the mouse abdomen over a sterile Eppendorf tube. Urine was mixed 10:1 with Turk’s stain (0.05 mg/ml crystal violet, 3% glacial acetic acid in distilled water), and neutrophils were counted with a Bürker chamber under a microscope.

- **Neutrophil MPO Assay**

  Urine was collected aseptically and bladders were harvested for ELISA of myeloperoxidase (MPO) at the times following UPEC inoculation indicated in Fig. 3 (beginning 4 weeks after injection of STZ or vehicle). Bladders were homogenized, and urine and bladder tissue neutrophils were quantified using a myeloperoxidase (MPO) ELISA kit from Hycult Biotech (Plymouth Meeting, PA) as described by Haraoka et al [224] and the manufacturer’s protocol.

- **Quantitative real-time RT-PCR (qRT-PCR)**

  Bladders were harvested for RNA isolation and qRT-PCR 4 weeks after injection of STZ or vehicle (before UPEC inoculation), and in the case of the chemokines, at the times following UPEC inoculation indicated in Fig. 3. Total RNA was isolated using
Trizol (Life Technologies, Grand Island, NY), and first-strand complementary DNA was synthesized from 1 µg of total RNA using a high capacity cDNA synthesis kit (Life Technologies). cDNA was amplified in an ABI PRISM 7500 Sequence Detection System, using SYBR Green PCR master mix with primer pairs for chemokines, HDACs, and β-actin (Life Technologies). The primer sequences are shown in Table 2.

Table 2. Primer sequences used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>MiP-2α</td>
<td>5’- AAAATCATCCAAAGATACCTGAACAA</td>
<td>5’- CTTTTGTTCTTCGCCGAGG</td>
</tr>
<tr>
<td>KC</td>
<td>5’- AGACTCCAGCCACACTCAAA</td>
<td>5’- TGACAGCGCAGCTCATTG</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5’- CATCCACGTGTGGCTCA</td>
<td>5’- GATCATCTTGCTGGTAGAATG</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’- GCTACCAAAACTGGAATAATCAGGA</td>
<td>5’- CCAGGTAGCTATTGACTCCAGAA</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’- GGTGTCATCACTATGGCAACG</td>
<td>5’- ACGGATGTCACAGCTCAACT</td>
</tr>
<tr>
<td>HDAC1</td>
<td>5’- TGGTCTCTACCGAAAAATGGAG</td>
<td>5’- TCATCACTGTGTCATTGGTCAG</td>
</tr>
<tr>
<td>HDAC2</td>
<td>5’- CTCCACGGGTGGCTCAGT</td>
<td>5’- CCACAGACAGGCTTCACTATA</td>
</tr>
<tr>
<td>HDAC3</td>
<td>5’- TTAACCGTGGGTGATGACCTG</td>
<td>5’- TTAGCTGTGGCTCTCTTGC</td>
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<tr>
<td>HDAC4</td>
<td>5’- AATCCTGCCCCGTGGAAC</td>
<td>5’- GTAGGGGCACTTGCAGA</td>
</tr>
<tr>
<td>HDAC5</td>
<td>5’- GCATGAAACTCCTCCACACAG</td>
<td>5’- TTAACCCCTCCACGTCCAGA</td>
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<td>HDAC6</td>
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<td>5’- CCCATCCATAAGTTGTGGCTG</td>
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<td>HDAC7</td>
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<td>HDAC10</td>
<td>5’- ACCCTTCGAGATGAGCTGGGA</td>
<td>5’- GTCAAGAACCCTCCAGTTG</td>
</tr>
<tr>
<td>HDAC11</td>
<td>5’- TGCAGACATCACACTGGCTAT</td>
<td>5’- GGTGGGCATCGAGATCAA</td>
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Chemokine ELISA

Bladders were harvested for ELISA of chemokines at the times following UPEC inoculation indicated in Fig. 3 (beginning 4 weeks after injection of STZ or vehicle). Bladders were homogenized, and levels of MIP-2, KC, MCP-1 and IL-6 (RayBiotech, Norcross, GA) were quantified by ELISA as indicated in the manufacturer’s protocols.

Statistical Analysis

Data are expressed as means ± SEM. Area under the curve (AUC) analysis was used to compare urine (serial) measures in diabetic and control groups. Comparisons of measures in bladder tissues were performed with the Student’s t test for each time point. Differences were considered significant for p<0.05.
3. RESULTS

At the time of euthanasia, the mean blood glucose level in diabetic mice was more than 5 times higher than in controls, while there were no statistically significant differences in body weights, bladder weights, or bladder weight/body weight ratios between the two groups (Table 1).

Table 3. General characteristics of age-matched DM and control mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/dl)</th>
<th>Body wt (g)</th>
<th>Bladder wt (mg)</th>
<th>Bladder wt/body wt (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>106.4±11.2</td>
<td>22.4±2.2</td>
<td>21.5±1.3</td>
<td>0.96</td>
</tr>
<tr>
<td>DM</td>
<td>524.2±30.6</td>
<td>21.6±1.8</td>
<td>23.2±4.1</td>
<td>1.07</td>
</tr>
</tbody>
</table>

Values are means ± SEM, except for the ratio of mean bladder weight (wt) to mean body wt. n = 10 mice/group.
Reduced bacterial clearance in diabetic mice during UTI

Lower UTI is an acute disease with a typical clearance pattern within 2 weeks in healthy C57BL/6 mice, but the process is determined within first 5 days post-infection, with a gradual decrease in bacterial load after a peak at about 24 hours post-infection [44, 231]. Here we show that, during the course of UTI from 3 hours to 5 days post-infection, the overall bacterial count in urine was significantly higher in diabetic mice than in the control group (Fig. 13A; AUC analysis, p<0.0001). Control mice cleared the majority of UPEC from the bladder by day 5, whereas diabetic mice had significantly higher numbers of bacteria in the bladder at all time points (p<0.0001 at 1, 2, 3, and 5 days) (Fig. 13B). The data indicate less efficient bacterial clearance ability in these diabetic mice, resulting in prolonged infection.
Figure 13: *In vivo* kinetics of UPEC infection and clearance in the bladder
Figure 13. *In vivo kinetics of UPEC infection and clearance in the bladder*. UPEC colony forming units in urine and bladder tissue at the indicated times after bacteria inoculation. LB agar plates were incubated at 37 °C overnight with serially diluted urine samples or Serial dilutions of bladder tissue homogenates or urine samples from 10 mice/group-time points were plated on LB agar and incubated at 37°C overnight. UPEC bacteria numbers were counted and calculated as per ml of urine, or per whole bladder. **A.** Bacterial count in urine. **B.** Bacterial count in bladder tissue. Asterisks indicate statistical significance (p<0.05) between DM and control at individual time points, calculated by Student’s t tests.
Neutrophil Recruitment Dynamics in UTI

To evaluate the effect of DM on neutrophil infiltration into the bladder in response to UTI, we evaluated the neutrophil numbers in urine and bladder tissues of diabetic and control mice at the times after UPEC inoculation indicated in Figure 14. In control mice, UPEC administration resulted in a strong increase in the number of neutrophils in urine, peaking at 6 h, followed by a sharp partial decrease by 12 hours, and then a gradual reduction through 72 hours. On the other hand, diabetic mice demonstrated a markedly attenuated and delayed influx of neutrophils in urine, peaking at 12h and then gradually decreasing through 72 hours (Fig. 14A, p<.0001 vs. controls by AUC analysis of the 3-12h period). Neutrophil infiltration after UPEC administration was also assessed by measuring the level of the neutrophil marker MPO in bladder and urine by ELISA. The time courses of MPO protein levels in bladder and urine paralleled the course of neutrophil counts in urine, with a robust peak at 6 hours post-UPEC installation in controls that was markedly attenuated and delayed in diabetic mice (Fig. 14B, bladder, p<0.0001 comparing DM with control at 3 and 6 hours; Fig. 14C, urine, p<0.0001 by AUC analysis of the 3-12h period in DM vs. control).
Figure 14: Influx of neutrophils to bladder and urine, with confirmatory MPO kinetics
Figure 14. Influx of neutrophils to bladder and urine, with confirmatory MPO kinetics. Urine was collected aseptically and bladders were harvested from diabetic and control mice at the indicated times after UPEC inoculation. A. Urine was stained with Turk’s stain, and neutrophils were counted using a hemacytometer. Inset: A representative microscopic view of TURK’s-stained neutrophils in urine from a control mouse 6 hours after UPEC administration; 100x oil-objective, bright field microscope, Zeiss. B. Results of ELISA quantification of the neutrophil marker MPO in bladder homogenates, shown as concentrations of MPO per mg total protein. *p<0.0001 by Student’s t test comparing DM with control at the indicated times after UPEC challenge. C. ELISA analysis of MPO concentrations in urine (ng MPO per ml of urine).
Diabetic Situation Attenuates Chemokine Expression in UPEC-UTI

Expression levels of MIP-2, KC, MCP-1, and IL-6 were measured at both the transcription and protein levels over time in response to UPEC installation (Fig. 15). Elevated chemokine mRNA levels were evident by 3h post-infection, and peaked at 3h (MCP-1), 6h (MIP-2 and IL-6), or 12h (KC) in both control and diabetic mice. However, the levels were significantly lower in the diabetic mice at multiple early time points (Fig. 15, left column). Chemokine protein levels followed the same pattern, except the MCP-1 protein level peaked later than its mRNA, at 6h (Fig. 15, right column). The results show that early release of neutrophil-attractant chemokines upon infection with type 1-fimbriated UPEC is severely attenuated in female diabetic mice.
Figure 15-1: Pro-inflammatory cytokine response to UPEC in the bladder: MIP-2 & KC
Figure 15-2: Pro-inflammatory cytokine response to UPEC in the bladder: MCP-1 & IL-6
Figure 15. Pro-inflammatory cytokine response to UPEC in the bladder.

A. qRT-PCR. Bladders were harvested from 4-wk diabetic mice and age-matched controls at the indicated time points following UPEC inoculation (n=5/group). Total RNA was isolated, cDNA was synthesized, and MIP-2, KC, MCP-1, and IL-6 mRNA levels were quantified by qRT-PCR as described in Materials and Methods. Using the comparative $C_T$ method, chemokine threshold cycles ($C_T$) were normalized to the corresponding $\beta$-actin $C_T$ values, and expression levels were calculated relative to the average normalized values in un-inoculated, non-diabetic control mice (time 0 in Figure 15A, set at 1.0). Statistical comparisons of DM vs. control groups at each time point were calculated by the Student’s $t$ test ($^*p<0.0001$, $^§p<0.005$, $^‡p<0.05$).

B. ELISA. Bladders were harvested from 4-wk diabetic mice and age-matched controls at the indicated time points following UPEC inoculation (n=10/group). Bladders were homogenized, and chemokine levels were quantified by ELISA. Absorbance at 450nm was measured using a FLUOstar OPTIMA Fluorescence plate reader (BMG LABTECH, GmbH). MIP-2, KC, MCP-1, and IL-6 MPO levels were calculated from the corresponding standard curves as pg/mg protein. Each bladder sample was assayed in quadruplicate. Statistical comparisons of DM vs. control groups at each time point were calculated by the Student’s $t$ test ($^*p<0.0001$, $^§p<0.005$, $^‡p<0.05$).
HDAC expression levels alters with DM

We also investigated the effect of DM on gene expression of HDACs 1-11 in the bladder in uninfected mice. The mRNA levels of HDACs 1-5 were significantly higher in diabetic mice bladders compared with controls, suggesting a potential inhibitory effect on the expression of chemokines during DM (Fig. 16). On the other hand, the expression levels of HDACs 9 and 11 were significantly lower in the bladders of diabetic mice compared with non-diabetic control mice.
Figure 16. Relative HDAC mRNA levels in diabetic and control mice. Bladders were harvested from 4-wk diabetic mice (n=8) and age-matched controls (n=8). Total RNA isolation, cDNA synthesis, and qRT-PCR were performed as described in Materials and Methods. The results, normalized to β-actin expression, are expressed relative to the level of HDAC 1 in control mice, set at 1.0. *p<0.0001, §p<0.0003, ‡p=0.003 for DM vs. control by the Student’s t test.
4. DISCUSSION

This study demonstrated that clearance of UPEC strain 53498 is impaired in diabetic female C57BL/6 mice, characterized by markedly attenuated induction of chemokine expression in the bladder, and diminished and delayed infiltration of neutrophils into the bladder compared with non-diabetic controls. In contrast, the bladder chemokine response to experimental UTI in healthy mice was rapid and robust, and was accompanied by substantial accumulation of active neutrophils in the bladder and urine.

In the experimental UTI model, type 1 fimbriae promote colonization of the urothelium and initiate an early innate host response [93]. The essential antibacterial role of neutrophils was shown by Haraoka et al. [224], who reported that pretreatment of female C3H/HeN mice with a granulocyte-specific antibody drastically impaired clearance of \textit{E. coli} from the bladder. Others have shown that UTI can trigger local mucosal expression of chemokines from the two main categories, C-X-C and C-C, referring to the position of the first two cysteine residues [204, 312]. Genetic disruption in mice of CXCR2, the only known receptor for the C-X-C family members MIP-2 and KC, resulted in impaired neutrophil infiltration into brain microvessels in response to i.p. injection of the TLR4 agonist LPS, a component of gram-negative bacteria [313]. More recently, neutrophil influx into and clearance of intratracheally instilled \textit{E. coli} from the pulmonary space was markedly reduced in mice by genetic disruption of the C-C family member MCP-1 [314]. Those results indicate essential roles for MIP-2 or KC, and MCP-1 in neutrophil recruitment. Both intratracheal installation of \textit{E. coli} in mice and LPS in humans resulted in increased MCP-1 levels in lung secretions 6 hours later, accompanied
by influx of neutrophils [314, 315] similar to our results of UPEC instillation into the bladder. Together, those reports suggest that the impaired chemokine responses to UTI that we observed in diabetic mice are a likely cause of the diminished and delayed neutrophil infiltration into the bladder and impaired bacterial clearance.

IL-6 is secreted into urine in response to UTI in women, peaking at an average of 4 hours after infection [210]. The time courses of responses of the four cytokines examined in our study differed somewhat, with IL-6 and MIP-2 expression peaking after MCP-1 and before KC, suggesting different regulation mechanisms. However, the finding that all four responses are impaired in diabetic mice implies a deficiency in those mice upstream of the differential regulation.

HDACs are important chromatin remodeling enzymes that regulate the expression of many genes including proinflammatory genes. It has been reported that DM affects inflammatory responses via altering the acetylation profile of certain lysine residues on the promoter sites of major proinflammatory genes [288]. Although a sustained proinflammatory state in DM has been suggested by studies using isolated immune cells [316], our results showing differential expression of HDACs in the bladder are in agreement with others examining histone acetylation/deacetylation at the tissue level, and suggest potential factors contributing to altered immune responsiveness against infection in diabetics. NF-κB is one of the most crucial transcription factors in the proinflammatory gene expression machinery upstream of IL-6, MIP-2, KC and MCP-1 [312], and its function is mostly dependent on the dynamic state of the chromatin
encompassing the promoters of those genes. In our study, increased expression of certain HDACs may explain the impaired inflammatory response to infectious stimuli in DM. Although class I HDACs are expressed in all mammalian tissues, whereas class 2 HDACs have more limited expression patterns, deletion studies have indicated that individual HDACs of both classes affect expression of limited sets of genes [317, 318]. The novel class IV HDAC, HDAC11, has been shown to reverse immune tolerance via down-regulation of IL-10, suggesting that decreased expression of HDAC11 in DM may promote immune tolerance [319]. Further studies are required to elucidate the roles of the specific HDACs up- or down-regulated in the bladder in diabetic mice in the gene expression machinery of the immune response to UTI.

In conclusion, we showed that bacterial clearance is impaired in diabetic mice, most probably due in part to attenuated expression of cytokines in response to UPEC challenge, resulting in lower recruitment of neutrophils to the bladder. Further characterization of the bacterial clearance defect in DM will include studies of upstream control of the expression of the related genes, as suggested by altered expression of HDACs.
CHAPTER 4

SUMMARY OF CONCLUSIONS, GENERAL DISCUSSION AND FUTURE DIRECTIONS
SUMMARY OF CONCLUSIONS & GENERAL DISCUSSION

The studies presented in this thesis have investigated a common complication of diabetes, increased susceptibility to infections, focusing on the most common infections in diabetics, those of the lower urinary tract. The studies of UPEC-induced UTI \textit{in vivo} in mice served as a model system of altered host-pathogen interactions in diabetic patients. Increased bacterial adherence to the bladder epithelium and an impaired host immune response to clear bacteria out of the urinary tract are two major drivers behind the etiopathogenesis of UTI in diabetic patients compared with non-diabetic individuals, and complete mechanisms to explain the problem have yet to be addressed. We have conducted a series of experiments to gain a better understanding of both UPEC adherence dynamics and alterations in the innate immune response in the diabetic background, employing \textit{in vitro} studies and an \textit{in vivo} murine UTI model. With regard to UPEC adherence, our results, in brief, are as follows. We observed increased bacterial adherence in the bladder in STZ-induced diabetic mice compared with controls after transurethral delivery of the major causative bacteria, UPEC. Notably, the increased adherence of that type-1-fimbriated \textit{E. coli} strain was associated with increased levels of AGEs on the urothelium of diabetic mice. The specificity of type 1 fimbria-AGE binding was confirmed with \textit{in vitro} and \textit{in vivo} binding assays. While many studies in both humans and animals have reported the involvement of AGEs in diabetes-related complications (reviewed extensively in [234]), our study is the first to postulate and provide evidence suggesting a novel role for accelerated AGE accumulation during DM, as a direct bacterial adherence mediator. Furthermore, we have shown that type 1 fimbria-AGE
binding is partially, but not completely inhibitable with mannose, an agonist of the type 1 fimbria adhesin FimH, suggesting that AGE binding is partially FimH independent and may involve an additional binding domain on type 1 fimbriae. Regarding the innate immune response to UPEC, we have confirmed a report of the Hultgren group [44] that bacterial clearance from the bladder and urine is defective in diabetic mice. It has also been reported that clearance of bacteria from other sites of infection including skin and bone is generally compromised in diabetics [320], but there is no consensus about the causes of the impairment. In our study, we have observed that prolonged clearance of UPEC during UTI in diabetic mice is associated with lower numbers of recruited neutrophils, the first line responders in the process, as well as markedly attenuated induction of expression of neutrophil-attracting chemokines in the bladder, compared with control mice. We also showed that diabetic mice overexpress several HDAC genes, which may play roles in the attenuated induction of chemokine expression. Although we are aware of the absolute need for further studies to establish causative roles for the observed factors associated with the phenotype of increased susceptibility to UTI, these encouraging results provide solid guidance for the direction and design of appropriate studies in the near future.

The type 1 fimbria is a well characterized organelle that is vital for adhesion of bacteria to urothelium, a necessary first step in the initiation of cystitis. The preeminence of UPEC type 1 fimbriae in UTI pathogenesis was shown in a study using signature-tagged mutagenesis and screening for infection-incompetent UPEC mutants [91]. The majority of genes identified in that study as required for virulence were genes encoding type 1 fimbrial proteins or proteins involved in type 1 fimbrial synthesis or its regulation;
the remaining genes encoded only a few proteins related to capsule formation, metabolism, or of unknown function.

Despite studies showing that the copious cystitis-causing UPEC strains are quite diverse genetically and biologically, expressing a wide range of virulence factors, [97] [321, 322], it has been emphasized that, “Investigations to unearth a novel bladder adhesin that is required for cystitis have not borne fruit” [106]. In a study of 247 UPEC strains isolated from women with acute cystitis, expression of the fim genotype was found in 96%, a far higher percentage than expression of other known virulence factors [323]. The type of fimbriae on the bacterium before the mucosal colonization is important, since evidence suggests that the trigger of UPEC cystitis in normal bladder urothelium mostly dependent on type 1 fimbriae regardless of the amount of fimbriation [91].

Accumulation of AGEs can be both intracellular and extracellular and can have wide-ranging effects on cell and tissue functions and structures, including cell metabolism, signaling pathways, and alteration of normal glycosylation patterns resulting in the elaboration of altered glycoproteins and glycolipids on cell surfaces. Many studies have shown that excessive AGE accumulation is involved in obesity- and diabetes-induced tissue injury. Our data revealed 6-fold increased binding of *E. coli* to the bladder urothelium of mice with type 1 DM compared with control mice, along with increased carbohydrate levels in the urothelium of diabetic mice. To determine if AGEs were among the increased carbohydrates in DM, we measured the levels of two common AGEs, CML and CEL, in the urothelium, and showed that the levels of both AGEs were significantly higher in diabetic mice compared with control mice. Furthermore, our *in vitro* studies repeatedly revealed that various AGEs and active Maillard intermediates are
recognized by and bind to type 1 fimbriae. Moreover, preincubating UPEC with exogenous AGE-modified proteins decreases the adherence of the bacteria \textit{in vivo}, suggesting the interaction of UPEC with AGEs on the urothelium. Furthermore, the binding of type 1 fimbriae to AGEs \textit{in vitro} was inhibited by preincubation with either the respective AGE or mannose, suggesting that either AGEs bind directly to FimH, or mannose bound to FimH interferes with the binding of AGEs. Those type 1 fimbria-AGE interactions may contribute to the increased UPEC colonization of the bladder in diabetic mouse models. Alternatively, AGEs might cause increased mannosylation or elaboration of uroplakins and/or other normally mannosylated proteins that enhance type 1 fimbria-mediated bacterial colonization. Further studies are required to unravel the structure and kinetics of AGE-type-1 fimbria binding to better understand the potential effect of accumulated AGEs in diabetic patients on susceptibility to UTI, and to establish a rationale and mechanistic foundation to create new therapeutic modalities based on AGE inhibition.

Bacterial adherence to mucosal surfaces is a well-described phenomenon in, not only the bladder, but in many other body organs including intestine, eye, kidney, and skin. \textit{In vitro} studies have shown that virulence of several important pathogenic bacteria, including Salmonella enteridis [324], Klebsiella pneumonia, and Candida albicans, depends on type 1 or related fimbriae, all of which normally attach to urothelium by binding to mannose [325]. Our data suggest that type 1 fimbria-mediated bacterial adherence through AGEs may be a new concept in infection susceptibility for diabetics, as well as for other vulnerable populations in which AGE accumulation is prevalent, such as elderly and obese individuals.
DM (type 1 & type 2) is associated with altered immune responses to bacterial infections, primarily involving innate immunity, since adaptive immune responses such as response to pneumococcal and hepatitis vaccines, and the lack of opportunistic infections seen in patients with adaptive immune dysfunction, seem to be intact in diabetics [326-328]. Recent studies suggest an alteration in sub-compartments in T cell family as a part of adaptive immune system, possibly contributing to the chronic inflammatory state seen in diabetics, but no such correlation related to the infection response have been documented [329]. One study attributed a cause of complications in type 1 DM to the impaired peripheral blood circulation that develops during prolonged periods of uncontrolled plasma glucose levels [330]. Uncontrolled hyperglycemia is strongly associated with many complications of DM, including UTI, which occurs with increased prevalence and greater severity in diabetics with poor glucose control [331, 332]. In the experimental UTI model, type 1 fimbriae promote colonization of the urothelium and initiate an early innate host response [93] involving many complex pathways, but the most prominent and essential response is robust neutrophil recruitment. The essential antibacterial role of neutrophils was shown by Haraoka et al. [224], who reported that pretreatment of female C3H/HeN mice, a TLR-4 responsive strain, with a granulocyte-specific neutralizing antibody drastically impaired clearance of E. coli from the bladder. We know that UTI can trigger urothelial expression of chemokines [204, 312], and genetic disruption in mice of CXCR2, the known receptor for the C-X-C family members MIP-2 and KC, resulted in impaired neutrophil recruitment to brain microvessels in response to i.p. injection of the TLR4 agonist LPS, a component of gram-
negative bacteria [313]. More recently, neutrophil influx into and clearance of intratracheally instilled *E. coli* from the pulmonary space was markedly reduced in mice by genetic disruption of the C-C family member MCP-1 [314]. Those results indicate essential roles for MIP-2 or KC, and MCP-1 in neutrophil recruitment. Together, those reports suggest that the impaired chemokine responses to UTI that we observed in diabetic mice are a likely cause of the diminished and delayed neutrophil recruitment and impaired bacterial clearance in those mice. Another important cytokine, IL-6, is secreted into urine in response to UTI in women, peaking at an average of 4 hours after infection. The time courses of responses of the four cytokines examined in our study differed somewhat, with IL-6 and MIP-2 mRNA expression peaking after MCP-1 and before KC, suggesting possible differences in the regulation of expression of the different chemokines. However, the finding that all four responses are impaired in diabetic mice implies a deficiency in those mice upstream of the differential regulation. Upstream regulation of the expression machinery of those chemokines could be affected at many steps in multiple pathways. For example, the well-studied TLR4 signaling pathway could be impaired by DM at the level of TLR-4 expression and activity, at the level of one or more proteins of the phosphorylation signaling cascade leading to NF-κB, or in regulation of NF-κB translocation into the nucleus. Delineating the precise defects in DM resulting in impaired neutrophil recruitment and bacterial clearance in response to UTI is a huge task to delineate the problem with a clear scientific answer needs well-planned studies, yet our results have evoked scientifically reasonable avenues to pursue. Increased HDAC expression in DM mice suggests possible epigenetic repression, through chromatin condensation, of the expression of genes leading to and/or including the genes
for MIP-2, KC, MCP-1, and IL-6. In a preliminary study, we measured the gene expression levels in the urothelium of several components of the TLR-4 signaling pathway leading to NF-κB activation, but did not detect any significant differences between diabetic and healthy mice (unpublished data). The importance of epigenetic regulation of gene expression in the impaired cytokine response to UTI in DM mice can be investigated further by determining if the response is restored by treatment of mice with an HDAC inhibitor. In addition, studies are planned to delineate whether NF-κB activation in response to UPEC challenge differs between diabetic and control mice, using NF-κB reporter mice that are transgenic for a construct containing two NF-κB response elements in front of a minimal promoter controlling expression of the luciferase gene.

Comprehensive studies of the neutrophil response to UTI in DM should include not just neutrophil recruitment, but also neutrophil activities such as motility and bacterial phagocytosis, since DM is a disease with known metabolic consequences affecting many crucial functional pathways [333]. Previous in vivo and in vitro studies of neutrophil recruitment, motility, and activity in diabetic mouse models of different types of infection have reported different results. It has been reported that neutrophils harvested from diabetic patients have attenuated functions such as bacterial killing capabilities, phagocytosis rolling, oxidative burst, and migration abilities (reviewed in [334]). Even though those extensive reports, studies on neutrophil migration defects are still lacking of a clear understanding. In some of these studies, chemokines such as MIP-2 is used as chemoattractant [335]. Our findings bring additional evidence that not just attenuated neutrophil migration functions, but also decreased expression of required
chemoattractants involve in the pathogenesis of infection susceptibility in diabetics. Pettersson et al recently reported that impaired clearance of subcutaneously injected *Staphylococcus aureus* in diabetic mice was due to decreased leukocyte phagocytic activity, which they observed using LPS-coated fluorescent beads *in vivo* [336]. They concluded that the impaired bacterial clearance was not due to reduced neutrophil recruitment or migration, based on their finding of higher leukocyte migration to muscle tissue exposed to recombinant MIP-2 in diabetic mice compared with healthy mice. However, they did not measure neutrophil recruitment in response to bacterial infection, in the absence of an exogenous chemokine, as we did in our study, which enabled us to detect the potential role of impaired induction of chemokine expression.

UPEC-induced UTI is also a common disease in domestic animals, and it has been reported that UPEC can spread between people and household pets, suggesting that the avenues of infection in the urinary tracts of different mammals are similar enough to not require different host-specific adaptations [337]. Mice are also naturally susceptible to UTI caused by *E. coli*, beyond their use as an experimental UTI model [55]. Furthermore, it is important to state that murine UPEC-induced UTI, especially cystitis models, closely resemble UTI in humans, with both displaying IBCs, QIRs, rapid urothelial exfoliation, and similar repertoires of innate immunity activators and players [112, 338].

UTI is a major potential problem in females throughout most of their lifespans, particularly when an infection becomes chronic, recurrent, or recalcitrant to treatment due to pathogenic mechanisms or antibiotic resistance [55]. Accompanying factors such as obesity and advanced age further complicate this disease, causing increased susceptibility
and severity and presenting an even a greater burden to both individuals and society. A better understanding of UPEC pathogenic mechanisms and host defenses during UTI, and how those processes are altered in conditions such as DM, will aid the biomedical community in improving current treatment and preventive modalities, and should open doors to the creation of new therapeutic approaches for certain risk groups with difficult disease outcomes.

**FUTURE DIRECTIONS**

UPEC strains possess an impressive repertoire of adhesins that enable them to aggregate and adhere to cell surfaces [113-116]. UPEC elaborate type 1 fimbriae on their surface, containing the mannose-binding FimH tip adhesin, which is critical for not only binding to and invasion of urothelium, but also the distinct process of intracellular bacterial colony (IBC) formation [4, 44, 93, 301]. The results of our studies presented in this dissertation provide evidence that type 1 fimbriae of UPEC bind to various AGEs and that increased levels of endogenous AGEs in the bladder urothelium of type 1 diabetic mice enhance type 1 fimbria-mediated binding to the urothelium. Competitive inhibition of FimH with mannose partially blocked binding of type 1 fimbriae to non-mannosylated AGEs, and preincubation of type 1 fimbriated UPEC with mannose or various AGEs markedly decreased the adherence of those UPEC to the urothelium in mice. These findings suggest that FimH and/or other as yet undefined components of type 1 fimbriae are capable of mediating binding to AGEs, and such fimbria-AGE interactions may result in increased UPEC colonization of the bladder in diabetic animals and
humans. Future studies are necessary to determine if excessive AGE accumulation occurs in the bladder mucosa in diabetic and elderly patients.

To date, many attempts to find more effective treatment modalities for cystitis due to UTI, especially in vulnerable populations with increased susceptibility to and morbidity from UTI such as diabetic and aging individuals, have focused on improving antibiotic regimens, reducing morbidity, limiting health-care costs, and combating increasingly antibiotic resistant bacterial strains [38, 302]. In an alternative or complementary approach to antibiotics, a family of synthetic, orally bioactive mannose derivatives called mannosides have been shown to work as FimH competitors with effectiveness in prevention and treatment of UPEC infection and chronic cystitis in mice, either alone or in conjunction with antibiotics [302]. Our novel findings that AGEs accumulate in urothelium of diabetic mice and bind to type 1 fimbriae, coupled with the reported efficacy of AGE inhibitors in alleviating some other diabetic complications [304-308], suggest that AGE inhibitors could be potent additions to the repertoire of treatments for UTI in vulnerable populations such as diabetic and aging individuals. The investigational new drug pyridoxamine, an orally bioactive vitamin B₆ derivative that inhibits AGE formation, has enabled the transition from animal studies to clinical trials for treatment of diabetic and aging-related complications, and has been shown to be effective in attenuating markers of diabetic nephropathy [309]. With the results of our studies in this dissertation, we believe that anti-AGE drugs such as pyridoxamine may have benefits in the treatment of chronic or recurrent UTI in diabetic individuals.

We have shown in our competitive binding studies of type 1 fimbriae with various AGE-modified protein products that binding to AGEs is partially inhabitable by mannose,
suggesting that either AGEs bind directly to FimH, or mannose bound to FimH interferes with the binding of AGEs to another fimbrial adhesion site. Further delineation of AGE-type 1 fimbria binding in terms of the precise binding domain(s) on type 1 fimbria for AGEs will help elucidate the potential efficacy of combining anti-AGE drugs with mannosides in therapeutic regimens for UTI in DM. Regarding that point, the dependency of AGE-type 1 fimbria binding on FimH can be tested by performing AGE binding assays using isolated fimbria from FimH null UPEC, as well as recombinant FimCH, a stable complex of FimC chaperone with FimH adhesin.

Regarding the innate immune response against UPEC adherence and invasion, we have demonstrated that induction of neutrophil-regulating and recruiting cytokines by UPEC is markedly attenuated in the bladder in diabetic mice and is likely responsible, at least in part, for the impaired neutrophil response in the bladder in those mice. A detailed investigation of mechanisms underlying the defective cytokine response in DM, including signaling pathways and epigenetic processes regulating cytokine expression, will provide a deeper understanding of DM-related alterations in host-pathogen interactions that may lead to novel therapeutic targets.

Bladder epithelial cells that have been invaded by type 1-fimbriated bacteria may exfoliate [140], or they may expel UPEC via a TLR4-dependent exocytic pathway regulated by cyclic AMP [99]. TLR4 also activates NF-κB-dependent and -independent signaling pathways that lead to production of cytokines such as IL-6 and IL-8, as well as recruitment of neutrophils to the bladder [105, 106]. TLR4 and neutrophils are essential for clearance of UPEC from the urinary tract [224]. Impaired expression of MIP-2, KC, MCP-1, and IL-6 in response to UPEC infection in diabetic mice implies a deficiency in
the upstream regulation of expression of those cytokines. The main signaling pathways stimulating expression of those chemokines start with TLR4 expression on the urothelial cell surface and its activation by UPEC. Active TLR4 stimulates a phosphorylation cascade through multiple secondary messenger proteins leading to translocation of NF-κB into the nucleus, where it can stimulate cytokine gene expression. In addition, our finding of increased expression of several HDACs in the bladder in diabetic mice suggests that epigenetic inhibition of gene transcription through chromatin condensation may also play a role in the repressed cytokine response to UPEC. Ascertaining the components of those pathways responsible for the disruption in DM will require future studies to test the functionalities of the secondary messengers in the TLR4 pathway, as well as evaluating possible epigenetic contributions by modulating HDAC activities. In a preliminary study, we measured the expression of TLR4 signaling intermediates in the urothelium at the mRNA level and did not detect any significant differences between diabetic and healthy mice. More definitive conclusions will require additional studies of the secondary messengers at the levels of protein expression and activation, as well as measurement of NF-κB nuclear translocation and activation in diabetic and control mice after UPEC challenge.

This dissertation investigated a common complication of DM, increased susceptibility to infections, focusing especially on lower UTI, the most common infection in diabetics, as an in vivo model system of altered host-pathogen interactions in DM. We have provided multiple lines of evidence that together suggest strongly that UPEC adherence increases in diabetic mice by virtue of increased accumulation of AGEs on the urothelial surface. Those data will lead the field to a better understanding of UPEC
adherence on mucosal surfaces and the importance of AGEs in diabetic subjects, and may lead to development of alternative or complementary therapeutic modalities including anti-AGE treatment in diabetic or elderly patients with chronic or recurrent UTI. We have also shown that neutrophil recruitment to UPEC-infected bladders is compromised in diabetic mice, at least in part because of attenuated induction of expression of neutrophil chemoattractants including MIP-2, KC, MCP-1, and IL-6 in the bladder. Those data increase our understanding of the innate immune response to UTI in DM and pave the way to more detailed studies that may identify new targets for therapy of this painful ailment.


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