CORRELATES OF PROTECTION IN RESPONSE TO MUCOSAL VACCINATION WITH UROPATHOGENIC *ESCHERICHIA COLI* ANTIGENS AND THE ROLE OF IL-17A DURING URINARY TRACT INFECTION

by

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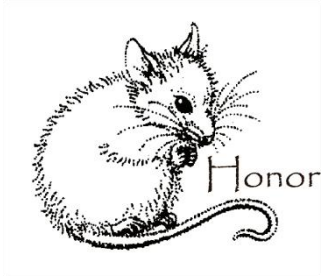
Honor

Dedication

This dissertation is dedicated to those individuals who have contributed to my education and therefore my path in life thus far, especially: my parents, Roger and Donna Baney and Charles and Lori Sivick; my grade school educators, Roger Klotz, Carol Kowaleski, Nancy Wilkin, and Debbie Anthony; my college professors, Sara Ades, Don Bryant, Dave Gilmour, Phillip Mohr, and Carl Sillman; my colleagues at the Cerus Corporation (circa 2007), Pete Lauer, Keith Bahjat, Dirk Brockstedt, and Justin Skoble; my Ph.D. mentor, Harry Mobley, and all members of the Department of Microbiology and Immunology and the Program in Immunology at the University of Michigan.

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Preface

This body of work represents a large transition in thought and interest, fostered not only by my mentor, but by my experiences throughout graduate school. I came to Michigan to learn about bacteria, and I leave considering myself both a microbiologist and immunologist. Several key components of my graduate tenure – the preliminary exam, an internship in biotech, an unwieldy biochemistry project, a knowledgeable and willing friend, a necessary yet unfilled niche in the lab, and an impressively supportive mentor – lead me to study immunology at the host-pathogen interface. Presented are the findings generated as a result of these people and events.

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List of Abbreviations

aa – amino acids
ABU – asymptomatic bacteriurial
AMP – antimicrobial peptide
APC – antigen presenting cell
BSA – bovine serum albumin
cAMP – cyclic adenosine monophosphate
CCL – (C-C) chemokine ligand
CD – cluster of differentiation or circular dichroism
CFT – ceftazidime
CNF – cytotoxic necrotizing factor
CRAMP – cathelin-related AMP
CREB – cAMP response element–binding protein transcription factor
CXCL – (C-X-C) chemokine ligand
CFU – colony-forming units
CT – cholera toxin
DC – dendritic cell
dpi – days post infection
EFC – E. coli fecal control
ELISA – enzyme-linked immunosorbent assay
ExPEC – Extraintestinal pathogenic *E. coli*
FBS – fetal bovine serum
FSC – forward scatter
G-CSF – granulocyte-colony stimulating factor
GSL – glycosphingolipid
h – hour
HA – hyaluronic acid
H&E – hematoxylin and eosin
HLA – human leukocyte antigen
hpi – hours post infection
IBC – intracellular bacterial community
ICAM – intracellular adhesion molecule
IEL – intraepithelial lymphocyte
Ig – immunoglobulin
IHC – immunohistochemistry
IL – interleukin
ILL – innate-like lymphocyte
IM – inflammatory monocyte
iNOS – inducible NO synthase
i.p. – intraperitoneal
IPTG – isopropyl β-D-1-thiogalactopyranoside
kD – kilodaulton
LPS – lipopolysaccharide
mAb – monoclonal antibody
MAPK – mitogen-activated protein kinase
MHC – major histocompatibility complex
min – minute
MPO – myeloperoxidase
MyD88 – myeloid differentiation primary response gene 88
NF-κB – nuclear factor of kappa light polypeptide gene enhancer in B-cells
NK – natural killer
nm – nanometers
NO – nitric oxide
OVA – ovalbumin
PAMP – pathogen-associated molecular pattern
PBS – phosphate-buffered saline
PCR – polymerase chain reaction
PKA – protein kinase A
PRR – pattern recognition receptor
OM – outer membrane
OMP – OM protein

QIR – quiescent intracellular reservoir
qPCR – quantitative RT-PCR
RT – reverse transcriptase
rt – room temperature
SCID – severe combined immunodeficient
sec – second
SSC – side scatter
TCR – T cell receptor
TCR $\delta^{-/-}$ – *Tcrd*^{tm1Mom} targeted mutation
Th – T helper
THP – Tamm-Horsfall protein
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-β
TU – transurethral
UPEC – uropathogenic Escherichia coli
UTI – urinary tract infection

Abstract

Urinary tract infection (UTI) caused by uropathogenic *Escherichia coli* (UPEC) is a substantial economic and societal burden – a formidable public health issue. Despite significant advances in our understanding of the biology of UPEC, the mechanism by which the host successfully responds to UTI and a full comprehension of genetic loci that influence susceptibility are not yet in place. While there is an appreciation for the role of classic innate immune responses that occur during UPEC-mediated UTI, there is a clear disconnect regarding how these factors stimulate acquired immunity that facilitates bacterial clearance upon reinfection. Unraveling the molecular details of this process is vital to understanding the host response to UTI and developing a successful vaccine to prevent human UTI.

Herein, the immune response to and efficacy of outer membrane iron receptor proteins delivered intranasally is demonstrated. Correlates of protection – the immunoglobulin "class switch index" and relative abundance of bladder urinary IgA – were identified by testing the immune responses to vaccination with both successful and unsuccessful vaccine candidates. In these experiments, splenocytes from mice vaccinated intranasally secreted high concentrations of the proinflammatory cytokine IL-17A in response to *in vitro* restimulation. From this observation, a study was conducted using IL-17A$^{-/-}$ mice as a murine model of ascending UTI to determine the role of IL-17A in both the innate and the adaptive immune response to experimental UPEC-mediated UTI.

While IL-17A appeared to be dispensable for the development of adaptive immune responses that result in enhanced clearance, IL-17A was clearly important to control the bacterial burdens of primary infection. Minimally, both γδ-positive cells and an inflammatory monocyte population appear to represent sources of IL-17A. Not surprisingly, IL-17A was found to play a role in regulating inflammatory responses in the murine bladder, crucial events for efficient clearance of UPEC during acute infection. These results advance our understanding of the innate immune response to UPEC-mediated UTI, and provide a means to more efficiently evaluate future vaccine candidates in an effort to develop an efficacious vaccine to prevent UTI.

Chapter 1
Introduction

1.1 Urinary tract infection (UTI)

UTI, one of the most common infections in humans, occurs by an ascending route (Figure 1.1). Bacteria present in fecal matter inoculate the periurethral area, then the bladder (191, 277, 461), causing symptoms clinically termed cystitis. Left untreated, bacteria ascend the ureters to the kidney and establish a secondary infection, acute pyelonephritis. At this juncture, there is risk of permanent renal scarring and bacteria can access the bloodstream (449). UTIs are divided into two broad categories: complicated and uncomplicated. Complicated UTI occurs due to anatomic, functional, or pharmacologic factors that predispose an individual to infection. Often these infections are caused by organisms other than *E. coli*, are resistant to many antibiotics (228), and spread to other parts of the body. Uncomplicated UTI occurs in otherwise healthy individuals that lack any urinary anatomical abnormalities (192).

1.1.1 Epidemiological statistics regarding UTI

In their lifetime, it is estimated that 40% of women and 12% of men will experience a symptomatic UTI, with incidences peaking in their early twenties or after age eighty-five, respectively (100, 292). In the year 2000, UTIs accounted for more than 6.8 million physician office visits and 1.3 million emergency room visits (247). Forty

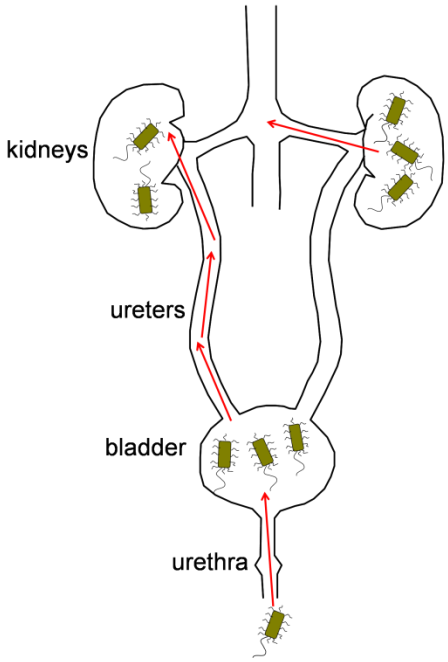

Figure 1.1 Ascending UTI. UPEC access the urethra, colonize the bladder, ascend the ureters, colonize the kidney, and penetrate the kidney epithelia to enter the blood. Note ascension, as indicated by the arrows, can occur in either (or both) ureters.

percent of nosocomial infections are complicated UTI, a direct result of urinary catheterization (398). Recurrence of UTI is a prevalent issue in the clinic. Approximately 25% of all women presenting with symptomatic UTI will experience another infection within six to twelve months (100, 292). The cause of recurrent infection is a hotly debated topic in the UTI community. Although UTI is not typically thought of as a life-threatening disease [only 1% were fatal in one study of nosocomial UTI (398)], 15% to 30% of all bacteremia and sepsis cases result from bacterial pyelonephritis (337). In the United States alone, the estimated annual societal cost of UTI is more than three billion dollars (247).

1.1.2 Risk factors of UTI

Risk factors for uncomplicated UTI are not always straightforward; however, epidemiological studies suggest that frequency of UTI can be attributed to sexual intercourse (49) and the use of spermicides (74, 75, 124) or antibiotics (382). In all age groups, women are more likely to experience a UTI than men, a fact attributed to differences in anatomy (101). Both sexes appear to be at high risk of UTI, regardless of age, in the event of hospitalization or catheterization (101). Young women in particular have a well-recognized association between recent vaginal intercourse and incidence of UTI (49). Suggested independent risk factors in this group of individuals include the use of spermicides with condoms or diaphragms, as opposed to an oral contraceptive (169). The mechanism of action responsible for the association of sexual intercourse and spermicides use with UTI is believed to be disruption of the normal vaginal flora (typically lactobacilli) and subsequent introduction of uropathogenic strains into the

vaginal and/or urethral meatus (389). Similar to uncomplicated UTI, the factors predisposing certain women to recurrence are not clear. Recurrent UTI patients often receive repeated antibiotic treatments, potentially leading to deleterious alterations of the normal microbiota (72) and contributing to the generation of antibiotic-resistant strains (130).

1.2 Bladder biology

The bladder mucosa is a transitional epithelium with large, highly differentiated, multinuclear superficial facet or umbrella cells lining the luminal surface (Figure 1.2A and B) (13). The apical side of umbrella cells, exposed to the lumen of the bladder, consists of a detergent-insoluble membrane containing a family of integral membrane proteins termed uroplakins (13). Uroplakins are partly responsible for the barrier function of the uroepithelium, and act as receptors for FimH, the tip adhesin of UPEC type 1 fimbriae (472). Beneath the basement membrane of the transitional epithelium lies the lamina propria, consisting of fibroblasts and blood vessels, and the site of edema and cellular infiltrate in response to infection (Figure 1.2). Like other surface epithelia of the body, the bladder mucosa is a impermeable barrier that is responsible for preventing passive diffusion of ions, solutes, and toxic metabolites (13). The bladder epithelium, however, must accomplish these tasks in the atmosphere of cyclical expansion and contraction to store and excrete urine waste, respectively (13). These dynamic fluctuations are achieved on a gross level by unfolding of the mucosal surface and on a molecular level by cellular membrane dynamics. Regarding these membrane dynamics, specialized “fusiform vesicles” present in superficial epithelial cells are thought to be

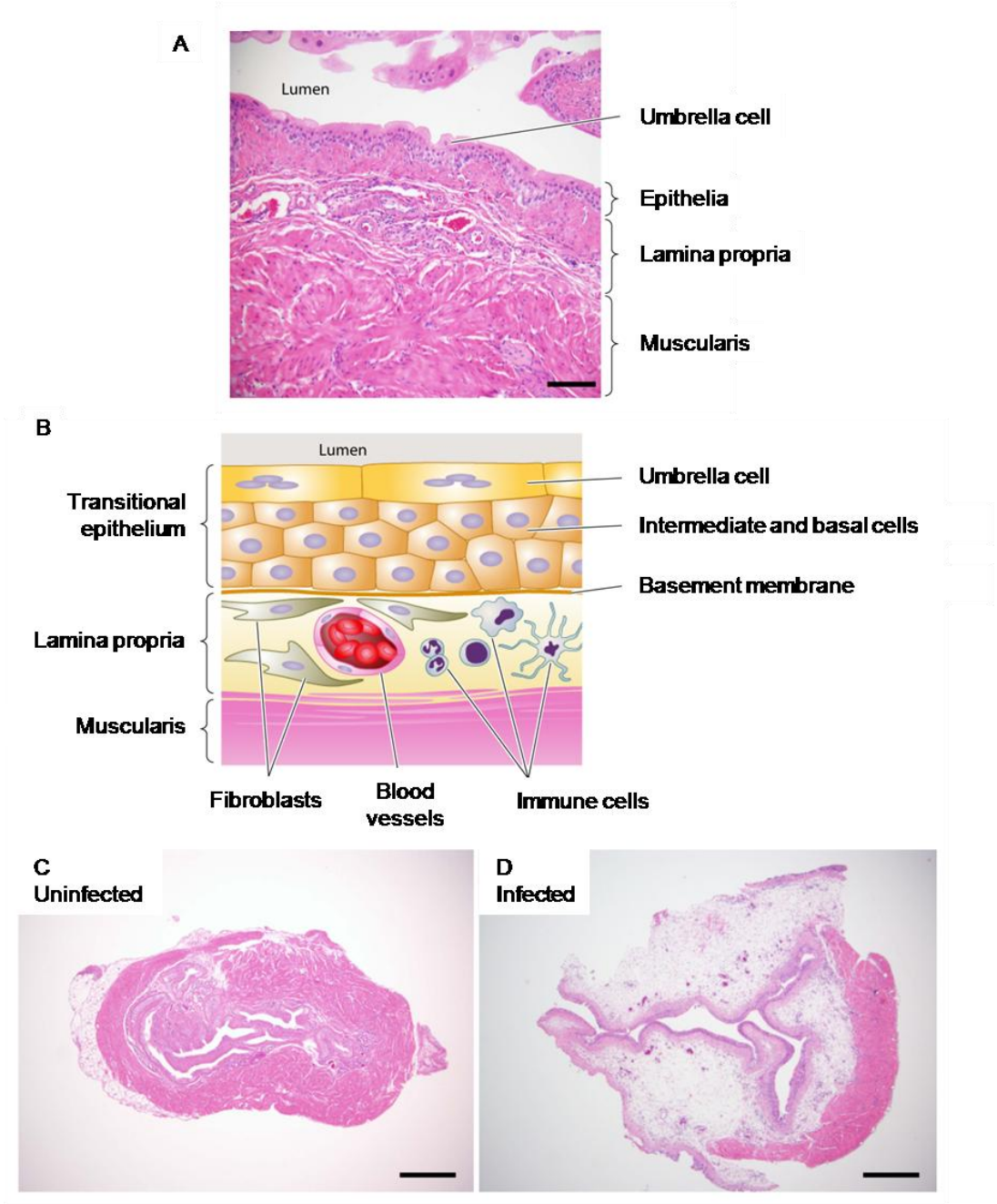

Figure 1.2 Histological and schematic views of the murine bladder. (A) Hematoxylin and eosin (H&E) stained section from a healthy wild type C57BL/6 female mouse. Magnification, 200x. Bar = 100 μm. (B) Schematic representation of bladder physiology shown in (A). (C and D) H&E-stained sections from wild type C57BL/6 mice that were (C) left untreated or (D) infected for 48 hours. Magnification, 40x. Bar = 500 μm. Artwork in (B) courtesy of Patrick Lane (Sceyence Studios).

endo- and exocytosed at a rate that provides additional membrane surface area during expansion (13).

1.3 Uropathogenic *Escherichia coli* (UPEC)

Escherichia coli is by far the most common etiological agent identified in patients experiencing uncomplicated UTI (129, 271) and is responsible for 80% of UTIs in women (70, 130, 271) and 70% of acute pyelonephritis cases in men (70). UPEC are not a completely random assortment of fecal *E. coli*. They appear to be a particular subset of strains, 75% of which belong to only six O-serogroups (208). UPEC is a member of a broader class of pathogenic *E. coli*, the extraintestinal pathogenic *E. coli* (ExPEC) (347), a grouping of *E. coli* strains that cause diseases other than gastroenteritis (346). Another defining feature of ExPEC is that they typically lack type III secretion systems utilized by diarrheagenic *E. coli* to inject host cells with an array of virulence effectors (267, 450, 451). UPEC strains themselves express an assortment of virulence and fitness factors which aid in successful colonization of the mammalian urinary tract (Figure 1.3) (193, 208).

1.3.1 UPEC virulence and fitness factors

To date, there are 14 *bone fide* virulence factors that have been shown, by molecular Koch's postulates (92), to aid in UPEC colonization. They are: type 1 fimbriae (66), Dr fimbriae (123), TonB (419), α-hemolysin (HlyA) (385), cytotoxic necrotizing factor (CNF)-1 (336), K2 capsule (52), PhoU (53), DegS (332), Deg P (333), FliC (365), RfaH (284), pckA (9), dppA (9), and OmpR (366). UPEC strains containing mutations in each of the genes encoding these factors had a colonization defect (in either independent

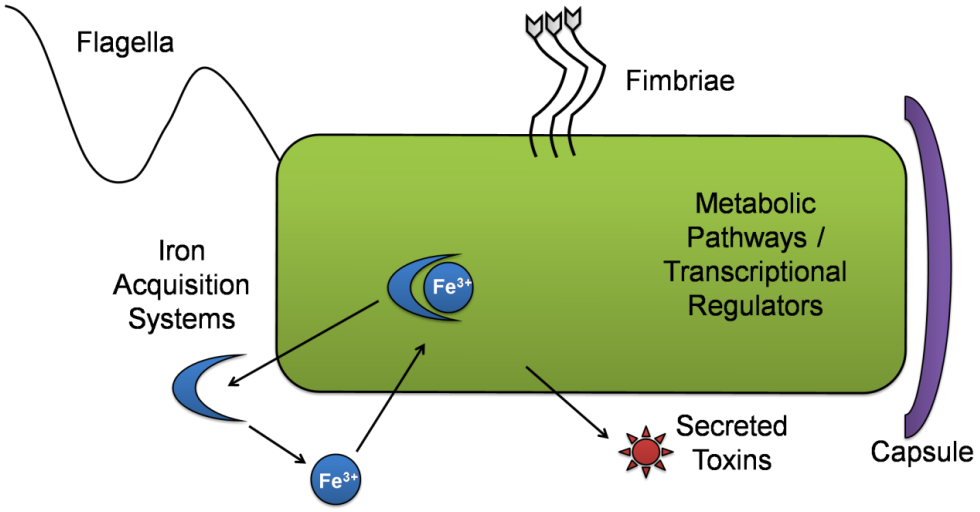

Figure 1.3 UPEC virulence and fitness factors. A schematic depicting the major classes of virulence and fitness factors utilized by UPEC during UTI. Adapted from (193).

or co-challenge) that could be complemented *in trans*. It should be noted that loss and complementation of the phenotype caused by the mutation of *hlyA* was shown histologically, and there was no difference in the ability to colonize the urine or bladder by wild type and isogenic Δ*hlyA* strains (385). Generally speaking, the requirement for these gene products reflects the environmental challenges presented by the urinary tract: osmotic stress, nutrient/metabolic restrictions, fluid flow, iron sequestration, and host immune factors (*i.e.*, complement). The inclusion of transcriptional regulators (RfaH) and proteins involved in sigma factor activity (DegS) also reflect the need to alter global transcriptional profiles to acclimate to the urinary tract. The inclusion of both hemolysin and CNF-1 illuminates the importance of toxins in UTI pathogenesis. Moreover, the genome of CFT073, a model UPEC (270), revealed the presence of seven putative autotransporter proteins (450). Autotransporter proteins are members of the type V secretion system (161), and with respect to UPEC, have pleiotropic functions both *in vivo* and *in vitro* (159, 305, 437, 439). The secreted autotransporters toxin (Sat) has both vacuolation and cytopathic effects on cell lines, and can cleave a number of host proteins that may contribute to the progression of UTI (131, 133, 252). Lastly, several of the aforementioned genes are encoded within large genomic elements termed "pathogenicity islands," acquired by UPEC via horizontal gene transfer (39, 60, 132, 248, 304, 328, 407). These islands are comprised of a number of genes that facilitate UPEC survival in both the gut and the urinary tract, including those regulated by iron, biosynthetic and metabolic operons, and genes encoding secretion systems, motility and adherence organelles, toxins, and hypothetical proteins.

1.3.2 Adherence and motility

Adherence is critical for establishment of UTI (451). UPEC stains have an impressive repertoire of factors that enable attachment to the uroepithelium. Fimbriae are filamentous organelles that allow bacterial cells adhere to a number of different tissue surfaces or cell types. To date, at least four types of fimbriae are known to be expressed by uropathogenic strains of *E. coli*. They include P (*pap*), type 1 (*fim*), S (*sfa*), and F1C (*foc*) fimbriae. Each type of fimbriae is encoded by at least one cluster of genes whose products are involved in synthesis and regulation (276). Uropathogenic strains also encode the highly heterologous family of Afa and Dr adhesins (314, 373) that mediate adherence to both urinary and intestinal niches (211, 373). Lastly, in addition to a number of uncharacterized non-polymeric adhesins that bind an assortment of host proteins, UpaG and antigen 43 autotransporter proteins have been shown to facilitate adherence to the extracellular matrix and be important for persistence in the urinary tract (437, 439).

Regulation of UPEC fimbriae expression is a complex process. Fimbrial expression is known to phase-vary, and expression of the different fimbrial operons must be coordinated with respect to each other and the environment (387, 388). Additionally, expression of fimbriae appears to influence flagellin expression and bacterial motility (234). Indeed, flagellar synthesis is negatively regulated during constitutive expression of fimbriae; however, the converse – fimbrial regulation in response to flagellar expression – does not appear to occur (234). Nonetheless, flagella are essential for propagation of the infection from the bladder to the kidneys (230, 232, 454). Using bacterial mutants lacking the major flagellar component, FliC, two different groups

demonstrated that flagella confer a fitness advantage for colonization (232) and persistence (454) in the urinary tract. In an elegant study looking at transcriptional activity from the *fliC* promoter using a luminescence reporter system *in vivo*, Lane *et al.* provided direct evidence that flagella are expressed at the time [four to six hours post infection (hpi)] and location (ureters) corresponding to UPEC ascension from the bladder to the kidneys (230).

1.3.3 UPEC iron acquisition systems

Iron is essential for UPEC growth and survival in the urinary tract. Sequenced genomes reveal that functional redundancy appears to be the theme for iron uptake by UPEC (Figure 1.4) (50, 60, 450). UPEC secretes several classes of siderophores: low molecular weight iron-chelating molecules that scavenge iron in the ferric (oxidized) form in the urinary tract (47, 290). In addition to aerobactin, enterobactin, salmochelin, and yersiniabactin siderophores, UPEC outer membrane (OM) receptors can also acquire iron bound by host heme/hemoglobin (46, 125). Energy for uptake of iron-bound substrates by highly-specific OM proteins is derived from the electrochemical potential of the inner (cytoplasmic) membrane; this energy is transmitted to the OM by the TonB-ExbB-ExbD system (46, 125). Transcription of iron-related proteins is controlled by Fur, a master regulator that exerts its activity based on its iron-bound state (146). Mutation of genes in several of the iron-uptake loci results in colonization defects during co-challenge with wild type in the mouse model, emphasizing the fitness advantage afforded by these systems (136, 195, 345, 348). However, mutation of any single iron uptake system does not result in a colonization defect in independent challenge, highlighting the functional

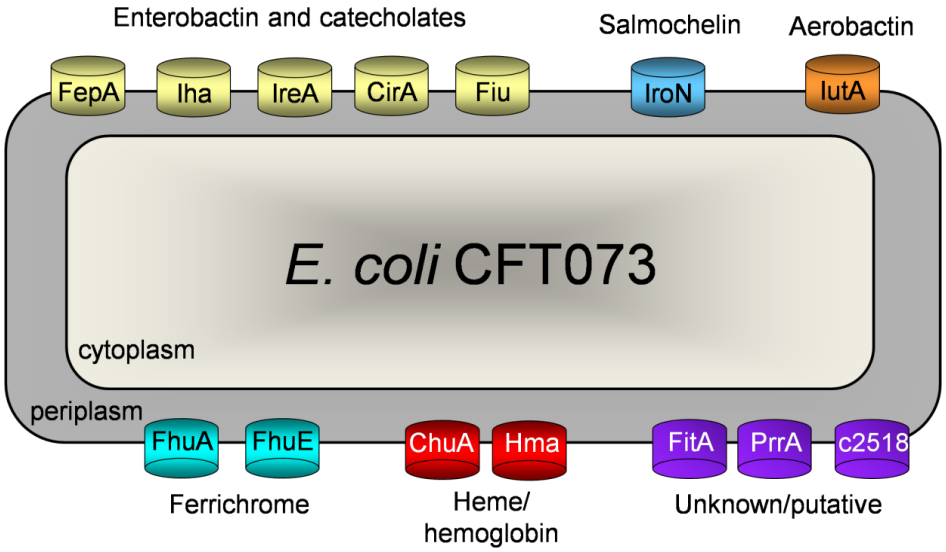

Figure 1.4 Putative and established outer membrane receptors utilized for iron acquisition by *E. coli* CFT073. Depicted are outer membrane receptors functioning in several different iron uptake systems, categorized by the iron-bound substrates (typically siderophores) they import.

redundancy of iron acquisition systems in UPEC.

1.3.4 UPEC central metabolism and pathogenesis

The metabolism field is generating an enriched understanding of bacterial pathogenesis by recognizing the importance of genes that are not classically associated with virulence (*i.e*. toxins and adherence organelles) (9, 91, 199). Specific to UTI, infection studies using bacterial mutants in key metabolic pathway enzymes revealed that the preferred carbon source for UPEC in the urinary tract is peptides, and that anaplerotic pathways (reactions that replenish metabolites) are also important (9). On the host side, genes important for glucose import were upregulated by the uroepithelium of C3H/HeJ mice experiencing UTI, possibly for either nutrient sequestration or energy to combat infection (334). The former prospect is unlikely, given that UPEC does not chemotax toward glucose *in vitro* (231) or utilize glucose as a primary carbon source *in vivo* (9). These data imply that, as a requisite for survival in the urinary tract, UPEC strains may have specifically evolved to use carbon sources other than glucose. These results indicate that nutrient acquisition is a crucial aspect of bacterial pathogenesis and the host response that may influence the outcome of UTI.

A role for metabolism involving D-serine has also been uncovered in the regulation of gene expression during UTI (151, 340). D-serine is present in high concentrations in human urine (179), and UPEC strains commonly encode a catabolic enzyme, D-serine deaminase (DsdA), that converts D-serine to pyruvate and ammonium (273, 340). Interestingly, a Δ*dsdA* mutant exhibits a hypervirulent phenotype in the mouse model when compared to an isogenic wild type strain, attributed to increased

expression of genes directly involved in urinary tract survival – flagella, fimbriae, hemolysin, an OM porin, a heat shock protein, and a dipeptide transporter (151, 340). It is not known if increased virulence gene expression in the Δ*dsdA* mutant is a result of direct transcription changes in response to intracellular accumulation of D-serine or indirect effects due to altered metabolic processes.

1.4 Treatments for UTI

1.4.1 Antibiotics

UTIs are typically treated with antimicrobial therapy; however, it is well established that treatment of bacterial infection with antibiotics selects for antibiotic resistance, a fact also applies to UPEC. Of equal but underappreciated importance, selection of host commensal bacteria by antibiotic treatment could potentially serve as a resistance reservoir for UPEC strains (103), thus making resistance an important factor in the appropriate antibiotic choice for UTI treatment. However, the choice of an antimicrobial agent for treatment of UTI is generally made empirically, especially in outpatient cases. In some geographical regions, both ampicillin (197) and trimethoprim-sulfamethoxazole have generated levels of bacterial resistance that render them inadequate for empirical treatment (279, 423). Fluoroquinolones are the preferred agent to treat complicated UTI, in addition to being an alternative for uncomplicated UTI (351, 397). Not surprisingly, increased use of fluoroquinolones has also correlated with elevated levels of fluoroquinolone resistance (70).

1.4.2 Prophylactic treatments

There has also been interest in non-antibiotic prophylactic treatments for UTI. Several reports indicate estrogen treatment of post-menopausal women alleviates recurrent UTI by virtue of its ability to restore a pre-menopausal vaginal pH (<4.5) and bacterial lactobacilli microbiota (45, 192, 309, 322, 331). These results, however, are controversial (56, 214). Prophylactic treatment for UTI using cranberry juice, while seemingly effective, has been somewhat anecdotal. One study, however, showed substantial clinical evidence – a daily cranberry juice regimen given to 153 women reduced bacteriuria and pyuria by 42% (18). Proposed mechanisms of action include acidification of urine (104) and interference with fimbriae expression or binding to the uroepithelium (311). Pre- and post-coital bladder voiding appears to be associated with prevention of sexual intercourse-associated UTI, although data regarding this practice and the incidence of UTI are conflicting (102, 169). Additionally, several studies have report that post-coital prophylactic treatment with antibiotics was found to be a successful means to prevent recurrent UTI (259, 399, 400).

1.4.3 Proposed new treatments

Specific targeting of particular virulence mechanisms is an exciting avenue of treatment with recent indications of translational use. Cyclic adenosine monophosphate (cAMP) was shown to play a role in both the cytokine response to and inhibition of invasion events by UPEC (392, 394). Forskolin, a drug causing increases in intracellular cAMP, was proposed as an effective treatment for the remove of UPEC contained in fusiform vesicles, which are the specialized vesicles dynamically endo- and exocytosed by bladder epithelial cells (37). Treatment of UPEC-infected mice with forskolin expels

UPEC harbored in intracellular vesicles, rendering the bacteria susceptible to immune responses and antibiotics (37). Similarly, exposing the bladder to protamine sulfate, a highly cationic protein, removes bound and intracellular UPEC by causing umbrella cells to exfoliate (280), unfortunately with a significant level of discomfort in volunteers (245). In addition to a number of non-specific chemical treatments (435), both small-molecule inhibitors (42) and specific antibody directed against FimH (412) have shown potential in that they interfere with FimH binding to its natural ligand in the urinary tract, the glycoprotein uroplakin Ia (263, 472).

Vaccine initiatives for UTI

While antibiotic therapy remains the standard treatment for UTI, overuse leads to deleterious alterations of the normal host microbiota (72) and selection for resistant strains (70, 103, 129, 130, 197, 279), prompting the need for vaccine-mediated prevention of UTI. Despite a relatively in-depth knowledge base for UPEC physiology and virulence mechanisms [reviewed in (74, 193, 208, 451)], no licensed vaccine to prevent UTI exists in the United States. A more thorough understanding of the mechanisms involved in the natural immune response to UTI, however, may direct a new approach to harness these responses in a vaccination setting. Nonetheless, small successes have been observed in both animal models and in clinical trials. These efforts are summarized in the following sections and in Table 1.1.

1.4.4 Vaccines based on O- and K-antigens

Early vaccine studies targeted the lipopolysaccharide (LPS) side chain (O)-antigen (436). There are trends regarding the frequencies of particular O-antigens among

Table 1.1 Previously tested vaccines for UPEC-mediated UTI.

Category/ Vaccine Type	Tested in[a]	Route[b]	Adjuvant[c]	Measured immune response[d]	Protection[e]	Ref(s)
Surface Structures						
O-antigen	R	B, SC	ND[f]	H	B	(436)
OMP fractions	Swiss	IM, O	CFA	C, H	B	(238)
Adherence						
Dr fimbria	C3H/HeJ	ND	CFA, IFA	H	-	(122)
Type 1 fimbria	BALB/c	IM, SC	CFA	H	-	(294)
FimC-H or FimHt	C3H/HeJ	S	CFA, IFA	H	B, K	(236)
FimHt	BALB/c	IM, IN	CFA, IFA, CpG	H	B	(317)
FimC-H	P	IM	MF59	H	U	(235)
Fim peptides	Swiss	IM, SC	CFA, IFA	H	B	(412)
P fimbria	BALB/c	IM, SC	CFA, IFA	H	K, U	(293, 294, 360)
PapD-G	P	IP	AP	H	K	(339)
Pap peptides	BALB/c	IM, SC	CFA, IFA	H	U, K	(360)
Toxin						
Denatured HlyA	BALB/c	IM	CFA, IFA	H	K	(293)
Iron acquisition						
Denatured IroN	BALB/c	SC	ND	H	K	(349)
Native ChuA	CBA/J	IN	CT	C, H	-	(7)
Native Iha	CBA/J	IN	CT	C, H	-	(7)
Native IroN	CBA/J	IN	CT	C, H	-	(7)
Native Hma	CBA/J	IN	CT	C, H	B, K	(7)
Native IreA	CBA/J	IN	CT	C, H	B, K	(7)
Native IutA	CBA/J	IN	CT	C, H	B, K	(7)
Complex						
SolcoUrovac	BALB/c	IP, V	MO	C	B, K	(432)
SolcoUrovac	C57BL/6	IP, V	MO	C	B, K	(432)
SolcoUrovac	Swiss	IP	ND	H	ND	(223)
SolcoUrovac	R	IP	AP	ND	K	(222)
SolcoUrovac	P	IM, V	MO	H	B	(430)

SolcoUrovac	H	IM, V	ND	H	Y	(126, 171, 174, 350, 426-429)
Uro-Vaxom	BALB/c	IP, O	ND	C, H	ND	(21, 180, 367)
Uro-Vaxom	H	O	ND	H	U, Y	(28, 71, 134, 241, 251, 362, 409)
Live (L), live attenuated (LA), or killed (K) *E. coli*						
L NU14	C57BL/6 J	TU	ND	C, H	B	(416)
L and K CP9	C57BL/6 J	IN	ND	H	ND	(344)
L and K CP923	C57BL/6 J	IN	ND	H	ND	(344)
LA Δ*waaL*	C57BL/6 J	B	ND	ND	B	(35)
K J96	BALB/c	IM, SC	CFA	H	-	(294)
K P678-54	BALB/c	IM, SC	CFA	H	-	(294)
K O6	R	V	IFA	N	B, K	(433)
K 1677	P	V	MO, MDP	H	U	(425, 431)

[a] If tested in mice, strain specified; R=rats; P=Non-human primates; H=humans

[b] B= bladder, IM=intramuscular, IN=intranasal, IP=intraperitoneal, O=oral, SC=subcutaneous, TU=transurethrally, V=vaginal

[c] AP=Aluminum phosphate, CFA=complete Freund's adjuvant, CpG= CpG oligodeoxynucleotides, CT=cholera toxin, IFA=incomplete Freund's adjuvant, MDP=muramyl di-peptide, MO=Mineral oil (for vaginal route only)

[d] C=cellular or H=humoral

[e] K=reduction in kidney colonization/histopathology, B= reduction bladder colonization, -=no protection, Y=significant decrease in UTI incidence, U= reduction of UTI as determined by urinalysis

[f] None disclosed

UTI isolates (90, 403, 451), and O-antigen-specific antibodies demonstrate an anti-adhesive effect (403). Despite this finding and the fact that antigen-specific antibodies were detected in their urine, mice injected (subcutaneously or directly into the bladder) with O-antigen displayed only a modest reduction in bacterial colonization upon challenge (436). Additionally, significant structural heterogeneity may represent an insurmountable obstacle for development of an O-antigen-based vaccine.

Similar to the trends regarding particular O-antigen serotypes and uropathogenic strains, a number of virulence determinants associated with UTI appear to be associated with strains that have the K1 serotype (90). There is evidence that capsular antigen specific antibodies are generated in response to UTI (145, 201), and the protective nature of K-specific antibodies has been demonstrated in an mice challenged the intraperitoneal (i.p.) route with *E. coli* (202). Studies specific to UTI, however, have shown that K-specific antibodies have only a minor role in preventing *E. coli* from binding to human epithelial cells (403). Considering that adherence is a key virulence determinant for uropathogenicity (79, 443), this result was not promising. Lastly, a study evaluating antibody responses in mice intranasally vaccinated with a killed *E. coli* lacking capsule and O-antigen demonstrated that these surface features actually obstruct optimal humoral responses to other surface proteins (344).

1.4.5 Vaccines based on kidney-specific virulence and fitness factors: P fimbriae and α-hemolysin

Later studies involved vaccines directed against particular virulence factors. P fimbriae (also known as P-pili) are adherence organelles that play a role in kidney colonization in mice and humans (294, 295, 457, 470); the pore-forming toxin HlyA and

P fimbriae are the proposed minimal factors required for colonization of and dissemination from the kidney (293). There are convincing data using both murine (293, 294, 360) and primate models (338, 339) that vaccination against P fimbriae or HlyA prevents renal colonization and damage. Additionally, to overcome P fimbrial allelic variability, linear peptide sequences that generated cross-reactive antibodies were evaluated as protective antigens (300, 360). While P-fimbriae vaccination strategies were shown to decrease colonization levels in the kidney upon challenge, α-hemolysin vaccination specifically protects mice from renal injury after challenge (293). Beyond this study, the secretion system of E. coli α-hemolysin, a prototypical type I system with secretion mediated by the HlyB, HlyD, and TolC proteins, has been used to deliver heterologous vaccine antigens from several different bacterial, parasitic, and viral antigens with some success (116). Despite these successes, vaccines targeting P fimbriae may not be effective because of the kidney tropism of this fimbriae type. In other words, while complicated UTI risk patients may avoid kidney damage and risk of bacteremia as a result of a vaccine based on P fimbria and HlyA, patients at risk of uncomplicated cystitis may benefit more from a vaccine that targets antigens expressed in the bladder, the site of initial infection.

1.4.6 Vaccines based on bladder specific virulence factors: Type 1 fimbriae

Type 1 fimbria is a *bone fide* virulence factor of UPEC and, in contrast to P fimbria, is critical for bladder colonization (15, 66, 127, 412). Animals vaccinated with various components of type 1 fimbriae had increased levels of antigen-specific antibodies and decreased levels of colonization upon challenge (235, 236, 303, 317, 412).

Unfortunately, type 1 fimbriae are subject to phase variation, allowing UPEC to evade immune responses by modulating gene expression (81, 388). Additionally, since non-pathogenic isolates also express type 1 fimbriae (140, 193), targeting this population may result in detrimental disruption of the host microbiota. Targeting uropathogenic strains expressing type 1 fimbriae may also select for strains of *E. coli* that have been described to adhere to the epithelia by alternative adherence systems (233) or other mechanisms altogether (443). Also of note, both P and type 1 fimbriae were not necessary for colonization of the human neurogenic bladder, indicating the need for alternative targets in certain high-risk patient groups (181).

1.4.7 Vaccines targeting proteins involved in iron acquisition

Iron is essential for nearly all organisms (112, 468) and UPEC encode a battery of genes whose products are involved in iron acquisition. Vaccination with UPEC OM protein (OMP) fractions that are enriched for iron receptors protects against experimental sepsis in both turkey and a murine models (41, 78). Additionally, mice vaccinated subcutaneously with denatured IroN, an OMP siderophore receptor and urovirulence factor (348), had both increased levels of antigen-specific serum IgG and significantly reduced kidney colonization upon challenge (349). Undetectable levels of IgA in the bladder mucosa may explain why IroN-vaccinated animals were not protected from cystitis (349). This study also demonstrated an important caveat in generating an effective vaccine for UTI: systemic and mucosal antibodies are necessary to protect both the bladder and kidney from infection, respectively (349). Chapter 2 describes a recent broad functional vaccinology initiative that was conducted using an “omics” approach to

identify *PASivE* vaccine candidates: UPEC proteins that are pathogen-specific, antigenic, surface-exposed, and *in vivo*-expressed (Table 1.2) (7, 380). Strikingly, the top targets identified by this approach were all OMPs functioning in iron uptake. Intranasal vaccination with three of six candidates afforded protection from cystitis and pyelonephritis (7), suggesting that combining antigenic motifs found in these proteins may be an effective multivalent vaccine for UTI.

1.4.8 Complex vaccines

Vaccines consisting of bacterial components or whole cells have also been assessed. Often, these types of vaccines result in adverse affects and non-protective immune responses (192). Nonetheless, they have been the focus of several studies over the last few decades. Transurethral immunization of mice with a live-attenuated UPEC strain lacking the ability to persist in the urinary tract resulted in heterologous protection (35), a potential platform for further development. In addition, SolcoUrovac (of the former Solco Basle Ltd./ICN Pharmaceuticals/Valeant Pharmaceuticals) is a whole-cell vaccine containing ten heat-killed uropathogenic strains, including six *E. coli*, and one each of *Proteus mirabilis*, *Morganella morganii*, *Enterococcus faecalis*, and *Klebsiella pneumoniae* (223). SolcoUrovac has been tested in mice (223, 432), non-human primates (430), and in clinical trials (126, 171, 174, 350, 426-429). Although SolcoUrovac showed efficacy in the kidneys in rats immunized intramuscularly and challenged by i.p. injection (222), the results of this study are non-translatable since there are clear toxicity concerns when delivering unknown levels of LPS systemically in humans. In response to this concern, SolcoUrovac was tested using a vaginal suppository in primates (430) and

Table 1.2 Screens used to identify *PASivE* UPEC vaccine candidates.

Criteria	Screen(s)	Reference
Pathogen-specific	Comparative genomic hybridization (CGH)	(248)
	In silico comparative genomics	(60)
	Southern blot	(137)
Antigenic	Two-dimensional SDS-PAGE and Western blot	(137)
	In vivo-induced antigen technology (IVIAT)	(P. Vigil *et al.*, unpublished)
Surface-exposed	*In silico* mining for genes containing predicted OM protein signatures (transmembrane domains, signal peptide cleavage sites, etc.)	(D. Rasko, unpublished)
	Identification of surface peptides by limited proteolysis and tandem mass spectrometry	(446)
in vivo Expressed	Gene expression microarray	(386)
	Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)	(8)
	IVIAT	(P. Vigil *et al.*, unpublished)
	Recombinant *in vivo* expression technology (RIVET)	(M. Walters, unpublished)
	Quantitative metabolomics	(162)
	Signature-tagged mutagenesis (STM)	(20)

in human clinical trials (426-429). While safe, vaginal vaccination with SolcoUrovac did not result in appreciable increases in local specific antibody (427, 429, 430), nor did it afford long-term protection levels in women with recurrent UTI (427, 429). Reasons for this result may include but not be limited to strain-specific (non-heterologous) immune responses, destruction of immunogenic antigens during vaccine preparation, and the lack of a mucosal adjuvant. Human leukocyte antigen (HLA) genes, encoding major histocompatibility (MHC) complexes present on the surface of immune cells and responsible for antigen presentation in humans, influence an individual's ability to recognize and respond to pathogens. SolcoUrovac was not efficacious in women with the HLA-DR2 phenotype (174), indicating that this or other UPEC vaccine formulations may not be efficacious in this patient population.

Uro-Vaxom (Om-89, OM Pharma) is a daily oral capsule containing a lyophilized mix of membrane proteins from eighteen *E. coli* strains (134, 409). The formulation elicits a number of immunological effects *in vitro* (359, 444, 458, 459) and generates specific antibodies in mice and humans (21, 71, 180, 367). As of 2002, twelve studies had been completed testing the efficacy of Uro-Vaxom (29), and in general, patients receiving Uro-Vaxom exhibited a reduced frequency of UTI than those receiving a placebo (28, 71, 134, 241, 251, 362, 409). Unfortunately, complications can occur due to toxicity, and the exact mechanism of action is difficult to delineate. The necessity of daily administration of Uro-Vaxom also presents supply and compliance issues.

1.5 Innate host defenses to UPEC-mediated UTI

1.5.1 Non-specific adherence prevention

Uroepithelial adherence is critical for establishment of UTI (451). UPEC strains possess an impressive repertoire of adhesins that enable them to aggregate and adhere to cell surfaces (160, 211, 314, 373, 439). Consequently, the first line of host defense against UTI is concentrated on preventing UPEC adherence to the bladder mucosa. The luminal surface of the bladder is lined with highly sulfated and anionic glycosaminoglycans that contribute to bladder wall impermeability and afford an antimicrobial anti-adherence property (183, 245, 307, 308, 310, 377). Intuitively, urine flow seems to be a convenient defense mechanism; however, FimH binds to mannose moieties using "catch-bonds," interactions that are actually strengthened by the sheer stress induced by urine flow (413).

1.5.2 Urothelial cell apoptosis

More active mechanisms such as umbrella cell exfoliation (14, 82, 108, 262, 277, 278, 298) also function to remove adherent UPEC. Exfoliation occurs by an apoptosis-like mechanism that is promoted by FimH (216, 277). An *in vitro* investigation of the apoptotic cascades suggested that FimH acts like a tethered toxin, inducing cellular events consistent with activation of both extrinsic (death receptor) and intrinsic (mitochondrial) apoptotic pathways, with cross-talk between the two signaling cascades mediated by the pro-apoptotic Bid protein (215). UPEC-induced urothelial cell death correlates with increased bladder cell differentiation and is also dependent on expression of the uroplakin IIIa receptor, a terminal differentiation marker (281, 414). Nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) is a transcription factor known for induction of proinflammatory genes concomitant with anti-apoptotic

genetic programs (26, 447). UPEC, independent of type 1 fimbriae, is able to suppress NF-κB and thereby promotes host cell apoptosis (216). Given the role for apoptotic cell exfoliation in UPEC host defense, promoting this sloughing activity may appear counter-productive for the bacteria. Nonetheless, cellular apoptosis may be an acceptable side effect of inhibiting the proinflammatory gene expression and the ensuing cellular responses initiated by NF-κB.

1.5.3 Tamm-Horsfall protein (THP)

THP (*a.k.a.*, mucoprotein, uromucoid, uromodulin) was first described in the early 1950s as a high molecular weight protein present in human urine (408); its ability to bind *E. coli* type 1 fimbriae was not recognized until some thirty years later (299, 301, 306). THP was shown to be associated with (and thus secreted by) cells of the ascending limb of the loop of Henle and the distal convoluted tubule in the kidney (378). A detailed biochemical analysis revealed that soluble THP from both mouse and human urine was able to bind type 1 fimbriae by virtue of its mannose moieties, inhibiting fimbrial interaction with uroplakin Ia and Ib receptors (269, 302). THP was also shown to bind efficiently to S fimbriae (306). Definitive studies using $THP^{-/-}$ mice confirmed the ability to help control bacterial burdens in the lower urinary tract. Compared to wild type mice, bacteria were present for longer durations and in higher numbers in the urine of $THP^{-/-}$ mice, and at twenty-four hpi, bladder bacterial burdens were increased in $THP^{-/-}$ mice (27, 268). The use of lower inocula for infection exacerbated the phenotype (268), and there was no phenotype attributable to UPEC expressing P fimbriae (27, 268), further evidence implicating THP as a type 1 fimbriae-specific effector protein. THP also appears to act

as an innate-adaptive immunoregulatory molecule that can activate dendritic cells (DCs), as exemplified by surface marker expression, cytokine secretion, and the ability to stimulate T cells (353). Both the aberrant presence of the THP itself and the generation of anti-THP-specific antibodies have implicated THP in many urological diseases (99, 289, 353, 370). The connection between UTI-induced THP expression and THP-mediated renal disease is currently unexplored (109).

1.5.4 Innate recognition of UPEC

1.5.4.1 UPEC recognition by TLR4

The late Charlie Janeway originally described the concept of pathogen-associated molecular patterns (PAMPs) being recognized by pattern recognition receptors (PRR) on host cells (420). Upon successful adherence to the uroepithelium, PAMP recognition by toll-like receptors (TLRs) (190, 258) generates signaling cascades to direct innate and adaptive immune responses (75, 391). It has been known for over two decades that C3H/HeJ mice, harboring a mutation in the Toll/Interleukin (IL)-1 receptor (TIR) domain of TLR4 (319), cannot resolve UTI as efficiently as LPS-responsive C3H/HeN counterparts (405). In accordance, $TLR4^{-/-}$ mice had significantly higher bacterial burdens in their bladders compared to similarly-infected wild type mice (16). This clearance defect is the result of both insufficient downstream cytokine and chemokine production and neutrophil recruitment (139, 170, 312, 374). Data from mouse chimeras disclosed that TLR4 on both stromal and hematopoietic cells is critical for normal inflammatory responses and clearance of UPEC in the bladder (356) and kidney (312). The importance of TLR4 in mediating the immune response to UTI has also been

demonstrated by studies of human patients. Asymptomatic bacteriuria (ABU) is the presence of bacteria in urine specimens with no clinical presentation (symptoms) of acute UTI (271). Children with low TLR4 expression on their neutrophils display the ABU carrier state, lacking both inflammation and bacterial clearance (325). A similar response is exhibited by C3N/HeJ (LPS-non-responsive) mice following UPEC inoculation (324). While no mutations or polymorphisms of the TLR4 gene appeared to explain the expression defect in the affected children, there were alterations in the level of adaptor and regulatory protein expression that may affect TLR4 expression (325).

1.5.4.1.1 UPEC molecular ligands recognized by TLR4

TLR4-mediated signaling in the urinary tract does not appear to be the result of the archetypal interaction with LPS. Both the role of LPS in and the molecular trigger of TLR4 signaling by UPEC are topics of debate (19, 158, 357). Studies using the A498 human kidney cell line indicate that TLR4 signaling in response to UPEC requires P fimbriae and can be mediated independently of LPS (106, 156, 158). Mechanistic details regarding this phenomenon include P fimbriae binding to surface glycosphingolipids (GSLs) and subsequent release of the GSL membrane-anchoring domain, ceramide (95). Ceramide appears to act as a TLR4 agonist and the putative intermediate for TLR4 signaling initiated by P fimbriae (95). In contrast to LPS-independent signaling by P fimbriae, there appears to be a cooperative stimulation of TLR4 by LPS and type 1 fimbriae (157, 358). This cooperative stimulation directly correlates with the level of cluster of differentiation (CD)14 expression on bladder cells (357). CD14 is an accessory molecule required for optimal TLR4 signaling in response to LPS (266).

Immunohistochemical (IHC) analysis of human bladder biopsies revealed that CD14 expression is localized to the submucosa (158), suggesting that uroepithelial cells exposed to the lumen have little to no CD14 expression and therefore may not respond efficiently to LPS alone. These results support a role for both independent and cooperative TLR4 stimulation by UPEC fimbriae. Lastly, the FimH tip adhesin of type 1 fimbriae was recently shown to directly interact with TLR4, an additional means for LPS-independent stimulation by UPEC fimbriae (16, 275).

1.5.4.1.2 Signaling downstream of TLR4

Infection of knockout mice has revealed critical roles for myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM) in signaling for UPEC clearance (96). It is also apparent that different fimbrial types influence the corresponding downstream signaling pathways (96). Regardless of the fimbrial type involved in stimulation, all pathways involving these adaptor molecules result in activation of NF-κB and proinflammatory gene expression. Song and colleagues identified an accompanying proinflammatory bladder cell signaling pathway that is also dependent on TLR4, but results in a spike in intracellular calcium levels (394). This calcium spike leads to adenylyl cyclase 3-mediated increases in cAMP, protein kinase A (PKA) activation, phosphorylation of the cAMP response element–binding protein transcription factor (CREB), and proinflammatory gene expression (394). In response to UPEC inoculation, cytokine secretion by the CREB pathway occurs faster (one hour) than NF-κB

translocation to the nucleus (two hours) and can also be activated by TLR2 and TLR3 ligands (394).

1.5.4.2 UPEC recognition by other TLRs

Other TLR pathways have been implicated in host defense during UTI (Figure 1.5). TLR2$^{-/-}$ mice appear to respond normally to acute UTI (324). Conversely, TLR11$^{-/-}$ mice are more susceptible to UPEC kidney infection compared to wild type mice (469). The TLR11 ligand is a profilin-like molecule that was isolated from *Toxoplasma gondii* (463). While structurally-related proteins are present in other apicomplexan protozoa (463), a UPEC-encoded homolog has yet to be identified. The fact that there is a stop codon in the open reading frame of human genomic and cell line *tlr11* sequences may help explain acute and recurrent UTI susceptibility in humans (469). In contrast to the kidney-specific role for TLR11 during UTI (469), TLR5 appears to play a UPEC-recognition role in the bladder (10). TLR5 recognizes flagellin, the structural subunit of flagella (152), which are essential for UPEC motility in the urinary tract (230, 232, 454). Flagellar expression peaks at four to six hours post inoculation, coinciding with UPEC ascension of the ureters (230). At this time point, there is TLR5-dependent induction of inflammatory cytokines and chemokines (10). By day five post inoculation, TLR5$^{-/-}$ mice have increased inflammation and bacterial burdens compared to wild type controls (10), highlighting the importance of early recognition of UPEC by TLR5 to contain the infection.

1.5.4.3 Non-TLR UPEC recognition

Surface molecules other than TLRs are also involved in host-UPEC interactions. Upon

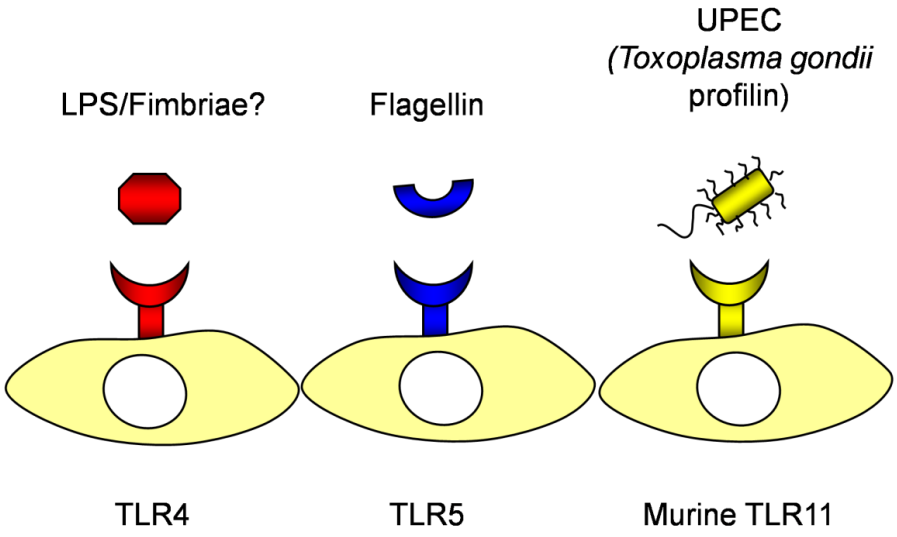

Figure 1.5 TLRs important in UTI host defense. Depicted are the three TLRs and their proposed or confirmed ligands have been definitively shown (through the use of knockout mice) to play a role in recognition of UPEC and host defense during UTI. Note that the UPEC-specific ligand for TLR11 is unknown.

UPEC exposure, the cytoplasmic tail of uroplakin IIIa undergoes phosphorylation and intracellular calcium levels increase, important events for uroepithelial cell apoptosis and exfoliation (415). Although uroplakin Ia is thought to be the main receptor for UPEC FimH *in vivo* (263, 412, 455), type 1 fimbriae may bind to a number of host molecules, including uroplakin complexes (415), extracellular matrix proteins (225, 320, 390), CD molecules (24, 115, 213), and integrins (87). The role of the CD44 receptor and its ligand, hyaluronic acid (HA) polysaccharide, in experimental UTI has been elucidated (342). Although UPEC themselves do not appear to express HA, they can bind it *in vitro* (342). Typically involved in cell-cell and cell-extracellular matrix interactions, HA accumulates in the urinary tract in response to infection, and likely facilitates the interaction between UPEC and CD44 (342). This interaction was shown to potentiate UPEC migration across the epithelium in a Transwell system and be important for murine kidney colonization (342). CD44 expression is ubiquitous throughout the uroepithelium and $CD44^{-/-}$ mice were more resistant to UTI, likely due to the inability of the bacteria to efficiently penetrate the kidneys (342). Neutrophil migration and cytokine secretion was found to be independent of CD44, indicating that this surface receptor likely does not play a role in innate immune recognition (342). However, because of its role in lymphocyte activation, it would be interesting to see if there are deficiencies in the adaptive immune response in $CD44^{-/-}$ mice. Lastly, there are still unidentified players in inflammation and clearance of UPEC. For example, LPS-responsive C3H/OuJ mice were found to be equally susceptible to UTI as non-LPS responsive C3H/HeJ mice, yet demonstrated elevated levels of inflammation (170), revealing a susceptibility locus to map.

1.5.4.4 UPEC defenses against host recognition

UPEC has evolved mechanisms to counter host recognition and signaling. Clinical UPEC isolates encode the gene for TcpC, which has structural homology to the TIR domain of human TLR1 and binds to MyD88, thereby inhibiting cytokine responses (64). While other *sequenced* genomes of UPEC and ExPEC model strains do not appear to encode the *tcpC* gene (34), a molecular epidemiology survey revealed the presence of the gene in *clinical* isolates is associated with severity of UTI; that is, 40% of pyelonephritis isolates encoded homologous genes as compared to only 21% cystitis, 16% ABU, and 8% commensal (64). TcpC-mediated interference with MyD88 signaling is an immune evasion strategy particular to acute pathogens; targeting this major signaling "hub" rapidly deteriorates innate immune responses (48). UPEC also encodes genes that modify bacterial proteins to evade detection. Billips and colleagues noted that a type 1-fimbriated K12 strain elicited more robust cytokine secretion from cultured urothelial cells than UPEC strains (33). The authors used a genetic screen to isolate bacterial mutants that resulted in enhanced secretion of IL-8 from bladder epithelial cell lines (34). They identified a peptidoglycan permease (*ampG*) and an O-antigen ligase gene (*waaL*) – enzymes that modify PRR targets – responsible for the dulled cytokine secretion in response to uropathogenic strains (34). A similar screen also identified the *rfa*/*rfb* operons and *surA*, encoding genes important for LPS biosynthesis and OMP biogenesis, respectively (182). These results suggest that UPEC utilizes gene products that modify bacterial membrane (especially LPS) to evade immune recognition and highlight the potential importance of TLR stimulation involving fimbriae and other organelles.

1.5.5 Host iron sequestration and active inhibition of UPEC iron acquisition

Considering the essential role of iron in both host and pathogen cellular processes, there are remarkably low concentrations of free iron in mammalian hosts. This is not only a mechanism to avoid iron toxicity; it is a way to prevent the growth and establishment of infection by invading pathogens. Indeed, as early as the 19th century it was noticed that supplementing tuberculosis sufferers with iron worsened the outcome of disease, a notion that was later confirmed in experimental animal models (329).

That *E. coli* strains causing UTI have several functionally redundant systems dedicated to iron uptake (50, 60, 450) suggests that the urinary tract, like other host niches, is an iron-limited environment requiring dedicated acquisition systems for survival (25). For instance, enterobactin, a siderophore encoded by UPEC, can bind free ferric ions with a higher affinity than transferrin (93), a host iron transport protein responsible for regulating the free iron concentration in serum (330, 445). A transferrin family member, lactoferrin, evokes antimicrobial activity by sequestering iron over a range of pH (445). Lactoferrin is secreted by kidney cells (3) and found in other bodily secretions and neutrophil granules (69), thus could be involved in combating UTI. Both transferrin and lactoferrin have been shown to mediate direct antimicrobial activity by disrupting Gram-negative bacterial membranes (83, 124).

In addition to iron sequestration, there are host factors that directly counter the action of siderophores. Early studies indicated that serum albumin, alone or in concert with other serum proteins, can impede bacterial siderophore function (218). In addition, the 24 kDa mammalian protein lipocalin 2 (Lcn2) can bind and sequester enterobactin and similar catecholate siderophores (93, 97, 120, 168). Accordingly, Lcn2 inhibits

enterobactin-dependent propagation of *E. coli in vitro*, and Lcn2$^{-/-}$ mice are unable to control systemic *E. coli* burdens as well as wild type mice (97). Production of Lcn2 is induced by ligation of TLR4, implicating iron regulation as a part of the immune response to infection (97). Murine GeneChip and quantitative reverse transcriptase (RT) polymerase chain reaction (PCR) (qPCR) analyses confirmed that Lcn2 mRNA is upregulated by the uroepithelium of infected mice (334). Interestingly, these results were obtained in C3H/HeJ mice, indicating a TLR4-independent signaling pathway can activate transcription of *lcn2* in response to UTI. Not surprisingly, UPEC has evolved a mechanism to counter Lcn2 siderophore sequestration. Encoded within the *iroA* gene cluster are glycosyltransferases that modify enterobactin in such a way that it cannot be bound by Lcn2 (Figure 1.6) (38, 94, 384). Thus, both the host and UPEC have systems in place to manage their own iron stores and to inhibit iron acquisition by the other – a molecular arms race for an essential nutrient.

1.5.6 Antimicrobial peptide (AMP) secretion

AMPs are short [20-60 amino acid (aa)] positively charged peptides secreted by both epithelial and hematopoietic cells that disrupt bacterial membranes and can be chemotactic for certain immune cells (395, 466, 467). Human β- defensin-1 mRNA and protein was found in kidney tissue, implicating this AMP in host defense against UPEC (440). More convincingly, mice deficient in *defb1*, a murine homolog of human β-defensin, have a significantly higher incidence of bacteriuria (274). Murine β-defensin is also a DC ligand that instigates upregulation of costimulatory molecules and maturation

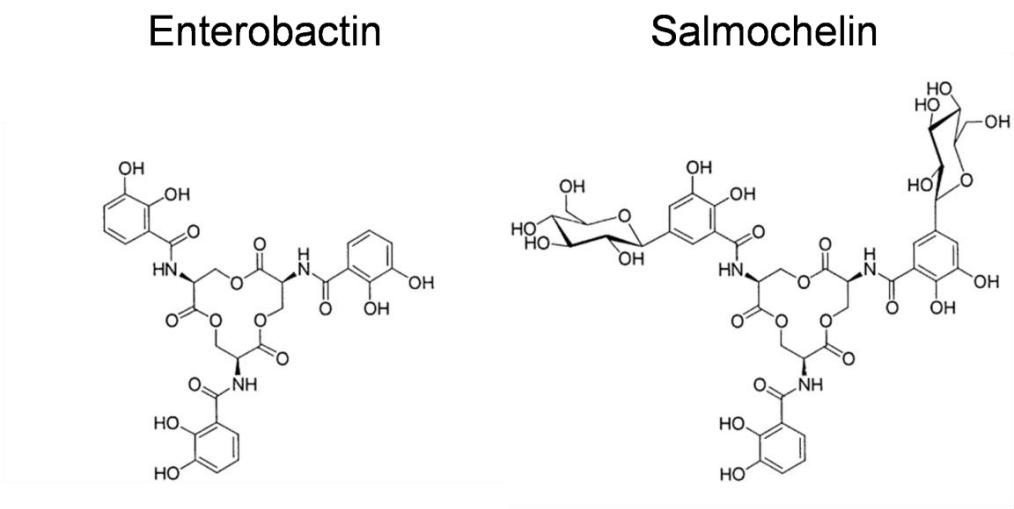

Figure 1.6 Chemical structures of enterobactin and one member of the salmochelin family. Adapted from (38).

(36). Alpha-defensins are specific to neutrophil granules, and in some cases other innate acting leukocytes (369, 467); however, murine neutrophils lack α-defensin (80, 369), thus the role, if any, of this AMP in UTI is not clear. The human cathelicidin, LL-37, and its murine homolog, cathelin-related AMP (CRAMP), are secreted in response to UPEC exposure (63). Studies using CRAMP-mutant mice revealed that epithelial-derived CRAMP is important during the early stages of UTI, while leukocyte-derived CRAMP likely functions later when bacteria penetrate the kidney epithelium (63).

1.5.7 Cytokine and chemokine secretion

1.5.7.1 Neutrophil-associated cytokine ligands and receptors

Human C-X-C ligand 8 (hCXCL8, IL-8) is the main chemoattractant for neutrophils in humans, and mCXCL1 and mCXCL2 [also known as keratinocyte-derived chemokine (KC) and macrophage inflammatory protein (MIP)-2, respectively] are the functional mouse homologs of IL-8 (189). Bladder and kidney cell lines secrete IL-8 in response to UPEC (154, 358, 456). Human and murine studies demonstrated that neutrophil migration to the UPEC-infected urinary tract is dependent on IL-8 (4, 5, 144, 265). Additionally, mCXCL2 secretion is dependent on TLR4, as it was deficient in infected C3H/HeJ mice (147). hCXCR1 and hCXCR2 are receptors for a number of chemokines, including IL-8 (189). Both are expressed in bladder and kidney biopsies, and transmigration studies indicated that hCXCR1 plays a dominant role in IL-8-dependent neutrophil migration (118). Consistent with this, children prone to pyelonephritis tend to have low hCXCR1 expression and heterozygous hCXCR1 polymorphisms (105, 324). hCXCR1 deficiency results in impaired bacterial clearance,

but unlike TLR4 deficiency, with intact inflammatory signaling that ultimately results in tissue damage (Figure 1.7) (324). Similarly, mice lacking mCXCR2 (the functional homolog for hCXCL1) experience subepithelial accumulation of neutrophils, increased bacterial titers, and renal scarring after UPEC inoculation (105, 118, 143). These data indicate that normal function of neutrophils, their chemotactic ligands, and their chemokine receptors are required for bacterial clearance without post-inflammatory sequelae.

1.5.7.2 Secretion of other cytokines and chemokines in response to UPEC

Despite ample information on IL-8 *in vitro* and *in vivo*, a complete picture of the cytokine and chemokine dynamics during UTI was lacking. In response, a longitudinal assessment using a Bio-Plex format was conducted by Ingersoll and colleagues (186). Chemokine (C-C motif) ligand 2 (CCL2) [macrophage chemotactic protein (MCP)-1], CCL4 [macrophage inflammatory protein (MIP)-1b], CCL5 [regulated on activation normal T-expressed and secreted (RANTES)], CXCL1, IL-1β, IL-6, IL-12p40, IL-17, tumor necrosis factor-α (TNF-α), and granulocyte-colony stimulating factor [G-CSF, colony-stimulating factor 3 (csf3)] were all upregulated in bladder homogenates from UPEC-infected C57BL/6 mice when compared to phosphate-buffered saline (PBS)-treated counterparts (186). These results agreed with patient and cell line data regarding upregulation of IL-6 in response to UPEC (155, 156). In mice, TNF-α expression was elevated at one hour post inoculation for rapid mobilization of acute responses (186); this waned at later time points which likely prevents deleterious effects of uncontrolled TNF

	$TLR4^{+/+}$ $mCXCR2^{+/+}$	$TLR4^{-/-}$ $mCXCR2^{+/+}$	$TLR4^{+/+}$ $mCXCR2^{-/-}$
			
Chemokines	+++	–	++
Chemokine receptors	+++	+++	–
Neutrophil recruitment	+++	–	Slow
Bacterial clearance	YES	Delayed	Delayed
Symptoms	Transient mild disease	Asymptomatic carriage	Acute pyelonephritis with bacteremia
Tissue damage	NO	NO	YES, renal scarring

Figure 1.7 Genetic susceptibility of mice lacking TLR4 or CXCR2. In both cases, bacterial clearance is delayed; however, the lack of inflammatory signaling in mice lacking TLR4 results in asymptomatic carriage whereas the lack of a neutrophilic chemokine receptor results in dysregulated inflammatory and sequelae. From (324).

-α signaling (22). Generally speaking, expression of most cytokines and chemokines weeks (186). These dynamics correlated well with the peak and resolution of bacterial burdens (186).

Similar to TLR adaptor molecule usage (96), the type of fimbriae expressed also seems to influence the repertoire of chemokines secreted. Specifically, kidney cells exposed to type 1 fimbriated-UPEC secrete neutrophil-associated chemokines, while P fimbriae-stimulated cells secrete chemokines targeting antigen presenting cell (APC)- and Th1-specific cytokines, exemplified by CCL2 and CCL5 expression (119). In addition, IFN-γ and IL-4 (signature cytokines of the Th1 and Th2 lineages, respectively) and IL-10 [a T regulatory cell (Treg) effector cytokine] knockout mice were tested for susceptibility to both acute cystitis and pyelonephritis (198). While IL-$4^{-/-}$ and IL-$10^{-/-}$ mice appear to experience infection dynamics similar to wild type, IFN-$\gamma^{-/-}$ mice had increased incidence and severity of UTI (198), implying a role for IFN-γ and Th1-mediated inflammatory responses to UTI.

1.5.7.3 IL-17 and host defense against microbes

One notable exception in the aforementioned study by Ingersoll and colleagues was IL-17A (also referred to as IL-17), which was highly upregulated from six hours to one week post inoculation, and remained above baseline through the two-week experimental duration (186). Aside from the canonical $CD4^+$ Th17 cells, other cell types have been found to secrete IL-17A, including cytotoxic T cells, γδ T cells, natural killer (NK)T cells, neutrophils, eosinophils, and monocytes (32). IL-17A or the Il-17 receptor (IL-17R) has been shown to play a critical role in autoimmune disease (32, 261) and in

bacterial (142, 187, 363, 376, 438, 465, 471), fungal (68, 178, 343, 442), and even viral (141, 177, 209) infection. Several studies have also demonstrated a role for IL-17A in adaptive immune responses (165, 250, 285, 286, 438). Because of the roles played in both arms of the immune system, IL-17A has emerged as an innate-adaptive immunomodulatory cytokine.

IL-17A plays a role in the innate immune response to infection by enhancing neutrophil migration to infected tissue. Specifically, IL-17A acts indirectly by stabilizing mRNA transcripts for cytokines involved in granulopoiesis and chemotaxis (148-150, 163). Although the exact PAMP that triggers secretion of IL-17A is not defined, components of microbial cell wall, host receptors that recognize such components (*i.e.* the mannose receptor), and TLR-related signaling pathways are all implicated (442, 471). NF-κB activation by IL-17A has been documented (150, 375, 462), as has a synergistic upregulation of cytokine expression by IL-17A and TNF-α or IL-1β (12, 148-150, 163, 375). This upregulation appears to be mediated by the activity of mitogen-activated kinases (MAPKs), namely p38 and extracellular signal-related kinase (ERK) (149, 150, 163); however, it seems that MAPK effects may be specific to the activation of NF-κB, while IL-17A effects on mRNA stability may be independent of MAPK signaling (149). IL-17A-specific signaling from the IL-17 receptor (IL-17R) was shown to be dependent on the adaptor protein NF-κB activator 1 (Act1) (58, 148, 243). Pathogen interference with this pathway has already been described (452), reflecting the importance of IL-17A signaling in host defense. Binding sites for the NF-κB, CCAATT enhancer-binding protein (C/EBP), activator protein-1 (AP-1), and octamer transcription factor (OCT1)

were overrepresented in IL-17 target promoters (375), indicating that these transcriptions factors likely control downstream gene expression initiated by IL-17A signaling.

1.6 Host factors involved in intracellular UPEC

1.6.1 Intracellular UPEC and persistence

Over the past twelve years there has been a growing body of literature revealing that UPEC, in addition to its extracellular luminal or adherent lifestyle, appears to have three distinct intracellular lifestyle components within the urinary tract (88). The first is uptake by apical endocytosis of $Rab27b^{+}/CD63^{+}$ fusiform vesicles which are subsequently recycled back to the cell surface and exocytosed (37). The other two pathways both begin with uptake into a membrane-bound compartment which can lead to either a quiescent non-replicative existence (89, 280) or escape from compartmental life to undergo a highly replicative phase in the cell cytoplasm (200, 278). While internalization via the fusiform vesicle pathway may be a side effect of normal bladder function, the latter pathways are perhaps intentional processes undertaken by UPEC to establish a persistent reservoir (89, 200, 212, 278, 280, 355). Indeed, UPEC have been shown to exist in the urinary tract for weeks following infection (278, 355), even after antibiotic treatment (212).

After umbrella cells exfoliate in response to UTI, intracellular infection of the less differentiated underlying cells is suggested to lead to formation of antibiotic-resistant "quiescent intracellular reservoirs" (QIRs) (200). Induction of urothelial renewal may lead to reinfection by UPEC contained in QIRs (282); specifically, cell proliferation and terminal differentiation may cause bacterial excision from the QIR, possibly in response

to the drastic changes in host cell actin (89, 212, 280). As an argument against a fecal reservoir, housing naïve mice with infected ones did result in transmission of UPEC to uninfected mouse bladders – persistent colonization of the bladder was only seen after prior UTI, although transmission of fecal positivity to uninfected animals was noted (likely through the practice of coprophagia) (355). Finally, infection of ten genetically distinct mouse strains also revealed that some strains were more susceptible to persistence than others, indicating that host hereditary components may also contribute to the ability of UPEC to persevere in the urinary tract (172).

1.6.2 *In vivo* characterization of intracellular UPEC

Infected mouse bladder explants monitored by time-lapse fluorescence videomicroscopy generated a model for the intracellular UPEC life cycle instigated after uptake in a membrane-bound compartment (the non-fusiform vesicle route) (200). While the mechanism of compartmental escape remains undefined, once contained in cytoplasmic "intracellular bacterial communities" (IBCs), UPEC can undergo several changes in morphology categorized as early, middle ("pod"), and late IBC stages (11, 200). In the pods, immunofluorescence microscopy showed expression of antigen 43 and type 1 fimbriae, and use of the periodic acid-Schiff reagent indicated the presence of polysaccharides, suggesting a biofilm-like state (11). Late IBCs that escape exfoliation with umbrella cells contain filamentous UPEC that are not present in C3H/HeJ mice, indicating that this morphological change may be a bacterial stress response to TLR4-mediated immune activation (200, 278). This murine background also experienced increased incidence and severity of IBCs compared to immmunocompetent mice (11,

114, 200). Although kinetics and morphology differences were noted, IBCs were formed by different UPEC patient isolates in several additional murine backgrounds, including C57BL/6J, CBA/J, and FVB/NJ (114). Images resembling findings in murine studies have been shown in the urine of females suffering from UTI (341); however, all the data validating IBCs involves microscopy and therefore must be interpreted with caution. Urothelial cells proximal to IBCs in C3H/HeJ mice upregulate transferrin receptor, Lcn2, complement system components (C3, factor B, CD55), and lysozyme (334). Involucrin and suprabasin transcripts were also increased indicating that, in addition to gene products that function to eradicate bacteria, proteins important for epithelial integrity may be an imperative host response in response to intracellular UPEC (334).

1.6.3 *In vitro* characterization of intracellular UPEC

In vitro treatment of either 5637 cells with a small amount of the detergent saponin (89) or immortalized pediatric bladder cells with the cholesterol-sequestering drug filipin (31) recapitulates some of the observed features of intracellular UPEC *in vivo*. Additionally, much work has been done using the 5637 bladder epithelial cell line to further delineate molecular components and mechanisms surrounding UPEC intracellularity (37, 77, 86, 87, 89, 253, 254, 278, 358). UPEC internalization does not require bacterial viability (358), but is dependent on FimH (254). β1 and α3 integrins were shown to be receptors for Fim-mediated UPEC internalization, mediated by signaling through focal adhesion kinase (FAK) and, in contrast to an earlier study, Src family kinases (87, 254). FimH-dependent uptake requires microtubules, histone deacetylase 6 (HDAC6), the kinesin-1 light chain, and aurora A kinase (73). In addition

to the involvement of cytoskeletal proteins, tyrosine kinases, and phosphoinositide 3-kinase (PI3K) (254), UPEC engulfment has also been reported to be cholesterol- and dynamin-dependent and modulated by calcium levels, clathrin, and clathrin adaptors (86). Additional work on dynamin revealed that the nitric oxide synthase (NOS) enzyme is responsible for chemically modifying dynamin, redistributing it to the membrane for bacterial internalization (448). As hinted by the cholesterol dependence, UPEC internalization is often reported to be associated with lipid rafts (24, 204), dependent on caveolin-1 and Rho-family GTP binding proteins (77, 253). The association with lipid rafts was confirmed *in vivo;* UPEC inoculation in the presence of a lipid raft-disrupting chemical decreased the number of intracellular bacteria in the murine bladder (77).

Notably, TLR4 also plays a non-inflammatory role in host defense against UPEC by modulating the activity of the observed secretory and vesicular internalization pathways. TLR4-mediated PKA activation suppresses the lipid raft endocytic pathway (392), a possible effort to prevent the establishment of persistence reservoirs. Also along these lines, UPEC exocytosis in fusiform vesicles was actually accelerated by TLR4-mediated recognition of LPS and dependent on the activities of cAMP, Rab27b, caveolin-1, and the scaffolding protein MyRIP (393).

1.7 Host response to UPEC-mediated UTI by innate inflammatory cell types

1.7.1 Neutrophils

Infected mouse bladders examined histologically display thickening of epithelium accompanied by robust infiltration of inflammatory cells and edema in the lamina propria (Figure 1.2C and D) (191). Neutrophils are the most rapid and abundant responders to

the infected urinary tract (5, 147, 191, 374). Infected individuals often experience an inflammatory response involving pyuria, or the presence of white blood cells in the urine. Since greater than 95% of the cells present in urine are neutrophils (374), counting urinary neutrophils is a quick measure of the innate immune response in mice and an indicator of UTI in humans. Antibody-mediated knockdown of the neutrophil population revealed their crucial role in bacterial clearance during UTI, especially within the kidney (147). There does not appear to be a resident population of neutrophils in the bladder (167, 356), likely due to the short-lived and tissue-damaging nature of these cells (383). Efficient migration of neutrophils requires intracellular adhesion molecule-1 (ICAM-1) expression by epithelial cells and β2 integrin (CD11b/CD18) expression by neutrophils (4, 356). G-CSF is also required for the neutrophil response, and unexpectedly, mice with neutralized G-CSF are more *resistant* to UTI (186). Although monocyte/macrophage numbers were similar in anti-G-CSF-treated and wild type mice, cytokines important for macrophage activation were upregulated in the knockdown animals, potentially leading to accelerated clearance by enhanced phagocytic killing (186). Of note, the electrostatic properties of the P fimbrial tip adhesin may interfere with neutrophil binding, allowing UPEC to evade neutrophil killing (40, 410).

1.7.2 Macrophages and DCs

Compared to the neutrophil response, relatively little is known about APCs in the context of UTI. In mice, resident $CD11c^+$ cells that express low to intermediate levels of F4/80 and CD11b macrophage markers were found in the kidney (221), while $CD11c^+$ cells expressing the MHC Class II activation marker were found in the bladder (167,

356). In spite of macrophage marker expression, $CD11c^+$ kidney cells had physical and functional characteristics of DCs (221). At twenty-four hours post inoculation, $CD11c^+$ cells that migrate to the bladder did not express CD8α, Gr-1, or B220, thus were not plasmacytoid or lymphoid, but appeared to be $CD11b^{intermediate}$ TNF-α- and iNOS-producing (Tip)-DCs (84). Infection studies in mice lacking Tip-DCs suggested that they are not necessary for the host response to acute UTI (84). Since Tip-DCs are necessary for the generation of mucosal IgA (411), their role may lie in mediating the humoral response to UPEC. Similar to what was observed for DCs, there appears to be a resident population of macrophages in bladder tissue that increase by several orders of magnitude in response to UTI (85, 167, 186). $Gr\text{-}1^{high}$ monocytes, a population of cells that include inflammatory monocytes (IMs) (17, 371) and can give rise to macrophages or DCs, are also recruited to the bladder in response to UPEC infection. Release of these cells from the bone marrow was dependent on CCR2 (85), and correspondingly CCL2 is upregulated in the bladder response to UTI (186).

1.7.3 Neutrophil and DC-associated factors

Some of the factors utilized by neutrophils, macrophages, and DCs for pathogen uptake and destruction have been described during UTI. iNOS generates the antimicrobial compound nitric oxide (NO) from L-arginine, and was originally reported to be secreted by macrophages (164, 287, 401). Although iNOS is rapidly upregulated in the inoculated bladder (282), $iNOS^{-/-}$ mice are equally as susceptible to UTI as wild type mice (198, 318). Neuronal NOS, endothelial NOS, or myeloperoxidase (MPO) may compensate for the lack of iNOS in these mice (203, 318). Alternatively or in addition,

iNOS $^{-/-}$animals may lack a colonization phenotype because there are several factors (Hfq and Nsr-regulated genes, polyamines, flavohemoglobin) expressed by UPEC that enhance tolerance to reactive nitrogen species *in vitro* (43, 44, 226, 406), suggesting NO production may be an ineffective host defense against UPEC. With respect to the complement system, it appears that UPEC is able to bind C3 to enter host uroepithelial cells via the surface receptors Crry or CD46 (242, 396). Correspondingly, C3$^{-/-}$ mice are more resistant to renal damage and infection (396). As C3 levels are significantly higher in the urine of UTI patients (242), UPEC may stimulate C3 production for pathogenic means, or at least evolved to exploit this host defense factor.

1.8 Innate-like lymphocytes (ILLs) in the innate immune response to UPEC-mediated UTI

Infection studies using severe combined immunodeficient (SCID) mice that lack functional B and T cells and nude mice that lack thymically-derived T cells provide preliminary evidence of a role for ILLs in acute UTI host defense (175). Epithelial γδ T cells, B-1 cells, and NKT cells are ILLs: cellular subsets that have relatively invariant receptors and reside in specific locations of the body (189). After a two-day primary infection, SCID mice had significantly higher bacterial counts in their bladder and kidneys, while nude mice were colonized similarly to wild type animals (175). The lack of a colonization phenotype in nude mice suggests that either antibody responses independent of thymus-derived T cell help or extrathymically-produced T cells may play a role in innate clearance of UPEC. The latter suggestion has some experimental support. γδ T cells can be produced extrathymically and rapidly secrete cytokines in response to stimulation (2, 23, 55, 62). In Chapter 3, we confirm that resident γδ T cells found in the

bladder increase in response to UTI (255), and T cell receptor (TCR) $\delta^{-/-}$ mice lacking expression of γδ T cells are more susceptible to UTI than isogenic controls (198). We go on to demonstrate that γδ TCR^+ cells express IL-17A during UPEC-mediated UTI, a rapid-response by γδ T cells that may function in concert with other innate factors to mediate neutrophil influx for clearance of UPEC. B-1 cells spontaneously secrete large quantities of polyspecific IgM against bacterial and self antigens, and in contrast to conventional (B-2) B cells, do not require T cell help (30). While IgM secreted by B-1 cells might play a role in innate clearance of UPEC, current evidence suggests otherwise. J_HD mice, lacking both B-1 and B-2 cells (59, 327), infected and monitored over a fourteen-day time period exhibited no significant increases in incidence or severity of cystitis (198). On a final note regarding ILLs, administration of α-GalCer, a ligand for CD1d-restricted NKT cells, alleviates renal UPEC infection (264). Consistent with this, we have observed a resident population of $NK1.1^+$ cells (potentially NK or NKT cells) in the bladder of C57BL/6 mice that increases in response to UTI. Studies using a systemic *E. coli* infection model suggested that, similar to γδ T cells, NKT cells may act as early amplifiers of the innate immune response to UTI by rapid cytokine secretion (283).

1.9 Adaptive host defenses to UPEC-mediated UTI

Existing data regarding adaptive immune responses to UPEC are relatively limited. In a seminal study, Thumbikat and colleagues engineered a strain of UPEC to express ovalbumin (OVA) to examine mechanisms behind antigen-specific adaptive immune responses during experimental UTI (416). In response to reinfection, $CD4^+$ and $CD8^+$ cells infiltrated the bladder and expressed the CD69 activation marker in the spleen

(416), extending the findings of early IHC studies probing T and B cell populations in infected bladders (167). Furthermore, splenocytes, enriched splenic T cells, or serum antibodies from previously infected donor mice each protected wild type naïve recipient mice against UPEC challenge (Figure 1.8) (416). This result suggests that protection derived from natural infection is antibody-mediated, as UPEC-specific antibody-secreting plasma cells could be present in both splenocyte and enriched T cells preparations. As expected, transfers from naïve donor mice did not facilitate enhanced protection to recipients (416). This result is in contrast to a previous murine adoptive transfer study where SCID recipients receiving splenocytes from either naïve or vaccinated wild type donors exhibited equal levels of enhanced clearance, despite the presence of antigen-specific plasma cells in the vaccinated donor cells (175). This result suggests that simply reconstituting immunosuppressed mice with lymphoid cells provides the means (likely stimulatory cytokines for phagocytic cells) for enhanced clearance. Conversely, wild type recipient mice used in the former study only exhibited enhanced clearance when given cells or serum from antigen-educated vaccinated donors (416), indicating that enhanced protection in individuals with intact immune systems will only be provided by stimulation of an effective adaptive immune response.

1.9.1 T cells and the adaptive immune response to UPEC-mediated UTI

T helper (Th) cells are a key component in adaptive immunity and the generation of protective responses involving antibodies. Th cell subsets are characterized by transcription factors and cytokines involved in their differentiation and the particular effector cytokines they secrete (Figure 1.9). In the past decade, the Th1 and Th2

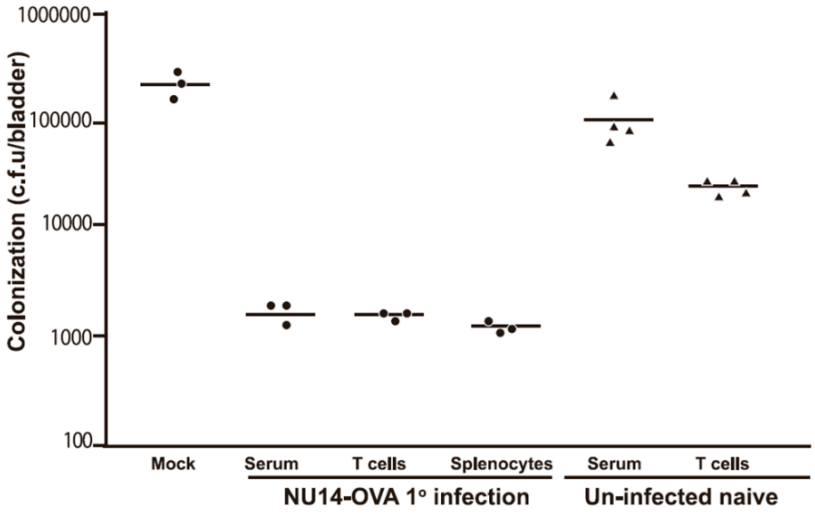

Figure 1.8 Passive and adoptive transfer of protection from mice vaccinated via the natural route of infection. Naive mice received adoptive transfer of 5 x 10^6 T cells, 1 x 10^7 splenocytes, or 200 µl of whole serum, and were then challenged with NU14-OVA, a UPEC strain expressing ovalbumin, followed by plating of bladder homogenates twenty-four hours later. Donor cells and serum were derived from mice that were infected with NU14-OVA two weeks previously (1°), or that were unexposed. Horizontal bars indicate the mean. From (416).

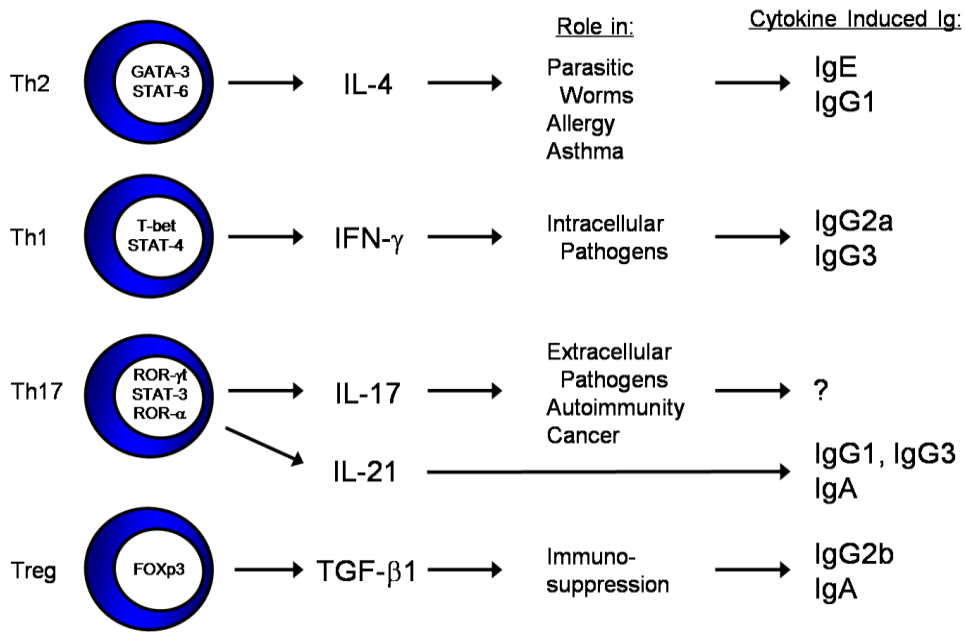

Figure 1.9 Summary of Th cell attributes. Depicted are the four major classes of Th cells, transcription factors responsible for their development, cytokines they secrete, and their effector functions. Also shown are the antibody isotypes secreted by B cells that have received T cell help from the corresponding Th cell subclass.

dichotomy of helper T cell differentiation has been modified by the discovery of other Th cell subgroups, namely Th17 and Treg cells. While IFN-γ, IL-4, and TGF-β are the signature cytokines secreted by Th1, Th2, and Treg cells, respectively, Th17 cells are characterized, among other factors, by the ability to secrete IL-21, IL-22, IL-17A (the signature cytokine), and IL-17F (61). Additionally, RORγt, RORα, and STAT3 are the transcriptional factors responsible for development and regulation of the Th17 lineage (220), whereas development of the inducible Treg, Th1, and Th2 lineages are primarily controlled by FoxP3, T-bet, and GATA3, respectively, among the activity of additional transcription factors (32).

To date, studies have not implicated a skew toward Th1- or Th2-mediated UTI immunity (7, 416). DC phagocytosis of infected apoptotic cells is the key event required for DCs to secrete the unusual cytokine milieu (predominately accepted to be TNF-β and IL-6) necessary for Th17 development (418). As previously mentioned, a number of DC subsets and UPEC-infected apoptotic cells are present in bladder tissue during UTI. Similar to other cell populations, we and others have observed a resident $CD8^+$ cell population in the bladder that increases in response to infection (416). We can speculate that these are classical cytotoxic T cells or an intraepithelial lymphocyte (IEL) population that may exert cytotoxic effects upon UPEC- or virus-infected cells or rapidly secrete cytokines to mobilize the innate immune response (1, 153). Finally, the role of Treg subsets in UTI host defense has not been formally examined.

1.9.2 Humoral adaptive immune responses to UPEC-mediated UTI

Despite the lack of detail regarding T cell responses to UTI, there is ample evidence for antibody-mediated clearance of UPEC. Since the 1970s, the genitourinary tract has been recognized as part of the secretory immune system (111, 417). UPEC-specific antibodies are detected in the urine of infected patients (313) and in the urine or serum of animals exposed to UPEC antigens (176, 339, 416, 424). Urinary IgG and IgA from UTI patients are capable of inhibiting UPEC adherence (79, 403, 422). Patient studies have also suggested that antibody responses to pyelonephritis are, in general, stronger and last longer than humoral responses to cystitis (107, 205, 206). In a study involving experimental UTI using ten different inbred mouse strains, initial infection led to an innate response marked by inflammation, followed by an antibody response to the infecting organism (172). There was a modest correlation between bacterial colonization and inflammation; however, and a positive correlation between splenic antibody-forming cells and bladder and kidney infection levels was detected (172). In Chapter 2, we describe analysis of murine urine and serum samples collected before and after vaccination with OMP iron receptors, allowing identification of immunological correlates of mucosal vaccine-induced protection against UTI (7). Specifically, levels of either urinary IgA or serum IgG (relative to serum IgM, denoted the "class switch index") inversely correlated with bladder colonization in vaccinated mice (7). Presumably urinary IgA plays a direct role in UPEC clearance from the bladder mucosa, while IgG may be a marker for class switching by B cells or also play a direct role in mucosal bacterial clearance. That stated, as previously mentioned, analysis of bacterial burdens in J_HD mice, which cannot produce antibodies (59), suggested no role for the humoral response in response to acute UTI (198). However, this result is not unexpected since

antibody-mediated protection by B-2 cells (canonical B cells) would likely play a role in memory responses, beckoning re-evaluation of these mice in UPEC-reinfection and vaccination-challenge models.

1.10 Urothelial regeneration in response to UPEC infection

One of the consequences of UPEC infection is exfoliation of the superficial facet cell layer that lines the surface of the bladder lumen (14, 82, 108, 262, 277, 278, 298). At this juncture, the host not only needs to rid the invading bacterial onslaught, but repair the damaged uroepithelium, as the impermeability of this barrier is crucial for protection from toxic substances and pHs found in urine. Mysorekar and colleagues have conducted a number of studies to understand the molecular events and players involved in urothelial regeneration (281, 282). Microarray analysis revealed that genes involved in cell differentiation, proliferative and immediate-early responses, apoptosis, stress response, signal transduction, and cell-cell contact are generally upregulated in response to UPEC infection, as compared to mock infection or bladders experiencing cell non-inflammatory exfoliation by chemical treatment (281, 282). Importantly, cytokines, chemokines, signaling molecules, and transcription factors involved in inflammation are highly upregulated and may also contribute to uroepithelial regeneration (281, 282). Regeneration itself appears to be a function of uroepithelial stem cells present in the basal and mesenchymal layers of the uroepithelium, and depends on downstream signaling mediated by downregulation of bone morphogenic protein 4 (Bmp4) though the Bmp4 receptor 1a (281). Studies of the gut epithelium unveiled macrophages act as "cellular transceivers" that relay MyD88-dependent inputs from the epithelium to colonic

epithelial progenitors via direct contact (323). Whether or not macrophages play a similar role in the urinary tract remains unknown.

1.11 Summary

From epidemiology studies delineating risk factors to cellular molecular studies concretely characterizing roles of particular immunological facets in UTI, research in the field is critical to ameliorate the societal and economic costs associated with this disease of morbidity. While many general concepts regarding the immune response to UTI are understood, molecular details of these responses are only beginning to be discovered and appreciated. Such details are likely going to be the key in the development of a successful vaccine for use in humans or more effective and directed treatments in lieu of antibiotic therapy. The results presented in this dissertation add to the current understand of both the innate and the adaptive immune response to UTI. Regarding adaptive immune mechanisms playing a role in protection against UPEC-mediated UTI, potential vaccine candidates and their corresponding immunological correlates of protection are tested and determined, respectively, in Chapter 2. In Chapter 3, the role of the innate-adaptive immunomodulatory cytokine IL-17A is defined in the context of both primary and secondary UTI. Speculations about and future directions stemming from these findings are given in Chapter 4.

Chapter 2
Mucosal immunization with iron receptor antigens protects against UTI

2.1 Abstract

Uncomplicated infections of the urinary tract, caused by UPEC, are among the most common diseases requiring medical intervention. A preventive vaccine to reduce the morbidity and fiscal burden these infections have upon the healthcare system would be beneficial. Here, the results of a large-scale selection process that incorporates bioinformatic, genomic, transcriptomic, and proteomic screens to identify six vaccine candidates from the 5379 predicted proteins encoded by uropathogenic *E. coli* strain CFT073 is described. The vaccine candidates; ChuA, Hma, Iha, IreA, IroN, and IutA, all belong to a functional class of molecules that is involved in iron acquisition, a process critical for pathogenesis in all microbes. Intranasal immunization of CBA/J mice with these outer membrane iron receptors elicited a systemic and mucosal immune response that included the production of antigen-specific IgM, IgG, and IgA antibodies. The cellular response to vaccination was characterized by the induction and secretion of IFN-γ and IL-17A. Of the six potential vaccine candidates, IreA, Hma, and IutA provided significant protection from experimental infection. In immunized animals, class-switching from IgM to IgG and production of antigen-specific IgA in the urine represent immunological correlates of protection from *E. coli* bladder colonization. These findings are an important first step toward the development of a subunit vaccine to prevent urinary

tract infections and demonstrate how targeting an entire class of molecules that are collectively required for pathogenesis may represent a fundamental strategy to combat infections.

2.2 Introduction

2.2.1 Epidemiological statistics, treatment of UTI, and the need for a vaccine

UTI caused by UPEC is a considerable public health issue. In addition to symptoms of acute cystitis and pyelonephritis caused by UTI, a number of more serious conditions are often associated with these infections. Upper UTIs in young children can cause permanent kidney damage. An estimated 57% of children with acute pyelonephritis develop renal scarring (246). As with other significant pathogens, increased resistance to antibiotics by UPEC poses a treatment issue (128). Even with treatment, recurrent infections frequently occur, with same-strain episodes making up between 25-100% of recurrent UTI cases [reviewed in (196)]. Consequently, these imply that a vaccine to prevent UTI would alleviate this source of morbidity and economic burden. Indeed, a number of groups have sought to stimulate protective immunity against UPEC. For example, immunization with the type 1 fimbrial adhesin, FimH, conjugated to its periplasmic chaperone, FimC, reduced murine bladder colonization by 99.9%, as well as provided protection in a primate model (235, 236). Additionally, subunit vaccines based on several other surface-exposed molecules have been shown to induce at least some immune response in immunized animals (122, 227, 293, 339, 349). However, although much research has focused on the development of a vaccine against UPEC, none are currently available in the United States.

2.2.2 A new strategy to identify novel UPEC vaccine candidates

Large-scale reverse vaccinology approaches offer an alternative to traditional vaccine design. Pioneered by successful work using *Neisseria meningitidis*, this technique applies genomic and bioinformatic methods to identify novel vaccine targets (315). Recently applied to extraintestinal pathogenic *E. coli* (ExPEC), a pathotype to which UPEC belongs, a subtractive hybridization study identified surface-exposed antigens specific to ExPEC and found that several of these proteins protected immunized mice from lethal sepsis (78). Due to the limited success of previous UTI vaccine design strategies, we hypothesized that a functional vaccinology approach – utilizing "omics" technologies to identify "*PASivE"* vaccine candidates – would identify vaccine targets of UPEC in an unbiased manner that could elicit protective immunity. Described here is the use of previously established genomics and proteomics data to identify six pathogen-associated OM iron receptors (ChuA, Hma, Iha, IreA, IroN and IutA) that are *Pathogen-specific, Antigenic, Surface-exposed, In vivo-Expressed* and therefore ideal putative UPEC vaccine targets. Each of these 71-84 kDa proteins is predicted to form a transmembrane beta-barrel in the OM, with a series of loops extending extracellularly (51). Facilitating import of specific iron sources, these receptors mediate uptake of siderophores, secreted bacterial iron-chelating molecules, or host heme-derived iron. Because iron acquisition is necessary for bacterial pathogenesis and it is well known that the urinary tract is an iron-limited environment, iron acquisition via these receptors is crucial for UPEC infection (419). Consequently, deletion of the siderophore receptor IreA, heme receptors ChuA or Hma, enterobactin receptor Iha, salmochelin receptor

IroN, or aerobactin receptor IutA all decrease the fitness of UPEC in the murine urinary tract (136, 195, 345, 348, 419).

This Chapter describes the use of an unbiased, rational vaccinology approach to identify suitable UPEC vaccine candidates; the results implicated a class of molecules involved in iron acquisition. Intranasal immunization with these UPEC OM iron receptors provides protection from UTI. Additionally, antigen-specific antibody and cytokine responses are generated in response to vaccination with iron receptor proteins, of which, the former correlated with protection. Therefore, this class of molecules is promising as protective vaccine targets against UPEC and, because of their conserved function of iron acquisition for pathogenesis, could potentially be adopted for the development of vaccines against other Gram-negative bacterial infections.

2.3 Materials and Methods

2.3.1 Bacterial strains and culture conditions

Escherichia coli CFT073, a prototypic UPEC strain, was isolated from the urine and blood of a patient with acute pyelonephritis (270) and has been fully sequenced and annotated (450). *E. coli* strain 536 was isolated from a patient with acute pyelonephritis (135). Unless otherwise noted, bacteria were cultured in Luria broth containing appropriate antibiotics (100 μg/ml ampicillin, 25 μg/ml kanamycin, and/or 20 μg/ml chloramphenicol) at 37°C with aeration.

2.3.2 Murine model of ascending UTI

Mice were maintained in specific pathogen-free conditions and all experiments were conducted according to protocols approved by University Committee on the Use

and Care of Animals at the University of Michigan. Female CBA/J mice were transurethrally inoculated as previously described (138). Six- to eight-week old mice were used for these studies and animals were ≤15 weeks old at the conclusion of all experiments. For manipulation, mice were anesthetized with an i.p. injection of 100 mg ketamine, 10 mg xylazine per kg body weight. Prior to inoculation, overnight *E. coli* CFT073 cultures were harvested by centrifugation (3000 x *g*, 30 min, 4°C) and resuspended in PBS to an OD600 of 4.0, equivalent to $4x10^9$ colony-forming units (CFU)/ml. Bacterial suspension (50 μl/mouse) was delivered transurethrally using a sterile 0.28 mm inner diameter polyethylene catheter connected to an infusion pump (Harvard Apparatus), with total inoculum of $1x10^8$ CFU/mouse. When necessary, mice were euthanized using a lethal dose of isoflurane and appropriate organs were harvested for further analysis. For determination of CFUs, organs were harvested from euthanized animals at 48 hours (h) post-inoculation and homogenized in PBS with a GLH homogenizer (Omni International). Bacteria in tissue homogenates were enumerated by plating on Luria-Bertani agar containing 0.5 g/L NaCl using an Autoplate 4000 spiral plater (Spiral Biotech). Colonies were enumerated using a QCount automated plate counter (Spiral Biotech). Blood was collected as necessary from anesthetized mice by an infraorbital bleed using 1.1 to 1.2 mm Micro-Hematocrit Capillary Tubes (Fisher) and serum was separated using Microtainer Serum Separator Tubes (Becton Dickinson).

2.3.3 Antigen purification

Genes encoding the selected antigens were PCR-amplified from CFT073 genomic DNA and cloned into either pBAD-*myc*-HisA (Invitrogen) or pET30b+ (Novagen).

Recombinant protein expression from pBAD (Hma, IutA, ChuA) was induced in *E. coli* TOP10 cultured to OD_{600} = 0.8 by addition of L-arabinose to 100 μM for 4 h. Proteins expressed from pET (Iha, IreA) were over-expressed in *E. coli* BL21(DE3) pLysS cultured in Terrific broth (12 g/L tryptone, 24 g/L yeast extract, 2.3 g/L KH_2PO_4, 12.5 g/L K_2HPO_4, 4% glycerol) to OD_{600} = 1.0 at 37°C and induced overnight with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Induced cultures were harvested by centrifugation (8,000 × *g*, 4°C, 10 min), resuspended in 10 mM HEPES, pH 7, and 100 U Benzonase nuclease (Sigma). Bacteria were lysed by two passages though a French pressure cell (20,000 psi) and the lysate was cleared by centrifugation (8,000 × *g*, 4°C, 10 min). Bacterial membranes were pelleted from the cleared lysate by ultracentrifugation (112,000 × *g*, 4°C, 30 min) and the membrane pellet resuspended in 5 ml 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, 1% ASB-14, pH 8.0. His_6-tagged proteins were purified on nickel-nitriloacetic acid-agarose columns (Qiagen) using urea- or imidizole-based methods according to the manufacturer's instructions (The QIAexpressionist). Eluted purified proteins were renatured by dialysis at 4°C into a final solution containing 0.05% Zwittergent in PBS, pH 7.5 and quantified using the BCA protein assay (Pierce). LPS was tested for in purified protein preparations using the Limulus Amebocyte Lysate PYROGENT Single Test Vials (Cambrex).

2.3.4 Peptide synthesis

Putative extracellular loops of IroN and IutA were predicted using the PRED-TMBB program (http://biophysics.biol.uoa.gr/PRED-TMBB/). 30-mer peptides corresponding loop 6 of IutA and loop 7 of IroN (IroN: YLLYSKGNGCPKDITS

GGCYLIGNKDLDPE; IutA: VDDIDYTQQQKIAAGKAISADAIPGGSVD) were synthesized to ≥96% purity by Invitrogen.

2.3.5 Vaccination

Purified antigens were chemically cross-linked to cholera toxin (CT) (Sigma) at a ratio of 10:1 using *N*-succinimidyl 3-(2-pyridyldithio) propionate (Pierce) according to the manufacturer's recommendations. Peptide antigens were dissolved in 1 mM EDTA in PBS, mixed with reduced CT, and incubated at 4°C for 18 h. All immunizations were administered intranasally in a total volume of 20 μl/animal (10 μl/nare). Animals received a primary dose on day 0 of 100 μg crosslinked antigen (containing 10 μg CT) or 10 μg CT alone. Two boosts of 25 μg antigen (crosslinked to 2.5 μg CT) or 2.5 μg CT alone were given on days 7 and 14, and mice were challenged as described above.

2.3.6 Tissue culture

Single-cell suspensions (1.5×10^6 cells/ml) were made from spleens by forcing organs though 40 μm Cell Strainers (BD Falcon). Red blood cells were lysed for 2 min using 8.02 mg/ml NH_4Cl, 0.84 mg/ml $NaHCO_3$, 0.37 mg/ml EDTA in distilled water. Final suspensions were made in RPMI (supplemented with L-Glutamine, Gibco) with 1% sodium pyruvate, 1% L-glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids, 10% fetal bovine serum (FBS), 0.001% 50 mM β-mercaptoethanol. Splenocytes were cultured with either medium alone or with 1 μg/ml purified antigen. After incubation of cells at 37°C, 5% CO_2 for 48 h, supernatants were harvested and stored at -20°C.

2.3.7 qPCR

RNA was isolated from pelleted splenocytes using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA was quantified using the ND-1000 Spectrophotometer (NanoDrop) and 1 μg was used for cDNA synthesis. cDNA was synthesized from 1 μg RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Oligo dT primers (0.5 μg/μl stock) and RNase inhibitor (10 U/μl stock) used in the reaction were obtained from Invitrogen. The cDNA reaction mix consisted of (per 10 reactions) 81 μl RNase-free water, 30 μl 10x buffer, 6 μl oligo dT primers, 12 μl dNTP mix, 6 μl RNase inhibitor, and 15 μl Multiscribe RT. The cDNA reaction conditions were: 25^{o}C for 10 min, 37^{o}C for 120 min, and 85^{o}C for five sec in a PTC-200 thermal cycler (MJ Research). The qPCR reaction mix consisted of (per 1 reaction) 8.75 μl RNase-free water, 12.5 μl 2x Taqman Universal PCR Master Mix (Applied Biosystems), 1.0 μl 20x target primers and FAM probe (Applied Biosystems), 0.75 μl 20x control GAPDH primers and VIC probe (Applied Biosystems), and 2.0 μl cDNA. The qPCR reaction conditions were: 50^{o}C for two min, 95^{o}C for 10 min, and 55 cycles of 95^{o}C for 15 sec followed by 60^{o}C for one min in a Mx3000P thermal cycler (Stratagene). Fluorescence was read at 60^{o}C at the end of each cycle. qPCR reactions were conducted in duplicate wells with appropriate no template and no RT controls for cDNA and qPCR reactions. Data were analyzed with MxPro Software (Stratagene) and were expressed using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

2.3.8 Enzyme-linked immunosorbent assay (ELISA)

For indirect serum ELISAs, EIA/RIA medium binding 96-well ELISA plates (Corning) were coated at room temperature (rt) overnight with 5 μg/mL purified protein (for serum) or 10 μg/mL purified protein (for urine) diluted in carbonate buffer, pH 9.8 (2.33 mg/mL Na_2CO_3, 2.86 mg/mL $NaHCO_3$, 0.2 mg/mL MgCl). Non-specific binding sites were blocked with blocking buffer (10% FBS, 0.04% NaN_3 in PBS) at rt for 1 h. 1:256 dilutions of mouse serum in blocking buffer were applied to wells at rt anywhere from 2 h to overnight. Isotype-specific secondary antibodies conjugated to alkaline phosphatase were diluted 1:2000 in Tris buffer, pH 8.0 (17.8 mg/mL Trizma HCl, 10.6 mg/mL Tris base, 10 mg/mL bovine serum albumin (BSA), 0.2 mg/mL MgCl, 0.2 mg/mL NaN_3) and bound to wells at rt for at least 4 h to overnight. As a note for total immunoglobulin (Ig) detection, a 1:2000 dilution of goat anti-mouse Ig was mixed with 1:4000 dilutions of goat anti-mouse IgA and IgM to ensure all serum antibodies were detected. Goat anti-mouse IgA, IgG, and IgM were obtained conjugated to alkaline phosphatase from Southern Biotech. Alkaline phosphatase substrate, *p*-nitrophenyl phosphate (1 mg/mL; Sigma), was diluted in carbonate buffer and applied to wells at rt until color developed (10 to 30 min). The reaction was stopped by addition of 0.2 N NaOH and read with a μQuant plate reader (Bio-Tek Instruments, Inc.) at a wavelength of 405 nanometers (nm). For all steps, 100 μL was applied to each well and plates were washed by flooding all wells four times with wash buffer (0.05% Tween 20 in PBS) between all steps.

To detect IL-17A and IFN-γ cytokines, a sandwich ELISA was performed. Purified IL-17A, IFN-γ, and their corresponding matched antibody pairs were obtained

from R&D Systems. 96-well ELISA plates were coated with 50 μL of 5 μg/mL capture antibody diluted in borate-buffered saline (7.0 mg/mL NaCl, 3.1 mg/mL H_3BO_3, 0.64 mg/mL NaOH) overnight at 4°C. Non-specific binding sites were blocked with blocking buffer (2% BSA in PBS) at 37°C for 1 h. Dilutions of purified IL-17A, IFN-γ, or test samples were made in dilution buffer (0.05% Tween 20, 2% FBS in PBS) and 50 μL applied to wells at 37°C for 1 h. Biotinylated detection antibodies were diluted to either 0.5 μg/mL or 0.25 μg/mL for IFN-γ and IL-17A, respectively, in dilution buffer and applied to wells at 37°C for 45 min. Streptavidin-HRP (Southern Biotech) was diluted 1:5000 in dilution buffer and 100 μL was applied to wells at 37°C for 30 min. OPD Easy-tablets (2 mg/tablet, Acros Organics) were diluted (4 tablets, 5 μL H_2O_2 in distilled H_2O) and 100 μL applied to wells at rt until color developed (5 to 20 min). The reaction was stopped by addition of 100 μL 6 N H_2SO_4 and read with a plate reader at a wavelength of 490 nm. Between all steps, plates were washed by flooding all wells four times with wash buffer.

2.3.9 Statistical analysis

All graphing and statistical analyses were done using GraphPad Prism 5. Significance was determined using Mann-Whitney tests. Correlates of protection were determined using the Pearson correlation coefficient with linear regression to generate a best fit line. All statistics were conducted using 95% confidence intervals where applicable.

2.4 Results

2.4.1 Candidate antigen selection

To identify bacterial proteins that could be used as vaccine targets against UPEC infection, a functional vaccinology approach was utilized, combining both genomics and proteomics techniques. To begin, criteria defining UPEC vaccine targets were established and data from previously-described studies assembled to identify proteins meeting these criteria. Of the 5379 predicted proteins in *E. coli* pyelonephritis strain CFT073, only 6 (Table 2.1) met all 5 of our established *PASivE* criteria.

Transcriptomic and proteomic data were evaluated to identify candidates meeting the *in vivo* expression and antigenicity criteria. Data from an *in vivo* transcriptome study indicated that genes encoding iron acquisition system components were among those most highly upregulated in RNA isolated from the urine of experimentally infected CBA/J mice (386). When all genes were ranked in order of expression level *in vivo*, these iron acquisition genes, specifically OM iron receptors, were among the top 18% most highly expressed in the murine urinary tract (Table 2.1). Similarly, when OM proteins (OMPs), which are partially surface-exposed, were isolated from CFT073 cultured in human urine *ex vivo* and compared with OMPs isolated from bacteria cultured in Luria broth *in vitro*, these iron receptors were the most highly induced proteins (8). Further, the vaccine candidates listed in Table 2.1 comprised 6 of the top 7 human urine-induced OMPs. In addition to bacteria isolated from urine, bladder cell-associated UPEC has been observed to express OM receptor, both *in vitro* (137) and *in vivo* (334). Finally, an immunoproteomics study identified these iron receptors as antigenic; that is, they

Table 2.1 Candidate antigens and selection criteria[a].

Gene	Locus tag	% UPEC (*n*=55)[f]	% fecal (*n*=30)[f]	Antigenic[e]	Fold change *in vivo*[b]	*in vivo* percentile[c]	Fold change in human urine[d]
		(*P*)	(*P*)	(*AS*)	(*ivE*)	(*ivE*)	(*ivE*)
chuA	c4308	87	30	+	7.06	95.0	27.8
hma	c2482	69	17	+	6.56	94.5	14.8
iha	c3610	45	37	+	18.9	89.1	5.87
ireA	c5174	20	17	+	23.3	95.2	7.81
iroN	c1250	71	33	+	22.7	82.7	7.63
iutA	c3623	65	17	+	5.57	97.0	49.2

[a] Table courtesy of Erin Hagan.
[b] Transcript fold change in the urine of experimentally infected mice as compared to growth in LB (386)
[c] Of 5379 genes ranked in order of *in vivo* transcript microarray signal intensity (386)
[d] Protein fold change following growth in pooled filter-sterilized human urine as compared to growth in LB (8)
[e] Reacts with sera from mice chronically infected with *E. coli* CFT073 (137)
[f] Prevalence of indicated gene in UPEC and fecal isolates (137)

reacted with antisera from mice chronically infected with UPEC strain CFT073, indicating that these proteins elicited a humoral response during experimental UTI (137).

As UPEC represent a subset of strains that are genetically distinct from commensal *E. coli*, a vaccine directed against UPEC should specifically target pathogenic strains. Toward this end, a comparative genomics hybridization study identified 131 genes that were present in all UPEC isolates analyzed (*n*=10), but none of the fecal-commensal strains (*n*=4) (248). Among these UPEC-specific genes, two – *chuA* and *hma* – encoded OM heme receptors,. Furthermore, dot blot analysis of a collection of UPEC and fecal-commensal *E. coli* isolates identified several of these iron OMP genes (*chuA, hma, iroN,* and *iutA*) as present more frequently among pathogens than non-pathogens (137). Even genes that were not statistically more frequent among uropathogens (*iha* and *ireA*) were nonetheless present at relatively low frequencies in commensals. Of the 5379 predicted proteins in UPEC strain CFT073, 6 candidate antigens, all OM iron receptors, emerged from our series of genomics and proteomics studies as uniformly highly ranked *PASivE* vaccine targets (Table 2.1). This screening process strongly suggested that broadly targeting an entire class of molecules involved in iron acquisition could be an effective strategy to develop a protective UTI vaccine.

2.4.2 Vaccination confers protection against experimental UTI

The 6 iron receptor vaccine candidates, ChuA, Hma, IutA, IreA, Iha, and IroN, were expressed and purified as 6xHistidine affinity-tagged recombinant proteins (Figure 2.1A and B). Consistent with the predicted structure of these antigens, the circular

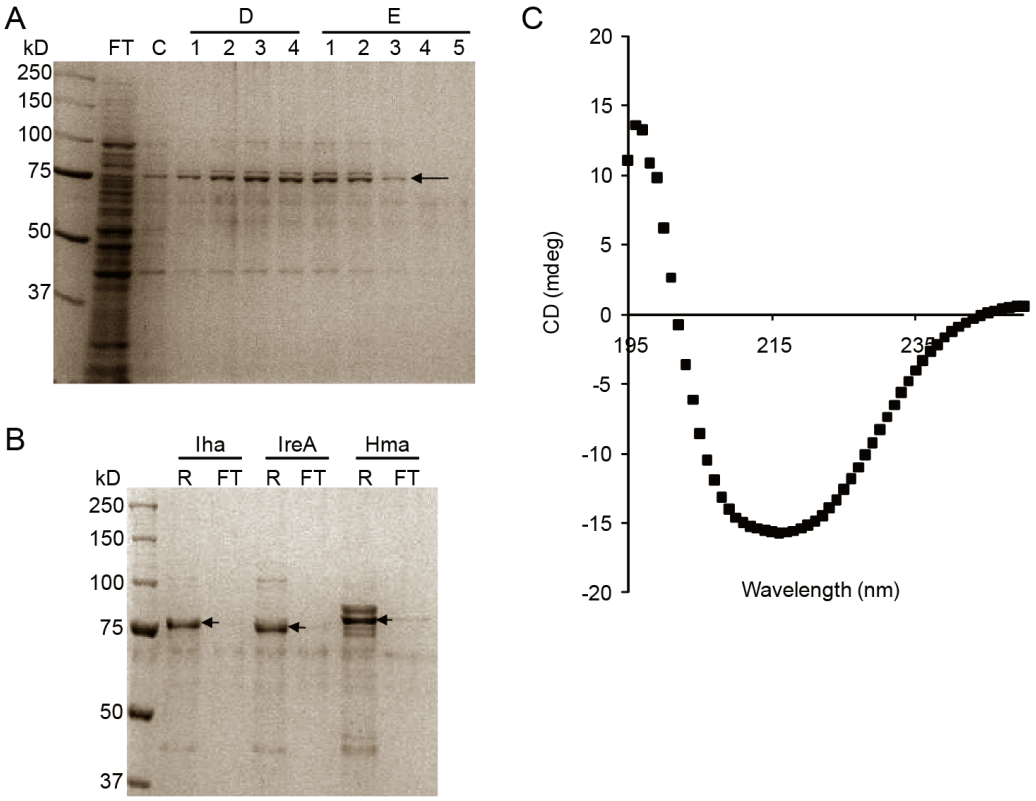

Figure 2.1 Purification and CD spectrum of OM iron receptors. (A) Example purification of IreA (arrow). Numbers represent the elution fraction and buffers C, D, and E have decreasing pH. The majority of IreA elutes off the column in fractions D2-4 and E1-2. (B) Purified Iha, IreA, and Hma (arrows). (C) Far UV CD spectral analysis of purified Hma (800 μg/ml) following buffer exchange and renaturation. Spectrum was measured from 195 to 250 nm at 25°C using a Jasco Co. (Tokyo, Japan) J-810 Rev. 1.00 instrument. CD conducted by Neal Hammer. R=retentate, FT=flow through.

dichroism (CD) spectrum of refolded purified Hma displayed a trough at 218 nm, which is characteristic of a β-sheet-rich conformation (Figure 2.1C). The six purified protein antigens were each biochemically cross-linked to the CT adjuvant at a ratio of 10:1 (antigen:CT) and mice were intranasally inoculated with either an antigen-CT complex or CT alone. Following primary immunization (day 0) and booster doses (days 7 and 14), the animals were transurethrally challenged with UPEC strain CFT073 and protection was assessed at 48 hpi by determining CFUs in the urine, bladder, and kidneys. The vaccination schedule is outlined in Figure 2.2.

Of the 6 candidates, 3 conferred protection against experimental challenge with UPEC (Figure 2.3B, D, and E). The heme receptor, Hma, protected mice against colonization of the kidney. Hma-vaccinated mice demonstrated nearly a 3-log reduction in median CFU/g in the kidney ($P = 0.008$) and 13/20 mice had undetectable levels of bacteria (<100 CFU/g) in the kidneys (Figure 2.3B). The putative siderophore receptor IreA showed significant protection and demonstrated a 3-log reduction in median CFU/g in the bladder ($P = 0.035$) (Figure 2.3D). For IreA, 9/15 vaccinated mice had undetectable levels of bacteria in the bladder. The siderophore receptor for aerobactin, IutA, conferred significant protection against UPEC challenge in both the bladder and kidneys. Mice vaccinated with full-length IutA displayed 1-log CFU/g reduction in both the bladder ($P = 0.009$) and kidneys ($P = 0.007$) (Figure 2.3E). This showed that mucosal immunization in the nares generates a protective effect at distal sites, the bladder and kidneys. Not all antigens selected as candidates provided protection against experimental UTI. Both ChuA and Iha recombinant proteins failed to elicit significant

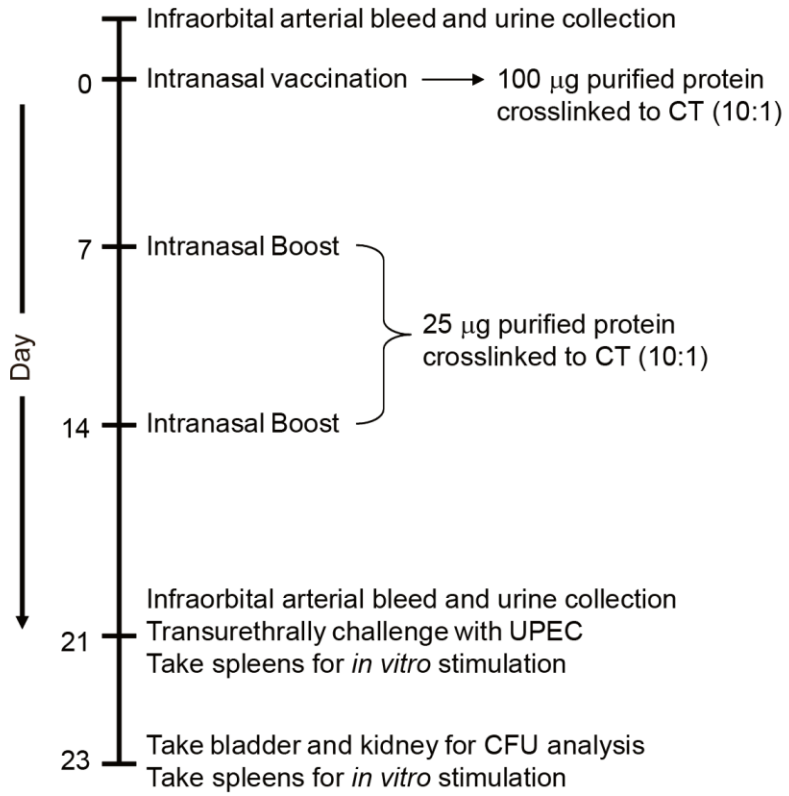

Figure 2.2 Vaccination schedule for OM iron receptor antigens. Mice were vaccinated and manipulated according to the outlined schedule. Blood and urine were collected before and after vaccination for antigen-specific antibody analysis while splenocytes were harvested after vaccination and both before and after challenge for analysis of cellular cytokines responses *in vitro*.

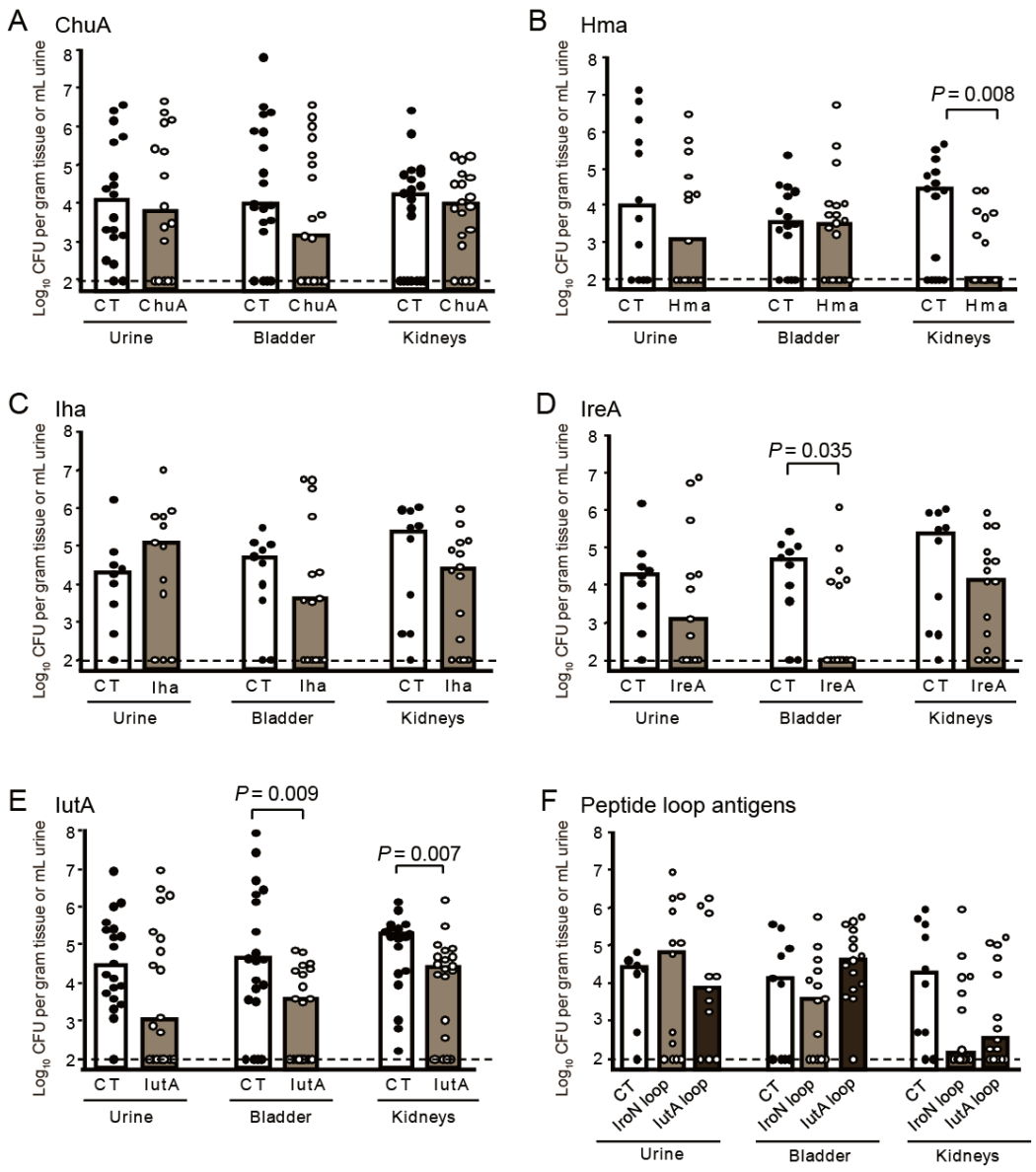

Figure 2.3 Immunization with OM iron receptor antigens. Vaccinated animals (*n* = 15-20 mice per experiment, 2 experiments combined) were transurethrally challenged with 1×10^8 CFU of *E. coli* CFT073 and colonization was measured 48 hpi. Symbols represent CFU/g tissue or /ml urine of individual mice and bars indicate median values. Clear bars, mock vaccinated with CT alone; gray bars, vaccinated with purified (A) ChuA, (B) Hma, (C) Iha, (D) IreA, or (E) IutA, or (F) peptides corresponding to the extracellular loops of IroN or IutA. The dotted line indicates the 100 CFU/gram tissue limit of detection. Infections and sacrifices conducted in collaboration with Chris Alteri, Erin Hagan, and Sara Smith.

protection against challenge (Figure 2.3A and C). With the exception of ChuA, which was lethal in 11/30 mice (surviving mice were not protected), all of the full-length protein and peptide vaccines were well tolerated in immunized mice and no animals died following vaccination. Of note, full-length IroN was never successfully cloned and expressed for efficacy trials.

Since a significant reduction in post-challenge CFU was observed for Hma and IreA vaccinated mice, these antigens were tested to determine if similar levels of protection could be generated following heterologous challenge with another UPEC isolate. Mice were immunized as described in Figure 2.2 with Hma, IreA, or CT alone and CFUs were determined at 48 hpi with UPEC strain 536. In these experiments, Hma vaccinated mice were significantly protected from heterologous challenge and displayed >10-fold reduction in median CFU/g in the bladder ($P = 0.0287$) (Figure 2.4). Vaccination with IreA significantly protected mice from challenge with UPEC strain 536 and these mice demonstrated a similar log-fold decrease in median CFU/g in the kidneys ($P = 0.0379$) (Figure 2.4).

Peptides (30 aa) corresponding to portions of the extracellular loops in both the salmochelin siderophore receptor IroN (aa 491-520) and aerobactin receptor IutA (aa 467-498) were also assessed to determine their use as potential immunogens. Extracellular loops were selected based on their predicted topology to direct an antibody response against surface-exposed residues. These peptides also represent highly conserved loops within the salmochelin and aerobactin receptors; the IroN peptide has >90% identity to 37 siderophore receptor sequences including urinary tract isolates 536, UTI89, and 83972. The IutA peptide has >76% identity (23/30 aa) among 15 aerobactin

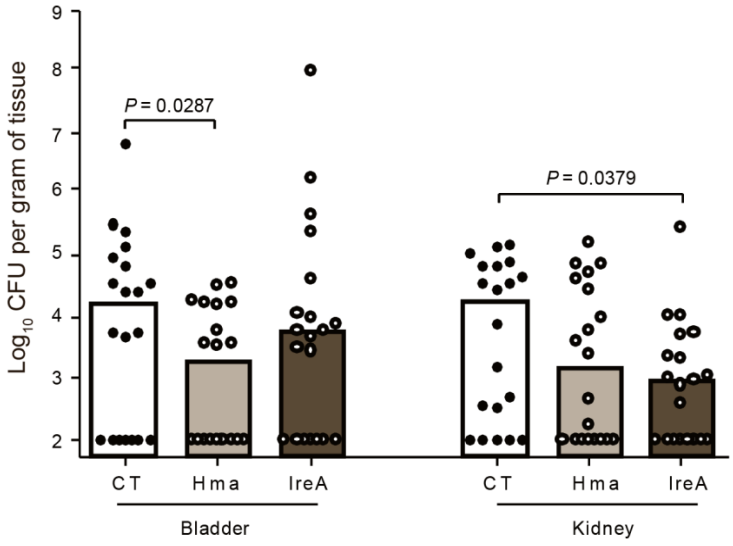

Figure 2.4 Immunization with Hma and IreA reduces kidney colonization by *E. coli* 536. Vaccinated animals (*n*=10 mice per experiment, 2 experiments combined) were transurethrally challenged with 1×10^8 CFU of *E. coli* 536 and colonization was measured 48 hpi. Symbols represent CFU/g tissue or /ml urine of individual mice and bars indicate median values. Clear bars, mock vaccinated with CT alone; gray bars, vaccinated with purified Hma or IreA. The dotted line indicates the 100 CFU/gram tissue limit of detection. Experiment conducted by Chris Alteri and Erin Hagan.

receptor sequences present in various pathogenic *E. coli*, including UPEC and ExPEC isolates UMN026 (240), IAI39, and S88 (316). Mice were inoculated as previously described with peptide mixed with CT or CT alone and protection was assessed at 48 hpi following infection with UPEC strain CFT073. These peptides failed to elicit significant protection in the urine, bladder, or kidneys of mice, however, there was nearly a 2-log reduction in CFU/g in the kidneys for both IroN ($P = 0.053$) and IutA ($P = 0.078$) peptides, demonstrating a strong trend towards protection (Figure 2.3F). These results imply that peptides may be suitable for development of a UTI vaccine but maybe more efficacious if cross-linked to or fused in a carrier molecule. Together, these findings show that targeting members of an entire functional class of molecules involved in iron acquisition is an effective strategy to identify protective vaccine candidates that significantly reduce bacterial colonization during ascending UTI following experimental challenge with UPEC.

2.4.3 Splenocytes from vaccinated mice upregulate transcripts for several proinflammatory cytokines

Proinflammatory cytokines are crucial for orchestrating protective immune responses against pathogens. To gain insight on what cytokines and cell types might be important for protection, the relative quantities of a panel of cytokine transcripts were analyzed in our CT control- and OMP antigen-vaccinated mice by qPCR analysis. Splenocytes were isolated from protein vaccinated and adjuvant (CT)-only controls and cultured with or without increasing concentrations of OMP fraction *in vitro.* At the time, the crude OMP extract (containing the vaccine candidates) was used for *in vitro* stimulation because purified protein quantities were limited. After 6 h of incubation,

cells were harvested and prepared for qPCR analysis. Figure 2.5 shows dose-dependent IFN-γ transcript upregulation by splenocytes from mice vaccinated with both full-length OM iron receptors and peptide (IroN and IutA) loops. Splenocytes from IreA- and Hma-vaccinated (protected) mice had the highest upregulation of IFN-γ, while only the highest concentration of peptide stimulation (100 μg/ml) fostered significant upregulation of IFN-γ transcript from splenocytes taken from peptide-vaccinated mice. Splenocytes from mice vaccinated with CT only did not upregulate IFN-γ in response to *in vitro* stimulation, indicating that vaccination with OM iron receptors (or antigenic peptides) stimulates the production of antigen-specific immune cells capable of responding to subsequent antigen exposure. Note that splenocytes taken from IreA- and Hma-vaccinated (protected) mice showed the strongest IFN-γ responses. In addition to IFN-γ transcript, we tested upregulation of other pertinent cytokine transcripts in response to OM iron receptor vaccination and *in vitro r*estimulation. Of the panel of cytokines tested, only IL-6 transcript was consistently upregulated by splenocytes from all of the vaccinated animals and not the CT controls (Figure 2.6). Lastly, peptide antigens IutA and IroN elicited a strong TNF-α response upon stimulation of splenocytes from vaccinated mice *in vitro*.

2.4.4 Splenocytes from vaccinated mice upregulate IL-17A transcript in an antigen-dependent manner

At the time these assays were being performed, information on the importance of the recently-described proinflammatory cytokine IL-17A in the innate and the adaptive

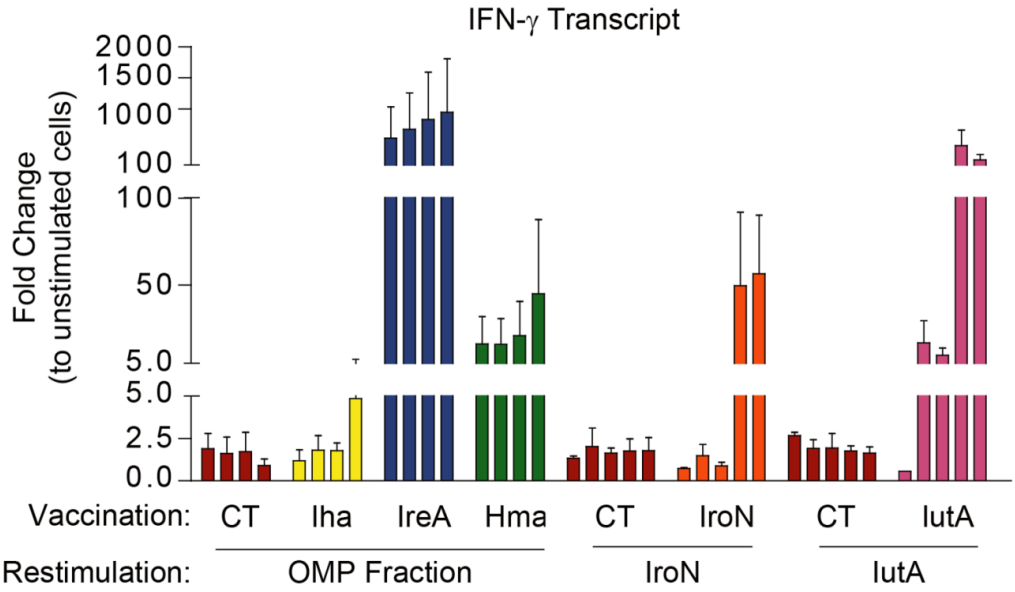

Figure 2.5 Splenocytes from vaccinated mice upregulate IFN-γ transcript in response to antigen stimulation. Splenocytes purified from mice vaccinated with recombinant Iha, IreA, Hma or peptides representing loops from IroN and IutA were restimulated with the OM fraction of *E. coli* CFT073 or the corresponding purified peptides, respectively (*n*=2 samples per condition per concentration). Bars indicate increasing concentrations of stimulant used (from left to right: 0.1 μg/mL, 0.5 μg/mL, 1.0 μg/mL, 10 μg/mL, 100 μg/mL).

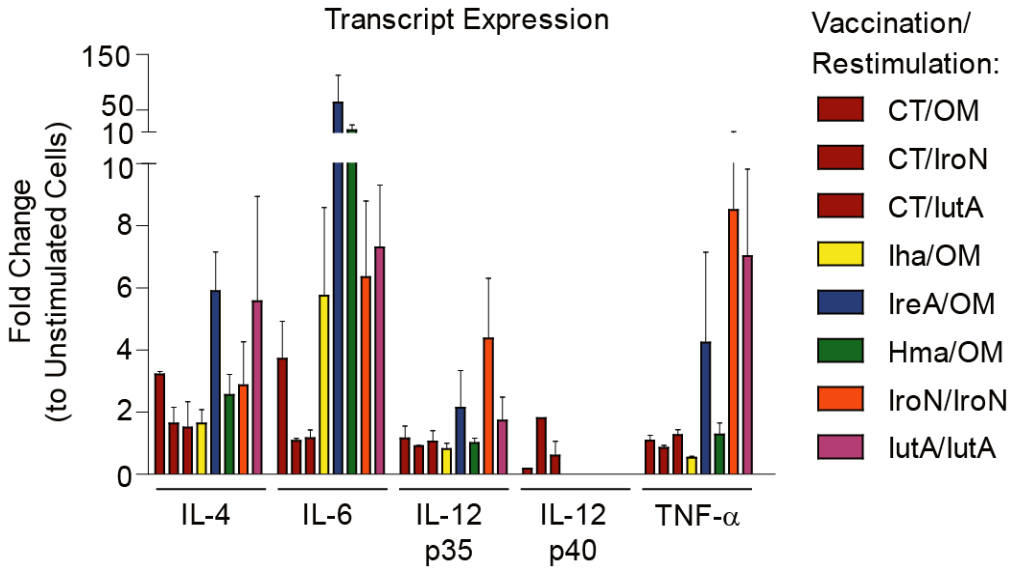

Figure 2.6 Splenocytes from vaccinated mice upregulate other cytokine transcripts in response to antigen stimulation. Splenocytes purified from mice vaccinated with recombinant Iha, IreA, Hma or peptides representing loops from IroN and IutA were restimulated with the OM fraction of *E. coli* CFT073 or the corresponding purified peptides, respectively (*n*=3 samples per condition). For these experiments, splenocytes from full-length receptor-vaccinated mice were stimulated with 10 μg/mL OM fraction while splenocytes from peptide-vaccinated mice were stimulated with 100 μg/mL of the corresponding peptide.

immune response to pathogens was rapidly emerging. In response to this, the upregulation of IL-17A transcript was also tested in the vaccinated mice. Coinciding with this, purification techniques were improved and larger yields of purified iron receptors were generated, facilitating *in vitro* stimulation of splenocytes with the purified antigen used for vaccination (rather than crude OMP extract). As shown in Figure 2.7, IL-17A transcript was the only proinflammatory transcript significantly upregulated compared to both unstimulated controls and to Hma-stimulated cells from CT-vaccinated animals ($P = 0.0051$). Significant upregulation of IL-17A transcript was also observed when analyzing antigen-stimulated splenocytes from mice vaccinated with the other iron receptor vaccine candidates (combined Hma studies shown, Figure 2.8A). Notably, the most protective vaccine against UPEC bladder colonization, IreA, showed the largest increase in IL-17A transcription (when compared to splenocytes from CT control mice), 128-fold, $P = 0.029$ (Figure 2.8A).

Testing splenocytes from mice vaccinated with different antigens revealed a trend: IL-17A transcript responses by OM iron receptor vaccinated mice were significantly different from those of CT control mice (Figure 2.8A), whereas IFN-γ transcripts appeared to be equally upregulated by splenocytes from both control and vaccinated mice (Figure 2.8B). This trend extended to other LPS-responsive proinflammatory genes (*i.e.* IL-6 and TNF-α), suggesting that splenocytes were responding to contaminating LPS in the purified iron receptor preparations, and that IL-17A transcripts were not subject to this innate LPS- responsiveness. Additional facts support this notion. First, purified OM iron receptor preparations all tested positive for LPS as measured by coagulation in the Limulus test. Additionally, over the course of

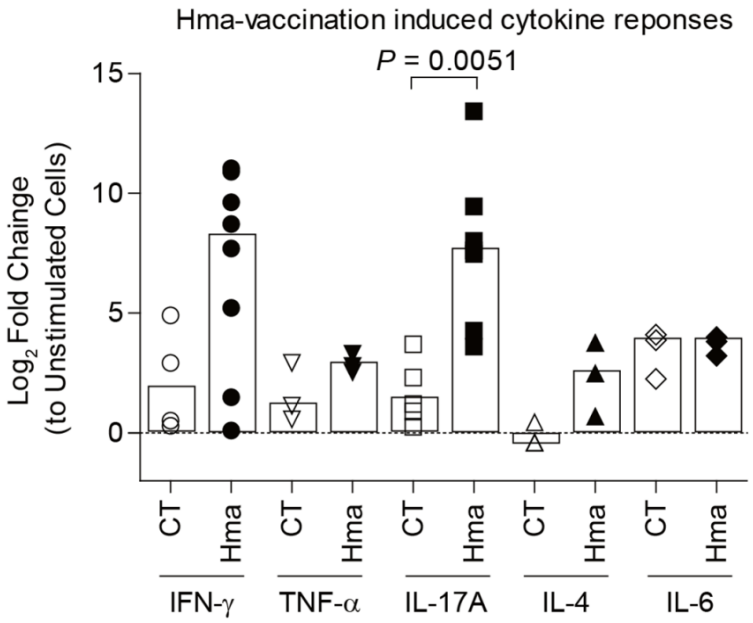

Figure 2.7 Splenocytes from Hma-vaccinated mice upregulate cytokine transcripts in response to stimulation with Hma *in vitro*. Splenocytes purified from mice intranasally vaccinated with recombinant Hma were restimulated with 1 μg purified Hma (*n*=2-7 samples per condition). Note the use of a $\log_2$-transformed y-axis. Each symbol represents an individual animal while bars represent the median.

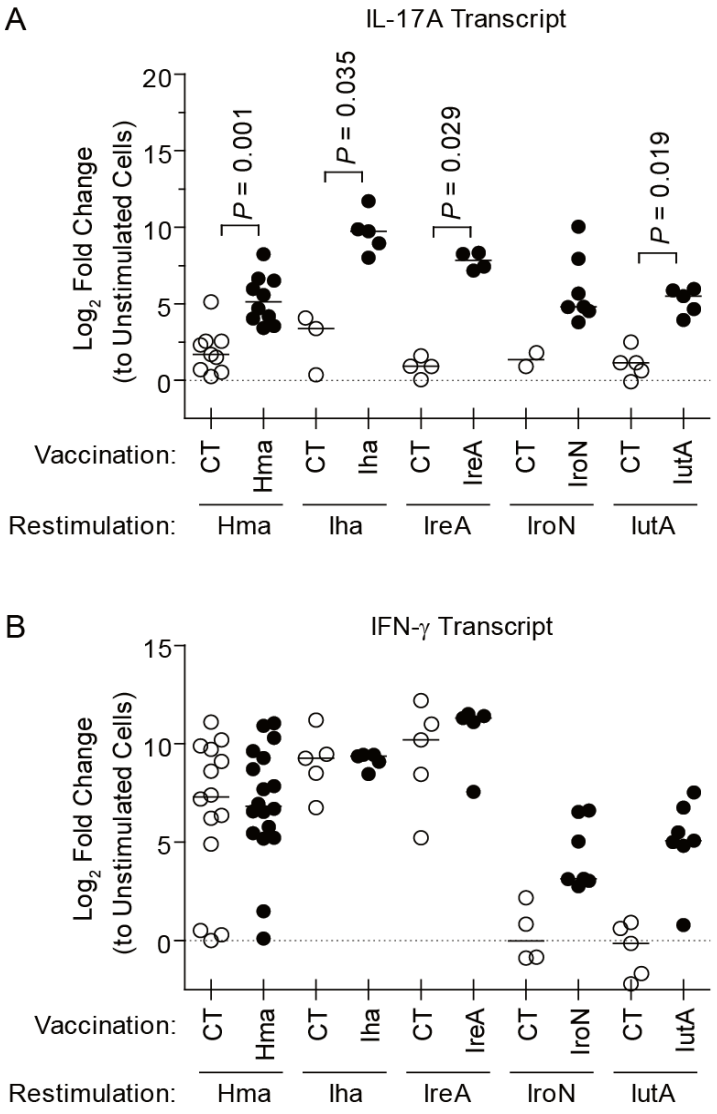

Figure 2.8 Splenocytes from vaccinated mice only upregulate IL-17A transcripts in an antigen-specific manner *in vitro*. Splenocytes taken from vaccinated mice 2 days post challenge were stimulated with corresponding antigen *in vitro* (*n*=4-10 samples per experiment, 1 or 2 experiments combined). After 6 hours incubation, RNA was isolated and (A) IL-17A or (B) IFN-γ transcript levels were quantified by qPCR. IroN and IutA represent data corresponding to extracellular loop peptides. Each symbol represents an individual animal and bars represent the median.

these experiments, purification protocols moved from a urea-based method to an imidizole-based method, and the level of cytokine transcripts upregulated non-specifically by splenocytes from CT-vaccinated mice shifted with this transition. One last piece of evidence suggesting that splenocytes were upregulating IFN-γ transcript non-specifically (in response to LPS) is that splenocytes that were stimulated with the peptide preparations, which were ultra-pure and did not test positive for LPS, only upregulated IFN-γ transcripts in an antigen-dependent fashion (Figure 2.8B). Surprisingly, OM preparations used for stimulation of cells in Figure 2.5, Figure 2.6, and Figure 2.7 did not result in a similar trend, suggesting that the purification scheme used in the latter experiments (Figure 2.8), which was imidizole-based rather than urea-based, either enriched for LPS or fostered contamination with LPS. Thus, the upregulation of IFN-γ and IL-17A transcripts in response to purified LPS was directly tested; the results of this experiment are shown in Figure 2.9. Indeed, IFN-γ transcripts are upregulated similarly by splenocytes from both CT-treated and antigen- (ChuA in this representative experiment) vaccinated mice in response to either ChuA or LPS (Figure 2.9, left four columns). In contrast, only when splenocytes from ChuA-vaccinated mice were stimulated *in vitro* with purified ChuA was IL-17A transcript generated (Figure 2.9, right four columns). These results demonstrate that, in this system, LPS does not stimulate upregulation of IL-17A transcript and antigen-specific stimulation is necessary to initiate IL-17A transcription, suggesting the involvement of $CD4^+$ Th17 memory cells.

2.4.5 Splenocytes from vaccinated mice secrete IFN-γ and IL-17A in an antigen-dependent manner

In addition to cytokine transcripts, secretion of cytokine protein was also

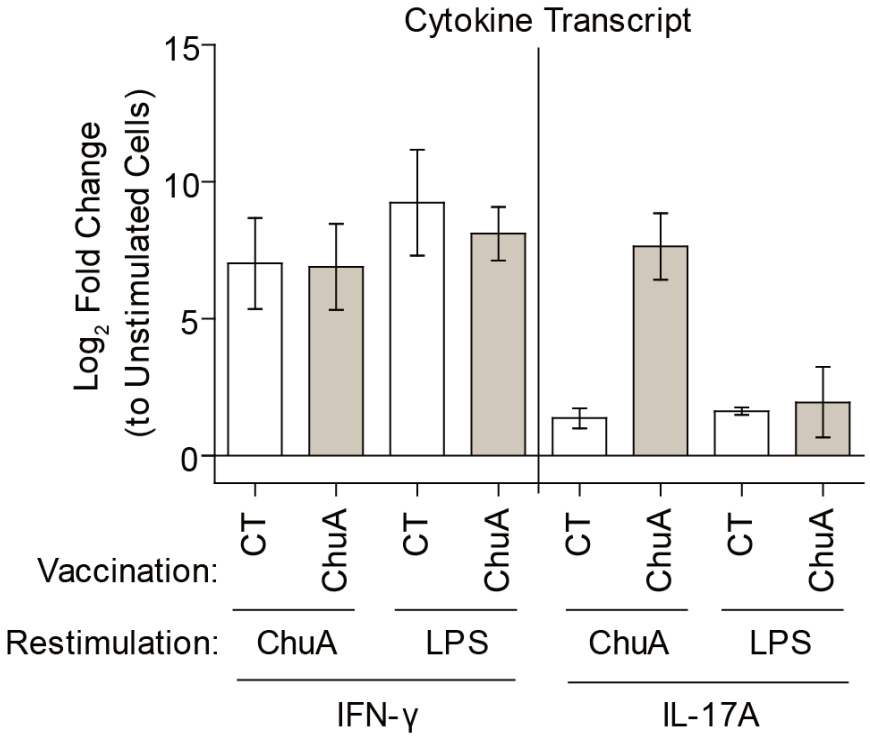

Figure 2.9 Splenocytes from vaccinated mice upregulate IFN-γ transcripts in a non-specific response to LPS and IL-17A transcripts in an antigen-specific response to OM iron receptor. Splenocytes taken from CT-control or ChuA-vaccinated mice 2 days post challenge were stimulated with ChuA or LPS *in vitro* (*n*=2-4 samples per condition). After 6 hours incubation, RNA was isolated and IFN-γ and IL-17A transcript levels were quantified by qPCR. Bars and error represent the mean and SEM.

investigated. Secretion of two major proinflammatory cytokine mediators, IFN-γ and IL-17A, were measured from the splenocytes of mice immunized with adjuvant alone (CT), IreA (a protective antigen), or Iha (a non-protective antigen) following vaccination. Cells were analyzed both pre- and post-challenge (days 21 and 23, respectively, Figure 2.2) to gauge the immune response to *in vivo* antigenic stimulation (*i.e.*, UPEC challenge). Splenocytes were cultured *in vitro* in the presence of 1 μg/ml of the purified antigen used for immunization. Splenocytes from Iha-vaccinated and IreA-vaccinated mice collected both before (-) and after (+) challenge demonstrated significant antigen-specific secretion of IFN-γ and IL-17A when compared to the corresponding splenocytes from CT control mice ($P < 0.05$, bars not shown) (Figure 2.10A and B). Supernatants from unstimulated splenocytes from both CT-treated and antigen-vaccinated mice had undetectable levels of both IFN-γ and IL-17A. Interestingly, splenocytes derived from Iha- and IreA-vaccinated mice post-challenge secreted less pro-inflammatory cytokines than pre-challenge cells ($P < 0.003$) (Figure 2.10 A and B). Both IFN-γ and IL-17A secreting splenocytes were significantly decreased post-challenge ($P <0.003$) in IreA-vaccinated mice (Figure 2.10B), whereas only IL-17A secreted splenocytes were significantly decreased ($P = 0.0007$) in mice vaccinated with Iha (Figure 2.10A). In other words, a decrease in both IFN-γ and IL-17A secretion from vaccinated mice post-challenge as compared to pre-challenge was only observed following immunization with a protective antigen (IreA), suggesting that IFN-γ or potentially Th1-mediated responses may play a role in protection. Overall, these results indicated that vaccination with OM iron receptors generates antigen-specific cells that are capable of secreting IFN-γ and IL-17A,

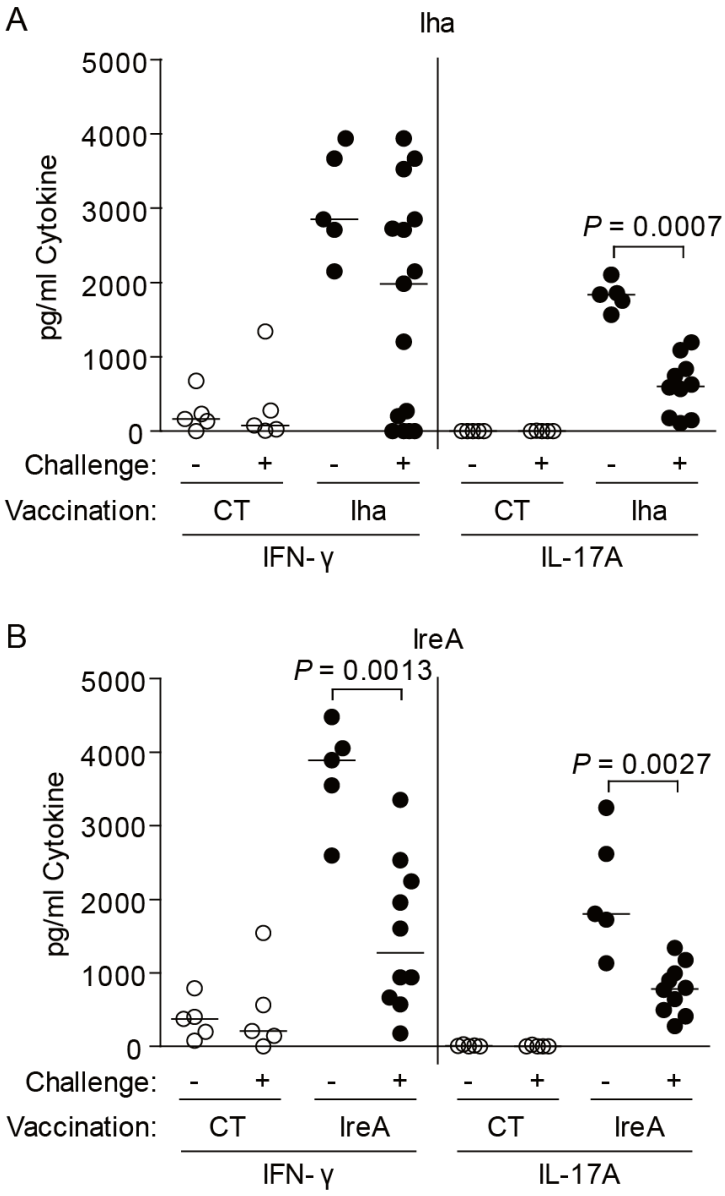

Figure 2.10 Antigen-specific splenocytes from vaccinated mice secrete IFN-γ and IL-17A. Splenocytes harvested from (A) Iha-vaccinated or (B) IreA-vaccinated without challenge (-) or 2 days after transurethral challenge with *E. coli* CFT073 (+) were stimulated with the corresponding antigen *in vitro* (*n*=5-10 samples per condition). Cytokines were measured in culture supernatants by ELISA. Each symbol represents an individual animal and bars represent the median.

two important pro-inflammatory cytokine mediators; unfortunately, no significant correlation was found between cytokine production and reduction in CFU post-challenge. Lastly, unlike transcript upregulation, IFN-γ was only secreted in the splenocyte supernatants when cells were exposed to specific antigen, not LPS or LPS-contaminated antigen preparations. This finding reveals antigen-specific regulation of proinflammatory immune responses at the level of translation and or secretion. The mechanism behind this specificity remains to be tested.

2.4.6 Vaccinated mice excrete antigen-specific IgA in urine

To evaluate the humoral immune response at the primary site of infection – the bladder mucosa – levels of antigen-specific IgA were measured in urine by ELISA. To account for variability in the amount and concentration of urine from each mouse, collections were pooled for use in an indirect ELISA to measure antigen-specific IgA. Urine from IreA- (Figure 2.11A) and IutA-vaccinated (Figure 2.11B) cohorts, which had significantly decreased bladder colonization upon challenge, had the highest fold increases in IgA (34-fold and 6.2-fold, respectively) post-vaccination. Antigens that did not generate significant decreases in colonization in the bladders of vaccinated mice had more modest fold-increases in urine IgA post-vaccination (2.0-fold for ChuA, and 1.7-fold for Iha) (Figure 2.11D and E). Correspondingly, peptide antigens representing extracellular loops of IroN and IutA did not significantly protect from infection in the bladder, and IgA was at background levels in the urine of these animals (Figure 2.11F). Hma, which was protective in the bladder when challenged with strain 536, but not CFT073, had an intermediate 2.2-fold increase in urinary IgA (Figure 2.11C). These

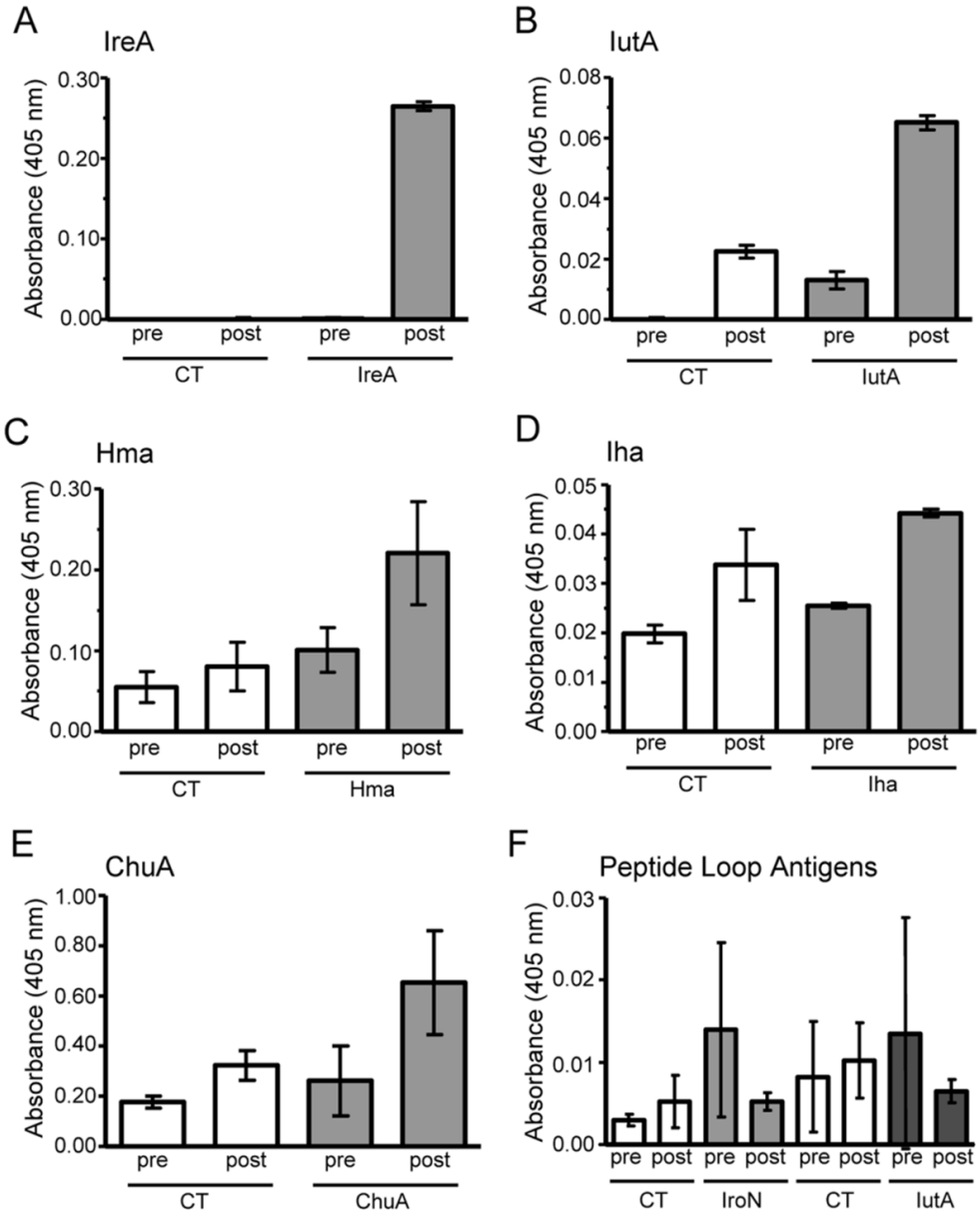

Figure 2.11 Mice produce antigen-specific urinary IgA in response to intranasal immunization. Urine from (A) IreA-vaccinated, (B) IutA-vaccinated, (C) Hma-vaccinated, (D) Iha-vaccinated, (E) ChuA-vaccinated, (F) peptide-vaccinated, and corresponding CT control mice was collected prior to vaccination (pre) and after vaccination but before transurethral challenge with UPEC strain CFT073 (post). Pooled samples (*n*=5-10 mice per group) were plated in appropriately coated ELISA plates and probed for antigen-specific IgA. Absorbance reflects relative quantity of IgA. Error bars indicate the mean +/- SEM of 2 experiments.

findings demonstrate that vaccination with, IreA and IutA, which provide significant protection in the bladder from experimental challenge with UPEC strain CFT073 (Figure 2.3), generates production of antigen-specific IgA that is secreted in urine.

2.4.7 Vaccinated mice produce antigen-specific serum antibodies

To evaluate the systemic humoral immune response to our vaccine candidates, the levels of serum antigen-specific antibodies generated by vaccinated mice were compared to those of CT control mice. Serum was collected by infraorbital ocular bleed before vaccination and after vaccination prior to transurethral challenge with UPEC strain CFT073 (Figure 2.2). Note that collections were also taken after challenge (day 23) in some experiments; the relative antigen-specific antibody measurements were not different from those on day 21. For each protein and loop peptide antigen tested, there was a significant increase of antigen-specific IgG in post-vaccination serum compared to the corresponding pre-immune serum ($P = 0.0002$) (Figure 2.12A-F). In most cases, there was no difference in IgG levels between the pre- and post-vaccinated sera from CT control mice. Additionally, all post-vaccination sera had significantly higher levels of IgM than pre-vaccination sera ($P < 0.05$) (Figure 2.12A-F). Although both IgG1 and IgG2a increased from pre- to post-vaccination, there was no definitive skew toward production of IgG1 or IgG2a by any antigen (Figure 2.13). This phenomenon is not unusual for protein antigens, as they can stimulate production of both Th1 and Th2 cells by virtue of their broad MHC class II peptide repertoire (67). Serum IgA or IgE was undetectable in all samples tested. These findings show that

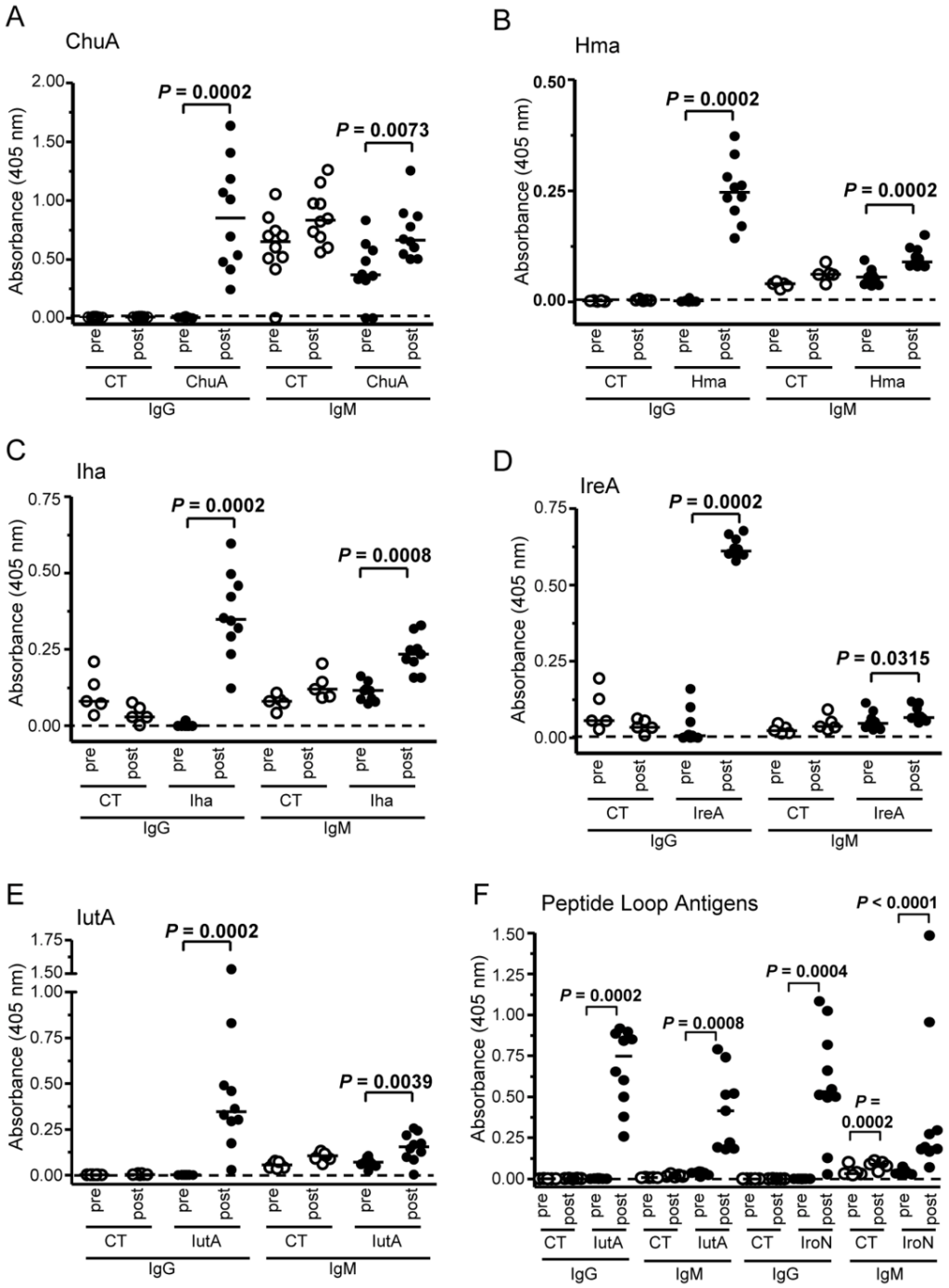

Figure 2.12 Antigen-specific serum IgM and IgG is produced by vaccinated mice. Serum from the indicated antigen-vaccinated mice and corresponding CT control-treated mice was collected prior to vaccination (pre) and after vaccination before transurethral challenge (post) (*n*=5-10 mice per experiment, representative experiments shown). Each symbol represents an individual animal and bars represent the median.

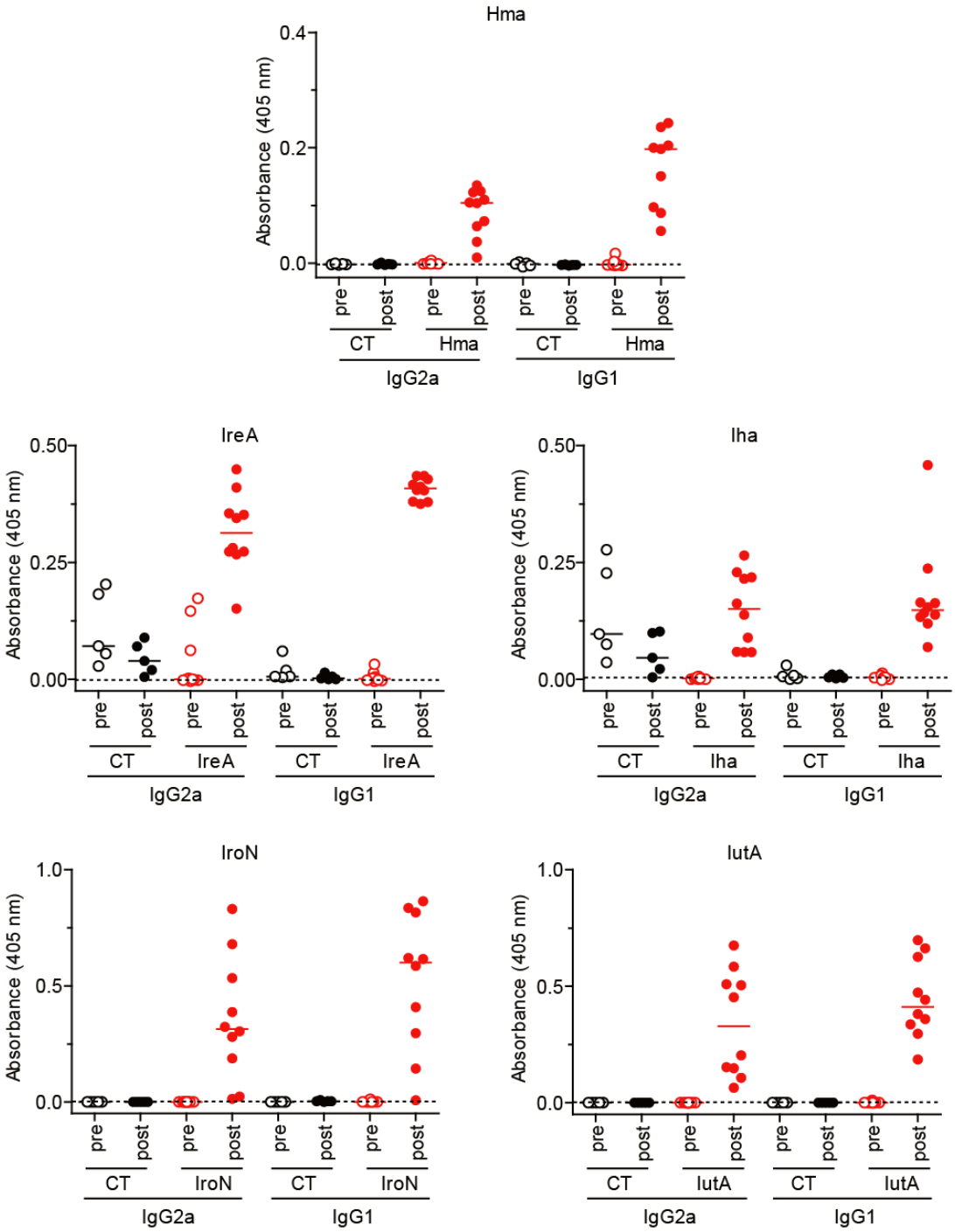

Figure 2.13 Antigen-specific serum IgG2a and IgG1 is produced by vaccinated mice. Serum from the indicated antigen-vaccinated mice and corresponding CT control-treated mice was collected prior to vaccination (pre) and after vaccination before transurethral challenge (post) (*n*=5-10 mice per experiment, representative experiments shown). Each symbol represents an individual animal and bars represent the median. IroN and IutA show data for peptide antigens.

vaccinated mice generate target-specific antibodies in response to intranasal immunization with iron-receptor antigens.

2.4.8 Antibody class-switching and mucosal IgA correlates with protection

IgG and IgM are often monitored to determine the level of class switching undergone by B cells. In our studies, all vaccinated animals demonstrated significant increases in both serum IgG and IgM from pre- to post-vaccination. However, Hma-, IreA-, and IutA-vaccinated (protected) animals displayed more dramatic IgG increases than IgM when compared to ChuA-, Iha-, and peptide loop antigen-vaccinated (unprotected) animals (compare Figure 2.12B, D, and E to Figure 2.12A, C, and F). This finding suggested that these animals have been sufficiently stimulated to class switch to more effective antibody isotypes. To more quantitatively assess this trend and the relationship of serum IgG and IgM to protection from UTI, we first calculated a "class switch index" using the data from all of the immunized mice. The class switch index is the ratio of the median change in sera IgG to the median change in sera IgM for each group of vaccinated mice. To account for non-specific antibody increases, the median changes in sera IgG and IgM of CT-treated mouse cohorts were subtracted from the corresponding antigen-vaccinated group values. The class switch indices were then plotted against the normalized post-challenge median bladder CFU/g for each individual study to determine if any relationship existed between antibody class-switching and protection from cystitis. Because infectivity can vary between experiments, the median CFU/g from each antigen-vaccinated group was divided by the median CFU/g of the CT control group for normalization. Finally, both the class switch index and normalized

median CFU/g values were $\log_{10}$ transformed for comparison on a linear scale. This analysis demonstrated that there was a significant correlation between the class switch index and the CFU/g in the bladder ($P = 0.0014$) (Figure 2.14A), suggesting that a decrease in bladder colonization may be attributed to the relative amount of antibody class-switching from IgM to IgG in mice vaccinated with protective antigens. No significant correlation was found between antibody class-switching from IgM to IgG and CFU/g in the kidneys. However, as predicted, there is also a strong correlation between the amount of antigen-specific IgA in urine and protection from UPEC infection in the bladder (Figure 2.14B); specifically, increases in urine IgA corresponded to decreases in bladder CFU ($P = 0.0165$). These findings show that antibody class-switching and urinary IgA are each an immunological correlate of protection against UTI caused by *E. coli*.

2.4.9 Passive and adoptive transfer of protection

The fact that the class switch index correlated with protection suggested that either IgG itself may mediate protection, or isotypes other than IgM (IgA, for instance) may mediate the protective response to UPEC infection of the urinary tract. To further establish the immune factor(s) involved in protection by intranasal vaccination with UPEC antigens, we conducted pilot passive (sera) and adoptive (cellular) transfer experiments. Groups of 10 CBA/J mice were vaccinated with either or Hma or IreA (protective antigens) as outlined in Figure 2.2, and subsequently their serum was harvested by cardiac puncture and pooled for i.p. administration to 10 naïve recipient animals (~250 μl sera per mouse). We also vaccinated mice with CT alone for control

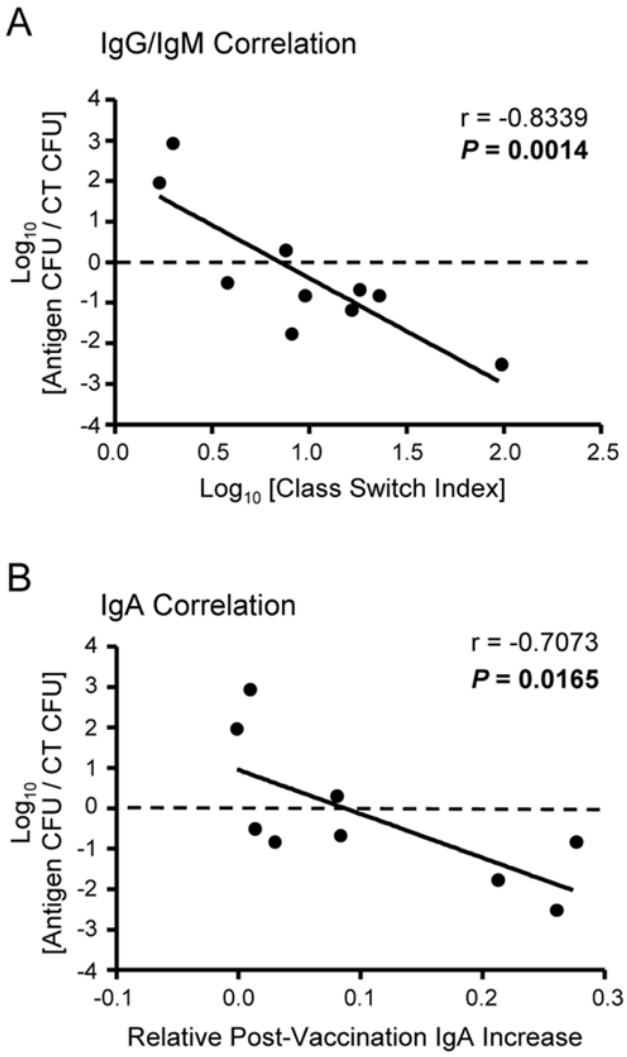

Figure 2.14 Isotype class-switching and urinary IgA correlates with vaccine-mediated protection from infection. (A) The class switch index [(ΔIgG_{VAC} - ΔIgG_{CT}) / (ΔIgM_{VAC} - ΔIgM_{CT}), where VAC indicates antigen vaccinated mice and CT indicates adjuvant control mice] was plotted against the normalized bladder CFU (group median CFU_{VAC} / group median CFU_{CT}) for each vaccination experiment (n = 203 mice total). Thus, each individual point reflects the collective data from an experiment involving at least 10 vaccinated and 5 CT mice. In most cases, experiments were repeated twice. Lower normalized CFU values indicate enhanced bacterial clearance after challenge, where higher class switch indices represent superior B cell activation. (B) Normalized bladder CFU values from each vaccination experiment are plotted against the median change in absorbance of antigen-specfic IgA detected in the urine (ΔIgA_{VAC}). Pearson correlation coefficient (r) and P values are shown.

sera to transfer to naïve mice. In addition to the sera, we collected spleens from CT control or iron receptor antigen-vaccinated animals. After lysing the red blood cells, $2.5x10^6$ splenocytes from each of the three groups (pooled from 4 spleens) were administered to 10 naïve mice by intravenous tail vein injection (in 100 μl PBS per animal). At the time of transfer, mice receiving either sera or splenocytes from CT-treated, IreA-vaccinated, or Hma-vaccinated animals were transurethrally challenged with $1x10^8$ CFU of UPEC strain CFT073. After 48 hours, recipient animals were sacrificed and colonization of their bladders and kidneys was assessed. While none of the mice receiving sera or splenocytes from IreA- or Hma-vaccinated animals had statistically significant protection when compared to mice receiving sera or splenocytes from CT-treated animals, there were some trends toward protection exhibited by "IreA recipient mice" (Figure 2.15A and B). Specifically, naïve mice receiving splenocytes from IreA-vaccinated animals showed a trend toward protection in the bladder, and naïve mice receiving sera from IreA-vaccinated animals showed a trend toward protection in the kidney. One interpretation of these results is that antigen-specific IgA-secreting plasma cells in the splenocyte population afforded some protection in the bladder, while antigen-specific IgG contained in the sera from vaccinated animals afforded some level of protection in the kidney tissue. Additional experiments involving passive transfer of higher volumes of sera or adoptive transfer of either higher numbers of splenocytes or plasma cells purified from the spleens and inguinal lymph nodes of vaccinated mice will further establish whether or not antigen-specific antibodies not only correlate with protection, but mediate the protective response to UPEC infection of the urinary tract.

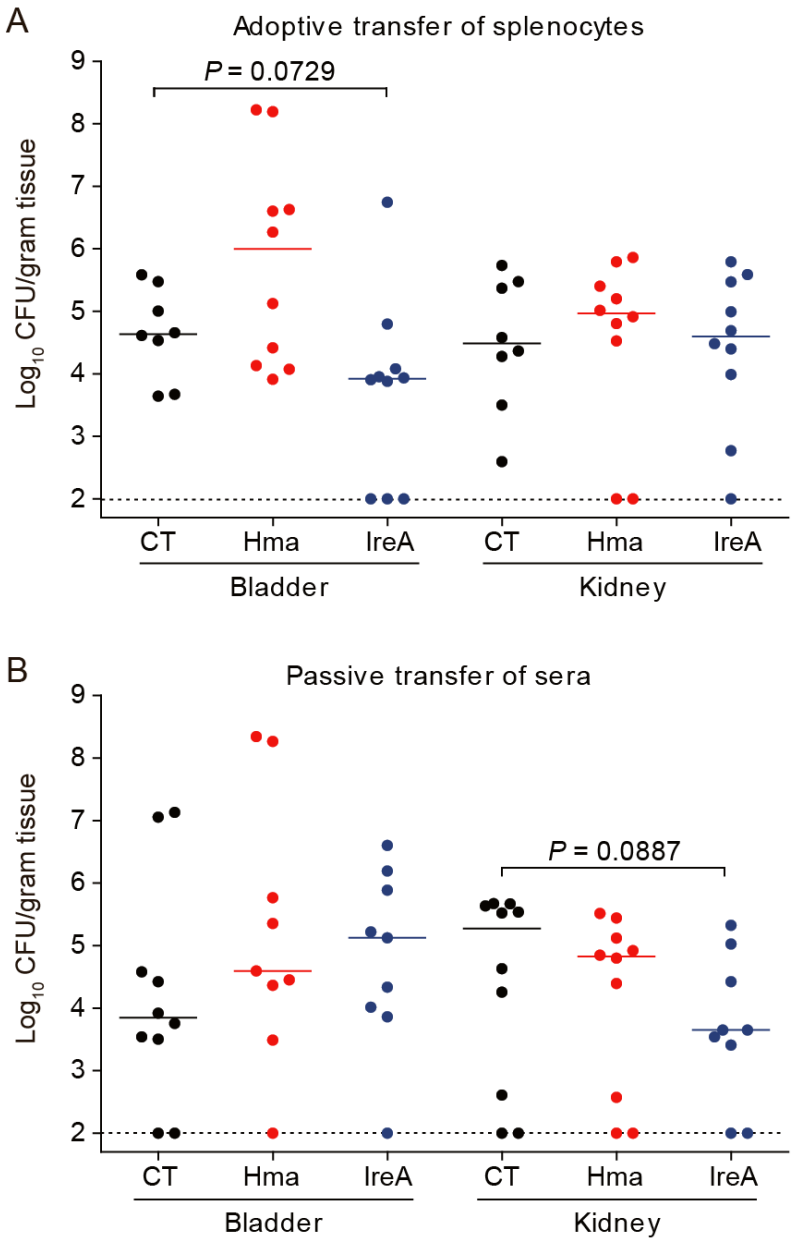

Figure 2.15 Transfer of immunity from iron receptor antigen-vaccinated animals to naïve recipients. Either sera or splenocytes from CT control or iron receptor antigen-vaccinated animals (*n*=10 each) were transferred to naïve recipients (*n*=10 each) as described in the text. (A) Colonization of mice receiving splenocytes from adjuvant-treated or iron receptor-vaccinated animals, indicated on the x-axis. (B) Colonization of mice receiving sera from adjuvant-treated or iron receptor-vaccinated animals, indicated on the x-axis. Note the *P*-values are trends, and not statistically significant.

2.5 Discussion

2.5.1 Summary

UTIs caused by UPEC represent a significant healthcare burden that could be alleviated by the development of a protective vaccine. Toward this aim, a multi-pronged, functional vaccinology approach was used, and this approach uniformly singled out one class of molecules, those involved in iron acquisition, as *PASivE* protein targets. The screening process selected six vaccine candidates from the 5379 predicted proteins encoded within the *E. coli* CFT073 genome. The six vaccine candidates meet the criteria for *PASivE* vaccine candidates. They were conserved among UPEC strains (137, 248), antigenic (137), predicted to be surface-exposed and present in the bacterial OM (8, 137), transcriptionally upregulated *in vivo* (386), and induced during culture in human urine (8). These vaccine targets, ChuA, Hma, Iha, IreA, IroN, and IutA, are all OM β-barrel proteins that function as receptors for iron-containing compounds. Of these, we found that intranasal immunization with Hma, IreA, or IutA generates an antigen-specific humoral response, antigen-specific upregulation of IL-17A transcript and secretion of IL-17A and IFN-γ, and provides significant protection against experimental infection with UPEC.

2.5.2 Protection and physiology

Significant protection was observed in mice vaccinated with IutA, Hma, or IreA (Figure 2.3). While protection by IreA and Hma appeared to be site-specific in response to challenge with CFT073 (IreA in the bladder and Hma in the kidney, Figure 2.3B and D), heterologous challenge with UPEC strain 536 revealed converse protection patterns

(Figure 2.4), perhaps due to differences in bacterial expression of IreA and Hma during infection. Alternatively, with respect to IreA, reduction in the level of bacterial colonization within the bladder may have resulted in reduced numbers of bacteria ascending to the kidneys, potentially accounting for the modest reduction in bacteria seen in the kidneys in the IreA-vaccinated mice following challenge with either UPEC strain CFT073 or 536 (Figure 2.4).

In contrast to the bladder protection seen with IreA, Hma significantly reduced the number of bacteria colonizing the kidneys and, in over half of the animals, prevented kidney colonization completely in response to challenge with CFT073 (Figure 2.3). The kidney-specific protection of the Hma vaccine may reflect the biological function of Hma for UPEC during colonization of the urinary tract; UPEC that are unable to produce Hma have reduced fitness only within the kidney during experimental UTI (136). This result suggests that the immune response may perturb the normal function of this OM heme receptor, perhaps by antibody interference with ligand-binding domains or selective immune targeting of bacteria that exhibit tissue-specific expression of Hma.

2.5.3 Protection and cytokines

To assess cellular responses to immunization, both antigen-specific upregulation of proinflammatory transcripts and antigen-specific secretion of IFN-γ and IL-17A, known to be critical for mediating anti-bacterial activities within the host, were analyzed. Production of IFN-γ has been previously shown to be important for the control of infection within the urinary tract (198) and IL-17A has been shown to promote the recruitment of neutrophils in response to bacterial infection (261). While a number of

transcripts were upregulated significantly, only IL-17A upregulation was shown to be antigen-dependent in this system (Figure 2.8 and Figure 2.9). IL-17A has been recently appreciated as an important mediator to control infection by *Salmonella*, *Listeria*, pathogenic *Mycobacterium*, *E. coli*, and *Klebsiella* (142, 363, 376, 438, 464). With regard to secretion, however, both IFN- γ and IL-17A were produced from splenocytes originating from IreA and Iha immunized mice in response *in vitro* stimulation (Figure 2.10). The fact that splenocytes obtained from vaccinated mice post-challenge showed a reduction in antigen-specific cytokine secretion compared to splenocytes obtained from mice pre-challenge suggests that antigen-specific lymphocytes may be homing from the spleen to the site of infection. Further, the post-challenge reduction in both antigen-specific cytokines tested was only significant for the IreA vaccine, which generates a protective response in the bladder.

2.5.4 Protection and antigen-specific antibodies

Mucosal immunization is considered the most effective means to develop a UTI vaccine and the Mobley Lab has shown previously that intranasal immunization with MR/P fimbria is effective in protecting mice against UTI caused by *P. mirabilis*, an agent of complicated UTI (244). Because local immunization of the urethra is not practical, the migration of immune cells between mucosal sites can be exploited during intranasal inoculation with antigen (207). We reasoned that intranasal immunization would generate a distant mucosal response in the genitourinary tract against UPEC in vaccinated animals. Consistent with this, we observed that the two vaccines, IreA and IutA, which conferred significant protection in the bladder, had the greatest antigen-specific

production of secretory IgA detectable in their urine (Figure 2.11). Conversely, antigens that did not induce protective immunity within the bladder did not generate a similar increase in the level of IgA. Based upon these findings, relative levels of IgA in the urine significantly correlated with protection in the bladder. That is, when examining all of the vaccine candidates and bacterial counts within the bladder, there is a direct relationship between a reduction in CFU in the bladder and increased IgA in the urine (Figure 2.14B). This immunological correlate suggests that a mucosal response and vaccine that generates IgA is sufficient to provide protection from UPEC colonization in the bladder. This finding is consistent with other studies that have shown that antibodies specific for the infecting *E. coli* strains leads to resolution of cystitis and that oral immunization with OM proteins generates an antigen-specific IgA mucosal response against UPEC (173, 238).

The finding that IgA in the urine is produced in response to intranasal immunization indirectly shows that class-switching of antibody isotypes is occurring in vaccinated animals. Analysis of serum antibodies from immunized mice shows that antigen-specific IgM is produced in response to all the vaccine candidates tested. Further, antigen-specific IgG is markedly increased in response to each vaccine when compared to pre-immune sera (Figure 2.12). For the Hma and IutA vaccines, which provided significant protection in the kidney, it is possible that circulating IgG or antigen-specific plasma cells migrate to the kidney to contribute to reduction of bacterial colonization. Interestingly, the vaccines that significantly protected mice against UPEC infection, Hma, IreA, and IutA, displayed a dramatic increase in antigen-specific IgG relative to IgM. To assess this effect, we mathematically defined a class switch index, a

value that significantly correlated with protection from bacterial colonization in the bladder (Figure 2.14A).

It is notable that both CT-treated and antigen-vaccinated mice experience increases on antigen-specific IgM, with increases in antigen-specific IgM being more dramatic in the antigen vaccinated mice (Figure 2.12). Increases in the control mice may be a result of CT stimulation of the immune system, whereas in the case of the antigen-vaccinated mice serum increases, naïve B cells that encounter antigen by ligation with surface IgM undergo proliferation (453). This proliferation results a clonal population of B cells specific for the iron receptor of interest and capable of secreting more antigen-specific IgM. In animals vaccinated with non-protective antigens, these cells are clearly not receiving additional signals (chemokines, for example) that are necessary for migration to lymphatic germinal centers and class-switching. Hence we observe more antigen-specific IgM in the serum of non-protected animals, and antigen-specific IgG in the serum of protected animals, indicating that class-switching of antibody isotypes is indicative of a protective immune response. The class switch index could potentially be further refined by the inclusion of total antibody (antigen-specific and non-specific) to account for this variable in the measurement of serum antibody.

Whether antibodies targeting OM iron-related receptors neutralize UPEC by interfering with iron acquisition or provoke a bactericidal immune response (*i.e.*, opsonization or activation of complement) is a topic to be investigated. Because of their physiological locales in the body, it is likely that IgA directly neutralizes UPEC, as phagocytes are not in excess in the mucosa, and IgG either opsonizes bacteria in the tissue or activates the complement system in the bloodstream. It is conceivable that

UPEC can evade the activity of these antibodies if the iron receptors are truly functionally redundant, as their expression could be modified to obtain iron in the case of neutralization or evade the immune system in the case of opsonization or complement activation. These ideas are also based on the assumption that there is no inherent killing property elicited by the antibody binding in itself, which the literature suggests otherwise (98). In any case, deciphering between these strategies using *in vitro* assays is an important next step to better understand what types of host response are necessary to overcome UPEC infection *in vivo*.

2.5.5 *PASivE* vaccine candidates in context of previous UPEC vaccination studies

There has been limited success to develop an efficacious UTI vaccine that would provide protection from infection by UPEC. Immunization with Solco-Urovac, a vaccine formulation that is comprised of inactivated uropathogenic bacteria, generates urinary IgA (288) and vaginal immunization with this vaccine provides protection that lasts eight to twelve weeks in humans (429). If administered more frequently, vaccination with Solco-Urovac can increase time to recurrence to six months (171). This suggests this vaccine might be useful to prevent recurrent UTIs in susceptible populations; however, the frequency of administration poses a problem with the general target population. One of the vaccine candidates we identified and tested, IroN, has been used previously to immunize mice, and similar to our results with IroN, was shown to generate antigen-specific IgG but not IgA (349). In contrast to our findings, IroN was previously found to protect mice against renal infection. This difference may be accounted for by the fact different forms of IroN and routes were used for vaccination: peptides representing IroN

extracellular loops mixed with CT given intranasally versus denatured IroN without adjuvant given subcutaneously (349). Lastly, using a lethal challenge model of extraintestinal pathogenic *E. coli* infection, it was found that vaccination with IroN, but not ChuA, increased survival in immunized mice (78). The results presented here are consistent with ChuA being non-protective (and unfortunately lethal); however, protection from UTI was not afforded by IroN vaccination. The discrepancy may be due to the ability of epitopes in the IroN sequence to stimulate class-switching to IgG, which would presumably be protective systemically, rather than mucosal IgA antibody, which likely mediates protection in the bladder mucosa.

2.5.6 Conclusions

Despite these advances, a UTI vaccine that confers long-term protection against uncomplicated UTIs is currently lacking. The rational large-scale screening process we described identified six surface-exposed OM receptors for iron compounds as *PASivE* vaccine candidates. Iron acquisition is well known to be a common trait necessary for bacterial pathogenesis, and by targeting receptors involved in this process, it may be possible to disrupt this critical function thereby neutralizing UPEC. Intranasal inoculation with three of six vaccine candidates (IreA, Hma, and IutA) provided protection from challenge with UPEC and this study provides the basis for the development of a subunit vaccine that would incorporate these protective antigens to provide broader efficacy within the urinary tract and across uropathogenic isolates. Importantly, this work shows that focusing on an entire class of proteins that are involved in a singular function necessary for pathogenesis rather than a single protein may be

fundamental strategy that could be generally adopted during the development of vaccines against pathogens. Administered together, the antigens may afford sterilizing immunity in both the bladder and the kidney. Lastly, this work also yielded immunological correlates of protection, a necessary asset in future UTI vaccine endeavors.

Chapter 3
The innate immune response to Uropathogenic *Escherichia coli* involves IL-17A in a murine model of UTI

3.1 Abstract

UPEC strains express a number of virulence and fitness factors that allow successful colonization of the mammalian bladder. To combat this, the host has distinct mechanisms, not only to prevent adherence to the bladder wall, but to detect and kill UPEC in the event of colonization. In this study, we investigated the role of IL-17A, an innate-adaptive immunomodulatory cytokine, during UTI using a murine model. Splenocytes, isolated from mice infected by the transurethral route, robustly expressed IL-17A in response to *in vitro* stimulation with UPEC antigens. Transcript expression of IL-17A in the bladders of infected mice correlated with a role in the innate immune response to UTI, and γδ-positive cells appear to be a key source of IL-17A production. While IL-17A appears to be dispensable for the generation of a protective response to UPEC, its importance in innate immunity is demonstrated by a defect in acute clearance of UPEC in IL-17A$^{-/-}$ mice. This clearance defect is likely a result of deficient cytokine and chemokine transcripts and impaired macrophage and neutrophil influx during infection. These results show that IL-17A is a key mediator for the innate immune response to UTI.

3.2 Introduction

Uncomplicated UTI occurs in otherwise healthy individuals that lack any urinary anatomical abnormalities. The fact that recurrence of uncomplicated UTI is so prevalent argues against the thought that an acquired immune response if formed in response to natural infection. Alternatively or in addition, high recurrence rates also suggest that UPEC are highly heterogeneous group of pathogenic strains. The importance of the innate immune response has been recognized in governing an effective adaptive immune response. When UPEC adheres to the uroepithelium, thereby establishing itself in the urinary tract, a robust innate immune response is generated. TLRs have been shown to be important for UPEC recognition and immune mobilization (10, 374, 469). A number of secreted factors such as AMPs, THP, cytokines, and chemokines are detected in the mammalian bladder upon infection (63, 166, 186, 352, 354, 404). Among cellular infiltrate, neutrophils are the most abundant early responders in infected bladders (147). Additionally, macrophages (167), DCs (84), and ILLs such as γδ T cells (198) have been implicated in innate host defense to UPEC-mediated UTI. Despite this knowledge, the role of each of these factors in the generation of a sterilizing recall response is not understood, and there are likely other undescribed factors that also regulate this process.

IL-17A or the IL-17R has been shown to play a critical role in autoimmune disease (32, 261) and in bacterial (142, 187, 363, 376, 438, 465, 471), fungal (68, 178, 343, 442), and even viral (141, 177, 209) infection. Because of the roles played in both arms of the immune system, IL-17A has emerged as an innate-adaptive immunomodulatory cytokine. Regarding the innate immune response to infection, IL-17A acts by indirectly enhancing neutrophil migration to infected tissue. Specifically,

transcripts for cytokines involved in granulopoiesis and chemotaxis in cells treated with IL-17A have enhanced stability (148-150, 163). Aside from being the signature cytokine secreted by $CD4^+$ Th17 helper cells, other cell types have been reported to secrete IL-17A, including cytotoxic T cells, γδ T cells, NKT cells, neutrophils, eosinophils, and monocytes (32). Although the exact PAMPs that trigger secretion of IL-17A are not defined, components of microbial cell wall, host receptors that recognize such components (*i.e.*, the mannose receptor), and TLR-related signaling pathways are implicated (442, 471).

In an effort to characterize the immune response to known UPEC antigenic OM proteins (137), we discovered that IL-17A was secreted by *in vitro* stimulated splenocytes derived from UPEC iron receptor-vaccinated mice (Figure 2.10) (7). Given the importance of IL-17A in controlling mucosal infection (76), the role of IL-17A during the innate and adaptive immune response to UTI was formally investigated. In the murine model, IL-17A is upregulated by *in vivo* sensitized secondary lymphoid tissue cells in response to *in vitro* stimulation, yet IL-17A appears to be dispensable for the generation of protective immunity. IL-17A is also upregulated in the bladder in response to acute infection, and both γδ-positive cells and IMs are a major source of secreted IL-17A in the bladder tissue. Lastly, IL-17A appears to play a role in regulating the innate immune response to UTI; mice lacking IL-17A exhibit both deficient cytokine transcript upregulation and cellular responses during acute UTI, resulting in suboptimal clearance of UPEC.

3.3 Materials and Methods

3.3.1 Animals

3.3.1.1 Strains

For these studies, C57BL/6 mice were used instead of the CBA/J model because knockout mice used were in this background. Of note, the CBA/J mouse model of UTI typically involves ascension of bacteria from bladder to the kidneys (138), whereas C57BL/6 mice usually exhibit colonization confined to the bladder, with more modest ascension to the kidney (172, 198). The factor(s) involved in this phenotype are not known. C57BL/6 wild type mice TCR $\delta^{-/-}$ mice deficient in $\gamma\delta$ TCR expression in all adult lymphoid and epithelial organs (188) were purchased from Jackson Laboratories (Bar Harbor, ME). Breeding pairs of IL-17A$^{-/-}$ mice were obtained as a gift from Yoichiro Iwakura (University of Tokyo) (285). IL-17R$^{-/-}$ mice were obtained as a gift from Weiping Zou (University of Michigan). All experiments were conducted when animals were 6 to 15 weeks old, and C57BL/6 wild type mice with birth dates within one week of the knockout mice (all in the C57BL/6 background) were used.

3.3.1.2 Manipulation

All infections (including sensitizations and challenges) consisted of $5x10^7$ CFU per mouse were administered as described in 2.3.2. CFU was also determined as described in 2.3.2, and in some experiments, homogenates were centrifugated (16,100 x *g*, 10 min, 4°C) for collection and storage (-20ºC) of supernatants for ELISA.

3.3.2 Bacterial strains and whole cell lysate preparation

E. coli CFT073 was described in 2.3.1. EFC4 is a fecal strain isolated from a healthy woman (270). For whole cell lysate preparation, a single colony of *E. coli* strain CFT073 was inoculated into 250 ml sterile human urine (pooled from 5 healthy donors) and cultured statically at 37°C until late stationary phase. Bacterial cells were harvested by centrifugation (8,000 x *g*, 5 min, 4°C), washed, resuspended in 10 ml PBS, and incubated at room temperature with 2 μl benzonase solution (10 U/μl, Sigma) for 30 min. Bacterial lysis and protein quantitation was done as described in 2.3.3. Lysate was cleared by centrifugation (8,000 x *g*, 5 min, 4°C) and sterilized using a 0.22 μm filter (Millipore).

3.3.3 Tissue culture

Spleens ($1.5x10^6$ cells/well) or the inner inguinal (lumbar) lymph nodes ($5x10^5$ cells/well), responsible for draining the pelvic viscera, were harvested, made into single cell suspensions, and cultured as described in 2.3.6. Lymph nodes from the same groups of animals were pooled and plated in replicate wells. Cells were cultured with medium, 5 μg/ml α-CD3 [clone 145-2C11, a gift from Dr. Cheong-Hee Chang (University of Michigan)], or 25 μg/ml *E. coli* CFT073 whole cell lysate, and incubated at 37°C, 5% CO_2 for 72 h at which point supernatants were harvested and stored at -20°C for ELISA.

3.3.4 Kinetic MPO assay

Groups of 10 mouse bladders were homogenized in 1 ml 2x Homogenization Buffer (in ddH_2O: 0.5 g HTAB, 0.1 ml EDTA, 43.9 ml 1.0 M monobasic potassium phosphate, 6.2 ml 1.0 M dibasic potassium phosphate, pH 6.0) and kept on ice. 0.1 ml of homogenate was then sonicated to complete cellular lysis. Samples were centrifugated

(15,700 x *g*, 15 min, 4°C), and 0.01 ml of supernatant was aliquotted into 96-well plates. 0.140 ml of Assay Buffer (in ddH_2O: 4.39 mL 1.0 monobasic potassium phosphate, 0.615 ml 1.0 M dibasic potassium phosphate, 0.0833 ml H_2O_2, 0.834 ml ODH reagent) to each sample, mix briefly, and read absorbance at 460 nm immediately using a kinetic setting with collections every 10 sec for 3 min. Homogenization buffer alone was used as a black, and the slopes (A460/min) were used to report MPO Units. Control and infected mouse lungs from Thomas Moore (University of Michigan) were used as negative and positive controls, respectively.

3.3.5 ELISAs

Cytokine ELISAs were performed as described in 2.3.8. For detection of MPO in bladder homogenates, a Mouse MPO ELISA Kit (Hycult Biotechnology) was used. Tissue homogenate preparation and ELISA protocol were done according to manufacturer's instructions.

3.3.6 RNA isolation, cDNA synthesis, and PCR

3.3.6.1 qPCR

For qPCR analyses, RNA was isolated from bladder tissue using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. cDNA synthesis, qPCR reactions, and subsequent analysis were done as described in 2.3.7. For G-CSF, forward (5'-tcc ctg gag caa gtg agg aa-3') and reverse (5'-gct tgt agg tgg cac aca act g-3') primers were designed using Taqman Probe and Primer Design Software (Applied Biosystems). This qPCR reaction consisted of: 9.5 μl RNase-free water, 12.5 μl 2x SYBR Green PCR Master Mix, 1 μl each of forward and reverse primer, and 1 μl cDNA. Cytokine

transcripts were normalized to that of GAPDH ($2^{-\Delta Ct}$, where ΔC_t equals the cycle threshold of test gene minus the cycle threshold of GAPDH).

3.3.6.2 RT-PCR

For RT-PCR analyses, RNA was isolated from bladder tissue using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Genomic DNA was removed from the samples using TURBO DNA-*free* (Ambion) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of DNase-treated RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) using gene-specific primers for common γ and δ chains [Cγ (256), Cδ (326)] according to the manufacturer's instructions. PCR for γ and δ variable chain transcripts was conducted using 2 μl Cγ or Cδ-generated cDNA, respectively, and either Taq Polymerase (Invitrogen) or Phire Hot Start DNA Polymerase (Finnzymes) according to the manufacturer's recommendations with a melting temperature of 54ºC (with the exception of Vγ5 and Vδ8, which were amplified with a melting temperature of 51ºC). Primer sets used were as follows: Vγ1.1 with Jγ4 (460); Vγ1.2 with Cγ (255); Vγ2-4 (121) and Vγ5 (5'-cct act tct agc ttt ctt gc-3') with Jγ1 (5'-ctt acc aga ggg aat tac tat gag-3'); Vδ1-3 and 5-8 (460), and Vδ4 (255) with Jδ1 (326). Nomenclature used is according to Garman (113). As with qPCR, RT-PCR reactions were conducted with appropriate no template and no RT controls for cDNA and qPCR reactions.

3.3.7 Histology

Bladder tissue was harvested at necropsy and fixed in 10% neutral buffered formalin for 24 h. Histology was performed by the Pathology Cores for Animal Research in the Unit for Laboratory Animal Medicine at the University of Michigan. Histopathological assessment was performed by pathologist Dr. Ingrid Bergin, VMD, MS, DACLAM, DACVP. Tissues were trimmed and processed by standard histological methods and were stained with hematoxylin and eosin. Light microscopic histopathological assessment was performed blinded to the group assignment of the samples. The presence or absence of inflammation and the predominating type and tissue distribution of inflammatory cells was qualitatively assessed. Additional points assessed were the presence/absence of bacteria, subjective assessment of bacterial quantity and distribution, and mucosal changes.

3.3.8 Neutrophil counts

Urine was collected by massaging the mouse abdomen while holding the urethra 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 enumerated using a hemacytometer.

3.3.9 Cellular staining and flow cytometry

Bladders, isolated from euthanized mice, were cut into small pieces with a scalpel. Tissue was digested for 50 min at 37°C with agitation in 0.5% heat inactivated FBS, 20 mM HEPES pH 7, 0.057 Kunitz units/μl DNase I (Sigma), and 1 mg/ml collagenase A (Roche) in RMPI medium, with repeated passage through an 18½ gauge needle 25 min into the incubation. Erythrocytes were lysed as described in 2.3.6.

Erythrocyte-lysed homogenates were filtered through 40 micron cell strainers and washed once with flow cytometry buffer (1% FBS, 0.01% NaN_3 in PBS). After enumeration by hemacytometer, cellular suspensions were treated with mouse anti-CD16/CD32 (clone 2.4G2, eBioscience) for 10 min to block Fc receptors. Surface markers were stained for 30 min, and cells were fixed overnight in a 4% formalin solution. For intracellular staining, fixed cells were permeabilized with 1% FBS, 0.1% saponin in PBS, and stained for 60 min. Data were acquired using a BD FACSCanto flow cytometer and BD FACSDiva software, and analyzed using FlowJo v7.2.4 (Tree Star, Inc.). Mouse anti-CD4-FITC (clone GK1.5), anti-γδ-TCR-FITC (clone eBioGL3), anti-F4/80-FITC (clone BM8), anti-IL-17A-PE (clone eBio17B7), anti-CD8a-PE (clone 53-6.7), anti-CD4-PE-Cy7 (clone GK1.5), anti-Ly-6G(Gr-1)-PE-Cy7 (clone RB6-8C5), anti-CD45R(B220)-APC (clone RA3-6B2), anti-MHC Class II(I-A/I-E) (clone M5/114.15.2), anti-CD8a(Ly-2)-APC-Cy7 (clone 53-6.7), and anti-CD11b-APC-Cy7 (clone M1/70) used staining and calibration controls were obtained from eBioscience.

3.3.10 Statistics

Graphing and statistical analyses were done using GraphPad Prism 5. Data were represented as mean or median values based on the D'Agostino & Pearson omnibus normality test. Where applicable, the Mann-Whitney test, Paired *t*-test, or Fisher's exact test was used to determine statistical significance with two-way analysis of variance and 95% confidence intervals.

3.4 Results

3.4.1 Adaptive immune responses and IL-17A

3.4.1.1 IL-17A is secreted by spleen and lymph node cells from C57BL/6 mice in response to transurethral infection with UPEC

To determine if lymphoid cells taken from infected mice secrete IL-17A in response to stimulation with UPEC antigens *in vitro*, wild type C57BL/6 mice were inoculated the transurethral route with either UPEC strain CFT073 or PBS according to the outlined schedules (Figure 3.1). Cells harvested from the spleen and the inner inguinal lymph nodes were stimulated *in vitro* with medium alone, α-CD3 monoclonal antibody (mAb, as a positive control), or UPEC strain CFT073 whole cell lysate and incubated for 72 h before harvesting supernatants for ELISA. Seventy-two hours was chosen because it was the peak of cytokine secretion from spleen and lymph node cells in dose dependence experiments (Figure 3.1C). Figure 3.1C also demonstrates that whole cell lysate was a superior stimulation agent compared to whoel killed bacterial cells. As expected, splenocytes treated with α-CD3 mAb had high expression of IL-17A, regardless of whether they originated from PBS- or CFT073-treated animals, and unstimulated cells from either treatment group did not secrete IL-17A (Figure 3.1A). However, in response to *in vitro* stimulation with UPEC whole cell lysate, only the splenocytes from UPEC-infected mice showed significant secretion of IL-17A compared to unstimulated controls (P=0.0147) (Figure 3.1A). Additionally, inner inguinal lymph node cells from UPEC-infected mice secreted significantly higher amounts of IL-17A in response to *in vitro* lysate stimulation than lymphoid cells from PBS-treated animals (P=0.0105) (Figure 3.1B). These results indicate that treatment with UPEC antigens

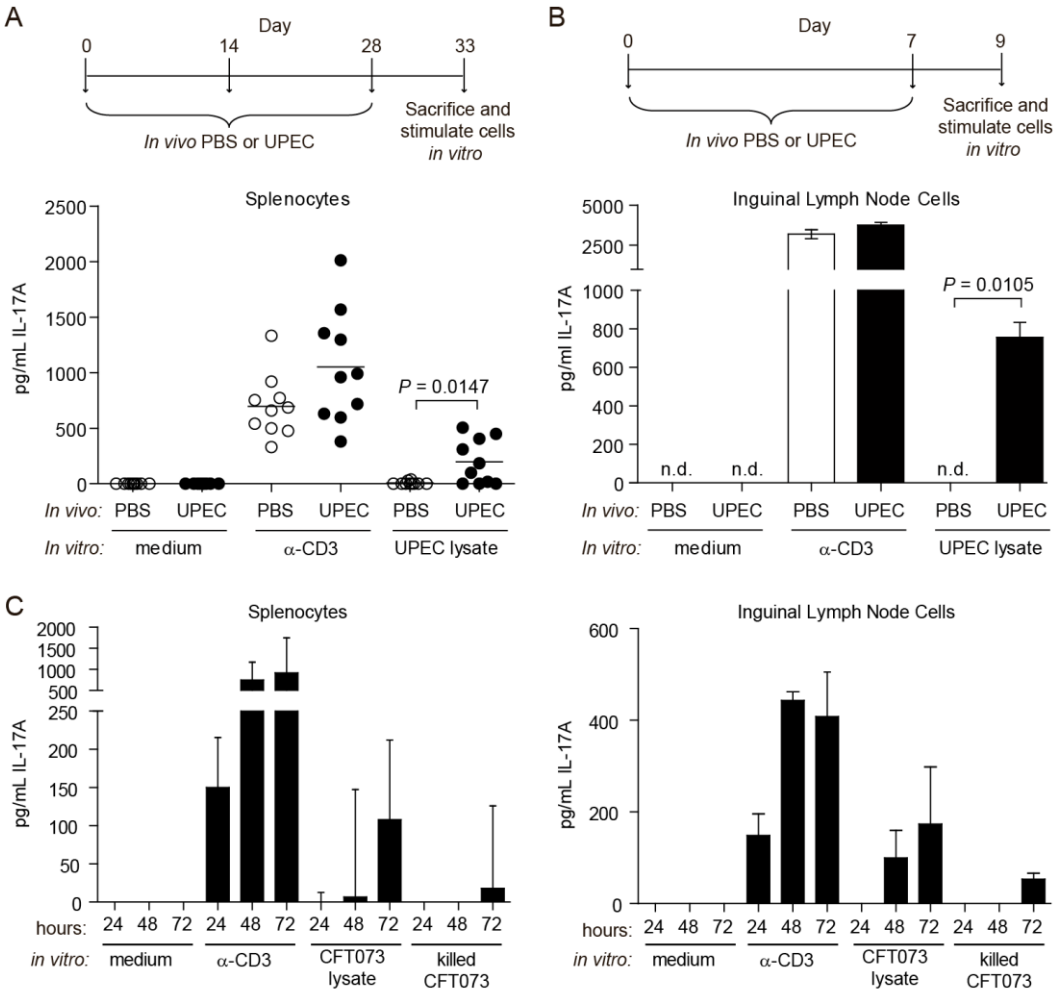

Figure 3.1 Spleen and inner inguinal lymph node cells from C57BL/6 mice receiving UPEC infection secrete IL-17A. (A) Mice were sensitized transurethrally with either PBS (open symbols) or UPEC (filled symbols) on days 0, 14, and 28, and were sacrificed on day 33. Upon sacrifice, splenocytes from individual animals (*n*=10) were stimulated with medium alone, 5 μg/ml α-CD3 mAb, or 25 μg/ml whole cell lysate from UPEC strain CFT073 for 72 h. Each symbol represents an individual animal while bars represent the median values in a representative experiment. (B) Mice were sensitized transurethrally with either PBS (open bars) or UPEC (filled bars) on days 0 and 7 and sacrificed 2 days after the second sensitization, day 9. Pooled inguinal lymph node cells from each group of animals (*n*=6) were plated in replicate wells and stimulated as in (A). Bars and error represent the mean ± SEM of triplicate ELISA wells in a representative experiment. n.d., not detectable. (C) Time-dependence of experiments shown in (A) and (B). Bars and error represent the median and range.

stimulates adaptive secretion of IL-17A from *in vivo*-sensitized cells from both systemic and local lymphoid origins.

3.4.1.2 IL-17A is not necessary for the adaptively-acquired protective immune response to UTI

Because IL-17A was secreted in response to UPEC antigens (Figure 3.1) and the role that IL-17A plays in bacterial vaccination models (165, 250), we wanted to determine whether IL-17A is required for the generation of a protective immune response to UTI. To do this, a reinfection model based on one presented by Thumbikat *et al.* was utilized (416). Wild type and IL-17A$^{-/-}$ mice were transurethrally infected once (1x), twice (2x), or not at all (N), prior to a 48 h challenge infection with UPEC (Figure 3.2). Both wild type sensitization groups had significantly fewer bacteria in their bladders when compared to wild type naïve mice (P=0.0193 for 1x and P=0.0016 for 2x compared to N) (Figure 3.2), consistent with a previously published study (416). Sensitized IL-17A$^{-/-}$ mice also exhibited a similar pattern of accelerated clearance (P=0.0071 for 1x and P=0.0062 for 2x compared to N) (Figure 3.2). Of note, a primary infection with UPEC is typically cleared from the urinary tract by day 7 post infection (Figure 3.3B). These results demonstrate that both wild type and IL-17A$^{-/-}$ mice experience enhanced clearance in response to a secondary infection, and suggest that IL-17A is not required for the generation of a protective immune response to UTI.

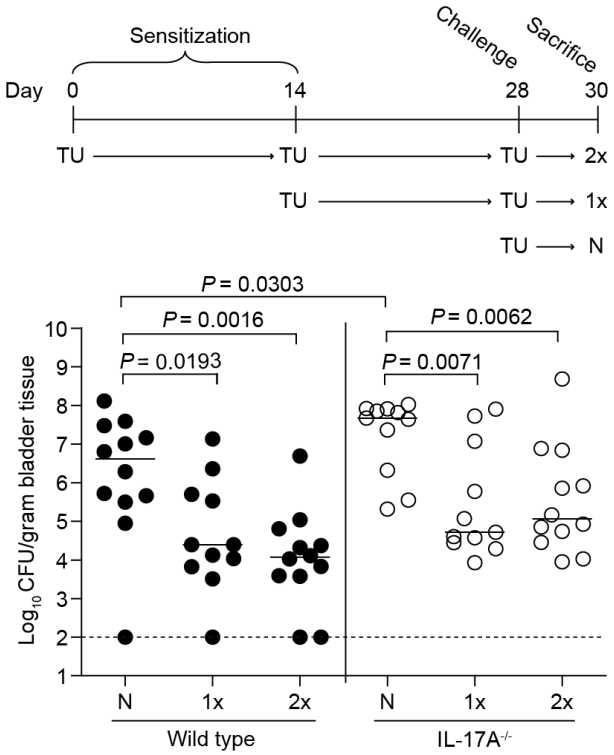

Figure 3.2 IL-17A is not necessary for the protective (re-challenge) response to UTI. Cohorts of wild type and IL-17A$^{-/-}$ mice (*n*=11-12 each, 2 experiments combined) were sensitized by the transurethral route with UPEC strain CFT073 once (1x), twice (2x), or not at all (N for naïve) on the indicated days. Subsequently, all mice were challenged with CFT073 on day 28. Bladder colonization levels 48 h post challenge for wild type (filled symbols) and IL-17A$^{-/-}$ (open symbols) mice are shown. The dotted line indicates the 100 CFU/gram tissue limit of detection. Each symbol represents an individual animal while bars represent the median values. TU=transurethral infection.

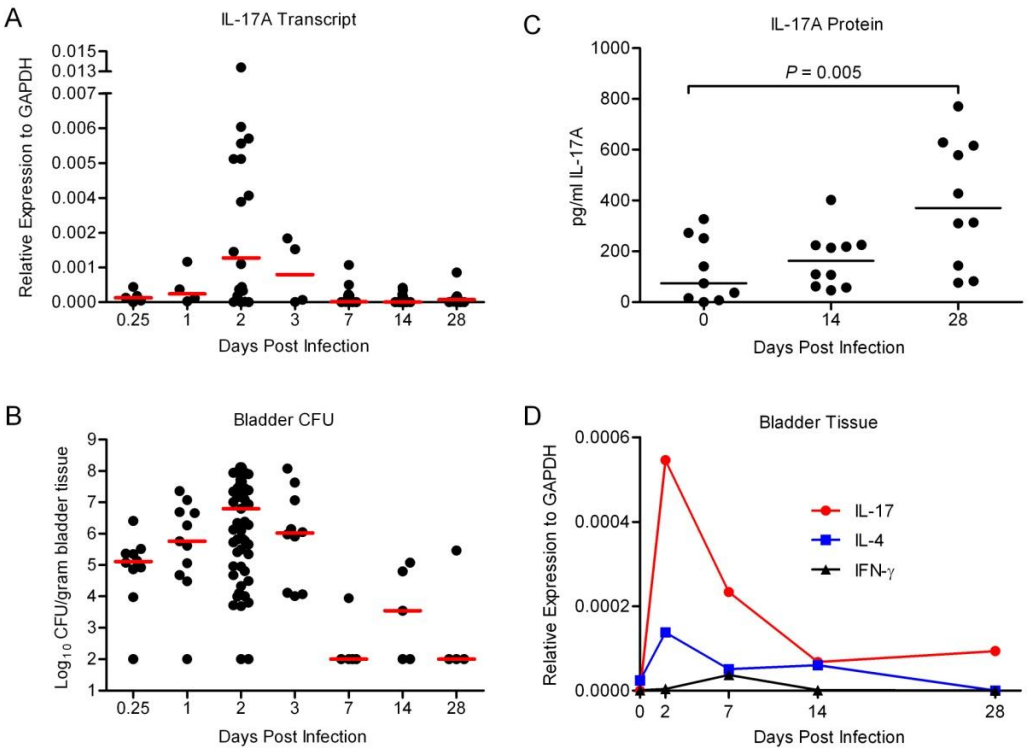

Figure 3.3 IL-17A transcript dynamics in response to acute infection. Mice were transurethrally inoculated with UPEC strain CFT073 and bladders were collected at specified time points for analysis of (A) cytokine transcript levels by qPCR (*n*=5-18 per time point, several experiments combined), (B) bladder CFU (*n*=3-55 per time point, several experiments combined), (C) IL-17A protein in bladder homogenate (*n*=9-10 mice per time point, 2 experiments combined), and (D) cytokine transcript levels by qPCR (*n*=5 mice per time point).

3.4.2 Innate immune responses and IL-17A

3.4.2.1 IL-17A transcript is upregulated in response to acute bladder infection by UPEC

Since there was no overt defect in the generation of protective immunity in IL-17A$^{-/-}$ mice, we sought to determine if IL-17A played a role in the innate response to UTI. To do this, we first examined IL-17A transcript dynamics during acute infection. Mice were inoculated transurethrally with UPEC and their bladders were collected for qPCR analysis at 7 time points during a 28-day period. UPEC-infected mice demonstrated a dramatic increase in IL-17A mRNA with median values peaking at 48 hpi (Figure 3.3A), suggesting a role for IL-17A in the innate immune response to UTI. As depicted in Figure 3.3B, peak levels of IL-17A transcript expression coincide with CFU dynamics in the infected C57BL/6 bladder, a phenomenon that is further examined in the next section. To see if protein expression reflected transcript dynamics, bladders from infected mice were probed for secreted IL-17A by ELISA. Surprisingly, IL-17A protein levels did not mirror transcript expression, as the amount of IL-17A detected in infected murine bladder homogenates increased over time, with a significant increase over baseline at 28 days post infection (dpi) ($P = 0.005$) (Figure 3.3C). This time point corresponds to the resolution of infection in the majority of animals, as measured by the lack of CFU counts in the bladder (Figure 3.3B). These data demonstrate that IL-17A transcript is highly upregulated at 2 days post bladder inoculation, and that significant protein levels are detectable after the infection has been cleared.

To put the dynamics of IL-17A transcript expression into perspective, RNA samples from the longitudinal IL-17A transcript analysis (Figure 3.3A) were also

analyzed for IFN-γ and IL-4 transcript expression over time. These particular transcripts were chosen because they are the signature cytokines of the Th1 and Th2 cell subsets. While IL-17A transcripts peak at 2 dpi and remain above baseline throughout the experimental duration, IFN-γ transcription appears to peak around 7 dpi, coinciding with the infiltration of adaptive immune cells (Figure 3.3D). IL-4 transcript peaks similarly to IL-17A; however, unlike IL-17A, IL-4 transcript expression is back to baseline by day 28 post infection (Figure 3.3D). These results indicate that transcription of IL-17A is acutely upregulated in response to infection, and expression appears to correlate with the innate immune response to UTI.

3.4.2.2 Innate expression of IL-17A appears to depend on UPEC colonization

The results in Chapter 2 revealed that expression of IL-17A transcript by splenocytes from UPEC antigen-vaccinated mice was not sufficiently stimulated by exposure to LPS (Figure 2.9). Moreover, the exact molecular ligand that triggers activation of the signaling pathway responsible for secretion of IL-17A during acute bacterial infection is not established. Perhaps TLR4 recognition of FimH (16, 275), or a different TLR-PAMP interaction such as TLR2 recognition of peptidoglycan components (441) or lipopeptide recognition by an unknown receptor (185), may trigger IL-17A secretion in response to UPEC. Another alternative, however unlikely considering the paradigm of conserved PAMPs recognized by innate PRRs, is that there is a UPEC-specific ligand that triggers transcription of IL-17A in the bladder in response to UTI. Indeed, studies on TLR11 were not able to pinpoint the exact ligand for TLR11; however, these experiments did demonstrate some specificity to UPEC (469). To see if

IL-17A expression was specific to UPEC, C57BL/6 mice were infected with either UPEC or fecal strain EFC4. EFC4 does not have the capacity to robustly colonize the murine urinary tract (270) (Figure 3.4A), and therefore cannot be considered an uropathogenic strain. While both strains stimulated similar upregulation of IFN-γ transcripts at 6 hpi (0.25 dpi), 2 dpi, and 7 dpi, it appeared that only mice infected with uropathogenic strain CFT073 upregulated IL-17A transcripts over time (Figure 3.4B). However, while the levels did not increase with time, infection EFC4 did result in upregulation of IL-17A transcripts over baseline, as no IL-17A transcripts are detected in naïve animals (Figure 3.4B). This result suggested that colonization is likely necessary for robust upregulation of IL-17A transcripts that increase in the initial days of infection.

3.4.2.3 γδ T cells are a significant source of IL-17A during acute UTI in the mouse model

3.4.2.3.1 qPCR for IL-17A in infected wild type and TCR $\delta^{-/-}$ mice

The cell type(s) responsible for the high amount of IL-17A transcript generated in response to UPEC-mediated UTI needed to be identified. Since TCR $\delta^{-/-}$ mice are more susceptible to UTI (198) and γδ TCR-positive cell populations are known to express IL-17A in the context of bacterial infection (142, 249, 363, 376, 438), we wanted to see if TCR $\delta^{-/-}$ mice had a deficiency in IL-17A transcript expression during bladder infection. Wild type C57BL/6 and TCR $\delta^{-/-}$ mice were inoculated transurethrally with PBS or UPEC and their bladders were analyzed by qPCR at 48 hpi. Both wild type and TCR $\delta^{-/-}$

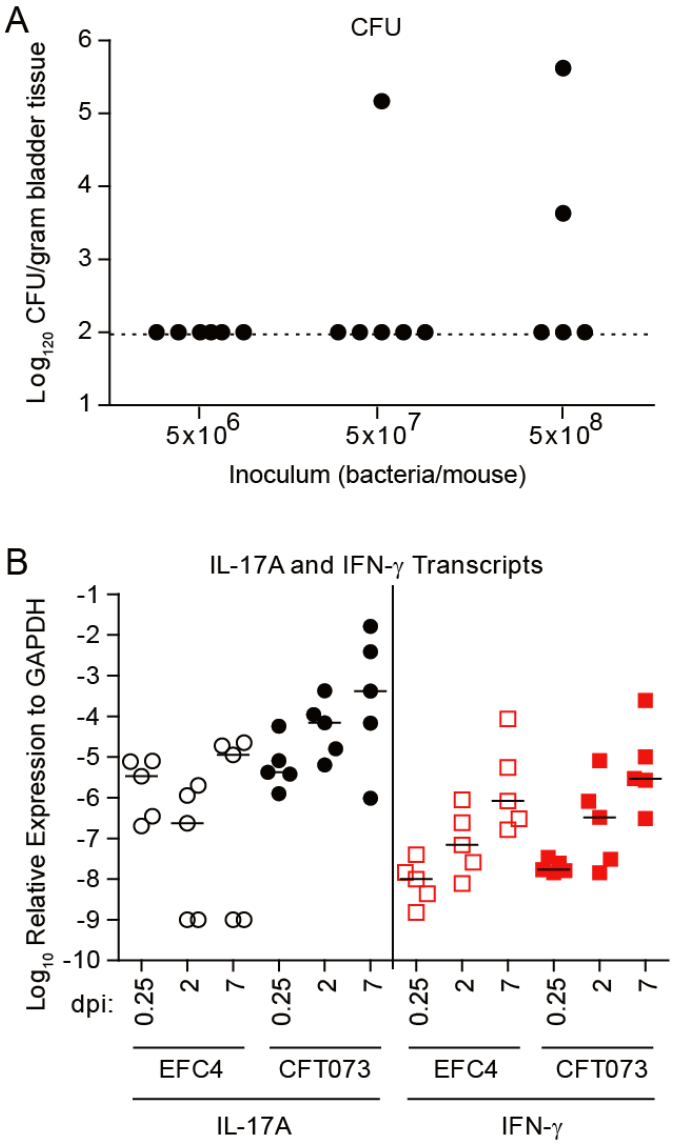

Figure 3.4 Robust upregulation of IL-17A may be UPEC-specific or depend on efficient bladder colonization. (A) Mice (*n*=5-6 per inoculum) were transurethrally infected with the indicated inoculums of EFC4 and bladder CFU was analyzed at 48 hpi. Figure and experiment done by Sara Smith. (B) Mice were transurethrally infected with UPEC strain CFT073 or fecal isolate EFC4 (*n*=5 per strain). At 0.25, 6, or 7 days dpi, bladders were harvested for qPCR analysis. Values of $1x10^{-9}$ were given to mice that had no detectable cytokine expression.

mice exhibited significant upregulation of IL-17A compared to PBS-treated controls, which were undetectable. However, the median value of IL-17A expression was 3.7-fold higher in the wild type mice ($1.16x10^{-3}$ relative to GAPDH for wild type compared to $3.1x10^{-4}$ for TCR $\delta^{-/-}$ mice) (Figure 3.5). These results demonstrate that mice deficient in the γδ TCR tend to have lower expression of IL-17A than wild type mice at 48 hpi, suggesting that γδ T cells are a source of the IL-17A secreted in response to UPEC bladder colonization.

3.4.2.3.2 Intracellular staining and flow cytometric analysis of IL-17A-producing γδ T cells

Because TCR $\delta^{-/-}$ mice express less IL-17A in response to experimental UTI, we sought to quantify the level of IL-17A expression by γδ T cells in infected wild type animals by flow cytometry. Bladders were isolated from PBS- or UPEC-inoculated wild type C57BL/6 mice at 48 hpi and made into single cell suspensions for staining and flow cytometric analysis. For comparison, the expression of IL-17A by $CD4^+$ cells was also analyzed. To best represent the level of IL-17A expression *in vivo*, these cells were not stimulated *in vitro* prior to staining and analysis by flow cytometry. While the number of infiltrating $CD4^+$ cells was an order of magnitude higher than that of γδ TCR^+ cells, increases in both populations were statistically significant (P=0.0021 and P=0.0229, respectively) (Figure 3.6A). Each population was then interrogated for IL-17A positivity, as depicted by representative plots (Figure 3.6B). Only γδ TCR^+ cells exhibited statistically significant increases in IL-17A positivity after UPEC infection (P=0.0225) (Figure 3.6C). In addition, the median frequency of γδ TCR^+ cells also staining positive

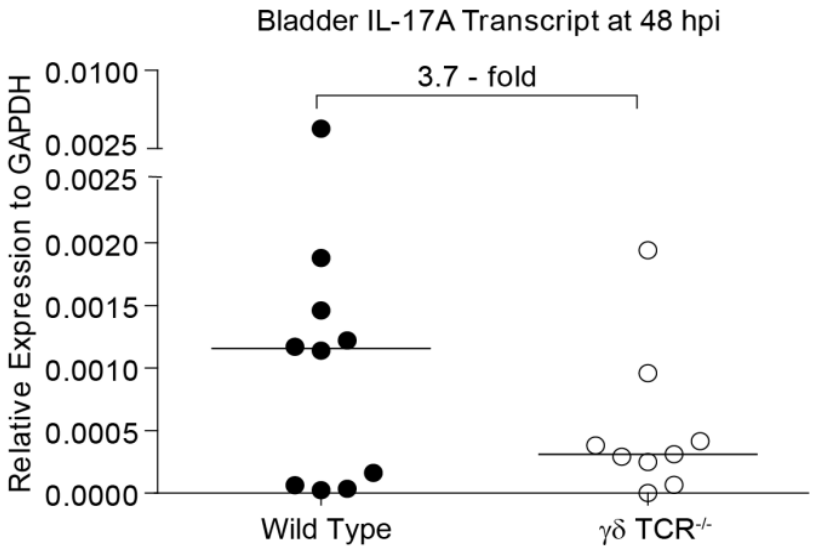

Figure 3.5 IL-17A transcript dynamics in wild type and γδ TCR$^{-/-}$ mice. Wild type (filled symbols) and γδ TCR$^{-/-}$ (open symbols) mice (*n*=9 each, two experiments combined) were inoculated transurethrally with UPEC and sacrificed at 48 hpi for analysis of bladder mRNA by qPCR. Each symbol represents an individual animal while bars represent the median values.

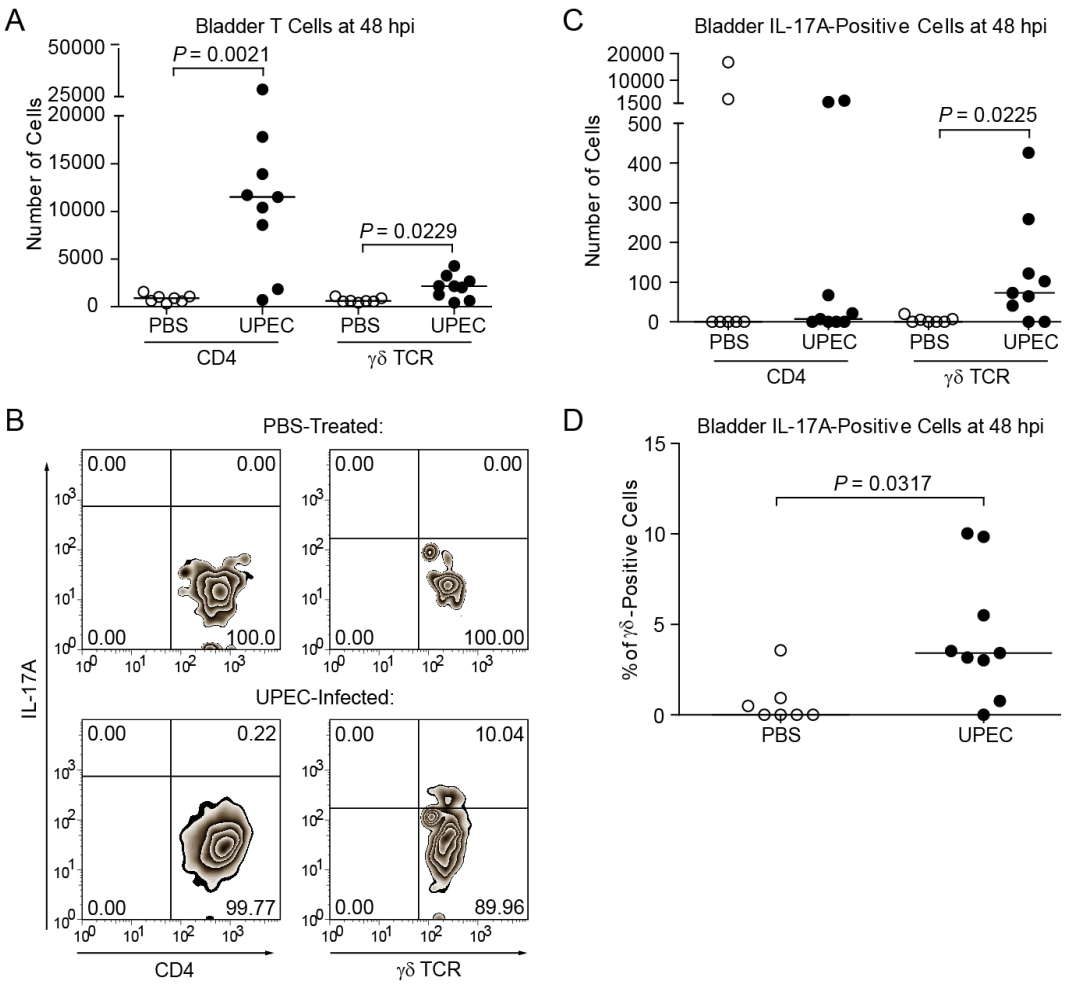

Figure 3.6 γδ T cells are a source of IL-17A during acute UTI. Bladders from mice treated transurethrally with PBS (open symbols, *n*=7, 2 experiments combined) or UPEC strain CFT073 (filled symbols, *n*=9, 2 experiments combined) were collected at 48 hpi, made into a single cell suspension, and stained (without *in vitro* stimulation) for flow cytometry. Forward- versus side-scatter (FSC vs. SSC) plots were gated to include all $CD4^+$ and γδ TCR^+ cells as determined by backgating. $CD4^+$ and γδ TCR^+ gates were then interrogated for IL-17A positivity individually based on unstained and singly-stained bladder control samples. (A) Total numbers of $CD4^+$ and γδ TCR^+ cells. (B) Flow plots showing $CD4^+$ (left) and γδ TCR^+ (right) cells also staining positive for IL-17A from representative PBS-treated (top) and UPEC-infected (bottom) animals. (C) The total number of $CD4^+$ and γδ TCR^+ cells also staining positive for IL-17A. (D) Frequency of γδ $TCR^+IL\text{-}17A^+$ cells in PBS treated and UPEC-infected animals. In (A), (C), and (D) each symbol represents an individual animal while bars represent the median values.

for IL-17A was ~5%; in some animals, up to 12% of the γδ cell population expressed IL-17A when compared to PBS group (*P*=0.0317) (Figure 3.6D). These results indicate that at 48 hpi, γδ-positive T cells are responsible fora portion of the upregulated IL-17A transcripts seen in UPEC-infected mouse bladders.

3.4.2.3.3 Expression of IL-23R in the murine bladder

Recently, it has been shown that responsiveness to IL-23 is important for the expression of IL-17A by γδ T cells in a non-TCR-dependent fashion (402). To see if IL-23R was expressed in the bladder and thus could be mediating a role in IL-17A expression by γδ T cells in response to UTI, we quantified IL-23R expression in bladder tissue by qPCR. IL-23R transcript was detected in the bladder of C57BL/6 wild type mice (Figure 3.7A). Additionally, unlike the inducible expression of IL-23R by peritoneal exudates cells in response to i.p. infection with *E. coli* (376), expression levels in untreated animals were not significantly altered after UTI (Figure 3.7A) and were similar in wild type and IL-17A$^{-/-}$ mice (Figure 3.7B). These results indicate that the IL-23R is present in the bladder tissue of mice and may mediate rapid expression of IL-17A in response to UTI.

3.4.2.3.4 Expression of γδ TCR variable chains in the murine bladder

In addition to IL-23R expression, we wanted to examine γ and δ variable chain expression in the bladder during UTI. Bladders from C57BL/6 mice that were transurethrally infected with CFT073 for 48 h were harvested and prepared for RT-PCR. cDNA was synthesized using gene specific primers for the common γ or common δ

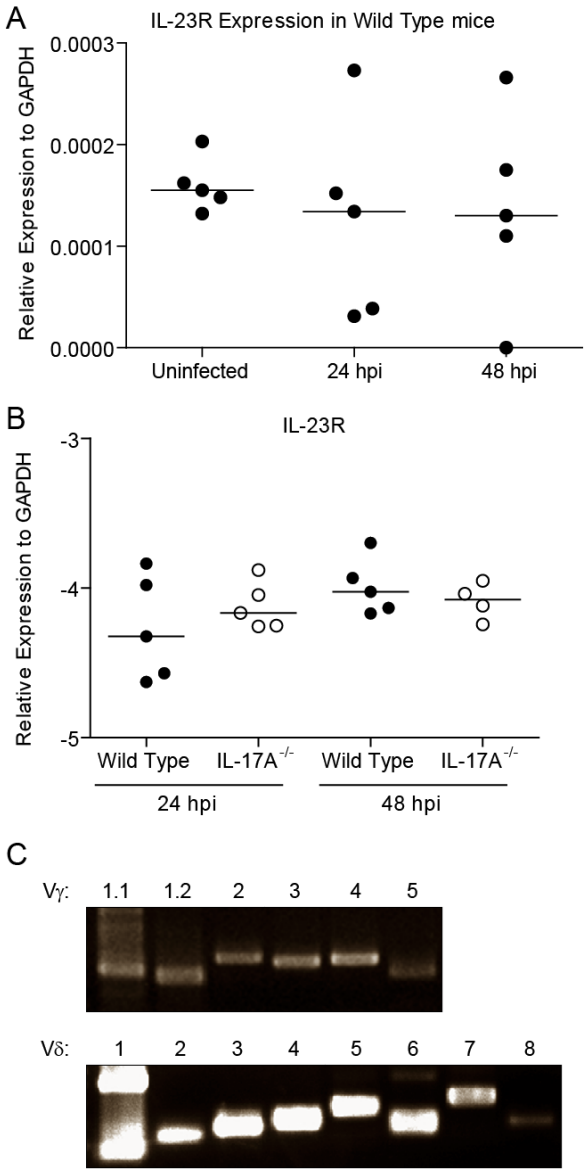

Figure 3.7 IL-23R and all of the known γ and δ TCR variable chains are expressed in the murine bladder. Wild type mice were left uninfected or transurethrally infected with UPEC. (A and B) At 24 or 48 hpi, bladders (*n*=5 each) from wild type of IL-17A$^{-/-}$ mice were harvested for RNA isolation and analysis of IL-23R expression by qPCR. Each symbol represents an individual animal while bars represent the median values. (C) Bladders were harvested for RNA isolation and analysis of γ and δ variable chain expression by RT-PCR. The presence of two products in the Vδ1 lane may reflect the junctional diversity capabilities of δ chain rearrangements (297). Shown are representative gels.

chain, and PCR using 5' Vγ with 3' Jγ or Cγ primers and 5' Vδ specific primers with 3' Jδ specific primers was performed on the respective products. We did not detect a preference for Vγ or Vδ chain expression in the murine bladder in response to UTI; that is, we detected expression of all known Vγ chains [with the exception of the Vγ1.3 pseudogene (55)] and Vδ chains in the bladder tissue of infected mice (Figure 3.7C), indicating that any or all of the γδ T cells expressing these variable chains could be responsible for the expression of IL-17A during UTI.

3.4.2.4 $CD11b^+$ $Gr\text{-}1^+$MHC Class II^+ inflammatory cells also produce IL-17A in response to UTI

While TCR $\delta^{-/-}$ mice appeared to have deficient upregulation of IL-17A transcript in response to UTI, these mice still had some residual expression (Figure 3.5), indicative of an additional source of IL-17A. To investigate what additional cells could be producing IL-17A, intracellular staining and flow cytometry was used. Similar to the experiments done with γδ T cells, cells were harvested and prepared directly from the mouse (no *in vitro* restimulation) to best represent populations of cells producing large amounts *in vivo*. Using this methodology, it was concluded that $CD8^+$ (which includes cytotoxic T cells and IELs), $B220^+$ (which includes B cells and plasmacytoid DCs), and $F4/80^+$MHC Class II^+ (including activated macrophages) cells were each not a significant source of IL-17A during UPEC-mediated UTI. However, upon investigation of the neutrophil population, another cell type was found to express high amounts of IL-17A. Figure 3.8 depicts the gating strategy on representative flow plots from PBS-mock treated (left panels) and transurethrally infected mice (right panels). This strategy is normally

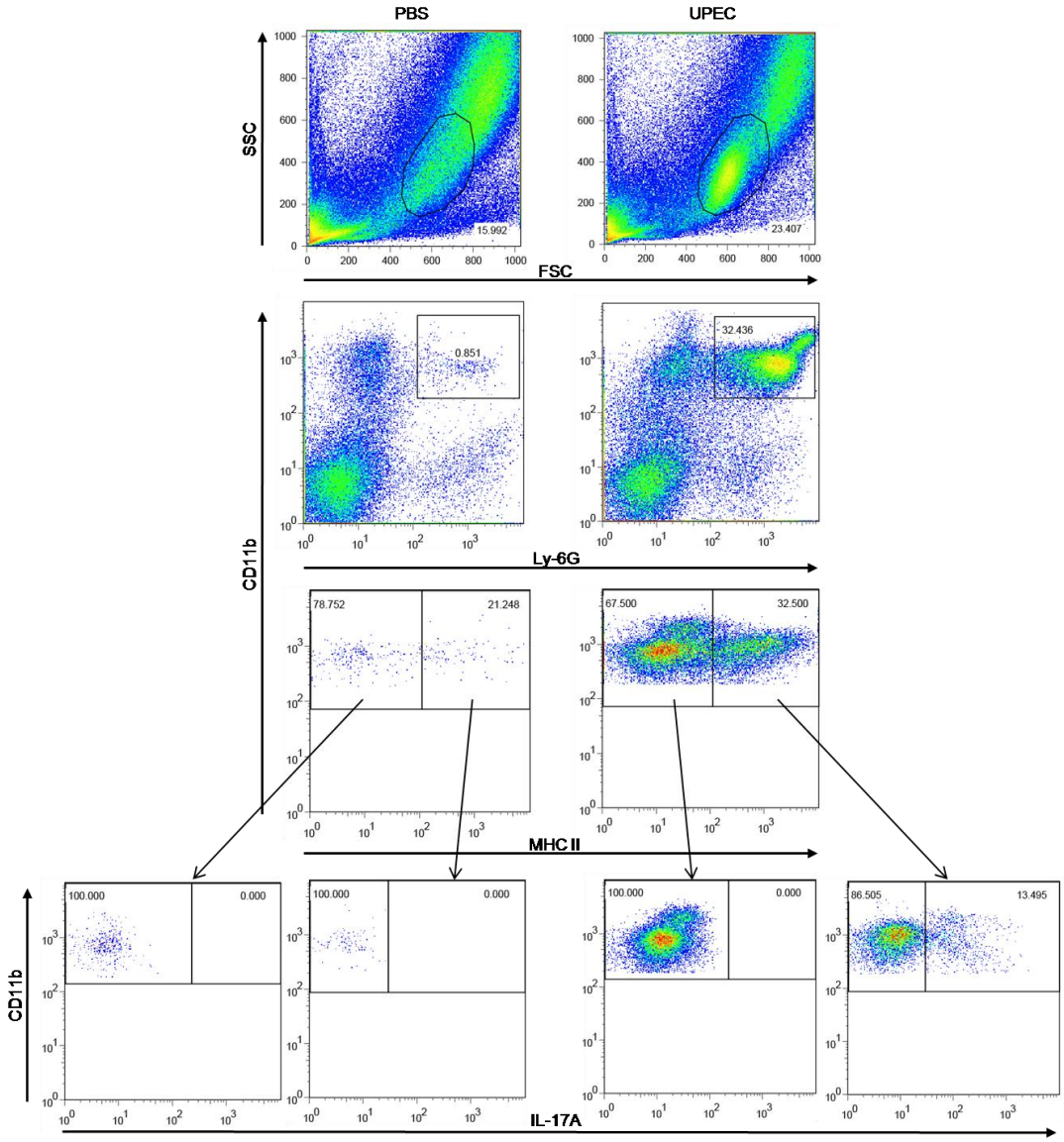

Figure 3.8 Representative flow cytometry plots of IL-17A-expressing CD11b^{+}Gr-1^{+}MHC Class II^{+} inflammatory cells. C56BL/6 mice were transurethrally infected with UPEC strain CFT073 or mock infected with PBS for 48 h before bladders were harvested and prepared for flow cytometric analysis. Shown is the gating strategy of a representative PBS-mock treated mouse (left side) and an UPEC-infected mouse (right side). FSC versus SSC plots were gated to include most myeloid cells as determined by backgating. Each successive row is the cell population gated in the previous row, and the arrows in the third row indicate the last sets of gates.

used to examined neutrophils, which are considered to be $CD11b^{+}Gr\text{-}1^{+}$ and MHC Class II^{-}. This cell population does not express IL-17A during the steady-state or in response to infection (Figure 3.8, Row 4, panels 1 and 3). However, the $CD11b^{+}Gr\text{-}1^{+}$MHC Class II^{+} cell population appears express high amounts of IL-17A in response to UPEC-mediated UTI (Figure 3.8, Row 4, panels 2 and 4). Indeed, while there is a statistically significant increase in $CD11b^{+}Gr\text{-}1^{+}$ cells both positive and negative for MHC Class II (P =0.0286) (Figure 3.9A), only the MHC Class II^{+} population significantly expressed IL-17A in response to UTI when compared to either $CD11b^{+}Gr\text{-}1^{+}$MHC Class II^{+} cells from mock-infected animals or $CD11b^{+}Gr\text{-}1^{+}$MHC Class II^{-} cells (P = 0.0294) (Figure 3.9B). As about 15% of the $CD11b^{+}Gr\text{-}1^{+}$MHC Class II^{+} cell population expresses IL-17A in response to UPEC in the bladder (Figure 3.9C), these inflammatory myeloid cells are also a significant source of IL-17A in response to UTI.

3.4.2.5 IL-17A plays a role in defending the urinary tract from acute UPEC colonization

To examine the role of IL-17A in innate control of UTI, bladder and kidney homogenates from wild type and $IL\text{-}17A^{-/-}$ mice were cultured at the reported peak of bacterial colonization in C57BL/6 mice, 24 hpi (186). At this time point, $IL\text{-}17A^{-/-}$ mice had a 3-fold higher median CFU/gram bladder tissue (Figure 3.10). By 48 hpi, the peak of IL-17A transcript expression in the bladder (Figure 3.3A), this trend increased 10-fold to a 35-fold higher median CFU per gram tissue in the bladders of $IL\text{-}17A^{-/-}$ mice (Figure 3.10). While these trends were reproducible, we sought to investigate later time points, presuming the colonization phenotypes resulting from the lack of IL-17A may be exacerbated. Indeed, at both 72 and 96 hpi, $IL\text{-}17A^{-/-}$ mice had significantly more

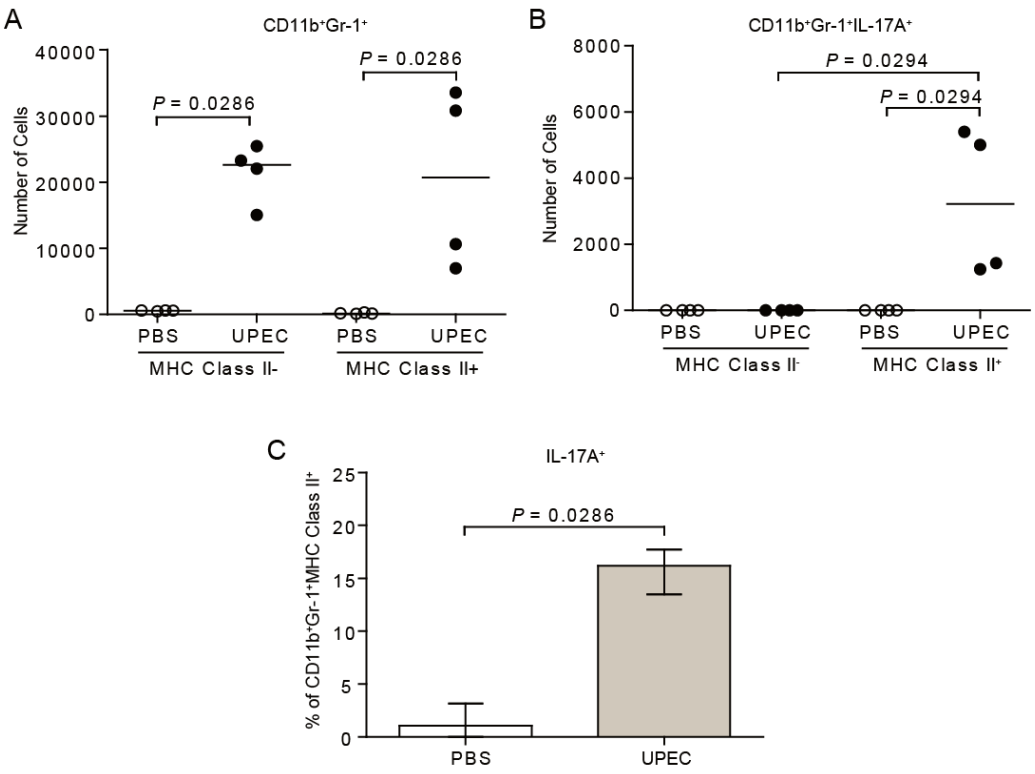

Figure 3.9 CD11b$^+$Gr-1$^+$MHC Class II$^+$ inflammatory cells expressing IL-17A. C56BL/6 mice (*n*=4) were transurethrally infected with UPEC strain CFT073 or mock infected with PBS for 48 h before bladders were harvested and prepared for flow cytometric analysis. (A) CD11b$^+$Gr-1$^+$ cells positive and negative for MHC Class II. (B) MHC Class II-negative and -positive CD11b$^+$Gr-1$^+$ cells expressing IL-17A as detected by intracellular staining. (C) Frequency of CD11b$^+$Gr-1$^+$MHC Class II$^+$ cells also positive for IL-17A in PBS treated and UPEC-infected animals. Each symbol represents an individual animal while bars and error represent the median values and range.

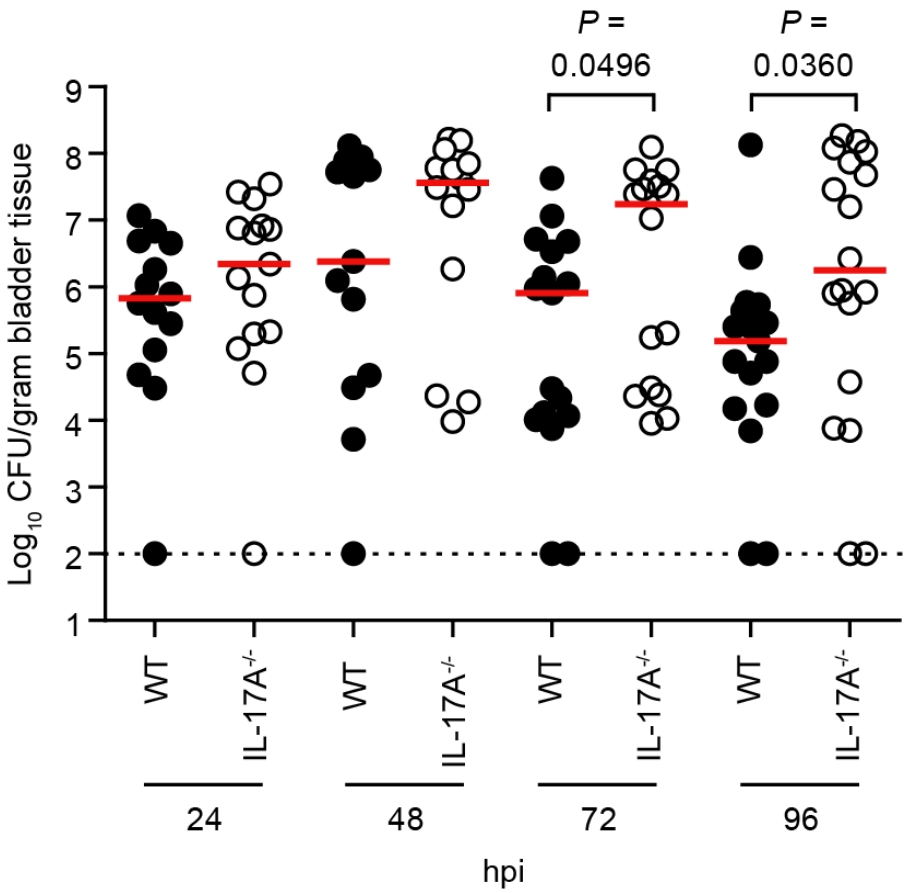

Figure 3.10 IL-17A is necessary for clearance of acute UTI. Wild type (WT, filled symbols) and IL-17A$^{-/-}$ (open symbols) mice were infected transurethrally with UPEC strain CFT073. At 24, 48, 72, and 96 hpi mice were sacrificed and bladder homogenates were plated on LB agar to determine the bacterial burden (*n*=14-19 mice per group per time point, 2 or 3 experiments combined). The dotted line indicates the 100 CFU/gram tissue limit of detection. Each symbol represents an individual animal while bars represent the median values.

bacteria colonizing their bladders ($P < 0.05$) (Figure 3.10). Of note, the naïve group of mice in the adaptive immune experiments also demonstrated a significant difference in colonization, with the IL-17A$^{-/-}$ having a higher bacterial load at 48 hpi ($P = 0.0303$) (Figure 3.2). These results indicate that IL-17A$^{-/-}$ mice are more susceptible to cystitis than wild type mice.

3.4.2.6 IL-17A is necessary for proinflammatory transcript upregulation in response to UTI

Given that IL-17A mediates inflammatory responses largely by influencing mRNA levels of cytokines and chemokines posttranscriptionally (148-150, 163), we wanted to determine if such effects were present in the context of UTI. Wild type and IL-17A$^{-/-}$ mice were inoculated transurethrally with UPEC and their bladders were collected at 48 hpi for transcript analysis by qPCR. We measured mRNA levels of a panel of chemokines, the antimicrobial effector protein inducible NO synthase (iNOS), and cytokines previously shown to be affected by IL-17A expression (110). At 48 hpi, the peak of IL-17A transcript expression in the infected murine bladder, transcripts for all of the genes investigated were expressed at a significantly lower level in IL-17A$^{-/-}$ mice compared to their wild type counterparts (Figure 3.11). Of note, expression of the AMP β-defensin was not found to significant change in response to infection in either genetic background. These results indicate that animals lacking IL-17A$^{-/-}$ signaling are not able to efficiently upregulate the appropriate mRNA transcripts in infected bladder.

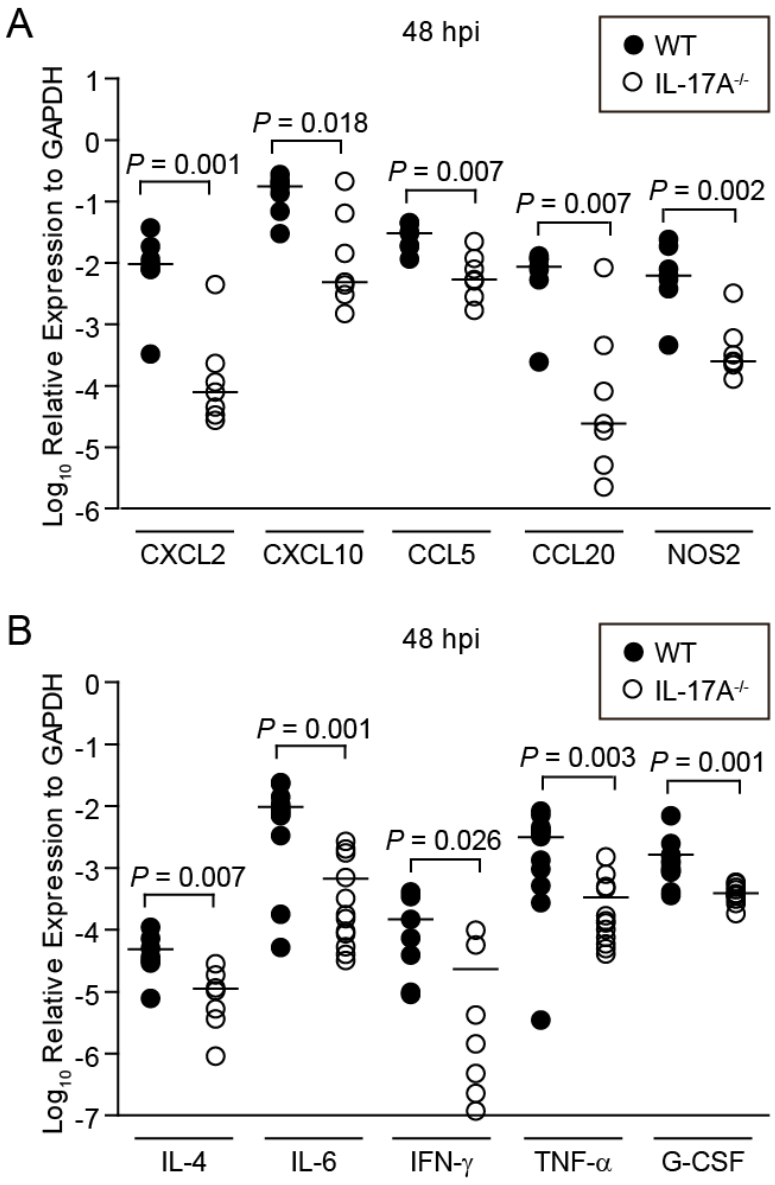

Figure 3.11 Proinflammatory transcript upregulation in response to acute UTI is defective in IL-17A$^{-/-}$ mice. Wild type (filled symbols) and IL-17A$^{-/-}$ (open symbols) mice were inoculated transurethrally with UPEC strain CFT073. Mice (*n*=7-12, 1 or 2 experiments combined) were sacrificed at 48 hpi and their bladders were harvested for qPCR analysis of (A) chemokines CXCL2, CXCL10, CCL5, CCL20, and iNOS (NOS2) and (B) cytokines IL-4, IL-6, IFN-γ, TNF-α, and G-CSF. Each symbol represents an individual animal while bars represent the median values.

3.4.2.7 Infected wild type and IL-17A$^{-/-}$ exhibit qualitatively similar responses to UTI when examined histologically

Since IL-17A$^{-/-}$ mice had decreased cytokine and chemokine expression, we decided to examine the bladders of wild type and IL-17A$^{-/-}$ histologically to see if there were any gross pathological differences or qualitative differences in inflammation. Longitudinal sections of bladders from wild type and IL-17A$^{-/-}$ mice that were either uninfected or infected for 48 h (the peak of IL-17A expression) were H&E stained and visualized microscopically at low (Figure 3.12) or high (Figure 3.13) magnification. The sections revealed similar histopathological effects in response to UTI both backgrounds. More specifically, bladders from the uninfected mice were histologically within normal limits, without inflammation or other alteration (Figure 3.12A, C, E, G). Bladders from the infected mice, however, had expansion of the lamina propria by edema fluid accompanied by perivascular and interstitial inflammation (Figure 3.12B, D, F, H, black filled arrowheads). These occurrences ranged from mild to severe in both wild type and IL-17A$^{-/-}$ mice. Additionally, umbrella cell sloughing was also apparent in the infected animals [compare apical surface of the transitional epithelium in Figure 3.12E and G to that in Figure 3.12F and H]. Inflammatory infiltrates consisted of primarily neutrophils; although, sometimes a mixed monocytic and neutrophilic infiltrate was observed (Figure 3.13A-F). Occasional intraepithelial inflammation was also noted in both backgrounds (Figure 3.12H and Figure 3.13B, open arrowhead). Small numbers of adherent bacteria were observed in slides from infected animals (Figure 3.13E, on UC; Figure 3.13F, "B" with arrow) while larger numbers of both adherent and intraluminal bacteria were present in sections from IL-17A$^{-/-}$ mice (Figure 3.12H, "B" with arrow; Figure 3.13B,

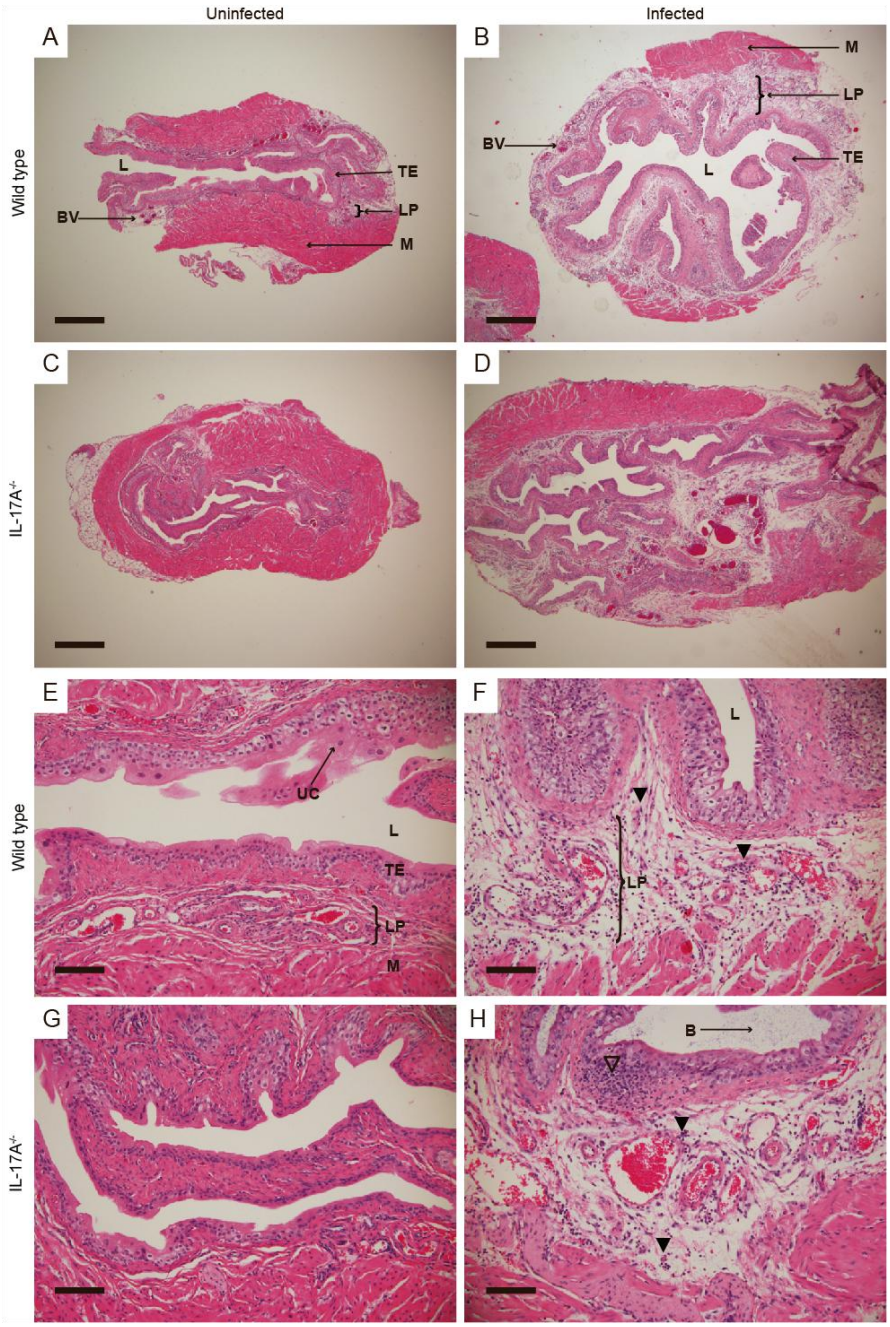

Figure 3.12 Wild type and IL-17A$^{-/-}$ exhibit similar histological profiles in response to UTI. Wild type (A, B, E, F) and IL-17A$^{-/-}$ (C, D, G, H) mice were left untreated (A, C, E, G) (*n*=1 each background) or infected with UPEC for 48 h (B, D, F, H) (*n*=3 each background). (A-D) 40x magnification, bar=500 μm; (E-H) 200x magnification, bar=100 μm. L=lumen, TE=transitional epithelium, LP=lamina propria, BV=blood vessel, M=muscularis, UC=umbrella cell, B=bacteria, open arrowhead= intraepithelial inflammation, closed arrowheads= perivascular and interstitial inflammation.

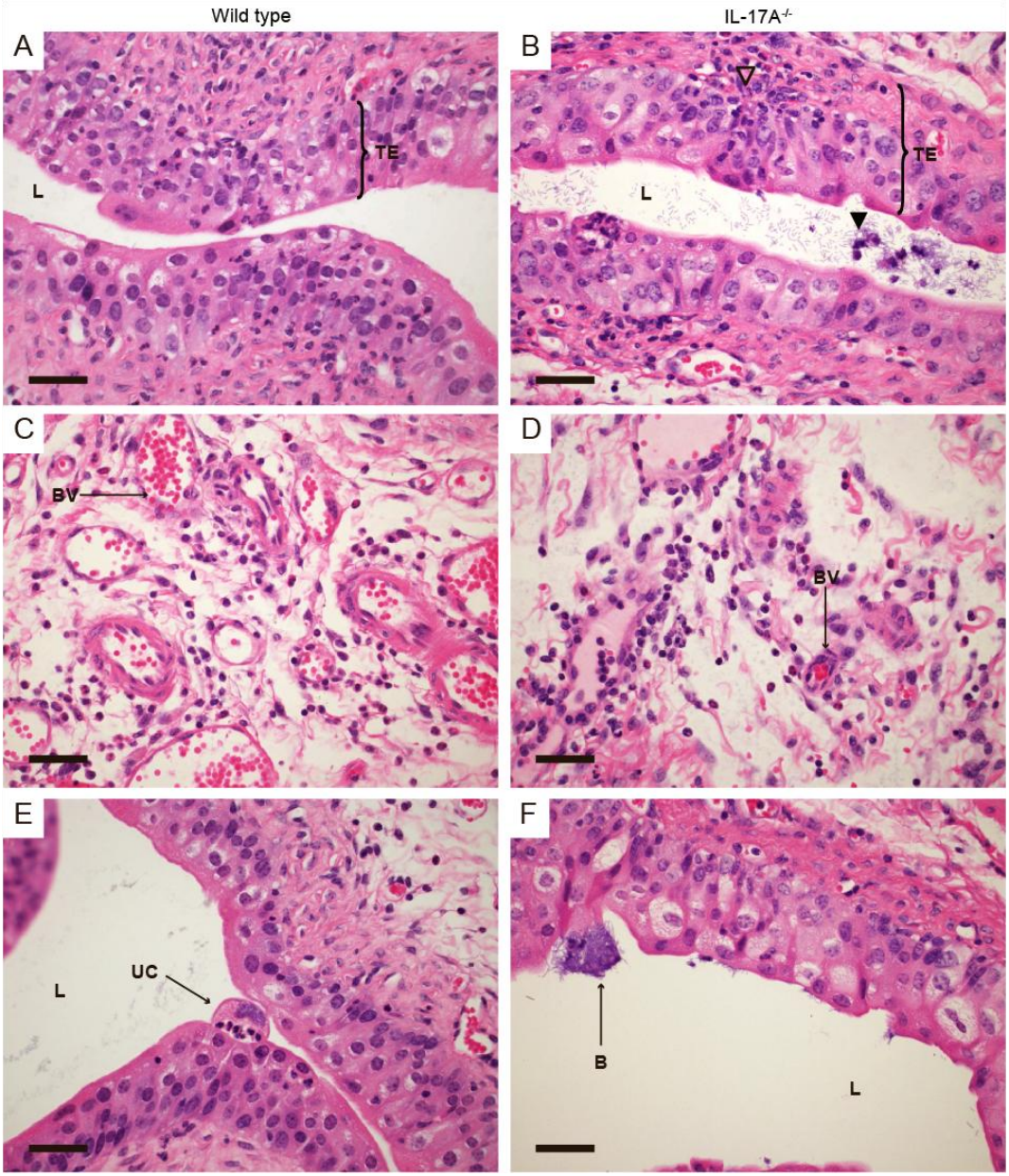

Figure 3.13 Wild type and IL-17A$^{-/-}$ exhibit similar histological profiles in response to UTI. Wild type (A, C, E) and IL-17A$^{-/-}$ (B, D, F) mice were infected with UPEC for 48 h (*n*=3 each background). Subsequently, bladders were harvested and prepared for histological examination. Shown are representative images of the transitional epithelium (A and B), lamina propria (C and D), and bacteria present in the bladder (E and F). All images: 600x magnification, bar=20 μm. L=lumen, TE=transitional epithelium, BV=blood vessel, UC=umbrella cell, B=bacteria, open arrowhead= intraepithelial inflammation.

arrowhead), indicative of a decreased ability to eliminate bacteria and consistent with higher bacterial loads in the knockout animals (Figure 3.10). Also depicted is a sloughing umbrella cell with adherent UPEC (Figure 3.13E, UC) and inflammatory cells accompanying luminal UPEC (Figure 3.13B, arrowhead). Collectively, histological analysis did not reveal any gross pathological defects that could explains the clearance defect exhibited by IL-17A$^{-/-}$ animals.

3.4.2.8 IL-17A is required for optimal macrophage and neutrophil infiltration in response to UTI

3.4.2.8.1 Indirect measures of MPO

Neutrophils are the first cell type to migrate to the bladder in the event of UTI, and they are crucial for controlling infection at early time points (5, 147, 374). Since no qualitative differences in neutrophil numbers were seen histologically, a more quantitative approach was needed to determine the neutrophil response in wild type and IL-17A$^{-/-}$ mice was taken. The levels of MPO, a neutrophil-associated enzyme, in mouse bladders were indirectly probed using both a kinetic assay and ELISA. While detectable, the signal for enzymatic activity of MPO in infected mouse bladders (pooled groups of 10) was extremely weak (Figure 3.14A). Moreover, while ELISA was able to detect MPO in a single mouse bladder, MPO data collected by ELISA was highly variable and inconsistent, and there was no significant difference in MPO levels at 6, 24, or 48 hpi in infected wild type and IL-17A$^{-/-}$ bladders (Figure 3.14B). Given the aforementioned complications, it was concluded that indirect measurement of MPO by either kinetic assay or ELISA was not a reliable approach to determine neutrophil numbers in UPEC-infected murine bladders.

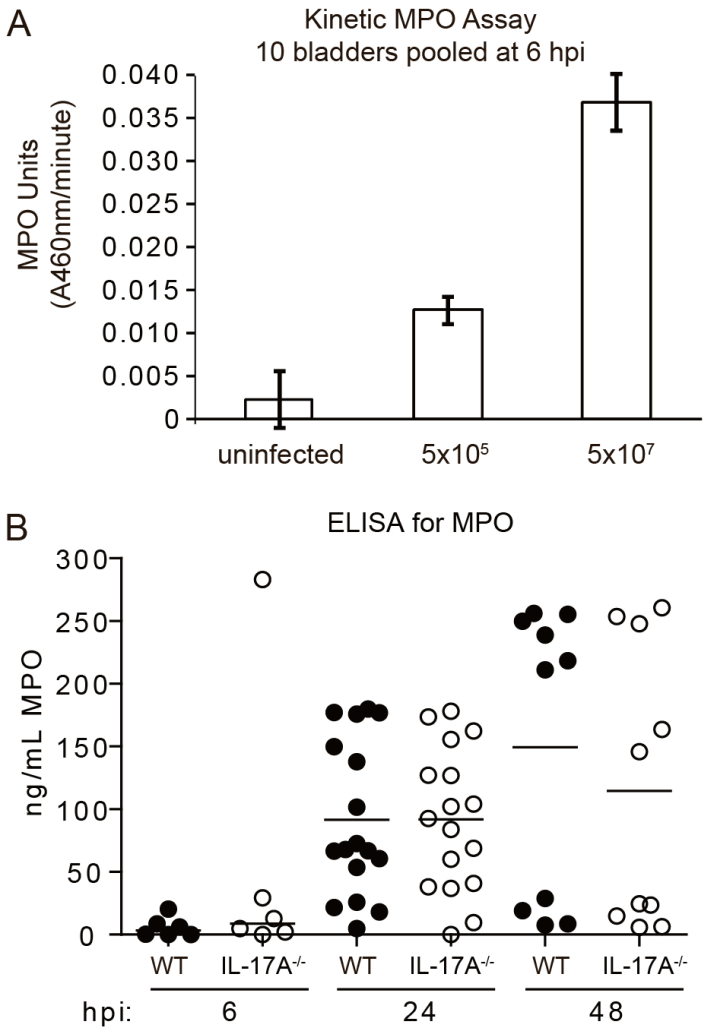

Figure 3.14 Measurement of MPO levels in the murine bladder by kinetic assay and ELISA. (A) Wild type mice were transurethrally infected with varying amounts of CFT073 and sacrificed at 6 hpi. Ten bladders were pooled for each condition and prepared for kinetic ELISA assay. (B) Wild type and IL-17A$^{-/-}$ mice (*n*=5-17 each background, 1 to 3 experiments combined) were transurethrally infected with UPEC strain CFT073. At 6, 24, and 48 hpi, their bladders were harvested for MPO-specific ELISA. Concentrations were calculated using a standard curve. Each symbol represents an individual animal while bars represent the median values.

3.4.2.8.2 Direct neutrophil urinalysis

In lieu of indirect MPO analysis, more direct quantitative assays were employed to determine the neutrophilic infiltrate in wild type and IL-17A$^{-/-}$ mice. One quick way to quantitatively measure neutrophil recruitment to the bladder is to directly count the neutrophils in urine using a hemacytometer; the peak of this measurement occurring at 6 hpi in both mice and humans (4, 147). At this time point, IL-17A$^{-/-}$ mice had significantly fewer neutrophils in their urine (P=0.0480) (Figure 3.15). Additionally, IL-17A$^{-/-}$ mice lacked a population of "high-responder" mice present in the wild type cohort; these animals had greater than 1.5x10^{6} neutrophils per ml present in their urine at 6 hpi (compare 45% of wild type animals to 17% of IL-17A$^{-/-}$ animals, P = 0.0262) (Figure 3.15). Since IL-17A transcript and protein are detectable in the bladder at this early (6 h) time point (Figure 3.3A) (186), these results indicate that IL-17A may be important for very early neutrophil migration to the bladder in response to UPEC infection.

3.4.2.8.3 Flow cytometry for neutrophils and macrophages

To investigate the innate cellular response to UTI in the absence of IL-17A at a later time point (48 hpi), we quantified the number of macrophages and neutrophils in wild type and IL-17A$^{-/-}$ mice localized to the bladder tissue using flow cytometry. Macrophages were determined by interrogating the bladder for F4/80^{+}MHC Class II^{+} cells while neutrophils were, as discussed in section 0, defined as Gr-1^{+}CD11b^{+}MHC Class II^{-} cells. Representative plots showing the gating strategy for macrophages and neutrophils in wild type and IL-17A$^{-/-}$ bladder cells are shown (Figure 3.16A and C). After quantification, both the macrophage (P=0.0145) (Figure 3.16B) and the neutrophil

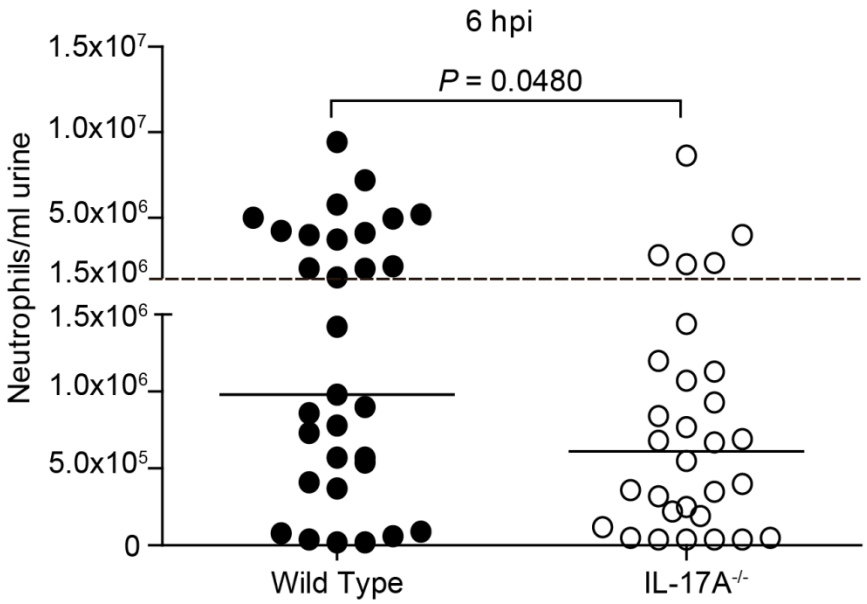

Figure 3.15 Early neutrophil infiltration is deficient in IL-17A$^{-/-}$ mice. Wild type (filled symbols) and IL-17A$^{-/-}$ (open symbols) mice were transurethrally infected with UPEC strain CFT073. At 6 hpi, urine was collected from live mice (*n*=30-31, 5 experiments combined). Cells in the excretion were stained and neutrophils were counted by hemacytometer. Each symbol represents an individual animal while bars represent the median values. Dashed like indicates cutoff for high-responder mice.

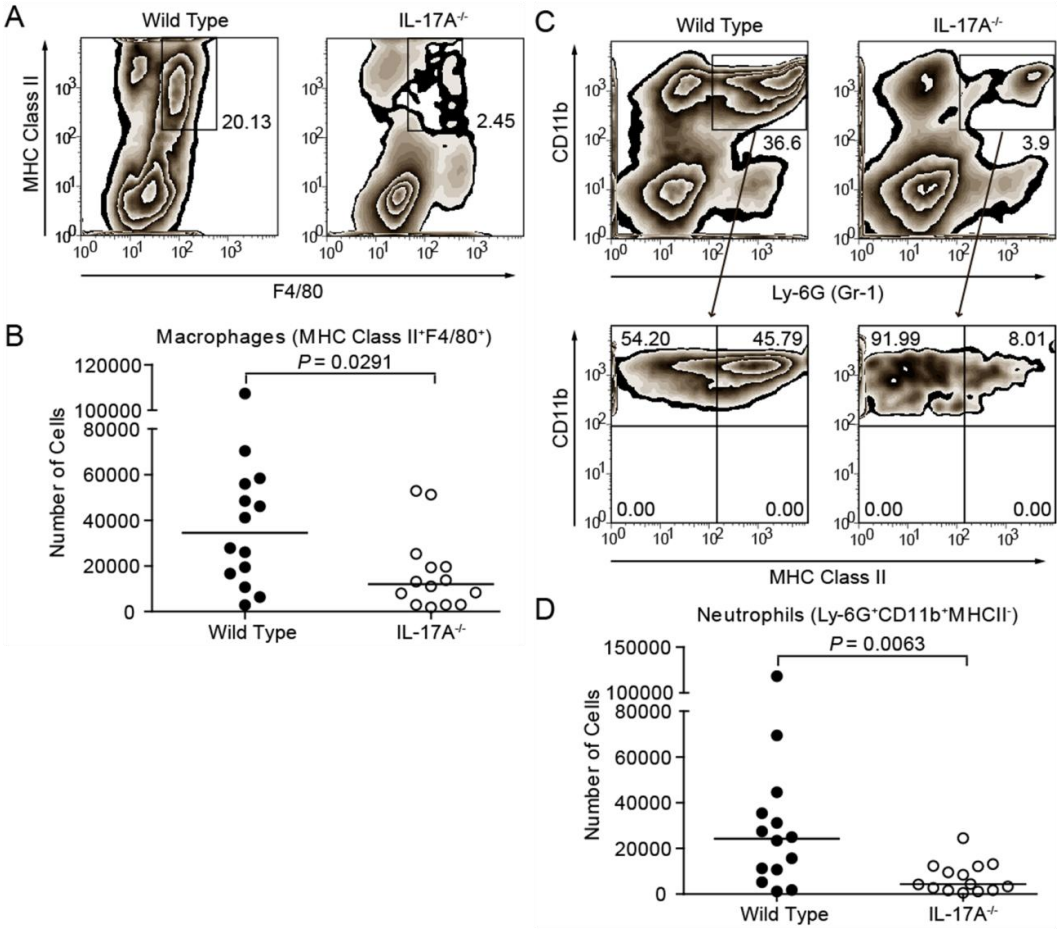

Figure 3.16 Macrophage and neutrophil infiltration is deficient in IL-17A$^{-/-}$ mice in response to UTI. Wild type (filled symbols) and IL-17A$^{-/-}$ (open symbols) mice were infected with UPEC strain CFT073 via the transurethral route. Mice (*n*=14 per background, two experiments combined) were sacrificed at 48 hpi. FSC versus SSC plots were gated to include all MHC Class II^{+}F4/80^{+} and Gr-1^{+}CD11b^{+} cells as determined by backgating. The Gr-1^{+}CD11b^{+} population was further interrogated for MHC Class II negatively to enumerate neutrophils. (A) Representative flow plots showing MHC Class II^{+}F4/80^{+} cells from wild type (left) and IL-17A$^{-/-}$ animals (right). (B) The total number of MHC Class II^{+}F4/80^{+} cells. (C) Representative flow plots showing Gr-1^{+}CD11b^{+} cells from wild type (left) and IL-17A$^{-/-}$ animals (right). The bottom plots show MHC Class II expression by the boxed population in the top plots. (D) The total number of Gr-1^{+}CD11b^{+}MHC Class II^{-} cells. In (A) and (C) numbers are the percent of the parent population and in (B) and (D) each symbol represents an individual animal while bars represent the median values.

(P=0.0031) (Figure 3.16D) cell populations were significantly lower in IL-17A$^{-/-}$ mice as compared to wild type. F4/80^{+}MHC Class II^{-} cells likely represent a monocyte or macrophage population that has not undergone activation (Figure 3.16A), which correlates with the lack of activated macrophages (F4/80^{+}MHC Class II^{+}) in IL-17A$^{-/-}$ mice. Moreover, the presence of the MHC Class II^{-} cell population in the IL-17A$^{-/-}$ mice suggests that there is not a defect in macrophage migration to the bladder in response to infection, but a defect in macrophage activation, which could potentially be due to inadequate expression of IFN-γ in this background (Figure 3.11B). Uninfected mice were not examined in this particular study, as knockout mice were limited and the inflammatory cells in question are not found in naïve animals (similar to the CD11b^{+}Gr-1^{+} population in PBS-treated animals in Figure 3.8). These data reveal that IL-17A plays an important role in the recruitment of neutrophils to the bladder, and potentially activation of macrophages, in response to UPEC infection.

3.4.3 IL-17R$^{-/-}$ mice lacking the receptor for IL-17A display enhanced clearance of UPEC during experimental UTI

Due to impaired proinflammatory cytokine and cellular responses, mice lacking IL-17A$^{-/-}$ appear to have an inability to clear UPEC-mediated UTI as well as their wild type counterparts (Figure 3.10). Among the 5 IL-17 cytokine family members, IL-17F has the highest degree of amino acid homology to IL-17A (~50%) (184, 217). IL-17F has also been shown to function similarly to IL-17A in terms of stimulating neutrophil recruitment during infection, albeit with less efficiency (184, 217). Given the role of IL-17A in clearance (Figure 3.10) and the redundant function of IL-17A and IL-17F, one may predict that mice lacking both IL-17A and IL-17F would be even more susceptible

to UPEC-mediated UTI than IL-17A$^{-/-}$ mice. IL-17A and IL-17F both signal though IL-17R (or IL-17RA) (257, 381, 421), and another IL-17R family member, IL-17RC, is necessary for optimal signaling (421). Since we had access to IL-17R$^{-/-}$ mice, the aforementioned prediction was indirectly tested [IL-17F$^{-/-}$ and IL-17A$^{-/-}$IL-17F$^{-/-}$ mice have since been generated by a group in Japan (187)]. IL-17R$^{-/-}$ mice were transurethrally infected with CFT073 and sacrificed at 48 hpi to determine the bladder and kidney bacterial burden. Surprisingly, mice lacking IL-17R$^{-/-}$ were *more* resistant to UPEC-mediated UTI than wild type mice, as both their bladders and kidneys had significantly less bacteria ($P = 0.0065$ and $P = 0.0479$, respectively) (Figure 3.17A). Upon examination of the cellular infiltrate in these animals, no statistically significant differences were seen when examining either neutrophil (Gr-1^{+}CD11b^{+}MHC Class II^{-}) or macrophage (F4/80^{+}MHC Class II^{+}) populations (Figure 3.17B); however, there was a trend toward deficient infiltration of phagocytes in the receptor knockout mice. These results suggest an additional unanticipated level of regulation in signaling by the IL-17 ligand and receptor family members, as a lack of signaling through IL-17R results in an environment where UPEC are more efficiently eradicated.

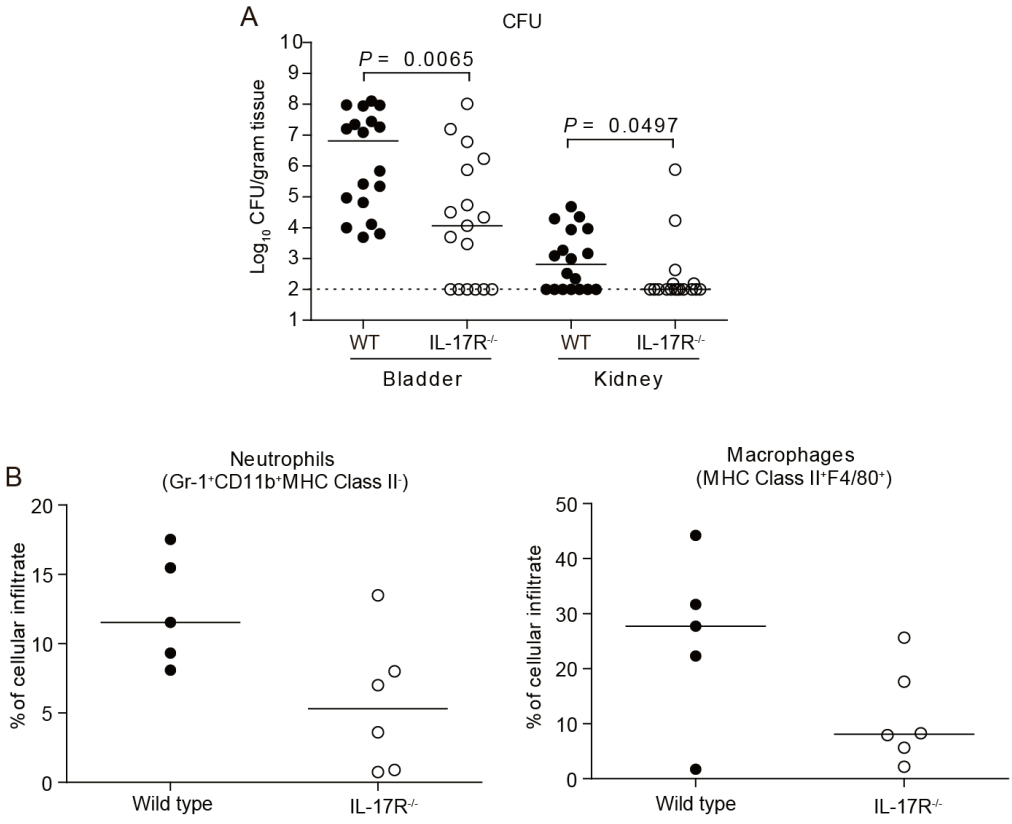

Figure 3.17 IL-17 receptor knockout (IL-17R$^{-/-}$) mice are less susceptible to UTI than wild type animals. Wild type and IL-17R$^{-/-}$ mice were transurethrally infected with CFT073. (A) Bladder and kidney CFU (*n*=17-18 each background, 3 experiments combined) was enumerated at 48 hpi. (B) Bladders were harvested for examination of cellular infiltrate by flow cytometry (*n*=5-6 each background). FSC versus SSC plots were gated to include all positive neutrophil and macrophage events in the infiltrating cell population. Each symbol represents an individual animal while bars represent the median values.

Discussion

3.5.1 Summary

With the exception of ABU (449), the bladder mucosa has been widely accepted as a sterile environment. Mammalian hosts employ a number of mechanisms to keep this niche microbe-free, as infection by bacterial and fungal pathogens can lead to serious clinical consequences. Here we characterized the role of the cytokine IL-17A during UTI using a murine model. IL-17A was upregulated specifically in response to UPEC antigens by secondary lymphoid tissue cells from UPEC-infected C57BL/6 mice. IL-17A transcripts were also highly upregulated in the bladders of acutely infected mice, γδ T cells and $CD11b^{+}Gr\text{-}1^{+}$MHC Class II^{+} inflammatory monocyte (IM) cells being major sources. Although no role for IL-17A in infection-induced protection was observed, we noted a deficiency in bladder neutrophil influx during the very early stages of acute cystitis, and higher bacterial burdens in IL-$17A^{-/-}$ mice. IL-$17A^{-/-}$ mice also had impaired proinflammatory transcript expression and fewer macrophages and neutrophils infiltrating the bladder tissue days after UTI. Taken together, these results define IL-17A as an important factor in the innate immune response to UPEC-mediated UTI.

3.5.2 Adaptive immunity and IL-17A

Although the presence of pathogen-specific antibodies in the urine and serum of infected humans and experimental animals has been documented for decades (434), the cascade of immunological events that occurs during the generation of adaptive immunity during UTI has not been established. Although cells from UPEC-sensitized mice highly upregulate IL-17A *in vitro* (Figure 3.1), IL-17A was not necessary for the generation of

vaccine-induced protective immunity (Figure 3.2). This result is in contrast to vaccination models for *Streptococcus pneumoniae, Bordetella pertussis,* and *Pseudomonas aeruginosa*, where IL-17A was required for a protective immunity (165, 250, 321). As the immune system features redundant pathways, it is unclear if there is a compensatory factor acting in bladder or lymphoid tissue or if the downstream effects of IL-17A are indeed not necessary for a protective response to UTI.

3.5.3 Innate immunity and IL-17A

3.5.3.1 Dynamics and stimulants of IL-17A transcription during UTI

In addition to secretion in a recall response setting, IL-17A is upregulated in an innate fashion (Figure 3.3A). Similarly, airway IL-17A peaked innately in response to intranasal infection with *Chlamydia muridarum*, another mucosal pathogen, and this was dependent on bacterial replication (471). In experimental UTI, IL-17A upregulation was also shown to be either UPEC-specific or dependent on the ability of UPEC to successfully colonize the urinary tract, as transurethral inoculation of a fecal strain EFC4 does not induce IL-17A transcript to the levels of the uropathogenic strain (Figure 3.4B). As shown in Figure 3.3A and B, expression dynamics of IL-17A during the first week of infection superimposes with bladder colonization by UPEC. Using either gDNA or cDNA as a template, PCR could be conducted on samples from individual mice to determine if there is a direct correlation between the level of bacterial infection (as measured by the amount of *fimH* in semi-quantitative or qPCR, for example) and expression of IL-17A mRNA by the host. The apparent dose-dependency of this response (based on separate experiments evaluating colonization and IL-17A mRNA

expression) supports the latter idea that upregulation of IL-17A in response to UTI is colonization-dependent and stimulated by recognition of PAMPs, not pathogen-specific motifs. As a future study, it would be interesting to determine which of these suppositions is accurate by stimulating primary bladder cells with *E. coli* strains EFC4 and CFT073 *in vitro* and assessing IL-17A transcript upregulation or secretion by qPCR or ELISA, respectively. However, this type of approach will exclude the cells that migrate to the bladder in response to infection to secrete IL-17A (Figure 3.9B). Alternatively, EFC4 and CFT073 lysates or killed cells could be transurethrally instilled into mice to determine if stimulation with strain-specific antigens is sufficient for migration of IL-17A-secreting cells and secretion of IL-17A. Additional strains that are known to colonize or not colonize the urinary tract ("uropathogenic" to different extents) could also be tested to shed light on this topic. While the more likely explanation for stimulation of IL-17A transcription by CFT073 and not EFC4 has to do with the ability to colonize the urinary tract, at this time one cannot rule out the possibility of a UPEC-specific ligand that stimulates IL-17A.

3.5.3.2 The discrepancy between IL-17A transcript and IL-17A protein expression

A recent study by Ingersoll and colleagues surveyed cytokines and chemokines in the bladder of mice during a two-week experimental UTI (186). While most of the protein levels examined peaked around two dpi and returned to near baseline levels after a week, IL-17A peaked accordingly but remained elevated (relative to control mice) throughout the experiment (186). Our IL-17A transcript data was consistent with these findings (Figure 3.3A). Despite this agreement, when we used ELISA to quantify IL-

17A protein in bladder homogenates from infected mice, a different pattern of expression was detected: low, but detectable, baseline levels in uninfected animals that steadily increased in response to infection throughout the duration of the experiment (Figure 3.3C). These results differed from the transcript data in that: 1) transcript levels are completely undetectable in the absence of infection, and 2) transcript levels demonstrate a signature spike in expression at two dpi while in protein steadily increases over time. The methodology used in the study by Ingersoll *et al.* negates comparison with the protein data in Figure 3.3C, as they used cytokine bead arrays rather than ELISA and expressed the data as a ratio compared to mock-treated animals rather than as calculated concentrations using a standard curve. This fact aside, the protein data we generated (Figure 3.3C) may not correlate with our transcript data (Figure 3.3A) due to rapid receptor-ligand internalization and subsequent degradation of IL-17A during the early time points. This phenomenon has been demonstrated for other cytokines (361). While receptors for IL-17A are expressed ubiquitously (217), the lack of the additional inflammatory cell infiltrate at later time points may hasten this process, allowing protein levels to linger and appear to increase over time. Alternatively, detergent was not used during homogenization of the bladder tissue, thus intracellular IL-17A may not have been detected due to incomplete cell lysis in the absence of a cellular disruption agent. Regardless, it is not known if IL-17A persists in the bladder longer than 28 dpi, or if the presence of IL-17A is beneficial or deleterious to host tissue, as residual IL-17A could conceivably aid in either priming the urinary tract in the event of reinfection or lead to autoimmune-related pathology.

3.5.4 Cell types responsible for upregulation of IL-17A during UTI

3.5.4.1 γδ T cells

A number of cell types have been shown to secrete IL-17A (32). Unlike classical αβ T cells, which recognize antigen that is processed and presented in the context of self MHC molecules, γδ T cells harbor the ability to directly recognize cognate antigen, allowing for rapid production of effector molecules (55, 62). Therefore, because of the early upregulation of IL-17A in the bladder (Figure 3.3A) (186), we reasoned that CD4-positive Th17 cells are not the principal source of IL-17A. Despite being in the T cell minority (Figure 3.6A), intracellular staining and flow cytometric analyses demonstrated that γδ T cells were a major source of IL-17A during UTI (Figure 3.6B-D). We also confirmed expression of IL-23R transcript in the bladder tissue of infected mice (Figure 3.7A), an important factor in secretion of IL-17A by γδ T cells (402).

It was previously reported that the main IL-17A-producers in the liver after *L. monocytogenes* infection were γδ T cells of Vγ4 and/or Vγ6 type(s) (142). Even more rapid production of IL-17A by γδ T cells was reported in a murine *E. coli* i.p. infection model, and in this model, the responsive γδ T cells were found to be mainly the Vγ6Vδ1-expressing subset (376). Thus, we wanted to investigate if similar repertories of γδ T cells are involved in UTI host defense. As antibodies for the known γ and δ variable chains were not readily available, RT-PCR was performed on RNA collected from UPEC-infected mouse bladders. Using previous established primer sets, all the variable chains probed were found to be expressed in the bladder (Figure 3.7C). Follow-up studies include qPCR analyses to quantitatively determine expression levels of the variable chains and flow cytometry studies using variable chain-specific antibodies to

determine expression of IL-17A by γδ T cell subsets. On a final note, TCR $\delta^{-/-}$ mice were still able to generate some IL-17A transcript over background (Figure 3.5), demonstrating that additional cell types make IL-17A in the bladder of UPEC-infected mice.

3.5.4.2 CD11b^{+}Gr-1^{+}MHC Class II^{+} inflammatory myeloid cells

Indeed, upon exploring the capability of neutrophils to secrete IL-17A in this model, we uncovered the ability of CD11b^{+}Gr-1^{+}MHC Class II^{+} cells to upregulate IL-17A in response to infection (Figure 3.8 and Figure 3.9). This cell population is only present in the bladder in significant numbers after mice have been infected with UPEC (Figure 3.9A) and may represent an IM population (17, 371). Approximately 15% of this cell population makes IL-17A, translating to a median of $4x10^{3}$ bladder cells (roughly 0.8% of the total cells in the bladder) (Figure 3.9B and C). Aside from their ability to migrate to the infected murine bladder and synthesize IL-17A, the role of CD11b^{+}Gr-1^{+}MHC Class II^{+} cells during UTI is unclear and warrants further investigation. In total, these results suggest that γδ-positive cells and an uncharacterized inflammatory myeloid population secrete IL-17A in response to UPEC infection.

3.5.5 IL-17A and control of bacterial infection

3.5.5.1 The role of IL-17A in controlling bacterial burdens

In the context UTI, IL-17A appears to play a role in optimal restriction of bacterial burden, especially at time points after the peak of IL-17A expression in wild type mice (Figure 3.10). Infection models for *L. monocytogenes,* disseminated *E. coli*, *K. pneumoniae,* oral and systemic *Candida albicans*, oral *T. gondii*, *C. muridarum*, *Bacillus subtilis*, and others also show that IL-17A signaling is required for acute clearance of the

invading organism (68, 142, 178, 210, 376, 379, 465, 471). This collection of data demonstrates the breadth and versatility of IL-17A-mediated pathways in handling various classes of microbes. In contrast, IL-17A has been shown to be dispensable for clearance in infection models for systemic *S. enterica* serovar Enteritidis and pulmonary *M. bovis* bacille Calmette-Guérin (363, 364, 438). The factors determining whether or not IL-17A signaling is important for clearance in a particular infection model are not clear. They may depend on anatomical location, inherent qualities of the infectious agent, or innate immune signaling in response to pathogen recognition. Lastly, mice treated with IL-17A neutralizing antibody experience exacerbated bacterial loads in the liver and peritoneal exudates in response to i.p. infection with *E. coli* (376), suggesting that this background may generally lack the ability to control infection with *E. coli*. Other routes of infection with *E. coli* (for example, gut infections with enterohemorrhagic or enteropathogenic strains) or UTI caused by other uropathogens (for example, *Klebsiella, Pseudomonas, Proteus,* or *Enterococcus* species) need to be examined to determine if the lack control of UPEC-mediated UTI by mice unable to signal through IL-17A is specific to the bacterium or to the biological niche.

3.5.5.2 The role of IL-17A in regulating cytokine and chemokine expression

IL-17A has a well-documented role in regulating cytokine, chemokine, and antimicrobial expression (110). Thus, it was not surprising that proinflammatory bladder transcripts probed in infected IL-17A$^{-/-}$ mice were not expressed as well as in wild type counterparts (Figure 3.11). The lack of chemokines important for the infiltration of neutrophils (CXCL2 and weakly CCL20), T cells (CXCL10, CCL5), and DCs (CXCL10,

CCL20) may each or all contribute to the defect in bacterial clearance seen in the IL-17A$^{-/-}$ animals. Additionally, cytokines crucial for activation and mobilization of innate immune responses (IL-6 and IFN-γ) were also lacking in this background. While deficiency in these transcripts may be the result of inadequate stimulation of epithelial cells and resident macrophages, lower iNOS levels may be a reflection of impaired neutrophil infiltration in the IL-17A$^{-/-}$ mice (Figure 3.15 and Figure 3.16D). Unexpectedly, IL-4 was also significantly lower in the bladders of IL-17A$^{-/-}$ mice. IL-4 is a canonical Th2 cytokine (291), and given that IL-4$^{-/-}$ mice do not have an acute UPEC clearance defect (198), a function in UTI immunity has not been defined. Nonetheless, the role of IL-4 in B cell activation (219) suggests that IL-4 may be upregulated to stimulate B cell antibody generation in an adaptive response to UTI. In total, it appears that there is a general inflammatory defect in IL-17A$^{-/-}$ mice, and that genes of both appreciated and unknown importance are affected by the absence of IL-17A during UTI.

3.5.5.3 The role of IL-17A and recruitment of neutrophils and macrophages

Since IL-17A orchestrates neutrophil recruitment to infected tissue, there is precedence for an innate immune mechanism involving rapid secretion of large amounts of IL-17A to bolster neutrophil killing of UPEC (147, 217). We could not detect any qualitative differences in inflammatory cell recruitment by histological examination of bladders from wild type and IL-17A$^{-/-}$ mice (Figure 3.12 and Figure 3.13), nor was quantitation of MPO a sensitive enough assay to reveal differences in neutrophil infiltration between wild type and IL-17A$^{-/-}$ mice (Figure 3.14B). However, when counted directly in the urine, a lack of early neutrophil infiltrate in IL-17A$^{-/-}$ mice was

apparent (Figure 3.15). This result was potentially due to both impaired neutrophil exit from the bone marrow and migration to the bladder tissue, as growth factor G-CSF and neutrophil-specific chemokine transcripts were both decreased in infected IL-17A$^{-/-}$ animals. Interestingly, antibody-mediated knockdown of G-CSF rendered mice more resistant to UTI, and the authors of that study suggested that macrophage activation status may be responsible for this surprising phenotype (186). Although deficient in G-CSF expression, IL-17A$^{-/-}$ mice do not exhibit an enhanced clearance phenotype, possibly owing to the pleiotropic effects of IL-17A deficiency or signaling by residual G-CSF.

We also examined neutrophil levels in wild type and IL-17A$^{-/-}$ mice at later time points during infection. Macrophages were included in the analysis to see if their total numbers varied similarly to neutrophils. Upon UPEC infection, numbers of both cell populations increased several fold over PBS mock-infected mice; however, both neutrophil and macrophage counts were lower in the IL-17A$^{-/-}$ mice (Figure 3.16B and D). These data reveal that the defect IL-17A$^{-/-}$ mice exhibit in UPEC clearance may be due to less macrophages and neutrophils present in the bladder to execute bacterial clearance.

3.5.6 The unexpected clearance phenotype exhibited by IL-17R$^{-/-}$ mice

While one may expect that removing a particular ligand or its corresponding receptor from a system would yield the same phenotype, redundancy and broad functionality of the immune system appears to meddle with the simplicity of this concept. In this work, IL-17A$^{-/-}$ mice and IL-17R$^{-/-}$ mice were found to have opposing phenotypes in response to UPEC-mediated UTI; ligand knockouts were deficient in clearing bacteria

while the receptor knockouts more efficiently eliminated UPEC (Figure 3.10 and Figure 3.17A). Unfortunately, we only had access to a small group of IL-17R$^{-/-}$ mice for a follow-up analysis of inflammatory cell infiltration by flow cytometry, which was inconclusive (Figure 3.17B). With no knowledge of UTI-specific cytokine or cellular responses in the IL-17R$^{-/-}$ animals, we will make predictions regarding the hyperclearance phenotype based on the literature. Similar to IL-17R$^{-/-}$ animals, we mentioned in section 3.5.5.3 that mice treated with an anti-G-CSF antibody and infected transurethrally with UPEC exhibited enhanced bacterial clearance (186). Mice lacking G-CSF did, however, also have increased levels of cytokines and chemokines that influence macrophage activity and recruitment (186). Moreover, mice lacking both IL-17A and IL-17F had increased amounts of several proinflammatory cytokines in response to *Citrobacter rodentium* infection (187). These results suggest that signaling though IL-17R may negatively regulate expression of proinflammatory cytokines. In support of this, IL-17R$^{-/-}$ mice infected with *Helicobacter pylori* had a marked increase in gastric B cells, plasma cells, and lymphoid follicles, indicating that IL-17R signaling down-regulates the chronic mononuclear inflammation elicited by *H. pylori* infection (6). Therefore, a lack of signaling through IL-17R may result in an exacerbated upregulation of proinflammatory cytokines in UPEC-infected IL-17R$^{-/-}$ mice, and this milieu may incite enhanced UPEC killing by both myeloid and lymphocytic cellular infiltrate. It is possible that in the IL-17A$^{-/-}$ mice, residual signaling by IL-17F keeps this hyperinflammatory phenotype in check, at the cost of both deficient proinflammatory responses and bacterial clearance. To this end, some *in vitro* studies were conducted where RAW or primary bone marrow macrophages were treated with different combinations of IL-17A, IL-17F, and IFN-γ and

analyzed for their ability to engulf and destroy UPEC. While having issues with repeatability, these experiments did hint at an inhibitory role for the IL-17 family of cytokines.

IL-17R can interact with several members of the IL-17 cytokine family leading to differential signaling cascades and biological outcomes. As mentioned earlier, both IL-17A and IL-17F have been shown to interact with IL-17R when in a complex with IL-17RC, leading to varying levels of signaling and cellular responses (421). Furthermore, in a complex with the IL-17RB receptor, IL-17R has been shown to be able to bind to IL-25, resulting in the induction of IL-5 and IL-13, a Th2 skewing milieu (335). In the absence of IL-17R, Th2-type immune responses may be inhibited, allowing Th1-type responses (and activation of proinflammatory responses and phagocytosis by the corresponding cytokines) to predominate, materializing as a hyperclearance phenotype. There also may be a cellular component to this regulation, as IL-17R is highly expressed on hematopoietic cells, while the other IL-17R family members and their splice variants, like IL-17RC, are highly expressed on non-hematopoietic cells (224). The lack of IL-17R expression on immune cells may negate inhibitory signaling received via this receptor, leaving these cells unchecked in their bactericidal functions. All told, there appears to be an intricate balance of signaling necessary for optimal clearance of UPEC. Mice experiencing enhanced bacterial clearance due to hyperinflammatory responses may also sustain permanent damage to the epithelia or chronic inflammation. Future studies examining the inflammatory responses of IL-17R$^{-/-}$ mice during UTI should address these ideas.

3.6 Conclusion

Collectively, these data demonstrate that IL-17A plays a role in the innate immune response to experimental UTI in a mouse model. As many of the genes influenced by IL-17A have similar function during UTI in mice and men (324), we expect that IL-17A also plays a role in controlling the bacterial burdens early during UTI in humans. IL-17A accomplishes such control by enhancing the presence of mRNA transcripts important for the infiltration of neutrophils and other inflammatory mediators. The presence of such cell types is crucial to the defense of the urinary tract from epithelial cell adherence and subsequent invasion by UPEC.

Chapter 4
Discussion and Future Directions

4.1 Overview and Primary Findings

UPEC induces a robust innate immune response that involves, among other facets, cytokine and chemokine secretion and immune cell infiltration. While it has been observed that both transurethral infection with live UPEC and intranasal vaccination with UPEC antigens afford mice with an acquired immune response that results in accelerated clearance, little has been established regarding the nature of adaptive immune effectors (*i.e.* specific T cell subsets and antibody isotypes) and innate mediators of this acquired immune response. In this work, the efficacy of UPEC OM iron receptor antigens as mucosal vaccine candidates was determined; in this process immunological correlates of protection were established, a significant contribution to field of UTI vaccinology. Both the relative levels of urinary IgA in the bladder and relative levels of antigen-specific IgG in the serum are linked to protection from UPEC-mediated UTI. Additionally, the role of IL-17A was evaluated in the innate and adaptive host response to UTI. While upregulated in both arenas, infections in IL-17A$^{-/-}$ mice revealed that IL-17A, while dispensable for the formation of acquired immunity, was necessary for optimal control of the innate immune response to UPEC in the bladder. IL-17A was shown to be secreted by $\gamma\delta$ T cells and an IM population. The presence of IL-17A was required for upregulation of proinflammatory cytokines and chemokines, and neutrophil and

macrophage migration to the bladder, events that likely result in the clearance defects exhibited by IL-17A$^{-/-}$ mice. Taken together, these findings contribute significantly to the understanding and of innate and adaptive immune responses during UTI.

4.2 Development of a vaccine against UPEC-mediated UTI

4.2.1 A new approach to identifying UPEC vaccine candidates

Conventional vaccinology approaches targeting *bone fide* virulence factors of UPEC, namely FimH of type 1 fimbriae, have generated promising data in animal models (235). Regardless of these efforts, there is no licensed vaccine available for prevention of UTI humans in the United States. This result is likely due to the challenge of targeting a relatively heterogeneous group of pathogenic strains in a unique physiological niche. A new approach was necessary to identify novel vaccine targets suitable for animal trials. Despite the advances afforded by reverse vaccinology, an *in silico* genomics-based approach to vaccine development (54), it was not practical at the time due to a lack of high-throughput methods to test for protection. A more optimal approach to UPEC vaccine discovery would be one that is broad and unbiased, yet has some aspect of selectivity to narrow the list of candidates. An example of this has been employed in Pneumococcal vaccinology (117). Applied to UPEC, we thought it would be useful to target only *PASivE* candidates, UPEC proteins that are pathogen-specific, antigenic, surface-exposed, and *in vivo* expressed. These particular traits are attractive because they ensure that the targets are not widely expressed by commensal *E. coli*, are accessible to and recognized by the host immune system, and are synthesized specifically *in vivo* and likely important for infection and pathogenesis.

4.2.2 The "omics" screening approach to identify *PASivE* UPEC vaccine candidates applied

Somewhat to our surprise, the six top ranking *PASivE* vaccine candidates (those that repeatedly ranked highest in all the screens) all belong to the same functional class of proteins. The hits were all membrane proteins involved in iron acquisition, a process critical for microbial pathogenesis. Other, lower-ranked targets identified by these studies included metabolic transporters, putative adhesins, and hypothetical proteins that may represent novel virulence factors. While not representative of a class of critical virulence factors, these candidates still warrant testing in the murine model. Intranasal immunization of CBA/J mice with three of the six OM iron receptors provided significant protection from experimental infection. There were no apparent characteristics hinting at why some of the OMP iron receptors were protective and some were not. Putative reasons may include the solubility of the proteins (affecting APC uptake) or the presence of preferential epitopes for antigen presentation in some aa sequences and not others. Alternatively, each of the iron receptors may have similar potential in stimulating efficacious acquired immunity; however, temporal and quantitative expression of these receptors by UPEC may dictate the ability of antigen-specific antibodies to interfere with iron uptake or target bacteria for destruction.

4.2.3 Immune responses to OM iron acquisition receptors

With regard to cytokine responses, all six candidates similarly induced transcription and secretion of proinflammatory cytokines and chemokines. In addition, regardless of protective effect, systemic humoral responses were elicited by all candidates, specifically production of antigen-specific IgG antibody. What differed

dramatically was the robustness and magnitude of these responses. Both antigen-specific class-switching from IgM to IgG and production of antigen-specific IgA in the urine were found to represent immunological correlates of protection from *E. coli* bladder colonization. The discovery of immunological correlates of protection is a significant contribution to UTI vaccine research, as a reverse vaccinology approach can now be applied to identify additional vaccine candidates. Rather than conducting challenges on large numbers of animals, larger pools of vaccine candidates can be screened on a small group of animals and be evaluated for efficacy by their class switch indices and ability to generate antigen-specific IgA in urine.

4.2.4 Targeting a class of functionally redundant virulence factors in a multivalent vaccine

The results of the "omics" approach to identify *PASivE* vaccine candidates clearly indicated the importance of iron uptake in uropathogenesis. By default, these results brought forth the idea of targeting an entire class or classes of molecules important for virulence. Given the absolute requirement of iron and the presumed functional redundancy of proteins involved in its acquisition, in hindsight, targeting iron uptake systems as a whole should have been considered an obvious approach. Indeed, a multivalent vaccine that contains several antigens, each targeting a difference iron uptake system, would presumably curb bacterial evasion of host iron sequestration and is therefore more inclined to generate sterilizing immunity.

While potent immunologically, the OMP iron receptors themselves are not attractive vaccine candidates because of their biochemical nature: they are relatively large integral membrane proteins that often encounter solubility issues during expression and

purification. Rather than combining each protein candidate in its entirety, this current theme may evolve to incorporate potent antigenic motifs from each target in an inert carrier protein backbone. Additionally, we predict that the lengthy extracellular loops that protrude beyond the LPS and capsule layers contain the antigenic epitopes that are recognized by the host immune system. The fact that mice vaccinated with synthetic peptides representative of the extracellular loop sequences demonstrates a trend toward protection accompanied by some, admittedly modest, antigen-specific immune responses supports this prediction. These modest immune responses may be due to inefficient uptake of soluble peptides by APCs, resulting in poorer antigen processing and presentation (237) – a process that could be improved by delivery in more formulations that facilitate optimal modes of phagocytosis for antigen presentation.

Regardless of whether the loops actually contain aa sequences of interest, epitopes from IreA, Hma, and IutA (protective candidates) must be mapped and cloned for combinatory administration in a multivalent platform. To do this, splenocytes from mice immunized with the protective candidates need to be harvested and fused to myeloma cells. Hybridoma supernatants can then be evaluated for secretion of specific antibody by ELISA, and hybridomas secreting OMP iron receptor-specific antibody cloned (by limiting dilution or plating in soft agar), expanded, and preserved in frozen stocks. Once mAbs are isolated, they can be evaluated for antimicrobial activity in a number of *in vitro* assays and in mice for their ability to passively protect naïve donors from transurethral challenge with UPEC. After identification of biologically relevant clones, the epitopes of interest can be mapped by screening overlapping synthetic peptides corresponding to the exposed loops of OMP iron receptors by ELISA. If this screen proves unsuccessful,

sequences representing other parts of the protein should also be analyzed. It is also possible that the epitopes of interest may be discontinuous conformational epitopes, in which case a much more laborious effort involving predictions based on crystal structures and screening point mutations may be necessary. Testing the reactivity of the mAbs of interest against unfolded and re-folded protein will also help discern if the epitopes of interest are conformation-dependent. After this extensive effort, potent epitopes from the different receptors can be cloned and expressed in combination or combined in another type of platform or evaluation of efficacy in mouse trials.

Other systems that could potentially be used in a multivalent vaccine strategy are proteins involved in adherence or effector secretion. Capsular and O-antigens are also likely multivalent vaccine targets; however, their heterogeneity approaches a realm where vaccine design is impractical, if not impossible. Thus, the next horizon in vaccine development may involve the identification of genes previously unknown to be responsible for essential virulence phenotypes and combination of the respective gene products into a single cocktail for vaccination.

4.2.5 Testing routes and adjuvants

The testing phase presents a new challenge for UPEC vaccinology. As discussed, the field should advance towards a multi-subunit vaccine directed against *PASivE* proteins. Additionally, being a mucosal pathogen, the vaccine must be administered with an appropriate adjuvant. The involvement of TLRs in the immune response to UTI and current knowledge of their ability to incite innate and direct adaptive responses make them attractive adjuvant candidates for UTI vaccines (229, 391). These and other

mucosal adjuvants and variations in vaccination routes and schedules must be tested in an effort to generate UPEC-specific local and systemic antibodies (238, 317) and optimize production of immunological memory, not tolerance (57, 260). To this end, animal trials must be used to address these issues and also to distinguish details regarding the molecular and cellular factors that play a role in the adaptive immune response to UTI. There is considerable work to be done to better understand the mechanisms of protective immunity against UPEC in the bladder. Specifically, available knockout mouse strains could be used to systematically evaluate the role of various receptors (CD series), cytokine and chemokine ligands and receptors (*e.g.*, CXC or CC families), innate signaling molecules (*e.g.*, MyD88, TRIF, TRAM, "3d" mice lacking intracellular TLRs) and cell types (for example, $CCR2^{-/-}$ mice lack IMs) in controlling UPEC-mediated UTI and eliciting potent adaptive and memory immune responses. Ideally, the field can acquire insights on UTI immunity at a level suitable to rationally develop a much-needed vaccine that elicits sterilizing immunity against UPEC in the human urinary tract.

4.2.6 Recurrent UTI and immunological memory

Despite immunity to natural infection, reinfection is a rampant problem. There is much controversy in the field as to the cause of reinfection. Same-stain reinfection has been documented, indicative of a reservoir (191, 277, 461). The source of this reservoir, however, is debatable, and there appears to be two main schools of thought. The first is that UPEC strains exist in the gut among benign fecal flora and fecal contamination of the vaginal meatus and the urethra result in reinfection. The second is that, during the initial episode of infection, UPEC undergoes an intracellular life cycle, part of which

involves the formation of QIRs that foster reemergence of UPEC and symptomatic infection upon an uncharacterized epithelial damage event (280). While either or both of these theories may be partly or entirely accurate, the simple fact of the matter is that if clonal reinfection is occurring, lasting effective immunity is not generated by natural infection. As it has been demonstrated in the mouse model, an adaptive immune response *is* generated during natural infection, the longevity of which is unknown. The fact that same-strain reinfections have been documented in patients indicates that while exposure of the bladder mucosa to UPEC antigens may stimulate an adaptive immune response, this response is not lasting. The results of the SolcoUrovac trials also support this conclusion, as ceasing the administration of uropathogenic antigen vaginal suppositories correlates with recurrence of UTI in patients (427, 429). Since there was no "official" adjuvant used in the Solco trials (it is assumed that the sample preparations were ripe with LPS and other TLR stimulants), it is possible that administration with a more effective (perhaps non-TLR-based) adjuvant may stimulate lasting effects. Support for this idea is found in the *Salmonella* literature, as there is a report documenting the generation of acquired humoral and cellular immunity independent of TLR4, TLR2, and MyD88 signaling (368). Ultimately, the appropriate adjuvant would be one that stimulates the activation of dendritic cells in such a way to mobilize production of a protective immune response. Regardless of adjuvant choice, the resonating idea is the use of multivalent vaccine formulations containing epitopes from *PASivE* candidates. Ideally, potent administration of a few tailored targets (rather than an thousands of antigens found on whole cells, some in limiting concentrations) will increase the likelihood of an effective and lifelong memory response.

4.2.7 Outlook on vaccination

While treatments have been proposed to expel intracellular UPEC from the bladder (37, 280), an *E. coli* reservoir harboring potential UPEC strains will always be present in the intestine (194, 272, 461). From epidemiology studies delineating UTI risk factors to cell and molecular studies characterizing the host response during UTI, research in the field is critical to ameliorate the societal and economic costs associated with this disease of morbidity. While many general concepts regarding the immune response to UTI are understood, many molecular details are only beginning to be discovered and appreciated. Such details are likely going to be the key in the development of a successful vaccine for use in humans or more effective and directed treatments in lieu of antibiotics.

4.3 Determining the role of IL-17A during UTI

4.3.1 Adaptive immunity: If IL-17A isn't important, what is?

Given the identification of humoral correlates of protection in a vaccine setting and the vast literature documenting the antibody response to UPEC, the role of the humoral response in clearance of UPEC infection is obvious. What is not clear is the Th cell population that mediates activation of B cells and development of antibody-secreting plasma cells. Chapter 2 highly implicated Th17 cells in mediating the immune response to intranasal vaccination with UPEC antigens. Since these experiments were conducted, literature has emerged indicating that both the cholera toxin adjuvant and the intranasal route of vaccination stimulate Th17-mediated immune responses (239, 473), indicating that we were unknowingly skewing the iron receptor vaccination studies to this arm of

the Th cell response. Thus, it is not surprising that, in Chapter 3, IL-17A was found not to be necessary for the formation of an effective adaptive immune response to natural (transurethral) infection. Considering IL-17A is the signature cytokine of the Th17 subset of T cells, this result could suggest that Th17 cells do not play a role in acquired immunity to UTI. Despite this notion, Th17 cells are known to secrete a number of other cytokines (*i.e.*, IL-21, IL-22) that may play a role in adaptive immunity to UTI. Moreover, both DCs and infected apoptotic cells are present in the infected mouse bladder. This is pertinent information as DC phagocytosis of infected apoptotic cells is the cellular event that triggers secretion of the cytokine milieu (TGF-β and IL-6) that promotes development of the Th17 lineage, indicating that Th17 cells have potential to develop in the bladder in response to UPEC infection. Future studies to test the role of Th17 cells in UTI may involve evaluating IL-22$^{-/-}$ mice in the protective model of UTI. Regarding the Th1/ Th2 dichotomy, in response to intranasal vaccination, no Th1 or Th2 skewing was evident upon examination of indicative antibody isotypes (IgG2a verses IgG1). Nonetheless, cytokine secretion dynamics by splenocytes from protected and non-protected animals suggested that Th1 cells were involved for acquired UTI immunity. To help discern which Th subsets contribute to the formation of humoral responses to UTI, it would be useful to purify CD4^{+} cells from the bladders of *in vivo*-stimulated (infected) and UPEC-antigen vaccinated mice to probe Th cell skewing in response to repeated transurethral infection with UPEC. Evaluating adaptive immune responses in both IL-6 and IL-12p35 knockout mice, which would not be able to generate Th17 or Th1 lineages, respectively, would be helpful to delineate which subset functions

to orchestrate the recall response to UTI. This facet of the adaptive immune response needs to be delineated both during natural infection and during mucosal vaccination.

4.3.2 Innate UTI immunity

4.3.2.1 Cytokine responses and IL-17A in the context of UTI

Upon infection with UPEC, the bladder epithelium mobilizes in every sense of the term. Umbrella cells undergo apoptosis and slough from the surface; meanwhile, a robust cytokine and chemokine response accompanied by edema and cellular inflammation fill the bladder epithelium and underlying lamina propria. While IL-17A was shown to be necessary for an optimal innate response to infection, IL-17A$^{-/-}$ animals were still able to mount a response, albeit a subpar one, highlighting the functional redundancy of the innate immune system. The fact that, amongst this redundancy, IL-17A$^{-/-}$ mice still exhibit a clearance defect further illustrates the importance of IL-17A in controlling bacterial infection in the bladder. This defect is probably attributable to the fact that expression of IL-17A is intimately linked to the neutrophil recruitment (217). Neutrophils have been both indirectly and directly shown to be critical for control of UPEC infection in the mammalian bladder (5, 147, 191, 374). What's more, the downstream effectors of IL-17A signaling (*i.e.*, IL-8, G-CSF, AMPs) have also been shown to be important in UTI host defense, thus the investigation of IL-17A in UTI was an obvious next step.

4.3.2.2 Role of IMs during UTI

In the process of exploring the role of IL-17A in the host response to UTI, it was discovered, for two very different reasons, that γδ T cells and CD11b^{+}Gr-1^{+}MHC Class

II^+ cells, or IMs, make IL-17A in response to infection. The involvement of γδ T cells in UTI host defense was heavily supported by the literature and therefore an immediate beacon of interest. In addition to being required for optimal clearance of a primary UTI episode (198), a large majority of the γδ T cells present in the bladder (migration was marginal, most of this small population was resident) during infection were making IL-17A. On the other hand, similar to the neutrophil population, IMs are not present in the bladder at high numbers, but migrate from the bone marrow in response to UTI. In the case of the γδ T cells, the majority of a minority cell population is making IL-17A, which translates to hundreds of cells, whereas with the IMs, a fraction of a majority cell population is making IL-17A, which translates to thousands of cells. This IM population was detected purely by coincidence and as a result of ample numbers and robust synthesis of IL-17A, as neutrophils were actually the cell population being probed. Therefore, two very different cell lineages, ILLs and myeloid cells, make IL-17A in response to UPEC-mediated UTI.

Monocytes are largely divided into two subsets, those involved in homeostasis (surveillance) and those involved in inflammation (17). Aside from the fact that they migrate in large numbers to the inflamed bladder and they make IL-17A, the function of the presumed IMs – $CD11b^+Gr1^+$MHC Class II^+ cells – in the context of UTI is unknown. In the future, it would be useful to further characterize this cell type. First, they should be probed by flow cytometry for expression of CD115, CCR2, CD62L, and CX_3CR1 surface markers to further confirm the identity of this population as that of IMs (17, 371). They should also be interrogated for Ly-6G (Gr-1) and Ly-6C expression, as the RB6-8C5 antibody clone used in these studies detects both markers. Additionally, it

has been described in the *L. monocytogenes* literature that these IMs can develop into Tip-DCs, suggestive of a function in the secretion of NO and TNF-α (372). Tip-DCs are not present in mice lacking CCR2, and transurethral infection of $CCR2^{-/-}$ mice demonstrated that Tip-DCs are dispensable for UPEC clearance from the urinary tract (84). This result also suggests that the IM population may also not be necessary for the innate immune response to UTI. Combined with the fact that TCR $\delta^{-/-}$ and IL-$17A^{-/-}$ mice are more susceptible to UPEC infection, these results may suggest that secretion of IL-17A by the $\gamma\delta$ T cell population is more critical than secretion by the IM population. Alternatively, by virtue of their ability to phagocytose pathogens and present antigens, IMs and Tip-DCs may play a role in the adaptive immune response to UTI, justifying testing of $CCR2^{-/-}$ mice in the reinfection model.

4.3.2.3 Turning off the innate immune response to UTI: regulators and therapeutic potential

While deciphering the dynamics of and players in IL-17A upregulation is important to understanding the host response to UTI, what is even more crucial (and perhaps underappreciated) is the cessation of IL-17A expression and signaling in inflamed tissue. Indeed, IL-17A has been implicated in several autoimmune diseases including rheumatoid arthritis, inflammatory bowel disease, psoriasis, multiple sclerosis, and asthma (220). As demonstrated in this study, IL-17A is a potent inflammatory cytokine, further illustrated by the fact that transcript is not expressed basally in the steady state. Treg cells have emerged as the counterbalance to the Th17 subset (296). These cells secrete inhibitory cytokines such as IL-10, IL-35, and TGF-β, and functionally suppress immune responses, the induction of this activity being contact-

dependent (65). Suppression of the robust inflammatory response generated by UPEC infection and the presumable role that Treg cells play in this process should be a topic of future inspection. Related to this topic, the cause of interstitial cystitis is unknown and could potentially link to uncontrolled expression of IL-17A and chronic inflammation in response to UTI. Due to its role in regulating inflammatory responses to infection and correlation with autoimmune disease, IL-17A has gained much attention over the last ten years as a target of anti-inflammatory therapeutics. While beneficial in the realm of other diseases, treatment with antibodies or inhibitors that block the activity of IL-17A may render a patient more susceptible to bacterial cystitis. Nonetheless, considerations of the level of morbidity in autoimmune disease relative to UTI may negate this risk.

4.4 Closing statements

Modeled experimentally in mice, the role of the proinflammatory cytokine IL-17A is defined with respect to the innate and adaptive immune response in the natural route of infection. Regarding adaptive immune mechanisms playing a role in protection against UPEC-mediated UTI, potential vaccine candidates and their corresponding immunological correlates of protection were tested and determined, respectively. The results presented in this dissertation significantly contribute to the current understanding of both the innate and the adaptive immune response to UTI.

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