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Role of ATG16L1 in Uropathogenic E. Coli Pathogenesis

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Role of ATG16L1 in Uropathogenic *E. Coli* Pathogenesis

by

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Dedication

To my family for all of their support,
but especially to my role model Janey I
and my incredible husband Jessen.

ABSTRACT OF THE DISSERTATION

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Professor Indira Mysorekar, Chairperson

Urinary tract infections (UTIs) are among the most common infectious diseases and are primarily caused by uropathogenic *E. coli* (UPEC). Given the greater incidence of antibiotic resistance among UPEC isolates, it is vital to determine factors and pathways important for an effective host response to UPEC in order to improve therapeutic options for combating UTIs. Autophagy is a cellular degradation pathway that plays important roles in pathogen control and modulation of innate immunity. One essential autophagy protein, ATG16L1, has been further implicated in controlling inflammation due to a common variant of ATG16L1 being associated with increased risk of Crohn's disease, a disease of continuous excessive inflammation in the gut. Autophagy and ATG16L1 had been shown to play anti-pathogenic roles in response to a number of infections, yet little was known about their role in response to UPEC. In this thesis, I examined the role of ATG16L1 in response to UPEC pathogenesis.

We found ATG16L1-deficient mice clear bacteriuria faster, recruit more monocytes/

macrophages, and recover their epithelial barrier faster than WT mice in a well-established mouse model of UPEC induced UTI. ATG16L1 deficiency in the urothelium led to altered urothelial cell architecture, with accumulations of lysosomes and multivesicular bodies, and fewer UPEC quiescent intracellular reservoirs that can seed recurrent infections. Additionally, we found that immune cells lacking ATG16L1 were better able to clear their infection. Furthermore, ATG16L1 deficient macrophages were particularly adept at clearing their bacteria load. My work has revealed that ATG16L1-deficiency in fact improves the ability of macrophages derived from the bones of mice to take up more UPEC and enhances their secretion of IL-1β, a key pro-inflammatory cytokine, in response to UPEC. This increased IL-1β secretion by ATG16L1-deficient macrophages in response to UPEC was dependent on enhanced cleavage of pro-IL-1β to its active form by activated caspase-1 and the NLRP3 inflammasome. Finally, to confirm that enhanced IL-1β secretion was the key mechanism for UTI clearance in ATG16L1-deficient mice, I determined that the mutant mice secreted more IL-1β in their urine in response to UPEC infection, and inhibition of IL-1 signaling abrogated the ATG16L1-deficiency dependent protection from UTIs. Together, my work suggests that ATG16L1 dampens what is otherwise a protective increase in macrophage recruitment and IL-1β production in response to UPEC infection. Thus, ATG16L1 deficiency improved the host response to UPEC infection, challenging the paradigm that deficiency in autophagy proteins is detrimental to the host. Furthermore, the detrimental ATG16L1-deficiency induced inflammation against commensal bacteria that is associated with Crohn's disease may be the trade off for enhanced protection against acute infection and reservoir formation by UPEC and possibly other common infections.

Chapter 1: Host responses to Uropathogenic *E. coli* (UPEC) induced urinary tract infections (UTIs)

Clinical features and epidemiology of UTIs

Urinary tract infections (UTIs) are one of most common infections in the United States (Foxman, 2014). This infection predominantly affects women, and the youngest and oldest of us. 50% of all women will have at least one UTI in their lifetime and as many as 24% experience a second UTI within a 6 month period, and ~2% will have more than 2 within that time frame (Foxman et al., 2000). This high burden of disease and recurrence leads to UTIs being the primary complaint in 8 million visits to clinics or emergency departments every year (Dielubanza and Schaeffer, 2011). These visits result in a large number of antibiotic prescriptions to treat UTIs, accounting for 15% of all outpatient prescriptions (Dielubanza and Schaeffer, 2011). Thus, the annual cost of UTIs in the United States is estimated to be over $1.6 billion (Dielubanza and Schaeffer, 2011). Given this large economic burden and the high usage of antibiotics, it is important to better understand the etiology of this common disease and to determine ways to improve treatment.

Urinary tract infections are infections of any part of the urinary tract, which is made up of two kidneys which filter the blood and produce the urine, two ureters which move the urine from the kidney to the bladder, the bladder which collects the urine till a time when it is appropriate to expel, and the urethra which channels urine from the bladder to outside the body (Foxman, 2010) (Figure 1a). Infections of the urethra are called urethritis, those of the bladder are called cystitis, and those of the kidney are called pyelonephritis (Foxman, 2010). Cystitis is the most common of theses infections and is characterized by the following symptoms: frequency, urgency and painful urination (dysuria) (Foxman, 2014). Pyelonephritis can present with symptoms of cystitis but is additionally associated with fever, chills, and flank pain. The diagnosis is commonly made of patient history, urine dipstick which measures leukocyte esterase and nitrates, and urine cultures above a threshold (over 10^4 or 10^5 colony forming units per ml urine) (Foxman, 2014; Hooton, 2012). Bacteriuria alone without symptoms is not diagnostic of a UTI, but could be a condition called asymptomatic bacteriuria (ABU). ABU is not an indication for treatment unless the patient is pregnant, in which case ABU is treated to decrease the likelihood of it progressing

to pyelonephritis (Hooton, 2012; Smaill and Vazquez, 2007). Uncomplicated UTIs are treated by giving antibiotics in an outpatient setting, yet to reduce the burden of recurrent UTIs, women can be prescribed daily low dose prophylactic antibiotics for many months or prophylactic antibiotics to be taken after sexual intercourse (Hooton, 2012).There are a number of known risk factors for UTIs and recurrent UTIs including female sex, infrequent urination, anatomical abnormalities, genetics, lack of estrogen, maternal history of UTI and sexual activity (Hooton, 2012; Scholes et al., 2000).

Bacteria are constantly entering into the urinary tract and being eliminated by way of immune response and urination, yet some pathogens appear better able to cause infection than others. In fact, uropathogenic *Escherichia coli* (UPEC) account for ~80% of UTIs (Foxman, 2010) and will be further discussed in the subsequent sections of this thesis. Other bacterial causes of UTI include *Klebsiella pneumoniae* and *Enterococcus faecalis* (Hooton et al., 2010), and are more associated with catheter associated UTIs. It is thought that many of the bacteria that cause UTIs can be maintained in the gut flora and can enter the urinary tract and cause a symptomatic infection(Silverman et al., 2013). Yet recent studies in the mouse model have shown that after the initial infection with UPEC, subsequent recurrences of UPEC infection may be the result of reemergence of bacterial populations that have remained quiescent in the bladder since the resolution of the last infection (Chen et al., 2013; Mysorekar and Hultgren, 2006; Silverman et al., 2013). Examining the formation and role of different tissue reservoirs of uropathogenic *E.coli* in the host may help discover better ways to prevent recurrent UTIs.

We need to better understand the host response to UPEC in order to develop ways to harness the host response to clear infection, rather than relying mainly on antibiotics to which UPEC is becoming resistant. This is especially true for cystitis, which accounts for most of the outpatient treated UTIs and is a major reason for prophylactic UTIs and leads to increased morbidity (Foxman, 2014). To study UPEC induced cystitis and understand the underlying mechanism of host pathogen interaction, we use well-characterized strain of UPEC, UTI89 (Chen et al., 2013;

Mulvey et al., 2001), which was isolated from a patient with cystitis and is able to colonize and thrive within the bladder.

Bladder Structure and Function

To understand the etiology of cystitis, one must understand the structure and function of the bladder. The bladder is a hollow organ whose purpose is to store urine created by the kidney until it can be expelled (Wein et al., 2012). This task is more difficult given the harmful contents of the urine and the extreme range of dilation that the bladder must endure while maintaining an impermeable barrier. The structure of the bladder allows it to have this flexibility while maintaining its function. The bladder is made of an external muscle layer, a submucosa or mesenchyme, and the inner epithelium (urothelium) that surrounds the bladder lumen that stores the urine (Wein et al., 2012) (Figure 1b).

The luminal surface of the bladder is lined by a pseudostratified transitional epithelium (urothelium) that is 3-4 cell layers thick (Hicks, 1975; Jost et al., 1989). The urothelium is made up of three primary cell types: basal cells, intermediate cells, and superficial facet cells \ (Hicks, 1975; Mysorekar et al., 2009) (Figure 1c). Basal cells are adjacent to the basement membrane and are thought to be the stem cell of the adult bladder (Gandhi et al., 2013; Mysorekar et al., 2009; Shin et al., 2011; Yamany et al., 2014). Intermediate cells are the cells between the basal and superficial facet cells that are more differentiated than basal cells, but are still able to divide in response to epithelial injury/barrier damage. Superficial cells are the multinucleated cells that cover large surface areas of the bladder lumen and are the most differentiated cells of the urothelium. Superficial facet cells must provide a barrier to the contents of the lumen, while dramatically changing surface area to adjust to the filling and voiding of the bladder. These cells have a complex vesicle trafficking system to provide the needed dynamic capabilities (Wu et al., 2009). The apical surface of superficial facet cells contains hexagonal plaques of uroplakin proteins that are transported in fusiform vesicles that are exocytosed in response to apical stretch to increase the surface area and are then endocytosed in response to stretch of the basal surface

when the surface area should be reduced (Truschel et al., 2002). Endocytosed fusiform vesicles can either be re-exocytosed, or can pass through sorting endosomes to multivesicular bodies and finally can be degraded by fusion with lysosomes (Wu et al., 2009). One of the uroplakin proteins, uroplakin 1a, is also the receptor for UPEC (Zhou et al., 2001).

Covering the surface of the urothelium is an extracellular proteoglycan mucin layer made up of glycosaminoglycans (GAGs) that protects the urothelium from exposure to luminal contents (Parsons et al., 1980; Parsons et al., 1988). These gylcosaminoglycans are protein cores covered in glycans, that can be modified in multiple ways to change the properties of these GAGs (Hurst

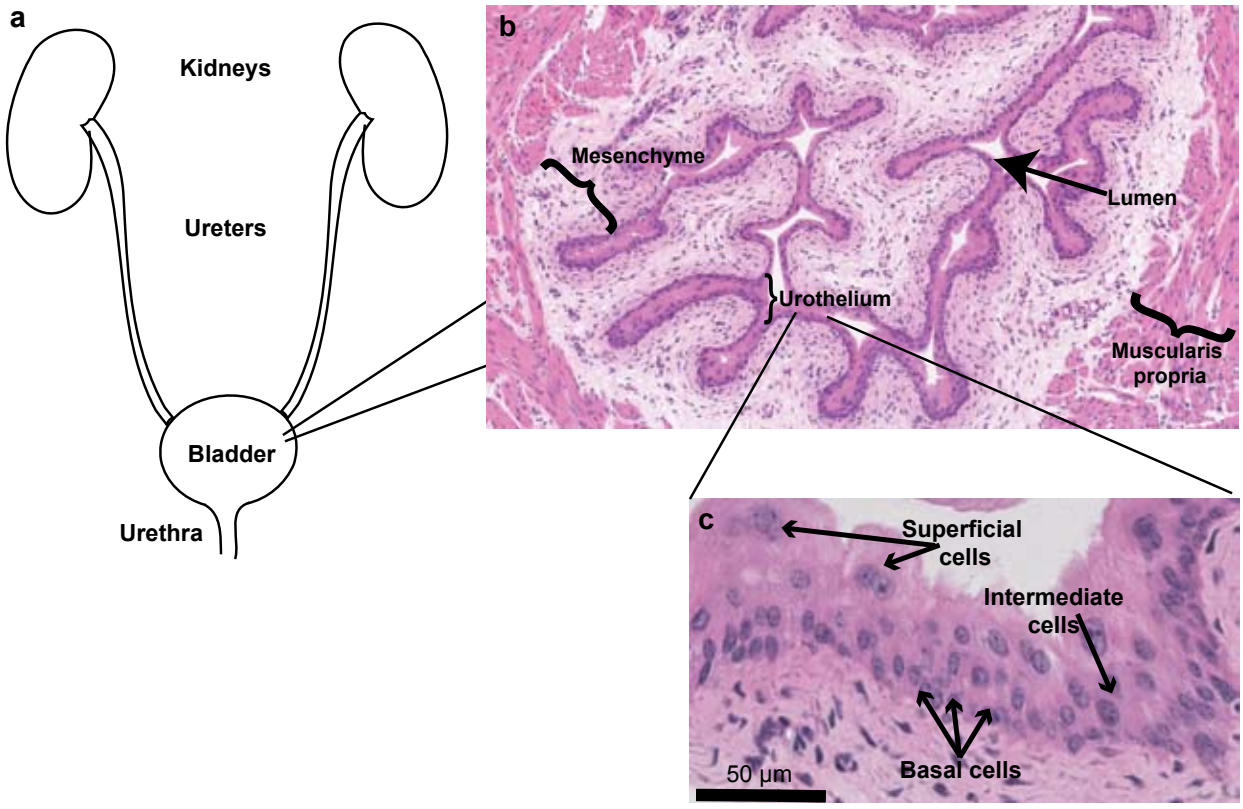

Figure 1: The Urinary Tract and Bladder Structure
(a) Diagram of the urinary tract. (b) Histological structure of the bladder depicting the inner lumen, the urothelium which surrounds the lumen, the lamina propria (mesenchume) and the outer muscle layer (muscularis propria). (c) Histology of the bladder urothelium showing the three main cell types: the superficial cells that are exposed to the lumen, the intermediate cells that are located below the superficial cells, and the basal cells which border the basement membrane. b and c are adapted from Stemler KA and Mysorekar IU. Infectious diseases – Urinary Tract Infections (bacterial). In Press 2014.

et al., 1987). Alterations in this barrier have been implicated in a number of bladder conditions and in susceptibility to diseases of the bladder (Anand et al., 2012; Nickel et al., 2009; Nickel et al., 1993).

The urothelium turns over very slowly under normal conditions (Jost, 1989), unlike other epithelial tissues like the gut. The urothelium can regenerate faster in response to stimuli that damage the barrier, such as a UPEC infection, and in those cases it can regenerate the barrier in as little as one week (Mulvey et al., 1998; Mysorekar et al., 2002). Thus, much of our understanding of the molecular mechanisms involved in the regeneration and proliferation/ differentiation of the bladder epithelium has come from studies of UPEC infection (Mysorekar et al., 2009; Mysorekar et al., 2002; Shin et al., 2011).

UPEC pathogenesis and lifecycle within the host

Uropathogenic *E. coli* are extra intestinal pathogenic *E. coli* that have adapted for optimal survival in the urinary tract with a complex lifecycle depicted in Figure 2. Originally it was thought that UPEC were extracellular pathogens, yet work in the last two decades in a well-established mouse model has shown that UPEC can survive and flourish within host epithelial cells.

Pathogenic cycle of UPEC

To survive in the bladder, UPEC must first attach to epithelial cells to avoid being eliminated by urinary flow. UPEC possess adhesive pili or fimbriae, which are surface organelles that have adhesive proteins on their ends. Two of the most important pili are type1 pili and pap pili, which allow UPEC to bind to the surface of different cell types in the urinary tract, and are associated with cystitis and pyelonephritis respectively (Hunstad and Justice, 2010). The adhesin on the end of Type 1 pili, FimH, can bind to uroplakin 1a on the surface of superficial facet cells in the bladder and induce the internalization of UPEC, enabling UPEC to subvert the normal uroplakin-recycling pathway to gain entry into urothelial cells. (Wu et al., 2009; Zhou et al., 2001). Upon

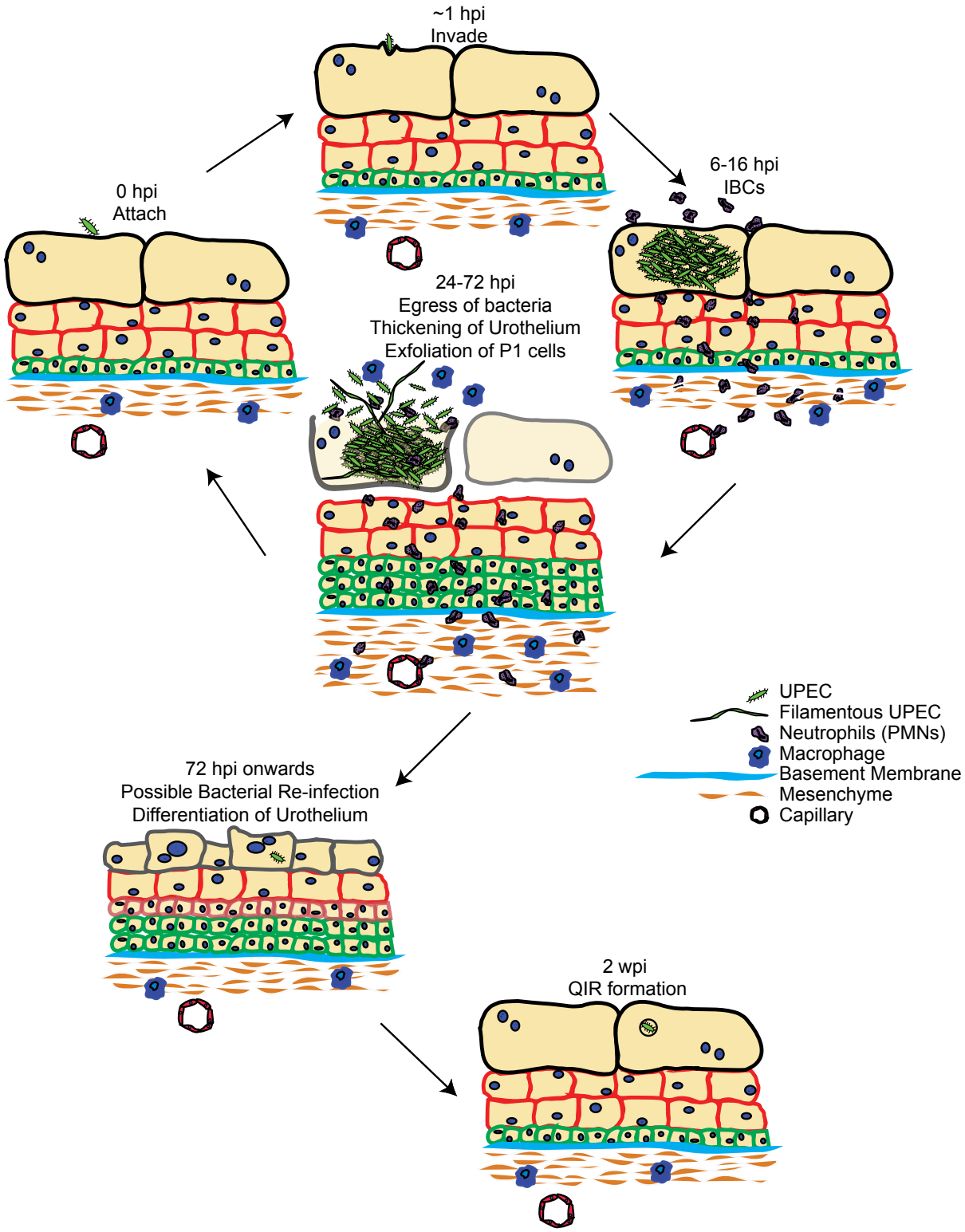

Figure 2: UPEC pathogenic cycle

Diagram showing the pathogenic cycle of UPEC and the host response to UPEC infection. The superficial cells are outlined in black, the intermediate cells in red, and the basal cells in green. This diagram was adapted from Stemler KA and Mysorekar IU. Infectious diseases – Urinary Tract Infections (bacterial). In Press 2014

UPEC binding to these surface proteins, a series of signal cascades involving other uroplakin proteins leads to endocytosis fusiform vesicles with attached UPEC, giving the bacteria access to the intracellular environment (Bishop et al., 2007; Song et al., 2007). The internalization of UPEC is also associated with actin rearrangement, with membrane zippering around the bacteria in a process dependent on both tyrosine kinases and phosphoinositide 3-kinases (PI 3-kinase) (Martinez and Hultgren, 2002).

By entering into host epithelial cells, UPEC are able to avoid initial conflict with immune cells and antibacterial compounds in the urine (Justice et al., 2004). UPEC that have entered superficial cells can escape into the cytoplasm and replicate to form Intracellular Bacterial Communities (IBCs) of between 10^5 and 10^6 bacteria in biofilm-like communities (Anderson et al., 2003; Justice et al., 2004; Mulvey et al., 2001). IBCs are not limited to murine urothelial cell, because they have also been observed in the urines of women who came to the clinic for UTIs (Rosen et al., 2007). UPEC can then exit these host cells and enter neighboring cells and continue the cycle, until the bacteria is cleared from the urine.

Even after the urine is sterile by culture, some bacteria can be found within the bladder in gentamicin-protected environments. These persistent colonies were only found in bladder that were exposed to FimH+ UPEC, but FimH+ expression on the surface of beads was not sufficient to form a long term reservoir (Mulvey et al., 2001). The existence of intracellular bladder reservoirs of bacteria was further confirmed by Schilling et al who followed urine and gut titers of UPEC after transurethral infection and found that mice could have sterile urines followed by recurrences in the absence of reinfection (Schilling et al., 2002). It was not until Mysorekar et al examined the bladders histologically using immunofluorescence, that they discovered that those bacteria reservoirs were actually membrane bound UPEC that could be found within the bladder for many weeks and thus were name quiescent intracellular reservoirs (QIRs) (Mysorekar and Hultgren, 2006). UPEC in QIRs are predominantly non-replicating (they do not incorporate BrdU) and are enclosed within a Lamp1+ membrane unlike bacteria within IBCs

(Mysorekar and Hultgren, 2006). Bacteria in these reservoirs are able to reemerge and cause a recurrence in urine titers, even when mice had previously had negative urine cultures for many days (Mysorekar and Hultgren, 2006). This finding has major implications for the cause and treatment of UPEC infections in humans. This work suggested that many recurrences may be the result of reemergence of a quiescent pathogen rather than a reinfection from a gut reservoir. This also suggests that appropriate treatment of UPEC infections may not be complete unless we can understand how to inhibit the formation of these reservoirs or if we can induce their reemergence to allow the host immune response to kill the extracellular bacteria.

***UPEC modulation of the host response**:*

UPEC is able to modulate the host and thrive by producing a number of virulence factors including adhesins, toxins and siderophores, and by expressing them at different times throughout its lifecycle within the bladder (Hunstad and Justice, 2010). UPEC inhibits, evades, and modifies the host immune response using many techniques and molecules. One of the first lines of defense for UPEC, and other pathogenic *E. coli* produces, is a capsule that can inhibit opsonization and phagocytosis. UTI89 has K1 capsule which protects from serum proteins (Opal et al., 1982), is currently a target for therapeutic intervention (Goller et al., 2014). UPEC can also inhibit cytokine production and reduce the recruitment of immune cells. For example, UPEC can reduce the epithelial cells expression of IL-6 (Hunstad et al., 2005), and neutrophil migration *in vitro* (Lau et al., 2012; Loughman and Hunstad, 2011). UPEC also appears to suppress macrophage and monocyte secretion of certain cytokines, including TNFα, IL-6, and IL-1α, even though it elicits increased transcription of TLR4 and NFκB activation (Bhushan et al., 2011; Bhushan et al., 2008).

Host response to UPEC induced UTIs

The host has developed many countermeasures to effectively combat UPEC and other infections of the bladder. These defense mechanisms include exfoliation of infected cells, regeneration

of the barrier, recruitment of innate immune cells, secretion of antimicrobial and anti-adhesive compounds. In this section I will discuss a number of these strategies used by the host to eliminate the bacterial threat.

In order to mount an immune response, the host must first detect the pathogen. This is commonly done by pattern recognition receptors (PRRs) on the surface or within the cytosol of host cells such as toll-like receptors (TLRs) and nod-like receptors (NLRs) which each recognize common pathogen associated molecular patterns (PAMPs) or danger associated molecular pattern (DAMPSs) (Newton and Dixit, 2012). One of the most important PRRs for recognizing bacteria is TLR4 that recognizes bacterial lipopolysaccharides (LPS). LPS are large molecules found on the outer membrane of gram-negative bacteria that are made up of a lipid and a polysaccharide, which has an o-antigen, and outer core and inner core. TLR4 is expressed by many urothelial cell lines (Schilling et al., 2003b), mouse urothelium and in human urothelial cells from the bladder and the kidney from biopsy samples (Samuelsson et al., 2004). TLR4 expression is important for control of UPEC in the bladder and is needed in both the hematopoietic and non-hematopoietic compartments for most effective clearance of bacteria (Schilling et al., 2003a). This supports the argument that the recognition of UPEC by both innate immune cells (hematopoietic cells) and urothelial cells (non-hematopoietic cells are important for effective clearance. The role of each of these cell types in the response to UPEC will be discussed below.

Urothelial cells are the first cells to come into contact with UPEC and can be infected by UPEC. After UPEC infection, there many changes in gene expression in urothelial cells, including those involved in differentiation and proliferation as well as proinflammatory genes (Mysorekar et al., 2002; Reigstad et al., 2007). Urothelial cells can secrete cytokine to recruit innate immune cells, their own exfoliation to eliminate intracellular bacterial communities, and a renewal and regeneration program to reestablish the urothelial barrier (Mulvey et al., 1998; Schilling et al., 2003b). Studies show that the greatest cytokine secretion and exfoliation responses happen in response to internalized UPEC rather than LPS alone or extracellular bacteria. (Schilling

et al., 2001). Following exfoliation, the bladder must induce restoration of the barrier by the differentiation of intermediate cells to superficial cells and proliferation of underlying basal cells (Colopy et al., 2014; Mysorekar et al., 2009; Shin et al., 2011). The ability of the urothelium to proliferate, differentiate, and restore the barrier post UPEC infection is dependent upon bone morphogenetic protein 4 (BMP4) an important regulator of differentiation in many tissues and a member of the transforming growth factor-B family of secreted signaling molecules (Mysorekar et al., 2009; Mysorekar et al., 2002). BMP4 expression was suppressed early in UPEC infection of the bladder, but its signaling is essential for appropriate proliferation and terminal differentiation of the urothelium in response to UPEC infection (Mysorekar et al., 2009).

Upon infection there is a rapid infiltrate of innate immune cells, followed by a tapering off of inflammation (Engel et al., 2006; Horvath et al., 2011). This robust innate immune cell response is thought to be a main reason for UPECs survival adaptation to enter and proliferate in the relatively protected environment of the bladder epithelial cell. The cellular infiltrate is made up mainly of neutrophils, macrophages, and neutrophils, of which neutrophils have been the most studied. Neutrophils are recruited to the bladder of mice with intact TLR4 expression (Shahin et al., 1987) and cross the epithelial in response to IL-8 derived by epithelial cells in response to UPEC or IL-1α (Godaly et al., 1997). Neutrophils have been shown to be essential for the clearance of UPEC (Haraoka et al., 1999). The role of macrophages in the host response to UPEC was not as well defined. There is a population of resident macrophages in the bladder even before infection, and upon infection more monocytes and macrophages are recruited to the bladder (Engel et al., 2006; Engel et al., 2008; Horvath et al., 2011). Macrophages may be important in the host response to UPEC, since inhibiting neutrophil recruitment to the bladder by blocking GM-CSF improved the clearance of the infection through the recruitment of more monocytes and macrophages and the production of more macrophage inducing cytokines (Ingersoll et al., 2008). Yet, how macrophages aid in the clearance of UPEC from the bladder and if they are essential for clearance of UPEC was not known.

Some initial studies have investigated the nature of macrophages and UPEC interactions. Recently published work by Bokil et al has shown that UPEC can survive in bone marrow derived macrophages from mice and monocyte derived macrophages from humans for up to 24 hours and that the UPEC strain we study, UTI89, was able to survive longer than other UPEC strains (Bokil et al., 2011). Although UTI89 was still found in the macrophages by 24 hours, the intracellular bacterial load was dramatically reduced from the 2 hour bacterial load, suggesting that even though they survive, UPEC is not adapted to thrive within macrophages unlike many other bacterial pathogens. Two groups examined the early trafficking of UPEC within macrophages and suggested that UPEC do not undergo normal phagocytosis, and instead enter into compartments that do not acidify as quickly or produce many reactive oxygen species(Amer et al., 2005; Baorto et al., 1997). How internalization affected inflammatory signaling was not investigated. Thus, there are still major gaps in our understanding of how macrophages handle intracellular UPEC and what affect that has on the activation of and secretion by macrophages.

The host response to UPEC relies on the coordination of many host pathways in different cell types to effectively clear the infection and regenerate the barrier. Prolonged inflammation damages the epithelium and leads to incomplete restoration of the barrier, but insufficient inflammation leads persistent bacteriuria. Although we have learned a great deal from our current mouse models, there are many key host pathways whose roles have not been determined in this important disease and which may drastically change the host response or may be subverted by UPEC. One such pathway is autophagy.

Autophagy and the autophagy proteins: genetics, regulation and role in homeostasis and response to infection.

Autophagy is a conserved host pathway for targeting and delivering cytoplasmic contents to the lysosomes for degradation, and is important for homeostasis and response to stress, starvation, and intracellular pathogens. Autophagy constitutes at least three distinct pathways: microautophagy, chaperone mediated autophagy, and macroautophagy (referred to in this

document as autophagy) (Levine et al., 2011). During autophagy, damaged mitochondria, other organelles, bulk cytoplasm, protein amyloids, or intracellular pathogens, are taken up by autophagosomes and the degraded after fusion with lysosomes (Levine et al., 2011). This happens in a coordinated pathway: first, there is initiation of autophagosome formation after the activation by some stimulus such as starvation (Choi et al., 2014; Suzuki and Ohsumi, 2010), then the nascent double membraned autophagosomes are targeted to the cytoplasmic contents, next the autophagosome is extended to fully engulf the contents, and finally autophagosomes fuse with lysosomes to degrade their contents (Figure 3) (Fujita et al., 2008; Yang and Klionsky, 2009).

The autophagy pathway was originally discovered as a response to starvation and stress, but has recently been shown to be involved in many aspects of physiology. Most important for this discussion, are the effects of autophagy and its proteins on intracellular pathogens, inflammation, and immune responses. One role of autophagy in pathogen control is xenophagy, the selective-

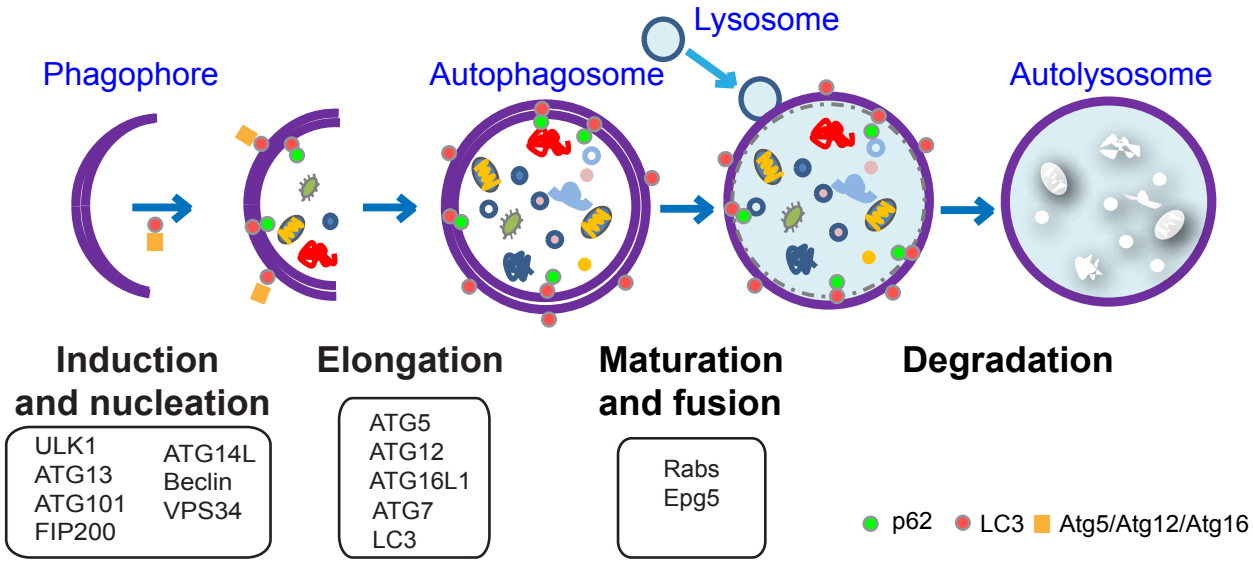

Figure 3: Autophagy Pathway Diagram
This diagram depicts the steps of the canonical autophagy pathway: induction and nucleation of the process in response to a stimuli, the elongation of the membranes to form an autophagosome, the fusion of that autophagosome with lysosomes, and subsequently the degradation of the contents of the autophagosome by the lysosome. The major autophagy proteins involved in each step are also labeled.

autophagy that directly targets intracellular pathogens (Levine et al., 2011). Intracellular bacteria, such as *Mycobacterium tuberculosis* (Gutierrez et al., 2004) and *Salmonella enterica* Typhimurium (Birmingham et al., 2006), can be targeted by autophagy proteins and inhibition of autophagy pharmacologically or genetically can decrease their survival. During an infection, autophagy machinery can target bacteria in all compartments within the cell, within intact vacuoles and damaged vacuoles as well as free bacteria in the cytoplasm (Shahnazari and Brumell, 2011).

Our understanding of autophagy pathway proteins has greatly changed in the last 10 years. Initially, functions of any one individual essential autophagy protein were ascribed to the pathway as a whole. Now we are discovering that autophagy proteins or protein complexes can have important anti-pathogenic functions independent of the complete canonical autophagy pathway (Choi et al., 2014; Sanjuan et al., 2007; Zhao et al., 2008). Additionally, autophagy and its proteins are generally considered to play anti-pathogenic roles in host response, yet some pathogens have developed ways to utilize parts of the pathway. Autophagy is used by the host to recycle host proteins in times of starvation and so could provide nutrients to intracellular pathogens during stress (Deretic and Levine, 2009). Additionally, autophagy may provide a cellular niche for replication or persistence (Checroun et al., 2006). These *in vitro* findings suggested that autophagy proteins may be used by intracellular pathogens to survive within host cells and thus may play pro-pathogenic roles in infection, yet at the time I started my thesis, there were no *in vivo* examples of autophagy proteins playing pro-pathogenic roles.

We were interested in investigating the roles of autophagy proteins in the host response to UPEC. Autophagy proteins could affect UPEC's acute intra-epithelial stage, its persistent reservoir formation, its interaction with phagocytic cells, and the general inflammatory response it elicits. Examining the course of UPEC infection *in vivo* using a genetic deletion or deficiency would be necessary in order to uncover the role of autophagy proteins in the complex host response to UPEC.

ATG16L1 and the association of its T300A variant and Crohn's Disease

One autophagy protein, ATG16L1 became of particular interest when a common polymorphism in the human allele ATG16L1, the T300A nonsynonymous mutation (Prescott et al., 2007), was positively linked to Crohn's Disease (Hampe et al., 2007; Rioux et al., 2007). The T300A variant made ATG16L1 an attractive protein to study, because along with being innately of interest as an essential autophagy protein, it provided possibly direct human relevance. These initial findings were then supported by numerous subsequent GWAS studies in a variety of populations, leading to increased interest in understanding the structure of ATG16L1, its function in diverse aspects of biology, and the changes induced by the T300A allele.

ATG16L1 is a key protein in the elongation phase of autophagy and forms a complex with ATG5 and ATG12 that conjugates LC3 to phosphatidylethanolamine (PE) (Hanada et al., 2007), converting it from LC3I to LC3II, in a specific membrane location (Fujita et al., 2008). This allows the ATG16L1 complex to target the autophagic isolation membrane and specify the site of LC3 lipidation (Fujita et al., 2008; Mizushima et al., 2003). The mammalian ATG16L1 protein has an N-terminal domain that is important for binding to ATG5, a middle coiled-coil domain that is essential for self-oligomerization, and a C-terminal tryptophan-aspartic acid (WD) repeat domain (which is not conserved between mammalian cells and yeast) (Fujita et al., 2009; Mizushima et al., 2003) (Figure 4). The Crohn's disease associated polymorphism, T300A is located in a structural unclassified region adjacent to the WD repeat domain (Hampe et al., 2007; Lassen et al., 2014; Rioux et al., 2007). The structure suggested that ATG16L1 may have other

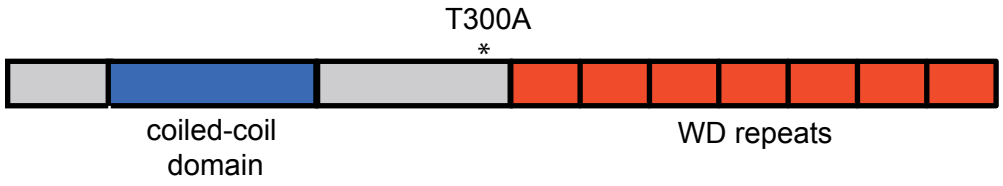

Figure 4: ATG16L1 protein
Diagram of the domains of mammalian ATG16L1, including the coiled-coil domain and the WD repeat domain. The location of the T300A variant is also noted.

functions in addition to the canonical autophagy conserved from yeast to human, and that T300A could be an important player in these other functions.

Initial studies sought to determine how ATG16L1 affects the host response to infections, and how deficiency or mutation in ATG16L1 could lead to inflammation as is seen in Crohn's disease. Cadwell et al conducted one of the first studies of ATG16L1's role in the intestine using mice that were hypomorphic for ATG16L1. These mice were made using gene-trap mediated disruption of *Atg16l1*(Cadwell et al., 2008), where the gene-trap vector inserts a splice acceptor into an intron followed by a reporter cassette and a polyadenylation site that results in the early termination of transcription of the gene. They used two gene-traps embryonic stem cells that were commercially available that had gene-trap mutations in introns on the 3' ends of either exon 6 or 10. Mice from both of these sets of ES cells were hypomorphic for ATG16L1; they exhibited decreased production of ATG16L1 in tissues throughout the body, but enough ATG16L1 remained to prevent death during the neonatal starvation period as was observed in the full knockout mice created by Saitoh et al (Saitoh et al., 2008). Using these mice, they were able to show that ATG16L1 deficiency resulted in reduced autophagy levels in the intestine, and although they did not exhibit gross abnormalities of the intestine, they had abnormal Paneth cells. Paneth cells are specialized epithelial cells in the small intestine that secrete antimicrobial factors. Paneth cells of Atg16L1 deficient mice exhibit mislocalization of lysozyme (a key antimicrobial enzyme), degenerating mitochondria and fewer granules (which normally contain the antimicrobial peptides and factors for secretion) (Cadwell et al., 2008). The Paneth cell abnormality found in ATG16L1 deficient mice was also found in humans with the T300A mutation, providing another similarity between ATG16L1 deficiency model and Crohn's disease (Cadwell et al., 2008).

ATG16L1 also appears to play a role in suppressing the over exuberant production of cytokines, specifically IL-1β, because murine macrophages deficient in ATG16L1 and human monocytes with the T300A variant both produced more IL-1β in response to inflammatory stimuli. (Plantinga et al., 2011; Saitoh et al., 2008). These findings suggested that ATG16L1 deficiency

may worsen inflammatory conditions. Why does a detrimental polymorphism in ATG16L1 remain common in the population even though it increases the risk to a devastating inflammatory condition? Could it be that this allele, that is harmful in the setting of inflammatory disease (Saitoh et al., 2008), may be beneficial in response to common infections such as UTIs.

IL-1β: production, secretion, and regulation by the inflammasome

The host coordinates an effective immune response to pathogens by eliciting an initial pro-inflammatory response followed by an equally important resolution phase. Much of this intricate coordination is done by the production of different cytokines, small proteins that are integral for cell signaling, at distinct times within the course of the infections. Each cytokine can help skew immune responses to resolve the inflammation or to recruit more immune cells. One of the most important pro-inflammatory cytokines is interleukin 1β (IL-1β). IL-1β is part of the IL-1 family of cytokines along with IL-1α and IL-18 and eight other proteins (Garlanda et al., 2013). IL-1β induces the production of key effector molecules, enhance cytokine production, and aid immune cell migration. IL-1β can induce the expression of many genes and synthesis of cyclooxygenase type 2 (COX-2), type 2 phospholipase A, and inducible nitric oxide synthase (iNOS) (Dinarello, 1991). Additionally, IL-1β can increase expression of adhesion molecules that helps promote the infiltration of immune cells into the tissue. IL-1β also induces the production of IL-6, another key pro-inflammatory cytokine (Dinarello, 2009).

The cytokine that we now call IL-1β was first discovered as a small protein that caused fevers, and thus was originally named endogenous pyrogen, among other names (Dinarello, 1994b). Eventually it was renamed IL-1, after it was found to effect many other systems and not simply induce fevers. The variety of tissues and cells that produced IL-1 and the plethora of functions was finally better understood when it was determined that IL-1 was actually two distinct proteins IL-1α and IL-1β which both signal through the IL-1 receptor (IL-1R) (Dinarello, 1994b; Garlanda et al., 2013). IL-1α and Il-1β are encoded by different genes, have distinct forms of activation, and are produced cells (Garlanda et al., 2013). IL-1β is mainly produced by

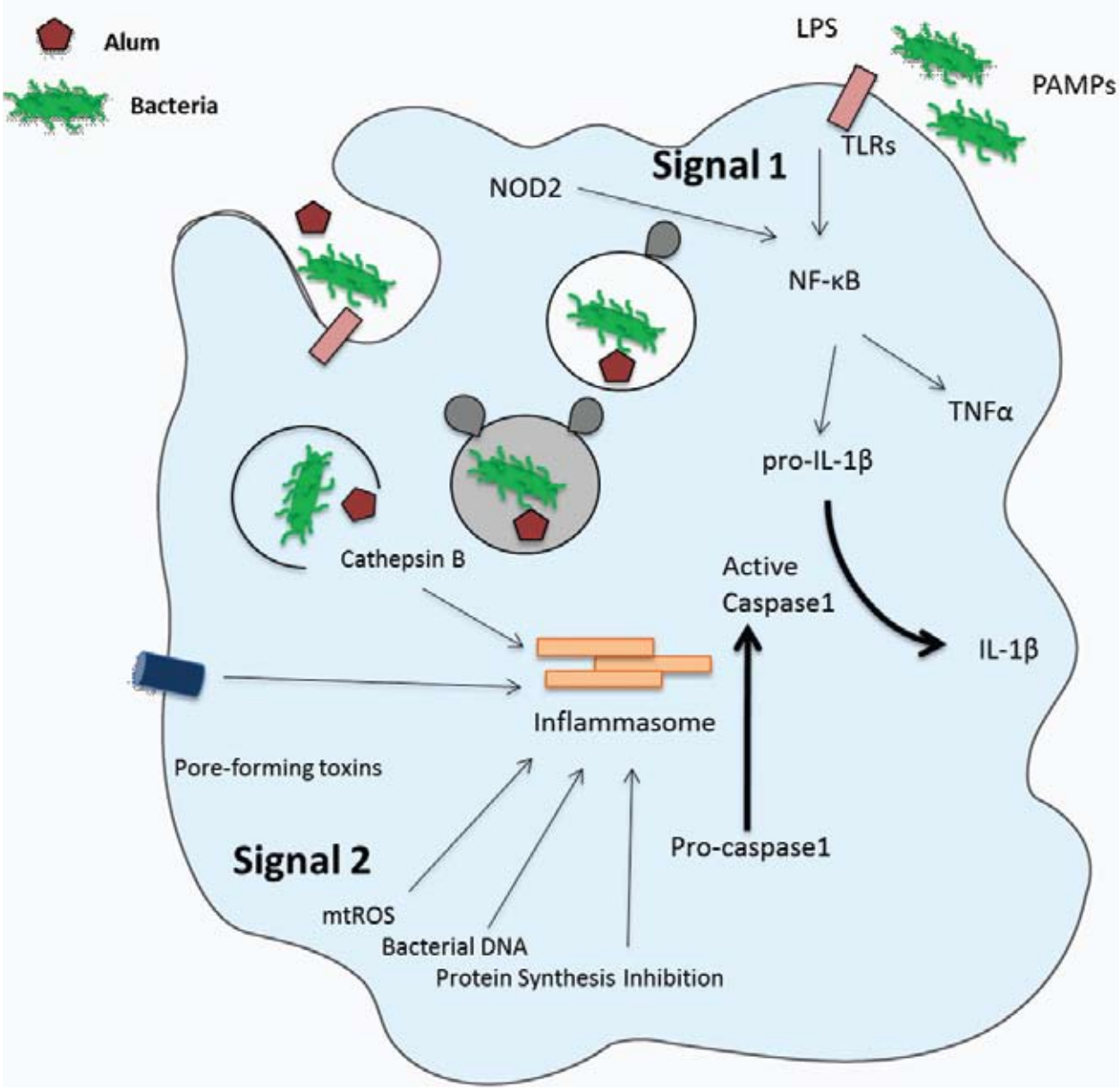

Figure 5: IL-1β processing
Model showing how IL-1β is produced and activated before secretion. IL-1β is secreted in response to 2 signals. The initial signal that induces NF-KB dependent production of pro-IL-1β can be LPS or another TLR or NLR agonist. The second signal leads to inflammasome activation which activates caspase-1 to cleave pro-IL-1β to the active IL-1β. Depicted in this model are methods to activate the NLRP3 inflammasome.

macrophage and monocytes, where as IL-1α is mainly release by dying cells that are not limited to innate immune cells. Both IL-1α and IL-1β are produced in a pro-form, yet the pro-form of IL-1α is functional, whereas the pro-form of IL-1β must be further cleaved by proteases to be active. Even small quantities of IL-1β elicits a robust immune response in the host, thus the two-part activation process for IL-1β secretion ensures that it is released in response intracellular pathogens and major danger signals, rather than the simple presence of TLR activators like LPS. The tight regulation of IL-1β's secretion is essential and will be discussed in greater detail below (modeled in Figure 5).

The first step in the secretion of IL-1β is the transcription of the mRNA encoding pro-IL-1β. This happens in response to a first signal (signal 1), which could be activation of the TLRs, or binding of TNFα or even IL-1. These signals then result in the activation of NFκB and the transcription of pro-IL-1β. This pro-IL-1β mRNA can be found as early as 15 min after a stimulus (Dinarello, 1994a). The mRNA is not efficiently translated unless the stimulus remains. Once the pro-IL-1β is produced, it will remain inactive until it is cleaved or it can be degraded. Pro-IL-1β is cleaved to IL-1β primarily by caspase-1, although it is possible for IL-1β to be cleaved by other enzymes (Dinarello, 2011). Caspase-1 is produced as a zymogen and to be activated must form part of inflammasomes, which are multimeric protein platforms that are assembled upon activation by a large variety of stimuli, which are considered the second signal (signal 2).

Inflammasomes are made up of ASC (Apoptosis-associated speck-like protein containing a card), Caspase-1, and a member of the NLR family (NLRP 1, 3, 6, 7 12, or NLRC4), Aim2 or Pyrin from which each inflammasome derives its name (Latz et al., 2013). Many NLRs are important in inflammasome activation, while others are involved in other aspects of signaling in response to intracellular pathogens or danger signals. The structure of NLRs is generally conserved with 3 domains, an N terminus effector domain either caspase recruitment domain (CARD) or pyrin domain (PYD), a central nucleotide binding (NACHT) domain and a C terminus leucine rich-

repeat (LRR) domain which senses ligands (Schroder and Tschopp, 2010). The only domain that is common among all NLRs is the NACHT domain which stands for NAIP, CIITA, HET_E and TP1 domain. Some NLRs are involved in sensing bacterial products in the cytosol and activating NFKB signaling, like NOD1 and NOD2, while others are involved in forming inflammasomes, such as NLRP3 and NLRC4. NLR family, CARD domain containing 4 (NLRC4) is activated in response to cytosolic bacterial flagellin and components of type 3 secretion systems (T3SS) (Zhao et al., 2011). On the other hand, NLR family, Pyrin domain containing 3 (NLRP3) responds to a much broader array of stimuli and was initially named cryopyrin because patients with a mutation in this gene would develop fevers and systemic inflammation upon exposure to cold in a disease named cold-induced autoinflammatory syndrome-1 (Hoffman et al., 2001). The NLRP3 inflammasome is induced by lysosomal damage, pore-forming toxins, reactive oxygen species (ROS), changes in potassium ions (K^+), and ATP, among other stimuli. Although the definitive mechanism of NLRP3 stimulus is not known, it have been suggested to be changes in K^+. Although not an NLR, Aim2 is the other major inflammasome protein that recognizes bacteria. Aim2 is activated by cytosolic DNA. Thus, the CARD and pyrin domain containing proteins allow inflamamsomes to be activated by a large variety of bacterial stimuli and thus provide excellent protection to the cell from intracellular pathogens.

Inflammasome activation and IL-1β release are also associated with an inflammatory form of cell death called pyroptosis that is commonly seen upon infection. Like apoptosis, pyroptosis is a programmed cell death, but unlike apoptosis it results in a robust inflammatory response to the released cytosolic contents (Miao et al., 2011). Pyroptosis is an important innate immune defense when macrophages cannot clear intracellular pathogens, because it results in the release of these bacteria and of inflammatory signals which can recruit neutrophils to the site of the infection to eliminate the bacteria (Miao et al., 2010). Pyroptosis and IL-1β release can be elicited by both canonical (discussed in the previous paragraphs) and non-canonical inflammasome activation, the later involving caspase-11 (Aachoui et al., 2013). During non-canonical inflammasome activation, caspase-11 is activated by cytosolic bacteria and can in turn induce the cleavage

of caspase-1 to its active form and subsequently the processing and secretion of activated IL-1β (Aachoui et al., 2013; Kayagaki et al., 2011). Caspase-11's affect on IL-1β is through its activation of caspase-1, but it can independently activate pyroptosis (Kayagaki et al., 2011). What role canonical and non-canonical inflammasome activation might play in the host response to UPEC is not known, nor is the role of pyroptosis in host response to UPEC.

Much of what we know about IL-1β processing and signaling has been determined in models of infection, but IL-1β also plays an important role in response to danger signals and thus in sterile inflammation. Because of this, the study of IL-1β's function has change drastically in the last few decades from focusing on its anti-microbial role to focusing on its role in the pathogenesis of inflammatory conditions. It is now thought that IL-1β mediates many common diseases including rheumatoid arthritis, goat, type 2 diabetes, smoldering multiple myeloma, post myocardial infarction heart failure, and osteoarthritis (Dinarello, 2011). Thus, whereas 20 years ago, many papers discussed the potential therapeutic value of increasing IL-1β signaling against pathogens, most current studies have been focused on the therapeutic benefits of inhibiting its function in auto-inflammatory conditions (Dinarello, 1994b). To that end, there are now clinically available drugs that modulate IL-1β signaling, such an IL-1R antagonist (anakinra), a soluble decoy receptor (rilonacept) and a neutralizing monoclonal anti IL-1β antibody (canakinumab) (Dinarello and van der Meer, 2013). These drugs are now indicated for treating a growing number of conditions, so understanding host pathways that modulate IL-1β production and signaling is increasingly important.

The autophagy pathway and its proteins have been implicated in the regulation of IL-1β production and activity. This was first documented by Saitoh et al who found that fetal liver derived macrophages from Atg16L1-/- mice produced more IL-1β in response to LPS than did wildtype macrophages (Saitoh et al., 2008). Other work has since documented the role of autophagy in this process, it suggests that inflammasome components are targeted by autophagy, possibly as a way to limit inflammation (Shi et al., 2012). Additionally, it appears that autophagy

can degrade pro-IL-1β that is already in the cell under certain circumstances thus reducing inflammation given certain signals (Harris et al., 2011). Thus, autophagy may be an important regulator of IL-1β production and signaling.

Even though IL-1β is one of the most important pro-inflammatory cytokines, not much is known about what role it plays in host response to UPEC induced cystitis. What is known is that IL-1β is transcribed early in the bladders of infected mice (Duell et al., 2012), that IL-1β is elevated in pediatric patients with UTIs (Sheu et al., 2007), and that both IL-1β and IL-1α are present in increased quantities in the urines of women compared to males (Sadeghi et al., 2005). Although it appears to be part of the innate host response to UPEC in the bladder, we do know how UPEC activates the inflammasome, what effect IL-1β has on the host response to UPEC, and what role autophagy or its proteins may play in this process.

Key questions addressed in this thesis.

The mouse model of UPEC induced UTI has greatly expanded our understanding of the host response to UPEC and of the *in vivo* pathogenic cycle of UPEC, yet there are many key questions that remain to be addressed. A major gap in our understanding is how these quiescent intracellular reservoirs are formed. We knew that QIRs existed and were membrane bound, but we did not know what host pathways were subverted to form these reservoirs. We hypothesized that UPEC may subvert the autophagy pathway to form QIRs and remain quiescent and unnoticed in the tissue. Thus, in the context of a UPEC infection, autophagy may be playing a pro-pathogenic role rather than an anti-pathogenic within the epithelium.

UPEC's pathogenic cycle allows us to address another important question: how do autophagy proteins regulate the host inflammatory response to extracellular bacteria? The role of autophagy proteins has primarily been studied in host response to intracellular infections, but the enhanced cytokine secretion and other inflammatory changes observed in other models of autophagy protein deficiency suggests that autophagy protein deficiency may change the host response to

luminal bacteria. Current studies suggest that there is an important balance maintained by the bladder, where too much sustained inflammation is detrimental, but insufficient inflammation leads to high bacterial titers (Hannan et al., 2010). Thus, autophagy deficiency may prove detrimental to the host, as has been seen in other models, or it may actually improve the host response to UPEC infection by eliciting a more robust response to luminal UPEC.

These questions address different aspects of an important question, is autophagy always protective to the host, or can it instead be utilized by the pathogen to survive or continue its lifecycle? This thesis will show that deficiency in the key autophagy protein ATG16L1 is protective to the host, and thus that normal levels of ATG16L1 actually improve UPEC's pathogenesis within the bladder. Thus, ATG16L1 plays a pro-pathogenic role in host response to UPEC infection, changing the paradigm that autophagy proteins are always protective to the host against infection.

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Chapter 2: Atg16L1 deficiency confers protection from uropathogenic *Escherichia coli* infection in vivo

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Atg16L1 deficiency confers protection from uropathogenic Escherichia coli infection in vivo

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ABSTRACT

Urinary tract infections (UTI), a frequent and important disease in humans, are primarily caused by uropathogenic *E. coli* (UPEC). UPEC form acute cytoplasmic biofilms within superficial urothelial cells and can persist by establishing membrane-enclosed latent reservoirs to seed recurrent UTI. The host responds with an influx of innate immune cells and shedding of infected epithelial cells. The autophagy gene, *ATG16L1* has a commonly occurring mutation that is associated with inflammatory disease and intestinal cell abnormalities in mice and humans. Here, we show that Atg16L1-deficient mice ($Atg16L1^{HM}$) cleared bacteriuria more rapidly and thoroughly than controls and showed rapid epithelial recovery. Atg16L1 deficiency was associated with a potent proinflammatory cytokine response with increased recruitment of monocytes and neutrophils to infected bladders. Chimeric and genetic studies showed $Atg16L1^{HM}$ hematopoietic cells alone could increase clearance, and that Atg16L1-deficient innate immune cells were required and sufficient for enhanced bacteriuric clearance. We further show that Atg16L1-deficient mice exhibit cell-autonomous architectural aberrations of superficial urothelial cells including increases in multivesicular bodies, lysosomes and expression of the UPEC receptor, Up1a. Finally, we show that $Atg16L1^{HM}$ epithelial cells contained significantly reduced number of latent reservoirs. Together, our results show that Atg16L1 deficiency confers protection *in vivo* to the host against both acute and latent UPEC infection, suggest that deficiency in a key autophagy protein can be protective against infection in an animal model of one of the most common diseases of women worldwide, and may have significant clinical implications for understanding the etiology of recurrent UTIs.

INTRODUCTION

Macroautophagy (henceforth 'autophagy') is a well conserved cellular pathway required to maintain cellular homeostasis, recycle cellular components, and eliminate intracellular pathogens(Levine et al., 2011; Virgin and Levine, 2009). In response to nutrient deprivation, stress or other signals, double membrane- bound autophagosomes are recruited to envelop bulk cytoplasm, damaged organelles, large cytoplasmic protein aggregates, or invading pathogens. These autophagosomes

then fuse with lysosomes so that their cargo may be degraded. Autophagy genes and proteins play a multiplicity of roles in both innate and adaptive immunity(Deretic et al., 2009; Levine and Kroemer, 2009; Saitoh et al., 2008). For example, in mice deficient for Atg5 in macrophages and neutrophils, there is increased susceptibility to infection with *L. monocytogenes* and *Toxoplasma. gondii,* and Atg5 deficiency is associated with decreased resistance to viral encephalitis(Orvedahl and Levine, 2009; Orvedahl et al.). Pathogens have also evolved sophisticated mechanisms to evade or subvert the host autophagy machinery for survival and persistence within cells(Campoy and Colombo, 2009) by blocking the xenophagic degradation of intracellular pathogens or the function of autophagy in innate and adaptive immunity. Although most work to date shows that autophagy as a whole and specific autophagy proteins are detrimental to the microorganism, they may also have 'proviral' or 'probacterial' effects. For example, autophagosomes may serve as a protected niche for intracellular bacteria and/or serve as a source of nutrients for intracellular pathogens(Deretic, 2010). *In vivo* evidence of such pro-pathogen role of autophagy is still lacking.

The autophagy gene/protein, Atg16L1 plays a key role in autophagosome maturation as part of a protein complex that directs the microtubule-associated protein light chain 3 (LC3), to autophagosomes en route to their fusion with lysosomes(Levine et al., 2011; Noda and Yoshimori, 2009). We previously demonstrated that mice deficient in Atg16L1 (Atg16L1^{HM}) display reduced autophagy. In addition, they develop, upon viral infection, intestinal abnormalities similar to pathologies found in Crohn's disease patients, including abnormalities in the integrity, architecture, and function of Paneth cells, specialized secretory epithelial cells of the small intestine(Cadwell et al., 2008; Cadwell et al., 2010). Atg16L1 has also been shown to play a role in modulating proinflammatory responses in mice and humans (Lee et al., 2012; Plantinga et al., 2011). Furthermore, population genetic studies have positively associated a common polymorphism in the *ATG16L1* gene with inflammatory bowel disease(Cadwell et al., 2008; Franke et al., 2010; Hampe et al., 2007). One explanation for a high prevalence of such harmful gene variants would be protection from common infections. However, such a protective role for mutation of an autophagy gene has not been reported.

Here we employ Atg16L1^{HM} mice as a genetically tractable model to determine whether mutation in an autophagy gene can increase resistance to a common infection. UTIs, primarily caused by UPEC, are among the most common infectious diseases in humans, resulting in an estimated 13 million outpatient visits yearly in the U.S.(Dielubanza and Schaeffer). Recurrent UTIs are a serious problem, with ~25% of patients experiencing multiple episodes per year despite appropriate antibiotic treatment and absence of bacteriuria between episodes(Foxman). In an *in vivo* mouse model, UPEC invades bladder superficial cells by binding uroplakin receptors expressed on the cell surface(Anderson et al., 2003; Zhou et al., 2001). During the acute stage of infection (0-72 hours), intracellular UPEC replicate rapidly and establish cytoplasmic biofilms termed intracellular bacterial communities (IBC) in bladders of both mice and humans(Garofalo et al., 2007; Rosen et al., 2007)}. Major mechanisms for control of UPEC-induced UTI include exfoliation of superficial epithelial cell layer containing IBCs into the urine and influx of innate immune cells(Hunstad and Justice; Hunstad et al., 2005; Ragnarsdottir et al.; Schilling et al., 2003). However, despite the innate immune host response, a subset of UPEC still survive and establish long term reservoirs within urothelial cells, termed quiescent intracellular reservoirs (QIRs)(Mysorekar and Hultgren, 2006), which are enclosed within vesicules decorated with late endosomal/lysosomal markers. Latent UPEC within QIRs can re-emerge to seed recurrent UTIs likely through cAMP-regulated exocytic processes to re-enter the bladder lumen concomitant with urothelial regeneration(Bishop et al., 2007; Mysorekar and Hultgren, 2006; Song et al., 2007).

RESULTS

Atg16L1 deficiency leads to superficial cell architectural abnormalities.

To determine if Atg16L1 plays a role in UPEC invasion and persistence within the bladder, we first examined the morphology of superficial urothelial cells, the target cells for UPEC invasion, in Atg16L1^{HM} mice. These cells are highly specialized to rapidly expand and shrink as the bladder fills and contracts and this function requires large plaques of hexagonal arrays of uroplakin protein complexes, which are transported by discoidal fusiform vesicles (FV) and multivesicular bodies

(MVB) to maintain cellular architecture and barrier properties (Guo et al., 2009; Khandelwal et al., 2009). UPEC use the uroplakins (Up1a) as receptors to facilitate invasion and entry into these urothelial cells(Guo et al., 2009; Khandelwal et al., 2009; Kreft et al., 2009). We reasoned that Atg16L1 deficiency might alter this membranous intracellular network because changes in intracellular membranes associated with granule exocytosis are observed in the intestinal Paneth cell in Atg16L1^{HM} mice(Cadwell et al., 2008). Here, we show that Atg16L1 deficiency governs urothelial cell architecture as follows: first, bladders of Atg16L1^{HM} mice exhibited decreased (by 78%) Atg16L1 expression (Fig. S1). Second, superficial epithelial cells of uninfected Atg16L1^{HM} mice showed dramatic accumulation of vesicles (Fig. 1B) compared to those of control (WT) mice (Fig. 1A). Transmission electron microscopy (TEM) confirmed this vesicular congestion (Fig. 1C-F, S2A-C) and revealed significantly greater numbers of organelles with the morphology of MVBs and lysosomes in Atg16L1^{HM} superficial cells (Fig. 1G-H; $p<0.05$ and $p<0.01$ respectively). Third, given that this vesicular system is responsible for uroplakin transport and recycling and that disruption in vesicular trafficking of FVs and MVBs is associated with aberrant MVB accumulation (Bishop et al., 2007; Guo et al., 2009; Sun et al., 1999), we next determined the effect of Atg16L1 deficiency on uroplakin receptor expression. We found increased Up1a expression in Atg16L1^{HM} bladders (Fig 1I-J), correlating with the increased MVBs seen by TEM in Atg16L1^{HM} superficial cells. These data show that Atg16L1 is a key player in modulating and/or maintaining the proper architecture of superficial cells, including membranes that are involved in uroplakin expression.

Autophagy machinery intersects with UPEC.

The autophagy pathway functions to sequester intracellular pathogens, following cellular invasion through recognition and decoration by LC3 and an adaptor protein p62 (Birmingham et al., 2007; Deretic, 2010; Levine et al., 2011; Zheng et al., 2009). To determine whether such autophagy-associated pathogen recognition is engaged upon UPEC infection *in vivo*, we infected bladders of adult female wild type mice by transurethral inoculation of a clinical cystitis isolate, UTI89(Hung et al., 2009). Upon UPEC invasion of bladder superficial cells, Atg16L1 and LC3 puncta colocalized

with UPEC within IBCs at 6 hours post inoculation (hpi) and QIRs at 14 days post inoculation (dpi) (Fig. S3A-Dand insets). p62 puncta, normally present throughout the urothelium in an uninfected state (Fig. S3E), were recruited to the site of bacterial entry (Fig. S3F). Thus, autophagy proteins associate with UPEC during the acute and latent stages of UPEC pathogenesis *in vivo*.

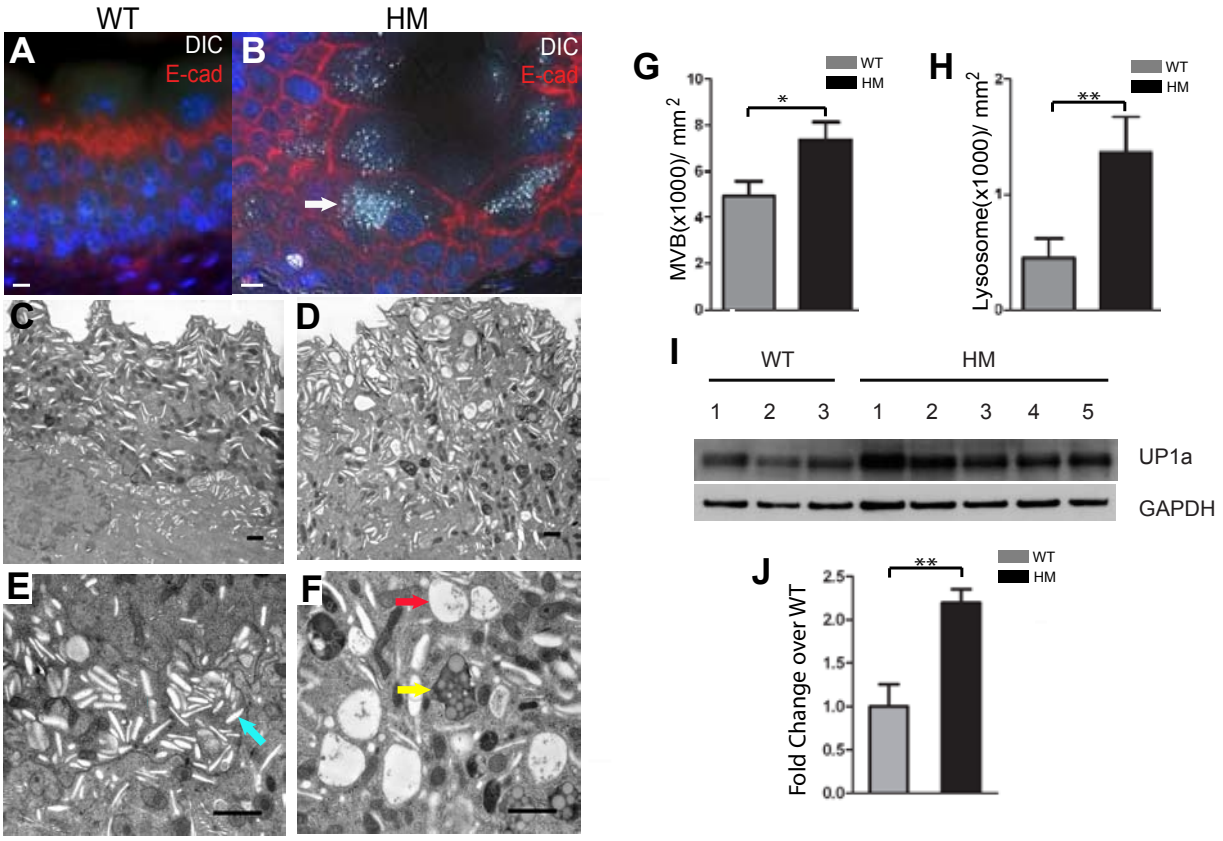

Figure 1. Atg16L1 deficiency leads to superficial cell architectural abnormalities: (A-B) IF and DIC imaging analysis showing WT (A) and Atg16L1HM (HM) (B) urothelium depicting enhanced vesicular congestion in HM (cyan, arrow). E-cadherin (red) outlines urothelial cells; nuclei are blue with biz-benzimide. Bar=10 µm. (C-D) TEM showing WT (C) and HM (D) superficial cell ultrastructure confirming architectural changes in HM urothelium. Panels representative of 10-15 sq. µm regions examined in n=3 mice. Bar=1 µm. (E-F) High magnification image of WT (E) and HM (F) superficial cells displaying fusiform vesicles (FV) (blue arrow), multi-vesicular bodies (MVB) (red arrow) and lysosomes (LY) (yellow arrow). Bar=1 µm. (G-H) Quantification of MVBs (G) and lysosomes (H) showing a significant increase in HM urothelium. Bars represent mean ± SEM. "*" $p<0.05$, "**" $p<0.01$ by unpaired two-tailed T test. (I-J) Western blot (I) of whole bladder protein indicates increased UP1a in HM mice relative to WT mouse (normalized to GAPDH) and quantification of UP1a expression relative to WT (J). Bars represent mean ± SEM. "*" $p<0.05$, "**" $p<0.01$ by unpaired two-tailed T test.

Atg16L1 deficiency leads to rapid clearance of bacteriuria and significant reduction in latent reservoirs.

To determine if Atg16L1 deficiency-induced alterations in superficial urothelial cell morphology and increased uroplakin expression affect UPEC invasion and persistence in superficial urothelial cells, we induced a UTI in adult female control and Atg16L1^{HM} mice. The progress of UPEC-induced UTI were monitored by measuring shedding of bacteria into the urine (*i.e.* bacteriuria), analyzing bacterial invasion and colonization in the bladder tissue and evaluating the number of QIRs. We found that UPEC invaded and colonized the bladders of Atg16L1^{HM} and control mice during the acute infection stage (6hpi) at similar levels (Fig. 2A-B). Strikingly, however, as early as 1dpi, Atg16L1^{HM} mice exhibit significantly reduced bacteriuria (Fig. 2A) and bladder tissue UPEC titers (Fig. 2B) and thus were more effective at clearing bacteriuria than controls ($p<0.01$). We further demonstrate that this enhanced bacteriuric clearance can be induced even in the presence of one hypomorphic *Atg16L1* allele: Atg16L1^{HM} heterozygote mice (HET) expressing 41% of Atg16L1 protein (Fig. S1) cleared UPEC faster compared to control mice by 3dpi ($p<0.05$, Fig. S4). Thus, the presence of a single hypomorphic *Atg16L1* allele is sufficient to confer protection from bacterial infection.

Given the abnormal accumulation of MVBs in the superficial bladder epithelial cells of Atg16L1^{HM} mice, we investigated whether these epithelial changes were altering early invasion events, thereby leading to the decreased bacterial burden. Examination at 6hpi for IBC formation (Fig. 2C) showed similar numbers of IBCs in Atg16L1^{HM} and control bladders (Fig. 2D), consistent with similar urine and bladder bacteria titers at 6hpi. Thus, decreased

Atg16L1 expression in Atg16L1^{HM} bladders is associated with decreased bacteriuria, which is not due to alteration of early epithelial invasion and colonization events.

We next examined control and Atg16L1^{HM} bladders at 14dpi to determine if enhanced acute clearance of bacteriuria from the bladders of Atg16L1^{HM} mice was associated with reduced establishment

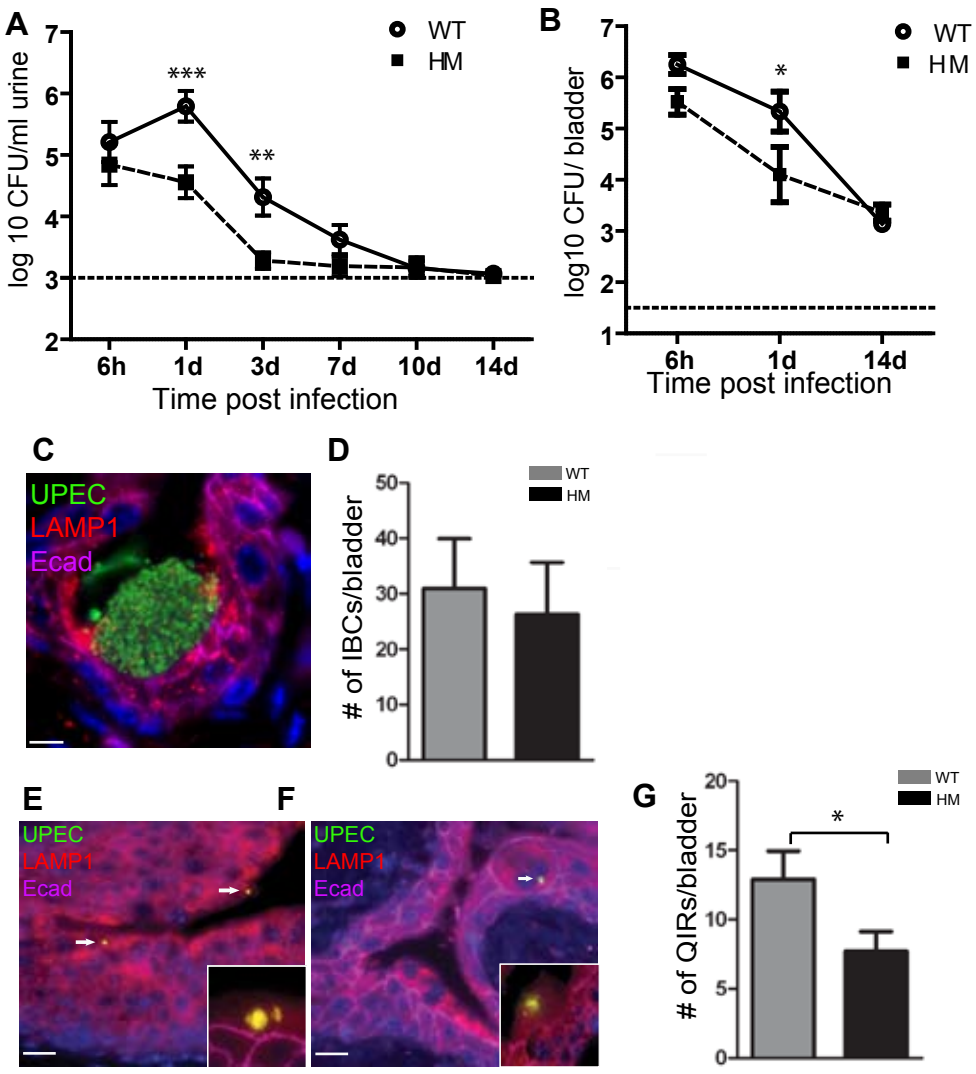

Figure 2. Atg16L1 deficiency leads to rapid clearance of bacteriuria, acute reduction in bladder colonization, and significant reduction of latent UPEC reservoirs: (A) CFU counts of bacteriuria plotted as mean ± SEM of the Log10 value at 6hpi-14dpi showing rapid reductions of bacteriuria in HM mice 24hpi and clearance by 3dpi. n=5-15 mice/timepoint/genotype; n=3 experiments, "***"p<0.001, "**" p<0.01 by two-way ANOVA with Bonferroni post test. (B) CFU counts from full bladder homogenates show similar titers in the HM and WT mice at 6hpi, and significantly lower levels in HM mice by 24hpi. n=3-5 mice/timepoint/genotype; n=3 experiments p<0.05 by two-way ANOVA with Bonferroni post test. (C) IF analysis of HM bladders at 6hpi with UPEC showing normal IBC formation. (D) Quantification of IBCs in WT versus HM bladders at 6hpi. (E-F) IF analysis of WT (E) and HM bladders (F) at 14dpi with UPEC showing QIRs (green, arrows). Insets show higher magnification image of a bacterial QIR enclosed by Lamp1 staining (red). E-cadherin (purple) outlines urothelial cells; nuclei are blue with biz-benzimide. (G) Quantification of QIRs in WT versus HM bladders at 14dpi indicating a significant decrease in QIR numbers in HM mice. n=6 sections/bladder, n=38-55 mice/genotype. Bar=10 μm. "*" p<0.05, by unpaired two-tailed T test.

of latent reservoirs QIRs. QIRs can seed recurrent infection, even though the reservoirs contain relatively small numbers of bacteria that are often at or below detection levels in urine and bladder CFU titers(Mysorekar and Hultgren, 2006). We found that bladders of Atg16L1^{HM} mice harbored significantly reduced numbers of Lamp1+ QIRs compared to bladders of control mice (Fig. 2 E-G; $p<0.05$). Thus, Atg16L1 deficiency resulted in increased resistance to the formation of protected niches containing UPEC during the latent stage of infection. Together, our data show that Atg16L1 deficiency is associated with decreased bacteriuria and bladder colonization by UPEC at acute stages of infection and protection from UPEC latency.

Superficial urothelial cell regeneration is hastened in Atg16L1 deficient bladders.

Urothelial tissue regeneration is an important step in the resolution of UTIs(Mysorekar et al., 2009). We analyzed bladder tissue sections from control and Atg16L1^{HM} mice at 6dpi and 14dpi and found that relative to uninfected bladders from control and Atg16L1^{HM} mice (Fig. S5A-B), the bladders of control mice at 6dpi displayed persistent stromal inflammation and proliferating transitional epithelial cells (arrow, Fig. S5C). In contrast, Atg16L1^{HM} bladders displayed newly regenerated, terminally differentiated and non-proliferating superficial cells (arrow) and resolution of inflammation (Fig. S5D). By 14dpi, both control and Atg16L1^{HM} bladders displayed regenerated superficial cells (Fig. S5E-F), but bladders of control mice continued to show inflammation (arrowheads, Fig. S5E). The proliferative index of stem/progenitor cells following infection was similar between control and Atg16L1^{HM} mice at 6dpi (Fig. S5G), suggesting that the stem cell niche was unaffected by Atg16L1 deficiency. However, by 14dpi, while continued proliferative activity was observed in bladders of control mice (arrow, Fig. 5E), it had returned to basal levels in bladders of Atg16L1^{HM} mice (Fig. S5F,H, $p<0.05$). Thus, early bacterial clearance in Atg16L1^{HM} mice correlated with faster epithelial recovery. Interestingly, TEM analysis of WT and Atg16L1^{HM} bladders at 14dpi (Fig. S6A-B) confirmed that the regenerated superficial cells in Atg16L1^{HM} bladders also exhibited the ultrastructural vesicular defects observed prior to infection, suggesting Atg16L1 deficiency induced cellular abnormality in superficial cells was autonomous to this cell type.

Atg16L1 deficiency in the hematopoietic compartment contributes significantly to clearance of bacterial load.

We next sought to determine the cellular mechanisms underlying the enhanced clearance of bacteria in Atg16L1^{HM} bladders and the resulting host-protective outcome. Prior work has shown that both hematopoietic and epithelial compartments are required to mount an efficient host response to UPEC infection in the bladder(Schilling et al., 2003). Although Atg16L1 deficiency in the epithelial compartment affected latent UPEC reservoir establishment, it did not show any differences in acute infection events, for e.g., in IBC formation, that could explain the more rapid clearance in Atg16L1^{HM} mice. Furthermore, Atg16L1 plays a role in modulating proinflammatory responses (13, 42). We reasoned therefore that Atg16L1 deficiency in immune cells could contribute to UPEC clearance directly by a potent pro-inflammatory response or indirectly by inducing the aberrant superficial urothelial cell architecture that we observed.

To elucidate contributions of Atg16L1 deficiency in hematopoietic cells to clearance of UTI, we generated reciprocal bone marrow (Fig. 3A-D). To examine the contribution of Atg16L1 hypomorphic hematopoietic compartment, we compared wild type recipients receiving Atg16L1^{HM} bone marrow with those receiving wild type bone marrow. We show that wild type mice receiving Atg16L1^{HM} bone marrow cleared bacteriuria faster: significantly more complete clearance was evident at 7dpi and 14dpi as indicated by the lower bacteriuria titers in the presence of Atg16L1^{HM} bone marrow (Fig. 3E). DIC imaging and TEM analysis revealed no aberrant changes in the epithelium of these wild type animals, suggesting that Atg16L1^{HM} hematopoietic cells do not contribute to the superficial epithelial cell abnormalities (Fig. S7A-B), consistent with those being cell autonomous, but that the presence of Atg16L1^{HM} bone marrow alone is sufficient for faster clearance of UTI infection.

Next, we sought to determine if Atg16L1 deficiency in the epithelium would contribute to the bacteriuric clearance and compared mice with wild type and Atg16L1^{HM} epithelium receiving wild type bone marrow. As expected, urothelial architectural abnormalities similar to those described

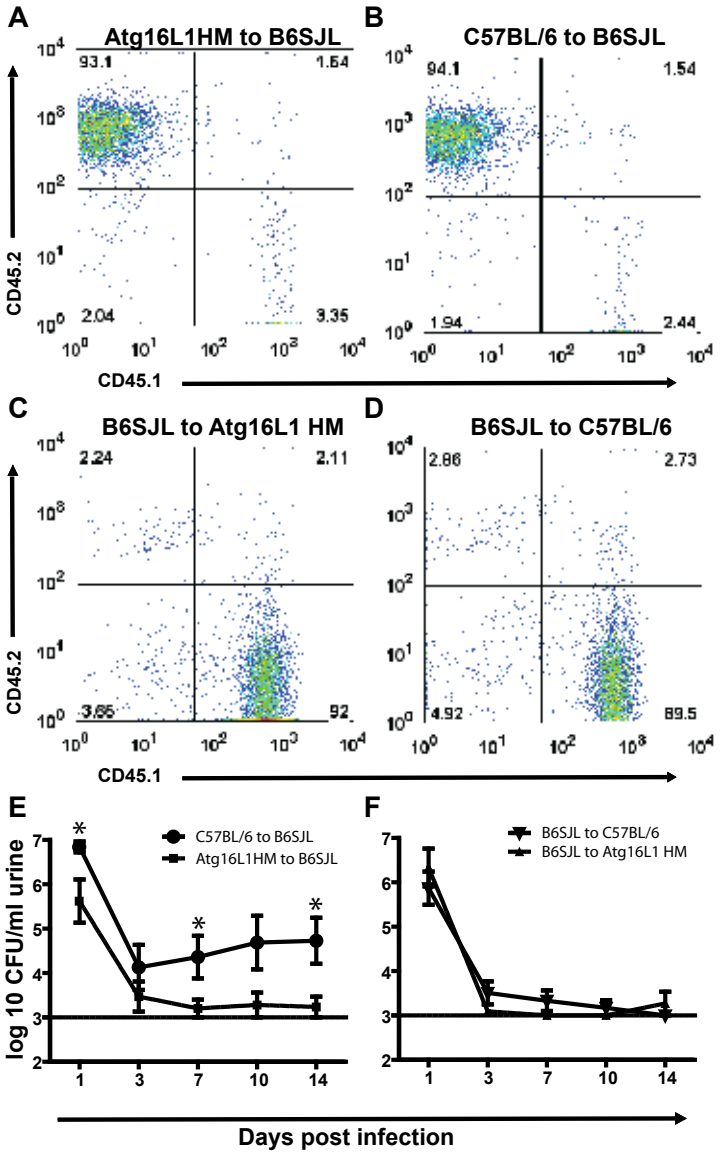

Figure 3. Atg16L1 deficiency in the hematopoietic compartment leads to increased bacteriuric clearance: (A-D) Lethally irradiated mice were given bone marrow from gender-matched donors and infected with UTI89 after 8 weeks of reconstitution. Flow cytometric analysis of splenocytes demonstrated effective engraftment of donor bone marrow for each bone marrow chimera animal. (E) Urinary UPEC CFU counts at 1-14dpi indicating more rapid and thorough clearance of bacteria in the B6SJL mice receiving Atg16L1HM bone marrow compared to those receiving wild type bone marrow. n=5-10 mice/timepoint/genotype; n=2 experiments. "*"$p<0.05$, "**" $p<0.01$, by two-way ANOVA with Bonferroni post test. (F) Urinary UPEC CFU at 1-14dpi showing similar UPEC clearance in wild type mice and Atg16L1HM mice receiving B6SJL bone marrow. n=5-10mice/timepoint/genotype; n=2 experiments.

in Atg16L1^{HM} mice were observed in bladders of Atg16L1^{HM} recipients receiving wild type bone marrow (Fig. S7A-B), however these mice did not display faster clearance of bacteriuria (Fig. 3F). Our findings indicate that the Atg16L1-deficient hematopoietic compartment is necessary for the clearance, that Atg16L1-deficiency in the epithelium alone with a wild type hematopoietic compartment is not sufficient for faster luminal clearance, and that Atg16L1 deficiency-induced epithelial architectural abnormality is not regulated by Atg16L1-deficiency in hematopoietic cells.

Atg16L1 deficiency in the innate immune compartment is necessary and sufficient for bacteriuric clearance.

Autophagy protein deficiency can have substantial effects on the adaptive and innate immune system(Levine et al., 2011). Thus, we sought to determine whether lymphocytes contributed to the UPEC pathogenesis phenotypes observed in Atg16L1^{HM} mice. We bred Atg16L1^{HM} mice onto a Rag1$^{-/-}$ background, which are T and B cell-deficient(Mombaerts et al., 1992) (henceforth termed Rag1$^{-/-}$Atg16L1^{HM}). We then compared the course of UPEC pathogenic cycle in Rag 1$^{-/-}$ mice and Rag 1$^{-/-}$/Atg16L1^{HM} mice that lack an intact adaptive immune system but have either an intact innate immune system (Rag1$^{-/-}$ mice) or Atg16L1^{HM} innate immune system (Rag1$^{-/-}$Atg16L1^{HM} mice). Whereas Rag1$^{-/-}$ mice exhibited chronic bacteriuria, Rag1$^{-/-}$/Atg16L1^{HM} mice were more efficient in clearing infection as evidenced by significantly lower urine bacterial titres 7dpi ($p<0.05$) and 14dpi ($p<0.01$)(Fig. S8). Thus, our data suggests that in the absence of an intact adaptive immune system, the Atg16L1^{HM} innate immune system is sufficient to lower the bacterial load.

Innate immune cells, particularly macrophages and neutrophils, constitute an early line of defense against UPEC infection *in vivo*(Ragnarsdottir et al.). We reasoned that Atg16L1 deficiency in these cells might contribute to the observed fast clearance of bacteriuria. This could be achieved by increased recruitment of the innate immune cells to the bladder lumen to clear infection in Atg16L1^{HM} mice, and/or increased proinflammatory cytokine expression by Atg16L1^{HM} innate immune cells. We tested these hypotheses as follows: first, we examined the urines of Atg16L1^{HM}

mice at acute stage of infection (6hpi and 24hpi) and found that urines from Atg16L1^{HM} mice contained significantly higher numbers of neutrophils (Fig. 4A, 6hpi) and monocytes upon infection (Fig. 4B, 24hpi). Next, we determined the proinflammatory cytokine response to infection in Atg16L1^{HM} mice. We conducted both qRT-PCR assays of bladder tissues as well as Bioplex assays of sera from infected control and Atg16L1^{HM} mice. We found significant increases in tissue IL-6 mRNA levels (Fig. 4C; $p<0.05$) and in serum IL-1α levels 6hpi (Fig. 4D; $p<0.01$) upon infection

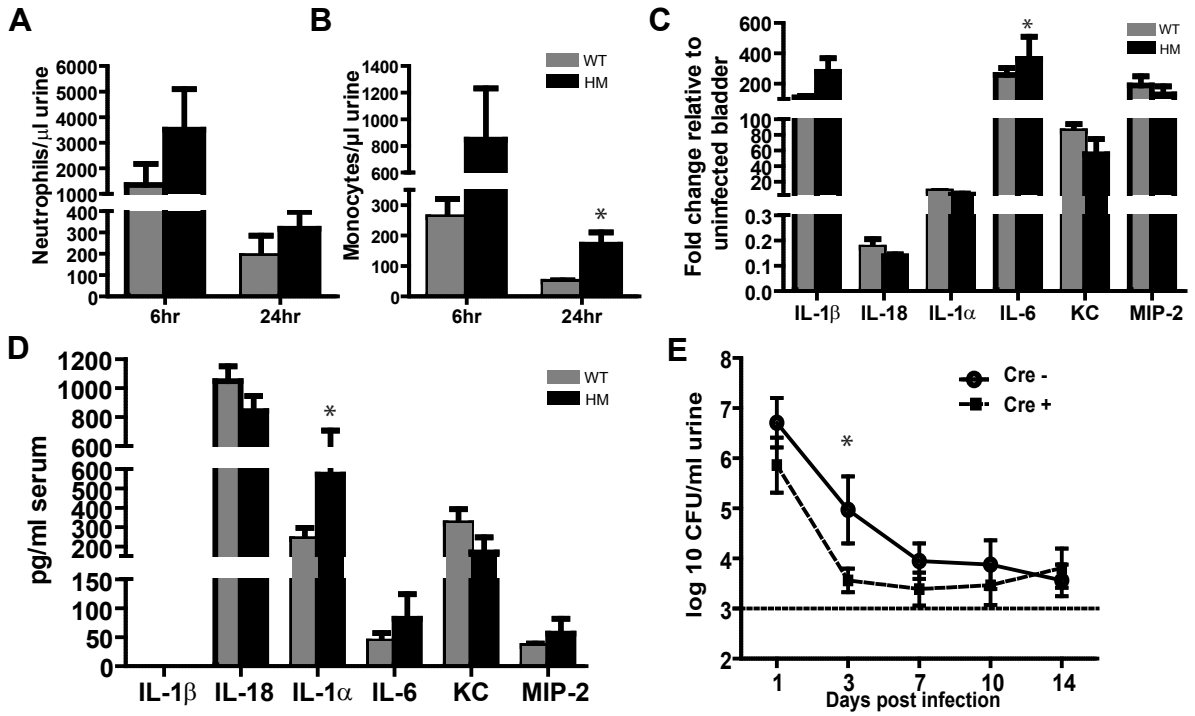

Figure 4. Deficiency in Atg16L1 or Atg5 specifically in innate immune cells is necessary and sufficient for increased bacteriuric clearance: (A) Neutrophil counts in the urine. Bars represent mean ± SEM n=4-5 mice/time point ,n=3 experiments. (B) Monocyte counts in the urine. Bars represent mean ± SEM, n=4-5 mice/time point, n=2 experiments. "*"$p<0.05$ by unpaired two-tailed T test. (C) Quantification of bladder tissue cytokines mRNA levels by qPCR at 6hpi indicating a significant increase in IL-6 in HM bladder response to UPEC infection compared to WT bladders. n=3 mice/genotype, Bars represent mean ± SEM. "*" $p<0.05$, by unpaired two-tailed T test. (D) Quantification of serum cytokine levels by Bioplex bead array at 6hpi showing a significant increase in IL-1α in HM mice in response to UPEC infection compared to WT mice. n=4-9 mice/genotype. Bars represent mean ± SEM. "*" $p<0.05$, by unpaired two-tailed T test. (E) CFU counts at 1-14dpi indicating faster clearance in Atg5fl/fl-Lyz-Cre+ mice as compared to Atg5fl/fl-Lyz-Cre- mice displayed as mean ±SEM of log10 data. n=13-18 mice/timepoint/genotype. "*" $p<0.05$ by two- way ANOVA with Bonferroni post test as unadjusted p-values.

in Atg16L1^{HM} mice relative to control mice, but which were not noted prior to infection (Fig S9). Together, our data show that Atg16L1 deficiency in the innate immune compartment is associated with increased recruitment of innate immune cells and a more robust pro-inflammatory response to infection.

To further dissect the role for autophagy protein deficiency in innate immune cells during an UTI, we used a mouse model that we previously generated, the Atg5$^{fl/fl}$-Lyz-Cre mice, which are deficient in the autophagy protein Atg5 specifically in macrophages and granulocytes(Zhao et al., 2008). We evaluated Atg5 in this system since Atg5 is an essential autophagy protein that forms a part of the Atg16L1-containing protein complex essential for autophagy(Levine et al., 2011). Consistent with Atg16L1 deficiency in immune cells causing enhanced clearance of infection, the Atg5$^{fl/fl}$-Lyz-Cre mice also resolved bacteriuria more rapidly than controls by 3dpi (Fig. 4E, $p<0.05$). Furthermore, bladders of Atg5$^{fl/fl}$-Lyz-Cre mice did not exhibit evidence of organelle congestion or aberrant uroplakin receptor expression in the epithelium (Fig. S10A-B). Thus, Atg5 protein deficiency in macrophages and granulocytes resulted in enhanced control of bacterial infection without indirect effects on the epithelium.

Together, our data show that Atg16L1 deficiency specifically in innate immune cells is the mechanism of enhanced clearance of bacteria from the urine. Atg16L1 deficiency is associated with increased recruitment of innate immune cells to the infected bladders and a robust pro-inflammatory response, which likely modulates the rapid UPEC clearance in Atg16L1^{HM} mice. This response is elicited even in the absence of an adaptive immune system. Finally, deficiency of another autophagy pathway protein, Atg5, exclusively in innate immune cells is sufficient to induce rapid UPEC clearance.

DISCUSSION

Autophagy has been recognized as an important defense system to combat intracellular pathogens, and been hitherto considered to play an anti-pathogenic role. Here, we use an *in vivo* model of a common infectious disease and describe for the first time a pro-pathogen role for an autophagy

protein. We show that deficiency in the key autophagy protein, Atg16L1, can confer protection against a non-obligate intracellular pathogen, UPEC, which causes recurrent UTIs and has been shown to have both intracellular and extracellular stages in its pathogenic cycle. Atg16L1^{HM} mice clear bacteriuria faster and renew their epithelial barrier faster. We show that this protection is mainly due to effects of Atg16L1 deficiency in hematopoietic cells, and specifically macrophages and neutrophils that are the main cellular drivers of the fast clearance of bacteriuria. Our findings suggest the presence of a more potent innate immune system in Atg16L1^{HM} mice with substantially increased pro-inflammatory cytokine expression as well as increased recruitment of innate immune cells to infected Atg16L1^{HM} bladders. We have previously demonstrated enhanced transcription of pro-inflammatory cytokines and adipokines in Paneth cells of Atg16L1^{HM} mice(Cadwell et al., 2008). Recently, Lee et al., delineated that Atg16L1 expression restricts IL-1β signaling cascades and the subsequent inflammatory response, such as IL-6 production, by constitutive autolysosomal and proteosomal degradation of p62, thus, the absence of Atg16L1 leads to a hyper-inflammatory response(Lee et al.). In the setting of Crohn's disease, Atg16L1 deficiency induces elevated proinflammatory cytokine levels, and the presence of commensal bacteria leads to intestinal pathology(Cadwell et al., 2010). In the Atg16L1 deficient urinary tract, however, the elevated proinflammatory cytokine levels may have a beneficial effect: to better combat UPEC infection. We speculate that the more effective innate immune compartment in Atg16L1^{HM} mice may promote rapid elimination of bacteria which may prevent further bacterial invasion into underlying urothelial cells and result in faster clearance of bacterial load(Horvath et al.; Hunstad et al., 2005; Schilling et al., 2003), and thereby lead to restoration of a normal urothelium(Mysorekar et al., 2002). UPEC has been shown previously to inhibit proinflammatory cytokine production after invasion into epithelial cells (Horvath et al.; Hunstad et al., 2005). Loss of Atg16L1 may impact UPEC's ability to dampen the innate immune response resulting in the enhanced clearance. Together, our data all point towards a protective role for Atg16L1 deficient innate immune compartment in UTI.

Complex membrane recycling events in superficial urothelial cells play an important role in UPEC pathogenesis. Atg16L1 deficiency was associated with striking abnormalities in the accumulation

of multivesicular bodies, lysosomes and Up1a receptor in Atg16L1^{HM} mice; all components of the epithelial cells that UPEC interact with during infection. These abnormalities appear intrinsic to the Atg16L1 deficient epithelial cells since autophagy protein deficiency in hematopoietic cells does not generate these abnormalities and these defects are found even in newly regenerated superficial cells. To our surprise, early invasion and colonization of the bladder were not dramatically altered by the Atg16L1 deficiency-induced urothelial ultrastructural changes. Strikingly, however, in the presence of these abnormalities, or perhaps because of them, UPEC appear less able to occupy their intracellular niches to persist within the urothelium as QIRs. Thus, our findings suggest that UPEC may subvert Atg16L1 and possibly other autophagy proteins to establish latency. Recent *in vitro* evidence supports our model. For example, Starr et al., showed *in vitro* that *Brucella* subverts autophagy complexes to facilitate its intracellular cycle and demonstrated that *Brucella* selectively co-opts autophagy-initiation complexes to subvert host clearance and promote infection (Starr et al.).

Our findings using the hypomorphic Atg16L1 mouse model reveal a striking benefit of Atg16L1 deficiency and suggest that deficiency in an autophagy gene that can promote inflammatory disease also protects against acute UTIs. This leads us to speculate that *ATG16L1* allelic variants in the human population, or other variants in autophagy-associated genes, might be under positive selection for their ability to protect from UTIs.

Delineating mechanisms of intracellular persistence during a UTI is critical to developing effective strategies for preventing recurrent UTIs. Traditional antibiotics are unable to penetrate bladder epithelial barriers, so bacteria sequestered in QIRs can survive long term in protected niches. In addition, antibiotic therapy itself risks driving the pathogens into quiescence(Blango and Mulvey, 2010). Targeting and eliminating the small yet significant intracellular pool of bacteria may have substantial clinical benefits to combat refractory and recurrent UTIs. Thus, our model provides a novel system for investigating the role for autophagy proteins in pathogenesis of bacterial infection *in vivo* and may have significant clinical implications for understanding the etiology of recurrent UTIs and for providing cellular targets for therapeutic intervention.

MATERIAL AND METHODS

Mice. Experiments were performed using protocols approved by the animal studies committee of the Washington University School of Medicine (Animal Welfare Assurance #A-3381-01). Mice were maintained under specified pathogen-free conditions in a barrier facility and under a strict 12 hr light cycle. The Atg16L1 HM2 strain (Atg16L1^{HM}) and Atg16L1 HET mice were generated as described(Cadwell et al., 2008). Rag1$^{-/-}$/Atg16L1^{HM} double mutants on a C57Bl/6 strain background were generated by first back-crossing Atg16L1^{HM} mice onto a C57Bl/6 background and then breeding with Rag1$^{-/-}$ mice. Atg5$^{fl/fl}$-Lyz-Cre mice were generated as described (Zhao et al., 2008).

Bacterial Strains, mouse inoculations and urinalysis. UTI89, a pathogenic UPEC strain(Mysorekar and Hultgren, 2006; Mysorekar et al., 2009), was used for all studies. Adult female mice (8-10 week old) were anesthetized and inoculated via transurethral catheterization with 10^7 CFU UTI89 in phosphate-buffered saline (PBS). Urinalysis and bacterial titering were performed as described(Hung et al., 2009; Rosen et al., 2007).

Histochemical and Immunofluorescence Analysis. For histological and immunofluorescence studies, bladders were processed as described(Mysorekar et al., 2009). The following primary antibodies were used: (1) rabbit and goat polyclonal antibodies to *E. coli* (United States Biological; Swampscott, MA), (2) mouse monoclonal antibody to E-cadherin (BD Bioscience; San Jose, CA), (3) goat polyclonal antibodies to BrdU, (4) rat monoclonal antibody to Lamp1(clone ID4B; Developmental Hybridoma Bank, NICHD; Bethesda, MD, (5) rabbit polyclonal to LC3B (Novus), (6) guinea pig polyclonal to p62 (Progen), (7) rabbit polyclonal to Atg16L1(Abgent). Antigen-antibody complexes were detected with Alexa Fluor 488, 594, and 647-conjugated secondary antibodies (Invitrogen; Carlsbad, CA). Images were obtained using Zeiss Apotome at 10-20x and 40-63x (oil) magnifications.

Immunoblotting Analysis. Bladders were isolated, processed, and immunoblotted using the following primary antibodies(Cadwell et al., 2008): (1) rabbit polyclonal to Atg16L1 (Abgent),

(2) goat polyclonal to UP1a (Santa Cruz), (3) rabbit monoclonal to GAPDH (Cell Signaling) and secondary antibodies: (1) goat anti-rabbit IgG-HRP, (2) donkey anti-goat IgG-HRP(Santa Cruz). The protein bands on the developed film were quantified by Image J (NIH: http://rsb.info.nih.gov/ij/).

QIR quantification. Six separate 5 μm serial sections over a thickness of 300 μm were immunostained with antibodies against *E. coli*, Lamp1 and E-cadherin. The total number of Lamp1+ UPEC reservoirs were imaged at 63x oil on the Zeiss scope and quantified per bladder. n= 6 sections/bladder, n=38-40 mice/genotype.

BrdU Labeling. BrdU labeling was performed as described(Mysorekar et al., 2009).

Transmission Electron Microscopy (TEM). The whole bladder was processed as described before. The number of lysosomes or MVBs and normalized to total surface area of region examined (n=33 TEM sections from 3 WT and 3 Atg16L1^{HM} mice, and bone marrow chimeras).

Quantitative real time-PCR analysis. RNA was isolated from bladders from uninfected and infected (6hpi) animals (n=3 mice/genotype) and processed as described (Mysorekar et al., 2009).

Bioplex Cytokine Bead Array Assay: Sera were obtained before infection and at 6hpi and cytokine levels were measured using Bioplex kit(BioRad, CA, USA)(Hannan et al., 2010)**.**

Neutrophil and monocyte counts in urine. The urines were collected before and after infection and neutrophil and monocyte counts were analyzed using HEMAVET® 950,Veterinary 5 part WBC Hematology System (Americas Drew Scientific Inc, Oxford CT 06478) by the diagnostic lab in the Department of Comparative Medicine, WUSM.

Bone marrow chimera generation. Seven-week-old recipients received 1000 Gy of gamma irradiation in divided doses over 2 sequential days and were injected i.v. with 1 x 10^7 T lymphocyte-depleted bone marrow cells from gender-matched donors. Mice were allowed 8 weeks for

reconstitution before use for experiments(Schilling et al., 2003), then UPEC-infected. The urine bacterial titers were examined at 0-14days. Appropriate reconstitution of lymphocyte compartments was examined by flow cytometry at the time of sacrifice.

Statistical Analysis. To assess the significance of a difference between groups, a two-sample, unpaired t-test was performed using Graph Prism software. For time-course studies, the standard error (SE) used in t-test was estimated by ANOVA and two-sample tests were performed at individual time points. To control for false positives, Bonferroni adjusted p-values at individual time points are reported. A p-value less than 0.05 (*) was considered to be significant, less than 0.01 (**) to be very significant, and less than 0.001 (***) to be very very significant**.**

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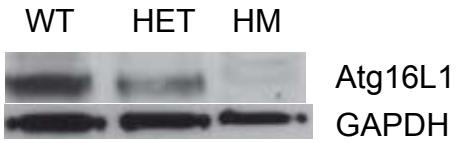
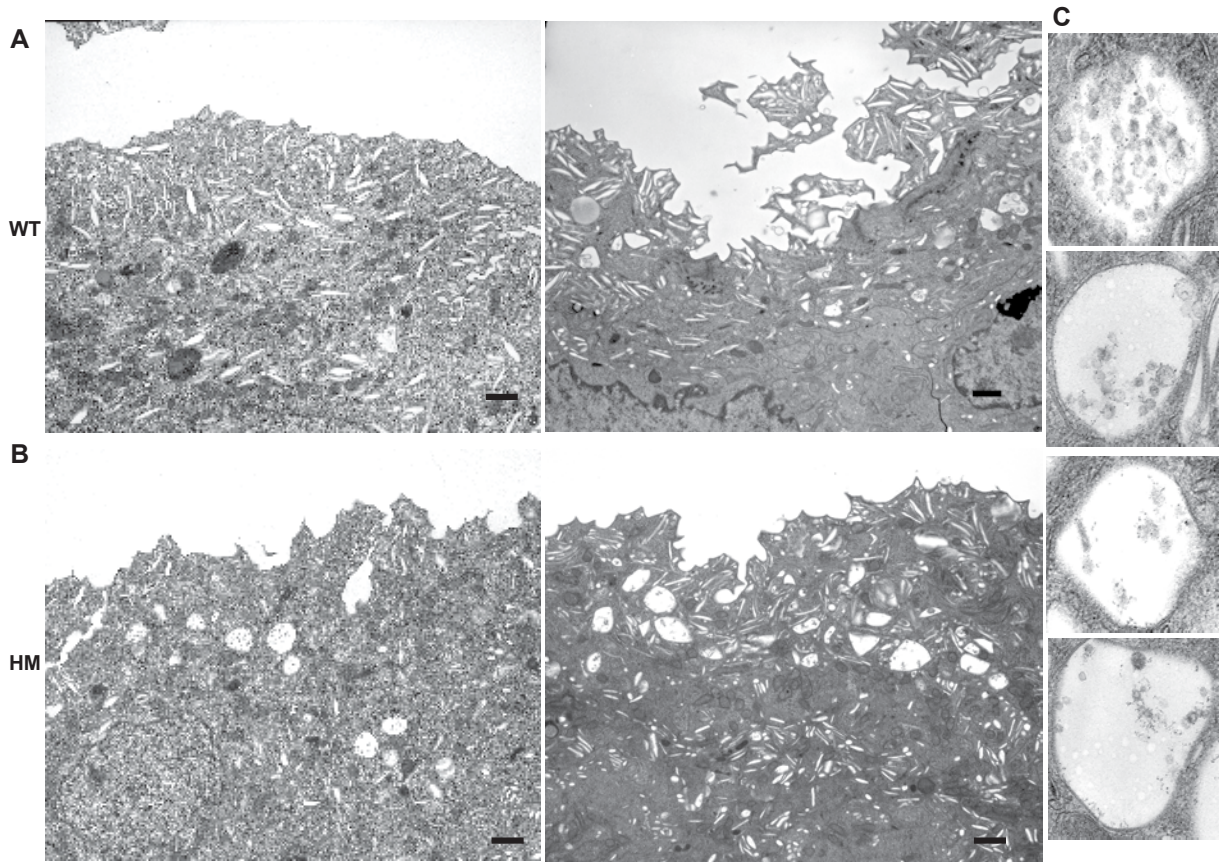
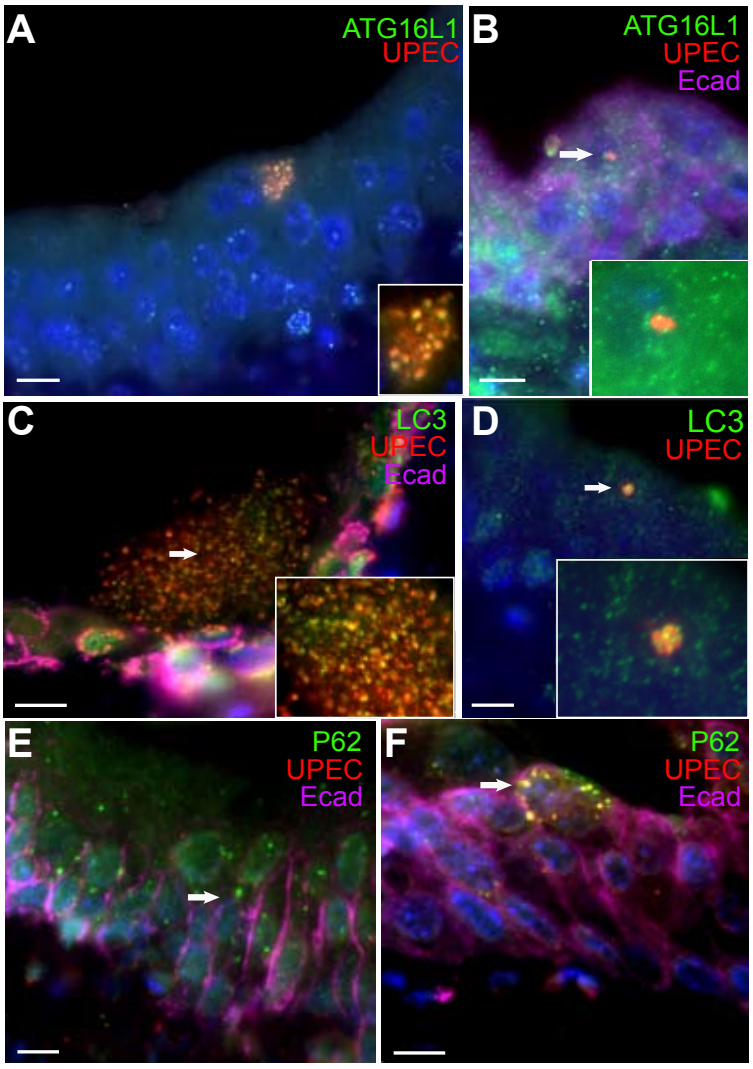
Supplementary figures.



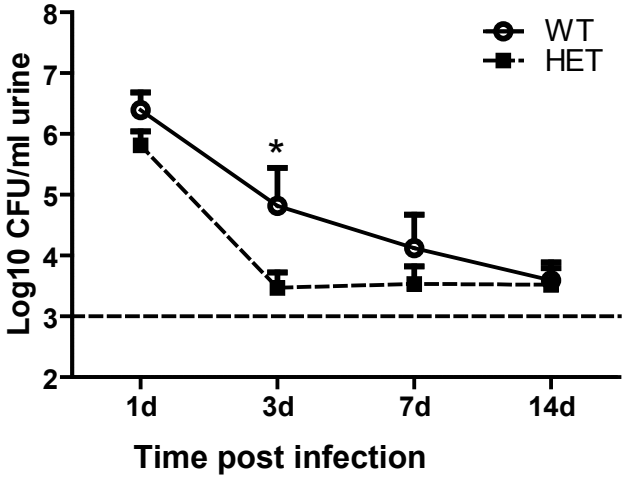
Supplementary Figure 1. Expression of Atg16L1 protein in Atg16L1^{HM} bladders. HM and HET (only one hypomorphic allele) mice demonstrate 78% and 59% knockdown of the Atg16L1 protein, respectively (~68kDa doublet) compared to WT. Knockdown was measured by immunoblotting and normalized to GAPDH loading control. n=3 experiments.



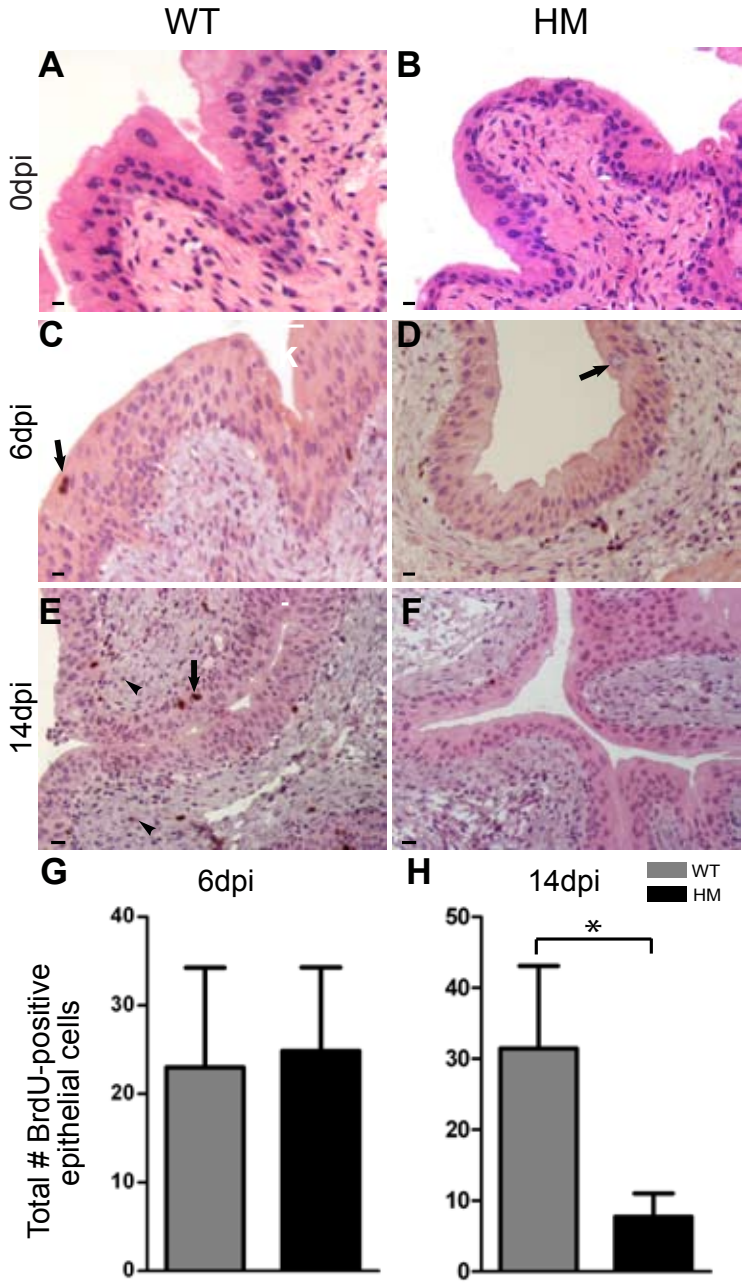
Supplementary Figure 2. Atg16L1 deficiency is associated with superficial cell ultrastructural abnormalities: (A-B) TEM showing WT (A) and HM (B) superficial cell ultrastructure depicting architectural changes in HM urothelium. Bar=1 μm. (C) High magnification images showing multi-vesicular bodies (MVB) from HM superficial cells.



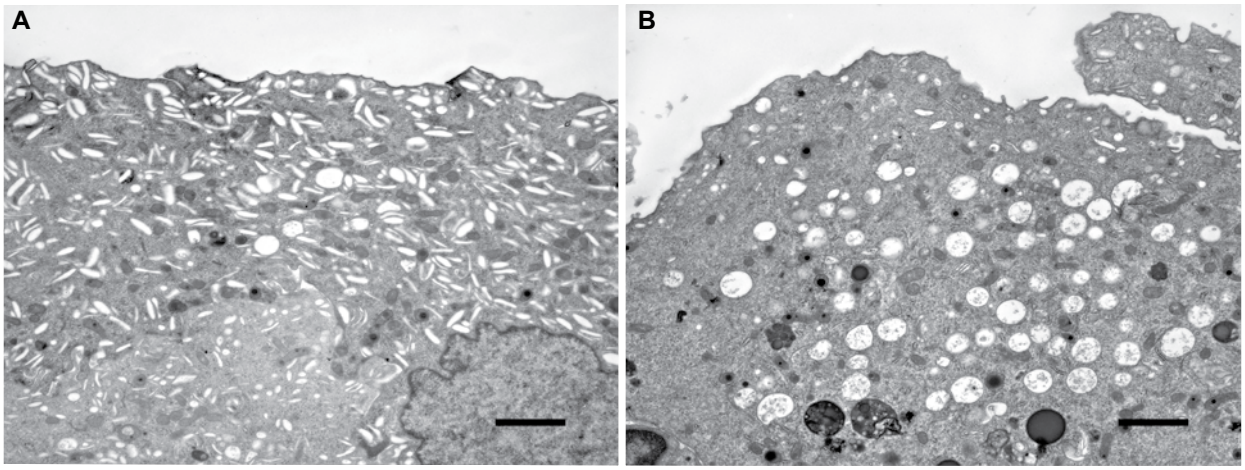
Supplementary Figure 3. Intracellular UPEC colocalizes with autophagic machinery. (A-B) Atg16L1 colocalizes with UPEC (red) within an IBC at 6hpi (A and inset) and a QIR at 14dpi (B, arrow and inset). (C-D) LC3 punctae (green) colocalize with UPEC (red) within an IBC at 6hpi (A and inset) and a QIR at 14dpi (B, arrow and inset). (E) p62 punctae (green, arrow) in uninfected bladders occur diffusely throughout the urothelium. (F) Upon UPEC infection (6hpi), p62 punctae (green) colocalize with invaded UPEC (red, arrow). n=5-10 mice/timepoint/ genotype, n=3 experiments. Bar=10 µm.



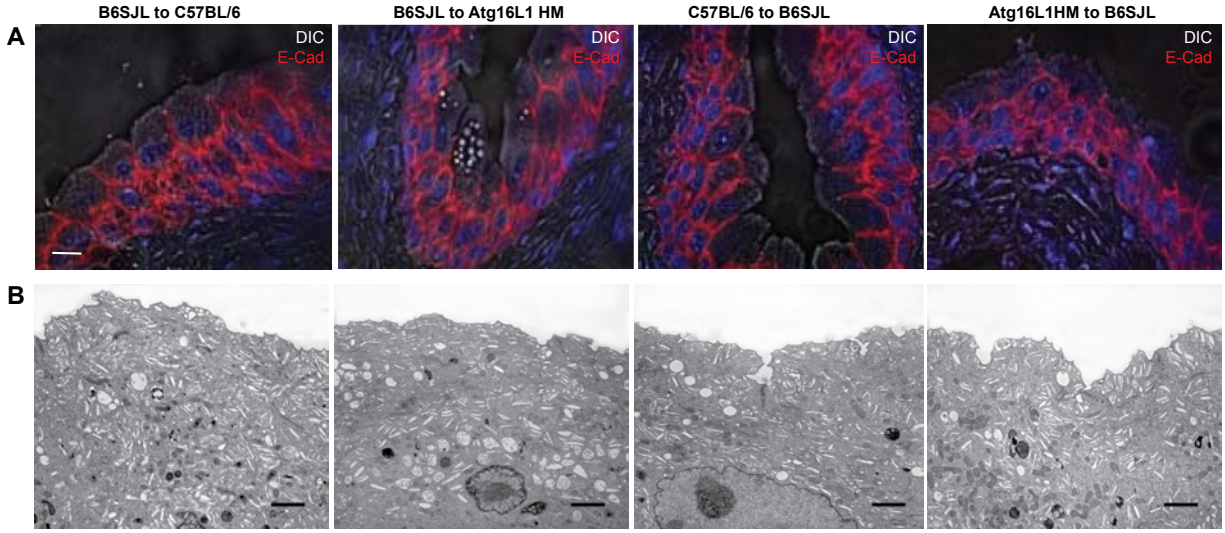
Supplementary Figure 4. A single hypomorphic *Atg16L1* allele is sufficient to confer protection to UPEC infection: CFU counts of bacteriuria plotted as mean ± SEM of the Log10 value 1dpi-14dpi indicating significant reductions of bacteriuria in Atg16L1 HET mice at 3dpi. n=5-15 mice/timepoint/genotype; n=2 experiments. "**" $p<0.01$ by two-way ANOVA with Bonferroni post test.



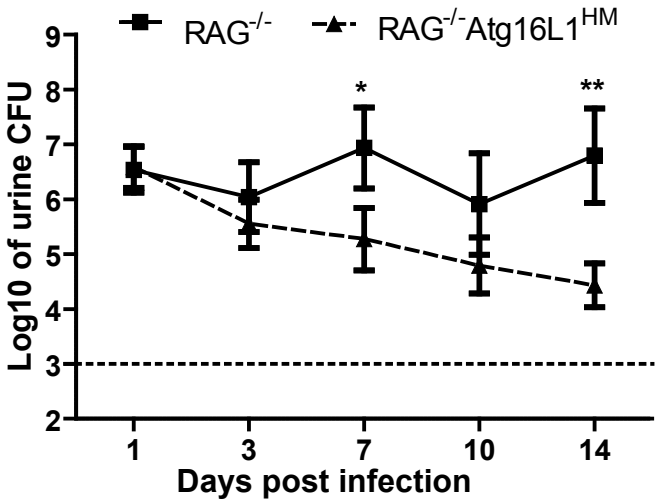
Supplementary Figure 5. Superficial urothelial cell regeneration postinfection is hastened in Atg16L1-deficient bladders. (A and B) H&E- and BrdU-stained WT (A) and HM (B) bladders at 0 dpi, respectively, showing normal morphology of the bladder before infection with no BrdU+ cells observed. (C–F) H&E- and BrdU-stained WT (C and E) and HM (D and F) bladders at 6 and 14 dpi, showing BrdU+ cells (brown, arrows in C and E), neutrophils (C and E, arrowhead), and superficial cells (arrow in D). (Scale bar: 10 μm.) BrdU counts at (G) 6 and (H) 14 dpi depicting rapid recovery from infection in HM mice. n = 15–20 mice per time point per condition. Bars represent mean ± SEM. *P < 0.05 by unpaired two-tailed t test.



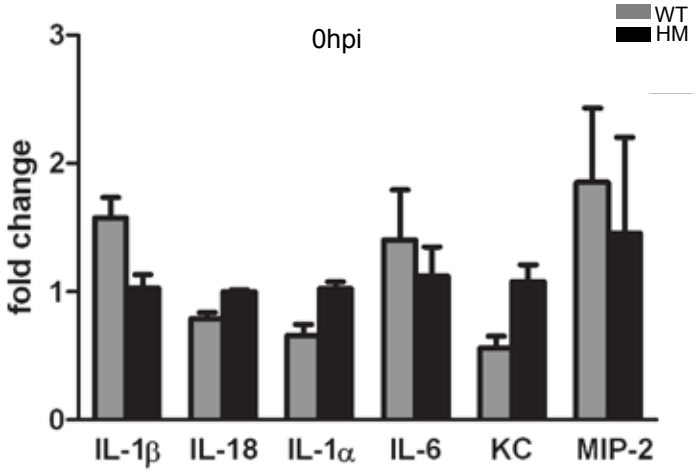
Supplementary Figure 6. Atg16L1 deficiency-induced alteration of superficial cell architecture is intrinsic to the deficiency and not altered by infection (A-B) TEM analysis showing WT (A) and HM (B) newly regenerated superficial cells at 14dpi, depicting ultrastructural abnormalities in HM urothelium. Bar=2 μm.



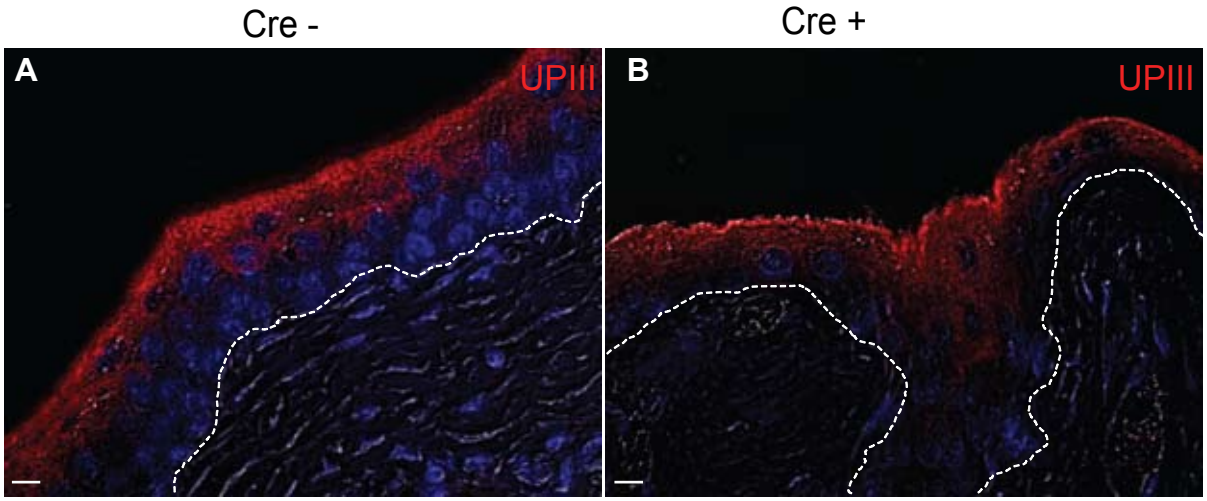
Supplementary Figure 7. Atg16L1 deficiency in the hematopoietic compartment does not induce epithelial abnormalities: (A) IF and DIC imaging analysis showing enhanced vesicular congestion in Atg16L1^{HM} recipients receiving wild type bone marrow, but not in mice with HM bone marrow. E-cadherin (red) outlines urothelial cells; nuclei are blue with biz-benzimide. Bar=10 μm. (B) TEM showing superficial cell ultrastructure of different groups of mice confirming presence of architectural defects in Atg16L1^{HM} recipients receiving wild type bone marrow urothelium. Bar=1 μm.



Supplementary Figure 8: Atg16L1 deficiency in the innate immune compartment induces enhanced bacteriuric clearance in mice lacking an intact adaptive immune system: CFU counts of bacteriuria plotted as mean ± SEM of the Log10 value 1dpi-14dpi indicating significant reduction in bacteriuria in $Rag1^{-/-}Atg16L1^{HM}$ mice at 7-14dpi compared to $Rag1^{-/-}$ mice. n=5-15 mice/timepoint/genotype; n=2 experiments. "**" $p<0.01$, "***" $p<0.001$ by two-way ANOVA with Bonferroni post test.



Supplementary Figure 9. Atg16L1 deficiency does not induce alterations in overall cytokine profiles prior to UPEC infection: qRT-PCR analysis of bladder tissue cytokine mRNA levels in uninfected WT and HM bladders do not exhibit any differences at baseline. Bars represent mean± SEM, n=3 mice/genotype.



Supplementary Figure 10. Atg5 deficiency in macrophages and granulocytes does not exhibit altered urothelial architecture: (A-B) IF and DIC imaging analysis showing $Atg5^{fl/fl}$-Lyz-Cre^{-} (A) and Cre^{+} (B) bladder superficial cells with normal uroplakin III(red) staining and no organellar congestion . Bar=10 μm.

Chapter 3: ATG16L1 and pathogenesis of urinary tract infections

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ATG16L1 and pathogenesis of urinary tract infections

Wang C, Symington JW, Mysorekar IU

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J.W.S co-wrote this chapter.

ABSTRACT

Autophagy is generally considered to be anti-pathogenic. The autophagy gene *ATG16L1* has a commonly occurring mutation associated with Crohn's disease (CD) and intestinal cell abnormalities. Mice hypomorphic for ATG16L1 (ATG16L1^{HM}) recreate specific features of CD. Our recent study shows that the same ATG16L1^{HM} mice that are susceptible to intestinal inflammatory disease are protected from urinary tract infections (UTI), a common and important human disease primarily caused by uropathogenic *E. coli* (UPEC). UPEC colonize the bladder and exhibit both luminal and intra-epithelial stages. The host responds by recruiting innate immune cells and shedding infected epithelial cells to clear infection. Despite these countermeasures, UPEC can persist within the bladder epithelium as membrane-enclosed quiescent intracellular reservoirs (QIRs) that can seed recurrent UTI. The mechanisms of persistence remain unknown. In this study, we show that ATG16L1 deficiency protects the host against acute UTI and UPEC latency. ATG16L1^{HM} mice clear urinary bacterial loads more rapidly and thoroughly due to ATG16L1-deficient innate immune components. Furthermore, ATG16L1^{HM} mice exhibit superficial urothelial cell-autonomous architectural aberrations that also result in significantly reduced QIR numbers. Our findings reveal a host-protective effect of ATG16L1 deficiency *in vivo* against a common pathogen.

REVIEW

Recent reviews highlight the complex interplay between autophagy and microbial adaptations governing host-pathogen interaction outcomes. Autophagic degradation of intracellular pathogens is a significant host defense. However, pathogens employ many strategies to evade or subvert the autophagy machinery for survival including retarding the maturation of autophagosomes, impairing fusion with lysosomes, escaping to the cytosol, and adapting to survive and replicate within the autophagosomal or lysosomal compartment. Recently, for example, Kim and coworkers demonstrated the importance of host autophagy in modulating effective antimicrobial responses to *Mycobacterium tuberculosis*, and the Celli group showed that *Brucella* subverts autophagy complexes to facilitate its intracellular cycle. However, little is known about how UPEC interact

with autophagy. We previously showed that UPEC are enclosed in LAMP1-positive vesicles (QIRs) that resemble LAMP1-positive spacious *Listeria*-containing phagosomes (SLAPs) described by Brumell's group. In this study, we show that UPEC are also targeted by the ATG16L1, LC3, and SQSTM1/p62 proteins. Our findings for UPEC, thus, resemble other studies on intracellular recognition of *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, and *Listeria monocytogenes*. We also find UPEC in double-membranous structures in transmission electron micrographs of infected bladders (**Fig. 1**).

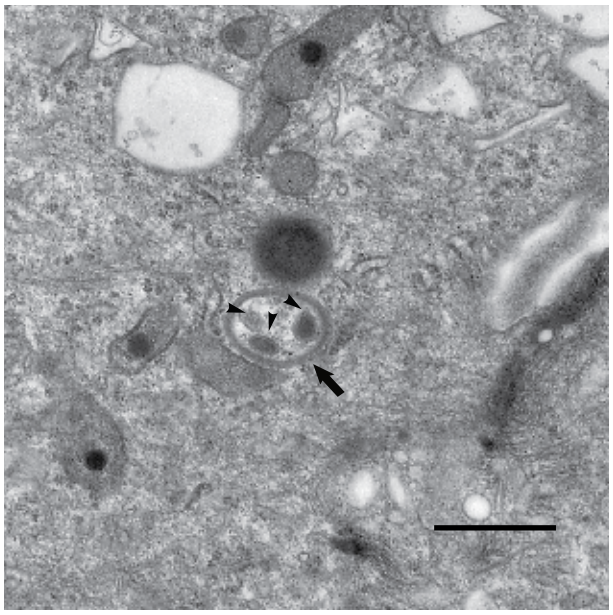

Figure 1: UPEC enclosed within a double-membraned autophagosomal structure. TEM of bladder tissue from ATG16L1HM mice 14 days post infection, depicting UPEC (arrowheads) enclosed in a double-membraned autophagosomal structure (arrow). Bar = 1 μm.

The Virgin group had previously demonstrated that ATG16L1^{HM} mice develop intestinal abnormalities in Paneth cells. Our work shows that ATG16L1 deficiency induces multiple, baseline abnormalities in cellular components that UPEC encounter during infection. ATG16L1-deficient cells dramatically accumulate multivesicular bodies, lysosomes and the UPEC receptor (UPK1A/UP1a). The aberrations are intrinsic to ATG16L1-deficient epithelial cells, because transferring ATG16L1-deficient hematopoietic cells does not induce them in wild-type epithelium, and even newly regenerating ATG16L1-deficient superficial cells show the same accumulations. Our study shows that UPEC are less able to occupy their intracellular niches to persist as QIRs in

the ATG16L1^{HM} epithelium. We propose, thus, that UPEC may normally utilize ATG16L1 and possibly other autophagy proteins to establish latency, thus ATG16L1 deficiency can be protective in this regard. In other words, UPEC may need the normal autophagic machinery to persist. The mechanisms underlying how UPEC avoid degradation or survive in the autophagosomal niches remain to be elucidated.

Autophagy plays multiple roles in both innate and adaptive immunity. Recent studies have suggested that autophagy governs the balance between defending against pathogens and modulating innate immunity to prevent excessive inflammatory responses and inflammasome signaling. Lee et al., demonstrated that ATG16L1 deficiency leads to a hyper-inflammatory response by removing the restriction on IL1B/IL-1β signaling cascades and IL-6 production. Similarly, Saitoh et al., showed that ATG16L1-deficient macrophages produce high amounts of the inflammatory cytokines IL1B and IL18. In our study, we observed significantly increased levels of IL-6 and IL1A/IL-1α in infected ATG16L1^{HM} mice relative to controls. Furthermore, our findings demonstrated that ATG16L1-deficient hematopoietic cells, especially neutrophils and macrophages, contribute critically to mounting a hypervigilant innate immune response, which likely promotes the rapid clearance of extracellular UPEC. Cadwell and Virgin previously demonstrated enhanced transcription of pro-inflammatory cytokines in aberrant Paneth cells of ATG16L1^{HM} mice. In the setting of CD and the presence of commensal bacteria, elevated proinflammatory cytokine levels induced by ATG16L1 deficiency lead to intestinal pathology, which is detrimental to the host. However, in the ATG16L1-deficient urinary tract, the elevated proinflammatory cytokine levels may have a beneficial effect, as UPEC has been shown previously to inhibit proinflammatory cytokine production. Thus, an ATG16L1 deficiency-induced hyperinflammatory response may help clear the infection by countering UPEC's ability to dampen innate immune responses.

It is important to determine if the protection we observed is ATG16L1-specific or represents a general effect of the autophagy pathway on UTI pathogenesis. The autophagy machinery comprises many essential proteins including ATG5, ATG7 and ATG12. We demonstrated that innate immune cell-

specific knockdown of *Atg5* induces a similar protective phenotype as that induced by ATG16L1 deficiency, suggesting that UPEC may hijack multiple autophagy components to colonize and persist in the urinary tract.

A polymorphism in *ATG16L1* associated with CD can be found in up to 50% of individuals in certain populations. It is unclear why a seemingly unfavorable allelic variant of *ATG16L1* would occur at such high frequency. One explanation is that the alleles were originally selected for a beneficial property such as protection against chronic or recurrent infectious diseases. Our results demonstrating that ATG16L1 mutation can confer protection against UTIs could explain why mutant alleles are unexpectedly frequent: protection against UTIs could be a mechanism to counter the negative selection imposed by the inflammation-promoting effects of the *ATG16L1* mutation. A better understanding of the link between autophagy, latency, and inflammation could lead to therapeutic approaches for example by targeting ATG16L1 or autophagy in general, to treat recurrent UTI. Thus, the use of inhibitors of autophagy or ATG16L1 to eliminate latent bacterial reservoirs may have substantial clinical benefits to combat refractory and recurrent UTIs, because conventional antibiotics are unable to penetrate urothelial barriers to clear bacteria sequestered as QIRs.

ACKNOWLEDGEMENTS:

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Chapter 4: ATG16L1 deficiency in macrophages drives clearance of Uropathogenic E. coli in an IL-1β dependent manner

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ATG16L1 deficiency in macrophages drives clearance of Uropathogenic E. coli in an IL-1β dependent manner

Symington JW, Wang C, Twentyman J, Owusu-Boaitey, Schwendener R, Nuñez G, Schilling JD, Mysorekar IU

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J.W.S performed all the experiments in this chapter unless otherwise stated.

ABSTRACT

Urinary Tract Infections (UTIs) are frequent, commonly recurrent, and costly. Deficiency in a key autophagy protein, ATG16L1, protects mice from infection with the predominant bacterial cause of UTIs, Uropathogenic *E. coli* (UPEC). Here, we report that loss of ATG16L1 in macrophages accounts for this protective phenotype. Compared with wild-type macrophages, macrophages deficient in ATG16L1 exhibit increased uptake of UPEC and enhanced secretion of interleukin-1β (IL-1β). The increased IL-1β production is dependent upon activation of the NLRP3 inflammasome and caspase-1. IL-1β secretion was also enhanced during UPEC infection of ATG16L1-deficient mice *in vivo*, and inhibition of IL-1β signaling abrogates the ATG16L1-dependent protection from UTIs. Our results argue that ATG16L1 normally suppresses a host-protective IL-1β response to UPEC by macrophages.

INTRODUCTION

Half of all women will suffer from a urinary tract infection (UTI) at some point in their lives. Moreover, in the US, UTIs account for 8 million visits to emergency departments and clinics every year and 15% of all outpatient antibiotic prescriptions(Dielubanza and Schaeffer, 2011). Despite effective clearance of the acute infection, as many as 10% of those who suffer from a UTI will go on to have recurrent infections that cannot be cured with antibiotics(Foxman, 2014). Thus, development of effective therapeutics will require an understanding of the ways in which bacteria infect the urinary tract and the defense mechanisms used by the host to combat the infection.

Uropathogenic *E. coli* (UPEC) is the causative agent in 75% of all UTIs(Foxman, 2014). Mouse models of UTI have revealed that clearance of UPEC from the bladder mucosa depends on the early recruitment of innate immune cells(Schilling et al., 2003; Song and Abraham, 2008). On the other hand, UPEC have evolved several mechanisms to actively subvert recruitment and response of host immune cells(Billips et al., 2007; Ulett et al., 2013). For example, UPEC suppress production of proinflammatory cytokines such as interleukin-6 (IL-6)(Hunstad et al., 2005) and attenuate the ability of neutrophils to migrate towards the sites of infection(Loughman and Hunstad, 2011).

However, the exact molecular mechanisms underlying the recruitment and functionality of innate immune cells, especially those of the monocyte/macrophages lineage, in response to UPEC infection remains unclear.

Host mucosal defense against pathogens requires coordination of multiple signaling pathways within innate immune cells. One such pathway is autophagy, a cellular recycling pathway responsible for lysosomal degradation of cytosolic components, including damaged organelles, protein aggregates and intracellular pathogens(Levine et al., 2011; Parzych and Klionsky, 2014). The autophagy pathway in general, and the autophagy gene/protein ATG16L1 in particular, has an important role in innate immune responses to infection(Deretic et al., 2013). A common polymorphism in *ATG16L1* (T300A) remains prevalent within the Caucasian population and is associated with Crohn's disease(Hampe et al., 2007; Rioux et al., 2007), a form of inflammatory bowel disease. An open question remains as how this 'bad' risk allele is prevalent in the population.

Recent studies have revealed that autophagy and ATG16L1 can dampen activation of the innate immune response to infection as well as activation of inflammasomes(Deretic et al., 2013; Levine et al., 2011), key signaling complexes that detect pathogenic microorganisms and which in turn activate the highly pro-inflammatory cytokine interleukin-1β (IL-1β)(Broz and Monack, 2011). In particular, ATG16L1 and autophagy have been suggested to have a role in the production of IL-1β(Harris et al., 2011; Saitoh et al., 2008) as well as another pro-inflammatory cytokine tumor necrosis factor alpha (TNFα)(Lapaquette et al., 2012; Sorbara et al., 2013). However, the mechanisms by which autophagy and ATG16L1 regulates the inflammasome in the context of infection are still uncertain.

We recently showed that mice deficient in ATG16L1 (Atg16L1^{HM} (Cadwell et al., 2008; Cadwell et al., 2010), carrying a hypomorphic allele that reduces *Atg16l1* expression) cleared UPEC infection more rapidly and thoroughly than controls(Wang et al., 2012). We further demonstrated that ATG16L1 deficiency in the hematopoietic compartment was the primary driver of increased clearance of UPEC, although the adaptive immune compartment did not contribute to the protective

phenotype(Wang et al., 2012). These findings argue that loss of ATG16L1 in the innate immune system is responsible for resistance to UTIs, yet the underlying mechanism for this resistance remains to be examined.

In this report, we demonstrate that macrophages are essential for the control and clearance of UPEC from the bladders of ATG16L1-deficient mice. Mechanistic studies using primary macrophages revealed that loss of ATG16L1 increases bacterial uptake and enhances release of the cytokine IL-1β, but not TNFα, in response to UPEC. The increased IL-1β production is independent of NOD2 or gross lysosomal damage, but is dependent on caspase-1 and the NLRP3 inflammasome. Finally, we show that augmented IL-1β signaling is the primary mechanism responsible for enhanced clearance of UPEC from the urinary tract in ATG16L1-deficient mice. Together, our findings show that ATG16L1 deficiency makes macrophages better able to control UPEC infection *in vivo* during a UTI by regulating levels of IL-1β. Our findings have implications for elucidating how UPEC is able to evade host innate defenses to cause a UTI and suggest that polymorphisms in ATG16L1 may be maintained in the population because of protective effects from a common infection.

RESULTS

Macrophages are required for UPEC clearance in Atg16L1^{HM} mice.

We previously found that monocyte recruitment was enhanced in the bladders of infected ATG16L1-deficient (Atg16L1^{HM}) mice compared with those of infected wild-type (WT) animals(Wang et al., 2012). Furthermore, Atg16L1^{HM} mice cleared their UTIs more rapidly than WT mice. To determine whether monocytes and macrophages were necessary for this effect, we depleted monocytes and macrophages from Atg16L1^{HM} mice by treating them with clodronate-containing liposomes (clodrolip)(Schilling et al., 2012; Zeisberger et al., 2006) followed by transurethral infection with a well-characterized clinical cystitis strain of UPEC (UTI89). Clodrolip treatment decreased the number of systemic macrophages and monocytes in the spleens of Atg16L1^{HM} mice by greater than 60% (Supplementary Figure 1), reduced the number of macrophages in the bladder mucosa after infection (Supplementary Figure 2), and significantly attenuated their enhanced ability to clear the

infection (Figure 1a and b). Furthermore, clodrolip treatment resulted in higher urine and bladder titers at 3 days post infection in Atg16L1^{HM} mice relative to WT mice (Figure 1a and b). Clodrolip treatment can also deplete CD11c+ dendritic cells; however, it has been previously shown that dendritic cells are dispensable for UPEC pathogenesis(Engel et al., 2006). Thus, we argue that macrophages or monocytes are essential for enhanced clearance of UTI89 in Atg16L1^{HM} mice.

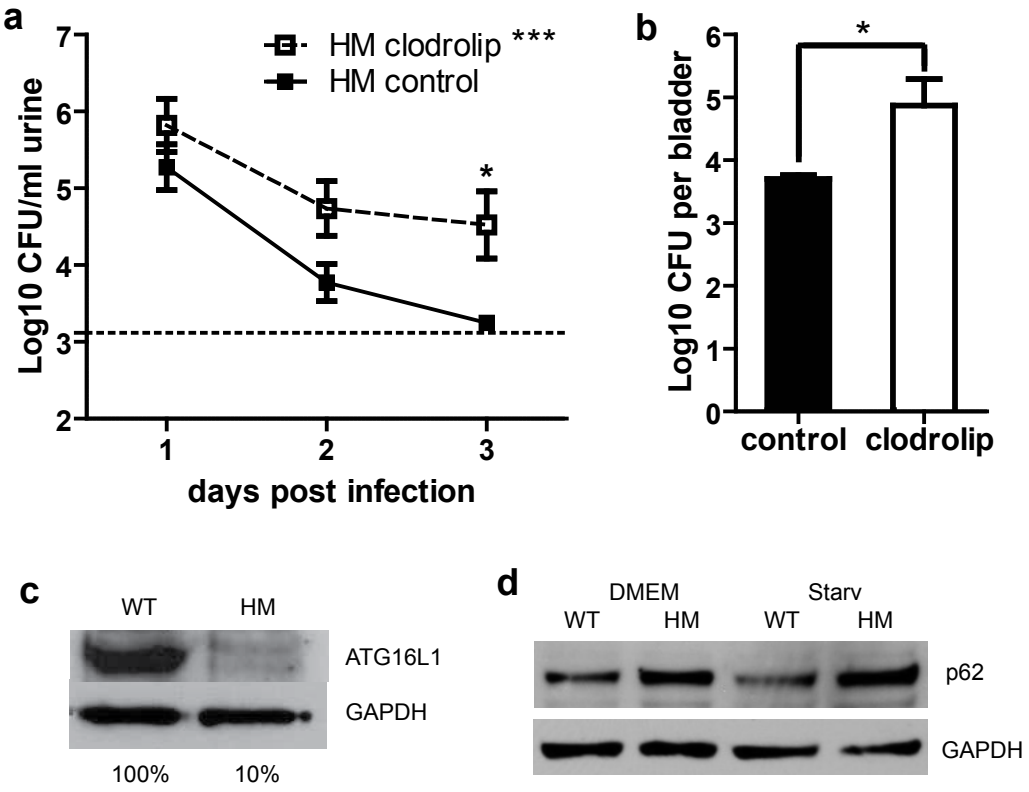


Figure 1. Macrophages and monocytes are essential for bacterial control in Atg16L1^{HM} mice. (a,b) Colony forming unit (CFU) counts of bacteriuria (a) or 3dpi bladder homogenates (b) from Atg16L1^{HM} mice treated with clodrolip or control liposomes as displayed as mean Log^{10}CFU/ml and SEM. For a, n=3 independent experiments, for a total of 12 clodrolip-treated mice and 13 control liposome-treated mice. For b, n=2 independent experiments, for a total of 9 clodrolip-treated mice and 9 control liposome-treated mice. Two-way ANOVA with matching by animal, ***P<0.001, with bonferroni post-test at individual time points (a), and unpaired T-test (b), * P<0.05. (c) ATG16L1 levels in BMDMs from WT and Atg16L1^{HM} mice as measured by western blot. (d) Western blot of p62 in samples of WT and ATG16L1-deficient BMDMs. Starvation (Starv) was used to induce autophagy. GAPDH, glyceraldehyde 3-phosphate dehydrogenase, used as a loading control in (c) and (d). Data are representative of 3 independent experiments (c and d).

Altered UTI89 uptake and processing by ATG16L1-deficient macrophages.

Macrophages have direct bactericidal functions and also secrete pro-inflammatory cytokines to orchestrate the host response to pathogens. Given the evidence that ATG16L1 deficiency in macrophages was crucial for the observed enhanced clearing of UTI89 *in vivo*, we sought to elucidate the molecular mechanisms underlying how ATG16L1 deficiency altered the response of macrophages to UTI89. To address this question, we isolated bone marrow-derived macrophages (BMDMs) from WT and Atg16L1^{HM} mice and challenged them with UTI89. As expected, BMDMs from Atg16L1^{HM} mice displayed a 90% reduction of ATG16L1 protein (Figure 1c). Consistent with a reduced rate of autophagic flux, they also accumulated the adaptor protein p62 (Figure 1d).

To assess the direct affects of ATG16L1 deficiency on bacterial clearance, we determined intracellular bacterial load at multiple time points postinfection and found that Atg16L1^{HM} BMDMs contained significantly more bacteria than WT BMDMs at 1 hour post infection (hpi) (Figure 2a). Likewise, immunofluorescence staining showed that more Atg16L1^{HM} BMDMs contained intracellular bacteria than WT macrophages (Figure 2b; Supplementary Figure 3a). To determine whether ATG16L1 deficiency affects degradation of intracellular bacteria, we quantified the intracellular bacterial load in BMDMs over time as compared with levels at 2 hpi. We observed a similar pattern of decrease in bacterial counts between WT and ATG16L1-deficient macrophages, arguing that the dynamics of bacterial killing over time were not altered by ATG16L1 deficiency (Figure 2c). Together, these findings indicate that ATG16L1 deficiency enhances killing of UTI89 by increasing bacterial uptake at early stages of infection.

To examine whether ATG16L1 deficiency affected intracellular trafficking of UTI89, we performed immunofluorescence and observed co-localization of UPEC with the lysosomal marker LAMP1 in both WT and ATG16L1-deficient macrophages (Supplementary Figure 3b). To further evaluate the intracellular localization of UTI89 within macrophages, we used transmission electron microscopy (TEM). At 1 and 3 hpi, consistent with the immunofluorescence results, UTI89 was consistently found in single-membrane compartments in both WT and ATG16L1-deficient macrophages (Figure 2d). We counted the numbers of bacteria in electron-lucent or highly electron-dense compartments.

At 1 hpi, the majority of bacteria appeared to be in highly electron-dense lysosomal compartments in both WT and ATG16L1-deficient macrophages (Figure 2e). However, by 3 hpi, whereas fewer bacteria were within highly electron-dense compartments in WT macrophages, the majority of bacteria still remained in these compartments in ATG16L1-deficient macrophages (Figure 2e). This suggests that UTI89 alters its phagocytic compartment in macrophages over time but does so more slowly in ATG16L1-deficient cells. Thus, ATG16L1-deficient macrophages may sense and respond to intracellular UTI89 differently than WT macrophages do.

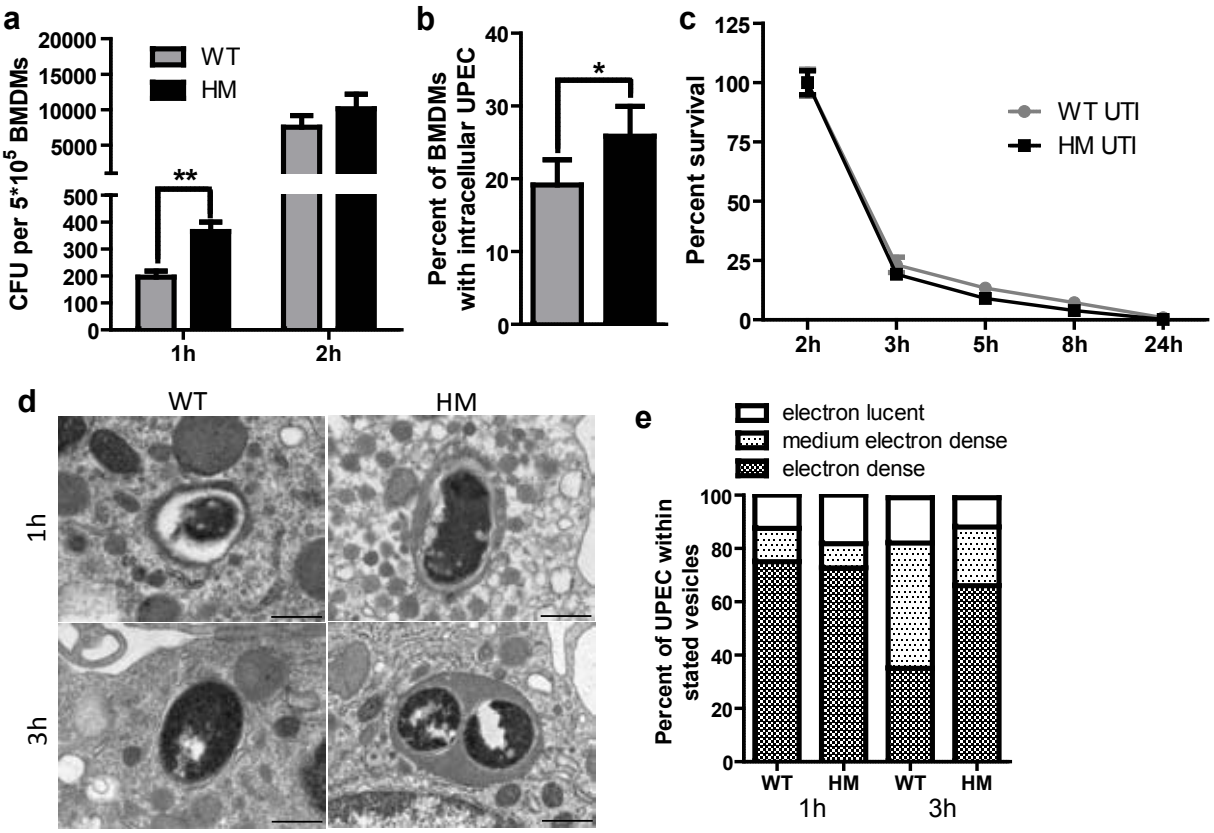


Figure 2. ATG16L1-deficient macrophages take up more bacteria than WT macrophages do. (a) Intracellular bacterial load of WT and ATG16L1-deficient macrophages at different times after UTI89 challenge. (b) Percent of macrophages that contained intracellular bacteria by immunoflourescence. (c) Intracellular bacterial survival displayed as percentage of intracellular CFU load at 2 hr. (d) TEM images of UTI89 within single-membrane vacuoles of different electron density at different time points (Scale bars = 500 nm), and (e) quantification of the distribution of vacuole types within which UPEC was found in WT and ATG16L1-deficient macrophages. $^{**}P<0.01$, $^{*}P<0.05$. Paired t-test (b) or two-way ANOVA with bonferroni post-tests (a and c). Data are from 3 independent experiments, with 3 replicates per experiment (a and c) or 4 fields per experiment (b) presented as mean and SEM.

To determine whether the increased uptake by ATG16L1-deficient macrophages was specific to a UPEC strain or would be elicited by any intracellular *E. coli*, we challenged the macrophages with a commensal *E. coli* (MG1655)(Blattner et al., 1997). At 1 hpi, macrophages of both genotypes took up the commensal bacteria more efficiently than UTI89 (compare Figure 3a to Figure 2a). However, no significant differences in uptake (Figure 3a) or survival rates of MG1655 (Figure 3b) were observed between WT and ATG16L1-deficient macrophages. TEM analysis revealed that, at 1 and 3 hpi, MG1655 was in single-membrane compartments, the majority of which were not highly electron dense (Figure 3c). Moreover, the distribution of types of compartments containing

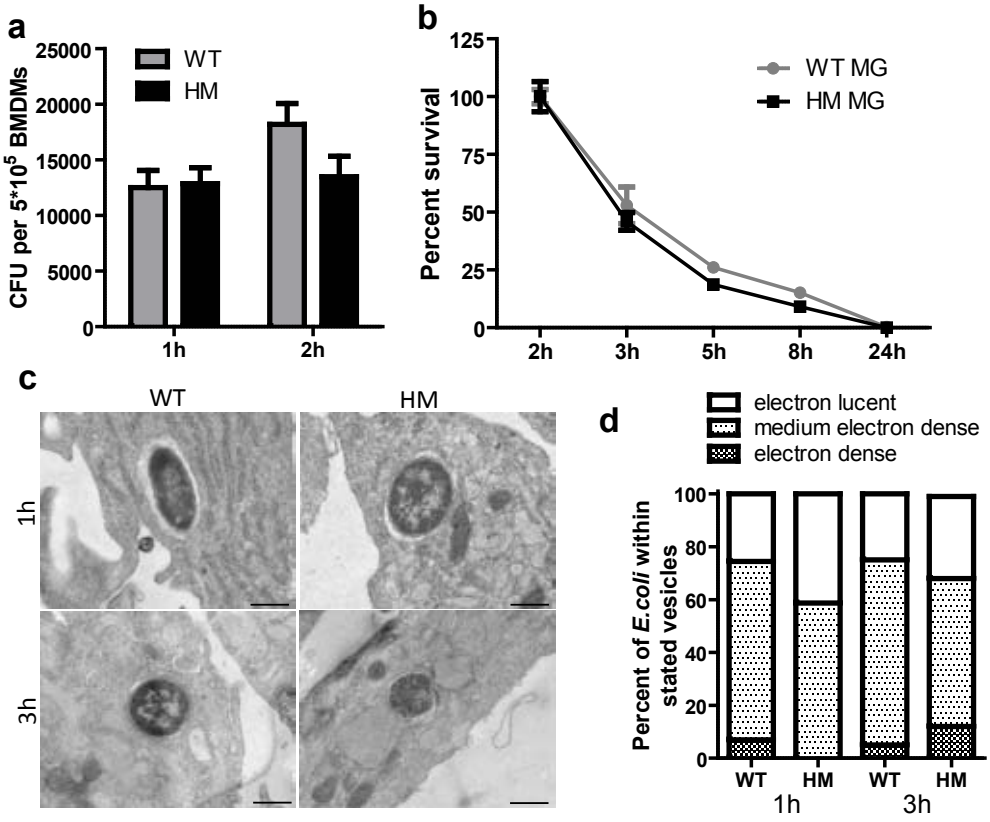


Figure 3. ATG16L1-deficient macrophages take up MG1655, an avirulent *E.coli* strain differently than the uropathogenic *E.coli* strain. (a) Intracellular bacterial load of WT and ATG16L1-deficient macrophages at 1 and 2 hours after MG1655 challenge. (b) Intracellular bacterial survival displayed as percentage of intracellular CFU load at 2 hours. (c) TEM images of MG1655 within single-membrane vesicles of different electron density at different time points, and (d) quantification of the distribution of vesicle types within which MG1655 was found in WT and ATG16L1-deficient macrophages. Not significant by two-way ANOVA with bonferroni post-tests (a and b). Data are from 3 independent experiments, with 3 replicates per experiment (a and b) presented as mean and SEM.

MG1655 did not differ between WT and ATG16L1-deficient macrophages (Figure 3d). We conclude that loss of ATG16L1 affects intracellular trafficking of UPEC but not of a commensal strain of *E. coli*.

ATG16L1-deficient macrophages secrete more IL-1β in response to UPEC challenge

Given the important link between autophagy and inflammation, we reasoned that the cytokine response elicited by UTI89 may be altered in macrophages deficient in ATG16L1. To test this hypothesis, we challenged WT and ATG16L1-deficient macrophages with UTI89, MG1655, or lipopolysaccharide (LPS). In response to these stimuli, WT and ATG16L1-deficient macrophages secreted similar amounts of TNFα at 24 hpi (Figure 4a). In contrast, IL-1β release was significantly higher in UPEC-infected ATG16L1-deficient macrophages compared with control macrophages at 24 hpi (Figure 4b) and this response could be detected as early as 8 hpi (Figure 4c). No IL-1β was detected in WT and ATG16L1-deficient macrophages at 2 hpi suggesting that increased IL-1β did not directly enhance UPEC uptake (Supplementary Figure 4a). Interestingly, IL-1β release only occurred

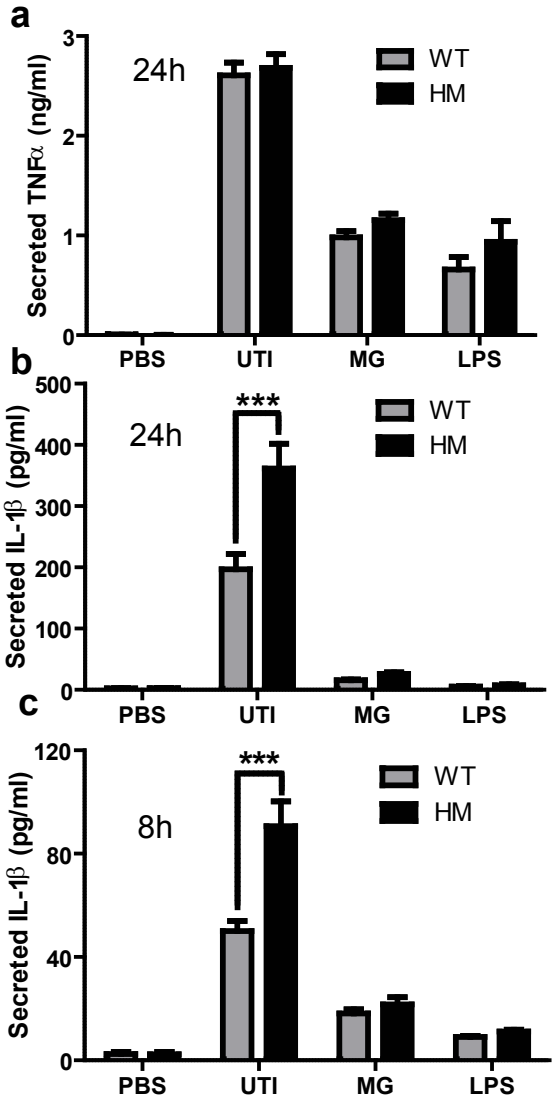


Figure 4. ATG16L1-deficient macrophages produce more IL-1β than WT macrophages: Levels of (a) TNFα and (b) IL-1β in the supernatants of WT and ATG16L1-deficient cells 24 hours after challenge with UTI89, MG1655, or LPS. (c) Levels of IL-1β in the supernatant after 8 hours of challenge with UTI89, MG1655 or LPS as determined by ELISA. ****P*<0.001. Two-way ANOVA with bonferroni post-tests. Data are from three (a and c) and four (b) independent experiments presented as mean and SEM.

following infection with the UPEC strains (UTI89 and CFT073, (Lloyd et al., 2007) a pyelonephritis strain, Supplementary Figure 4b), not in response to commensal *E. coli* or LPS (Figure 4b and c). Further, dose dependent increases in IL-1β production were observed in WT macrophages treated with varying concentrations of 3-MA, an autophagy inhibitor that targets VPS34, suggesting that the increased IL-1β production by ATG16L1-deficient macrophages in response to UTI89 may be the result of their autophagy deficiency (Supplementary Figure 5).

Increased IL-1β production by ATG16L1-deficient macrophages is independent of NOD2 activity and enhanced transcription of pro-IL-1β

IL-1β release is tightly controlled through the combination of two distinct triggers. First, activation of Toll-like receptors (TLRs) or Nod-like receptors (NLRs), such as by LPS, induces transcription of pro-IL-1β(Franchi et al., 2012; Latz et al., 2013). In a second step, pro-IL-1β protein is cleaved by caspase-1, a process that is regulated by inflammasomes, which are defined by their NLR component, which can include AIM2, NLRC4, NLRP1, and NLRP3(Latz et al., 2013). Thus, we wanted to know whether ATG16L1 deficiency affected pro-IL-1β generation (signal 1) or assembly of the inflammasome complex (signal 2).

No significant differences were observed in level of pro-IL-1β mRNA (Figure 5a) or protein (Figure 5b) between ATG16L1-deficient and WT macrophages challenged with UTI89, even though increased cleaved IL-1β was observed in the supernatants by western blot. Additionally, caspase-11 and NLRP3 protein levels increased upon infection, but there was no significant difference between WT and ATG16L1-deficient macrophages (Supplementary Figure 6). Furthermore, when we pretreated cells with LPS to activate transcription of pro-IL-1β before activation by UTI89, ATG16L1-deficient macrophages still produced more IL-1β in response to UTI89 than WT cells did (Figure 5c). These findings argue enhanced IL-1β release in ATG16L1-deficient macrophages does not occur via modulation of signal 1.

Recent studies have suggested that ATG16L1 is important for controlling secretion of IL-1β and TNFα in response to agonists of NOD2, an NLR that recognizes cytosolic bacterial

components(Carneiro and Travassos, 2013; Sorbara et al., 2013; Travassos et al., 2010). We thus asked whether NOD2 was required for the increased IL-1β secretion of UTI89-challenged ATG16L1-deficient macrophages. We found that macrophages from Nod2$^{-/-}$ and WT mice produced equivalent levels of IL-1β (Figure 5d). Likewise, upon challenge with UTI89, macrophages from Nod2$^{-/-}$; Atg16L1^{HM} mice produced similar amounts of IL-1β as macrophages from Atg16L1^{HM} mice (Figure 5d), suggesting that NOD2 does not contribute to ATG16L1-deficiency-mediated increased secretion of IL-1β upon UPEC infection.

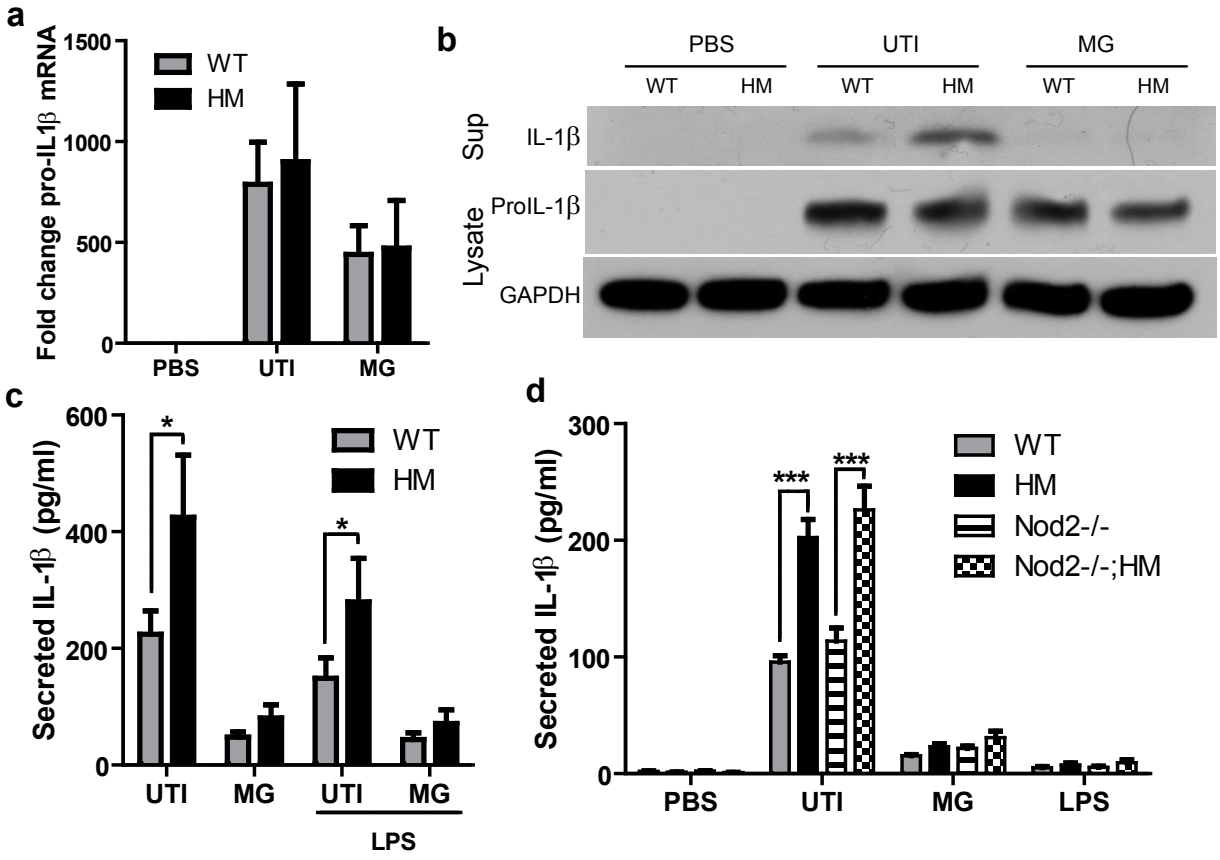


Figure 5. Increased IL-1β secretion by ATG16L1-deficient macrophages is not due to enhanced signal 1 or NOD2. (a) Levels of pro-IL1β mRNA determined by quantitative PCR from macrophages at baseline or after 3 hours of challenge with UTI89 or MG1655. (b) Representative western blot of cleaved IL-1β in the supernatant at 24 h and pro-IL-1β and GAPDH levels at 3 h from macrophages treated with PBS or challenged with UTI89 or MG1655. (c) IL-1β level in the 24 h supernatant of BMDMs pretreated with LPS or PBS for 4 h before challenge with UTI89 or MG1655. (d) Levels of IL-1β in the supernatants of BMDMs from WT, ATG16L1^{HM}, Nod2$^{-/-}$, and Nod2$^{-/-}$; Atg16L1^{HM} mice 24 h after challenge with UTI89. ***P<0.001. Paired t-test (c) or two-way ANOVA with bonferroni post-tests (a and d). Data are from two (c) or three (a and d) independent experiments with three replicates per experiment presented as mean and SEM.

UTI89 activates NLRP3 inflammasomes in macrophages

As our findings suggested that ATG16L1 deficiency likely impacts signal 2, we sought to define that mechanism by which UTI89 activates the inflammasome. Importantly, inflammasome activation in macrophages in response to UPEC had not been previously examined. Thus, we first confirmed that UTI89-induced IL-1β production was dependent on TLR4 activation; as expected, BMDMs from $TLR4^{-/-}$ mice produced significantly decreased levels of IL-1β (Figure 6a). Next, we determined whether both caspase-1 and caspase-11 were essential for IL-1β secretion in response to UTI89. BMDMs isolated from caspase-1/caspas-11 double knockouts had minimal secretion of IL-1β in response to UTI89 and MG1655, but caspase-11 deficient mice had no defect in IL-1β secretion in response to UTI89 (Figure 6b). This suggests that UTI89 activates the canonical inflammasome rather than the non-canonical caspase-11 activated inflammasome (Kayagaki et al., 2011). To further define the key inflammasome proteins activated upon UPEC challenge, we performed q-RTPCR analysis of genes encoding two inflammasome proteins that sense and respond to bacteria (NLRC4 and NLRP3) (Franchi et al., 2012; Miao et al., 2010b; Sauer et al., 2011) and found that whereas Nlrc4 transcription was minimally induced, Nlrp3 transcription was highly upregulated in response to UTI89 infection of both WT and ATG16L1-deficient macrophages (Figure 6c). We then examined IL-1β production by macrophages from mice lacking the inflammasome proteins NLRP3, NLRC4, or AIM2. Macrophages from $Nlrp3^{-/-}$ mice secreted significantly less IL-1β than WT macrophages (Figure 6d). In contrast, macrophages from $Nlrc4^{-/-}$ and $Aim2^{-/-}$ mice produced roughly the same amount of IL-1β as WT macrophages. Together, these findings demonstrate that most IL-1β secretion in response to UTI89 requires TLR4 and NLRP3 inflammasome activation.

In response to infection, macrophages can kill intracellular pathogens by undergoing a form of cell death called pyroptosis, which is associated with IL-1β release and caspase-1 activation(Miao et al., 2010a). It is not known if UPEC induce pyroptosis in macrophages. Thus, we investigated the effect of ATG16L1 deficiency on pyroptosis in response to UTI89 infection by using the CytoTox 96 assay, which measures the levels of release of the cytoplasmic enzyme lactate dehydrogenase

into the supernatant. We detected no difference in total cell death between WT and ATG16L1-deficient macrophages challenged with UTI89 or MG1655 (Figure 6e), suggesting that pyroptosis is not a mechanism of fast clearance of UTI89 in Atg16L1^{HM} mice.

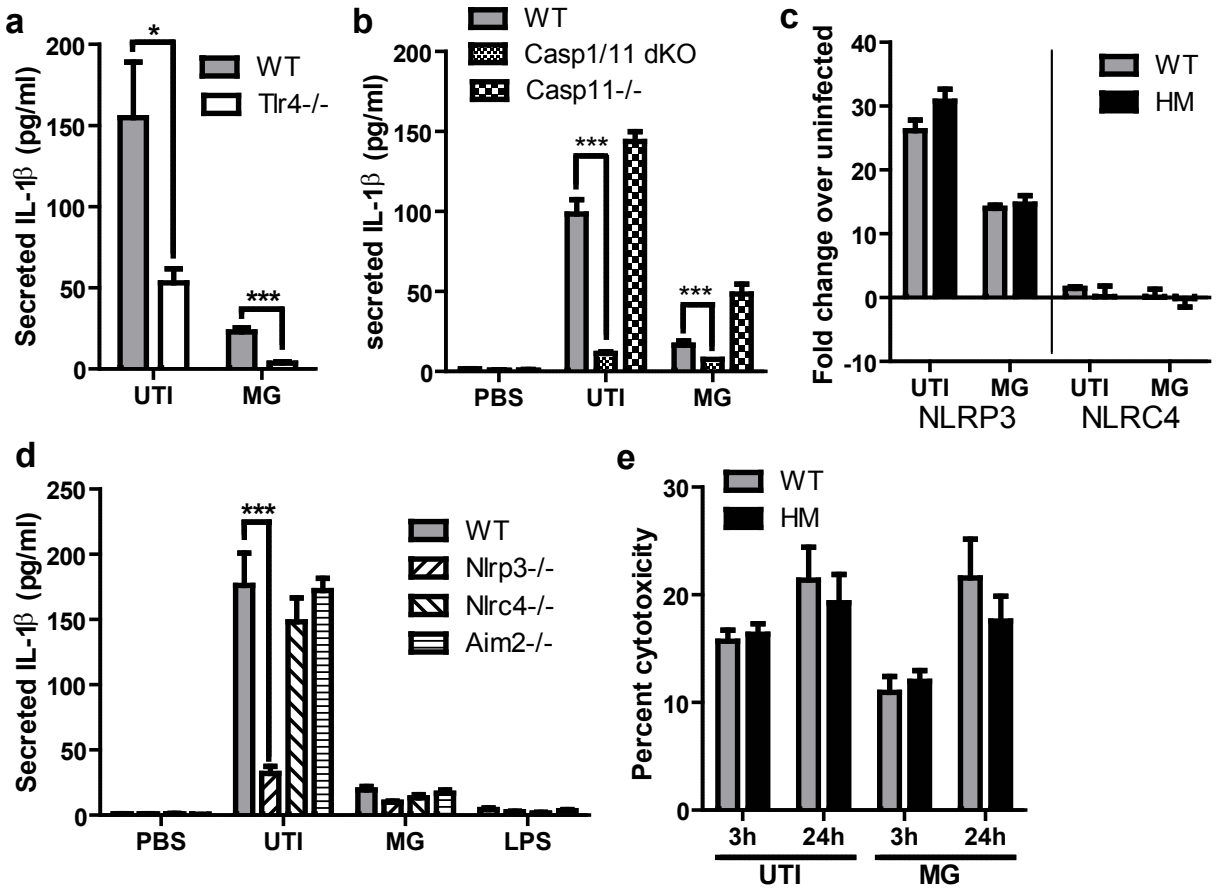
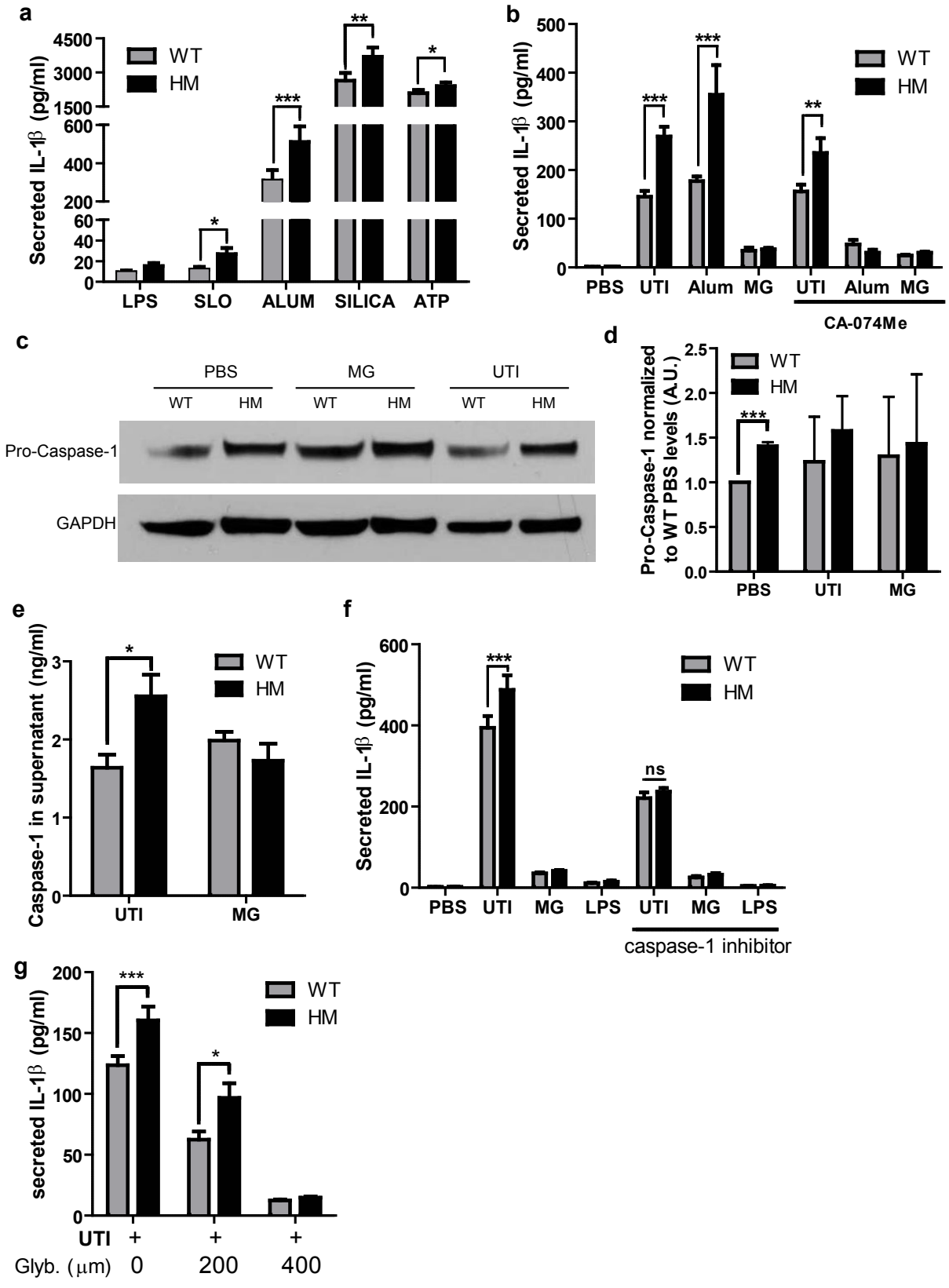


Figure 6. TLR4 and NLRP3 activation by UPEC is important for IL -1β production. (a) Amount of IL-1β secreted by WT and Tlr4$^{-/-}$ macrophages in response to UTI89 and MG1655. (b) Amount of IL-1β secreted by WT, caspase1/11 double knockouts, or caspase 11$^{-/-}$ macrophages in response to UTI89 and MG1655. (c) Levels of Nlrp3 and Nlrc4 mRNA (determined by quantitative PCR) in macrophages after challenge with UTI89 or MG1655 for 3 h, presented relative to the 0 h timepoint. (d) Secreted IL-1β detected by ELISA from macrophages from WT, Nlrp3$^{-/-}$, Nlrc4$^{-/-}$, and Aim2$^{-/-}$ mice in response to UTI89, MG1655, and LPS challenge. (e) Measurement of cytotoxicity as LDH release by BMDM exposed to UTI89 for 3 or 24 h (2 hour exposure to extracellular bacteria followed by incubation for 22 hours in gentamicin) as a percentage of total LDH release upon Triton-X treatment. * P <0.05, *** P <0.001. Two-way ANOVA and bonferroni post-test at for each pair. Data are from 2 (a,b, and d) or 3 (c and e) independent experiments with 3 individual replicates per experiment.

ATG16L1 deficiency enhances IL-1β secretion in response to UTI89 and other NLRP3 activators due to increased caspase-1 activation

Given that pro-IL-1β levels were similar between WT and ATG16L1-deficient macrophages, we hypothesized that the increased IL-1β secretion by ATG16L1-deficient macrophages was due to increased activation of the NLRP3 inflammasome and caspase-1. To determine whether ATG16L1 deficiency enhanced NLRP3 activation, we treated WT and ATG16L1-deficient macrophages with four known NLRP3 activators: Streptolysin-O, ATP, Alum, and Silica(Harder et al., 2009; Hornung et al., 2008). Each caused secretion of IL-1β by both types of macrophages, but in each case, ATG16L1-deficient macrophages produced more IL-1β than WT macrophages did (Figure 7a). Given that lysosome damage can trigger NLRP3 activation(Davis et al., 2011; Latz et al., 2013; Weber and Schilling, 2014) in conjunction with our data indicating that UPEC traffics to lysosomes in macrophages, we reasoned that UPEC might activate the NLRP3 inflammasome by impairing lysosome integrity and inducing release of cathepsin B and other vacuolar contents into the cytosol. To test this, we treated UTI89 infected WT and ATG16L1-deficient macrophages with the cathepsin B inhibitor CAO74-Me (Weber and Schilling, 2014). As expected, CAO74-Me decreased the secretion of IL-1β in response to Alum, a known inducer of lysosome damage(Hornung et al., 2008). In contrast, the release of IL-1β in response to UPEC was unaffected by cathepsin B inhibition (Figure 7b). Thus, inflammasome activation by UTI89 occurs without major lysosomal injury and cathepsin B release. This is consistent with our TEM observation that UTI89 was found within intact vacuoles in both WT and ATG16L1-deficient macrophages. These findings suggest that activation of the NLRP3 inflammasome occurs independently of gross lysosomal damage by UTI89.

We then determined the effect of ATG16L1 deficiency on caspase-1 activation. The levels of pro-caspase-1 in uninfected ATG16L1-deficient macrophages were higher than those observed in WT macrophages (Figure 7c and d). Upon challenge with UPEC, ATG16L1-deficient macrophages released more caspase-1 into the supernatant than WT macrophages, consistent with enhanced


a
Secreted IL-1β (pg/ml)
WT
HM
LPS
SLO
ALUM
SILICA
ATP
b
Secreted IL-1β (pg/ml)
PBS
UTI
Alum
MG
CA-074Me
c
PBS
MG
UTI
Pro-Caspase-1
GAPDH
d
Pro-Caspase-1 normalized to WT PBS levels (A.U.)
e
Caspase-1 in supernatant (ng/ml)
f
Secreted IL-1β (pg/ml)
ns
caspase-1 inhibitor
g
secreted IL-1β (pg/ml)
Glyb. (μm)

activation of this protease (Figure 7e; Supplementary Figure 6). To determine whether the difference observed in IL-1β secretion by WT and ATG16L1-deficient macrophages was due to changes in caspase-1 activity, we applied a caspase-1 inhibitor during infection. Caspase-1 inhibition, using 10μM Z-WEHD-FMK, eliminated the difference in secretion of IL-1β between WT and ATG16L1-deficient BMDMs (Figure 7f). Additionally, inhibiting NLRP3 with glyburide was able to inhibit IL-1β production in both WT and ATG16L1-deficient BMDMs in a dose-dependent manner (Figure 7g). We conclude that NLRP3 and caspase-1 activation is enhanced by ATG16L1 deficiency, and this pathway mediates the enhanced release of IL-1β by ATG16L1 deficient macrophages in response to UTI89 challenge.

Blocking IL-1 signaling in Atg16L1^{HM} mice reduces clearance of UPEC during a UTI

Our data suggest that Atg16L1^{HM} mice are better able to clear a UPEC infection than WT mice for two reasons. First, as previously shown, more macrophages are recruited to the bladder mucosa(Wang et al., 2012). Second, ATG16L1-deficient macrophages produce more IL-1β *ex vivo* in response to UPEC challenge due to increased caspase-1 activation (Figure 4b). In agreement with our mechanistic *ex vivo* studies, we found significantly more IL-1β in urines from Atg16L1^{HM} mice than in urines from WT mice at 6 hpi (Figure 8a). To determine the physiological importance

Figure 7. ATG16L1-deficient macrophages produce more IL-1β due to increased NLRP3 and caspase-1 activity. (a) Levels of IL-1β in the supernatants of WT and ATG16L1-deficient BMDMs after pretreatment with LPS followed by exposure to Streptolysin O (with 10 mM DTT), Alum, Silica, or ATP. (b) Levels of IL-1β in the supernatants of WT and ATG16L1-deficient BMDMs challenged with UTI89 for 24 h or the NLRP3 activator Alum for 8 h with or without the Cathepsin B inhibitor CA-074Me. (c) Representative western blot of pro-caspase-1 protein levels normalized to GAPDH values from BMDMs at baseline and after 3 h of UTI89 challenge, quantification (d) displayed normalized to baseline WT levels. A.U., arbitrary units. (e) Level of caspase-1 in the supernatants of BMDMs challenged with UTI89 for 24 h. (f) Level of IL-1β in the supernatants of BMDMs challenged with UTI89 for 24 h with or without a caspase-1 inhibitor during the entire experiment. (g) Level of IL-1β in the supernatants of BMDMs challenged with UTI89 for 24 h with increasing concentrations of glyburide, an NLRP3 inhibitor. *P<0.05, **P<0.01, ***P<0.001. Paired t-test (a) or two-way ANOVA and bonferroni post-test for each condition (b, d-g). Data are from 3 independent experiments with 3 replicates per experiment (a-b, e-g) or 1 replicate per experiment (d) presented as mean with SEM.

of IL-1β *in vivo*, we treated both WT and Atg16L1^{HM} mice with Anakinra (a recombinant form of the endogenous IL-1 receptor antagonist, IL-1Ra, which blocks both IL-1α and IL-1β signaling through IL-1 receptor (IL-1R)) to inhibit IL-1 signaling during infection(de Luca et al., 2014).

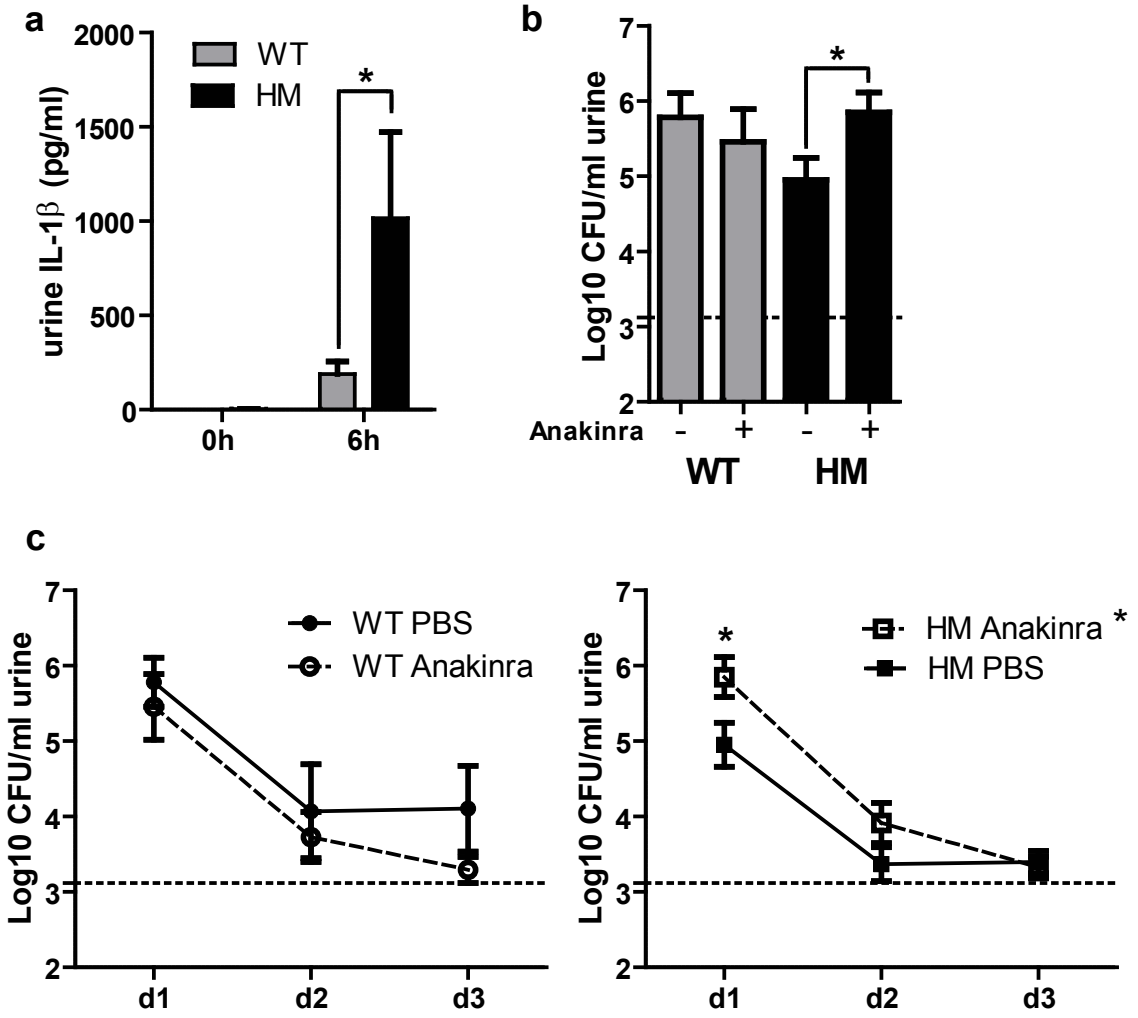


Figure 8. IL-1β production is enhanced in Atg16L1^{HM} mice and is important for faster clearance of UPEC. (a) Urine levels of IL-1β at baseline and 6 hours post infection as measured by ELISA (b) CFU counts of day one bacteriuria from WT and Atg16L1^{HM} mice given intraperitoneal (IP) PBS or Anakinra. (c) Bacteriuria over time from WT (left) or Atg16L1^{HM} (right) mice treated with Anakinra or PBS displayed as mean Log^{10}CFU/ml. *P<0.05, Two-way ANOVA with matching by animal and bonferroni post-test at each time point (a and c) and Mann Whitney test (b). Data are from (a) two independent experiments with a total of 7 WT and 6 Atg16L1^{HM} mice examined, and from (b,c) four independent experiments with 13 PBS treated Atg16L1^{HM} mice, 11 Anakinra treated Atg16L1^{HM} mice, 8 PBS treated WT mice, and 7 Anakinra treated WT mice, all are presented as mean with SEM.

Anakinra reduced bacterial clearance in Atg16L1^{HM} mice compared to vehicle treated controls at 1dpi, whereas bacteriuric titers were not significantly affected in WT mice given vehicle (phosphate-buffered saline, PBS) or Anakinra at 1dpi (Figure 8b). In fact, the infection phenotype of Anakinra-treated Atg16L1^{HM} mice was similar to WT animals. In addition, Anakinra-treated Atg16L1^{HM} mice had a significantly worse course of infection than vehicle-treated Atg16L1^{HM} mice (Figure 8c, right), whereas anakinra treatment did not significantly change the course of infection in WT animals (Figure 8c, left). Thus, our data argue that in the context of WT macrophages, subverting IL-1β production is not a part of the UPEC arsenal of host defense evasion. However, our results demonstrate that the augmented IL-1β release from macrophages in Atg16L1^{HM} mice is the primary driver of enhanced bacterial clearance in those mice.

DISCUSSION

Autophagy and its proteins are important for combating intracellular pathogens and have generally been considered to be anti-pathogenic(Deretic et al., 2013). However, emerging evidence suggests that autophagy proteins can also be pro-pathogenic(Marchiando et al., 2013; Starr et al., 2012; Wang et al., 2012). In previous work, we found that ATG16L1-deficient mice display reduced levels of bacteriuria and bladder titers early in infection(Wang et al., 2012). Our work suggested that hematopoeitic cells were important for ATG16L1 deficiency-mediated protection, but did not reveal the mechanism by which this occurred.

In this report, we showed that ATG16L1 deficiency-mediated protection from UTIs is dependent on the macrophage/monocyte lineage *in vivo*. ATG16L1 deficiency in the macrophage/monocyte lineage promoted a more robust IL-1β response to UPEC challenge, and blockade of IL-1 signaling *in vivo* abolished the protective phenotype mediated by ATG16L1 deficiency. Additionally, our findings show for the first time that the NLRP3 inflammasome is the principal inflammasome activated by UPEC during a UTI, with minimal IL-1β production as a result of activation of other inflammasomes including, NLRC4 or AIM2. Furthermore, our work has revealed that the IL-1β response mediated by NLRP3 was dependent on caspase-1 activation. Altogether, our results

indicate that loss of ATG16L1 promotes a beneficial innate immune response during a UTI.

UTIs are not the only type of infection in which loss of ATG16L1 can be beneficial. Deficiency of this gene was recently shown to provide significant protection from *Citrobacter rodentium* infection of the intestine(Marchiando et al., 2013). Moreover, ATG16L1-deficient macrophages and monocytes were found to be essential for the protection against both infections in these distinct mucosae. However, the underlying mechanisms differ in two ways. First, whereas an ATG16L1-deficient hematopoietic compartment is the main driver for the protective phenotype in UTI(Wang et al., 2012), deficiency in the non-hematopoetic compartment also contributes to reduced *Citrobacter* titers and increased survival(Marchiando et al., 2013). Second, although NOD2 has an essential role in ATG16L1 deficiency-induced resistance to *Citrobacter*, we found that NOD2 was dispensable for ATG16L1 deficiency-induced resistance to UPEC(Wang et al., 2014) and now show that NOD2 does not affect ATG16L1 deficiency-induced enhanced IL-1β secretion by BMDMs in response to UPEC. These differences suggest that ATG16L1 plays distinct roles in the pathogenesis of different infections.

Nonetheless, these studies indicate that ATG16L1 deficiency may be protective against a broader range of pathogens, particularly those that are not obligate intracellular pathogens and do not thrive within macrophages. For such pathogens, alterations in immune signaling and immune cell recruitment may outweigh any potential defects in controlling intracellular bacteria. Additionally, because UPEC do not thrive within macrophages and are primarily degraded(Bokil et al., 2011), we suggest that increased uptake of UPEC or similar pathogens by ATG16L1-deficient macrophages could itself be protective to the host. In contrast, increased uptake of intracellular pathogens that do thrive within macrophages could be detrimental.

IL-1β is a pro-inflammatory cytokine that plays a key role in the host response to infection and injury. This cytokine is tightly controlled at the transcriptional and post-translational level by two distinct signals, which prevent inappropriate activation and release(Sims and Smith, 2010). An additional level of control exists through production of an endogenous IL-1Ra that competes

with IL-1β for binding to the IL-1R. Our work adds to evidence that ATG16L1 and autophagy can modulate the production and release of active IL-1β. Saitoh et al. were the first to show this connection when they discovered that both ATG16L1-deficient fetal liver-derived macrophages and peritoneal macrophages in which basal autophagy was inhibited produced more IL-1β in response to LPS and commensal bacteria but not *Salmonella*(Saitoh et al., 2008). Later, Harris et al. showed that autophagy induction promotes the degradation of pro-IL-1β in response to LPS(Harris et al., 2011). Most recently, Lee et al. demonstrated that ATG16L1 regulates IL-1β signaling by controlling p62 levels by autophagosomal and proteosomal degradation(Lee et al., 2012). Our results suggest that increases in IL-1β in response to UPEC in our ATG16L1 deficient macrophages are primarily a result of altered caspase-1 activation rather than upregulation of transcription as was reported in studies of the T300A variant of ATG16L1(Plantinga et al., 2011). Interestingly, in contrast to what was observed previously(Saitoh et al., 2008), we found that LPS stimulation of $Atg16L1^{HM}$ macrophages was not sufficient to induce robust IL-1β secretion; this may be related to the defree of ATG16L1 deficiency. Nevertheless, our work provides the first evidence that, instead of harming the host, increased IL-1β secretion due to ATG16L1 deficiency can lead to a more effective response to infection.

The finding that elevated IL-1 signaling was important for the ability of $Atg16L1^{HM}$ mice to control UPEC suggests that ATG16L1 has a role in dampening the bladder mucosal production of IL-1β in response to UPEC infection. Such dampening may be helpful in preventing excessive inflammation in mucosae that cannot resolve the inflammation. For example, elevated levels of IL-1β due to ATG16L1 deficiency are detrimental in the context of chemically induced colitis(Saitoh et al., 2008). Inhibition of autophagy in cells infected with *Borrelia burgdorferi* has been shown to lead to increased IL-1β which is associated with worsened disease progression(Buffen et al., 2013). By contrast, increased macrophage-driven inflammation may be helpful in the bladder mucosa(Ingersoll et al., 2008), which is not permeable or absorptive like the gut mucosa and is usually sterile. Thus, the normal balance maintained by ATG16L1 may prevent infections by obligate intracellular pathogens in most cells but may lead to insufficient responses to other

pathogens, such as UPEC. Furthermore, the enhanced secretion of IL-1β due to loss of ATG16L1 appears to uncover a vulnerability in the ability of UPEC to evade early innate immune defenses and colonize and persist in the urinary tract. Enhanced secretion of IL-1β is protective in the Atg16L1^{HM} mice with inhibition of IL-1 signaling leading to bacteriuria levels similar to those of the WT mice at 1 dpi, yet inhibition of IL-1 signaling in WT mice did not make the infection worse, suggesting that the level and timing of IL-1β secretion may be important. Given the high numbers of UTIs and the increasing antibiotic resistance among UPEC isolates, the potential of this new knowledge to contribute to development of new treatment regimens to elicit an effective immune response to UPEC could be vital.

Our data and that of others suggests that the hyperinflammatory responses observed by ATG16L1-deficiency(Lapaquette et al., 2012; Sorbara et al., 2013) can be damaging or protective in different mucosal environments in response to different stimuli(Lassen et al., 2014; Saitoh et al., 2008). This may explain why the T300A polymorphism in *ATG16L1* is highly prevalent in the Caucasian population(Hampe et al., 2007) even though it increases susceptibility to Crohn's Disease(Hampe et al., 2007; Rioux et al., 2007). Recent studies have shown that the proteins produced by the T300A (human) or T316A (mouse) variants of ATG16L1 are more easily degraded by caspase-3, resulting in reduced expression of ATG16L1(Lassen et al., 2014; Murthy et al., 2014). ATG16L1 deficiency in the gastrointestinal tract is thought to cause intestinal dysbiosis because of an aberrant pro-inflammatory response to commensal gut flora(Cadwell et al., 2008; Cadwell et al., 2010; Kostic et al., 2014). Our work suggests that aberrant pro-inflammatory responses to commensals in the gut may occur as a trade-off for productive responses to pathogens in other tissues. The improved function of macrophages and increased production of IL-1β in response to UPEC by the hypomorphic ATG16L1 suggests that humans with the T300A variant may be more protected against pathogens such as UPEC where increased IL-1β would help clear the infection. Future genomic studies to investigate the possibility of associations between the T300A mutation and common infections such as UTI will help to reveal the selective pressure on maintenance of polymorphisms in *ATG16L1* in the human population.

METHODS

Mice, treatments and infections:

All protocols were approved by the animal studies committee of the Washington University School of Medicine (Animal Welfare Assurance #A-3381-01). Mice were maintained in a barrier facility under pathogen-free conditions and a strict 12-hour light/dark cycle. Atg16L1^{HM} and wild type (WT) mice on a C57BL/6 background were used at 7-9 weeks of age for infection (females) or to obtain bone marrow derived macrophages (males). Atg16L1^{HM} mice were given two intraperitoneal (IP) injections of 100 mg/kg of clodronate-containing liposomes (clodrolip) or the equivalent amount of control liposomes: two days and again 30 minutes (min) before infection. Mice were given two IP injections of 1 mg of Anakinra (Kineret, Swedish Orphan Biovitrum, Stockholm, Sweden) diluted in Dulbecco's Phosphate Buffered Saline (DPBS): 16 hours and again 30 min before infection. For infections, the UPEC strain UTI89 was grown statically for 17 hours in Luria-Bertani broth at 37°C; anesthetized mice were transurethrally inoculated with 10^7 colony forming units (CFU) of UTI89 in 50 µl of PBS as previously described(Hung et al., 2009). Urines from infected mice were collected at multiple time points, serially diluted in PBS, and plated on LB plates(Hung et al., 2009). Three days post infection, bladders were aseptically removed, homogenized in 0.1% Triton-X, serially diluted in PBS, and plated on LB plates (Wang C et al., 2012).

Flow cytometry analysis:

Single-cell suspensions from spleens were stained with the following antibodies (eBioscience, San Diego, CA) or flourescent dyes at 1:100 dilutions unless stated: anti-mouse GR1 FITC, anti-mouse F4/80 PE, anti-mouse CD11b APC, anti-mouse CD45 Pacific Blue, and 7AAD (1:500, AnaSpec Inc., Fremont, CA). Cells were fixed with 1% Paraformaldehyde (PFA) for 20 min with Actinomycin (ACROS Organics, Geel, Belgium). Stained cells were analyzed on a BD FACSCantoII using BD FACS DIVA software (BD Bioscience, San Jose, CA).

Isolation, differentiation and challenge of bone marrow-derived macrophages (BMDMs):

Bone marrow was aseptically isolated from Atg16L1^{HM}, Tlr4$^{-/-}$, Nlrp3$^{-/-}$, Nlrc4$^{-/-}$, Aim2$^{-/-}$, Nod2$^{-/-}$, Nod2$^{-/-}$; Atg16L1^{HM}, 129S6 (caspase-11-/-), Caspase-1/Caspase-11 double knockout, and WT mice and differentiated on non-tissue-culture-treated petri dishes for 7 days at 37°C with 5% ambient CO_2 in DMEM with 15% FBS, 30% L929 conditioned media, 1% Glutamax, 1% Na-Pyruvate, and 1% Penicillin and Streptomycin. Then on day 7 post-isolation, non-attached cells were aspirated, and the media was changed to DMEM with 10% FBS, 1% Glutamax, and 1% Na-Pyruvate. On day 8, cells were incubated with ice-cold DPBS, removed with a cell scraper, counted, and plated at $5x10^5$ cells/ml. On day 9, BMDMs were challenged with UTI89 or MG1655(Blattner et al., 1997) grown as described above for mouse infections at a multiplicity of infection (MOI) of 0.1.

Intracellular bacteria quantification:

Intracellular CFU were determined by serially diluting and plating cell lysates from BMDMs that had been challenged with bacteria for 1 or 2 hours and then treated with 100 μg/ml gentamicin for 15 min. For bacterial survival time course, BMDMs were challenged with UPEC for 2 hours, treated with 100 μg/ml gentamicin for 1 hour, and then 10 μg/ml gentamicin for the remaining time stated. For imaging analysis, cells were stained with rabbit anti-*E.coli* (1:500, US Biologicals, Swampscott, MA)(Wang et al., 2012) and rat anti-LAMP1 (1:50, clone ID4B; Developmental Studies Hybridoma Bank, Iowa City, IA)(Wang et al., 2012), and antigen-antibody complexes were detected with anti-rabbit Alexa Fluor-488-conjugated secondary antibody and anti-rat Alexa-594-conjugated secondary antibody (both at 1:500, Invitrogen; Carlsbad, CA). Images were obtained with a Zeiss Apotome microscope (Zeiss, Jena, Germany).

ELISAs:

BMDMs were incubated with UTI89, CFT073, or MG1655 for 2 hours, then treated with 100 μg/ml gentamicin containing medium for 1 hour and finally incubated with 10 μg/ml gentamicin containing medium for 5 hours (8-hr samples) or 21 hours (24-hr samples) at which point the supernatant was collected for ELISAs. IL-1β DuoSet ELISA kit (R&D systems, Minneapolis,

MN), TNFα DuoSet ELISA kit (R&D systems) and Caspase-1 (mouse) matched pair detection set (Adipogen, San Diego, CA) were used according to the manufacture's instructions. Where indicated, the Cathepsin B inhibitor CA-074 Me (Enzo Life Sciences, Inc., Farmingdale, NY) was used at 10 μM, the caspase-1 inhibitor Z-WEHD-FMK (R&D systems) was used at 10μM, and the VPS34 inhibitor 3-MA (Calbiochem, Millipore, Billerica, MA) was used at 2, 5, and 10μM. Alum- and Silica-treated samples were pretreated with 100 ng/ml LPS(*E. coli* strain 055:B5, Sigma) for 2 hours, and then treated with 200 μg/ml of Inject Alum (Thermo Scientific, Rockford, IL) or 100 μg/ml of Silica (Thermo Scientific) in fresh media for 6 hours. Streptolysin O (SLO) (Sigma) was used at 10 μg/ml with 10 mM DTT for 5 min, and then the medium was changed and collected 6 hours later.

qPCR:

TRIzol (Invitrogen) was used to isolate RNA from $4x10^6$ BMDMs that had been challenged with bacteria or LPS for 2 hours and then treated with gentamicin for 1 hour. RNA was treated with DNase1 (Ambion, Austin, TX), and Superscript II RNase H reverse transcriptase (Invitrogen) was used to synthesize cDNA from 1 μg of total RNA. Gene expression was determined by calculating ΔΔ CT with values normalized to 36B4 RNA levels and then to values from PBS-treated samples.

Western Blots:

Protein was isolated from cells as described(Wang et al., 2012). Briefly, cell lysates were electrophoresed on 4-20% Pierce Precise Protein Gels (Thermo Scientific) and transferred to nitrocellulose membranes. Membranes were probed with the following primary antibodies: rabbit-anti-ATG16L1 (1:500, Sigma) guineapig-anti-p62 (1:1000, Progen Biotechnik, Heidelberg, Germany), rabbit anti-Caspase-1 (1:5,000, Dr. Gabriel Nuñez), rat anti-Caspase-11 (1:250, Sigma), rat anti-NLRP3 (1:250, R&D Systems), and rabbit-anti-GAPDH (1:1000, Cell Signaling Technology, Danvers, MA). Goat-anti-rabbit IgG-HRP, goat-anti-guinea pig IgG-HRP, and goat-

anti-rat IgG-HRP secondary\ies (Santa Cruz Biotechnology, Dallas, TX) was used and detected by SuperSignal West Dura (Thermo Scientific).

Statistical analyses:

For time-course experiments, two-way ANOVAs with matching were used with Bonferroni post-tests for individual time points. To determine significance between two samples, unpaired t-tests, paired t-tests or non-parametric Mann-Whitney U tests were performed by using Graph Prism software (GraphPad Software, Inc., San Diego, CA). A value of $P < 0.05$ was used as the cut off for statistical significance.

Supplementary Material is linked to the online version of the paper at http://www.nature.com/mi

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Competing Financial Interests

The authors declare no competing financial interests

Author Contributions

JWS, CW, and IUM designed the research; JWS did research; JT did the TEM enumeration; NOB did immunostaining experiments; RS provided the clodronate-containing liposomes; GN

provided Nlrp3, Aim2, and Nlrc4 knockout mouse bones; JDS provided technical support and conceptual advice; JWS and IUM analyzed data; and JWS, CW, and IUM wrote the manuscript.

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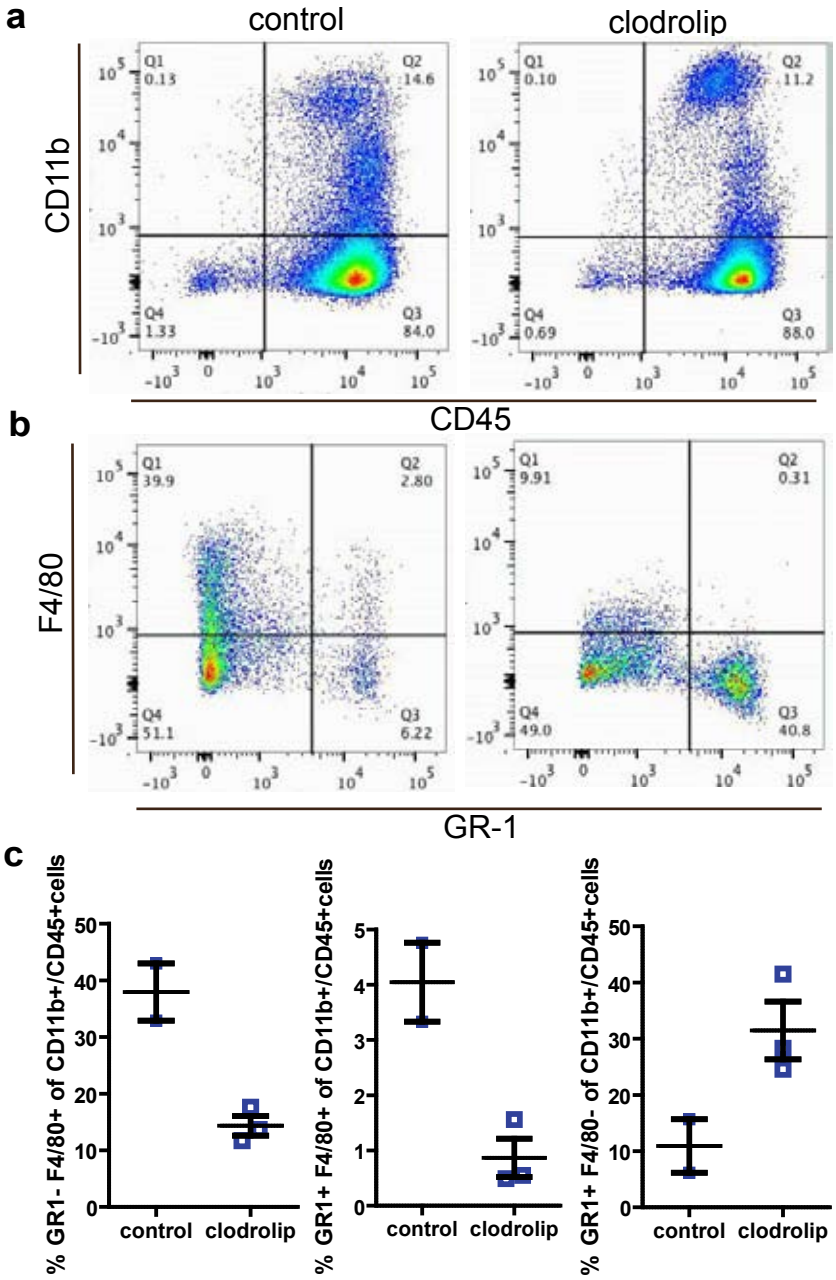
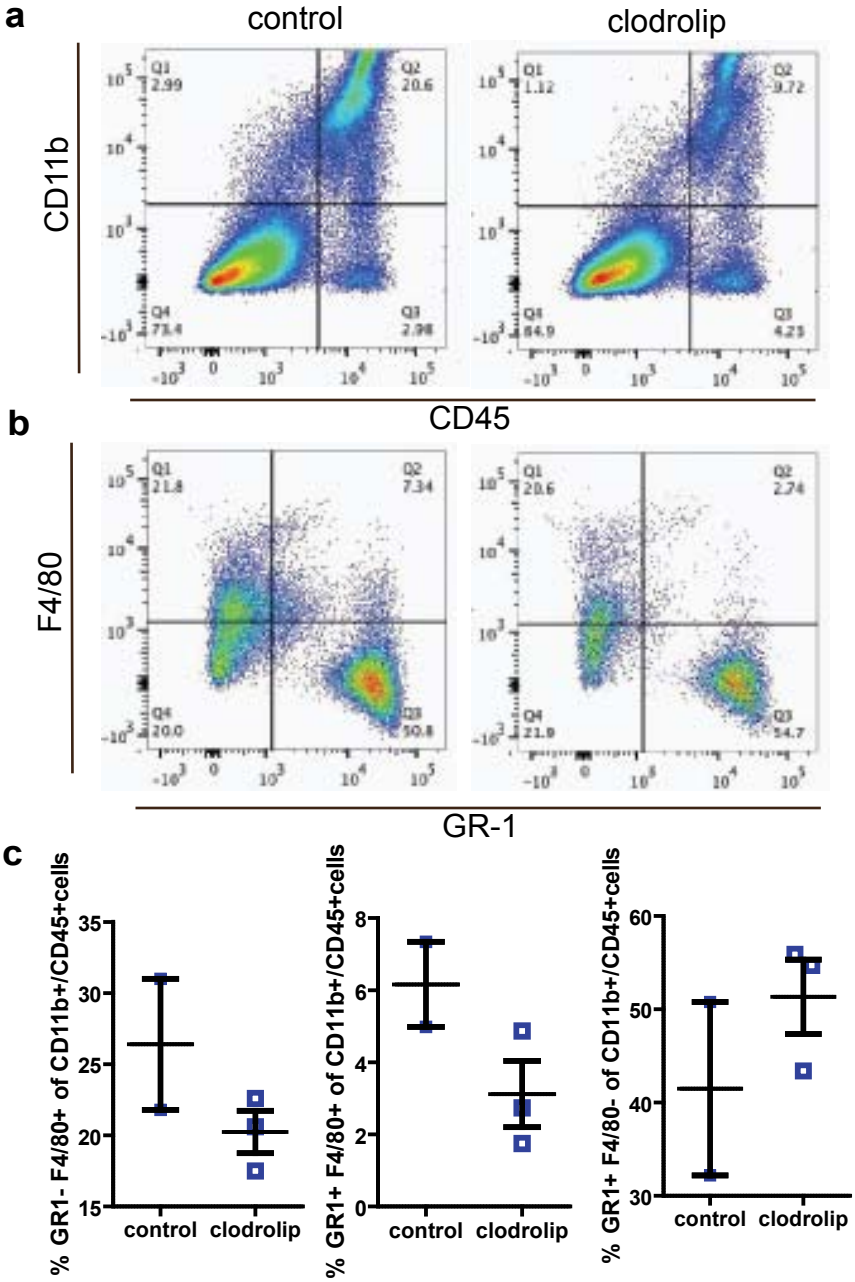
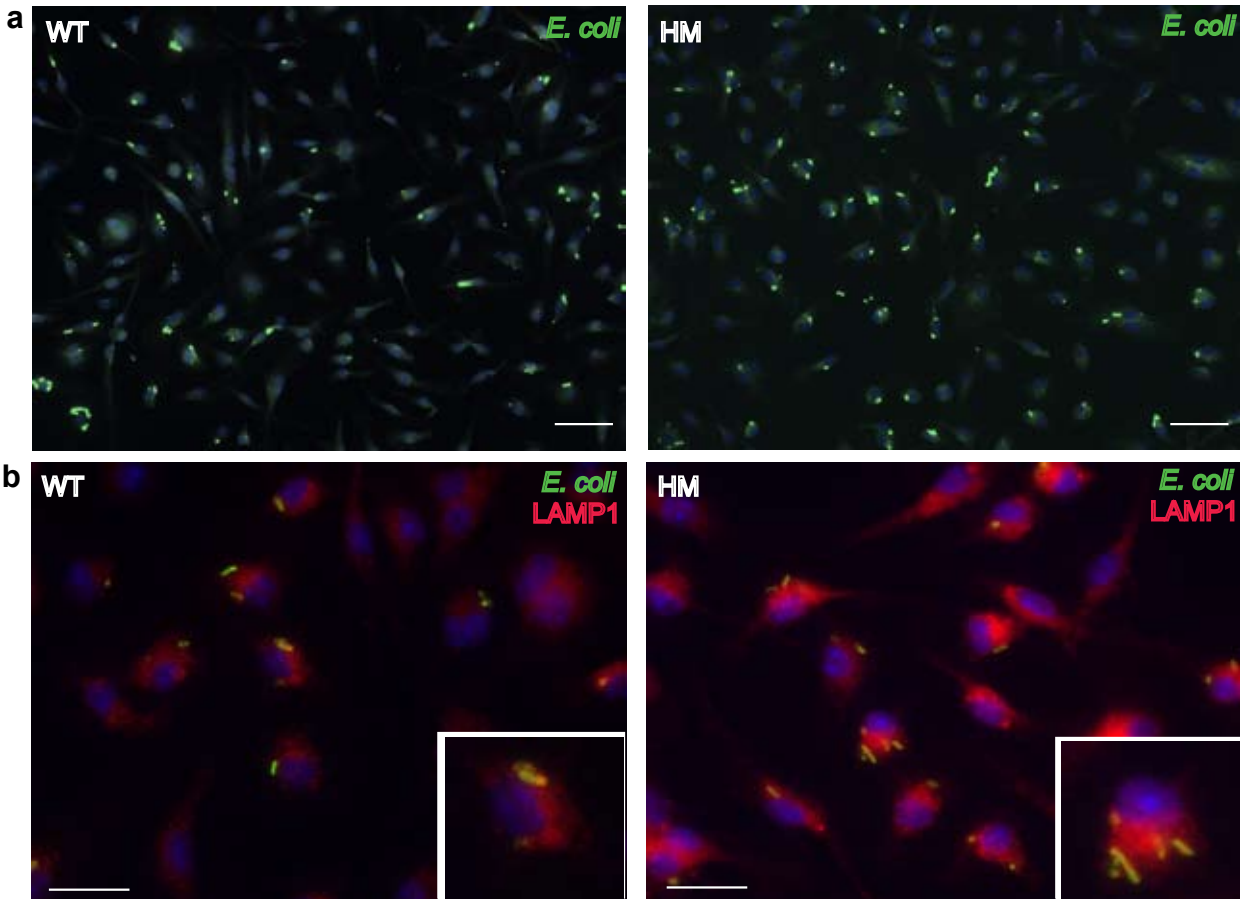
Supplementary figures.



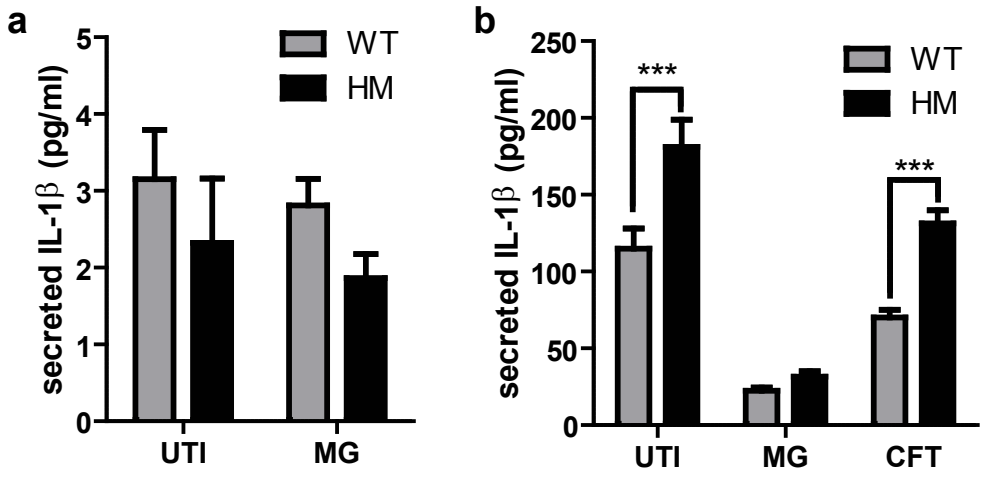
Supplementary Figure 1. Clodrolip depletes macrophages and monocytes from Atg16L1^{HM} mice. (a) Depletion of CD11b and CD45 double positive cells from the spleen of clodrolip-treated mice. (b) Depletion of F4/80 positive cells (both GR1 low, macrophages, and GR1 medium, monocytes) in spleens. (c) Quantification of macrophage (GR1 low, F4/80 positive), monocytes (GR1 medium, F4/80 positive), and neutrophil (GR1 hi, F4/80 negative) populations in spleens from multiple control or clodrolip treated animals.



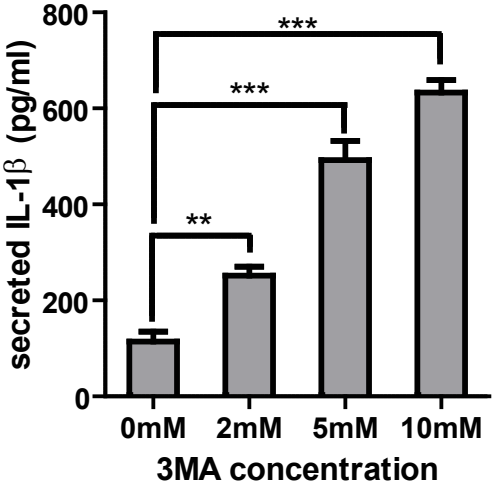
Supplementary Figure 2. Clodrolip depletes macrophages and monocytes from the bladders of Atg16L1^{HM} mice 24 hours post infection with UPEC. (a) Depletion of CD11b and CD45 double positive cells from the bladder of clodrolip-treated mice. (b) Depletion of F4/80 positive cells (both GR1 low, macrophages, and GR1 medium, monocytes) in bladders. (c) Quantification of macrophage (GR1 low, F4/80 positive), monocytes (GR1 medium, F4/80 positive), and neutrophil (GR1 hi, F4/80 negative) populations in bladders from multiple control or clodrolip treated animals.



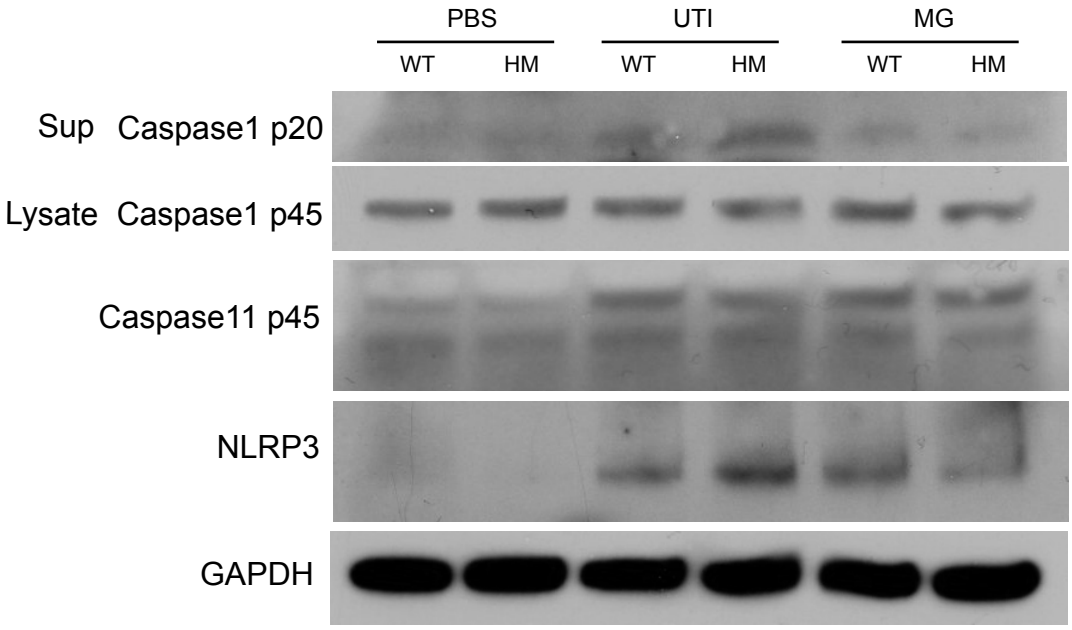
Supplementary Figure 3. Increased uptake of UPEC into LAMP1 positive compartments in ATG16L1- deficient macrophages. (a) Representative immunoflourescence images of UPEC within wild type (WT, left) and ATG16L1-deficient (HM, right) macrophages at 3 hours post challenge. E. coli (green) and nuclei are blue with biz-benzimide, quantification is presented in Fig. 2b. Scale bar = 40 μm. (b) Images showing co-localization of LAMP1 (red) with UPEC (green) in WT (left) and ATG16L1-deficient (right) BMDMs. Scale bar = 20μm.



Supplementary Figure 4. More IL-1β is secreted by ATG16L1-deficient macrophages in response to cystitis and pyelonephritis strains, after 24 hours. (a) Levels of IL-1β secretion by WT and HM macrophages at 2 hours in response to UTI89 and MG1655. (b) Levels of IL-1β secretion by WT and HM macrophages at 24 hours in reponse to UTI89 (cystitis strain), MG1655 (K-12 strain), or CFT073 (pyelonephritis strain). Two-way ANOVA with bonferroni post-tests. *** $P<0.001$.



Supplementary Figure 5. Inhibiting autophagy in WT macrophages leads to increased IL-1β secretion. Levels of IL-1β secretion by WT macrophages at 24 hours when treated with increasing concentrations of VPS34 inhibitor 3-MA and challenged with UTI89. Two-way ANOVA with bonferroni post-tests. ** $P<0.01$, *** $P<0.001$.



Supplementary Figure 6. More cleaved caspase-1 is found in the suprenatants of ATG16L1 deficient macrophages than WT macrophages. Western blot of cleaved caspase-1 (p20) in supernatants (24 hrs) and uncleaved caspase-1 (p45), caspase-11 (p45), and NLRP3 (~117) in cell lysates (3 hrs) of PBS, UTI89, and MG1655 challenged WT and ATG16L1-deficient macrophages.

Chapter 5: Conclusions and Future Directions

With increasing rates of antibiotic resistance among UPEC isolates from patients with UTIs, there is a greater need to understand the host response to UPEC and to find ways to improve it. To that end, we have helped define the role of the key host autophagy protein ATG16L1 in host-pathogen interactions of UPEC in the bladder. We have showed ATG16L1 deficiency can improve the host innate immune response to UPEC by increasing IL-1β production by macrophages. These studies and others like them will help us further define what makes a productive immune response to UPEC. New treatments and vaccines are being proposed and investigated to reduce our reliance on antibiotics to treat UPEC induced UTIs. One approach currently taken is to combat UPEC binding to bladder epithelial cells with mannosides (Cusumano et al., 2011; Guiton et al., 2012). Another approach being taken is the development of a vaccine, with a number of new candidates within the last few years targeting different parts of UPEC (Brumbaugh et al., 2013; Goller et al., 2014; Savar et al., 2014). By studying the immune response to UPEC, we hope to find ways to harness the host response to reduce the burden of disease and improve the outcomes of new forms of treatment.

Investigate mechanisms by which resident and recruited macrophages clear a UTI in normal mice

Macrophages are a set of cells that are found in diverse tissues throughout the body and are involved in many areas of biology (Wynn et al., 2013). Macrophages display incredible diversity in function and tissue distribution, challenging earlier thoughts of macrophage homogeneity (Gautier et al., 2012; Wynn et al., 2013). Recent studies have shown that tissue macrophages have diverse origins; some are derived from the yolk sac cells, others come from hematopoietic stems cells in the fetal liver, and yet others come from hematopoietic stem cells from the bone marrow (Ginhoux et al., 2010; Schulz et al., 2012). Little is known about macrophages in the bladder and urinary tract. Where do they come from? Are the bladder tissue macrophages one single population or are they a heterogeneous population with diverse functions? Are the resident macrophages a primarily self-sustaining population or are they renewed from monocyte populations? How does the bladder environment affect the function of these macrophages? These

questions can now be addressed given improved techniques for defining macrophages subsets. Our goal should be to determine the hallmarks of macrophages that are effectively responding to UPEC and those of macrophages that are helping clear dead cells to aid in the repair of the bladder epithelium. Guided by those findings, we may be able to modulate the different phases of the response to infection to get the more effective macrophage killing of the pathogen, but with quicker resolution of the inflammation to avoid unnecessary tissue damage (Epelman et al., 2014).

Our work and other recent studies are beginning to help us better understand the role of macrophages in the acute response to UPEC. Resident macrophages are present in the uninfected bladder, and may play a role in homeostasis. Upon UPEC infection there is a large increase in macrophages and recruited monocytes (Engel et al., 2006; Ingersoll et al., 2008; Wang et al., 2012a). Recent work from Schiwon et al described a coordinated response of multiple cytokines from monocyte and macrophage subtypes secreted in a specific order allowing the effective crossing of neutrophils through the epithelium to the bladder lumen and subsequent clearance of UPEC (Schiwon et al., 2014). Macrophages express different receptors on their surface that are associated with pathogen recognition, antigen presentation, and uptake of cellular debris. Another graduate student in the lab is now studying how the receptor profiles of these macrophages changes throughout the infection. These studies show provide important insight into the role of macrophages in the acute inflammatory response to UPEC and in the reparative phase after the resolution of infection.

Macrophages play key roles in the host response to pathogens, but are also important in development, homeostasis, regeneration, and wound healing (Wynn et al., 2013). Many tissues will not develop normally if not for macrophages providing key differentiation signals or eliminating dead and apoptotic cells or extruded components (Wynn et al., 2013). Macrophages also play a role in regeneration after injury. In the intestine, macrophages are important for the restoration of the epithelial barrier after dextran sodium sulfate induced wounding of the epithelium (Pull et al., 2005). Thus, it would be interesting to examine the role of macrophages in

resolution of UPEC infection. Macrophages may affect epithelial proliferation and differentiation after UPEC infection. Work from our lab has shown that different models of urothelial damage result in distinct proliferation and regenerative responses. UPEC infection leads to epithelial barrier disruption and inflammation resulting in proliferation of basal urothelial cells that are thought to be the putative stem cells (Mysorekar et al., 2009). On the other hand, protamine sulfate treatment causes epithelial barrier disruption, but results in proliferation of mostly intermediate cells. Macrophages and other immune cells may be responsible for some of the differences between these two responses (Wang et al., 2012b). Thus, as in other tissues, macrophages may play significant roles in the restoration of the urothelial barrier, and may integrate inflammatory signals to aid in different forms of regeneration post tissue injury.

Important questions remain. Do macrophages play both anti-pathogenic and reparative roles in the bladder throughout the course of a UPEC infection? What is the role of metals in macrophages response to infection, primarily the role of iron homeostasis in this process? Does UPEC modulate the response of macrophages in vivo to enable more efficient infection? What are the origins of bladder associated macrophages and how do they change upon infection?

Determine the effects of ATG16L1 deficiency on the ability of resident and recruited macrophages to combat a UTI

We have already shown that ATG16L1 modulates the macrophage response to UTIs as ATG16L1 deficiency resulted in an increased number of macrophages and monocytes in the urines of UPEC infected $Atg16L1^{HM}$ mice (Wang et al., 2012a) and those cells were essential for the clearance of bacteriuria (Symington et al., chapter 4). Additionally, I have shown that ATG16L1 deficiency can change the uptake of UPEC by BMDM and the production of IL-1β by BMDM in vitro (Symington et al., chapter 4). That increase in IL-1β was also observed in the urines of $Atg16L1^{HM}$ mice. BMDM provide an excellent model for studying macrophage UPEC interactions and simplifying the complex *in vivo* environment, but as mentioned in the earlier subsection not all macrophages are the same. ATG16L1-deficiency may have different effects on

the tissue macrophages as compared to the recruited monocytes. Determining which macrophage populations are responsible for the increased IL-1β production *in vivo* would provide additional insight into the normal and improved response in Atg16L1^{HM} mice. If these studies help define the key macrophages whose function is improved ATG16L1 deficiency *in vivo*, it may allow us to more elegantly change host responses than by global inhibition of the autophagy pathway to offer protection to UPEC infection.

ATG16L1 may also affect the role of macrophages in the resolution of infection. We observed faster reconstitution of the bladder epithelial barrier after infection in Atg16L1^{HM} mice (Wang et al., 2012a). This earlier restoration of the barrier may be due to better clearance of the infectious insult, but it may also be due to changes in macrophage epithelial crosstalk. Determining how ATG16L1 affects the reparative state of macrophages could provide valuable insight into macrophages role in the bladder and possibly other tissues where macrophages may play a reparative role.

Examine autophagy proteins in trafficking of UPEC within macrophages

UPEC is different from many of the pathogens studied in the autophagy literature, because it does not thrive within macrophages. Bokil et al recently showed that at early time points there may be many bacteria that are either taken up or enter into macrophages, but by 24 hours, although some bacteria remain viable within the cell, most have been eliminated (Bokil et al., 2011). Our data further clarified the dynamics of UPEC clearance from macrophages overtime, showing that the number of viable intracellular bacteria reduced 80% within the first hour of extracellular gentamicin treatment, and by 24 hours very few viable bacteria remain. This suggests that although UPEC is an excellent pathogen of bladder epithelial cells, it is not well suited to survive within macrophages. Autophagy proteins may affect how UPEC is taken up by macrophages, how UPEC is trafficked through the macrophage, and how UPEC is eventually degraded within the macrophage. Answering these questions may provide novel insights into autophagy's role in macrophages' response to other pathogens that do not thrive in macrophages.

Autophagy proteins may be directly involved in the trafficking of UPEC within the cell. In chapter 4 of this thesis, we observed that UPEC within Atg16L1^{HM} BMDM appeared to remain in a lysosome at 3 hour post challenge when UPEC in WT BMDM was increasingly found in compartments that were more electron lucent suggesting that UPEC may be altering the compartment under normal conditions in a way that depends on autophagy proteins, or that autophagy proteins. This difference may be because autophagy proteins are important for the trafficking of UPEC within macrophages. Early studies by Amer et al and Baorto et al showed that FimH positive E.coli are found in different compartments than FimH negative E. coli (Amer et al., 2005; Baorto et al., 1997). Baorto et al found that the compartments containing FimH positive E coli were not acidified as quickly, did not contain as many reactive oxygen species and were smaller than those compartments that contained FimH negative E coli (Baorto et al., 1997). This suggested that FimH dependent phagocytosis results in an altered initial compartment. Subsequently, Amer et al showed that FimH positive E. coli entered into cells and were found in vacuoles that colocalized with lipid raft markers and quickly recruited two autophagy proteins, first Atg7 and then Atg8 (LC3) (Amer et al., 2005). Our TEM data provides new insight into this process by showing that UPEC remain within intact single membrane bound compartments at 1 and 3 hours post challenge and that they are not within canonical double membraned autophagosomes. Thus, autophagy proteins appear to be recruited to compartments containing UPEC that has entered into macrophages through a FimH dependent phagocytosis, rather than canonical autophagy of UPEC, and may represent LC3-associated phagocytosis (LAP)(Sanjuan et al., 2007) or a similar process. This may be a conserved method to protect the host cell from pathogens that avoid canonical phagocytosis, by targeting bacteria for degradation that enter the cell in other ways. Defining the timeline of autophagy protein recruitment, the other adapters involved in the process, and the eventual outcome of UPEC tagged by autophagy proteins compared to those that are not would be valuable. Do autophagy proteins may play a role in the degradation of UPEC within macrophages?

Another important question for future studies is the following: is the trafficking of UPEC within macrophages altered by the activation state of the macrophage? Most of our studies were done in BMDM that had not been activated, thus they model the initial UPEC exposure of a naïve macrophage. In the bladder, UPEC may encounter both naïve and activated macrophages. One way to model the diversity of possible UPEC/macrophage interactions observed *in vivo* is to challenge macrophages that have been activated by different sets of cytokines and TLR agonists with UPEC *in vitro*. We have conducted a few experiments with LPS pretreated BMDM and have shown that BMDM from both WT and Atg16L1^{HM} mice were able to take up many more bacteria when pretreated with LPS for 24 hours then when they were not pretreated. Pretreatment with LPS did not result in increased secretion of IL-1β by WT or ATG16L1-deficient BMDM as compared to non-pretreated cells, and the difference between WT and ATG16L1-deficient cells remained (chapter 4, Figure 5c). This suggests that macrophages activation by LPS allows the macrophage to better sense UPEC and circumvent UPECs uptake avoidance mechanisms, such as the capsule, but may temper its inflammatory cytokine production. Intriguingly, we did not observe a difference in uptake of UPEC between WT and ATG16L1-deficient BMDM that had been pretreated with LPS, even though the ATG16L1-deficient BMDM still secreted significantly more IL-1β. This could be because when macrophages are activated they upregulate a number of receptors for pathogens and thus may speed up the uptake process and may not need autophagy proteins as much. Alternatively, uptake may have been equal at the times observed because the whole process occurs faster and thus the differences would only be observed within the first few minutes of the infection. Thus, depending on the activation of the macrophage, autophagy proteins may play distinct roles in the trafficking of UPEC within macrophages. Further examining the interaction of UPEC with macrophages that have been activated with different pro or anti-inflammatory signals could be better models of the *in vivo* reality. If macrophages play a role in regenerating the bladder epithelium, how would those reparative macrophages respond to a UPEC and how would that change their reparative response and their handling and degradation of UPEC.

Investigate the modulation of reactive oxygen species in host defense against UPEC by ATG16L1

Reactive oxygen species are important for host defense against many pathogens. They are produced in the phagosome in order to kill intracellular pathogens, and their production has been linked to inducing autophagy (Huang et al., 2011). Autophagy plays a key role in modulating reactive oxygen species in the host cell and reactive oxygen species themselves can lead to the induction of autophagy. During mitophagy, damaged mitochondria are degraded and thus the cellular levels of reactive oxygen species are reduced. Elevated levels of mitochondria ROS can activate the inflammasome and thus induce increased inflammation. Reactive oxygen species may also play a role in the inflammasome activation in macrophages by intracellular UPEC.
In preliminary work in our lab, IL-1β production by WT macrophages could be reduced by inhibiting mitochondrial ROS with MitoTEMPO in a dose dependent manner. Thus, some of the increased IL-1β production in our ATG16L1-deficient cells may be the result of increased ROS. Specifically, given that ATG16L1-deficient macrophages have reduced autophagic flux at baseline, there may be more damage mitochondria in ATG16L1-deficient cells that produce ROS and thus activate the NLRP3 inflammasome. By inhibiting VP34, a PI3K essential for autophagy, with a range of concentrations of 3-MA, we show reduced autophagy is sufficient to increase IL-1β production by macrophages in response to UPEC. UPEC may be inducing ROS production and specifically ROS produced by mitochondria. What roles those functions may play in the production of IL-1β and in the eventual degradation of UPEC is unknown and could be valuable to understanding UPEC macrophage interaction.

Determine the role of ATG16L1 and other autophagy proteins in the bladder epithelium and QIR formation

ATG16L1 deficiency in mice resulted in vesicular accumulation in the epithelium and in decreased numbers of QIRs after UPEC infection. These alterations could be specific for ATG16L1 or could be dependent on a greater number of autophagy proteins. To address this

question, we examined the role of another important autophagy protein ATG7 that is not part of the ATG12/ATG5/ATG16L1 complex. We used a mouse model with a tamoxifen inducible knockdown of Atg7, Atg7fl/fl; ER inducible B-actin-cre mice, and their cre negative littermate controls to examine the role of ATG7 in the epithelial response to UPEC. Cre+ and cre- controls were injected 3 times in 1week with tamoxifen by IP and allowed to rest a week. This resulted in a 70% knockdown of ATG7 in whole bladder lysates. Uninfected and infected bladders from these mice had no gross abnormalities, but were more likely to have vesicular congestion as observed by immunofluorescence and TEM. This confirms that autophagy proteins are important for normal vesicle recycling in bladder superficial cells. At 2 weeks post infection, the Atg7fl/fl cre positive mice had fewer QIRs per bladder than their cre negative littermate control mice. Thus, ATG7 and ATG16L1 both appear to be important for the formation of QIRs by UPEC, suggesting that UPEC is subverting the normal role of autophagy in the epithelial cell to create its own protective niche. Given that UPEC utilizes multiple components of the autophagy pathway to form reservoirs, we believe that inhibiting the autophagy pathway will be protective against recurrent UPEC infections.

Our work was the first to show that autophagy protein deficiency actually impaired the survival of a pathogen in vivo. Since then, a growing number of pathogens have been shown to utilize autophagy proteins to form specialized niches that facilitate their replication, proliferation or survival (Birmingham et al., 2008; Cemma and Brumell, 2012; Starr et al., 2012). This suggests that reductions in autophagy proteins and their functions may be protective to the host against pathogens that utilize this pathway to form reservoirs, such as UPEC.

Selective pressure on maintaining CD risk allele of ATG16L1

The associate of the T300A allele of ATG16L1 with Crohn's disease has made understanding the functional consequences of that variant an area of intense research. The first studies showed that T300A did not change basal autophagy levels, ATG16L1's homodimerization, or its binding with ATG5, in a number of cell types (HeLa, Caco2, and MEFS) (Fujita et al., 2009; Kuballa et

al., 2008), but there were conflicting reports on its role in anti-*Salmonella* autophagy Kuballa et al showed that the T300A allele led to decreased anti-*Salmonella* autophagy in HeLa and caco2 cells (Kuballa et al., 2008), whereas Fujita et al found no change in anti-*Salmonella* autophagy in MEFs (Fujita et al., 2009). Studies using another pathogen, the adherent and invasive *E.coli* (AIEC), found that the T300A variant resulted in increased intracellular bacterial replication and decreased the percent of AIEC in LC3 positive compartments in HeLa cells (Lapaquette et al., 2010). Thus, it appeared that in epithelial cells under normal conditions there is no defect in basal autophagy with expression of the T300A variant, but there may be changes in the anti-bacterial autophagy.

More recent studies have now shown that the T300A variant does not impair ATG16L1's role in autophagy, but it makes ATG16L1 more vulnerable to cleavage by caspase-3, thus possibly affecting autophagy functions in certain stressful conditions (Lassen et al., 2014; Murthy et al., 2014). Caspase-3 is a member of the caspase family that like caspase-1 and 11 is produced as a zymogen and must be cleaved to be activated. It is activated by cytochrome c from mitochondria interacting with capase-9, apoptosis activating factor 3 and ATP. The T300A allele creates a site for caspase-3 to cleave ATG16L1 and leads to lower levels of ATG16L1 in the cell, and concomitant decreases in autophagic flux (Lassen et al., 2014; Murthy et al., 2014). Thus, under conditions with stresses that induce apoptosis and caspase-3 activation in a cell, the T300A mutation may display similar phenotypes to those seen in our Atg16L1^{HM} mouse model.

ATG16L1 deficiency was protective in our model; Atg16L1^{HM} mice had reduced levels of QIRs and faster clearance of bacteriuria (Wang et al., 2012a). Enhanced IL-1β production by ATG16L1 deficient macrophages was protective in response to a UPEC infection, even though it had previously only been associated with worse outcomes in the host, such as in colitis models (Cadwell et al., 2010; Saitoh et al., 2008). This protective response is not limited to UPEC infection, since Atg16L1^{HM} mice are also protected from Citrobacter (Marchiando et al., 2013). The T300A variant may mimic ATG16L1 deficiency, and thus may be protective against pathogens like UPEC. This could help explain why the T300A allele, that has been

associated with increased risk of Crohn's disease in numerous studies, remains prevalent within the Caucasian population (Franke et al., 2010; Rioux et al., 2007). UTIs may provide selective pressure to maintain this otherwise harmful allele in the population.

Human studies to see if the T300A allele is protective in humans against UTIs may help explain why the allele is so prevalent. These studies had previously not been possible, because although many groups have conducted GWAS studies examining the T300A genotypes and different gut related problems of Crohn's disease patients, these databases rarely asked for data on urinary tract infection prevalence and recurrences. Also, many of those patients with Crohn's disease may have additional confounders due to medications taken to treat their disease. To avoid these problems, we are now collaborating with a group in the Netherlands that has genotypic data on the T300A allele in a general population that has filled out questionnaires about their history of urinary tract infections. Preliminary findings from this study suggest that males with the T300A allele are less likely to have had multiple UTIs, thus the T300A allele may in fact provide protection from recurrent UTIs *in vivo* (Figure 1). A larger population would provide additional power to this study, but these results are promising. UTIs may not be the only infection that is cleared better with ATG16L1 deficiency, thus looking at a number of common infections in different sites of the body will also be enlightening.

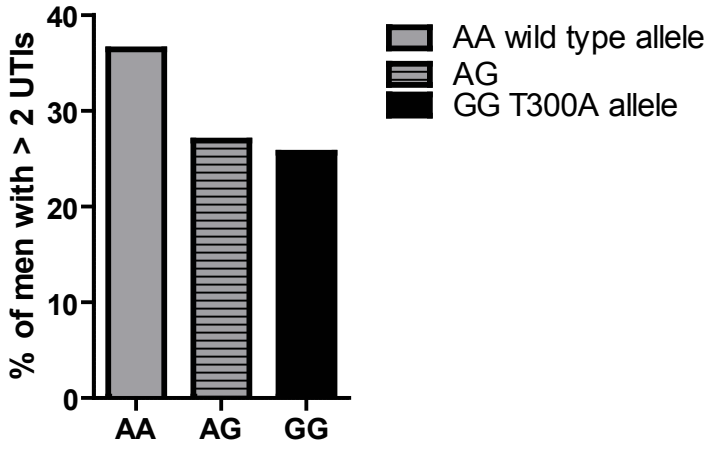


Figure 1. Human Population Studies. Data from our collaboration with members of the Nijmegen Biomedical Study showing the number of men who self-reported having more than 2 UTIs that had been treated with antibiotics as a function of their genotype.

In conclusion, our work has changed the paradigm that autophagy and autophagy proteins are always protective to the host against a pathogen. These studies provided the first in vivo evidence of an autophagy protein deficiency being protective to the host against a pathogen. In fact, ATG16L1 deficiency was protective against both intracellular and extracellular bacteria. Thus, inflammation that is detrimental in other tissues can enhance the clearance of bacteria from the bladder and may in fact be why the detrimental variant has remained within the population. These studies further highlight the need to study a diverse array of pathogens with different pathogenic cycles and tissue tropisms in order to fully understand the functions of this essential pathway in the body. If we do that, we might be able to modify autophagy components locally to improve host responses to many conditions without causing adverse reactions in other tissues.

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Appendix: Estrogenic modulation of uropathogenic Escherichia coli infection pathogenesis in a murine menopause model

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Estrogenic modulation of uropathogenic Escherichia coli infection pathogenesis in a murine menopause model

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ABSTRACT

Recurrent urinary tract infections (UTIs), primarily caused by uropathogenic *Escherichia coli* (UPEC), annually affect over 13 million patients in the US. Menopausal women are disproportionally susceptible, suggesting estrogen deficiency is a significant risk factor for chronic and recurrent UTI. How estrogen status governs susceptibility to UTIs remains unknown, and whether hormone therapy protects against UTIs remains controversial. Here, we used a mouse model of surgical menopause by ovariectomy and demonstrate a protective role for estrogen in UTI pathogenesis. We found that ovariectomized mice had significantly higher bacteriuria, a more robust inflammatory response, and increased production of the proinflammatory cytokine, IL-6, upon UPEC infection compared to sham-operated controls. We further show that response of the urothelial stem cell niche to infection, normally activated to restore homeostasis after infection, was aberrant in ovariectomized mice with defective superficial urothelial cell differentiation. Finally, UPEC-infected ovariectomized mice showed a significant increase in quiescent intracellular bacterial reservoirs, which reside in the urothelium and can seed recurrent infections. Importantly, this and other ovariectomy-induced outcomes of UTI were reversible upon estrogen supplementation. Together, our findings establish ovariectomized mice as a model for UTIs in menopausal women, pinpoint specific events during course of infection that are most susceptible to estrogen deficiency. These findings have profound implications for the understanding of the role of estrogen and estrogen therapy in bladder health and pathogen defense mechanisms and open the door for prophylaxis for menopausal women with recurrent UTIs.

INTRODUCTION

Urinary tract infections (UTIs), primarily caused by uropathogenic *E. coli* (UPEC), are among the most common frequently recurring infectious diseases in humans (Dielubanza and Schaeffer, 2011). Menopausal women, who have greatly reduced levels of the sex hormone estrogen, are more likely to have recurrent/chronic UTIs than any other group (Foxman, 1990, 1999; Hextall and Cardozo, 2001; Raz, 2011); 53% of menopausal women with a UTI will have at least one

recurrence (Ikaheimo et al., 1996). Consistent with evidence from animal and human studies that sex hormones have an important effect on the female lower urinary tract during adult life, estrogen receptors have been identified in the bladder, urethra, and pelvic floor. In addition, fluctuations in the circulating levels of estrogen during the menstrual cycle and in pregnancy influence the prevalence of urinary symptoms. Furthermore, decreased estrogen during menopause is a significant risk factor for UTIs (Foxman, 1990; Foxman and Brown, 2003; Foxman et al., 2000; Foxman et al., 2001) and bladder barrier dysfunction (Hass et al., 2009), but little is known about the mechanisms underlying this increased susceptibility.

A murine model of UTI shows that UPEC infection of the urinary bladder follows a multi-step pathogenic cycle: UPEC invade superficial urothelial cells by binding cell-surface uroplakin receptors (Hung et al., 2009). During the acute stage of infection (0-72 hours), intracellular UPEC replicate rapidly and establish cytoplasmic biofilms termed intracellular bacterial communities (IBCs), which are also observed in humans (Garofalo et al., 2007; Rosen et al., 2007). The host response includes induction of pro-inflammatory cytokines, including IL-6, exfoliation of the superficial urothelial cells containing IBCs into the urine, and influx of innate immune cells, particularly neutrophils. This rapid pro-inflammatory response aids in defending against pathogens(Hannan et al., 2012; Nielubowicz and Mobley, 2010). The damaged epithelial barriers are restored by urothelial stem cell niche activation and terminal differentiation of superficial cells (Mysorekar et al., 2009). Despite the host defense response however, a subset of UPEC can survive and establish long-term reservoirs, likely within autophagosomal compartments, in urothelial cells (Wang et al., 2012a; Wang et al., 2012b). These reservoirs, termed quiescent intracellular reservoirs (QIRs) can serve as seeds for recurrent infection (Mysorekar and Hultgren, 2006).

Estrogen has been used widely to treat urinary symptoms in postmenopausal women, but the evidence from randomized studies does not consistently show that estrogen therapy is effective in reducing recurrence of UTIs (Brown et al., 2001; Curran et al., 2007; Eriksen, 1999; Oliveria et al., 1998; Orlander et al., 1992; Perrotta et al., 2008; Raz et al., 2003; Raz and Stamm, 1993; Stamm, 2007).

These inconsistencies may be because the trials used variable dosages and durations of treatment. Given that the causes of UTIs are complex and multifactorial, gaining a clear understanding of the role of estrogen in UPEC pathogenesis requires development of an animal model with a defined genetic background in which estrogen levels can be manipulated in a controlled manner. However, there have been limited studies using animal models to examine the estrogenic modulation of UTI progression. One study has suggested that supplementation of 17β-estradiol increased the susceptibility of ascending UTI in the kidneys, but not in the bladder (Curran et al., 2007). Thus, the dynamic interaction between estrogen signaling and UPEC pathogenesis in the urothelium still remains to be elucidated. In this report, we employ a murine model of surgical menopause, ovariectomy, to directly test the hypothesis that changes in hormone levels play a role in regulating the course of UTIs in the bladder and present evidence that estrogenic deficiency adversely affects the course of UPEC pathogenesis, in particular UPEC persistence in the bladder wall and the urothelial regenerative response upon infection.

MATERIALS AND METHODS

Mice:

All protocols were approved by the animal studies committee of the Washington University School of Medicine (Animal Welfare Assurance #A-3381-01). Mice were maintained under pathogen-free conditions in a barrier facility under a strict 12 hr light/dark cycle.

Ovariectomy:

7-8 week old C57BL/6 female mice (NCI Mouse Repository; Frederick, MD) were anesthetized and the ovaries excised as described (Anand et al., 2012). For SHAM surgery, the same procedures were performed without removal of the ovaries. The animals were allowed at least two weeks to recover.

17 β-estradiol supplementation:

A 90-day time-release pellet containing 0.01mg of 17 β-estradiol (Innovative Research of America; Sarasota, FL) was implanted under the side of the neck of each animal. The mice were maintained on the pellets for 60-90 days until sacrifice.

Inoculations of Mice:

UTI89 (Mysorekar and Hultgren, 2006), a pathogenic UPEC strain, was grown statically in Luria-bertani (LB) broth for 17 hours at 37°C. Mice were anesthetized and inoculated, via transurethral catheterization, with 50µl of bacterial suspension (10^7 CFU) of UTI89 in phosphate-buffered saline (PBS) as previously described (Hung et al., 2009; Rosen et al., 2007).

Tissue histopathology and inflammation scoring:

Bladders were processed as described (Wang et al., 2012a). Briefly, bladders were aseptically removed immediately after sacrifice, fixed in methacarn (60% methanol, 30% chloroform and 10% acetic acid) and embedded in paraffin. 5µm thick tissue sections were stained with hematoxylin and eosin. Inflammation scores of infected bladders were determined as described (Stemler et al., 2013).

Histochemical and Immunofluorescence analysis:

Bladders were processed as described above. The following primary antibodies were used on bladder tissue sections: rabbit polyclonal antibody (pAb) to *E. coli* (1:500, United States Biological; Swampscott, MA), mouse monoclonal antibody (mAb) to Uroplakin III (1:100, Fitzgerald; Acton, MA), goat pAb to BrdU (Kim et al., 1993), rat mAb to Lamp1 (1:50, clone ID4B; Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit pAb to p27kip1 (1:500, Sigma; St. Louis, MO), rabbit pAb to Cytokeratin 5 (CK5) (1:500, Abcam; Cambridge, MA), and mouse mAb to E-Cadherin (1:500, BD Transduction Labs; San Jose, CA). After three, five-minute PBS washes at room temperature, antigen-antibody complexes were detected with species-specific Alexa Fluor-

488, -594, or -647-conjugated secondary antibodies (1:500, Invitrogen; Carlsbad, CA). Images were obtained using a Zeiss Apotome microscope.

QIR quantification:

Six separate 5-μm serial sections over a thickness of 300 μm were immunostained with antibodies against *E. coli*, Lamp1, and E-Cadherin (listed above) and imaged at 63X. The total number of Lamp1-positive UPEC reservoirs in the six sections was counted and reported as the number of QIRs per bladder. n=18-23 mice per group.

BrdU labeling:

BrdU labeling was performed as described (Mysorekar et al., 2009).

Urinalysis and bacterial titering:

Urines from infected mice were collected at 0-14 dpi and serially diluted in PBS; 5 μL of each dilution was spotted onto LB plates six times as described (Hung et al., 2009). Bacterial titers were calculated as CFUs/ml of urine. Urine sediments were obtained by cyto-centrifuging 50 μl of a 1:5 dilution of the urine onto poly-L-lysine-coated glass slides, which were then stained for inflammatory scoring as described (Stemler et al., 2013).

Bioplex cytokine bead array assay:

Sera were obtained at 6 hour post infection (hpi), and cytokine levels were measured using the Bioplex kit from BioRad (BioRad, Hercules, CA) as described previously (Hannan et al., 2010; Wang et al., 2012a).

Quantitative real time-PCR analysis:

Bladders from SHAM or OVX mice (3 mice per group) were removed at 6hpi. RNA was isolated from the bladders using TRIzol (Invitrogen, Carlsbad, CA) and treated with DNase I (Ambion, Austin, TX) to remove contaminating DNA. cDNAs were synthesized from 2 μg of total RNA,

using Superscript II RNase H reverse transcriptase (Invitrogen, Carlsbad, CA). Expression of Bmp4 and p27kip1 were detected by real-time PCR using an ABI Prism 7700 sequence detection system and SYBR Green PCR mastermix (Applied Biosystems, Foster City, CA). Expression of each target was measured in triplicate. Relative quantification was determined by using the comparative CT method with 18s expression as a control, as described in the ABI Prism 7700 sequence detection system user bulletin. The following primers were used for real time PCR: 18s, 5'-CGGCTACCACATCCAAGGAA-3' and 5'-GCTGGAATTACCGCGGCT-3'; Bmp4, 5′-CAACACCATGATTCCTGGTAACC-3′ and 5′-TCCCGGTCTCAGGTATCAAACT-3′; p27kip1, 5′-CGGCGGCAAGGTTTGGAGAGG-3′ and 5′GGAGGAGGCAGGAGGAGGTGG-3′.

Statistical analysis:

Two-sample, unpaired t-tests, non-parametric Mann-Whitney U tests, and one way ANOVA followed by Tukey's multiple-comparison posttest were performed using Graph Prism software. In cases where n>5, Shapiro-Francia test was performed for normality. t test was performed if the normality test was not significant. Mann-Whitney U test was used if normality test was significant or n<5. For time-course studies, the standard error (SE) used in the t-test was estimated by ANOVA, and two-sample tests were performed at individual time points. To control for false positives, Bonferroni adjusted p-values at individual time points are reported. A value of $p < 0.05$ was used as the cut off for statistical significance.

RESULTS

Ovariectomized mice exhibit delayed bacterial clearance from the bladder

To determine whether removal of ovaries affects the UPEC pathogenic cycle, we generated ovariectomized (OVX) mice, which exhibited reduced estrogen levels, and sham-operated controls (SHAM). We then infected the bladders of adult OVX and SHAM mice with UPEC and monitored the progress of the UTI by measuring shedding of bacteria into the urine (bacteriuria) for two weeks (schema in Fig. 1). We found that OVX mice exhibited significantly higher and

more sustained bacteriuria, evidenced by higher bacterial load at three to ten days post infection (dpi), than SHAM mice, who cleared the infection by day three (Fig. 2A). This finding indicates that removal of ovaries results in a prolonged UTI.

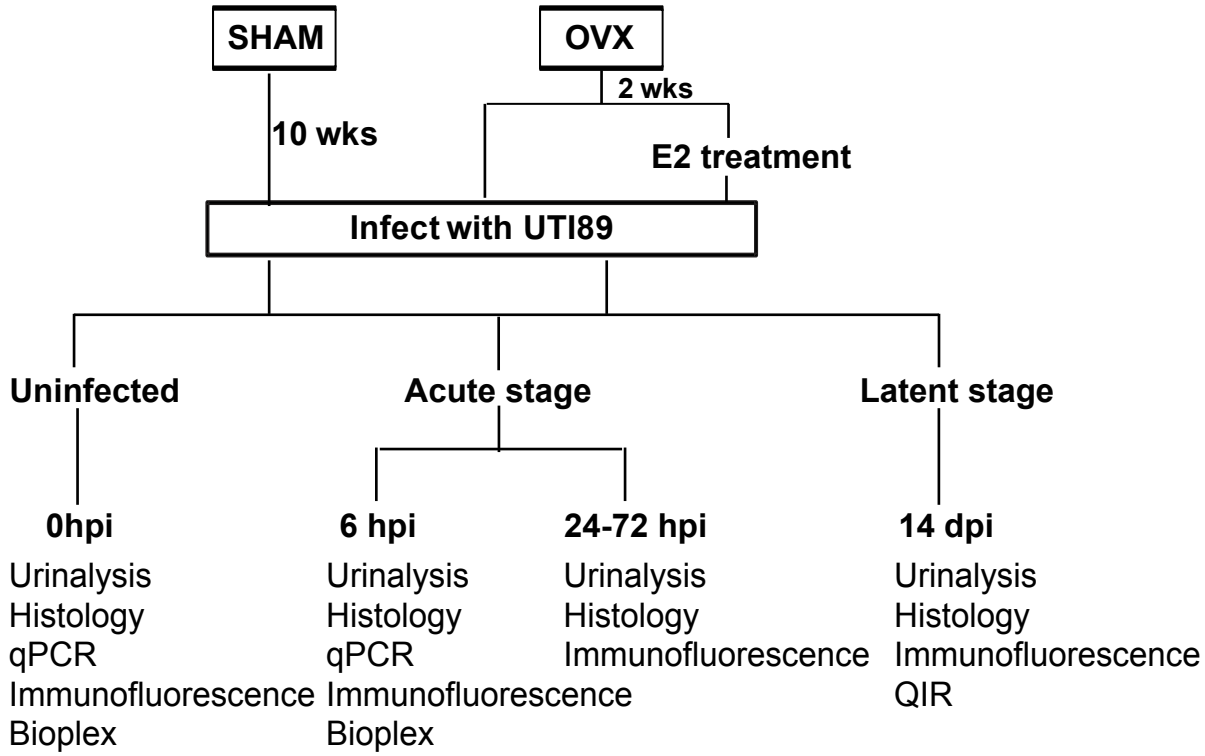


Figure 1: Experimental design: Diagram illustrating experimental strategy.

Ovariectomy decreases exfoliation of superficial urothelial cells

One of the first host responses to UPEC infection in the bladder is exfoliation of the superficial urothelial cells containing IBCs into the urine. Although the majority of superficial cells were sloughed into the urine in SHAM mice at 24 hours post infection (hpi) (Fig. 2B), a relatively unperturbed superficial layer was evident in OVX mice (arrow in Fig 2C; note the thickness of the superficial cell layer marked by expression of uroplakin III [UPIII]). Additionally, UPEC staining of the bladders indicated that more IBCs remained in the intact superficial cells in OVX mice (arrowheads in Fig. 2E) than in SHAM mice (Fig. 2D) at this time point. Thus, ovary removal resulted in decreased exfoliation of infected superficial cells at the acute stage (24 hpi), which might contribute to the prolonged UPEC infection.

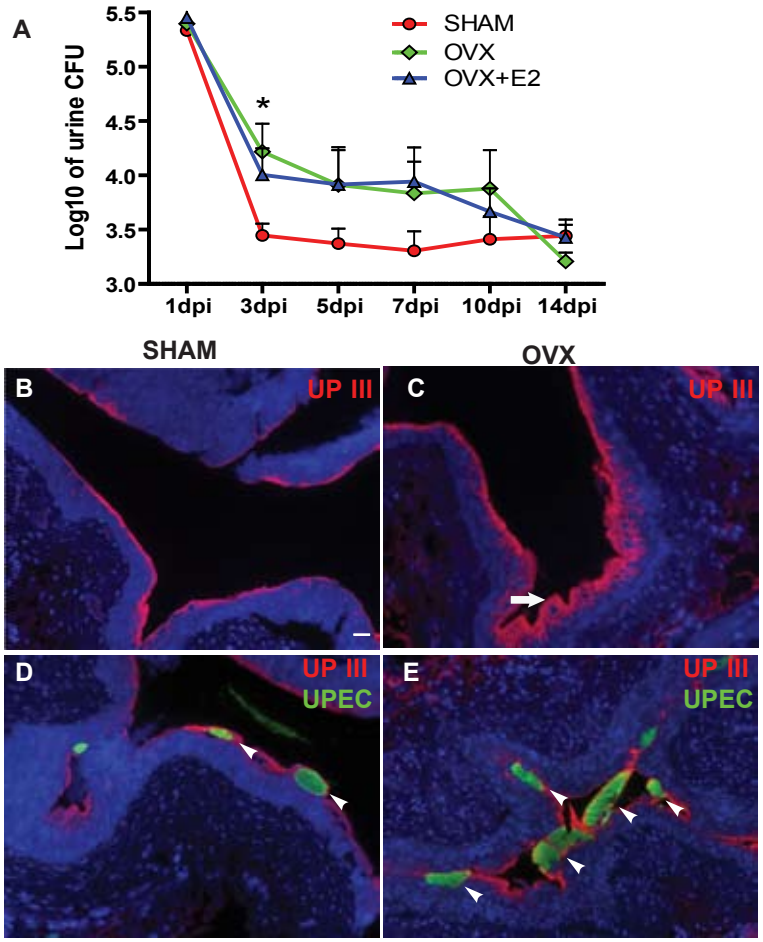


Figure 2: Ovariectomized mice exhibit prolonged infection: (A) CFU counts of bacteriuria over a timecourse in SHAM, OVX, and OVX+E2 mice plotted as mean ± SEM of the Log10 value. n=6-12 mice/timepoint/group in 2 experiments. * $p<0.05$ between SHAM and OVX mice by two-way ANOVA with Bonferroni post test. (B-E) immunofluorescence (IF) analysis reveals more UpIII+ (red) superficial cells in OVX mice than SHAM mice at 24 hpi (B-C) as well as more intracellular UPEC communities (green)(D-E). Bar=20µm.

Estrogen-deficient mice display a severe proinflammatory response upon UPEC infection

In addition to the sloughing of superficial cells, the host responds to the pathogen by inducing pro-inflammatory cytokines including IL-6 (Hunstad et al., 2005) and recruiting immune cells, such as neutrophils, to the infected site. Cytological analysis of urine samples of infected mice revealed greater extent of neutrophil infiltration in the urine at 24 and 72 hpi in OVX mice than in SHAM mice (Fig. 3A). Next, we performed cytokine assays using Bioplex bead arrays on sera

to determine whether increased influx of neutrophils was associated with higher production of cytokines in OVX mice. We observed significantly higher levels of IL-6 in sera from infected OVX mice than from SHAM mice at 6 hpi (Fig. 3B), suggesting an overall enhanced systemic and luminal pro-inflammatory response to infection in OVX mice.

To determine whether bladder tissue of OVX mice was prone to greater inflammation, we examined bladders from SHAM and OVX mice at 24 hpi and quantified the level of tissue inflammation. We found that bladders from UPEC-infected OVX mice exhibited higher inflammation scores than SHAM mice (Fig 3C). Histopathological analyses revealed more severely inflamed bladder tissue in OVX mice with greater immune/inflammatory influx including neutrophils (arrow in Fig. 3E) and severe edema (arrowhead in Fig. 3E) than bladders from SHAM mice (Fig. 3D). Together,

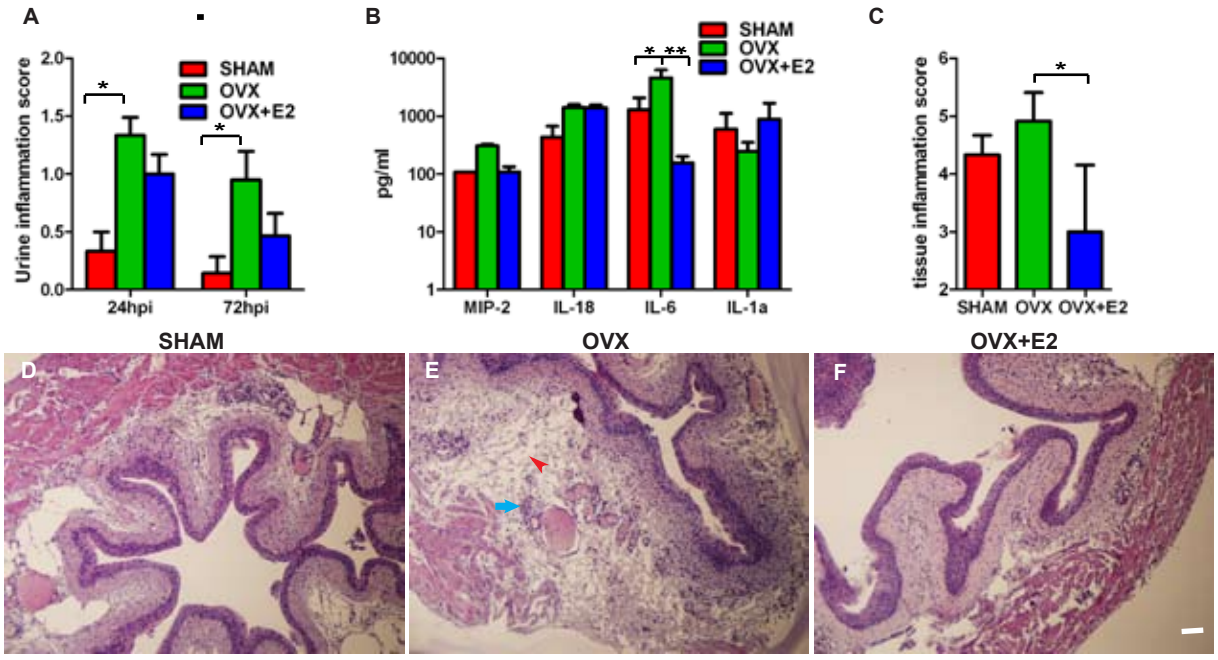


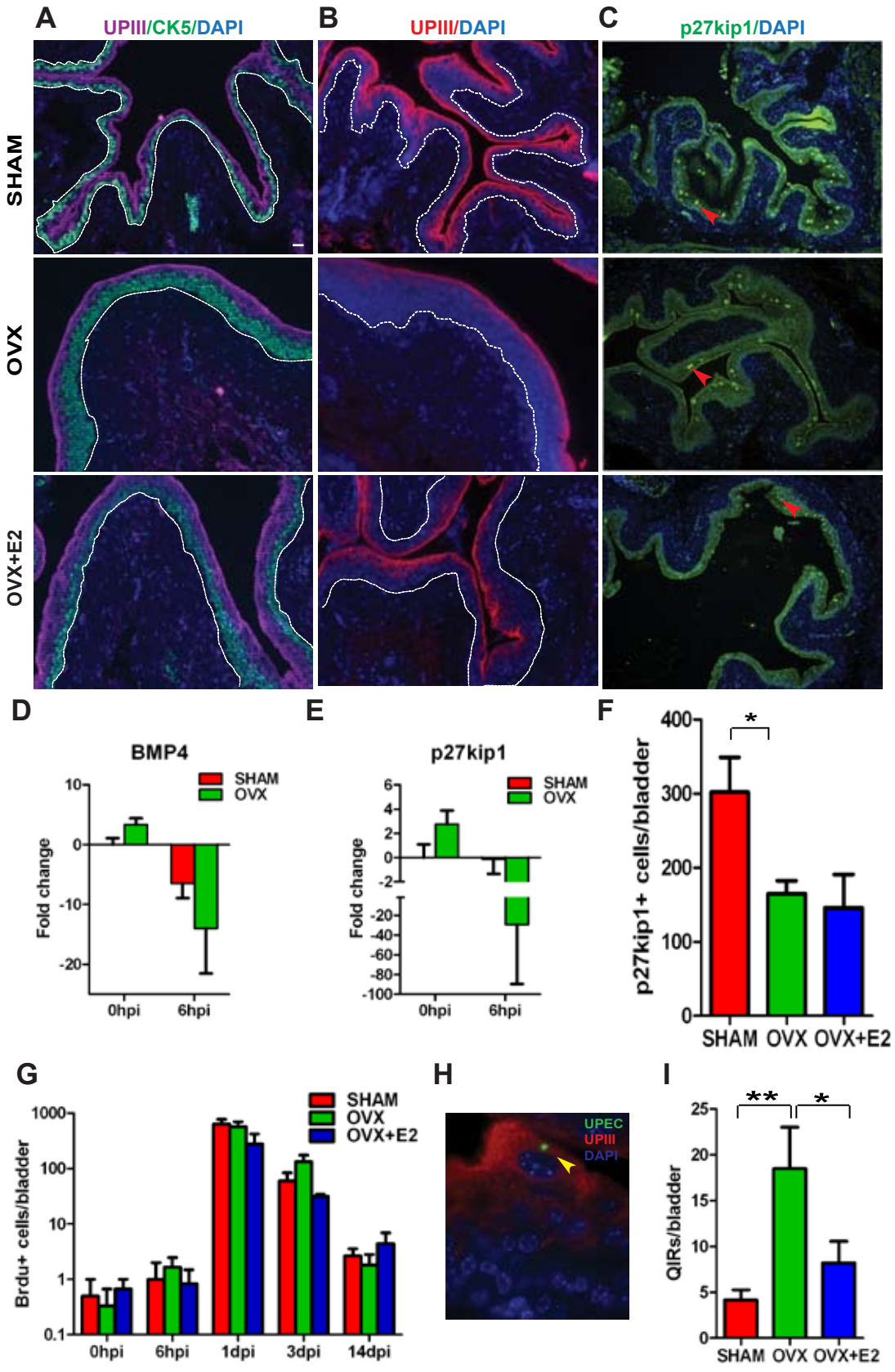
Figure 3: Ovariectomized mice display a severe proinflammatory response upon UPEC infection: (A) Urine inflammation scores are higher in UPEC-infected OVX mice at 24 and 72 hpi. * $p<0.05$ by Mann Whitney U test. (B) Bioplex cytokine assay of sera reveals more IL-6 secreted in UPEC-infected OVX mice at 6 hpi, reversible upon E2 supplementation. * $p<0.05$, ** $p<0.01$ by one way-ANOVA followed by Tukey's multiple-comparison posttest. (C) Inflammation scoring of bladder tissue at 24 hpi reveals higher scores in OVX mice, reversible upon E2 supplementation. * $p<0.05$ by Mann Whitney U test. (D-F) Hematoxylin & Eosin staining of bladders from SHAM, OVX and OVX+E2 mice at 24 hpi depicts greatest inflammation (arrow points to neutrophils) and edema (arrowhead) in OVX mice. Bar=40 µm.

these results suggest that OVX mice mount a more robust and prolonged proinflammatory response to UPEC infection than SHAM mice.

Ovariectomization affects USC niche response to UPEC infection

Superficial urothelial cell loss and associated inflammation induced by UPEC infection cause damage to urothelial barriers that are critical for bladder function. To restore homeostasis, the urothelium activates a regeneration response fueled by activation of the urothelial stem cell (USC) niche and proliferation of the basal urothelial layer. Niche activation and terminal differentiation into mature superficial urothelial cells is dependent on the Bmp4 signaling pathway (Miyazaki et al., 2003; Mysorekar et al., 2009). We sought to determine whether estrogen deficiency affected the USC response to infection. Because the predominant estrogen receptor in the bladder, ERβ, is mainly expressed in the basal USC layer (Imamov et al., 2007), we speculated that the basal USC layer is most likely to be affected by estrogen deficiency. We found that OVX mice exhibited a thickened CK5+ (a marker for basal cells (Mysorekar et al., 2009)) cell layer at 14 dpi than SHAM mice and correspondingly, a thinner UPIII+ superficial cell layer (Fig. 4 A-B).

The thickened basal cell layer in OVX mice could be a result of increased and sustained USC proliferation or a defect in terminal differentiation. To determine whether there was a block in terminal differentiation, we assessed expression of Bmp4 pathway components. q-PCR analysis revealed that expression of Bmp4 and its downstream target p27kip1 were both down-regulated in OVX mice after UPEC infection (Fig. 4D-E). We also found that bladders from OVX mice had fewer p27kip1-positive nuclei than SHAM mice (Fig. 4C, F), suggesting that these cells were not exiting the cell cycle to terminally differentiate. We assessed proliferation by BrdU labeling and found no significant differences in proliferative activity between SHAM and OVX mice (Fig. 4G), indicating that the thickened basal cell layer in OVX mice was not due to increased proliferation. Together, our findings suggest that the Bmp4 pathway was down-regulated, and the differentiation of basal cells to superficial cells was blocked by ovariectomy, thus impairing the USC niche adaptation to infection.


A
UPIII/CK5/DAPI
B
UPIII/DAPI
C
p27kip1/DAPI
SHAM
OVX
OVX+E2
D
BMP4
SHAM
OVX
Fold change
10
0
-10
-20
0hpi
6hpi
E
p27kip1
SHAM
OVX
Fold change
6
4
2
0
-2
-20
-40
-60
-80
-100
0hpi
6hpi
F
p27kip1+ cells/bladder
400
300
200
100
0
*
SHAM
OVX
OVX+E2
G
Brdu+ cells/bladder
1000
100
10
1
0.1
SHAM
OVX
OVX+E2
0hpi
6hpi
1dpi
3dpi
14dpi
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UPEC
UPIII
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QIRs/bladder
25
20
15
10
5
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SHAM
OVX
OVX+E2

Figure 4: Estrogen deficiency leads to aberrant urothelial regeneration and increased bacterial reservoir formation: (A) CK5 and Uroplakin III staining of bladders at 14 dpi depicting thickened CK+ (green) cell layer in the OVX urothelium. (B) IF analysis depicts reduced Uroplakin III + (red) staining in OVX bladders at 14 dpi. Dotted lines demarcate epithelium from bladder stroma. Bar=20μm. (C) p27kip1 staining of bladders at 14 dpi indicates fewer p27kip1+ cells in OVX urothelia. Bar=40μm. (D-E) q-RTPCR analysis reveals Bmp4 (D) and p27kip1 (E) gene expression is downregulated upon UPEC infection at 6 hpi in OVX mice. (F) Quantification of p27kip1+ nuclei reveals reduced numbers in OVX bladders at 14 dpi. Bars represent mean ± SEM. *$p<0.05$, by unpaired two-tailed T test. (G) BrdU counts at the indicated times after infection. n=3-6 mice/time point/condition. Bars represent mean ± SEM. (H) Representative image depicting a quiescent intracellular reservoir (QIR), Bar=10 μm. (I) Quantification of QIRs in bladders at 14 dpi reveals highly significant increased number in OVX mice and this is reversible upon E2 supplementation. n= 6 sections/bladder, 10-15 mice/group, n=3 experiments. * $p<0.05$, ** $p<0.01$ by one way-ANOVA followed by Tukey's multiple-comparison posttest.

Estrogen deficiency enhances UPEC persistence

Even after epithelial exfoliation and proinflammatory responses eliminate the majority of intracellular bacteria, a subset of UPEC can survive and establish long-term reservoirs, termed quiescent intracellular reservoirs (QIRs), which serve as seeds for recurrent infection (Mysorekar and Hultgren, 2006). We thus examined SHAM and OVX bladders at 14 dpi to determine whether enhanced bacterial colonization in the bladders of OVX mice at the acute stage of infection was associated with increased establishment of QIRs. Because UPEC can establish reservoirs containing small numbers of bacteria that are below the detectable limit in bladder titers (Mysorekar and Hultgren, 2006), we investigated QIR formation by immunostaining bladder sections with antibodies to UPEC and Lamp-1, a marker for the vesicles in which QIRs are found (Mysorekar and Hultgren, 2006; Wang et al., 2012a) (Fig. 4H). Bladders of OVX mice harbored significantly more QIRs in Lamp1-positive vesicles than bladders of SHAM mice (Fig. 4I). Thus, estrogen deficiency is associated with increased establishment of persistent bacterial reservoirs.

Estrogen replacement therapy overall restores bladder response to UPEC to pre-menopausal state

Ovariectomy results in decreases in both estrogen and progesterone levels. Thus, to establish whether the effects of OVX we observed were mediated exclusively by estrogen, we supplemented a cohort

of OVX mice with 17β-estradiol (OVX+E2) for 8 weeks before induction of a UTI and followed the pathogenic cycle as described above. Exogenous administration of E2 did not significantly affect the bacterial loads in the urine (Fig. 2A). We next asked whether the immunological responses of the OVX mice to infection were rescued by estrogen supplementation. We observed a slight, but not significant, decrease in neutrophil infiltration in the urine at 24 and 72 hours after UPEC infection (Fig. 3A). However, we observed significantly lower IL-6 serum levels (Fig. 3B) and low inflammation levels in the bladder mucosa (Fig. 3C, and compare 3F to 3E) in OVX+E2 mice than OVX mice. Additionally, we did not observe a thickening of the CK5+ basal cell layer in OVX+E2 mice (Fig. 4A). Together, these findings suggest that the increased and sustained inflammation and tissue damage observed in OVX mice is, at least in part, due to estrogen deficiency.

Finally, we asked whether estrogen supplementation could reduce the number of latent QIRs that formed in OVX mice. We found that bladders from OVX mice that received E2 supplementation contained fewer QIRs than mice that were ovariectomized but did not receive E2 supplementation (Fig. 4I). In fact, at 14 dpi, OVX mice receiving E2 before infection harbored similar numbers of QIRs as SHAM mice, indicating that the enhanced QIR formation we observed in OVX mice was largely due to estrogen deficiency.

DISCUSSION

Here, we employ a murine model of surgical menopause by ovariectomy to demonstrate that estrogen plays a protective role in regulating the host response to UPEC infection. We show that ovariectomization results in prolonged and more severe infection that is associated not only with increased and sustained bacteriuria, but also with elevated inflammation and significantly greater number of persistent bacterial reservoirs. We further demonstrate that estrogen deficiency is associated with an aberrant tissue regenerative response wherein restoration of urothelial barriers is delayed considerably. Thus, our model has shown that critical aspects of disease pathogenesis are under estrogenic control: namely, tissue restoration and regeneration following infection-induced injury, inflammatory response to UPEC, and importantly, UPEC persistence in the bladder wall.

We propose that our model lays the groundwork for exploring the mechanisms of estrogenic action and for testing hormone therapy efficacy.

Estrogen and immune response

High estrogen levels have potent anti-inflammatory functions, including repression of proinflammatory gene transcription and cytokine production such as IL-6 (Galien and Garcia, 1997; Liu et al., 2005; Ray et al., 1997; Straub, 2007). The anti-inflammatory effects of estrogens have been observed in several disease models, including autoimmunity, atherosclerosis, arthritis, inflammatory bowel disease, asthma and influenza (Jansson et al., 1994; Kim et al., 1999; Robinson et al., 2011). Our data are consistent with an inhibitory role of estrogen on IL-6 production (Deshpande et al., 1997; Galien and Garcia, 1997) and presence of systemic elevated IL-6 levels in menopausal women (Straub, 2007). A heightened IL-6 response may play a role in the pathogenesis of UTIs in menopausal patients similar to other chronic inflammatory diseases such as rheumatoid arthritis (Straub, 2007). Our data suggest that estrogen might contribute to the functional integrity of the bladder barrier by quenching the inflammatory response associated with UTI. Excessive IL-6 or production of other proinflammatory cytokines can result in urothelial damage that may affect disease outcome. It has been previously shown that elevated and sustained levels of serum cytokines including IL-6 along with IL-5, G-CSF, and KC (biomarkers of local and systemic acute inflammation) precede the development of chronic cystitis and that the early immune events serve as a 'checkpoint' for predicting infection outcomes (Hannan et al., 2010). The elevated early IL-6 response in OVX mice may predispose the mice to the prolonged high-titre UPEC urine load noted. This window of time may be key in setting the stage for disease outcome and/or UTI recurrence. Our data, together with other studies in postmenopausal women (Kim et al., 2012), suggests that IL-6 levels may be a major biomarker of chronic inflammatory activity in postmenopausal state.

Estrogen, urothelial stem cell niche activation, and recurrent UTIs

UPEC can persist indefinitely as quiescent reservoirs within the immature basal cells of the bladder and can re-emerge to seed recurrent UTIs (Mysorekar and Hultgren, 2006). The basal cells are

the early progenitor cells and serve as a protective niche in which UPEC can escape immune detection and evade exfoliation. Here, we showed that hypo-estrogenization induces a thickened basal cell layer due to the disrupted differentiation process of basal cells to superficial cells. OVX mice not only displayed low estrogen levels, but the expression of Bmp4 and its downstream target p27kip1 were down-regulated in these mice. Because the Bmp4 pathway is required for USC niche activation and differentiation (Mysorekar et al., 2009), this result may explain why the basal cell layer was thicker in OVX than SHAM mice after UPEC infection. The mechanisms by which Bmp4 signaling is modulated by estrogen in response to UPEC infection in the bladder are unclear, but one possibility is that Bmp4 signaling may be modulated by the glycosaminoglycan (GAG) layer covering the urothelium. GAGs are large linear polysaccharides with a high degree of structural heterogeneity mediated by GAG biosynthetic enzymes (Taylor and Gallo, 2006). GAGs are known to modulate growth factors (Esko and Lindahl, 2001; Garcia-Garcia and Anderson, 2003; Lamanna et al., 2007). We have previously shown that levels of a GAG-sulfating enzyme, HS6ST1, a key modulator of Bmp4 signaling, are increased upon infection (Mysorekar et al., 2002). Additionally, we have shown that estrogen plays a key role in influencing the GAG thickness and the increased expression of GAG sulfation enzymes over the course of UPEC infection (Anand et al., 2012). Together, this leads us to speculate that increased sulfation of GAGs may modulate the down-regulation of Bmp4 signaling and thereby the USC niche response to infection.

UTI recurrence may depend on UPEC's ability to manipulate differentiation and proliferation of USCs. Thus, the thickened USC layer may provide more protective niches for QIRs to form. These QIRs may hide in the bladder longer due to the slower turnover rate from basal cells to superficial cells. Traditional antibiotic therapies are not effective against bacteria sequestered in QIRs and can also increase the risk of driving the pathogens into quiescence (Blango and Mulvey, 2010). As these reservoirs are a source of recurrent UTIs, any reduction in their establishment and greater understanding of the interplay between latency and estrogen signaling will have great significance for studying infectious disease in aging female populations.

Estrogen therapy and UTIs

Our results suggest that hormone therapy is beneficial to OVX mice with UTI and this beneficial effect may be due to the downregulation of the proinflammatory response and the homeostasis of urothelium promoted by the presence of exogenous estrogen. While further studies using multiple UPEC strains would be valuable, we propose that our findings provide an explanation for why menopausal women may be at greater risk for recurrent UTIs. In postmenopausal women, efficacy of estrogen supplementation in UTI prevention has relied upon alterations in bacteriuria loads as a measure of success. Our findings showing that estrogen supplementation does not affect bacteriuria may offer an explanation for the contradictory reports and are consistent with those of Curran and co-workers who did not observe changes in bacterial load in the bladder (Curran et al., 2007) as well as another study demonstrating that estrogen's effect on disease outcome was independent of influenza viral load (Robinson et al., 2011). Although testing different levels of E2 supplementation and the short term and long term consequences of therapy remain to be determined, our work suggests that estrogen therapy may be beneficial to women with recurrent UTIs and could have implications for reducing the burden of this infectious disease in aging populations. For example, longer term estrogen therapy with transiently increased doses during an acute episode of UTI might be beneficial. Our data may have significant clinical implications for understanding the etiology of recurrent UTIs in menopausal women and warrant further studies for potential usage of estrogen as a therapeutic intervention.

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RESEARCH EXPERIENCE

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AWARDS AND HONORS

2013 **Travel Award** for ASCI/AAP/APSA Joint Meeting conference

2013 **Rita Levi-Montalcini Award for Best Student Poster Presentation,** Developmental, Stem cell, and Regenerative Biology Program Retreat, Washington University in St. Louis School of Medicine

2008 **MSTP Merit Award,** Washington University in St. Louis School of Medicine

2007 **William R. Belknap Prize** for Excellence in Biology, Yale University

MEMBERSHIPS AND SERVICE

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2009 **Coordinator, Summer Health Screenings,** Student National Medical Association, Washington University in St. Louis Medical School Chapter.

PRESENTATIONS

2014 July **Poster presentation,** Gordon Research Conference on Microbial Toxins and Pathogenesis, Waterville Valley, NH.

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PEER-REVIEWED PUBLICATIONS

Symington JW, Wang C, Twentyman J, Owusu-Boaitey N, Schwendener R, Nuñez G, Schilling JD, Mysorekar IU. ATG16L1 deficiency in macrophages drives clearance of uropathogenic E. coli in an IL-1β-dependent manner. Mucosal Immunol. 2015 Nov; 8(6):1388-99.

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