Immunity to Uropathogens: The Emerging Roles of Inflammasome

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Abstract

Urinary tract infections (UTIs) cause a huge burden of morbidity worldwide with recurrence of UTIs becoming significantly frequent due to the emergence of antibiotic-resistant bacterial strains. Recent research has focussed on interactions between the innate and adaptive immune responses to pathogens colonizing the urinary tract. Inflammasomes are part of the innate immune defense and respond rapidly to several infectious diseases. Assembly of the multiprotein inflammasome complex activates Caspase-1, processes proinflammatory cytokines IL-1β and IL-18, and induces pyroptosis. These effector pathways, in turn, act at different levels to either prevent or resolve infection, or eliminate the infectious agent itself. Whilst in certain instances inflammasome activation promotes tissue pathology, the precise functions of inflammasomes in UTIs remain unexplored. In this review, we discuss recent studies on the roles of inflammasomes in UTIs with a particular focus on common infections of the urinary tract. An improved understanding of inflammasomes may provide valuable novel approaches for the design of diagnostics and therapeutics for complicated UTIs, thus enabling us to counteract the challenge of drug resistance.

Introduction

Urinary tract infections (UTIs) represent some of the most common bacterial infections, affecting 150 million people each year worldwide. UTIs, which affect the bladder, ureters, kidneys, and urethra, are particularly common in women, and the associated total healthcare costs exceed US$3 billion per annum in the United States alone[1–4]. Recurrence of urinary infections, which occurs in approx. 25-30% of women[5–8], has particularly made it challenging to tackle infections of the urinary tract, with limited treatment options due to the emergence of multidrug resistant strains[9–13]. UTIs are clinically subdivided into uncomplicated and complicated infections. Uncomplicated UTIs typically occur in healthy individuals, and include those that affect the lower urinary tract (cystitis) or those that affect the upper urinary tract (pyelonephritis), whereas complicated UTIs typically affect those that have a compromised urinary tract or host defence[2]. The majority of complicated UTIs are catheter-associated, and are linked to an increase in morbidity and mortality. Complicated UTIs may also be associated with other predisposing conditions including urinary retention due to neurological diseases, renal failure and transplantation, urinary obstruction, and pregnancy[2]. A variety of Gram-positive and Gram-negative bacteria, as well as fungi, have been implicated in infections of the urinary tract. The most common causes of both uncomplicated and complicated UTIs are uropathogenic *Escherichia coli* (UPEC)[14,15]. Other common infectious agents include *Staphylococcus saprophyticus, Klebsiella species*, *Enterococcus faecalis*, group B *Streptococcus* (GBS), *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida species*[2,14].

The urothelium is the first line of defence against UTIs, as epithelial cells provide both a physical and an immunological barrier to avert infection and further trigger the innate immune system. Given the variety of pathogens that can infect the urinary tract, recent work has focused on the immune response during the establishment and clearance of UTIs, which is not only important to understand the mechanisms of recurrent infections, but also to identify

alternative strategies in light of increasing antimicrobial resistance. The innate immune system, consisting of pattern recognition receptors (PRRs) and their downstream effectors, helps to protect against pathogens in the urinary tract[16–19]. PRRs are germ-line encoded and recognise conserved microbial structures known as pathogen associated molecular patterns (PAMPs), or danger associated molecular patterns (DAMPs) released by damaged host cells. The PRRs can be classified into two major families based on their localization. The membrane-bound Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) primarily survey the extracellular space. Conversely, intracellular pathogens are sensed by cytoplasmic receptors, and includes RIG-I-like receptor (RLR), the AIM2-like receptor (ALR), and the nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs)[19–23]. Relevant to this review, NLRs have been shown to be expressed in the bladder epithelium, with expression of NLRP3 and NLRC4 being reported previously[24,25]. Further studies have confirmed these results, and additionally reported the expression of NLRs such as NLRP1, 6, 7 and 12 and ALR family member AIM2[26]. The location and expression of these receptors in the urothelium indicates that the bladder has the potential to initiate immune responses to a wide variety of uropathogens, in addition to being involved in sterile inflammatory responses. NLRs and few ALRs form important components of inflammasomes, multimeric protein complexes consisting of ASC and Caspase-1 that assemble in response to PAMPs and DAMPs.

Despite pivotal roles of inflammasomes in infection, our understanding of inflammasome signaling in different infections is imperfect, and is mainly limited to a few well-studied microbes. In particular, infections of the urinary tract remain a major cause of morbidity and mortality worldwide; however, the function of inflammasome signaling in UTIs has only recently been suggested. Below, we describe our present knowledge of the NLRP3 complex followed by current and emerging evidence on the impact of inflammasomes and their downstream effectors in the pathogenesis of UTIs. Notably, although several pathogens capable of colonizing the urinary tract activate inflammasomes in macrophages, not all

uropathogens have been investigated in the context of inflammasome activation in the urinary tract. Thus, we will limit the discussion to studies of three important uropathogens, *Escherichia coli*, Group B *Streptococcus* and *Pseudomonas aeruginosa*, where direct or indirect impact to the urinary tract has been reported. Understanding the role of the inflammasomes may also lead to new diagnostic and therapeutic approaches for UTIs, and these will also be briefly discussed with regards to IL-1β and NLRP3.

Inflammasomes and pyroptosis

The human genome contains 22 NLRs which are characterized into three different sub-families based on the N-terminal domain present. Members NLRP1, NLRP3 and NLRC4 form inflammasomes in response to their specific ligands or upstream stimuli. AIM2, which belong to the ALR family (PYHIN family), also forms an inflammasome upon sensing any double-stranded DNA (dsDNA) which is at least 80bp in length[27–31]. Other NLRs have also been reported to assemble inflammasomes under specific conditions but these await further characterization[32–36]. In the majority of cases, formation of the inflammasome assembly is dependent on apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), an adaptor protein that is crucial for the recruitment of Caspase-1 into the complex[37]. Activation of inflammasome results in the cleavage of the enzyme pro-Caspase-1 to its active form, which subsequently cleaves the precursor forms of inflammatory cytokines interleukin (IL) -1 beta (IL-1β) and IL-18[38–40]. Inflammasome activation also triggers a type of inflammatory cell death known as pyroptosis, a process dependent on Caspase-1 mediated cleavage of gasdermin D[41,42]. Pyroptosis, which is morphologically distinct from apoptosis, is characterised by cell swelling followed by membrane rupture, which releases inflammatory cell contents in the extracellular milieu; these features are not observed during apoptosis although molecules Caspase-8 and FADD have overlapping functions in both the processes[43–47]. Pyroptosis, and production of IL-1β and IL-18 cytokines, are caspase-1 dependent and these two events coincide upon inflammasome activation, although under certain conditions, cytokine secretion can also be observed without the induction of

pyroptosis[43]. While pyroptosis results in the elimination of the pathogen niche, IL-1β and IL-18 serve important proinflammatory and chemotactic functions including induction of adhesion molecules and influx of immune cells to the site of infection[43,44,48–50].

Activation of the NLRP3 inflammasome

Out of all inflammasomes, NLRP3 is by far the most well characterised inflammasome complex to date and plays vital roles in both infectious and inflammatory conditions. This inflammasome is triggered by a wide variety of microbial (lipopolysaccharide, bacterial hemolysins and toxins), endogenous (ATP, uric acid, cholesterol), and exogenous (silica, alum, asbestos) ligands[51–54], and requires 2 signals for its activation, at least in mouse macrophages. The first signal is provided upon ligation of TLRs by microbial stimuli, which initiates the production of pro-IL-1β and NLRP3 via NF-κB signalling and gene induction. The second signal is provided by above PAMPs and DAMPs, which convene the NLRP3 assembly for processing pro-Caspase-1. Recently, a non-canonical version of the NLRP3 inflammasome has been described that additionally involves Caspase-11 functioning upstream for Caspase-1 processing[55–57]. Non-canonical Caspase-11–dependent inflammasome is triggered in response to Gram-negative, but not Gram-positive, bacterial infection in mouse cells[55–57]; humans lack Caspase-11 but homologous Caspase-4 and Caspase-5 compensate its absence. Subsequent studies demonstrated direct sensing of LPS by Caspase-11 leading to the activation of the non-canonical inflammasome[58]. Notably, Caspase-11, even in the absence of the NLRP3 complex, can directly cleave gasdermin D leading to pyroptosis[41] **(Figure 1)**. While a plethora of molecules are known to trigger canonical NLRP3, it is unlikely that such a wide variety of ligands are all able to bind NLRP3 directly. Instead, it is generally accepted that these stimuli induce one or more downstream cellular events, which in turn prompt NLRP3 complex formation. While the exact pathway of NLRP3 activation is yet to be elucidated, several mechanisms have been proposed. These include the release of cathepsins as a result of phagosomal membrane destabilisation[52],

potassium efflux[59,60], the production of mitochondrial reactive oxygen species (ROS)[61–63], and release of mitochondrial DNA or cardiolipin[64,65]. Undoubtedly, elucidation of the activation mechanisms is crucial to gain further understanding into the role of the NLRP3 inflammasome in several infectious diseases. Equally, the key role of NLRP3 in inflammatory and autoimmune diseases cannot be ignored. Naturally occurring mutations in the NLRP3 (*CIAS1*) gene are associated to the progression of autoinflammatory conditions known collectively as *C*ryopyrin-*A*ssociated *P*eriodic *S*yndromes (CAPS), which are characterized by recurrent rash, fever/chills, conjunctivitis, and fatigue[66]. Besides CAPS, studies in the past decade have also implicated NLRP3 in many other inflammatory diseases, including type 2 diabetes, gout, atherosclerosis, rheumatoid arthritis and inflammatory bowel disease[40,51,53,67–69]. Therefore, not only does the NLRP3 inflammasome serves as a defence mechanism against invading pathogens, its dysregulation may also be a contributing factor in inflammatory diseases.

Innate immune responses during UPEC infection

Uropathogenic *E. coli* (UPEC) is estimated to cause up to 80% of all UTIs[2]. The pathogenesis of UPEC invasion in the urinary tract has been well characterized. UPEC mediates adhesion to specific receptors on the uroepithelium by utilizing Type I fimbriae and a variety of surface associated adhesins. Although predominantly extracellular, several reports agree that UPEC do invade superficial bladder epithelial and kidney epithelial cells and the internalization does not appear to be actively directed due to the absence of any identified bacterial effectors [70,71]. Once intracellular, it has been suggested that UPEC are harboured within specialized exocytic vesicles which, upon increase in urine volume, fuse with the apical membrane of epithelial cells in a TLR4-triggered cAMP-dependent mechanism. While this process plays an important role in host defence mechanism leading to bacterial expulsion by the epithelial cells, it also performs a critical physiological function by providing the requisite membranes for bladder expansion[72]. More frequently, UPEC elude expulsion by escaping into the cytoplasm, where they replicate within biofilm-like intracellular bacterial communities (IBCs)[73]. Thus, exfoliation

of UPEC infected superficial epithelial cell layer is the predominant mechanism for termination of acute UPEC infection. However, exfoliation exposes deeper urothelial layers for invasion, wherein a subset of UPEC may establish themselves into quiescent intracellular reservoirs (QIRs). Such QIRs possess characteristics of a membrane-bound compartment and allow bacteria to survive for months[74,75]. UPEC in QIRs remain refractory to systemic antibiotic therapy and serve as a source of recurrent UTIs.

The innate immune responses to UPEC in the urinary tract have been extensively investigated[76]. Introduction of *E. coli* in the mammalian urinary tract elicits a robust inflammatory response resulting in the release of inflammatory cytokines such as TNF-α and IL-6, in addition to the recruitment of neutrophils and macrophages[77,78]. This immune response is mainly dependent on TLRs, with studies showing an importance for TLR4, TLR5, TLR11 (in mice) and downstream cytokines, such as IL-8, in the pathogenesis of UTIs[79–82]. Indeed, deficiency in *Tlr4* and *Tlr5* exacerbates urinary infection in experimental mouse models, highlighting the importance of these PRRs in UPEC recognition and immune activation[77,80,83]. Furthermore, individuals with genetic mutations in these pathways are more susceptible to UTIs[84]. Additionally, polymorphism in TLRs has also been associated to UTI susceptibility[85]. Thus, it is evident that innate immune recognition of UPEC and other urinary pathogens by PRRs is vital for host clearance and resolution.

Activation and suppression of inflammasome by UPEC

Although our understanding of TLR-pathways critical to UTIs has developed over the past few decades, a role for inflammasome activation has only recently been highlighted in UPEC infections. Studies have previously shown that other strains of *E. coli*, such as enterohemorrhagic *E. coli,* are able to activate the NLRP3 inflammasome[86]. Recently, Schaale *et al* demonstrated that the pore-forming cytotoxin α-hemolysin of several strains of UPEC activates the NLRP3 inflammasome and induces IL-1β production[87] **(Figure 1)**. Interestingly,

it was revealed that inflammasome activation between different strains of UPEC varied, with those that did not activate the inflammasome being either highly drug-resistant or associated with asymptomatic infection. In contrast, UPEC strains which did activate the inflammasome caused symptomatic infections leading to enhanced urinary tract pathology[87]. Approx. 50% of UPEC isolates encode hemolysin, and in agreement with the above findings, strains expressing the virulence factor display more extensive damage to the urothelium and bladder haemorrhage during infection[88,89]. Consequently, α-hemolysin –expressing strains may contribute to severe clinical outcomes in some individuals. However, whether some of the UPEC strains have lost the ability to activate the inflammasome, or developed means to inhibit it, in order to avoid immune detection remains to be determined. Nevertheless, inflammasome activation appears to contribute to the inflammatory pathology in several UPEC strains and may be responsible for the signs and symptoms observed in human cystitis.

Activation of the NLRP3 by UPEC was also demonstrated in a recent study which further identified a putative inflammasome inhibitor expressed by UPEC[90]. Infection of macrophages with UPEC CFT073 strain, which expresses a major virulence factor and a TLR-signaling inhibitory protein, Toll/IL-1 receptor-containing (TIR-containing) protein C (TcpC), triggered marginal NLRP3 assembly. CFT073 infection at low multiplicity of infection resulted in minimal IL-1β secretion, which increased dramatically upon infection with a mutant bacteria that was not expressing TcpC (Δ*tcpC*) **(Figure 1)**. Mechanistically, TcpC dampened pro-Caspase-1 processing and thus IL-1β production by chelating directly with NLRP3 and Caspase-1, thereby effectively prohibiting the NLRP3 complex formation[90]. In a murine model of UTI, challenge with Δ*tcpC* resulted in elevated IL-1β secretion in the urine compared to mice infected with wild-type UPEC strain suggesting that UPEC TcpC dampens multiple immune pathways. The role that the NLRP3 inflammasome plays against UPEC infection remains to be fully determined, however it appears that IL-1β may contribute to host defence, as IL-1β deficient mice revealed higher bacteria titres in the urine. Therefore, inhibition of NLRP3-

dependent IL-1β production by TcpC may be beneficial to UPEC during bladder infection, and may help establish acute cystitis in individuals. Whether this response is common to all strains of UPEC is yet to be determined. However, 40-50% of UPEC strains isolated from patients with pyelonephritis, and around 20% of UPEC strains isolated from patients with cystitis are known to harbour the TcpC gene[91,92]. Hence, the frequency of TcpC-positive UPEC isolates seems to correlate with the severity of the urinary tract disease. Though, whether the UPEC TcpC is expressed differentially in a tissue-specific manner is not known, thus precluding identification of the disease where TcpC-dependent inflammasome inhibition may be most relevant.

Inflammasome-mediated pyroptosis promotes urothelium exfoliation

In comparison to its detrimental role described above, α-hemolysin and inflammasome-dependent pyroptosis has been suggested to be advantageous to the host in the early response to UPEC. α-hemolysin has been observed to activate caspase-1/caspase-4, leading to pyroptosis of urothelial cells[93]. Exfoliation and regeneration of the urothelium is thought to eliminate adherent IBCs. The shedding of urothelial cells is dependent on Caspases[94], and additionally relies on serine proteases to degrade cytoskeletal scaffold proteins[95]. Recent studies have highlighted a role for NLRP3 and IL-1β in this response. Indeed, overexpression of α-hemolysin triggered NLRP3-dependent urothelial cell death, which was inhibited in the presence of Caspase-1 and Caspase-4 inhibitor Ac-YVAD-CHO[93] **(Figure 2)**. This overexpression reduced the incidence of chronic cystitis, indicating that exfoliation of the urothelium serves to eliminate UPEC and thereby prevent bacterial persistence[93]. In contrast, it has been argued that exfoliation may infrequently allow UPEC to attach to and invade the exposed urothelial layers[96] **(Figure 2)**. Supporting this, studies have demonstrated that inhibition of Caspase-1/11 is protective against chronic cystitis in mouse models of UPEC superinfection[97]. Superinfection, or repeat introduction of bacteria, augments the risk of chronic cystitis, and in part explains the increased risk of UTI with frequent sexual intercourse,

as this may serve to reintroduce bacteria from periurethral flora into the urinary tract. Thus, it has been argued that Caspase-1/Caspase-11 -dependent exfoliation may have a role in predisposition to recurrent UTI infection. Overall, these studies suggest that inflammasome-dependent pyroptosis promotes urothelium exfoliation which may enable clearance or latency. Additionally, exfoliation may also be advanced by Caspase-3 dependent apoptosis, and this further contributes as a defence mechanism against bacterial attachment and invasion[94,98]. Therefore, urothelium exfoliation is supported by distinct cell death pathways. It is intriguing to consider that compromise in barrier integrity at the time of exfoliation may also permit unresolved UPEC infection access to the underlying urothelial layers.

Autophagy and inflammasome activation in UPEC clearance and latency

Autophagy is an intracellular degradation system that enables the cell to recycle cytosolic components such as damaged organelles and proteins. In the last decade, studies have also proposed autophagy as a major mechanism for the removal of intracellular pathogens, in addition to its role in the inflammatory process and the adaptive immune response[62,99–102]. Activation of autophagy (macroautophagy) is a multi-stage process that initiates with the formation of a membrane phagophore which elongates to a preautophagosomal structure which finally matures into an autophagolysosome. Each stage of autophagosome elaboration requires concerted effort of a number of autophagy-related proteins. The Atg12-Atg5-Atg16L1 complex and the LC3 lipidation system are critical for expansion and closure of the autophagosomal membrane structure, and deficiency in any of the molecules in these pathways leads to defective autophagic degradation[103,104]. By employing genetic tools lacking key components of the autophagosome formation, a vital role for autophagy has been demonstrated in microbial clearance, inflammasome activation and IL-1β production[105]. Recently, a role for autophagy has been proposed in UPEC infection[106,107]. Mice carrying an ATG16L1 hypomorphic allele ($Atg16L1^{HM}$), causing reduced ATG16L1 expression, cleared UPEC more effectively than wild-type mice, indicating a detrimental role of autophagy in this infection. Further, Atg16L1 expression was found to be critical in radio-sensitive cells of the

hematopoietic compartment, and macrophages carrying the defective allele (Atg16L1^{HM} macrophages) demonstrated increased bacterial uptake at 1h post-infection. Intriguingly, comparable UPEC load was observed at later times post-infection arguing that the dynamics of bacterial killing are not altered, and that Atg16L1^{HM} macrophages enhance UPEC degradation only during the early stages of infection.

In addition to processing damaged organelles and infectious agents, specific autophagic pathways have been demonstrated to target ubiquitinated inflammasomes and pro-IL-1β for degradation[108,109]. In agreement with this observation, ATG16L1^{HM} macrophages and mice secreted more IL-1β, which was dependent on the NLRP3 inflammasome, and played key roles in UPEC clearance *in vitro* and in mice infected transurethrally[106,107]. Consequently, treatment with anakinra, an IL-1 receptor antagonist (IL-1Ra), abrogated protection to UPEC in Atg16L1^{HM} mice. Besides suggesting an important role of IL-1β signaling in UPEC clearance, these data add to increasing evidence that autophagy dampens down the exaggerated inflammatory response that cannot be resolved. In contrast, in other models of infection and inflammation, defective autophagic function may be detrimental. In particular, defective autophagy was shown to enhance susceptibility to bacterial infection, and worsened dextran sulphate sodium (DSS)-induced acute colitis[102,105,110]. Therefore, the role of autophagy in UPEC colonization may be specific to pathogen and the experimental model employed. Indeed, the above response was not observed in Atg16L1^{HM} macrophages infected with commensal *E. coli,* which displayed comparable invasion and growth. Another argument favouring the detrimental role of autophagy in UPEC infection is supported by the apparent function of autophagy in promoting UPEC persistence. UPEC establishes latency in membrane-bound QIRs (discussed above), and these were found to be less abundant in the epithelial cells of *Atg16l1*-deficient mice[107]. This implies that UPEC survive in autophagy-competent cells by establishing QIRs **(Figure 3)**, although it remains unclear if UPEC hijacks some of the pre-existing autophagosomes to gain this advantage. Overall, the data indicate a

role for autophagy in the persistence of UPEC, with the loss of ATG16L1 preventing UPEC latency. Thus, reduced persistence and enhanced inflammasome activation observed in *Atg16l1*-deficiency may potentially work together to alleviate UTI burden. It is intriguing to speculate that IL-1β signaling may have a direct role in the destabilisation of QIRs. Furthermore, although the associated detrimental features of lack of autophagic process cannot be ignored, these studies suggest that humans with naturally occurring mutations in the *Atg16l1* gene may have advantage in resolving UPEC infection.

IL-1β secretion and NLRP3 activation in Group B Streptococcal infections

Group B *Streptococcus* (GBS) or *Streptococcus agalactiae*, a Gram-positive bacterium, is a leading cause of disease in neonates, pregnant women, and immunocompromised individuals[111–115]. A limited number of serotypes are associated with invasive infection, and cervicovaginal colonisation can result in transmission from mother to child during pregnancy. Several GBS serotypes are also associated with UTIs, resulting in bacteraemia, cystitis and pyelonephritis[116,117]. Predisposing factors for urinary GBS infection include diabetes mellitus and chronic renal failure[116]. The innate immune system plays a significant role in anti-GBS host defences *in vivo*, and has been shown to induce the production of proinflammatory cytokines TNF-α and Type I IFNs[118,119]. Recently, studies have begun to highlight the importance of inflammasome activation and signalling in GBS infections. Ulett *et al.* first highlighted the induction of IL-1α and IL-1β in the initial stages of GBS urinary infections in mice[114]. The role of IL-1α in inflammasome mediated responses has not been fully established, however its secretion has been shown to coincide with IL-1β following stimulation with known inflammasome activators[120]. This secretion was shown to occur both in an NLRP3-dependent and inflammasome-independent manner[120]. Mice challenged with uropathogenic GBS induced robust production of IL-1β, to an extent similar to that seen upon infection with UPEC. Interestingly, IL-1α production in response to GBS was significantly higher than that induced by UPEC, and could be induced by both uropathogenic and non-uropathogenic GBS strains[114], therefore indicating that the response may be unique to this pathogen. Alternatively,

less IL-1α secretion following UPEC infection may be due to the ability of this pathogen to suppress some of the inflammatory pathways in order to avoid innate immune responses[121]. The critical role for the NLRP3 inflammasome *in vivo* was described by subsequent studies where it was revealed that mice deficient in core components of the NLRP3 complex display enhanced mortality concomitant with elevated GBS burden in the blood and kidneys of intraperitoneally-infected mice[122]. Similar to this, a separate study demonstrated increased susceptibility of *Il-1r* –deficient mice to GBS infection[123]. Collectively, these studies indicate that inflammasome activation by GBS is not only important in the initial inflammatory response, but is also required to control the infection *in vivo*.

The mechanism of NLRP3 activation during GBS infections has been examined in detail. GBS is able to induce both the synthesis of pro-IL-1β, and secretion of IL-1β and IL-18 cytokines in bone-marrow derived macrophages and dendritic cells (DCs), with increased cytokine production observed in DCs[122]. Although live GBS has been shown previously to induce Type-I interferon production following release of GBS DNA in the cytosol[124], IL-1β secretion by GBS was absolutely dependent on the NLRP3 inflammasome with no role observed for the DNA-triggered AIM2 inflammasome[122]. Furthermore, activation of the NLRP3 by GBS is dependent on β-hemolysin as GBS strains lacking this toxin were unable to process pro-Caspase-1 or secrete IL-1β **(Figure 1)**. In contrast, infection with hyperhemolytic GBS strains resulted in significantly enhanced IL-1β secretion. Other studies have emphasized the importance of β-hemolysin-mediated lysosomal leakage in inflammasome activation following interaction between GBS RNA and cytosolic NLRP3[125] **(Figure 1)**. Accordingly, transfection of biotinylated-RNA pulled-down more NLRP3 from cells infected with GBS NEM 316 wild-type strain than following infection with β-hemolysin-deficient NEM 2459 strain[125]. These experiments suggest the indispensable nature of β-hemolysin-mediated lysosomal damage in NLRP3 inflammasome activation by GBS, although the direct contribution of lysosomal cathepsins needs reassessment. Moreover, NLRP3 has never been demonstrated to bind

RNA directly and DExD/H-box RNA helicase family members, particularly DDX19A and DHX33, have been proposed to mediate this interaction[126,127]. Overall, further studies are needed to determine the precise activation mechanisms of inflammasomes and their impact on disease pathogenesis during GBS infection of the urinary tract.

NLRC4 inflammasome activation by *Pseudomonas aeruginosa*

Risk factors for infections with non-UPEC organisms include recurrent UTIs, the presence of foreign bodies, obstruction, urinary catheters and male gender[128]. *Pseudomonas aeruginosa* is the third most common infectious agent associated with nosocomial catheter-associated UTIs, in addition to causing a variety of other acute infections[129,130]. Because of their ability to form biofilms on the surface of catheters, *P. aeruginosa* are able to access and colonise the bladder. In addition, they pose a significant challenge as such biofilms are more resistant to host defence mechanisms and antimicrobial agents[131], leading to persistent infections and an overall habitat that fosters establishment of antimicrobial resistant strains[132,133].

NLRC4 forms a well-characterized inflammasome by interacting with Caspase-1, inducing the production of IL-1β and leading to cell death via pyroptosis[134]. Studies have shown that Gram negative bacteria such as *Legionella pneumophila* and *Salmonella typhimurium* are able to activate the NLRC4 inflammasome, which was later attributed to detection of flagellin in the cytoplasm[135–138]. Further studies demonstrated that NLRC4 could also be activated via type III secretory system (T3SS), which is used to inject effector proteins into the cytoplasm, thereby enhancing their bacterial virulence[37,139,140]. *P. aeruginosa* has been shown to activate the NLRC4 in a manner similar to *L. pneumophila and S. typhimurium* via introduction of flagellin into the cytoplasm, a process that is dependent on the T3SS[141]. Later studies revealed that distinct NAIP family members, NLR proteins which contain a BIR domain, are the true cytosolic receptors that act upstream of NLRC4 to trigger this complex. NAIP5 and NAIP6 recognize the cytosolic presence of flagellin, while NAIP1 and NAIP2 recognize bacterial needle and

inner rod proteins of the T3SS, respectively[142,143]. Rod proteins from the T3SS apparatus, such as PrgJ from *S. typhimurium,* share a similar secondary structure to the D0 domain of flagellin, which is the domain responsible for activation of NLRC4[144,145]. However, flagellin-deficient strains of *P. aeruginosa* also activated the NLRC4 suggesting the presence of other NAIP-NLRC4 triggers[146]. This was attributed to the T3SS inner rod protein PscI of *P. aeruginosa* **(Figure 1)**, which is homologous to *Salmonella* PrgJ[145]. Additionally, pilin, the major component of type IV pilus of *P. aeruginosa*, has also been suggested to activate NLRC4 leading to IL-1β secretion[147]. Inflammasome activation has been examined in a lung model of acute *P. aeruginosa* pneumonia where activation of the NLRC4 was associated with increased lung pathology, cell death, and impairment of microbial clearance[148]. The increased pathology in the lung diminished upon infection with *P. aeruginosa* mutants that lacked flagellin b (Δ*fliC*) or in *Nlrc4*-deficient mice infected with wild-type strain. In accordance, mice deficient in *Il-1r* cleared lung infection rapidly compared to control wild-type mice[149]. These studies suggest that reduced inflammasome signalling may be beneficial for *P. aeruginosa* clearance and immune homeostasis in the lung tissue.

Similar detrimental roles of IL-1β have been observed in a mouse model of ascending pyelonephritis following infection with *P. aeruginosa.* Elevated levels of IL-1β were observed in both the urine and renal tissue starting at day 1 post-infection which peaked at day 5[150]. Intriguingly, mice infected with biofilm cells of *P. aeruginosa* produced more IL-1β (and TNF-α) than those infected with planktonic bacteria. While this correlated with the influx of neutrophils, the recruited neutrophils did not contribute to resolution of infection. Instead, higher bacteria titres were found in mice infected with biofilms, suggesting that biofilms are resistant to neutrophil-mediated cell death[150]. Closer histopathological evaluation associated increased neutrophil infiltration to severe inflammation in the renal tissue along with shedding of cells and vascular permeability in biofilm-infected mice. Again, enhanced IL-1β may be implicated in the pathogenesis of *P. aeruginosa* infections. Since this study also demonstrated

the elevated levels of TNF-α, inflammasome-independent effects of IL-1β on the renal tissue cannot be excluded. Whether *P. aeruginosa* major virulence factor, flagellin, could be used for protection against ascending infection of the urinary tract has been investigated by a study. Immunisation of mice with non-adjuvanted flagellin 'b', which is encoded by the *fliC* gene and does not undergo antigenic variation, led to reduced IL-1β production in mice subsequently infected with *P. aeruginosa* compared to control unimmunised mice[151]. Consequently, immunised mice also displayed enhanced clearance of the bacteria in renal tissue which was associated to improved gross morphology of kidney sections. In addition, neutrophil recruitment to the kidney seemed to fade after the second booster dose of flagellin b, and the kidney tissue displayed no signs of any degradative or inflammatory signs compared to greater inflammation observed in unimmunised mice[151]. Therefore, vaccination with flagellin in this context seems to limit inflammation and help prevent UTI infection. Whether this represents a viable target for prophylactic therapy, particularly in the setting of recurrent UTIs, remains to be determined.

IL-1β as a diagnostic marker in acute pyelonephritis

Acute pyelonephritis is a severe infection of the kidney and can lead to long-term local and systemic effects, including renal scarring and hypertension especially in children[152,153]. Acute pyelonephritis is also a major cause of end-stage renal failure globally, therefore prompt diagnosis and accurate treatment is key. However, clinical diagnosis of acute pyelonephritis can be difficult as symptoms are similar to those experienced during severe infection of the lower urinary tract, such as urinary frequency, urgency and painful urination (dysuria). Patients with acute pyelonephritis may also experience nausea, vomiting, fever and flank pain, in addition to general malaise[154]. As with other bacterial infections, an increase in leukocyte counts, neutrophil counts, erythrocyte sedimentation rate, and C-reactive protein values may help to differentiate acute pyelonephritis from a severe lower UTI, but with varying sensitivity and specificity[155].

It has been suggested that IL-1β production could be useful as a marker to discriminate between the acute pyelonephritis and lower UTIs[155]. Numerous clinical studies have demonstrated increased production of IL-1β in acute pyelonephritis and other urinary infections[156–158], although patients with acute pyelonephritis appear to have significantly higher levels of IL-1β in their urine than patients with lower UTI[155,158]. A similar increase in IL-1β levels have been observed in the serum of patients with acute pyelonephritis, and the elevated cytokine, in both serum and urine, returns to normal levels following antibiotic therapy. Interestingly, these findings also corroborate with studies conducted in the late 1990s that reported increased IL-1β mRNA levels in the kidneys of mice infected with *E. coli* and subjected to urethral obstruction[159]. Additionally, studies in a mouse model of experimental pyelonephritis, where infection was performed by urethral catheterization, suggest a favourable role for IL-1β, as deficient mice displayed more frequent and widespread inflammatory changes than their wild type counterparts 48 hours after infection. Similarly, while IL-1β is important for the induction of inflammatory mediators and thus might be expected to promote renal scarring, the cytokine was unexpectedly found to reduce scarring, which increased in mice lacking *Il-1β*[160]. Remarkably, these effects were due to the enhanced production of neutrophil chemoattractant MIP2 (also known as CXCL-2) in *Il-1β* deficient mice[160]. The increased influx of neutrophils may therefore play a role in the inflammatory changes seen in *Il-1β*-deficient mice following acute pyelonephritis. These studies thus suggest that IL-1β promotes not only pro-inflammatory chemokines, but may also induce anti-inflammatory mediators. Additionally, this suggests an important role for IL-1β in the initial stages of pyelonephritis. Although cleavage, and thus maturation, of pro-IL-1β to its bioactive form depends on Caspase-1 in the inflammasome, inflammasome-independent pathways of IL-1β processing have been suggested. These include neutrophil- and macrophage-derived serine proteases such as proteinase 3 (PR3), elastase, Cathepsin G, and chymotrypsin amongst others[161,162]. Whether IL-1β production in pyelonephritis is indeed dependent on inflammasome activation or is processed by Caspase-1-independent mechanism has not

been examined. Similarly, whether renal tubular cells possess enhanced propensity for inflammasome activation or IL-1β production is not known. Taken together, these studies indicate the central role for IL-1β in the acute inflammatory response to a variety of urinary pathogens and potential for IL-1β levels to be used as a diagnostic marker to distinguish between severe lower UTI and acute pyelonephritis.

NLRP3 as a pharmacological target in bladder infection

Inflammation of the bladder, or cystitis, is a contributing factor in many bladder-associated pathologies. The most common bladder-associated condition are UTIs, although the bladder is also susceptible to sterile inflammation induced by bladder outlet obstruction, bladder stones, chemotherapy treatment, and chronic inflammation caused by conditions such as interstitial cystitis[163]. These conditions share similar symptomology, with frequency, urgency and pelvic pain being common, making it difficult to distinguish the more complicated interstitial cystitis. Whether these diseases converge on common inflammatory pathways is yet to be established, however recent research has focussed on the contributing factors of the innate immunity, in particular the role of TLRs and NLRs. NLRs are expressed in the bladder, and particularly NLRP3, NLRC4, and key inflammasome components were demonstrated to be expressed in the bladder urothelia[26]. In agreement with their roles, introduction of NLRP3- and NLRC4-specific PAMPs and DAMPs into the bladder lumen of mice induced the activation of these receptors resulting in pro-Caspase-1 processing[26]. Again, these results demonstrate the ability of the uroepthelium to mount an inflammatory response to pathogens, in particular to flagellin, which is a common virulence factor among uropathogens[164].

Lipopolysaccharide (LPS) is also an important virulence factor of uropathogens[165], leading to the discovery of a role for NLRP3 activation in an LPS-induced rat model of cystitis[166]. LPS injected directly into the bladder epithelium, in order to bypass the glycosaminoglycan layer lining the bladder lumen, elicited increased Caspase-1 activation in the urothelium and elevated levels of IL-1β in the urine compared to rats injected with saline control. In these

experiments, levels of IL-1β were detectable in the urine 24h post LPS exposure, which coincides with separate studies showing the production of IL-1β mRNA in the bladder in the cystitis model[166,167]. Furthermore, inhibition by glyburide, a NLRP3 inflammasome inhibitor, attenuated levels of both Caspase-1 activation and IL-1β production[166]. Additionally, glyburide treatment reduced bladder weight and extravasation of Evan's blue dye into bladder tissue, two well-documented indices of bladder inflammation, which were significantly increased in rats treated with LPS alone[166]. Moreover, exposure to LPS (and also saline), significantly reduced void volume and intercontraction intervals; these parameters were restored upon treatment with glyburide. In addition, voiding- and threshold- pressure also decreased upon LPS injection, although only the former was found to be NLRP3-dependent. These data indicate that inflammasome activation may play a role in bladder dysregulation, however its exact role in urodynamic changes, particularly in humans, warrants further investigation. Similar to the above study, NLRP3 activation was also observed in a cyclophosphamide-induced rat model of cystitis[25]. Although no infectious agent is involved in this model, cyclophosphamide metabolite acrolein induces necrosis and apoptosis in urothelia resulting in the generation of DAMPs which trigger NLRP3. Collectively, these data indicate a role for the NLRP3 inflammasome in induction of inflammation within the bladder regardless of the nature of the trigger. Glyburide, which is a sulfonylurea drug approved for the treatment of diabetes, is also a NLRP3 inflammasome inhibitor and represents a viable pharmacological agent to target bacterial cystitis. However, it is important to be aware that the precise functions of inflammasomes are context dependent. Thus, whether these results are also faithfully recapitulated during actual infection by uropathogens needs to be investigated before the translational potential of targeting inflammasomes can be exploited.

Conclusions

UTIs remain a major health problem worldwide with recurrence occurring in approximately 25% of all patients. The cellular architecture of the urinary tract, which is enforced with tight

epithelial barriers, is assembled to oppose pathogen invasion. In addition, the mild antiseptic properties of urine contribute to inhibition of microbial growth. While most microbes do not survive, some of them colonize and cause infection in the urinary tract. The innate immune responses to uropathogens are chiefly mediated by TLRs and NLRs. In particular, the roles of inflammasome-assembling NLRs in UTIs are becoming apparent, and it is vital to continuously evaluate their therapeutic potential as we make further progress. Can inflammasomes be targeted for treatment of UTIs? What are the possible approaches? Should we focus on the direct inhibition of inflammasome assembly, or does targeting downstream effector signaling offers better therapeutic outlook? Our current state of understanding requires significant expansion before this can be realized. Inflammasome activation leads to processing of biologically inactive forms of cytokines IL-1β and IL-18. In patients with UTIs, elevated IL-1β levels in the urine had been recognised for some time; however, the precise role that this cytokine played in the pathogenesis of urinary infections remained enigmatic. Studies over the past two decades have indicated vital roles of IL-1β in resolving UTI and in dampening inflammatory changes in the kidney. Meanwhile, lack of *Il-1β* is linked to elevated renal scarring in mouse models of experimental pyelonephritis. Whether IL-1β secretion in these studies is absolutely dependent on inflammasomes is not known. In contrast, activation of the NLRP3 inflammasome in the murine bladder epithelium enhances inflammatory pathology. Thus, the variability in these studies needs to be addressed to develop inflammasomes as a potential pharmacological target.

Drugs that directly target inflammasomes have been identified. Type 2 diabetes drug glyburide is a specific NLRP3 inhibitor and abrogates pathology in a mouse model of UTI[166]. Likewise, a compound, MCC950, has been found to selectively inhibit both the canonical and non-canonical NLRP3 inflammasomes[168]. However, these drugs inhibit all inflammasome functions. Notably, inflammasomes play both favourable and detrimental roles. Consequently, it is imperative to design therapeutics targeting each of the distinct inflammasome functions, thereby allowing systematic management and treatment of UTIs while still retaining the

inflammasome-dependent host-beneficial functions. Furthermore, a comprehensive portrayal of the NLR responses in the urinary tract is required in order to design improved therapeutics. Finally, an emerging theme from the studies suggest that a balance of inflammasome activation is key to effectively respond to uropathogens. Understanding fundamental mechanisms that regulate inflammasomes in the urinary tract, and the impact of inflammasome signaling in disease pathogenesis during UTIs should be the ultimate goal for future studies.

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Key Points

- NLRP3 inflammasome is activated during infection with UPEC in an α-hemolysin-dependent manner, and exaggerated activation of this inflammasome may cause symptomatic infection.
- Inflammasome-dependent pyroptosis promotes exfoliation of UPEC-infected urothelial cells, thus serving to resolve the infection and reduce the incidence of chronic cystitis. However, this may also increase latency by permitting microbial access to deeper urothelial layers.
- Autophagy dampens IL-1β release and endorses UPEC latency by promoting the establishment of quiescent intracellular reservoirs (QIRs), which are significantly reduced in cells defective in autophagic process.
- Group B *Streptococcus* activates the NLRP3 inflammasome which is dependent on β-hemolysin-mediated lysosomal leakage and RNA escape; however, the mechanistic details remain unclear.
- IL-1β levels are markedly enhanced during acute pyelonephritis, and may serve as a diagnostic marker for acute pyelonephritis.
- Inflammasome activation and IL-1β secretion play significant roles in various murine models of bladder inflammation, and represent viable pharmaceutical targets.

Figure Legends

Figure 1. Activation of inflammasomes by uropathogens.

Uropathogenic *E. coli* enters the urinary tract and can activate TLR4 and MyD88-dependent signaling. Phagocytosis of UPEC activates the NLRP3 inflammasome in a α-hemolysin-dependent manner, leading to Caspase-1 dependent maturation of pro-inflammatory cytokines IL-1β and IL-18. Significant percentage of UPEC strains also encode an inhibitory protein TcpC, which may directly inhibit NLRP3 assembly formation. Group B *Streptococcus* also activates the NLRP3 inflammasome and this process is dependent on β-hemolysin mediated lysosomal rupture. While it remains unknown whether the release of Cathepsins augment inflammasome activation, NLRP3 activation by the released RNA has been observed. *Pseudomonas aeruginosa* activates the NLRC4 inflammasome and this is accomplished by the introduction of flagellin and the presence of PscI, the inner rod protein of *P. aeruginosa* T3SS. IL-1β production is significantly elevated upon infection with *P. aeruginosa* biofilms. Activation of inflammasomes also leads to Caspase-1 mediated cleavage of gasdermin D, which triggers pyroptotic cell death. Notably, in the non-canonical inflammasome, Caspase-11 can also directly cleave gasdermin D leading to pyroptosis without the assembly of the NLRP3 complex.

Figure 2. Pyroptosis promotes urothelium exfoliation in UPEC infection.

Recognition of UPEC by TLRs at the urothelial cell surface activates NF-kB and MAP-kinase pathways leading to the production of pro-IL-1β. Activation of the NLRP3 inflammasome by UPEC α-hemolysin triggers pyroptosis of urothelial cells and subsequent shedding of the urothelium thereby eliminating adherent and intracellular bacterial communities (IBCs). Though exfoliation is the predominant mechanism for termination of acute UPEC infection, this may also enable dissemination of UPEC to inner urothelial layers where the pathogen

may reside in quiescent intracellular reservoirs (QIRs), which serve as a site of latency for recurrent UPEC infections.

Figure 3. Autophagy dampens IL-1β release and promotes recurrent UPEC infection. Infection by UPEC activates the NLRP3 inflammasome. Mechanisms of NLRP3 inflammasome activation include potassium efflux, release of Cathepsin B from damaged lysosomes, or generation of reactive oxygen species (ROS). The NLRP3 inflammasome or the precursor form of IL-1β can be degraded by autophagosomes. Thus, autophagy negatively regulates inflammasome activation and IL-1β release. In the case of UPEC infection, autophagy may promote persistence and establishment of quiescent intracellular reservoirs (QIRs) which enable recurrent UPEC infections.

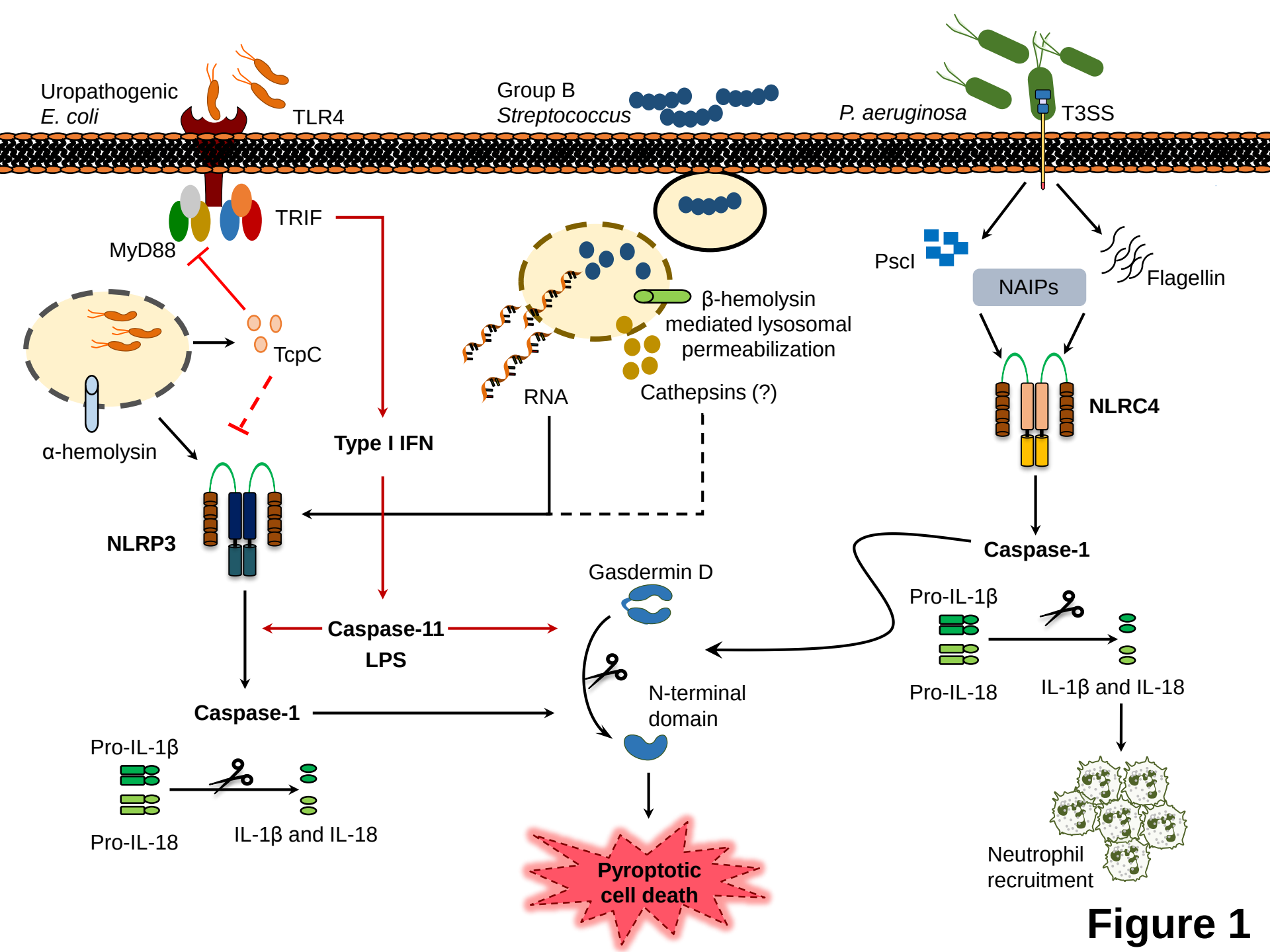
Uropathogenic
E. coli
TLR4
Group B
Streptococcus
P. aeruginosa
T3SS
TRIF
MyD88
PscI
NAIPs
Flagellin
β-hemolysin
mediated lysosomal
permeabilization
TcpC
RNA
Cathepsins (?)
α-hemolysin
Type I IFN
NLRC4
NLRP3
Caspase-1
Gasdermin D
Caspase-11
LPS
Pro-IL-1β
Pro-IL-18
IL-1β and IL-18
Caspase-1
N-terminal
domain
Pro-IL-1β
Pro-IL-18
IL-1β and IL-18
Pyroptotic
cell death
Neutrophil
recruitment

Figure 1

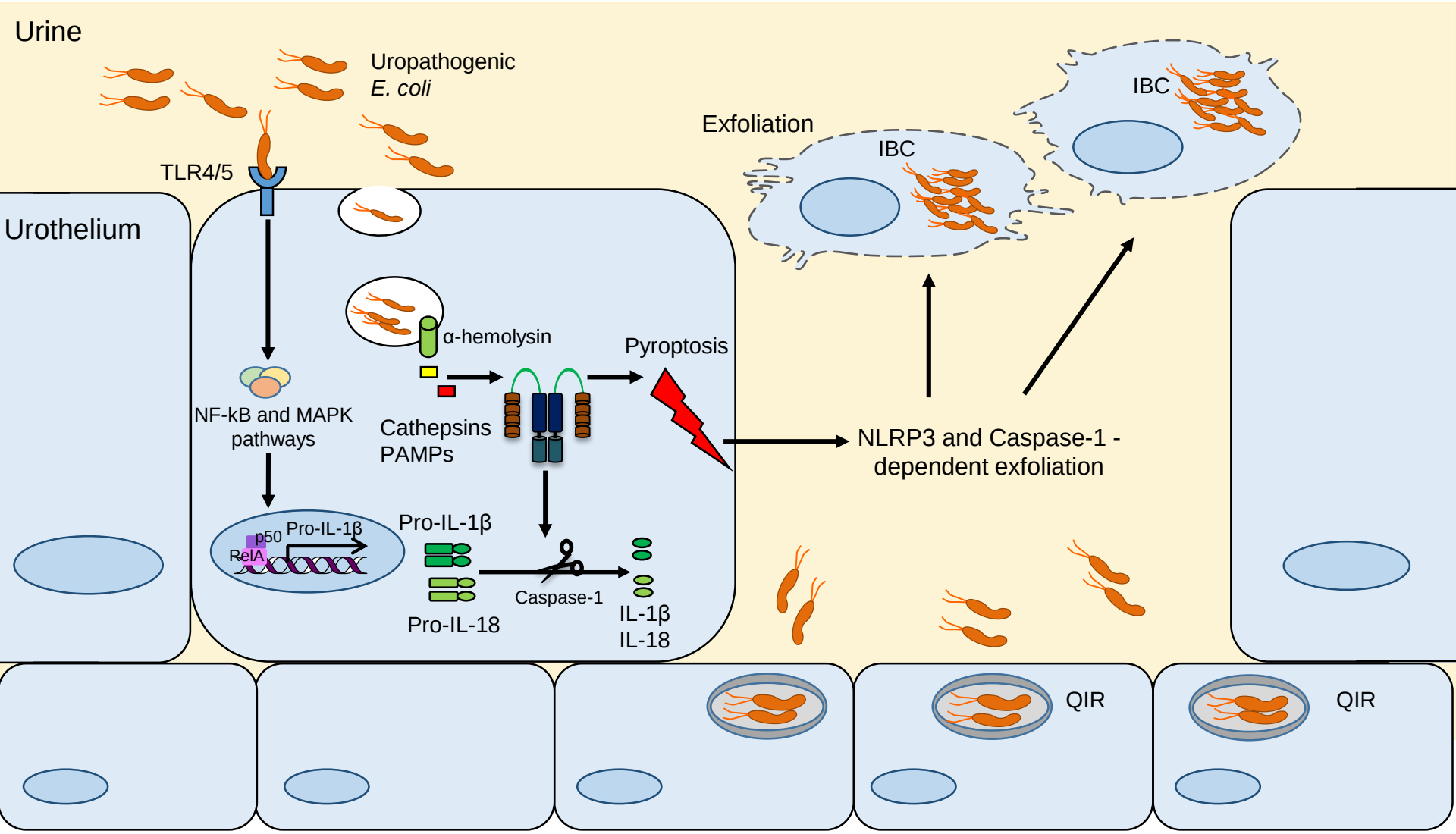
Urine
Uropathogenic
E. coli
TLR4/5
Urothelium
α-hemolysin
Pyroptosis
NF-kB and MAPK
pathways
Cathepsins
PAMPs
p50
RelA
Pro-IL-1β
Pro-IL-1β
Caspase-1
Pro-IL-18
IL-1β
IL-18
Exfoliation
IBC
IBC
NLRP3 and Caspase-1 -
dependent exfoliation
QIR
QIR

Figure 2

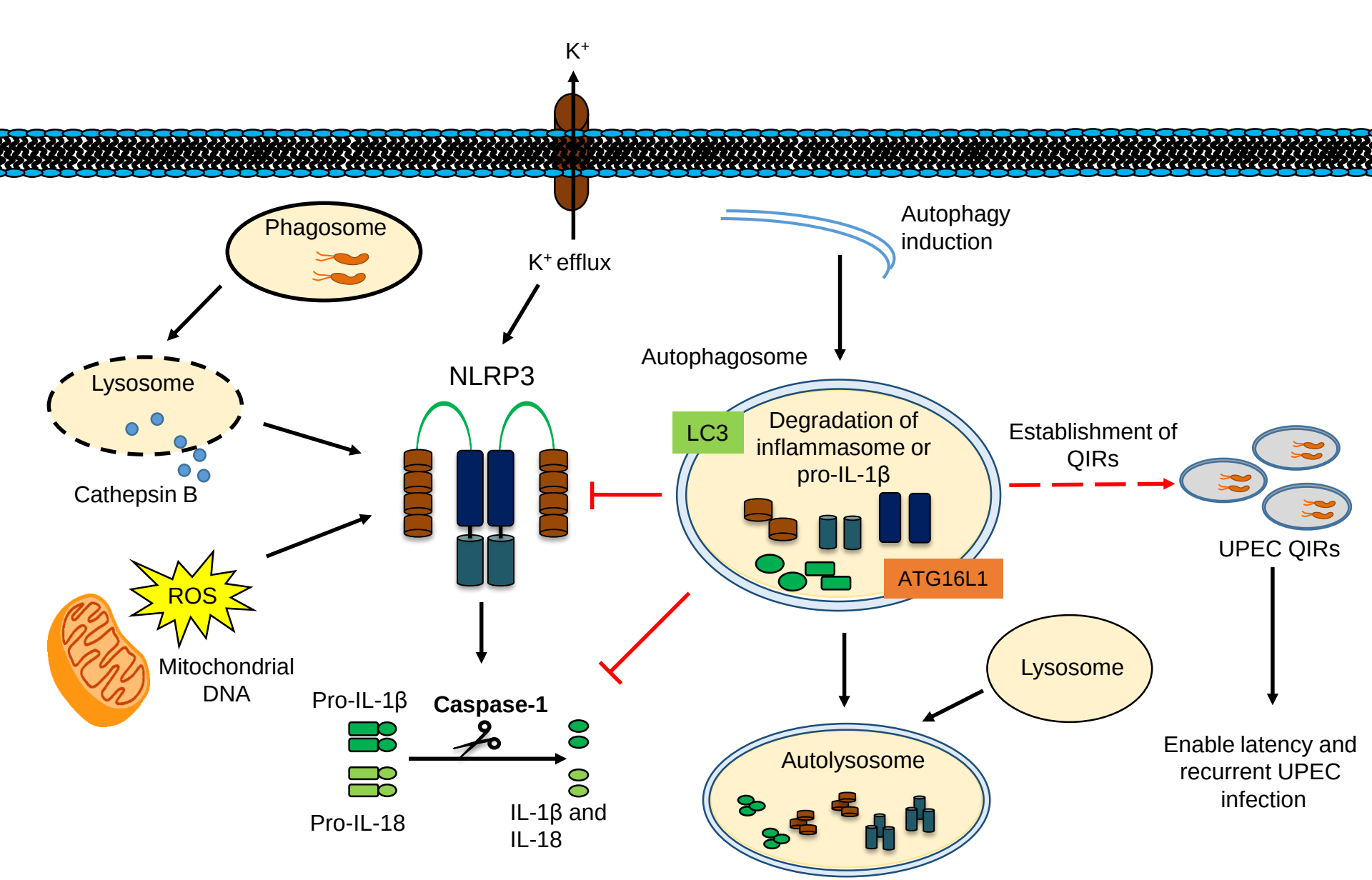
K+
Phagosome
K+ efflux
Autophagy induction
Lysosome
Cathepsin B
NLRP3
Autophagosome
LC3
Degradation of inflammasome or pro-IL-1β
ATG16L1
Establishment of QIRs
UPEC QIRs
ROS
Mitochondrial DNA
Pro-IL-1β
Caspase-1
Pro-IL-18
IL-1β and IL-18
Lysosome
Autolysosome
Enable latency and recurrent UPEC infection

Figure 3