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# Strain- and host species-specific inflammasome activation, IL-1β release and cell death in macrophages infected with uropathogenic *Escherichia coli*

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## Abstract

Uropathogenic *E. coli* (UPEC) is the main etiological agent of urinary tract infections (UTI). Little is known about interactions between UPEC and the inflammasome, a key innate immune pathway. Here we show that UPEC strains CFT073 and UTI89 trigger inflammasome activation and lytic cell death in human macrophages. Several other UPEC strains, including two multidrug resistant ST131 isolates, did not kill macrophages. In mouse macrophages, UTI89 triggered cell death only at a high MOI, and CFT073-mediated inflammasome responses were completely NLRP3-dependent. Surprisingly, CFT073- and UTI89-mediated responses only partially depended on NLRP3 in human macrophages. In these cells, NLRP3 was required for IL-1β maturation, but contributed only marginally to cell death. Similarly, caspase-1 inhibition did not block cell death in human macrophages. In keeping with such differences, the pore forming toxin α-hemolysin mediated a substantial proportion of CFT073-triggered IL-1β secretion in mouse but not human macrophages. There was also a more substantial α-hemolysin-independent cell death response in human versus mouse macrophages. Thus, in mouse macrophages, CFT073-triggered inflammasome responses are completely NLRP3-dependent, and largely α-hemolysin-dependent. In contrast, UPEC activates an NLRP3-independent cell death pathway and an α-hemolysin-independent IL-1β secretion pathway in human macrophages. This has important implications for understanding UTI in humans.

## Introduction

Uropathogenic *Escherichia coli* (UPEC) is estimated to cause up to 80% of community-acquired and 65% of nosocomial urinary tract infections (UTI), making it the single most important etiological agent of this highly prevalent infectious disease[1]. UTI typically involves infection of the bladder (cystitis) or kidneys (pyelonephritis), and can also lead to renal scarring and sepsis[1, 2]. Asymptomatic bacteriuria (ABU), caused by various etiologic agents, is also common[3].

Mouse UTI models, as well as genetic associations within patient cohort studies, have helped to pinpoint the roles of specific innate immune pathways in defense against uropathogens[2]. Such studies have highlighted the importance of members of the Toll-like Receptor family in controlling bacterial growth and dissemination, as well as causing symptoms and pathology. At the cellular level, roles of the urothelium and neutrophils in innate defence and host subversion during UTI are well established. However, surprisingly little is known about the roles of monocytes and macrophages in these processes[4]. A recent study by Schiwon *et al.* dissected the role of different macrophage populations in a mouse UTI model and unraveled complex interactions of sentinel and helper macrophages governing antimicrobial actions of neutrophils[5]. We previously demonstrated that some UPEC strains can survive for up to 24 h in murine bone marrow-derived macrophages (BMM) within LAMP1$^+$ compartments[6], reminiscent of quiescent intracellular reservoirs observed in epithelial cells that may facilitate recurrent infection. Thus, the role of myeloid cells in UPEC infection may not always be protective.

Upon detection of cellular stress and/or microbial products, some Nod-like-receptor (NLR) family members, as well as the PYHIN-family member AIM2, form large cytoplasmic

multiprotein complexes known as inflammasomes. Inflammasomes have important functions in many bacterial infections[7], as well as in chronic diseases[8, 9]. The NLR-family comprises twenty-two genes in humans and more than thirty in mice, and can be phylogenetically grouped into the NLRP, IPAF and NOD sub-families[10]. The NLRP family members NLRP1 and NLRP3, as well as the IPAF family member NLRC4, can all initiate inflammasome formation. Most NLRP family members contain a C-terminal leucine rich repeat that is involved in danger sensing, a central nucleotide-binding and oligomerisation domain, and an N-terminal pyrin domain that relays downstream signalling. NLRC4 has a similar domain structure, but contains an N-terminal caspase recruitment domain (CARD), rather than a pyrin domain. Upon activation, NLRPs oligomerise and cluster into a cytoplasmic complex with the adapter protein ASC and the protease caspase-1, facilitating its autocatalytic cleavage and activation. Active caspase-1 is required for maturation and secretion of the pro-inflammatory IL-1 family cytokines, IL-1β and IL-18. One of the many functions of IL-1β is to facilitate neutrophil and macrophage recruitment to sites of infection. In addition to mediating cytokine processing, inflammasome activation also initiates a programmed, pro-inflammatory form of cell death called pyroptosis. Pyroptotic cell death is thought to eliminate the intracellular replication niche of pathogens that infect macrophages (e.g. *Shigella*, *Salmonella*, *Legionella* and *Listeria*) and to re-expose them to antimicrobial effector functions[7, 11].

Among the different pathogenic *E. coli* subtypes, enterohemorrhagic *E. coli* O157:H7, which causes severe enteritis, triggers inflammasome activation[12]. However, until very recently no studies had investigated inflammasome involvement in UPEC recognition or UTI. In this study, we show that the genome-sequenced UPEC reference strains CFT073 and UTI89 trigger

inflammasome activation and rapid cell death in macrophages, whereas others do not. Moreover, we define key mechanistic differences between human and mouse macrophages in the host recognition pathways and bacterial factors that initiate these responses. Our findings of fundamental differences between different UPEC strains in inflammasome engagement, as well as between human and mouse innate immune recognition pathways for UPEC, have major implications for understanding and modeling UTI pathogenesis.

## Results

### UPEC strains CFT073 and UTI89 cause rapid cell death in macrophages

Given the paucity of information on interactions between UPEC and macrophages, we investigated whether the survival of human monocyte-derived macrophages (HMDM) was affected by different UPEC strains. We analyzed strains associated with different UTI severity, including the reference strains CFT073 (a blood culture isolate from a patient with pyelonephritis)[13] and UTI89 (a urine isolate from a patient with recurrent cystitis)[14], the sequence type (ST) 131 strains EC958[15, 16] and MS3179 (urine isolates from patients with UTI) and the asymptomatic bacteriuria (ABU) strains 83972 and VR50[17, 18]. These experiments revealed that only CFT073 and UTI89 caused rapid, lytic cell death by 2 h post-infection as assessed by LDH release (**Fig. 1A**). Cell death was further increased by 24 h post-infection (**Fig. 1B**). A direct comparison of HMDM with murine BMM over a multiplicity of infection (MOI) range confirmed that mouse macrophages were also susceptible to CFT073-induced cell death (**Fig. 1C**). In BMM, UTI89 did not trigger cell death, except at the highest MOI used (MOI 100, **Fig. 1C**). This is consistent with our previous findings that UTI89 can survive for up to 24 h within BMM[6, 19]. To investigate whether UPEC-mediated cell death is a macrophage-specific phenomenon, the response to CFT073, UTI89 and MS3179 was also analyzed in PMA-differentiated THP-1 cells (a human macrophage-like cell line), murine peritoneal cavity cells (PCC, which are predominantly comprised of resident peritoneal macrophages[20]) and two human bladder epithelial cell lines (5637 and T24) commonly used to study UPEC infection *in vitro* (**Fig. 1D**). PMA-differentiated THP-1 cells showed a similar response to HMDM. CFT073 also triggered cell death in PCC, whereas the effect of UTI89 was much weaker, similar to the

findings with mouse BMM (compare **Fig. 1C**). The ST131 isolate MS3179 did not trigger cell death in any of the cell types tested, and the two epithelial cell lines were not killed efficiently by any of the UPEC strains at an MOI of 10 at 2 h post-infection (**Fig. 1D**). However, 24 h exposure of epithelial cell lines to a very high MOI (MOI 1000) of all UPEC strains did result in some cell death (**Fig. S1**). Collectively, these data demonstrate substantial variability in the capacity of different UPEC strains to elicit macrophage cell death.

**UPEC-mediated macrophage cell death correlates with inflammasome activation**

To investigate potential involvement of the inflammasome pathway in cell death, we first examined the capacity of UPEC strains to trigger IL-1β release from LPS-primed macrophages. LPS priming was performed to boost pro-IL-1β levels, allowing the use of IL-1β release as a marker for inflammasome activation with minimal interference by rapid cell death or by other confounding processes such as suppression of cytokine production by some UPEC strains[21]. Indeed, we found that, whereas CFT073, UTI89 and MS3179 all elicited similar levels of secreted TNF-α from BMM, this response was greatly reduced in HMDM infected with strains triggering rapid cell death (CFT073, UTI89) as compared to MS3179 that did not cause cell death (**Fig. S2A**). Thus, TNF-α release inversely correlated with UPEC-induced rapid cell death in HMDM, as might be expected given that this cytokine must be synthesized prior to its release. In the case of IL-1β release from LPS-primed cells that already express pro-IL-1β, there was a clear correlation with induction of cell death in all cases. In human macrophages (HMDM and PMA-differentiated THP-1 cells), both CFT073 and UTI89 (MOI 10) triggered IL-1β release (**Fig. 2A and S2B**). In contrast, CFT073 but not UTI89 (MOI 10) elicited IL-1β release from LPS-primed mouse macrophages (BMM and PCC) (**Fig. 2A and S2C**), consistent with the

failure of UTI89 to trigger robust cell death in mouse macrophages at low MOI (compare **Fig. 1C and 1D**). Also consistent with the cell death data, the ST131 strain MS3179 did not trigger IL-1β release from LPS-primed macrophages of either human or murine origin. LPS-primed epithelial cell lines did not release IL-1β in response to any of the UPEC strains tested (**Fig. S2B**). Similar patterns were observed for caspase-1 cleavage; both CFT073 and UTI89 triggered comparable capase-1 cleavage in human macrophages (**Fig. 2B**), whilst in mouse macrophages the response to CFT073 was much more pronounced than for UTI89 (**Fig. 2B**). Another hallmark of inflammasome activation, the formation of ASC specks, was also apparent in CFT073- and UTI89-infected human macrophages, whereas the ST131 strain MS3179 did not elicit this effect (**Fig. 2C**). Furthermore, infection with the two ASC speck-inducing UPEC strains (i.e. CFT073 and UTI89) appeared to induce morphological changes and loss of nuclear integrity in HMDM, as visualized by actin and DNA staining in the same samples (**Fig. 2C**).

**CFT073-mediated cell death in murine macrophages is completely dependent on the NLRP3 inflammasome**

Causality of inflammasome activation and cell death in BMM was analysed using macrophages deficient for NLRP3 and NLRC4 (two NLRs most commonly activated by bacterial infection[7]), the inflammasome adaptor protein ASC, or the inflammatory caspases, caspase-1 and -11. Since UTI89 did not trigger pronounced inflammasome activation in mouse macrophages at an MOI of 10, only CFT073 was assessed. CFT073-mediated caspase-1 cleavage was completely dependent on NLRP3 and ASC, but did not require NLRC4 (**Fig. 3A**). As expected, the positive controls nigericin and *Salmonella enterica serovar* Typhimurium (*S.* Typhimurium) strain SL1344 acted via NLRP3 and NLRC4, respectively (**Fig. 3A**). Analysis of LDH release confirmed that

NLRP3, ASC and Caspase-1/11 were required for CFT073-mediated cell death (**Fig. 3B**). These inflammasome components were also indispensable for CFT073-triggered IL-1β release from LPS-primed BMM (**Fig. 3C**). As with caspase-1 cleavage, NLRC4-deficiency did not affect LDH or IL-1β release upon infection with CFT073. Again, the positive controls for NLRP3, ASC and caspase-1 involvement (nigericin), and NLRC4 and caspase-1 involvement (*S.* Typhimurium), behaved as expected. ASC was dispensable for *S.* Typhimurium-mediated cell death (**Fig. 3B**) as previously reported[22], and NLRP3, ASC and caspase-1/11 were indispensable for nigericin-triggered IL-1β release (**Fig. 3C**). *S.* Typhimurium-triggered IL-1β release was partially dependent on NLRP3 and ASC, and completely dependent on NLRC4 and caspase-1/11. Hence, rapid cell death and IL-1β secretion triggered by CFT073 in mouse macrophages is dependent on NLRP3, ASC and caspase-1 and/or -11.

**In human macrophages, UPEC-mediated IL-1β secretion is dependent on NLRP3, whereas cell death is primarily NLRP3-independent**

We next investigated NLRP3 involvement in human macrophage responses to UPEC using MCC950, a recently described NLRP3 inhibitor that does not affect AIM2, NLRP1 or NLRC4-mediated inflammasome activation[23]. Surprisingly, in LPS-primed HMDM, MCC950 blocked nigericin-triggered cell death, but had little effect on CFT073-triggered cell death in cells from most donors examined (**Fig. 4A, Table S1**). In contrast, MCC950 significantly reduced both nigericin- and CFT073-triggered IL-1β release from LPS-primed HMDM (**Fig. 4B**). Similar observations were apparent for UTI89, where MCC950 had only modest effects in reducing UTI89-triggered cell death for HMDM from 3 out of 4 donors (**Table S1**). This suggests that there are differences between human and mouse macrophages in NLRP3 responses to UPEC.

Indeed, a direct comparison revealed that, whereas MCC950 completely inhibited responses to nigericin in both HMDM and BMM, CFT073-mediated cell death was only blocked in mouse macrophages (**Fig. S3A**). Moreover, LPS priming had no apparent effect on NLRP3-dependency of CFT073-mediated cell death in human or mouse macrophages. In contrast to differential effects on cell death, MCC950 inhibited CFT073-triggered IL-1β release in both LPS-primed HMDM and BMM, albeit more effectively in BMM (**Fig. S3B**). The level of NLRP3-dependence for CFT073-triggered IL-1β release varied between cells from different donors (**Table S1**), and MCC950 never completely abolished IL-1β release, as was the case for nigericin. We therefore investigated the possibility that the residual IL-1β response detected by ELISA might be due to the release of unprocessed IL-1β, as a consequence of cell death. Indeed, analysis of concentrated cell culture supernatants by immunoblotting revealed that treatment with MCC950 completely blocked release of mature IL-1β from CFT073-infected HMDM, whereas pro-IL-1β was still present in culture supernatants (**Fig. 4C**). We also monitored ASC speck formation upon NLRP3 inhibition in HMDM. In these experiments, the NLRP3 inhibitor MCC950 completely blocked nigericin- but not *S.* Typhimurium-triggered ASC speck formation (**Fig. 4D**). MCC950 substantially reduced, but did not ablate, CFT073- and UTI89-induced ASC speck formation in all experiments (**Fig. 4D**).

To further investigate inflammasome involvement in human macrophages, the effect of the caspase-1 specific inhibitor VX-765[24] on CFT073-triggered cell death in human and mouse macrophages was examined. VX-765 effectively blocked CFT073- and LPS/nigericin-triggered cell death and IL-1β release by LPS-primed BMM at 2 h post-infection (**Fig. 5A-B**). However, similar to the observations with MCC950, VX-765 reduced CFT073-mediated IL-1β release

from LPS-primed HMDM, but did not affect cell death. Control experiments confirmed that both inhibitors blocked cleavage and release of caspase-1 in response to CFT073 infection and LPS/nigericin stimulation in HMDM and BMM (**Fig. 5C-D**). Together, these findings indicate that (1) the NLRP3 inflammasome drives UPEC-triggered IL-1β maturation in human macrophages; and (2) an NLRP3-independent pathway is the primary mediator of UPEC-triggered cell death in human macrophages.

To independently verify that an NLRP3-independent pathway mediates UPEC-triggered cell death, we used THP-1 defNLRP3 cells, which stably express an NLRP3 shRNA and have reduced NLRP3 expression compared to a control cell line (THP-1 Null) transfected with an 'empty' construct (**Fig. 6A**). Caspase-1 processing in THP-1 defNLRP3 cells responding to CFT073, UTI89 or nigericin was greatly reduced, whereas caspase-1 p20 was still detectable at high levels after infection with *S.* Typhimurium. In these cells, CFT073 and UTI89 still triggered substantial cell death, whereas nigericin did not (**Fig. 6B**). Again, LPS priming had no effect on the degree of NLRP3 dependency of UPEC-mediated cell death, although it did appear to reduce the effect of NLRP3-knockdown in the nigericin control. As expected, no reduction in cell death was observed in defNLRP3 cells when using *S.* Typhimurium as an NLRP3-independent trigger for cell death. Collectively, these data suggest the involvement of another NLRP3-independent, cell death pathway triggered by UPEC.

**α-hemolysin is the main factor in CFT073 triggering cell death and IL-1β release in mouse but not human macrophages**

The capacity for CFT073 and UTI89 to trigger human macrophage cell death was also conferred by culture supernatants (**Fig. S4**). Crude biochemical analysis indicated that the factor(s) responsible were heat- and protease-sensitive, and with a likely molecular weight of greater than 30 kDa. Candidate proteins mediating cell death and/or IL-1β secretion included the pore forming toxin α-hemolysin, as well as serine-protease autotransporter toxins (Sat and Vat). Genes encoding all three toxins are present in CFT073, while UTI89 contains the *hlyCABD* (α-hemolysin operon) and *vat* genes. To test the involvement of these three toxins in triggering the inflammasome response, we generated a series of CFT073 mutants deleted for genes encoding each individual toxin and a triple-mutant deficient in the ability to produce all three toxins. Compared to wild type CFT073, the α-hemolysin mutant (CFT073Δ*hlyA*) was greatly impaired in its ability to trigger cell death of mouse macrophages (~30% of wild type) and, to a lesser extent, of human macrophages (~60% of wild type) when macrophages were exposed to an MOI of 10 (**Fig. 7A**). CFT073 *sat*- and *vat*- mutants were very modestly compromised for their ability to trigger mouse and human macrophage cell death. However, no additive effect was observed, as the triple-mutant showed no difference to the CFT073Δ*hlyA* single mutant in this assay. Similar observations were made at an MOI of 100 with both CFT073 and UTI89 (**Fig. 7B**). In this case, cell death induced by CFT073 and UTI89 was almost completely *hlyA*-independent in HMDM (cell death for *hlyA* mutants was ~85% of the wild type strains), whereas in BMM the response was largely *hlyA*-dependent (~30% and 40% of wild type strains). Intriguingly, analysis of IL-1β release revealed a striking difference between human and mouse macrophages with respect to triggering by α-hemolysin. Whereas IL-1β levels were substantially reduced in BMM responding to CFT073Δ*hlyA* compared to wild type CFT073, deletion of *hlyA* had no effect on HMDM responses (**Fig. 7C**). Again, we analysed cleavage of released IL-1β by western blot and

found that in HMDM levels of cleaved IL-1β were similar in samples infected with CFT073 and CFT073Δ*hlyA* (**Fig. 7D**). In contrast, CFT073Δ*hlyA* was drastically impaired in its ability to induce the release of cleaved IL-1β in BMM. Deletion of *sat* or *vat* had no effect on IL-1β release from either human or mouse macrophages, and the response to the triple-mutant was again identical to that of the *hlyA* single-mutant. Thus, α-hemolysin is the primary, but not only, mediator of cell death and IL-1β release in mouse macrophages. Moreover, generation of mature IL-1β was completely dependent on α-hemolysin. By contrast, in the human macrophage response to UPEC, α-hemolysin does not contribute to IL-1β release or cleavage, and plays a lesser role in rapid cell death. These differential effects of α-hemolysin further highlight the divergent UPEC recognition pathways of human and mouse macrophages. In summary, UPEC triggers both NLRP3-independent cell death and α-hemolysin-independent IL-1β processing in human macrophages, thus indicating that additional host and pathogen-derived factors are likely to be important in the macrophage response to UPEC.

## Discussion

In this study, we demonstrate that some UPEC strains can trigger both NLRP3-dependent inflammasome activation and rapid cell death in macrophages. We also provide important insights into these processes in the context of similarities and differences between human and mouse macrophage responses to UPEC. Other *E. coli,* including enterohemorrhagic *E. coli* isolates[12] as well as non-pathogenic or commensal stains[25, 26], have been reported to activate inflammasomes by a variety of different mechanisms involving several bacterial factors. These include nucleic acids[27] and protein toxins (enterohemolysin[12] and heat-labile enterotoxin[28]) acting via NLRP3, the T3SS rod protein EprJ[29] and flagellin[25] acting via NLRC4 and (intracellular) LPS[30] acting via non-canonical inflammasomes. Our study adds to this literature by identifying UPEC α-hemolysin-dependent and -independent mechanisms of inflammasome activation in macrophages.

It remains unclear as to what roles UPEC-mediated inflammasome activation has in different pathophysiological contexts. Two out of the four strains that did not elicit inflammasome activation are associated with ABU[17, 18], whilst the remaining two belong to the globally disseminated fluoroquinolone-resistant *fimH*30/clade C ST131 lineage that is frequently associated with symptomatic infection[16, 31]. The two inflammasome-activating strains are also associated with UTI pathology. Hence, the capacity for inflammasome activation is variable, further highlighting the genetic diversity that exists amongst different UPEC isolates. Since the capacity to trigger inflammasome activation and macrophage cell death was not common to all UPEC strains, some UPEC strains may have gained inflammasome-activating factors as a

component of their virulence armoury or lost these to avoid host detection. In the case of the former, candidate virulence factors included the pore forming toxin α-hemolysin[32], as well as serine-protease autotransporter toxins (Sat and Vat) that are known to elicit cytotoxic effects on epithelial cells[33], and for which the genes are present in CFT073 (*hlyA, sat, vat*) and UTI89 (*hlyA, vat*), but not 83972, VR50[34] or EC958[15]. Mutation of all three factors in CFT073 revealed that only the absence of α-hemolysin substantially reduced inflammasome responses in mouse macrophages. Intriguingly, however, CFT073-triggered IL-1β release and cleavage was completely independent of α-hemolysin in human macrophages, and there was also a pronounced α-hemolysin-independent cell death pathway. This finding points towards fundamentally different recognition mechanisms for these UPEC strains in human versus mouse macrophages, yet conservation in the overall outcomes. Whether this extends to other cell types needs to be further examined, given a recent report on *E. coli* α-hemolysin triggering IL-1β secretion in human urothelial cells[35], an earlier study showing a similar phenomenon in human monocytes[36] and well documented cell type-specific effects of α-hemolysin[32].

Our aim to characterize the specific inflammasome involved in UPEC recognition led us to study the response of mouse macrophages as a more tractable genetic system, as compared to human macrophages. Initial experiments revealed that mouse and human macrophages seemed to respond similarly with regard to induction of cell death, caspase-1 cleavage and IL-1β secretion upon infection with CFT073. In the case of UTI89, a much less pronounced response was observed in mouse macrophages compared to human macrophages when using a low MOI (MOI 10). Nonetheless, a 10-fold higher MOI did initiate some cell death in these cells. In contrast, both CFT073 and UTI89 had similar effects on human macrophages. The conservation between

human and mouse macrophage responses to CFT073 led us to focus on this particular strain for the identification of host mechanisms mediating cellular responses. NLRP3, ASC and the inflammatory caspases (1 and/or 11) were indispensable for CFT073-mediated rapid lytic cell death and IL-1β secretion in mouse BMM, while a role for NLRC4 was excluded. These findings are consistent with a very recent study showing that UTI89 induces moderate IL-1β release from mouse macrophages in an NLRP3-dependent manner, albeit under different experimental conditions[37].

The conclusion that the acute CFT073-mediated inflammasome response leading to cell death and IL-1β secretion in BMM was absolutely dependent on NLRP3 was also supported by experiments using small molecule inhibitors of NLRP3 (MCC950) and caspase-1 (VX-765). However, primary human macrophages that were analysed in parallel showed a remarkably different response. The NLRP3 and caspase-1 inhibitors substantially reduced or blocked IL-1β release from HMDM in response to UPEC infection or the NLRP3 agonist nigericin, respectively. In contrast, UPEC-triggered cell death was largely unaffected by either inhibitor. Although analysis of HMDM generated from several donors revealed some variation in the level of NLRP3-dependence, the overall conclusion is that in human macrophages cell death is NLRP3-independent, whereas IL-1β cleavage was shown to be completely NLRP3-dependent. Interestingly, LPS-primed HMDMs released unprocessed IL-1β upon UPEC infection, even when the NLRP3 inflammasome was blocked. Biologically, this may be of significance since it was shown that uncleaved IL-1β can be processed in the extracellular space by inflammasome complexes[38] or by enzymes such as cathepsin-G and elastase[39]. Consistent with the existence of an NLRP3-independent death pathway in human macrophages, stable knockdown of NLRP3 in

358 THP-1 cells blocked nigericin-induced cell death, but only marginally reduced CFT073- and
359 UTI89-mediated cell death. Whether NLRP3-independent cell death involves activation of
360 another inflammasome is unknown at this stage. However, given that NLRP3 inhibition ablated
361 both UPEC-induced IL-1β maturation and caspase-1 cleavage in HMDM, other modes of cell
362 death such as necroptosis would appear to be more likely.
363
364 Divergence in the repertoire of NLR family members between human and mouse can contribute
365 to differences in inflammasome responses between these species[10, 40]. However, differences in
366 the recognition of pathogens by orthologous human and mouse NLRs have also been reported.
367 For example, *F. tularensis* activates only the AIM2 inflammasome in mouse macrophages, but
368 triggers NLRP3- and AIM2-dependent responses in human macrophages[41]. Conversely, *L.*
369 *monocytogenes* was reportedly recognized by AIM2, NLRP3 and NLRC4 in mouse cells[42-44], but
370 exclusively by NLRP3 in human cells[45]. The causes for these differences are not fully
371 understood, but may be related to species differences in ligand recognition. Our study highlights
372 that one pathogen can activate NLRP3 in both human and mouse macrophages, but through
373 distinct mechanisms. Our demonstration of α-hemolysin-dependent IL-1β cleavage and cell
374 death in mouse macrophages is consistent with a recent study showing α-hemolysin-mediated
375 inflammasome activation in UTI89-infected mice[35]. In stark contrast however, our studies with
376 human macrophages identified an α-hemolysin-independent pathway to IL-1β maturation. This
377 suggests that another UPEC factor selectively promotes NLRP3 activation in human but not
378 mouse macrophages, or that its relative potency in triggering inflammasome responses differs
379 between these species or between different cell types.
380

Emerging evidence indicates that cytokine processing and pyroptosis can be uncoupled in some systems. For example, *Salmonella*-mediated NLRC4 activation promoted IL-1β maturation but not pyroptosis in mouse neutrophils[46]. Other studies have also reported distinct roles for individual inflammasomes in cytokine processing versus pyroptosis. For example, NLRP3 was shown to mediate cell death and IL-1β release in *S. aureus*-infected HMDM, whilst a novel NLRP7 inflammasome was shown to selectively promote IL-1β secretion[47]. Similarly, NLRP3 and NLRC4 were shown to mediate *B. pseudomallei*-induced IL-1β and IL-18 release in the mouse, whilst pyroptotic cell death was attributed only to NLRC4[48]. At present, there is no unifying model explaining why similar recognition systems lead to cytokine maturation in one setting, and pyroptosis in another. Broz *et al.* proposed that CARD-containing NLRs can initiate distinct complexes with different roles in mediating cytokine maturation versus pyroptotic cell death[22]. However, the above described mechanism does not apply for most NLRPs and AIM2, which contain a pyrin domain rather than a CARD[10], and does not explain how death and cytokine responses happen simultaneously in the presence of ASC. The fact that NLRP3 was causal for cytokine processing but not cell death in human macrophages might again be interpreted as another example of uncoupling of downstream inflammasome responses. However, it would seem more likely that the NLRP3-independent cell death pathway overrides NLRP3-dependent pyroptosis in our system.

A protective role for inflammasome activation and IL-1β production has been shown in many *in vivo* infection models including *S. Typhimurium*, *L. monocytogenesis* and *Burkholderia* species[11] On the other hand, the role of pathogen-induced cell death is ambiguous, having either protective or detrimental effects by either eradicating intracellular niches or promoting dissemination,

respectively[49]. Since UPEC can occupy both extracellular and intracellular niches, it is difficult to predict what role pyroptosis plays during UTI. Activation of caspase-1/11 was shown to facilitate clearance of UPEC in a mouse model, presumably by inducing pyroptosis and subsequent exfoliation of bladder epithelial cells[35]. In another study however, activity of caspase-1/11 was associated with chronicity and higher bacterial loads in the bladder in a model of recurrent UTI[50]. In the case of cytokine processing, previous studies have associated IL-1β release with renal pathology of UTI in patients[51, 52], and also in a mouse model[53]. Only very recently was IL-1β release in *atg16l1*-/- mice shown to be associated with protection from UTI[37]. While the effects of IL-1β can be studied simply by knockout or by blocking its interaction with receptors, new approaches for genetically and/or pharmacologically uncoupling pyroptosis from other inflammasome responses will be required to address the role of cell death in pathology. Whether NLRP3-dependent responses have a causal role in host defence or pathology remains to be elucidated.

In conclusion, our study highlights the complexity of interactions between UPEC and the innate immune system. Some UPEC strains trigger inflammasome activation and rapid, lytic cell death in macrophages, whereas others, including two strains from the multidrug resistant ST131 lineage, do not. This again highlights the genetic complexity that exists amongst different UPEC strains and that host response pathways engaged, as well as host colonization strategies employed, will vary depending on the specific UPEC strain encountered. For inflammasome-activating strains such as CFT073, NLRP3 drives IL-1β maturation in both human and mouse macrophages. However, this pathway only marginally contributes to cell death in human macrophages, despite its causal role in cell death in mouse macrophages. Finally, α-hemolysin is

427 the primary trigger for cell death and IL-1β release in mouse macrophages, whereas these
428 cellular responses are either primarily or completely independent of this toxin in human
429 macrophages. The yet-to-be-identified death pathway in human monocyte-derived macrophages
430 highlights a potential difference between human and mouse innate immune UPEC recognition
431 pathways and needs to be considered in future studies using macrophages and other cell types
432 from a variety of sources, as well as in *in vivo* studies. Given the importance of mouse UTI
433 models for understanding host colonization and pathology, and especially in the light of recent
434 studies showing detrimental and beneficial effects of inflammasome activation in mouse
435 models[37, 50], as well as a prominent role for α-hemolysin[35], our findings are likely to have broad
436 significance for understanding susceptibility and severity of UTI in humans.
437
438
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440

# Methods

## Bacterial strains and growth conditions

UPEC strains CFT073[13], UTI89[14], 83972[17], VR50[18] and EC958[15] have been described previously. MS3179 is an ST131 strain isolated from a patient presenting with UTI at the Royal Brisbane and Women's Hospital, Brisbane, Australia. *S.* Typhimurium strain SL1344[54] was used as a control for NLRP3-independent inflammasome activation in some experiments. All strains were routinely grown at 37˚C on solid or in liquid Luria-Bertani (LB) medium.

## Genetic manipulation procedures and generation of mutants

Mutation of the *hlyA*, *sat* and *vat* genes in CFT073, and the *hlyA* gene in UTI89, was performed using the λ-Red recombinase gene replacement system[55]. The primers used for amplification of the kanamycin resistance gene (*hlyA*) or chloramphenicol resistance gene (*vat, sat*), and subsequent insertion into the chromosome of CFT073 (or UTI89) were as follows: *vat* (3353: 5'-tcgtaatgaacacagttcatctgatctccacacaccaagacttgataagctcacgtcttgagcgattgtgtagg and 3354: 5'-gaaaccaccaccccatgattttgttttaccgctgtacaggcctgctgacgcgacatgggaattagccatggtcc), *sat* (3351: 5'-aagaaattccaatgattttgagattcagaggttaaataaatttgttgtggacacgtcttgagcgattgtgtagg and 3352: 5'-ccaggagtgggagctgtagtctctggtgccaaggccggcgaaagttgcggtgacatgggaattagccatggtcc), *hlyA* (2049: 5'-aaattaaaagcacactacagtctgcaaagcaatcctctgcaaataaattgtgtaggctggagctgcttc and 2050: 5'-tgctctgctgctttttttaatgcatctttcgtgctttgtcctgctgagtgcatatgaatatcctccttag). CFT073 *hlyA* (CFT073Δ*hlyA*), *sat* (CFT073Δ*sat*) and *vat* (CFT073Δ*vat*) mutants, as well as the UTI89 *hlyA* mutant (UTI89Δ*hlyA*), were confirmed by PCR and DNA sequencing. The CFT073*hly-sat-vat*

463 triple mutant was constructed by sequential deletion of each gene, as described above, and was
464 confirmed by PCR and DNA sequencing.
465
466 **Mammalian cell culture**
467 Approval for all experiments using primary human and mouse cells was obtained from the
468 University of Queensland Medical Research Ethics Committee or the Animal Ethics Committee.
469 Human monocytes were isolated from buffy coats of healthy donors (kindly provided by the
470 Australian Red Cross) by positive selection for CD14 using MACS technology (Miltenyi Biotec,
471 Bergisch Gladbach, Germany), as previously described[56]. HMDM were differentiated for 7 days
472 with CSF-1 (10,000 U/ml, Chiron Emeryville, CA, USA) from $CD14^+$ cells, as previously
473 described[56], but in the absence of antibiotics. Cells from a single donor were used in every
474 experiment. Murine BMM were differentiated using 10,000 U/ml CSF-1 (Chiron) from bone
475 marrow of C57BL/6 wild type, *Nlrp3*$^{-/-}$ , *Nlrc4*$^{-/-}$ , *Asc*$^{-/-}$ , and *Caspase-1/11*$^{-/-}$ mice (all described
476 in[46]), in the absence of antibiotics as previously described[6]. PCC were flushed from the
477 peritoneal cavity of C57BL/6 mice by injection of 5 ml PBS. THP-1 (TIB-202, ATCC,
478 Manassas, VA, USA), THP-1 Null and THP-1 defNLRP3 (InvivoGen, San Diego, CA, USA)
479 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1
480 mM Na-Pyruvate and 10 mM HEPES (all Life Technologies, Carlsbad, CA, USA). THP-1 cells
481 were differentiated into macrophage-like cells by culture for 48 h in medium containing 30 ng/ml
482 phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA). PMA or CSF-1
483 was removed 4 h prior to infection with UPEC strains. Human bladder epithelial cell lines 5637
484 (HTB-9, ATCC) and T24 (HTB-4, ATCC) were cultured in FBS (10%) and 2 mM L-glutamine
485 supplemented RPMI-1640 or McCoy's 5A medium (Life Technologies), respectively.

**Bacterial culture and macrophage infection assays**

UPEC strains were grown statically at 37˚C overnight in LB broth. *S.* Typhimurium strain SL1344 was grown overnight in LB broth (200 rpm, 37˚C), diluted 1:33 and grown for another 3 h (to ensure logarithmic growth). Cells were pelleted, washed and adjusted to the same optical density at 600 nm. A MOI of approximately 10 was used (unless indicated otherwise) and confirmed by enumeration of colony forming units following serial dilution. Mammalian cells were seeded at a density of 4-8x$10^4$/0.2 ml in 96-well plates or 2-4x$10^5$/ml in 24-well plates (Nunc, Roskilde, Denmark). Medium was changed for all cell types to RPMI-1640 supplemented with FBS (10%) and 2 mM L-glutamine (all Life Technologies) 4 h prior to infection. LPS-priming was performed by addition of 100 ng/ml Ultrapure LPS from *Salmonella minnesota* R595 (InvivoGen). Nigericin sodium salt (Sigma-Aldrich) at a concentration of 10 µM was used as a positive control for NLRP3-dependent responses. In some experiments, cells were pre-incubated for 1 h with the caspase-1 inhibitor VX-765 (Selleck Chemicals, Houston, TX, USA) or the NLRP3 inhibitor MCC950[23], prior to performing infections. At 1 h post-infection, 200 µg/ml gentamicin (Life Technologies) was added for 1 h to inhibit growth of extracellular bacteria. For infections over a 24 h time course, medium was replaced with fresh medium containing 20 µg/ml gentamicin for the remaining 22 h.

**Cytotoxicity assays**

Cell culture supernatants were collected at 2 or 24 h post-infection, centrifuged for 5 min at 500 g, and analysed for LDH release using the *In Vitro* Toxicology Assay Kit (Sigma-Aldrich). Cytotoxicity (%) was calculated by quantification of LDH in culture supernatants versus total

509 cellular LDH (present in S/N after cell lysis with 0.1% Triton X-100) according to the formula %
510 cell death = $(100/LDH_{total}-LDH_{spontaneous})x(LDH_{treatment}-LDH_{spontaneous})$. 24 h values represent
511 summed measurements of the same well at 2 and 24 h post-infection, since medium was changed
512 at 2 h post-infection as part of the gentamicin exclusion protocol (see: Bacterial culture and
513 macrophage infection assays).
514
515 **Confocal microscopy**
516 Confocal microscopy was performed as previously described[6]. Cells were stained with 200 ng/ml
517 Alexa Fluor® 594 Phalloidin (Life Technologies) to visualize cell morphology, and ASC was
518 detected with a rabbit anti-ASC Antibody (N-15)-R (Santa Cruz Biotechnology, Santa Cruz, CA)
519 (1:300) and Alexa Fluor® 647 or 688-conjugated chicken anti-Rabbit IgG (Life Technologies) as
520 a secondary antibody (1:150). For quantifying ASC speck formation, HMDM were cultured in
521 12 mM glycine to reduce loss of cells due to lytic cell death[57]. ASC specks were counted
522 manually in a blinded fashion (5 fields at 40x magnification per condition per replicate).
523
524 **Immunoblotting**
525 $4x10^5$ cells were lysed in 100 µl 2xSDS loading buffer (125 mM Tris-HCl, 20% glycerol (*v/v*),
526 4% SDS (*w/v*), pH 6.8). For analysis of secreted caspase-1 in cell culture supernatants, medium
527 was replaced with OptiMEM medium (Life Technologies) 4 h prior to infection. Cell culture
528 supernatants were precipitated by incubation with 4 volumes of acetone at -20˚C overnight and
529 centrifugation at 5300 g and -10˚C for 30 min. Pellets were taken up in 2xSDS loading buffer.
530 Western blotting was performed as previously described[56]. Membranes were stained with
531 cleaved IL-1β (Asp116) rabbit mAb, IL-1β (3A6) mouse mAb, cleaved caspase-1 (ASP297)

(D57A2) rabbit mAb (all Cell Signalling Technology, Danvers, MA, USA), mouse IL-1β/IL-1F2 affinity purified polyclonal Ab, Goat IgG (R&D Systems**,** Minneapolis, MN, USA**),** anti-caspase-1 (p20) (mouse) mAb (Adipogen, San Diego, CA, USA), anti-NLRP3/NALP3 mAb (Cryo-2) (Adipogen) or human anti-G3PDH antibody (Trevigen, Gaithersburg, MD, USA). All primary antibodies were diluted 1:1000 except for human anti-G3PDH antibody, which was used at 1:10000. As secondary antibodies, anti-mouse and anti-rabbit IgG, HRP-linked Antibodies (Cell Signalling Technology) (1:2500) and anti-goat IgG-peroxidase antibody (Sigma-Aldrich) (1:5000) were used. HRP was detected using ECL Plus substrate (GE Healthcare, Buckinghamshire, UK) and Super RX film (Fujifilm, Tokyo, Japan).

**ELISA**

Cell culture supernatants were analysed for IL-1β with the human or mouse IL-1**β**/IL-1F2 DuoSet ELISA kit (R&D Systems) (detection limit 4 and 15.6 pg/ml, respectively) and anti-human or mouse ELISA Ready-Set-Go! (eBiosciece, San Diego, CA, USA) (detection limit: 4 and 8 pg/ml, respectively). TNF-α was detected using the Mouse TNF OptEIA ELISA set (BD Biosciences, San Diego, CA, USA) (detection limit: 15.6 pg/ml) and the human TNF-α standard ELISA Developmental kit (Peprotech, Rocky Hill, NJ, USA) (detection limit: 16 pg/ml) .

**Statistical Analysis**

All LDH, ELISA and MTT assays were performed using duplicate or triplicate cell culture wells for individual experiments. Presented data are typically mean values combined from three or more independent experiments, unless otherwise indicated. For statistical analysis of datasets with N>4, two-sided Wilcoxon matched-pairs signed-rank tests were performed using GraphPad

555 Prism Version 6 (GraphPad software, Inc., La Jolla, CA, USA). For these datasets, the
556 differences between pairs were plotted and were generally distributed approximately
557 symmetrically around the median.
558

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572 ## Disclosure

573 The author declared no conflict of interest.

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804
805 ## Figure legends

806

807 **Figure 1: The UPEC strains CFT073 and UTI89 induce rapid cell death in macrophages.**
808 **A-C)** HMDM or BMM were infected with the indicated UPEC strains (MOI 1, 10 and 100).
809 Supernatants were analyzed by LDH release assays at the indicated times post-infection (p.i.).
810 Data represent the mean+SEM of three independent experiments. **D)** Human macrophage-like
811 cells (THP-1) and mouse peritoneal cavity cells (PCC), as well as human T24 and 5637 bladder
812 epithelial cells, were infected with the indicated UPEC strains (MOI 10) for 2 h. Supernatants
813 were analyzed by LDH release assay. Data represent the mean+range of two independent
814 experiments.

815

816 **Figure 2: The UPEC strains CFT073 and UTI89 promote IL-1β secretion, caspase-1**
817 **cleavage and ASC-speck formation. A)** HMDM and BMM were primed with 100 ng/ml LPS
818 for 4 h, then infected with the indicated UPEC strains (MOI 10) for 2 h. Supernatants were
819 analyzed by ELISA. Data represent the mean+SEM of three independent experiments. **B)**
820 HMDM and BMM were infected with the indicated UPEC strains for 1 h. Whole cell lysates and
821 concentrated supernatants were analyzed by western blot with antibodies detecting human or
822 mouse caspase-1 p20. GAPDH is shown as a loading control. Similar findings were apparent in
823 three independent experiments. **C)** HMDM were infected with the indicated UPEC strains (MOI
824 10) for 2 h, after which they were fixed and analyzed by confocal microscopy. Arrowheads
825 indicate ASC-specks. Similar results were apparent in two independent experiments.

826

827 **Figure 3: In murine macrophages, CFT073-induced caspase-1 cleavage and cell death is**
828 **dependent on the NLRP3 inflammasome.** BMM derived from wild type, *Nlrp3*-, *Nlrc4*-, *Asc*-
829 and *Casp1/11*-deficient mice were infected with the UPEC strain CFT073 or the *S.* Typhimurium
830 strain SL1344 (MOI 10), or were stimulated with nigericin (10 μM, pre-stimulated with 100
831 ng/ml LPS for 4 h). **A)** After 1 h, whole cell lysates and concentrated supernatants were analyzed
832 by western blot with antibodies detecting murine full-length caspase-1, as well as multiple
833 caspase-1 cleavage products. GAPDH was used as loading control. Data is representative of two
834 independent experiments. **B)** Supernatants were analyzed by LDH assay at 2 h p.i. Data is the
835 mean+range of two independent experiments. **C**) LPS-primed BMM (100 ng/ml, 4 h) were
836 treated as above, after which secreted IL-1β was measured by ELISA after 2 h. Data are
837 mean+SEM of three independent experiments.
838
839 **Figure 4: NLRP3 contributes to IL-1β release and maturation but does not mediate cell**
840 **death in human macrophages responding to CFT073. A-B)** HMDM were primed with LPS
841 (100 ng/ml, 4 h) or left untreated, then pretreated for 1 h with the NLRP3 inhibitor MCC950 (10
842 μM) and subsequently infected with the UPEC strain CFT073 or *S.* Typhimurium SL1344 (MOI
843 10), or stimulated with nigericin (10 μM). Supernatants were collected at 2 h p.i., and analyzed
844 by LDH release assay **(A)** and ELISA **(B)**. Data are from nine (CFT073, LPS/nigericin) and six
845 (*S.* Typhimurium) independent experiments (different donors), respectively. Statistical analysis
846 was performed using a Wilcoxon matched-pairs signed-rank tests: ns: p> 0.05, *: p<=0.05, **:
847 p<=0.01. **C**) Concentrated supernatants from cells treated as described above were analyzed by
848 western blot with antibodies detecting full-length or cleaved human IL-1β. **D)** HMDM were
849 treated as above, but in presence of 12 mM glycine, and analyzed by confocal microscopy. ASC

specks and nuclei were counted manually in a blinded manner. Data are mean+SEM of three independent experiments.

**Figure 5: UPEC-mediated cell death is blocked by caspase-1 inhibition in mouse but not human macrophages. A-B)** HMDM and BMM were primed with LPS (100 ng/ml, 4 h) or left untreated, then pretreated for 1 h with the caspase-1 inhibitor VX-765 (50 μM), and subsequently infected with the UPEC strain CFT073 (MOI 10) or stimulated with nigericin (10 μM). Supernatants were collected at 2 h p.i., and were analyzed for cell death by LDH release assay **(A)** and IL-1β release by ELISA **(B)**. Data represent the mean+SEM of three independent experiments. **(C-D)** HMDM and BMM were treated as above for 1 h. Cell lysates, as well as cell culture supernatants, were analyzed by western blot for cleavage of caspase-1. GAPDH served as loading control. Similar results were obtained in two independent experiments.

**Figure 6: UPEC triggers NLRP3-independent cell death in human macrophages.** Stable NLRP3 knockdown THP-1 cells (defNLRP3), as well as THP-1 control cells (Null) were infected with the indicated UPEC strains or *S.* Typhimurium (MOI 10), or were treated with nigericin (10 μM) for 1 h **(A)** or 2 h **(B)**. **A)** Cell lysates were analyzed by western blot for expression of NLRP3 and GAPDH as a loading control. Caspase-1 p20 was detected in cell lysates. Similar findings were apparent in two independent experiments. **B)** Supernatants of unprimed or LPS primed (100 ng/ml LPS, 4 h) THP-1 cells were analyzed by LDH assay. Data are the mean+SEM of four independent experiments.

872 **Figure 7: Differences between human and mouse macrophages in cellular responses to α-**
873 **hemolysin. A-B)** HMDM and BMM were infected with wild type UPEC strains or mutants
874 deficient for α-hemolysin (*ΔhlyA*), *sat* (*Δsat*), *vat* (*Δvat*) or all three genes (*ΔhlyA;Δsat;Δvat*) at
875 the indicated MOI for 2 h. Supernatants were analyzed by LDH assay. **C)** LPS–primed (100
876 ng/ml, 4 h) cells were treated as above (MOI 10) and analyzed by IL-1β ELISA. Data are the
877 mean+SEM of three (**A, C**) or four (**B**) independent experiments. **D**) Concentrated supernatants
878 from cells treated as described above were analyzed by western blot with antibodies detecting
879 cleaved human or mouse IL-1β. Similar observations were made in 2 independent experiments.
880

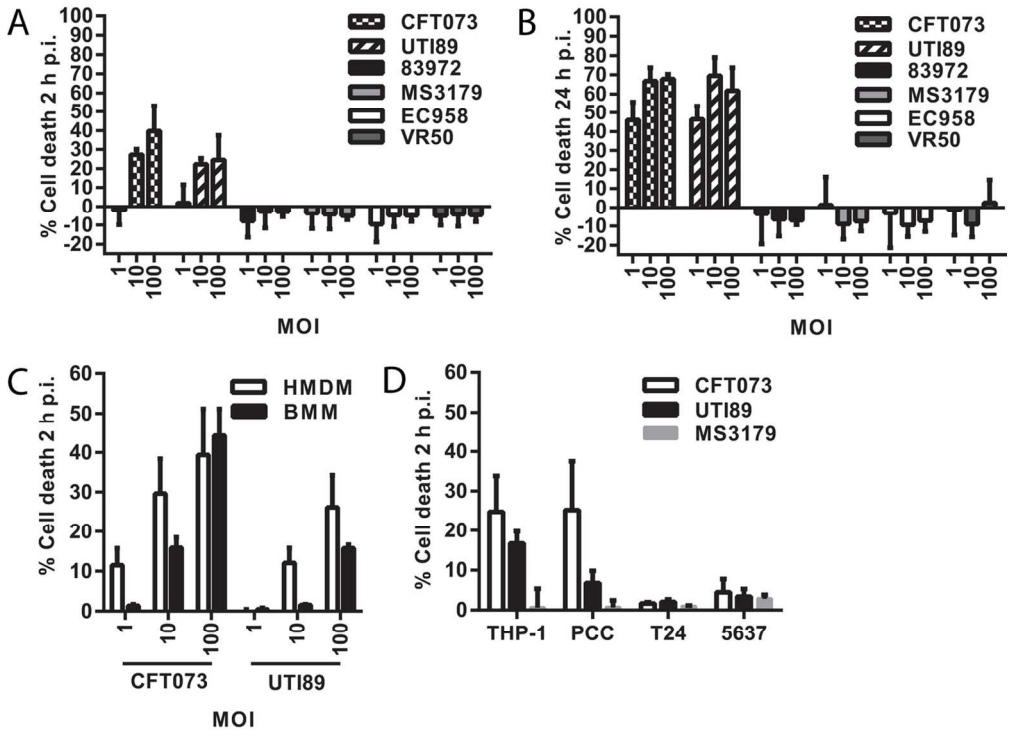


Fig. 1
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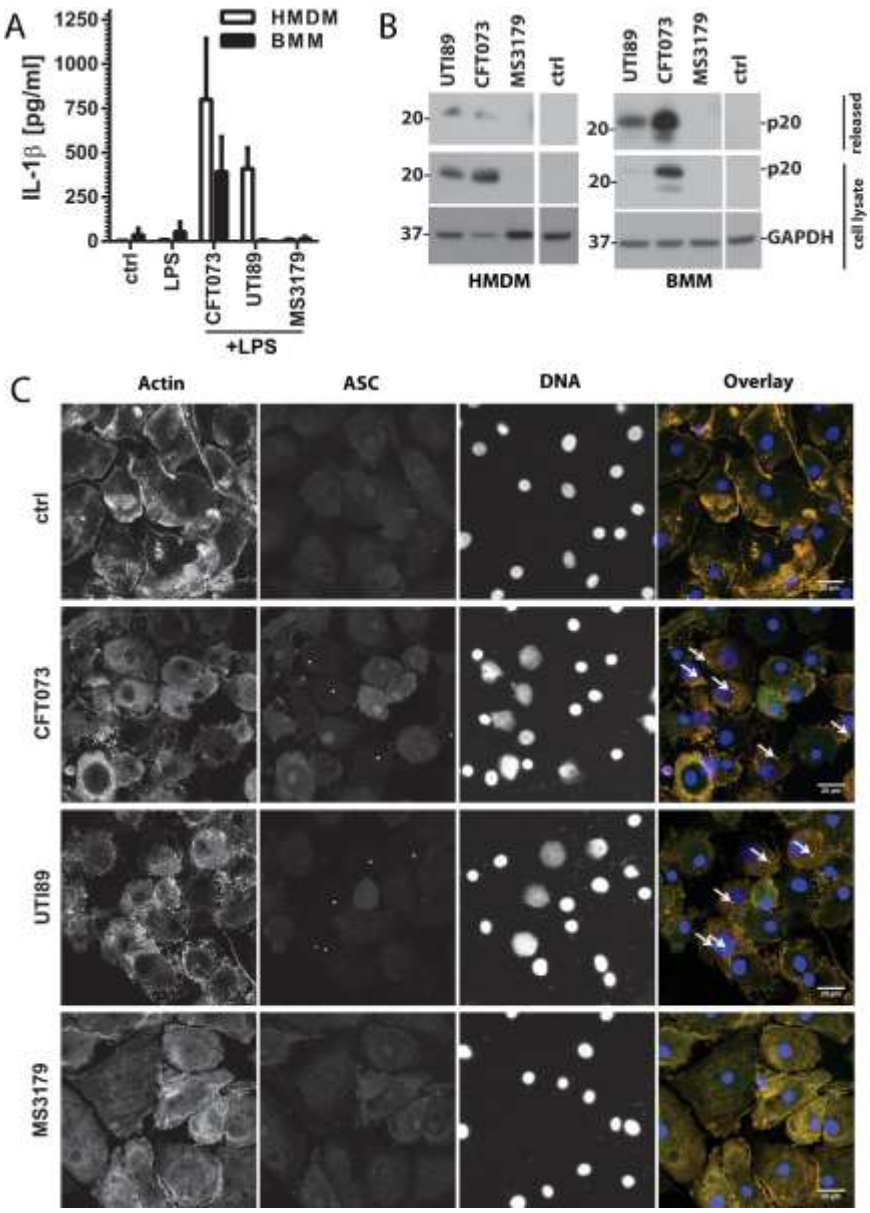


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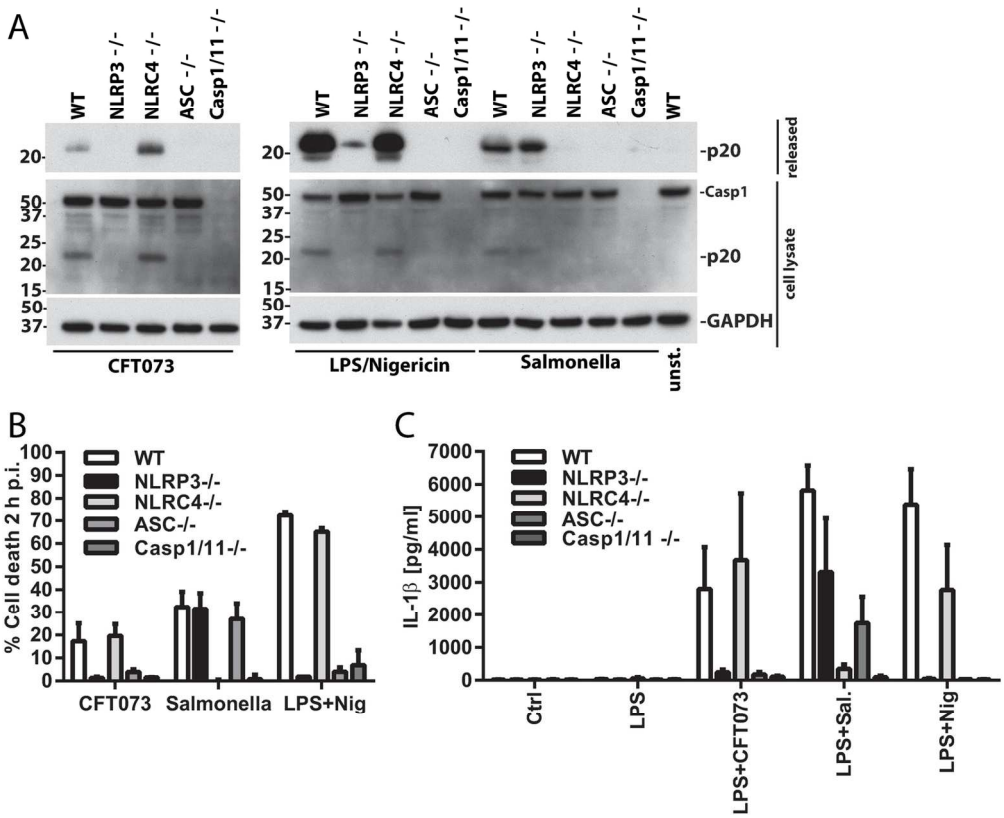


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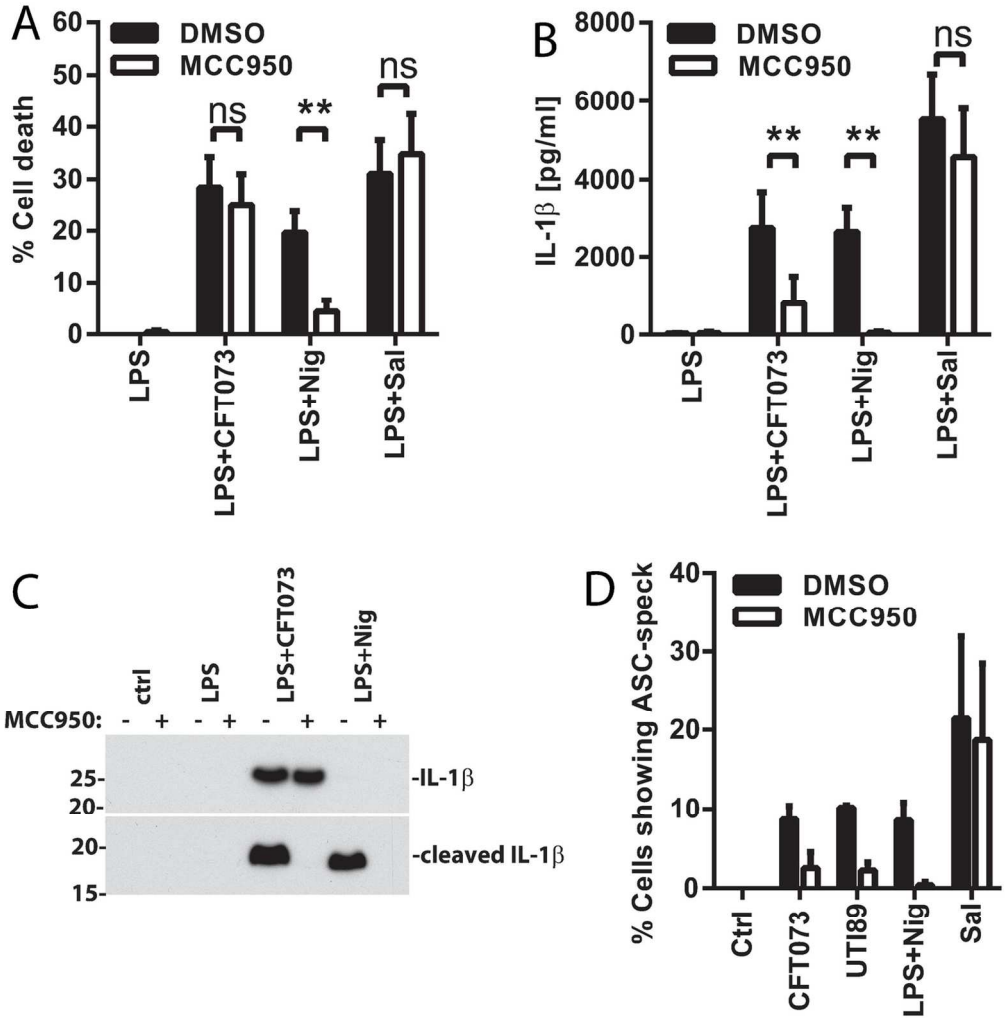


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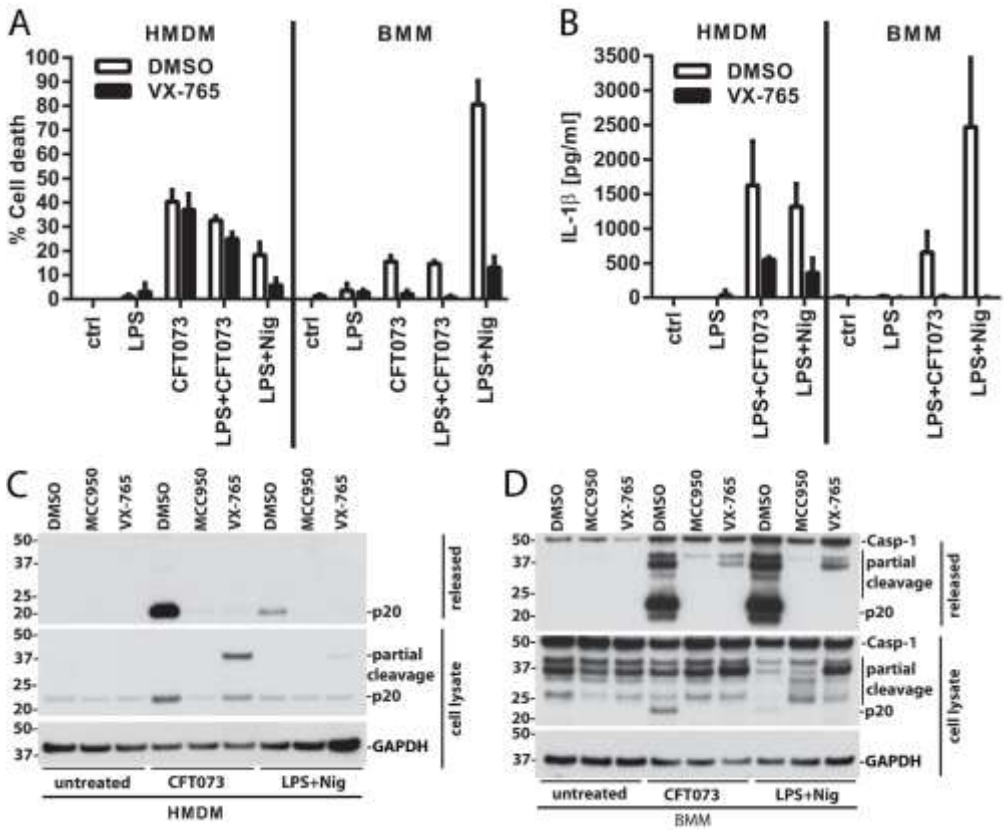


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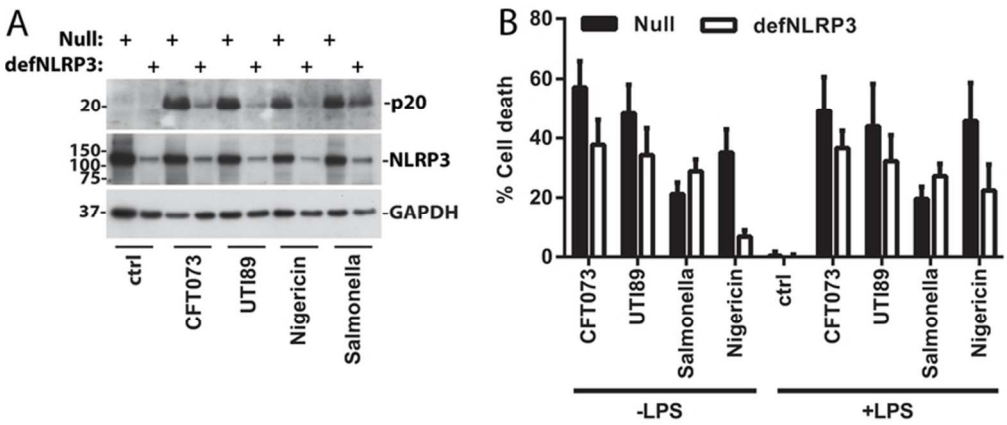


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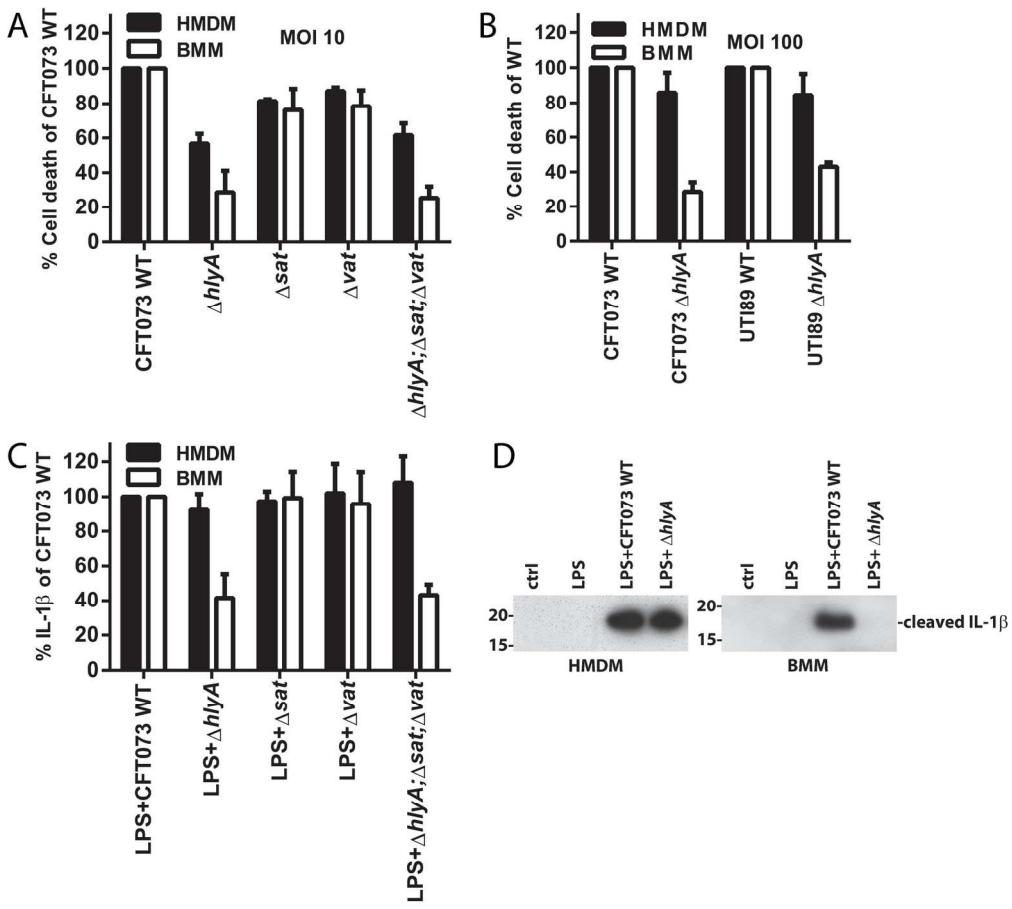


Fig. 7
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# Supplementary Figures

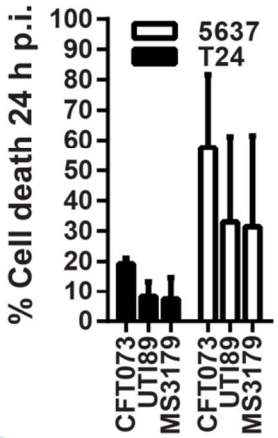


**Figure S1: UPEC strains CFT073 and UTI89 induce cell death in epithelial cells at high MOI.** Human bladder epithelial cell lines (T24 and 5637) were infected with the indicated UPEC strains (MOI 1000) for 24 h. Supernatants were analyzed by LDH release assay. Data are the mean + range of two independent experiments.

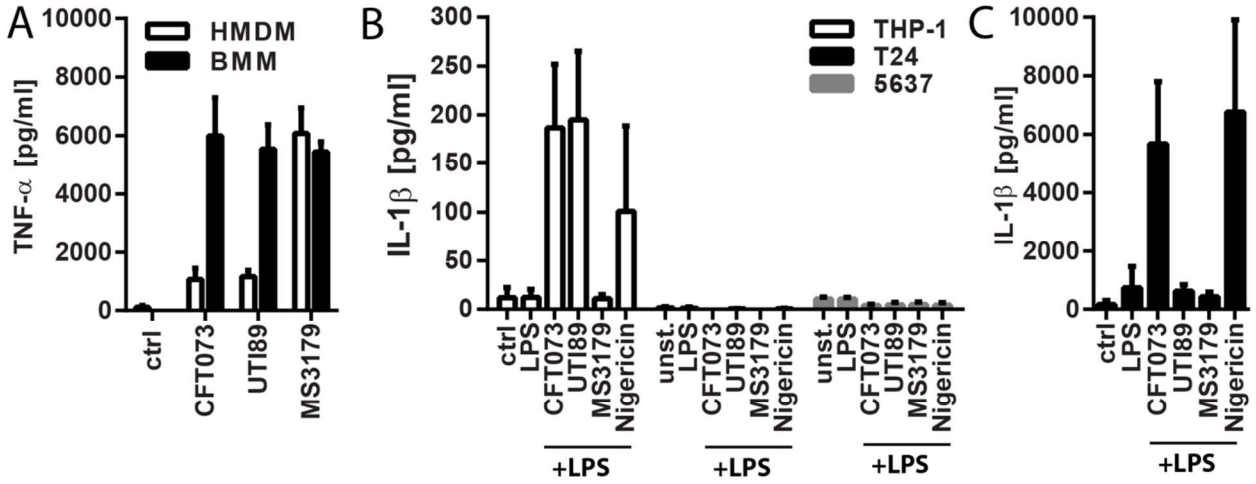


**Figure S2: TNF-α secretion inversely correlates with cell death in HMDM, whilst IL-1β secretion correlates with cell death in all cell types examined. A)** HMDM and BMM were infected with the indicated UPEC strains (MOI 10) for 2 h and supernatants were analyzed by ELISA. **B)** THP-1, T24 and 5637 cells and **C)** mouse peritoneal cavity cells (PCC) were primed with 100 ng/ml LPS for 4 h, then infected with the indicated UPEC strains for 2 h (MOI 10) or stimulated with nigericin (10 μM) for 2 h. Supernatants were analyzed by ELISA. Data in **A-C** are the mean + SEM of three independent experiments.

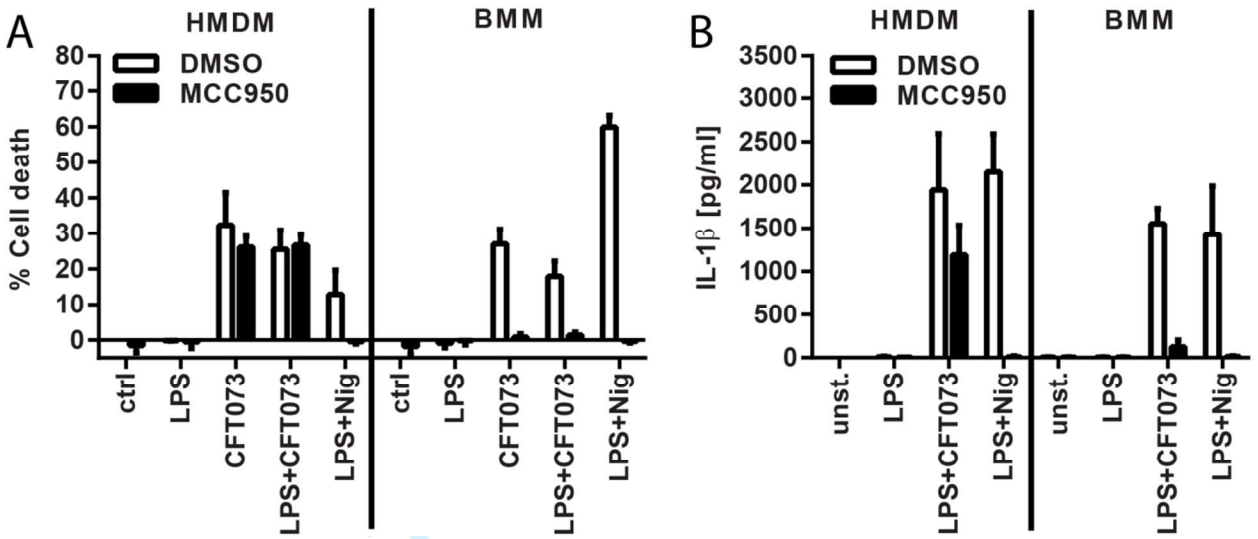


**Figure S3: NLRP3 inhibition has differing effects in CFT073-infected primary human versus mouse macrophages.** HMDM and BMM were primed with LPS (100 ng/ml, 4 h) or were left untreated, then pretreated for 1 h with the NLRP3 inhibitor MCC950 (10 µM), and subsequently infected with the UPEC strain CFT073 (MOI 10) or stimulated with nigericin (10 µM). At 2 h p.i., supernatants were collected and analyzed by **A)** LDH release assay and **B)** IL-1β ELISA. Data are the mean + SEM of three independent experiments.

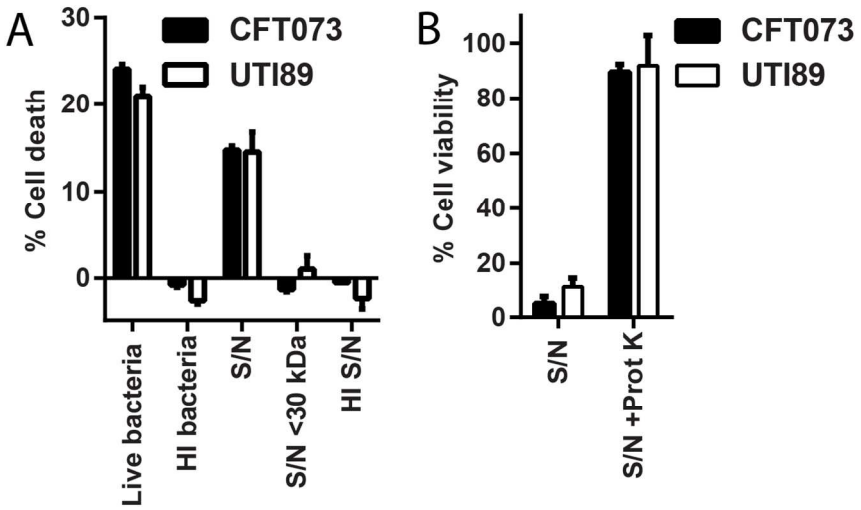


**Figure S4: CFT073- and UTI89-triggered cell death is mediated by a large, soluble factor(s) that is heat- and protease-sensitive. A)** PMA-differentiated THP-1 cells were treated with live bacteria at MOI 10, heat inactivated supernatants and bacteria (95˚C, 20 min) (HI bacteria), 10% filtered (0.45 µm) supernatants from bacterial overnight cultures (S/N), or flow though from filtration with Amicon Ultra-15 centrifugal fiter units with 30 kDa cutoff (S/N < 30 kDa). Cell culture supernatants were analyzed by LDH release assay after 2 h. Similar results were obtained in two independent experiments. **B)** PMA-differentiated THP-1 cells were treated with bacterial culture supernatants as above (S/N) or supernatants pretreated with proteinase K (100 µg/ml) for 45 min at 37˚C (S/N +Prot K). Cell viability was assessed by methylthiazolyldiphenyl-tetrazolium bromide (MTT) viability assays. Medium was replaced with medium containing 1 mg/ml MTT (Sigma-Aldrich) 2 h post-infection and incubated at 37˚C and 5% $CO_2$ for another 2 h. Cells were lysed in isopropanol and formation of formazan was assessed by measuring absorption at 570 nm. Cell viability was calculated as % of absorption at 570 nm of untreated cells.

| % Cell death | Donor : | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | MCC950 | | | | | | | | | | Average |
| LPS | - | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| | + | 0.34 | 0.47 | 1.21 | 2.83 | -0.23 | -1.16 | 0.00 | 0.00 | 0.00 | 0.38 |
| LPS+CFT073 | - | 6.93 | 34.49 | 27.71 | 39.05 | 8.08 | 63.04 | 17.32 | 23.25 | 35.83 | 28.41 |
| | + | 0.51 | 25.89 | 24.66 | 29.14 | 1.18 | 62.69 | 21.88 | 25.61 | 32.43 | 24.89 |
| LPS+Nig | - | 46.68 | 14.72 | 18.63 | 24.28 | 14.73 | 18.89 | 4.60 | 7.91 | 26.25 | 19.63 |
| | + | 1.06 | 14.98 | 16.85 | 3.19 | 1.89 | 2.63 | -0.45 | 0.50 | -1.54 | 4.35 |
| LPS+Sal | - | 10.71 | 49.15 | 32.57 | 27.43 | 17.73 | 48.54 | ND | ND | ND | 31.02 |
| | + | 10.95 | 43.51 | 45.27 | 27.51 | 20.24 | 61.45 | ND | ND | ND | 34.82 |
| LPS+UTI89 | - | 24.82 | 30.66 | 29.16 | 33.79 | ND | ND | ND | ND | ND | 29.61 |
| | + | -0.01 | 22.72 | 24.66 | 20.15 | ND | ND | ND | ND | ND | 16.88 |
| IL-1β [pg/ml] | Donor : | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
| | MCC950 | | | | | | | | | | Average |
| LPS | - | 41.74 | 42.49 | 37.23 | 15.48 | 43.21 | 12.56 | 0.00 | 25.94 | 0.00 | 24.29 |
| | + | 41.89 | 42.49 | 49.64 | 26.26 | 117.92 | 7.00 | 2.73 | 8.93 | 0.00 | 32.98 |
| LPS+CFT073 | - | 1877.28 | 232.20 | 2973.51 | 1084.81 | 2973.85 | 9706.29 | 685.04 | 2259.10 | 2876.68 | 2740.97 |
| | + | 363.65 | 67.74 | 1803.38 | 149.82 | 117.58 | 1093.71 | 501.10 | 1527.51 | 1539.38 | 795.98 |
| LPS+Nig | - | 1314.92 | 107.99 | 4872.90 | 1202.30 | 4629.54 | 5147.09 | 1497.69 | 2994.72 | 1949.71 | 2635.21 |
| | + | 46.94 | 43.10 | 74.98 | 44.01 | 106.71 | 33.27 | 3.47 | 27.50 | 0.00 | 42.22 |
| LPS+Sal | - | 5215.72 | 2708.92 | 7228.57 | 4618.81 | 3074.61 | 10308.71 | ND | ND | ND | 5525.89 |
| | + | 5701.27 | 2718.58 | 3973.76 | 4733.99 | 4182.30 | 6127.35 | ND | ND | ND | 4572.87 |
| LPS+UTI89 | - | 2235.10 | 238.90 | 4034.61 | 1220.35 | ND | ND | ND | ND | ND | 1932.24 |
| | + | 155.28 | 57.51 | 336.57 | 147.63 | ND | ND | ND | ND | ND | 174.25 |

**Table S1:** NLRP3 contributes to IL-1b release but contributes only marginally to cell death in human macrophages responding to the UPEC strains CFT073 and UTI89. HMDM were primed with LPS (100 ng/ml, 4 h), pretreated for 1 h with the NLRP3 inhibitor MCC950 (10 mM) and then subsequently infected with the UPEC strains CFT073 or UTI89 or *S.* Typhimurium SL1344 (MOI 10), or were stimulated with nigericin (10 mM). Supernatants were collected at 2 h p.i., and analyzed by LDH release assay and ELISA. Data are from nine (CFT073, LPS/nigericin), six (*S.* Typhimurium) or four (UTI89) independent experiments (different donors), respectively. ND = not determined.

# Strain- and host species-specific inflammasome activation, IL-1β release and cell death in macrophages infected with uropathogenic *Escherichia coli*

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## Abstract

Uropathogenic *E. coli* (UPEC) is the main etiological agent of urinary tract infections (UTI). Little is known ~~of the~~about interactions between UPEC and the inflammasome, a key innate immune pathway. Here we show that UPEC strains CFT073 and UTI89 trigger inflammasome activation and lytic cell death in human macrophages. Several other UPEC strains, including ~~globally disseminated~~two multidrug resistant ST131 ~~lineage~~ isolates, did not kill macrophages. In mouse macrophages, UTI89 triggered cell death only at a high MOI, and CFT073-mediated inflammasome responses were completely NLRP3-dependent. Surprisingly, CFT073- and UTI89-mediated responses only partially depended on NLRP3 in human macrophages. In these cells, NLRP3 ~~contributed to~~was required for IL-1β maturation, ~~and~~but contributed only marginally to cell death. Similarly, caspase-1 inhibition did not block cell death in human macrophages. In keeping with such differences, the pore forming toxin α-hemolysin mediated a substantial proportion of CFT073-triggered IL-1β secretion in mouse but not human macrophages. ~~Moreover, there~~There was also a more substantial α-hemolysin-independent cell death response in human versus mouse macrophages. Thus, in mouse macrophages, CFT073-triggered inflammasome responses are completely NLRP3-dependent, and largely α-hemolysin-dependent. In contrast, UPEC activates an NLRP3-independent cell death pathway and an α-hemolysin-independent IL-1β secretion pathway in human macrophages. This has important implications for understanding UTI in humans.

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## Introduction

Uropathogenic *Escherichia coli* (UPEC) is estimated to cause up to ~~75~~80% of community-acquired and 65% of nosocomial urinary tract infections (UTI), making it the single most important etiological agent of this highly prevalent infectious disease[1]. UTI typically involves infection of the bladder (cystitis) or kidneys (pyelonephritis), and can also lead to renal scarring and sepsis[~~1, 2~~1, 2]. Asymptomatic bacteriuria (ABU), caused by various etiologic agents, is also common[3].

Mouse UTI models, as well as genetic associations within patient cohort studies, have helped to pinpoint the roles of specific innate immune pathways in defense against uropathogens[2]. Such studies have highlighted the importance of members of the Toll-like Receptor family in controlling bacterial growth and dissemination, as well as causing symptoms and pathology. At the cellular level, roles of the urothelium and neutrophils in innate defence and host subversion during UTI are well established. However, surprisingly little is known about the roles of monocytes and macrophages in these processes[4]. A recent study by Schiwon *et al.* dissected the role of different macrophage populations in a mouse UTI model and unraveled complex interactions of sentinel and helper macrophages governing antimicrobial actions of neutrophils[5]. We previously demonstrated that some UPEC strains can survive for up to 24 h in murine bone marrow-derived macrophages (BMM) within LAMP1$^+$ compartments[6], reminiscent of quiescent intracellular reservoirs observed in epithelial cells that may facilitate recurrent infection. Thus, the role of myeloid cells in UPEC infection may not always be protective.

Upon detection of cellular stress and/or microbial products, some Nod-like-receptor (NLR) family members, as well as the PYHIN-family member AIM2, form large cytoplasmic multiprotein complexes known as inflammasomes. Inflammasomes have important functions in many bacterial infections[7], as well as in chronic diseases[8, 9]. The NLR-family comprises twenty-two genes in humans and more than thirty in mice, and can be phylogenetically grouped into the NLRP, IPAF and NOD sub-families[10]. The NLRP family members NLRP1 and NLRP3, as well as the IPAF family member NLRC4, can all initiate inflammasome formation. Most NLRP family members contain a C-terminal leucine rich repeat that is involved in danger sensing, a central nucleotide-binding and oligomerisation domain, and an N-terminal pyrin domain that relays downstream signalling. NLRC4 has a similar domain structure, but contains an N-terminal caspase recruitment domain (CARD), rather than a pyrin domain. Upon activation, NLRPs oligomerise and cluster into a cytoplasmic complex with the adapter protein ASC and the protease caspase-1, facilitating its autocatalytic cleavage and activation. Active caspase-1 is required for maturation and secretion of the pro-inflammatory IL-1 family cytokines, IL-1β and IL-18. One of the many functions of IL-1β is to facilitate neutrophil and macrophage recruitment to sites of infection. In addition to mediating cytokine processing, inflammasome activation also initiates a programmed, pro-inflammatory form of cell death called pyroptosis. Pyroptotic cell death is thought to eliminate the intracellular replication niche of pathogens that infect macrophages (e.g. *Shigella*, *Salmonella*, *Legionella* and *Listeria*) and to re-expose them to antimicrobial effector functions[7, 11].

Among the different pathogenic *E. coli* subtypes, enterohemorrhagic *E. coli* O157:H7, which causes severe enteritis, triggers inflammasome activation[12]. However, until very recently no

studies ~~have~~had investigated inflammasome involvement in UPEC recognition or UTI. In this study, we show that the genome-sequenced UPEC reference strains CFT073 and UTI89 trigger inflammasome activation and rapid cell death in macrophages, whereas others do not. Moreover, we define key mechanistic differences between human and mouse macrophages in the host recognition pathways and bacterial factors that initiate these responses. Our findings of fundamental differences between different UPEC strains in inflammasome engagement, as well as between human and mouse innate immune recognition pathways for UPEC, have major implications for understanding and modeling UTI pathogenesis.

## Results

### UPEC strains CFT073 and UTI89 cause rapid cell death in macrophages

Given the paucity of information on interactions between UPEC and macrophages, we investigated whether the survival of human monocyte-derived macrophages (HMDM) was affected by different UPEC strains. We analyzed strains associated with different UTI severity, including the reference strains CFT073 (a blood culture isolate from a patient with pyelonephritis ~~isolate~~)[13] and UTI89 (a urine isolate from a patient with recurrent cystitis ~~isolate~~)[14], the ~~ST131~~sequence type (ST) 131 strains EC958[~~15, 16~~15, 16] and MS3179 (urine isolates from patients with UTI) and the asymptomatic bacteriuria (ABU) strains 83972 and VR50[~~17, 18~~17, 18]. These experiments revealed that only CFT073 and UTI89 caused rapid, lytic cell death by 2 h post-infection as assessed by LDH release (**Fig. 1A**). Cell death was further increased by 24 h post-infection (**Fig. 1B**). A direct comparison of HMDM with murine BMM over a multiplicity of infection (MOI) range confirmed that mouse macrophages were also ~~similarly~~ susceptible to CFT073-induced cell death (**Fig. 1C**). In BMM, UTI89 did not trigger cell death, except at the highest MOI used (MOI 100, **Fig. 1C**). This is consistent with our previous findings that UTI89 can survive for up to 24 h within BMM[~~6, 19~~6, 19]. To investigate whether UPEC-mediated cell death is a macrophage-specific phenomenon, the response to CFT073, UTI89 and MS3179 was also analyzed in PMA-differentiated THP-1 cells (a human macrophage-like cell line), murine peritoneal cavity cells (PCC, which are predominantly comprised of resident peritoneal macrophages[20]) and two human bladder epithelial cell lines (5637 and T24) commonly used to

study UPEC infection *in vitro* (**Fig. 1D**). PMA-differentiated THP-1 cells showed a similar response to HMDM. CFT073 also triggered cell death in PCC, whereas the effect of UTI89 was much weaker, similar to the findings with mouse BMM (compare **Fig. 1C**). The ST131 isolate MS3179 did not trigger cell death in any of the cell types tested, and the two epithelial cell lines were not killed efficiently by any of the UPEC strains at an MOI of 10 at 2 h post-infection (**Fig. 1D**). However, 24 h exposure of epithelial cell lines to a very high MOI (MOI 1000) of all UPEC strains did result in some cell death (**Fig. S1**). Collectively, these data demonstrate substantial variability in the capacity of different UPEC strains to elicit macrophage cell death.

**UPEC-mediated macrophage cell death correlates with inflammasome activation**

To investigate potential involvement of the inflammasome pathway in cell death, we first examined the capacity of UPEC strains to trigger IL-1β release from LPS-primed macrophages. LPS priming was performed to boost pro-IL-1β levels, allowing the use of IL-1β release as a marker for inflammasome activation with minimal interference by rapid cell death~~.~~ or by other confounding processes such as suppression of cytokine production by some UPEC strains[21]. Indeed, we found that, whereas CFT073, UTI89 and MS3179 all elicited similar levels of secreted TNF-α from BMM, this response was greatly reduced in HMDM infected with strains triggering rapid cell death (CFT073, UTI89) as compared to MS3179 that did not cause cell death (**Fig. S2A**). Thus, TNF-α release inversely correlated with UPEC-induced rapid cell death in HMDM, as might be expected given that this cytokine must be synthesized prior to its release. In the case of IL-1β release ~~correlated~~from LPS-primed cells that already express pro-IL-1β, there was a clear correlation with induction of cell death in all cases. In human macrophages (HMDM and PMA-differentiated THP-1 cells), both CFT073 and UTI89 (MOI 10) triggered IL-

1β release (**Fig. 2A and ~~S2A~~S2B**). In contrast, CFT073 but not UTI89 (MOI 10) elicited IL-1β release from LPS-primed mouse macrophages (BMM and PCC) (**Fig. 2A and ~~S2B~~S2C**), consistent with the failure of UTI89 to trigger robust cell death in mouse macrophages at low MOI (compare **Fig. 1C and 1D**). Also consistent with the cell death data, the ST131 strain MS3179 did not trigger IL-1β release from LPS-primed macrophages of either human or murine origin. LPS-primed epithelial cell lines did not release IL-1β in response to any of the UPEC strains tested (**Fig. ~~S2A~~S2B**). Similar patterns were observed for caspase-1 cleavage; both CFT073 and UTI89 triggered comparable capase-1 cleavage in human macrophages (**Fig. 2B**), whilst in mouse macrophages the response ~~for~~to CFT073 was much more pronounced than for UTI89 (**Fig. 2B**). Another hallmark of inflammasome activation, the formation of ASC specks, was also apparent in CFT073- and UTI89-infected human macrophages, whereas the ST131 strain MS3179 did not elicit this effect (**Fig. 2C**). ~~Additionally~~Furthermore, infection with the two ASC speck-inducing UPEC strains (i.e. CFT073 and UTI89) ~~led~~appeared to ~~dramatic~~induce morphological changes and loss of nuclear integrity in HMDM, as visualized by actin and DNA staining~~.~~ in the same samples (**Fig. 2C**).

**CFT073-mediated cell death in murine macrophages is completely dependent on the NLRP3 inflammasome**

Causality of inflammasome activation and cell death in BMM was analysed using macrophages deficient for NLRP3 and NLRC4 (two NLRs most commonly activated by bacterial infection[7]), the inflammasome adaptor protein ASC, or the inflammatory caspases, caspase-1 and -11. Since UTI89 did not trigger pronounced inflammasome activation in mouse macrophages at an MOI of 10, only CFT073 was assessed. CFT073-mediated caspase-1 cleavage was completely dependent

on NLRP3 and ASC, but did not require NLRC4 (**Fig. 3A**). As expected, the positive controls nigericin and *Salmonella enterica serovar* Typhimurium (*S.* Typhimurium) strain SL1344 acted via NLRP3 and NLRC4, respectively (**Fig. 3A**). Analysis of LDH release confirmed that NLRP3, ASC and Caspase-1/11 were required for CFT073-mediated cell death (**Fig. 3B**). These inflammasome components were also indispensable for CFT073-triggered IL-1β release from LPS-primed BMM (**Fig. 3C**). As with caspase-1 cleavage, NLRC4-deficiency did not affect LDH or IL-1β release upon infection with CFT073. Again, the positive controls for NLRP3, ASC and caspase-1 involvement (nigericin), and NLRC4 and caspase-1 involvement (*S.* Typhimurium), behaved as expected. ASC was dispensable for *S.* Typhimurium-mediated cell death (**Fig. 3B**) as previously reported[~~21~~22], and NLRP3, ASC and caspase-1/11 were indispensable for nigericin-triggered IL-1β release (**Fig. 3C**). *S.* Typhimurium-triggered IL-1β release was partially dependent on NLRP3 and ASC, and completely dependent on NLRC4 and caspase-1/11. Hence, rapid cell death and IL-1β secretion triggered by CFT073 in mouse macrophages is dependent on NLRP3, ASC and caspase-1 and/or -11.

**In human macrophages, UPEC-mediated IL-1β secretion is ~~partially~~ dependent on NLRP3, whereas cell death is primarily NLRP3-independent**

We next investigated NLRP3 involvement in human macrophage responses to UPEC~~, using a recently described NLRP3 inhibitor, MCC950[22]~~. using MCC950, a recently described NLRP3 inhibitor that does not affect AIM2, NLRP1 or NLRC4-mediated inflammasome activation[23]. Surprisingly, in LPS-primed HMDM, MCC950 blocked nigericin-triggered cell death, but had little effect on CFT073-triggered cell death in cells from most donors examined (**Fig. 4A, Table S1**). In contrast, MCC950 significantly reduced both nigericin- and CFT073-triggered IL-1β

release from LPS-primed HMDM (**Fig. 4B**). Similar observations were apparent for UTI89, where MCC950 had only modest effects in reducing UTI89-triggered cell death for HMDM from 3 out of 4 donors (**Table S1**). This suggests that there are differences between human and mouse macrophages in NLRP3 responses to UPEC. Indeed, a direct comparison revealed that, whereas MCC950 completely inhibited responses to nigericin in both HMDM and BMM, CFT073-mediated cell death was only blocked in mouse macrophages (**Fig. S3A**). Moreover, LPS priming had no apparent effect on NLRP3-dependency of CFT073-mediated cell death in human or mouse macrophages. In contrast to differential effects on cell death, MCC950 inhibited CFT073-triggered IL-1β release in both LPS-primed HMDM and BMM, albeit more effectively in BMM (**Fig. S3B**). The level of NLRP3-dependence for CFT073-triggered IL-1β release varied between cells from different donors (~~**Fig. 4B, S3B**~~**Table S1**), and MCC950 never completely abolished IL-1β release, as was the case for nigericin. ~~This~~We therefore ~~suggests that CFT073 triggers both NLRP3-dependent and -independent inflammasomes in human macrophages. To examine this~~investigated the possibility that the residual IL-1β response detected by ELISA might be due to the release of unprocessed IL-1β, as a consequence of cell death. Indeed, analysis of concentrated cell culture supernatants by immunoblotting revealed that treatment with MCC950 completely blocked release of mature IL-1β from CFT073-infected HMDM, whereas pro-IL-1β was still present in culture supernatants (**Fig. 4C**). ~~, we~~We also monitored ASC speck formation upon NLRP3 inhibition in HMDM. In these experiments, the NLRP3 inhibitor MCC950 completely blocked nigericin- but not *S.* Typhimurium-triggered ASC speck formation (**Fig. 4D**)~~4C). In contrast to its effects on the nigericin response,~~ MCC950 substantially reduced, but did not ablate, CFT073- and UTI89-induced ASC speck formation in all experiments (**Fig. 4D**).

To further investigate inflammasome involvement in human macrophages, the effect of the caspase-1 specific inhibitor VX-765[23,24] on CFT073-triggered cell death in human and mouse macrophages was examined. VX-765 effectively blocked CFT073- and LPS/nigericin-triggered cell death and IL-1β release by LPS-primed BMM at 2 h post-infection (**Fig. 5A-B**). However, similar to the observations with MCC950, VX-765 reduced CFT073-mediated IL-1β release from LPS-primed HMDM, but did not affect cell death. Control experiments confirmed that both inhibitors blocked cleavage and release of caspase-1 in response to CFT073 infection and LPS/nigericin stimulation in HMDM and BMM (**Fig. 5C-D**). Together, these findings indicate that (1) the NLRP3 inflammasome ~~primarily~~ drives UPEC-triggered IL-1β maturation in human macrophages; ~~(2) another inflammasome also likely contributes to this response;~~ and (~~3~~2) an NLRP3-independent pathway is the primary mediator of UPEC-triggered cell death in human macrophages.

To independently verify that an NLRP3-independent pathway mediates UPEC-triggered cell death, we used THP-1 defNLRP3 cells, which stably express an NLRP3 shRNA and have reduced NLRP3 expression compared to a control cell line (THP-1 Null) transfected with an 'empty' construct (**Fig. 6A**). Caspase-1 processing in THP-1 defNLRP3 cells responding to CFT073, UTI89 or ~~LPS/~~nigericin was greatly reduced, whereas caspase-1 p20 was still detectable at high levels after infection with *S.* Typhimurium. In these cells, CFT073 and UTI89 still triggered substantial cell death, whereas ~~LPS/~~nigericin did not (**Fig. 6B**). Again, LPS priming had no effect on the degree of NLRP3 dependency of UPEC-mediated cell death~~.~~, although it did appear to reduce the effect of NLRP3-knockdown in the nigericin control. As

expected, no reduction in cell death was observed in defNLRP3 cells when using *S.* Typhimurium as an NLRP3-independent trigger for cell death. Collectively, these data suggest the involvement of another NLRP3-independent, cell death pathway triggered by UPEC.

**α-hemolysin is the main factor in CFT073 triggering cell death and IL-1β release in mouse but not human macrophages**

The capacity for CFT073 and UTI89 to trigger human macrophage cell death was also conferred by culture supernatants (**Fig. S4**). Crude biochemical analysis indicated that the factor(s) responsible were heat- and protease-sensitive, and with a likely molecular weight of greater than 30 kDa. Candidate proteins mediating cell death and/or IL-1β secretion included the pore forming toxin α-hemolysin, as well as serine-protease autotransporter toxins (Sat and Vat). Genes encoding all three toxins are present in CFT073, while UTI89 contains the *hlyCABD* (α-hemolysin operon) and *vat* genes. To test the involvement of these three toxins in triggering the inflammasome response, we generated a series of CFT073 mutants deleted for genes encoding each individual toxin and a triple-mutant deficient in the ability to produce all three toxins. Compared to wild type CFT073, the α-hemolysin ~~(*hlyA*)~~ mutant (CFT073Δ*hlyA*) was greatly impaired in its ability to trigger cell death of mouse macrophages ~~(~70%)~~(~30% of wild type) and, to a lesser extent, of human macrophages ~~(~40%)~~(~60% of wild type) when macrophages were exposed to an MOI of 10 (**Fig. 7A**). CFT073 *sat*- and *vat*- mutants were very modestly compromised for their ability to trigger mouse and human macrophage cell death. However, no additive effect was observed, as the triple-mutant showed no difference to the CFT073Δ*hlyA* single mutant in this assay. Similar observations were made at an MOI of 100 with both CFT073 and UTI89 (**Fig. 7B**). ~~*hlyA* single mutant in this assay. Intriguingly, analysis of IL-1β secretion~~ In

this case, cell death induced by CFT073 and UTI89 was almost completely *hlyA*-independent in HMDM (cell death for *hlyA* mutants was ~85% of the wild type strains), whereas in BMM the response was largely *hlyA*-dependent (~30% and 40% of wild type strains). Intriguingly, analysis of IL-1β release revealed a striking difference between human and mouse macrophages with respect to triggering by α-hemolysin. Whereas IL-1β levels were substantially reduced in BMM responding to ~~the *hlyA* mutant as~~CFT073Δ*hlyA* compared to wild type CFT073, deletion of *hlyA* had no effect on HMDM responses (**Fig. 7C**). Again, we analysed cleavage of released IL-1β by western blot and found that in HMDM levels of cleaved IL-1β were similar in samples infected with CFT073 and CFT073Δ*hlyA* (**Fig. 7D**). In contrast, CFT073Δ*hlyA* was drastically impaired in its ability to induce the release of cleaved IL-1β in BMM.~~**7B).**~~ Deletion of *sat* or *vat* had no effect on IL-1β release from either human or mouse macrophages, and the response to the triple-mutant was again identical to that of the *hlyA* single-mutant. Thus, α-hemolysin is the primary, but not only, mediator of cell death and IL-1β release in mouse macrophages. Moreover, generation of mature IL-1β was completely dependent on α-hemolysin. By contrast, in the human macrophage response to UPEC, α-hemolysin does not contribute to IL-1β release or cleavage, and plays ~~only~~ a ~~minor~~lesser role in rapid cell death. These differential effects of α-hemolysin further highlight the divergent UPEC recognition pathways of human and mouse macrophages. In summary, UPEC triggers both NLRP3-independent cell death and α-hemolysin-independent IL-1β processing in human macrophages, thus indicating that additional host and pathogen-derived factors are likely to be important in the macrophage response to UPEC.

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## Discussion

~~To our knowledge,~~In this ~~is the first report describing the ability of~~ study, we demonstrate that some UPEC ~~to~~strains can trigger both NLRP3-dependent inflammasome ~~responses~~ activation and rapid cell death in macrophages. We also provide important insights into these processes in the context of similarities and differences between human and mouse macrophage responses to UPEC. Other *E. coli,* including enterohemorrhagic *E. coli* isolates[12] as well as non-pathogenic or commensal stains[~~24, 25~~25, 26], have been reported to activate inflammasomes by a variety of different mechanisms involving several bacterial factors. These include nucleic acids[~~26~~27] and protein toxins (enterohemolysin[12] and heat-labile enterotoxin[27]~~) acting via NLRP3, the T3SS rod protein EprJ[28] and flagellin[24] acting via NLRC4 and (intracellular) LPS[29] acting via non-canonical inflammasomes. Here we showed that the UPEC strains CFT073 and UTI89 induce rapid lytic cell death in primary human macrophages and correlated this response with their ability to elicit inflammasome activation.~~[28]) acting via NLRP3, the T3SS rod protein EprJ[29] and flagellin[25] acting via NLRC4 and (intracellular) LPS[30] acting via non-canonical inflammasomes. Our study adds to this literature by identifying UPEC α-hemolysin-dependent and -independent mechanisms of inflammasome activation in macrophages.

It remains unclear as to what roles UPEC-mediated inflammasome activation has in different pathophysiological contexts. Two out of the four strains that did not elicit inflammasome activation are associated with ABU[~~17, 18~~17, 18], whilst the remaining two belong to the globally disseminated fluoroquinolone-resistant *fimH*30/clade C ST131 lineage that is frequently associated with symptomatic infection[~~16, 30~~16, 31]. The two inflammasome-activating strains are

also associated with UTI pathology. Hence, the capacity for inflammasome activation is variable, further highlighting the genetic diversity that exists amongst different UPEC isolates. Since the capacity to trigger inflammasome activation and macrophage cell death was not common to all UPEC strains, some UPEC strains may have gained inflammasome-activating factors as a component of their virulence ~~mechanisms~~armoury or lost these to avoid host detection. In the case of the former, candidate virulence factors included the pore forming toxin α-hemolysin[~~31~~32], as well as serine-protease autotransporter toxins (Sat and Vat) that are known to elicit cytotoxic effects on epithelial cells[32]~~, and for which the genes are present in CFT073 and UTI89 but not 83972, VR50[33] or EC958[15]~~.[33], and for which the genes are present in CFT073 (*hlyA, sat, vat*) and UTI89 (*hlyA, vat*), but not 83972, VR50[34] or EC958[15]. Mutation of all three factors in CFT073 revealed that only the absence of α-hemolysin substantially reduced inflammasome responses in mouse macrophages. Intriguingly, however, CFT073-triggered IL-1β release and cleavage was completely independent of α-hemolysin in human macrophages, and there was also a pronounced α-hemolysin-independent cell death pathway. This finding points towards fundamentally different recognition mechanisms for these UPEC strains in human versus mouse macrophages, yet conservation in the overall outcomes. Whether this extends to other cell types needs to be further examined, given ~~an earlier~~a recent report on *E. coli* α-hemolysin triggering IL-1β secretion in human urothelial cells[35], an earlier study showing a similar phenomenon in human monocytes[36] and well documented cell type-specific effects of α-hemolysin[~~31~~32].

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Our aim to characterize the specific inflammasome involved in UPEC recognition led us to study the response of mouse macrophages as a more tractable genetic system, as compared to human macrophages. Initial experiments revealed that mouse and human macrophages seemed to

respond similarly with regard to induction of cell death, caspase-1 cleavage and IL-1β secretion upon infection with CFT073. In the case of UTI89, a much less pronounced response was observed in mouse macrophages compared to human macrophages~~,~~ when using a low MOI (MOI 10). Nonetheless, a 10-fold higher MOI did initiate some cell death in these cells. In contrast, both CFT073 and UTI89 had similar effects on human macrophages. The conservation between human and mouse macrophage responses to CFT073 led us to focus on this particular strain for the identification of host mechanisms mediating cellular responses. NLRP3, ASC and the inflammatory caspases (1 and/or 11) were indispensable for CFT073-mediated rapid lytic cell death and IL-1β secretion in mouse BMM, while a role for NLRC4 was excluded. These findings are consistent with a very recent study showing that UTI89 induces moderate IL-1β release from mouse macrophages in an NLRP3-dependent manner, albeit under different experimental conditions[37].

The conclusion that the acute CFT073-mediated inflammasome response leading to cell death and IL-1β secretion in BMM was absolutely dependent on NLRP3 was also supported by experiments using small molecule inhibitors of NLRP3 (MCC950) and caspase-1 (VX-765). However, primary human macrophages that were analysed in parallel showed a remarkably different response. The NLRP3 and caspase-1 inhibitors substantially reduced or blocked IL-1β ~~secretion~~release from HMDM in response to UPEC infection or the NLRP3 agonist nigericin, respectively. In contrast, UPEC-triggered cell death was largely unaffected by either inhibitor. Although analysis of HMDM generated from several donors revealed some variation in the level of NLRP3-dependence, the overall conclusion is that in human macrophages cell death is NLRP3-independent, whereas IL-1β ~~release always shows at least some NLRP3-~~

~~dependence.~~cleavage was shown to be completely NLRP3-dependent. Interestingly, LPS-primed HMDMs released unprocessed IL-1β upon UPEC infection, even when the NLRP3 inflammasome was blocked. Biologically, this may be of significance since it was shown that uncleaved IL-1β can be processed in the extracellular space by inflammasome complexes[38] or by enzymes such as cathepsin-G and elastase[39]. Consistent with the existence of an NLRP3-independent death pathway in human macrophages, stable knockdown of NLRP3 in THP-1 cells blocked ~~LPS/~~nigericin-induced cell death, but only marginally reduced CFT073- and UTI89-mediated cell death. ~~Given~~Whether NLRP3-independent cell death involves activation of another inflammasome is unknown at this stage. However, given that NLRP3 inhibition ablated both UPEC-~~triggered ASC speck formation still occurred to some extent~~induced IL-1β maturation and caspase-1 cleavage in HMDM~~ after blocking NLRP3 function, a~~, other modes of cell death such as necroptosis would appear to be more likely~~ explanation is the existence of another UPEC-sensing inflammasome in~~.

Divergence in the repertoire of NLR family members between human ~~macrophages. Human versus~~and mouse can contribute to differences in ~~UPEC-triggered~~ inflammasome responses ~~could potentially be explained by divergence in the repertoire of NLR family members~~ between these species[10, 34,40]. However, differences in the recognition of pathogens by orthologous human and mouse NLRs have also been reported. For example, *F. tularensis* activates only the AIM2 inflammasome in mouse macrophages, but triggers NLRP3- and AIM2-dependent responses in human macrophages[35, 41]. Conversely, *L. monocytogenes* was reportedly recognized by AIM2, NLRP3 and NLRC4 in mouse cells[36, 38,42-44], but exclusively by NLRP3 in human cells[39,45]. The causes for these differences are not fully understood, but may be related to species differences in

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ligand recognition. ~~siRNA screens of likely inflammasome candidates in human macrophages have thus far failed to identify the responsible host recognition system. Furthermore, other modes of cell death such as necroptosis cannot be discounted at this stage.~~Our study highlights that one pathogen can activate NLRP3 in both human and mouse macrophages, but through distinct mechanisms. Our demonstration of α-hemolysin-dependent IL-1β cleavage and cell death in mouse macrophages is consistent with a recent study showing α-hemolysin-mediated inflammasome activation in UTI89-infected mice[35]. In stark contrast however, our studies with human macrophages identified an α-hemolysin-independent pathway to IL-1β maturation. This suggests that another UPEC factor selectively promotes NLRP3 activation in human but not mouse macrophages, or that its relative potency in triggering inflammasome responses differs between these species or between different cell types.

~~The selective role of NLRP3 in promoting IL-1β release but not cell death in human macrophages responding to UPEC suggests~~Emerging evidence indicates that cytokine processing and pyroptosis can be uncoupled~~. This is reminiscent of recent studies of mouse neutrophils, where~~ in some systems. For example, *Salmonella*-mediated NLRC4 activation promoted IL-1β maturation but not pyroptosis[40] in mouse neutrophils[46]. Other studies have also reported distinct roles for individual inflammasomes in cytokine processing versus pyroptosis. For example, NLRP3 was shown to mediate cell death and IL-1β release in *S. aureus*-infected HMDM, whilst a novel NLRP7 inflammasome was shown to selectively promote IL-1β secretion[41,47]. Similarly, NLRP3 and NLRC4 were shown to mediate *B. pseudomallei*-induced IL-1β and IL-18 release in the mouse, whilst pyroptotic cell death was attributed only to NLRC4[42,48]. At present, there is no unifying model explaining why similar recognition systems lead to cytokine maturation in one

setting, and pyroptosis in another. Broz *et al.* proposed that CARD-containing NLRs ~~form a cytokine processing inflammasome in the presence of ASC, but a death complex directly with caspase-1 in its absence[21].~~can initiate distinct complexes with different roles in mediating cytokine maturation versus pyroptotic cell death[22]. However, the above described mechanism does not apply for most NLRPs and AIM2, which contain a pyrin domain rather than a CARD[10], and does not explain how death and cytokine responses happen simultaneously in the presence of ASC. The fact that NLRP3 was causal for cytokine processing but not cell death in human macrophages might again be interpreted as another example of uncoupling of downstream inflammasome responses. However, it would seem more likely that the NLRP3-independent cell death pathway overrides NLRP3-dependent pyroptosis in our system.

A protective role for inflammasome activation and IL-1β production has been shown in many *in vivo* infection models including *S. Typhimurium*, *L. monocytogenesis* and *Burkholderia* species[11] On the other hand, the role of pathogen-induced cell death is ambiguous, having either protective or detrimental effects by either eradicating intracellular niches or promoting dissemination, respectively[43]~~. Since UPEC can occupy both extracellular and intracellular niches, it is difficult to predict what role pyroptosis plays during UTI. Approaches for genetically and/or pharmacologically uncoupling pyroptosis from other inflammasome responses will be required to address this question. In the case of cytokine processing, previous studies have associated IL-1β release with renal pathology of UTI in patients[44, 45] and in a mouse model[46]. Whether NLRP3 has a causal role in host defence or pathology is unknown at this stage. Given differences between human and mouse macrophages in NLRP3 dependence for UPEC responses, this is not a trivial task.~~[49]. Since UPEC can occupy both extracellular and intracellular niches, it is difficult

to predict what role pyroptosis plays during UTI. Activation of caspase-1/11 was shown to facilitate clearance of UPEC in a mouse model, presumably by inducing pyroptosis and subsequent exfoliation of bladder epithelial cells[35]. In another study however, activity of caspase-1/11 was associated with chronicity and higher bacterial loads in the bladder in a model of recurrent UTI[50]. In the case of cytokine processing, previous studies have associated IL-1β release with renal pathology of UTI in patients[51, 52], and also in a mouse model[53]. Only very recently was IL-1β release in *atg16l1*-/- mice shown to be associated with protection from UTI[37]. While the effects of IL-1β can be studied simply by knockout or by blocking its interaction with receptors, new approaches for genetically and/or pharmacologically uncoupling pyroptosis from other inflammasome responses will be required to address the role of cell death in pathology. Whether NLRP3-dependent responses have a causal role in host defence or pathology remains to be elucidated.

In conclusion, our study highlights the complexity of interactions between UPEC and the innate immune system. Some UPEC strains trigger inflammasome activation and rapid, lytic cell death in macrophages, whereas others, including two strains from the ~~globally disseminated~~multidrug resistant ST131 lineage, do not. ~~Further, whereas~~This again highlights the genetic complexity that exists amongst different UPEC~~-mediated cell death~~ strains and ~~IL-1β secretion are mediated by~~that host response pathways engaged, as well as host colonization strategies employed, will vary depending on the specific UPEC strain encountered. For inflammasome-activating strains such as CFT073, NLRP3 ~~in~~drives IL-1β maturation in both human and mouse macrophages. However, this pathway only ~~partly~~marginally contributes to ~~IL-1β maturation~~cell death in human macrophages, despite its causal role in cell death in mouse macrophages. Finally, α-hemolysin is

the primary trigger for cell death and IL-1β release in mouse macrophages, whereas these cellular responses are either primarily or completely independent of this toxin in human macrophages. The yet-to-be-identified death pathway in human monocyte-derived macrophages highlights a ~~key~~potential difference between human and mouse innate immune UPEC recognition pathways~~.~~ and needs to be considered in future studies using macrophages and other cell types from a variety of sources, as well as in *in vivo* studies. Given the importance of mouse UTI models for understanding host colonization and pathology, and especially in the light of recent studies showing detrimental and beneficial effects of inflammasome activation in mouse models[37, 50], as well as a prominent role for α-hemolysin[35], our findings are likely to have broad significance for understanding susceptibility and severity of UTI in humans.

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## Methods

### Bacterial strains and growth conditions

UPEC strains CFT073[13], UTI89[14], 83972[17], VR50[18] and EC958[15] have been described previously. MS3179 is an ST131 strain isolated from a patient presenting with UTI at the Royal Brisbane and Women's Hospital, Brisbane, Australia. *S.* Typhimurium strain SL1344[47] was used as a control for NLRP3-independent inflammasome activation in some experiments. All strains were routinely grown at 37˚C on solid or in liquid Luria-Bertani (LB) medium.

### Genetic manipulation procedures and generation of mutants

Mutation of the *hlyA*, *sat* and *vat* genes in CFT073, and the *hlyA* gene in UTI89, was performed using the λ-Red recombinase gene replacement system[48]. The primers used for amplification of the kanamycin resistance gene (*hlyA*) or chloramphenicol resistance gene (*vat, sat*), and subsequent insertion into the chromosome of CFT073 (or UTI89) were as follows: *vat* (3353: 5'-tcgtaatgaacacagttcatctgatctccacacaccaagacttgataagctcacgtcttgagcgattgtgtagg and 3354: 5'-gaaaccaccaccccatgattttgttttaccgctgtacaggcctgctgacgcgacatgggaattagccatggtcc), *sat* (3351: 5'-aagaaattccaatgattttgagattcagaggttaaataaatttgttgtggacacgtcttgagcgattgtgtagg and 3352: 5'-ccaggagtgggagctgtagtctctggtgccaaggccggcgaaagttgcggtgacatgggaattagccatggtcc), *hlyA* (2049: 5'-aaattaaaagcacactacagtctgcaaagcaatcctctgcaaataaattgtgtaggctggagctgcttc and 2050: 5'-tgctctgctgctttttttaatgcatctttcgtgctttgtcctgctgagtgcatatgaatatcctccttag). CFT073 *hlyA* (CFT073Δ*hlyA*), *sat* (CFT073Δ*sat*) and *vat* (CFT073Δ*vat*) mutants, as well as the UTI89 *hlyA* mutant (UTI89Δ*hlyA*), were confirmed by PCR and DNA sequencing. The CFT073*hly-sat-vat*

triple mutant was constructed by sequential deletion of each gene, as described above, and was confirmed by PCR and DNA sequencing.

**Mammalian cell culture**

Approval for all experiments using primary human and mouse cells was obtained from the University of Queensland Medical Research Ethics Committee or the Animal Ethics Committee. Human monocytes were isolated from buffy coats of healthy donors (kindly provided by the Australian Red Cross) by positive selection for CD14 using MACS technology (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described[49,56]. HMDM were differentiated for 7 days with CSF-1 (10,000 U/ml, Chiron Emeryville, CA, USA) from $CD14^+$ cells, as previously described[49,56], but in the absence of antibiotics. Cells from a single donor were used in every experiment. Murine BMM were differentiated using 10,000 U/ml CSF-1 (Chiron) from bone marrow of C57BL/6 wild type, *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$, *Asc*$^{-/-}$, and *Caspase-1/11*$^{-/-}$ mice (all described in[40,46]), in the absence of antibiotics as previously described[6]. PCC were flushed from the peritoneal cavity of C57BL/6 mice by injection of 5 ml PBS. THP-1 (TIB-202, ATCC, Manassas, VA, USA), THP-1 Null and THP-1 defNLRP3 (InvivoGen, San Diego, CA, USA) cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1 mM Na-Pyruvate and 10 mM HEPES (all Life Technologies, Carlsbad, CA, USA). THP-1 cells were differentiated into macrophage-like cells by culture for 48 h in medium containing 30 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA). PMA or CSF-1 was removed 4 h prior to infection with UPEC strains. Human bladder epithelial cell lines 563455637 (HTB-9, ATCC) and T24 (HTB-4, ATCC) were cultured in FBS (10%) and 2 mM L-glutamine supplemented RPMI-1640 or McCoy's 5A medium (Life Technologies), respectively.

**Bacterial culture and macrophage infection assays**

UPEC strains were grown statically at 37˚C overnight in LB broth. *S*. Typhimurium strain SL1344 was grown overnight in LB broth (200 rpm, 37˚C), diluted 1:33 and grown for another 3 h (to ensure logarithmic growth). Cells were pelleted, washed and adjusted to the same optical density at 600 nm. A MOI of approximately 10 was used (unless indicated otherwise) and confirmed by enumeration of colony forming units following serial dilution. Mammalian cells were seeded at a density of 4-8x$10^4$/0.2 ml in 96-well plates or 2-4x$10^5$/ml in 24-well plates (Nunc, Roskilde, Denmark). Medium was changed for all cell types to RPMI-1640 supplemented with FBS (10%) and 2 mM L-glutamine (all Life Technologies) 4 h prior to infection. LPS-priming was performed by addition of 100 ng/ml Ultrapure LPS from *Salmonella minnesota* R595 (InvivoGen). Nigericin sodium salt (Sigma-Aldrich) at a concentration of 10 µM was used as a positive control for NLRP3-dependent responses. In some experiments, cells were pre-incubated for 1 h with the caspase-1 inhibitor VX-765 (Selleck Chemicals, Houston, TX, USA) or the NLRP3 inhibitor MCC950[22]~~, prior to performing infections.~~[23], prior to performing infections. At 1 h post-infection, 200 µg/ml gentamicin (Life Technologies) was added for 1 h to inhibit growth of extracellular bacteria. For infections over a 24 h time course, medium was replaced with fresh medium containing 20 µg/ml gentamicin for the remaining 22 h.

**Cytotoxicity assays**

Cell culture supernatants were collected at 2 or 24 h post-infection, centrifuged for 5 min at 500 g, and analysed for LDH release using the *In Vitro* Toxicology Assay Kit (Sigma-Aldrich). Cytotoxicity (%) was calculated by quantification of LDH in culture supernatants versus total

559 cellular LDH (present in S/N after cell lysis with 0.1% Triton X-100) according to the formula %
560 cell death = (100/$LDH_{total}$-$LDH_{spontaneous}$)x($LDH_{treatment}$-$LDH_{spontaneous}$). ~~Values calculated at~~ 24 h
561 values represent summed ~~measures~~measurements of the same ~~wells~~well at 2 and 24 h post-
562 infection, since medium was changed at 2 h post-infection ~~(see above~~as part of the gentamicin
563 exclusion protocol (see: Bacterial culture and macrophage infection assays).
564
565 **Confocal microscopy**
566 Confocal microscopy was performed as previously described[6]. Cells were stained with 200 ng/ml
567 Alexa Fluor® 594 Phalloidin (Life Technologies) to visualize cell morphology, and ASC was
568 detected with a rabbit anti-ASC Antibody (N-15)-R (Santa Cruz Biotechnology, Santa Cruz, CA)
569 (1:300) and Alexa Fluor® 647 or 688-conjugated chicken anti-Rabbit IgG (Life Technologies) as
570 a secondary antibody (1:150). For quantifying ASC speck formation, HMDM were cultured in
571 12 mM glycine to reduce loss of cells due to lytic cell death[~~50~~ 57]. ASC specks were counted
572 manually in a blinded fashion (5 fields at 40x magnification per condition per replicate).
573
574 **Immunoblotting**
575 4x$10^5$ cells were lysed in 100 µl 2xSDS loading buffer (125 mM Tris-HCl, 20% glycerol (*v/v*),
576 4% SDS (*w/v*), pH 6.8). For analysis of secreted caspase-1 in cell culture supernatants, medium
577 was replaced with OptiMEM medium (Life Technologies) 4 h prior to infection. Cell culture
578 supernatants were precipitated by incubation with 4 volumes of acetone at -20˚C overnight and
579 centrifugation at 5300 g and -10˚C for 30 min. Pellets were taken up in 2xSDS loading buffer.
580 Western blotting was performed as previously described[~~49~~ 56]. Membranes were stained with
581 cleaved IL-1β (Asp116) rabbit mAb, IL-1β (3A6) mouse mAb, cleaved caspase-1 (ASP297)

(D57A2) rabbit mAb (all Cell Signalling Technology, Danvers, MA, USA), mouse IL-1β/IL-1F2 affinity purified polyclonal Ab, Goat IgG (R&D Systems, Minneapolis, MN, USA), anti-caspase-1 (p20) (mouse) mAb (Adipogen, San Diego, CA, USA), anti-NLRP3/NALP3 mAb (Cryo-2) (Adipogen) or human anti-G3PDH antibody (Trevigen, Gaithersburg, MD, USA). All primary antibodies were diluted 1:1000 except for human anti-G3PDH antibody, which was used at 1:10000. As secondary antibodies, anti-mouse and anti-rabbit IgG, HRP-linked Antibodies (Cell Signalling Technology) ~~were used (1:2500).~~(1:2500) and anti-goat IgG-peroxidase antibody (Sigma-Aldrich) (1:5000) were used. HRP was detected using ECL Plus substrate (GE Healthcare, Buckinghamshire, UK) and Super RX film (Fujifilm, Tokyo, Japan).

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**ELISA**

Cell culture supernatants were analysed for IL-1β with the human or mouse IL-1β/IL-1F2 DuoSet ELISA kit (R&D Systems~~, Minneapolis, MN, USA~~) (detection limit 4 and 15.6 pg/ml, respectively) and anti-human or mouse ELISA Ready-Set-Go! (eBioscience, San Diego, CA, USA~~).~~) (detection limit: 4 and 8 pg/ml, respectively). TNF-α was detected using the Mouse TNF OptEIA ELISA set (BD Biosciences, San Diego, CA, USA) (detection limit: 15.6 pg/ml) and the human TNF-α standard ELISA Developmental kit (Peprotech, Rocky Hill, NJ, USA) (detection limit: 16 pg/ml) .

**Statistical Analysis**

All LDH, ELISA and MTT assays were performed using duplicate or triplicate cell culture wells for individual experiments. Presented data are typically mean values combined from three or more independent experiments, unless otherwise indicated. For statistical analysis of datasets

with N>4, two-sided Wilcoxon matched-pairs signed-rank tests were performed using GraphPad Prism Version 6 (GraphPad software, Inc., La Jolla, CA, USA). For these datasets, the differences between pairs were plotted and were generally distributed approximately symmetrically around the median.

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## Disclosure

The author declared no conflict of interest.

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## Figure legends

**Figure 1: The UPEC strains CFT073 and UTI89 induce rapid cell death in macrophages. A-C)** HMDM or BMM were infected with the indicated UPEC strains (MOI 1, 10 and 100). Supernatants were analyzed by LDH release assays at the indicated times~~.~~ post-infection (p.i.). Data represent the mean+SEM of three independent experiments. **D)** Human macrophage-like cells (THP-1) and mouse peritoneal cavity cells (PCC), as well as ~~the~~ human T24 and 5637 bladder epithelial ~~cell lines (T24 and 5637),~~cells, were infected with the indicated UPEC strains (MOI 10) for 2 h. Supernatants were analyzed by LDH release assay. Data represent the mean+range of two independent experiments.

**Figure 2: The UPEC strains CFT073 and UTI89 promote IL-1β secretion, caspase-1 cleavage and ASC-speck formation. A)** HMDM and BMM were primed with 100 ng/ml LPS for 4 h, then infected with the indicated UPEC strains (MOI 10) for 2 h. Supernatants were analyzed by ELISA. Data represent the mean+SEM of three independent experiments. **B)** HMDM and BMM were infected with the indicated UPEC strains for 1 h. Whole cell lysates and concentrated supernatants were analyzed by western blot with antibodies detecting human or mouse caspase-1 p20. GAPDH is shown as a loading control. Similar findings were apparent in

three independent experiments. **C)** HMDM were infected with the indicated UPEC strains (MOI 10) for 2 h, after which they were fixed and analyzed by confocal microscopy. Arrowheads indicate ASC-specks. Similar results were apparent in two independent experiments.

**Figure 3: In murine macrophages, CFT073-induced caspase-1 cleavage and cell death is dependent on the NLRP3 inflammasome.** BMM derived from wild type, *Nlrp3*-, *Nlrc4*-, *Asc*- and *Casp1/11*-deficient mice were infected with the UPEC strain CFT073 or the *S.* Typhimurium strain SL1344 (MOI 10), or were stimulated with nigericin (10 μM, pre-stimulated with 100 ng/ml LPS for 4 h). **A)** After 1 h, whole cell lysates and concentrated supernatants were analyzed by western blot with antibodies detecting murine full-length caspase-1, as well as multiple caspase-1 cleavage products. GAPDH was used as loading control. Data is representative of two independent experiments. **B)** Supernatants were analyzed by LDH assay at 2 h p.i. Data is the mean+range of two independent experiments. **C**) LPS-primed BMM (100 ng/ml, 4 h) were treated as above, after which secreted IL-1β was measured by ELISA after 2 h. Data are mean+SEM of three independent experiments.

**Figure 4: NLRP3 ~~partially~~ contributes to IL-1β release and maturation ~~and~~but does not mediate cell death in human macrophages responding to CFT073. A-B)** HMDM were primed with LPS (100 ng/ml, 4 h~~),~~) or left untreated, then pretreated for 1 h with the NLRP3 inhibitor MCC950 (10 μM) and ~~then~~ subsequently infected with the UPEC strain CFT073 or *S.* Typhimurium SL1344 (MOI 10), or ~~were~~ stimulated with nigericin (10 μM). Supernatants were collected at 2 h p.i., and analyzed by LDH release assay **(A)** and ELISA **(B)**. Data are from nine (CFT073, LPS/nigericin) and six (*S.* Typhimurium) independent experiments (different donors),

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respectively. Statistical analysis was performed using a Wilcoxon matched-pairs signed-rank tests: ns: p> 0.05, *: p<=0.05, **: p<=0.01. ~~C~~C) Concentrated supernatants from cells treated as described above were analyzed by western blot with antibodies detecting full-length or cleaved human IL-1β. **D)** HMDM were treated as above, but in presence of 12 mM glycine, and analyzed by confocal microscopy. ASC specks and nuclei were counted manually in a blinded manner. Data are mean+SEM of three independent experiments**.**

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**Figure 5: UPEC-mediated cell death is blocked by caspase-1 inhibition in mouse but not human macrophages. A-B)** HMDM and BMM were primed with LPS (100 ng/ml, 4 h~~),~~) or left untreated, then pretreated for 1 h with the caspase-1 inhibitor VX-765 (50 μM), and subsequently infected with the UPEC strain CFT073 (MOI 10) or ~~were~~ stimulated with nigericin (10 μM). Supernatants were collected at 2 h p.i., and were analyzed for cell death by LDH release assay **(A)** and IL-1β release by ELISA **(B)**. Data represent the mean+SEM of three independent experiments. **(C-D)** HMDM and BMM were treated as above for 1 h. Cell lysates, as well as cell culture supernatants, were analyzed by western blot for cleavage of caspase-1. GAPDH served as loading control. Similar results were obtained in two independent experiments.

**Figure 6: UPEC triggers NLRP3-independent cell death in human macrophages.** Stable NLRP3 knockdown THP-1 cells (defNLRP3), as well as THP-1 control cells (Null) were infected with the indicated UPEC strains or *S.* Typhimurium (MOI 10), or were treated with nigericin (10 μM) for 1 h **(A)** or 2 h **(B)**. **A)** Cell lysates were analyzed by western blot for expression of NLRP3 and GAPDH as a loading control. Caspase-1 p20 was detected in cell

lysates. Similar findings were apparent in two independent experiments. **B)** Supernatants of unprimed or LPS primed (100 ng/ml LPS, 4 h) THP-1 cells were analyzed by LDH assay. Data are the mean+SEM of ~~three~~four independent experiments.

**Figure 7: Differences between human and mouse macrophages in cellular responses to ~~the toxin~~ α-hemolysin.** **A-B)** HMDM and BMM were infected with wild type ~~CFT073,~~ ~~CFT073~~UPEC strains or mutants deficient for α-hemolysin (*ΔhlyA*), *sat* (*Δsat*), *vat* (*Δvat*) or all three genes (*ΔhlyA;Δsat;Δvat*) at ~~a~~the indicated MOI ~~10~~ for 2 h. ~~A)~~ Supernatants were analyzed by LDH assay. ~~B~~**C)** LPS–primed (100 ng/ml, 4 h) cells were treated as above (MOI 10) and analyzed by IL-1β ELISA. Data are the mean+SEM of three (**A, C**) or four (**B**) independent experiments. **D**) Concentrated supernatants from cells treated as described above were analyzed by western blot with antibodies detecting cleaved human or mouse IL-1β. Similar observations were made in 2 independent experiments.

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