



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

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1 **Strain- and host species-specific inflammasome activation, IL-1 $\beta$  release and cell death in**  
2 **macrophages infected with uropathogenic *Escherichia coli***

3

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22

## 23 Abstract

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

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## 45 **Introduction**

46 Uropathogenic *Escherichia coli* (UPEC) is estimated to cause up to 80% of community-acquired  
47 and 65% of nosocomial urinary tract infections (UTI), making it the single most important  
48 etiological agent of this highly prevalent infectious disease<sup>1</sup>. UTI typically involves infection of  
49 the bladder (cystitis) or kidneys (pyelonephritis), and can also lead to renal scarring and sepsis<sup>1, 2</sup>.

50 Asymptomatic bacteriuria (ABU), caused by various etiologic agents, is also common<sup>3</sup>.

51  
52 Mouse UTI models, as well as genetic associations within patient cohort studies, have helped to  
53 pinpoint the roles of specific innate immune pathways in defense against uropathogens<sup>2</sup>. Such  
54 studies have highlighted the importance of members of the Toll-like Receptor family in  
55 controlling bacterial growth and dissemination, as well as causing symptoms and pathology. At  
56 the cellular level, roles of the urothelium and neutrophils in innate defence and host subversion  
57 during UTI are well established. However, surprisingly little is known about the roles of  
58 monocytes and macrophages in these processes<sup>4</sup>. A recent study by Schiwon *et al.* dissected the  
59 role of different macrophage populations in a mouse UTI model and unraveled complex  
60 interactions of sentinel and helper macrophages governing antimicrobial actions of neutrophils<sup>5</sup>.  
61 We previously demonstrated that some UPEC strains can survive for up to 24 h in murine bone  
62 marrow-derived macrophages (BMM) within LAMP1<sup>+</sup> compartments<sup>6</sup>, reminiscent of quiescent  
63 intracellular reservoirs observed in epithelial cells that may facilitate recurrent infection. Thus,  
64 the role of myeloid cells in UPEC infection may not always be protective.

65  
66 Upon detection of cellular stress and/or microbial products, some Nod-like-receptor (NLR)  
67 family members, as well as the PYHIN-family member AIM2, form large cytoplasmic

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

86

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

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

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For Peer Review

## 114 **Results**

115

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

117 Given the paucity of information on interactions between UPEC and macrophages, we  
118 investigated whether the survival of human monocyte-derived macrophages (HMDM) was  
119 affected by different UPEC strains. We analyzed strains associated with different UTI severity,  
120 including the reference strains CFT073 (a blood culture isolate from a patient with  
121 pyelonephritis)<sup>13</sup> and UTI89 (a urine isolate from a patient with recurrent cystitis)<sup>14</sup>, the sequence  
122 type (ST) 131 strains EC958<sup>15, 16</sup> and MS3179 (urine isolates from patients with UTI) and the  
123 asymptomatic bacteriuria (ABU) strains 83972 and VR50<sup>17, 18</sup>. These experiments revealed that  
124 only CFT073 and UTI89 caused rapid, lytic cell death by 2 h post-infection as assessed by LDH  
125 release (**Fig. 1A**). Cell death was further increased by 24 h post-infection (**Fig. 1B**). A direct  
126 comparison of HMDM with murine BMM over a multiplicity of infection (MOI) range  
127 confirmed that mouse macrophages were also susceptible to CFT073-induced cell death (**Fig.**  
128 **1C**). In BMM, UTI89 did not trigger cell death, except at the highest MOI used (MOI 100, **Fig.**  
129 **1C**). This is consistent with our previous findings that UTI89 can survive for up to 24 h within  
130 BMM<sup>6, 19</sup>. To investigate whether UPEC-mediated cell death is a macrophage-specific  
131 phenomenon, the response to CFT073, UTI89 and MS3179 was also analyzed in PMA-  
132 differentiated THP-1 cells (a human macrophage-like cell line), murine peritoneal cavity cells  
133 (PCC, which are predominantly comprised of resident peritoneal macrophages<sup>20</sup>) and two human  
134 bladder epithelial cell lines (5637 and T24) commonly used to study UPEC infection *in vitro*  
135 (**Fig. 1D**). PMA-differentiated THP-1 cells showed a similar response to HMDM. CFT073 also  
136 triggered cell death in PCC, whereas the effect of UTI89 was much weaker, similar to the



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

143

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

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

160 failure of UTI89 to trigger robust cell death in mouse macrophages at low MOI (compare **Fig.**  
161 **1C and 1D**). Also consistent with the cell death data, the ST131 strain MS3179 did not trigger  
162 IL-1 $\beta$  release from LPS-primed macrophages of either human or murine origin. LPS-primed  
163 epithelial cell lines did not release IL-1 $\beta$  in response to any of the UPEC strains tested (**Fig.**  
164 **S2B**). Similar patterns were observed for caspase-1 cleavage; both CFT073 and UTI89 triggered  
165 comparable caspase-1 cleavage in human macrophages (**Fig. 2B**), whilst in mouse macrophages  
166 the response to CFT073 was much more pronounced than for UTI89 (**Fig. 2B**). Another hallmark  
167 of inflammasome activation, the formation of ASC specks, was also apparent in CFT073- and  
168 UTI89-infected human macrophages, whereas the ST131 strain MS3179 did not elicit this effect  
169 (**Fig. 2C**). Furthermore, infection with the two ASC speck-inducing UPEC strains (i.e. CFT073  
170 and UTI89) appeared to induce morphological changes and loss of nuclear integrity in HMDM,  
171 as visualized by actin and DNA staining in the same samples (**Fig. 2C**).

172

### 173 **CFT073-mediated cell death in murine macrophages is completely dependent on the** 174 **NLRP3 inflammasome**

175 Causality of inflammasome activation and cell death in BMM was analysed using macrophages  
176 deficient for NLRP3 and NLRC4 (two NLRs most commonly activated by bacterial infection<sup>7</sup>),  
177 the inflammasome adaptor protein ASC, or the inflammatory caspases, caspase-1 and -11. Since  
178 UTI89 did not trigger pronounced inflammasome activation in mouse macrophages at an MOI of  
179 10, only CFT073 was assessed. CFT073-mediated caspase-1 cleavage was completely dependent  
180 on NLRP3 and ASC, but did not require NLRC4 (**Fig. 3A**). As expected, the positive controls  
181 nigericin and *Salmonella enterica serovar* Typhimurium (*S. Typhimurium*) strain SL1344 acted  
182 via NLRP3 and NLRC4, respectively (**Fig. 3A**). Analysis of LDH release confirmed that

183 NLRP3, ASC and Caspase-1/11 were required for CFT073-mediated cell death (**Fig. 3B**). These  
184 inflammasome components were also indispensable for CFT073-triggered IL-1 $\beta$  release from  
185 LPS-primed BMM (**Fig. 3C**). As with caspase-1 cleavage, NLRC4-deficiency did not affect  
186 LDH or IL-1 $\beta$  release upon infection with CFT073. Again, the positive controls for NLRP3,  
187 ASC and caspase-1 involvement (nigericin), and NLRC4 and caspase-1 involvement (*S.*  
188 *Typhimurium*), behaved as expected. ASC was dispensable for *S. Typhimurium*-mediated cell  
189 death (**Fig. 3B**) as previously reported<sup>22</sup>, and NLRP3, ASC and caspase-1/11 were indispensable  
190 for nigericin-triggered IL-1 $\beta$  release (**Fig. 3C**). *S. Typhimurium*-triggered IL-1 $\beta$  release was  
191 partially dependent on NLRP3 and ASC, and completely dependent on NLRC4 and caspase-  
192 1/11. Hence, rapid cell death and IL-1 $\beta$  secretion triggered by CFT073 in mouse macrophages is  
193 dependent on NLRP3, ASC and caspase-1 and/or -11.

194

195 **In human macrophages, UPEC-mediated IL-1 $\beta$  secretion is dependent on NLRP3, whereas**  
196 **cell death is primarily NLRP3-independent**

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

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

223  
224 To further investigate inflammasome involvement in human macrophages, the effect of the  
225 caspase-1 specific inhibitor VX-765<sup>24</sup> on CFT073-triggered cell death in human and mouse  
226 macrophages was examined. VX-765 effectively blocked CFT073- and LPS/nigericin-triggered  
227 cell death and IL-1 $\beta$  release by LPS-primed BMM at 2 h post-infection (**Fig. 5A-B**). However,  
228 similar to the observations with MCC950, VX-765 reduced CFT073-mediated IL-1 $\beta$  release

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

235  
236 To independently verify that an NLRP3-independent pathway mediates UPEC-triggered cell  
237 death, we used THP-1 defNLRP3 cells, which stably express an NLRP3 shRNA and have  
238 reduced NLRP3 expression compared to a control cell line (THP-1 Null) transfected with an  
239 ‘empty’ construct (**Fig. 6A**). Caspase-1 processing in THP-1 defNLRP3 cells responding to  
240 CFT073, UTI89 or nigericin was greatly reduced, whereas caspase-1 p20 was still detectable at  
241 high levels after infection with *S. Typhimurium*. In these cells, CFT073 and UTI89 still triggered  
242 substantial cell death, whereas nigericin did not (**Fig. 6B**). Again, LPS priming had no effect on  
243 the degree of NLRP3 dependency of UPEC-mediated cell death, although it did appear to reduce  
244 the effect of NLRP3-knockdown in the nigericin control. As expected, no reduction in cell death  
245 was observed in defNLRP3 cells when using *S. Typhimurium* as an NLRP3-independent trigger  
246 for cell death. Collectively, these data suggest the involvement of another NLRP3-independent,  
247 cell death pathway triggered by UPEC.

248  
249  **$\alpha$ -hemolysin is the main factor in CFT073 triggering cell death and IL-1 $\beta$  release in mouse**  
250 **but not human macrophages**

251 The capacity for CFT073 and UTI89 to trigger human macrophage cell death was also conferred  
252 by culture supernatants (**Fig. S4**). Crude biochemical analysis indicated that the factor(s)  
253 responsible were heat- and protease-sensitive, and with a likely molecular weight of greater than  
254 30 kDa. Candidate proteins mediating cell death and/or IL-1 $\beta$  secretion included the pore  
255 forming toxin  $\alpha$ -hemolysin, as well as serine-protease autotransporter toxins (Sat and Vat).  
256 Genes encoding all three toxins are present in CFT073, while UTI89 contains the *hlyCABD* ( $\alpha$ -  
257 hemolysin operon) and *vat* genes. To test the involvement of these three toxins in triggering the  
258 inflammasome response, we generated a series of CFT073 mutants deleted for genes encoding  
259 each individual toxin and a triple-mutant deficient in the ability to produce all three toxins.  
260 Compared to wild type CFT073, the  $\alpha$ -hemolysin mutant (CFT073 $\Delta$ *hlyA*) was greatly impaired  
261 in its ability to trigger cell death of mouse macrophages (~30% of wild type) and, to a lesser  
262 extent, of human macrophages (~60% of wild type) when macrophages were exposed to an MOI  
263 of 10 (**Fig. 7A**). CFT073 *sat*- and *vat*- mutants were very modestly compromised for their ability  
264 to trigger mouse and human macrophage cell death. However, no additive effect was observed,  
265 as the triple-mutant showed no difference to the CFT073 $\Delta$ *hlyA* single mutant in this assay.  
266 Similar observations were made at an MOI of 100 with both CFT073 and UTI89 (**Fig. 7B**). In  
267 this case, cell death induced by CFT073 and UTI89 was almost completely *hlyA*-independent in  
268 HMDM (cell death for *hlyA* mutants was ~85% of the wild type strains), whereas in BMM the  
269 response was largely *hlyA*-dependent (~30% and 40% of wild type strains). Intriguingly, analysis  
270 of IL-1 $\beta$  release revealed a striking difference between human and mouse macrophages with  
271 respect to triggering by  $\alpha$ -hemolysin. Whereas IL-1 $\beta$  levels were substantially reduced in BMM  
272 responding to CFT073 $\Delta$ *hlyA* compared to wild type CFT073, deletion of *hlyA* had no effect on  
273 HMDM responses (**Fig. 7C**). Again, we analysed cleavage of released IL-1 $\beta$  by western blot and

274 found that in HMDM levels of cleaved IL-1 $\beta$  were similar in samples infected with CFT073 and  
275 CFT073 $\Delta hlyA$  (**Fig. 7D**). In contrast, CFT073 $\Delta hlyA$  was drastically impaired in its ability to  
276 induce the release of cleaved IL-1 $\beta$  in BMM. Deletion of *sat* or *vat* had no effect on IL-  
277 1 $\beta$  release from either human or mouse macrophages, and the response to the triple-mutant was  
278 again identical to that of the *hlyA* single-mutant. Thus,  $\alpha$ -hemolysin is the primary, but not only,  
279 mediator of cell death and IL-1 $\beta$  release in mouse macrophages. Moreover, generation of mature  
280 IL-1 $\beta$  was completely dependent on  $\alpha$ -hemolysin. By contrast, in the human macrophage  
281 response to UPEC,  $\alpha$ -hemolysin does not contribute to IL-1 $\beta$  release or cleavage, and plays a  
282 lesser role in rapid cell death. These differential effects of  $\alpha$ -hemolysin further highlight the  
283 divergent UPEC recognition pathways of human and mouse macrophages. In summary, UPEC  
284 triggers both NLRP3-independent cell death and  $\alpha$ -hemolysin-independent IL-1 $\beta$  processing in  
285 human macrophages, thus indicating that additional host and pathogen-derived factors are likely  
286 to be important in the macrophage response to UPEC.

287

288

289

## 290 Discussion

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

302  
303 It remains unclear as to what roles UPEC-mediated inflammasome activation has in different  
304 pathophysiological contexts. Two out of the four strains that did not elicit inflammasome  
305 activation are associated with ABU<sup>17, 18</sup>, whilst the remaining two belong to the globally  
306 disseminated fluoroquinolone-resistant *fimH30*/clade C ST131 lineage that is frequently  
307 associated with symptomatic infection<sup>16, 31</sup>. The two inflammasome-activating strains are also  
308 associated with UTI pathology. Hence, the capacity for inflammasome activation is variable,  
309 further highlighting the genetic diversity that exists amongst different UPEC isolates. Since the  
310 capacity to trigger inflammasome activation and macrophage cell death was not common to all  
311 UPEC strains, some UPEC strains may have gained inflammasome-activating factors as a



312 component of their virulence armoury or lost these to avoid host detection. In the case of the  
313 former, candidate virulence factors included the pore forming toxin  $\alpha$ -hemolysin<sup>32</sup>, as well as  
314 serine-protease autotransporter toxins (Sat and Vat) that are known to elicit cytotoxic effects on  
315 epithelial cells<sup>33</sup>, and for which the genes are present in CFT073 (*hlyA*, *sat*, *vat*) and UTI89  
316 (*hlyA*, *vat*), but not 83972, VR50<sup>34</sup> or EC958<sup>15</sup>. Mutation of all three factors in CFT073 revealed  
317 that only the absence of  $\alpha$ -hemolysin substantially reduced inflammasome responses in mouse  
318 macrophages. Intriguingly, however, CFT073-triggered IL-1 $\beta$  release and cleavage was  
319 completely independent of  $\alpha$ -hemolysin in human macrophages, and there was also a  
320 pronounced  $\alpha$ -hemolysin-independent cell death pathway. This finding points towards  
321 fundamentally different recognition mechanisms for these UPEC strains in human versus mouse  
322 macrophages, yet conservation in the overall outcomes. Whether this extends to other cell types  
323 needs to be further examined, given a recent report on *E. coli*  $\alpha$ -hemolysin triggering IL-  
324 1 $\beta$  secretion in human urothelial cells<sup>35</sup>, an earlier study showing a similar phenomenon in  
325 human monocytes<sup>36</sup> and well documented cell type-specific effects of  $\alpha$ -hemolysin<sup>32</sup>.

326  
327 Our aim to characterize the specific inflammasome involved in UPEC recognition led us to study  
328 the response of mouse macrophages as a more tractable genetic system, as compared to human  
329 macrophages. Initial experiments revealed that mouse and human macrophages seemed to  
330 respond similarly with regard to induction of cell death, caspase-1 cleavage and IL-1 $\beta$  secretion  
331 upon infection with CFT073. In the case of UTI89, a much less pronounced response was  
332 observed in mouse macrophages compared to human macrophages when using a low MOI (MOI  
333 10). Nonetheless, a 10-fold higher MOI did initiate some cell death in these cells. In contrast,  
334 both CFT073 and UTI89 had similar effects on human macrophages. The conservation between

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

342  
343 The conclusion that the acute CFT073-mediated inflammasome response leading to cell death  
344 and IL-1 $\beta$  secretion in BMM was absolutely dependent on NLRP3 was also supported by  
345 experiments using small molecule inhibitors of NLRP3 (MCC950) and caspase-1 (VX-765).  
346 However, primary human macrophages that were analysed in parallel showed a remarkably  
347 different response. The NLRP3 and caspase-1 inhibitors substantially reduced or blocked IL-1 $\beta$   
348 release from HMDM in response to UPEC infection or the NLRP3 agonist nigericin,  
349 respectively. In contrast, UPEC-triggered cell death was largely unaffected by either inhibitor.  
350 Although analysis of HMDM generated from several donors revealed some variation in the level  
351 of NLRP3-dependence, the overall conclusion is that in human macrophages cell death is  
352 NLRP3-independent, whereas IL-1 $\beta$  cleavage was shown to be completely NLRP3-dependent.  
353 Interestingly, LPS-primed HMDMs released unprocessed IL-1 $\beta$  upon UPEC infection, even  
354 when the NLRP3 inflammasome was blocked. Biologically, this may be of significance since it  
355 was shown that uncleaved IL-1 $\beta$  can be processed in the extracellular space by inflammasome  
356 complexes<sup>38</sup> or by enzymes such as cathepsin-G and elastase<sup>39</sup>. Consistent with the existence of  
357 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-1 $\beta$  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<sup>10, 40</sup>. 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<sup>41</sup>. Conversely, *L.*  
369 *monocytogenes* was reportedly recognized by AIM2, NLRP3 and NLRC4 in mouse cells<sup>42-44</sup>, but  
370 exclusively by NLRP3 in human cells<sup>45</sup>. 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  $\alpha$ -hemolysin-dependent IL-1 $\beta$  cleavage and cell  
374 death in mouse macrophages is consistent with a recent study showing  $\alpha$ -hemolysin-mediated  
375 inflammasome activation in UTI89-infected mice<sup>35</sup>. In stark contrast however, our studies with  
376 human macrophages identified an  $\alpha$ -hemolysin-independent pathway to IL-1 $\beta$  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

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

399

400 A protective role for inflammasome activation and IL-1 $\beta$  production has been shown in many *in*  
401 *vivo* infection models including *S. Typhimurium*, *L. monocytogenes* and *Burkholderia* species<sup>11</sup>  
402 On the other hand, the role of pathogen-induced cell death is ambiguous, having either protective  
403 or detrimental effects by either eradicating intracellular niches or promoting dissemination,

404 respectively<sup>49</sup>. Since UPEC can occupy both extracellular and intracellular niches, it is difficult  
405 to predict what role pyroptosis plays during UTI. Activation of caspase-1/11 was shown to  
406 facilitate clearance of UPEC in a mouse model, presumably by inducing pyroptosis and  
407 subsequent exfoliation of bladder epithelial cells<sup>35</sup>. In another study however, activity of caspase-  
408 1/11 was associated with chronicity and higher bacterial loads in the bladder in a model of  
409 recurrent UTI<sup>50</sup>. In the case of cytokine processing, previous studies have associated IL-1 $\beta$   
410 release with renal pathology of UTI in patients<sup>51, 52</sup>, and also in a mouse model<sup>53</sup>. Only very  
411 recently was IL-1 $\beta$  release in *atg16ll1*<sup>-/-</sup> mice shown to be associated with protection from UTI<sup>37</sup>.  
412 While the effects of IL-1 $\beta$  can be studied simply by knockout or by blocking its interaction with  
413 receptors, new approaches for genetically and/or pharmacologically uncoupling pyroptosis from  
414 other inflammasome responses will be required to address the role of cell death in pathology.  
415 Whether NLRP3-dependent responses have a causal role in host defence or pathology remains to  
416 be elucidated.

417  
418 In conclusion, our study highlights the complexity of interactions between UPEC and the innate  
419 immune system. Some UPEC strains trigger inflammasome activation and rapid, lytic cell death  
420 in macrophages, whereas others, including two strains from the multidrug resistant ST131  
421 lineage, do not. This again highlights the genetic complexity that exists amongst different UPEC  
422 strains and that host response pathways engaged, as well as host colonization strategies  
423 employed, will vary depending on the specific UPEC strain encountered. For inflammasome-  
424 activating strains such as CFT073, NLRP3 drives IL-1 $\beta$  maturation in both human and mouse  
425 macrophages. However, this pathway only marginally contributes to cell death in human  
426 macrophages, despite its causal role in cell death in mouse macrophages. Finally,  $\alpha$ -hemolysin is

427 the primary trigger for cell death and IL-1 $\beta$  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<sup>37, 50</sup>, as well as a prominent role for  $\alpha$ -hemolysin<sup>35</sup>, our findings are likely to have broad  
436 significance for understanding susceptibility and severity of UTI in humans.

437

438

439

440

## 441 **Methods**

442

### 443 **Bacterial strains and growth conditions**

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

449

### 450 **Genetic manipulation procedures and generation of mutants**

451 Mutation of the *hlyA*, *sat* and *vat* genes in CFT073, and the *hlyA* gene in UTI89, was performed  
452 using the  $\lambda$ -Red recombinase gene replacement system<sup>55</sup>. The primers used for amplification of  
453 the kanamycin resistance gene (*hlyA*) or chloramphenicol resistance gene (*vat*, *sat*), and  
454 subsequent insertion into the chromosome of CFT073 (or UTI89) were as follows: *vat* (3353: 5'-  
455 tcgtaatgaacacagttcatctgatctccacacaccaagacttgataagctcacgtcttgagcgattgtgtagg and 3354: 5'-  
456 gaaaccaccacccatgattttgtttaccgctgtacaggcctgctgacgcgacatgggaattagccatggtcc), *sat* (3351: 5'-  
457 aagaaattccaatgattttgagattcagaggttaataaattgtgtggacacgtcttgagcgattgtgtagg and 3352: 5'-  
458 ccaggagtgggagctgtagtctctggtgccaaggccggcgaaagttgcggtgacatgggaattagccatggtcc), *hlyA* (2049: 5'-  
459 aaattaaagcacactacagtctgcaaagcaatcctctgcaaataaattgtgtaggctggagctgcttc and 2050: 5'-  
460 tgetctgctgcttttttaatgcatcttctgtgctttgtcctgctgagtgcatatgaatacctccttag). CFT073 *hlyA*  
461 (CFT073 $\Delta$ *hlyA*), *sat* (CFT073 $\Delta$ *sat*) and *vat* (CFT073 $\Delta$ *vat*) mutants, as well as the UTI89 *hlyA*  
462 mutant (UTI89 $\Delta$ *hlyA*), were confirmed by PCR and DNA sequencing. The CFT073*hlyA-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<sup>56</sup>. HMDM were differentiated for 7 days  
472 with CSF-1 (10,000 U/ml, Chiron Emeryville, CA, USA) from CD14<sup>+</sup> cells, as previously  
473 described<sup>56</sup>, 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*<sup>-/-</sup>, *Nlrc4*<sup>-/-</sup>, *Asc*<sup>-/-</sup>, and *Caspase-1/11*<sup>-/-</sup> mice (all described  
476 in<sup>46</sup>), in the absence of antibiotics as previously described<sup>6</sup>. 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.



486

487 **Bacterial culture and macrophage infection assays**

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

504

505 **Cytotoxicity assays**

506 Cell culture supernatants were collected at 2 or 24 h post-infection, centrifuged for 5 min at 500  
507 g, and analysed for LDH release using the *In Vitro* Toxicology Assay Kit (Sigma-Aldrich).  
508 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 =  $(100/LDH_{total}-LDH_{spontaneous}) \times (LDH_{treatment}-LDH_{spontaneous})$ . 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<sup>6</sup>. 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<sup>57</sup>. ASC specks were counted  
522 manually in a blinded fashion (5 fields at 40x magnification per condition per replicate).

523

### 524 **Immunoblotting**

525  $4 \times 10^5$  cells were lysed in 100  $\mu$ l 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<sup>56</sup>. Membranes were stained with  
531 cleaved IL-1 $\beta$  (Asp116) rabbit mAb, IL-1 $\beta$  (3A6) mouse mAb, cleaved caspase-1 (ASP297)

532 (D57A2) rabbit mAb (all Cell Signalling Technology, Danvers, MA, USA), mouse IL-1 $\beta$ /IL-1F2  
533 affinity purified polyclonal Ab, Goat IgG (R&D Systems, Minneapolis, MN, USA), anti-  
534 caspase-1 (p20) (mouse) mAb (Adipogen, San Diego, CA, USA), anti-NLRP3/NALP3 mAb  
535 (Cryo-2) (Adipogen) or human anti-G3PDH antibody (Trevigen, Gaithersburg, MD, USA). All  
536 primary antibodies were diluted 1:1000 except for human anti-G3PDH antibody, which was used  
537 at 1:10000. As secondary antibodies, anti-mouse and anti-rabbit IgG, HRP-linked Antibodies  
538 (Cell Signalling Technology) (1:2500) and anti-goat IgG-peroxidase antibody (Sigma-Aldrich)  
539 (1:5000) were used. HRP was detected using ECL Plus substrate (GE Healthcare,  
540 Buckinghamshire, UK) and Super RX film (Fujifilm, Tokyo, Japan).

541

## 542 **ELISA**

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

549

## 550 **Statistical Analysis**

551 All LDH, ELISA and MTT assays were performed using duplicate or triplicate cell culture wells  
552 for individual experiments. Presented data are typically mean values combined from three or  
553 more independent experiments, unless otherwise indicated. For statistical analysis of datasets  
554 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

For Peer Review

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570

571

## 572 **Disclosure**

573 The author declared no conflict of interest.

574

575

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803

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-1 $\beta$  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*-, *Nlrp4*-, *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  $\mu$ M, 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-1 $\beta$  was measured by ELISA after 2 h. Data are  
837 mean+SEM of three independent experiments.

838  
839 **Figure 4: NLRP3 contributes to IL-1 $\beta$  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  $\mu$ M) and subsequently infected with the UPEC strain CFT073 or *S. Typhimurium* SL1344 (MOI  
843 10), or stimulated with nigericin (10  $\mu$ M). 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:  $p > 0.05$ , \*:  $p \leq 0.05$ , \*\*:  $p \leq 0.01$ . **C)** Concentrated supernatants from cells treated as described above were analyzed by  
847 western blot with antibodies detecting full-length or cleaved human IL-1 $\beta$ . **D)** HMDM were  
848 treated as above, but in presence of 12 mM glycine, and analyzed by confocal microscopy. ASC

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

852

853 **Figure 5: UPEC-mediated cell death is blocked by caspase-1 inhibition in mouse but not**

854 **human macrophages. A-B)** HMDM and BMM were primed with LPS (100 ng/ml, 4 h) or left

855 untreated, then pretreated for 1 h with the caspase-1 inhibitor VX-765 (50  $\mu$ M), and

856 subsequently infected with the UPEC strain CFT073 (MOI 10) or stimulated with nigericin (10

857  $\mu$ M). Supernatants were collected at 2 h p.i., and were analyzed for cell death by LDH release

858 assay **(A)** and IL-1 $\beta$  release by ELISA **(B)**. Data represent the mean+SEM of three independent

859 experiments. **(C-D)** HMDM and BMM were treated as above for 1 h. Cell lysates, as well as cell

860 culture supernatants, were analyzed by western blot for cleavage of caspase-1. GAPDH served as

861 loading control. Similar results were obtained in two independent experiments.

862

863 **Figure 6: UPEC triggers NLRP3-independent cell death in human macrophages.** Stable

864 NLRP3 knockdown THP-1 cells (defNLRP3), as well as THP-1 control cells (Null) were

865 infected with the indicated UPEC strains or *S. Typhimurium* (MOI 10), or were treated with

866 nigericin (10  $\mu$ M) for 1 h **(A)** or 2 h **(B)**. **A)** Cell lysates were analyzed by western blot for

867 expression of NLRP3 and GAPDH as a loading control. Caspase-1 p20 was detected in cell

868 lysates. Similar findings were apparent in two independent experiments. **B)** Supernatants of

869 unprimed or LPS primed (100 ng/ml LPS, 4 h) THP-1 cells were analyzed by LDH assay. Data

870 are the mean+SEM of four independent experiments.

871

872 **Figure 7: Differences between human and mouse macrophages in cellular responses to  $\alpha$ -**  
873 **hemolysin. A-B)** HMDM and BMM were infected with wild type UPEC strains or mutants  
874 deficient for  $\alpha$ -hemolysin ( *$\Delta hlyA$* ), *sat* ( *$\Delta sat$* ), *vat* ( *$\Delta vat$* ) or all three genes ( *$\Delta hlyA; \Delta sat; \Delta vat$* ) 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-1 $\beta$  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-1 $\beta$ . Similar observations were made in 2 independent experiments.  
880

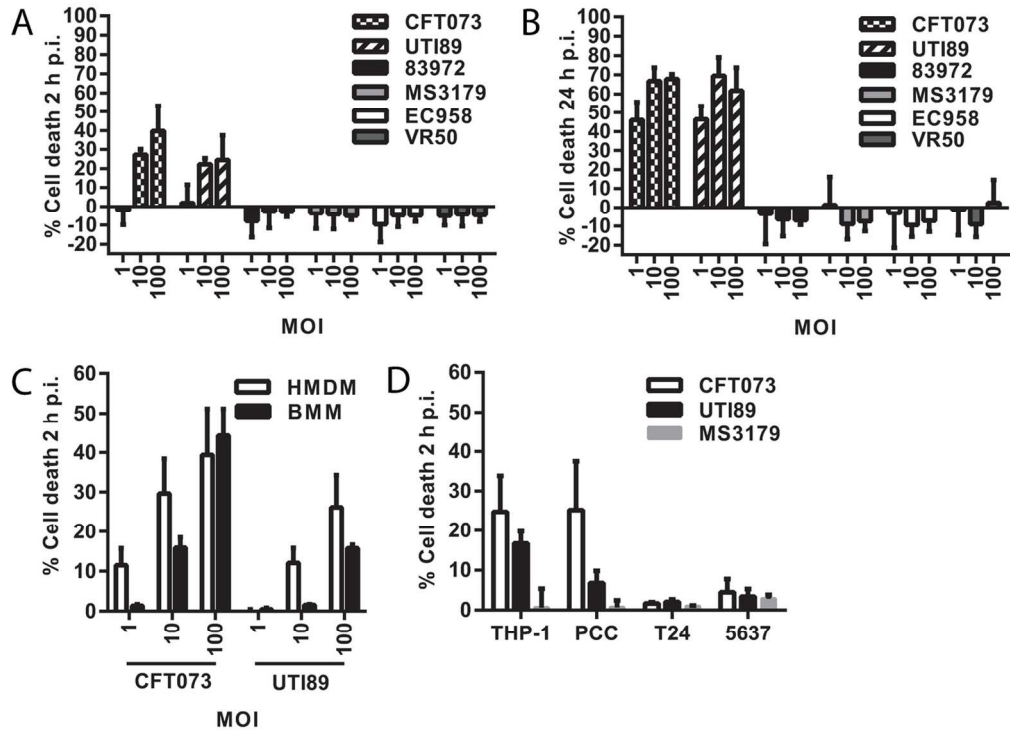


Fig. 1  
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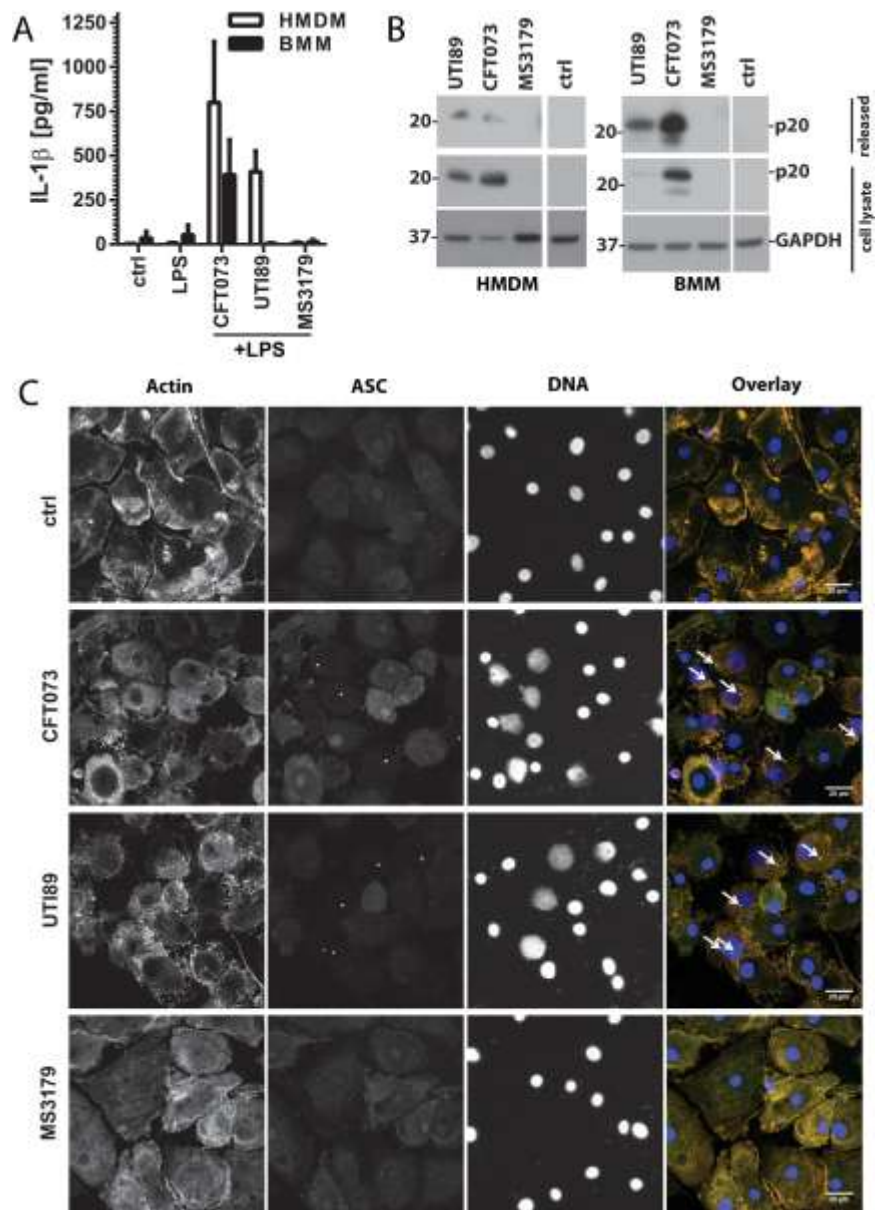


Fig. 2  
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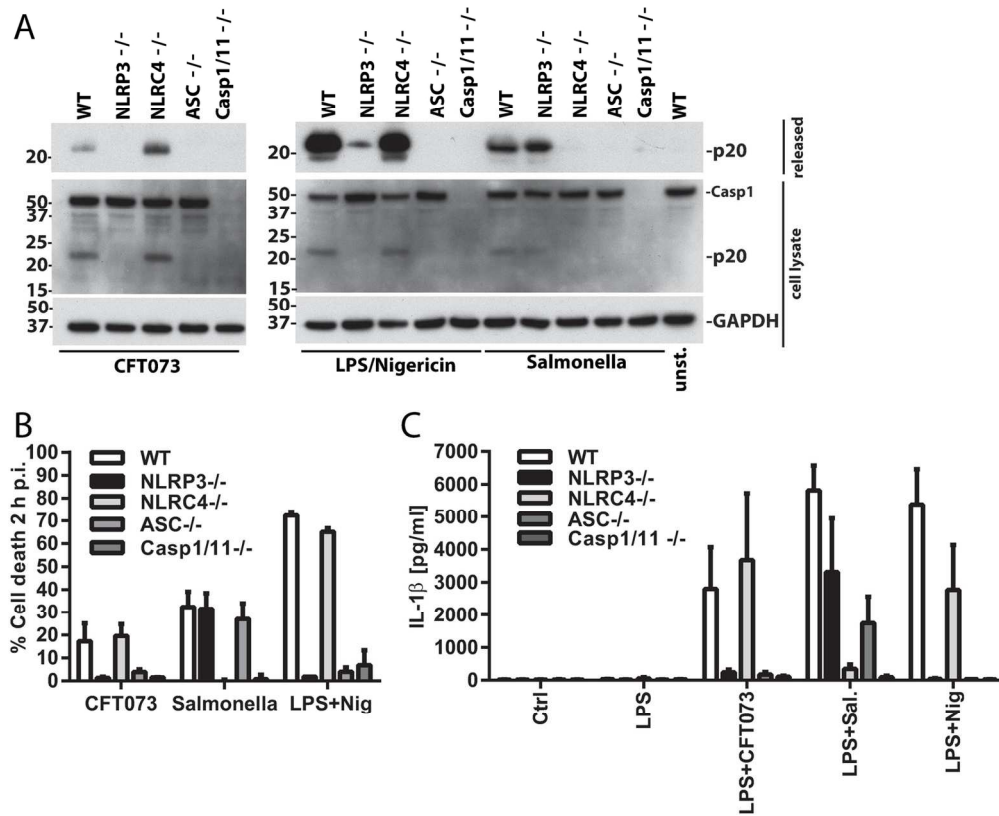


Fig. 3  
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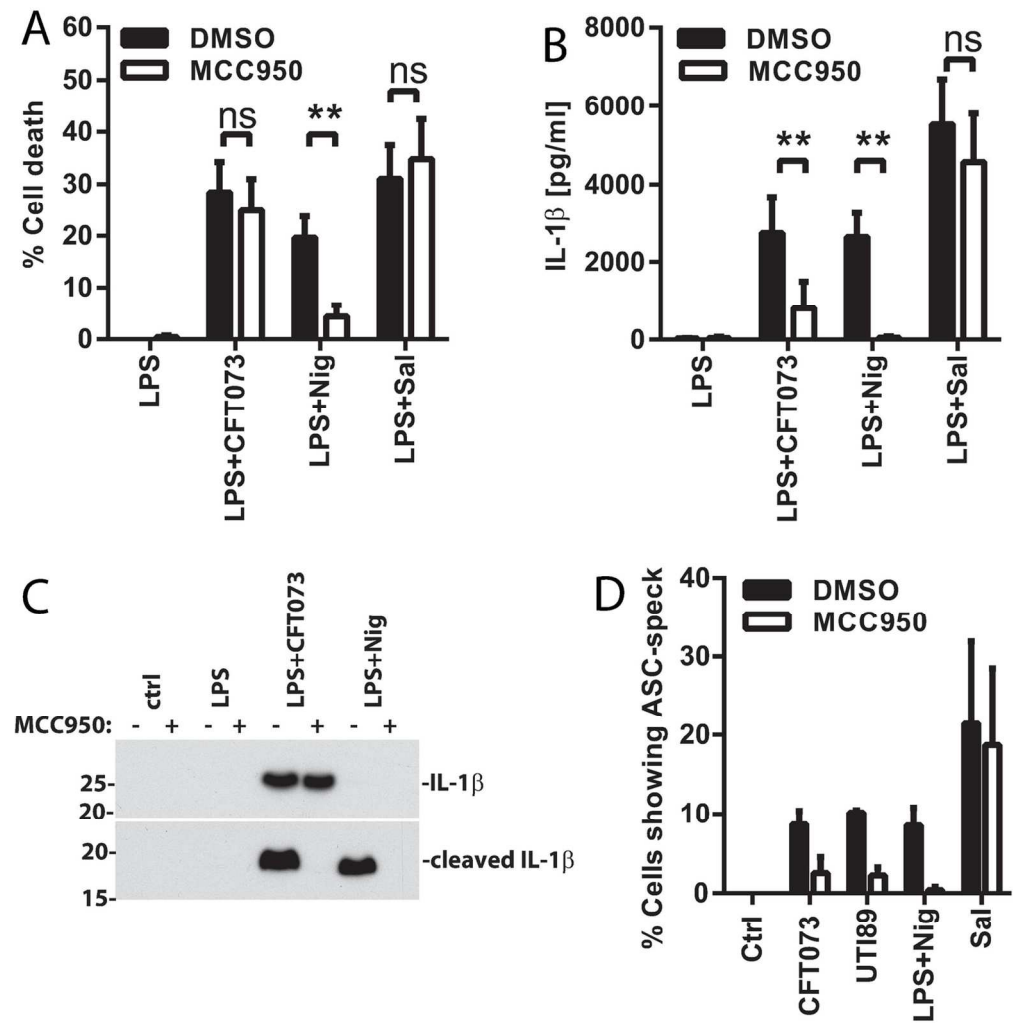


Fig. 4  
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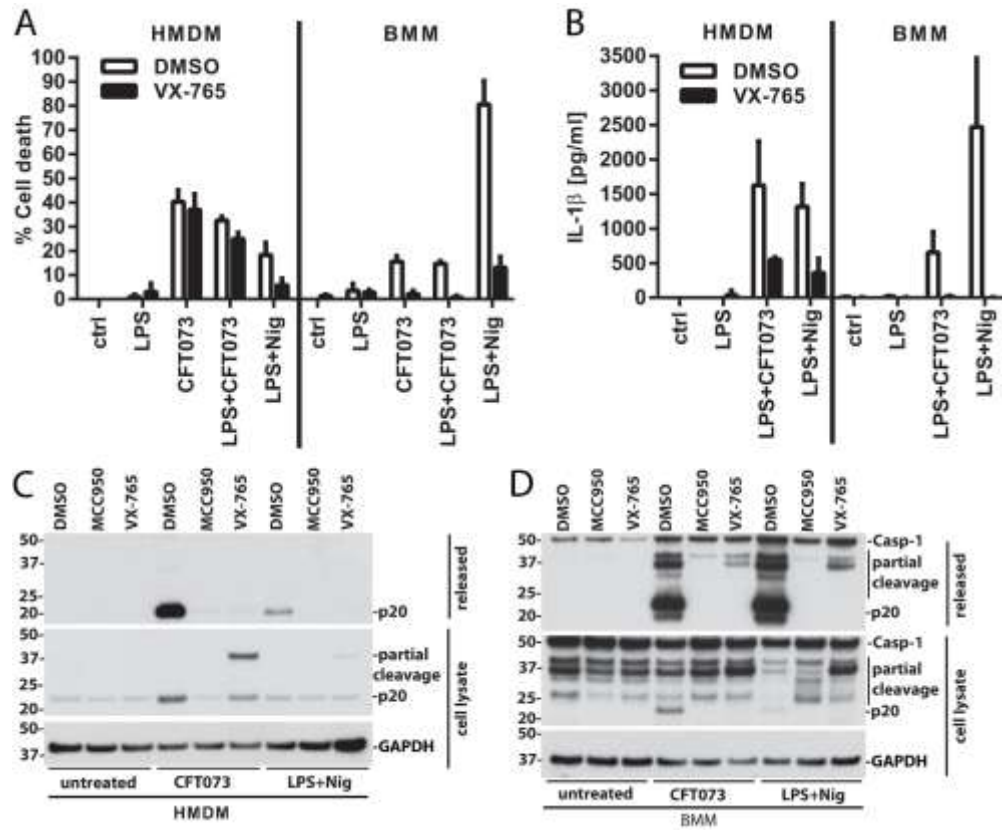


Fig. 5  
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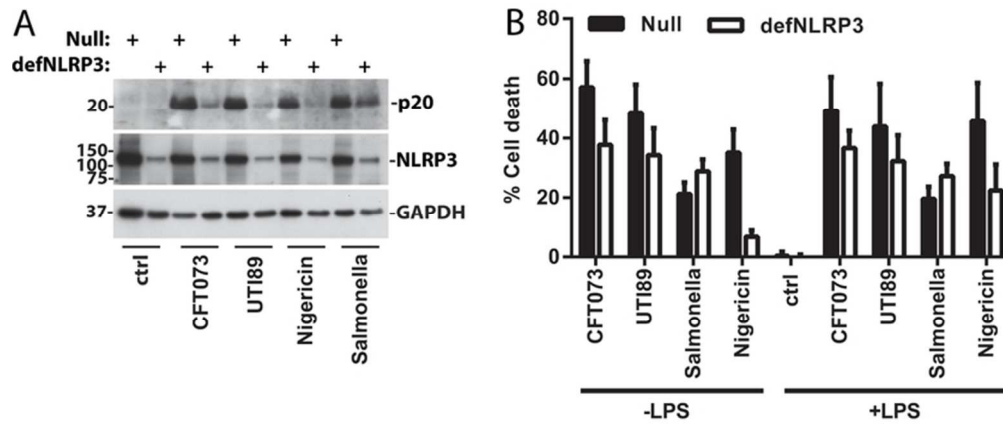


Fig. 6  
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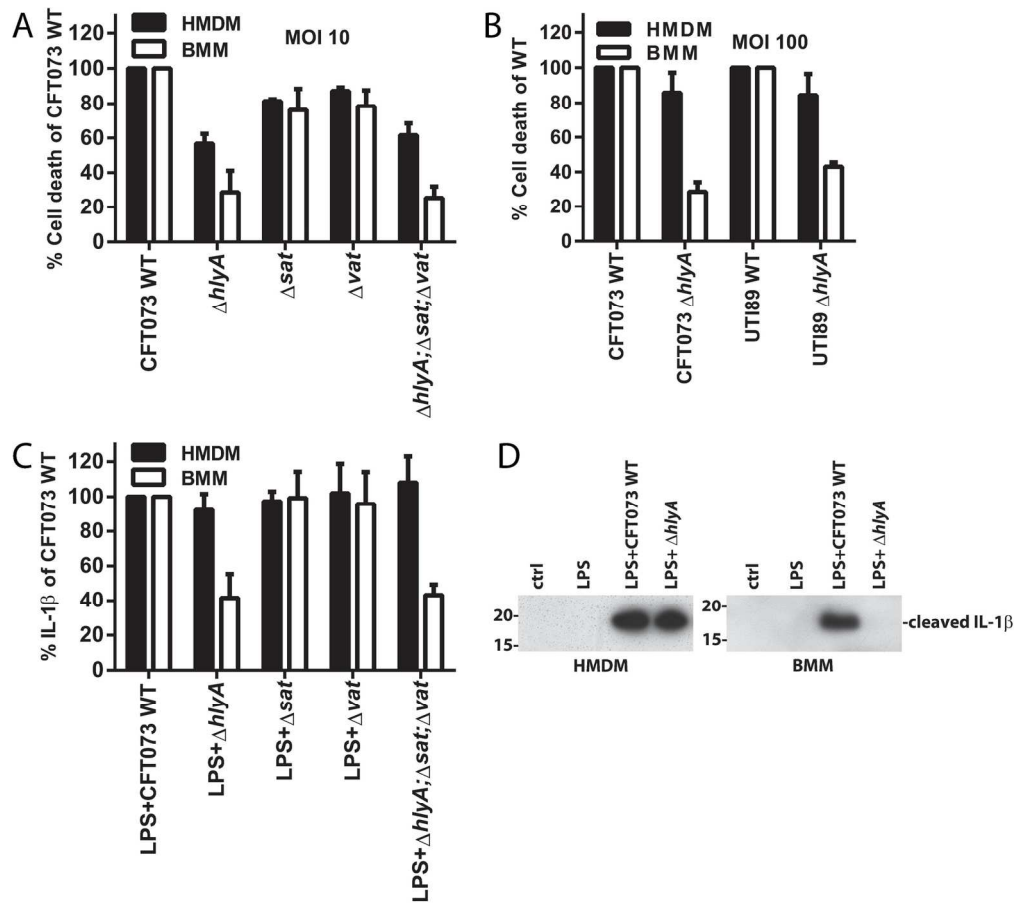
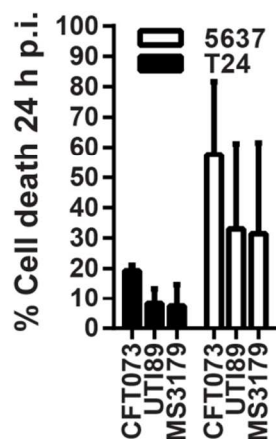
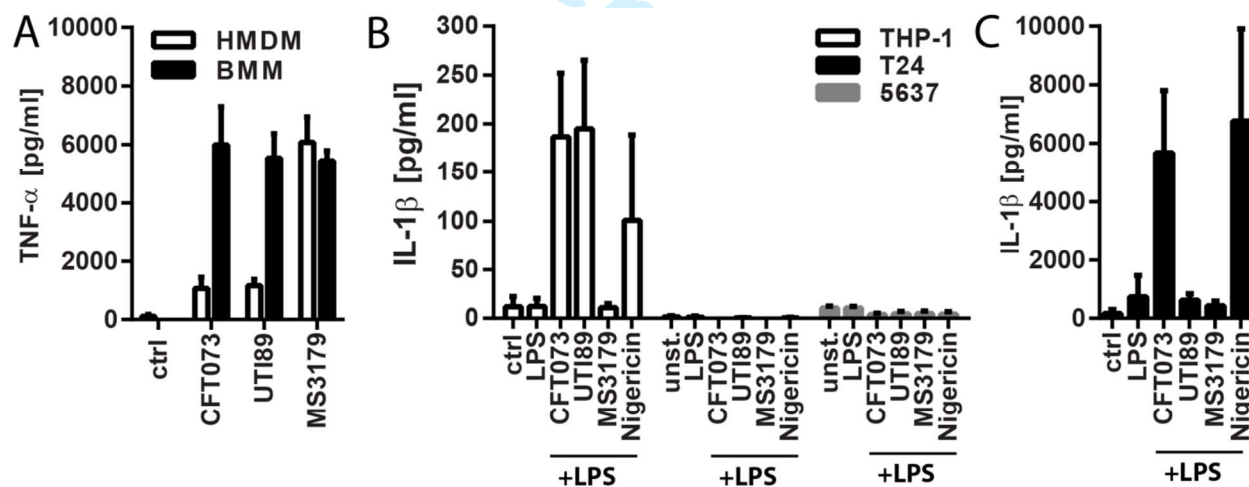


Fig. 7  
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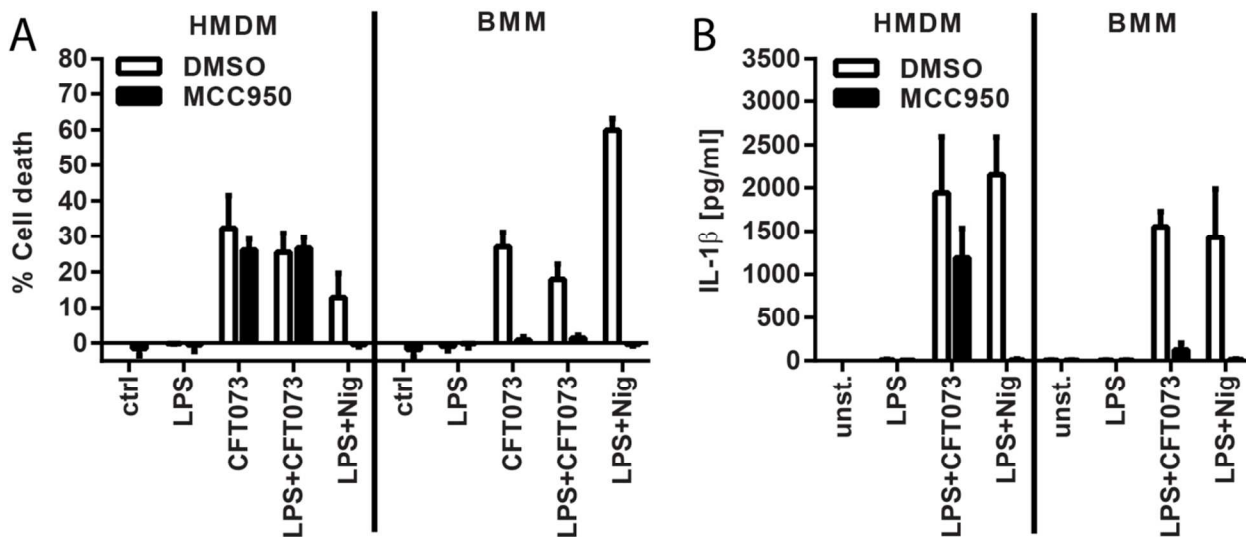
## Supplementary Figures



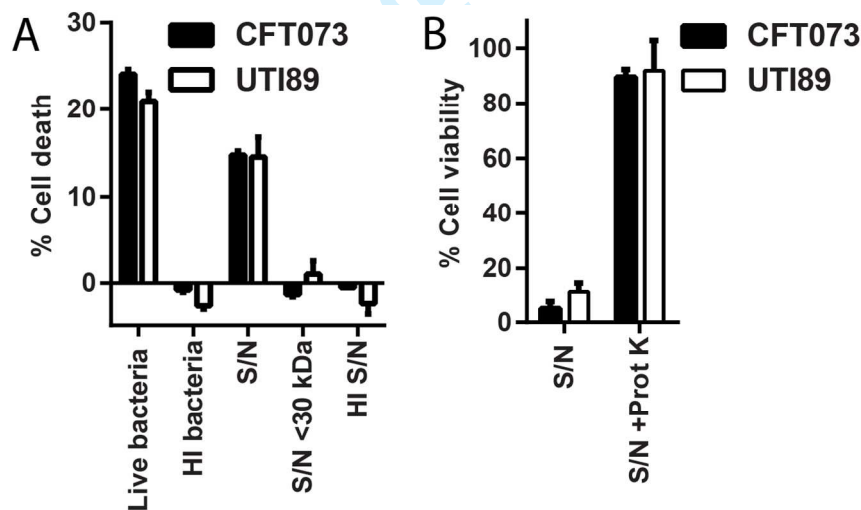
**Figure S1: UPEC strains CFT073 and UTI89 induce cell death in epithelial cells at high MOI.** Human bladder epithelial cell lines (T24 and 5637) were infected with the indicated UPEC strains (MOI 1000) for 24 h. Supernatants were analyzed by LDH release assay. Data are the mean + range of two independent 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  $\mu$ M), and subsequently infected with the UPEC strain CFT073 (MOI 10) or stimulated with nigericin (10  $\mu$ M). At 2 h p.i., supernatants were collected and analyzed by **A**) LDH release assay and **B**) IL-1 $\beta$  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  $\mu$ m) supernatants from bacterial overnight cultures (S/N), or flow through from filtration with Amicon Ultra-15 centrifugal filter 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  $\mu$ g/ml) for 45 min at 37°C (S/N +Prot K). Cell viability was assessed by methylthiazolyl-diphenyl-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<sub>2</sub> 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	Average
	MCC950										
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-1 $\beta$ [pg/ml]	Donor :	1	2	3	4	5	6	7	8	9	Average
	MCC950										
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-1 $\beta$  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  $\mu$ M) and then subsequently infected with the UPEC strains CFT073 or UTI89 or *S. Typhimurium* SL1344 (MOI 10), or were stimulated with nigericin (10  $\mu$ M). 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.



1 **Strain- and host species-specific inflammasome activation, IL-1 $\beta$  release and cell death in**  
2 **macrophages infected with uropathogenic *Escherichia coli***

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25

26 **Abstract**

27 Uropathogenic *E. coli* (UPEC) is the main etiological agent of urinary tract infections (UTI).

28 Little is known ~~of the~~about interactions between UPEC and the inflammasome, a key innate

29 immune pathway. Here we show that UPEC strains CFT073 and UTI89 trigger inflammasome

30 activation and lytic cell death in human macrophages. Several other UPEC strains, including

31 ~~globally disseminated~~two multidrug resistant ST131-~~lineage~~ isolates, did not kill macrophages.

32 In mouse macrophages, UTI89 triggered cell death only at a high MOI, and CFT073-mediated

33 inflammasome responses were completely NLRP3-dependent. Surprisingly, CFT073- and

34 UTI89-mediated responses only partially depended on NLRP3 in human macrophages. In these

35 cells, NLRP3 ~~contributed to~~was required for IL-1 $\beta$  maturation, ~~and~~but contributed only

36 marginally to cell death. Similarly, caspase-1 inhibition did not block cell death in human

37 macrophages. In keeping with such differences, the pore forming toxin  $\alpha$ -hemolysin mediated a

38 substantial proportion of CFT073-triggered IL-1 $\beta$  secretion in mouse but not human

39 macrophages. ~~Moreover, there~~There was also a more substantial  $\alpha$ -hemolysin-independent cell

40 death response in human versus mouse macrophages. Thus, in mouse macrophages, CFT073-

41 triggered inflammasome responses are completely NLRP3-dependent, and largely  $\alpha$ -hemolysin-

42 dependent. In contrast, UPEC activates an NLRP3-independent cell death pathway and an  $\alpha$ -

43 hemolysin-independent IL-1 $\beta$  secretion pathway in human macrophages. This has important

44 implications for understanding UTI in humans.

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## 49 Introduction

50 Uropathogenic *Escherichia coli* (UPEC) is estimated to cause up to ~~75~~80% of community-  
51 acquired and 65% of nosocomial urinary tract infections (UTI), making it the single most  
52 important etiological agent of this highly prevalent infectious disease<sup>1</sup>. UTI typically involves  
53 infection of the bladder (cystitis) or kidneys (pyelonephritis), and can also lead to renal scarring  
54 and sepsis<sup>1,21,2</sup>. Asymptomatic bacteriuria (ABU), caused by various etiologic agents, is also  
55 common<sup>3</sup>.

56  
57 Mouse UTI models, as well as genetic associations within patient cohort studies, have helped to  
58 pinpoint the roles of specific innate immune pathways in defense against uropathogens<sup>2</sup>. Such  
59 studies have highlighted the importance of members of the Toll-like Receptor family in  
60 controlling bacterial growth and dissemination, as well as causing symptoms and pathology. At  
61 the cellular level, roles of the urothelium and neutrophils in innate defence and host subversion  
62 during UTI are well established. However, surprisingly little is known about the roles of  
63 monocytes and macrophages in these processes<sup>4</sup>. A recent study by Schiwon *et al.* dissected the  
64 role of different macrophage populations in a mouse UTI model and unraveled complex  
65 interactions of sentinel and helper macrophages governing antimicrobial actions of neutrophils<sup>5</sup>.  
66 We previously demonstrated that some UPEC strains can survive for up to 24 h in murine bone  
67 marrow-derived macrophages (BMM) within LAMP1<sup>+</sup> compartments<sup>6</sup>, reminiscent of quiescent  
68 intracellular reservoirs observed in epithelial cells that may facilitate recurrent infection. Thus,  
69 the role of myeloid cells in UPEC infection may not always be protective.

70

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

91  
92 Among the different pathogenic *E. coli* subtypes, enterohemorrhagic *E. coli* O157:H7, which  
93 causes severe enteritis, triggers inflammasome activation<sup>12</sup>. However, until very recently no

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

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119 **Results**

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121 **UPEC strains CFT073 and UTI89 cause rapid cell death in macrophages**

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

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140 study UPEC infection *in vitro* (**Fig. 1D**). PMA-differentiated THP-1 cells showed a similar  
141 response to HMDM. CFT073 also triggered cell death in PCC, whereas the effect of UTI89 was  
142 much weaker, similar to the findings with mouse BMM (compare **Fig. 1C**). The ST131 isolate  
143 MS3179 did not trigger cell death in any of the cell types tested, and the two epithelial cell lines  
144 were not killed efficiently by any of the UPEC strains at an MOI of 10 at 2 h post-infection (**Fig.**  
145 **1D**). However, 24 h exposure of epithelial cell lines to a very high MOI (MOI 1000) of all UPEC  
146 strains did result in some cell death (**Fig. S1**). Collectively, these data demonstrate substantial  
147 variability in the capacity of different UPEC strains to elicit macrophage cell death.

148

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

150 To investigate potential involvement of the inflammasome pathway in cell death, we first  
151 examined the capacity of UPEC strains to trigger IL-1 $\beta$  release from LPS-primed macrophages.

152 LPS priming was performed to boost pro-IL-1 $\beta$  levels, allowing the use of IL-1 $\beta$  release as a

153 marker for inflammasome activation with minimal interference by rapid cell death- or by other  
154 confounding processes such as suppression of cytokine production by some UPEC strains<sup>21</sup>.

155 Indeed, we found that, whereas CFT073, UTI89 and MS3179 all elicited similar levels of  
156 secreted TNF- $\alpha$  from BMM, this response was greatly reduced in HMDM infected with strains  
157 triggering rapid cell death (CFT073, UTI89) as compared to MS3179 that did not cause cell  
158 death (Fig. S2A). Thus, TNF- $\alpha$  release inversely correlated with UPEC-induced rapid cell death

159 in HMDM, as might be expected given that this cytokine must be synthesized prior to its release.

160 In the case of IL-1 $\beta$  release ~~correlated~~ from LPS-primed cells that already express pro-IL-1 $\beta$ ,  
161 there was a clear correlation with induction of cell death in all cases. In human macrophages

162 (HMDM and PMA-differentiated THP-1 cells), both CFT073 and UTI89 (MOI 10) triggered IL-



163 | 1 $\beta$  release (**Fig. 2A and S2AS2B**). In contrast, CFT073 but not UTI89 (MOI 10) elicited IL-1 $\beta$   
164 | release from LPS-primed mouse macrophages (BMM and PCC) (**Fig. 2A and S2BS2C**),  
165 | consistent with the failure of UTI89 to trigger robust cell death in mouse macrophages at low  
166 | MOI (compare **Fig. 1C and 1D**). Also consistent with the cell death data, the ST131 strain  
167 | MS3179 did not trigger IL-1 $\beta$  release from LPS-primed macrophages of either human or murine  
168 | origin. LPS-primed epithelial cell lines did not release IL-1 $\beta$  in response to any of the UPEC  
169 | strains tested (**Fig. S2AS2B**). Similar patterns were observed for caspase-1 cleavage; both  
170 | CFT073 and UTI89 triggered comparable caspase-1 cleavage in human macrophages (**Fig. 2B**),  
171 | whilst in mouse macrophages the response ~~for~~to CFT073 was much more pronounced than for  
172 | UTI89 (**Fig. 2B**). Another hallmark of inflammasome activation, the formation of ASC specks,  
173 | was also apparent in CFT073- and UTI89-infected human macrophages, whereas the ST131  
174 | strain MS3179 did not elicit this effect (**Fig. 2C**). ~~Additionally~~Furthermore, infection with the  
175 | two ASC speck-inducing UPEC strains (i.e. CFT073 and UTI89) ~~led~~appeared to ~~dramatic~~induce  
176 | morphological changes and loss of nuclear integrity in HMDM, as visualized by actin and DNA  
177 | staining- in the same samples (Fig. 2C).

#### 178 179 **CFT073-mediated cell death in murine macrophages is completely dependent on the** 180 **NLRP3 inflammasome**

181 Causality of inflammasome activation and cell death in BMM was analysed using macrophages  
182 deficient for NLRP3 and NLRC4 (two NLRs most commonly activated by bacterial infection<sup>7</sup>),  
183 the inflammasome adaptor protein ASC, or the inflammatory caspases, caspase-1 and -11. Since  
184 UTI89 did not trigger pronounced inflammasome activation in mouse macrophages at an MOI of  
185 10, only CFT073 was assessed. CFT073-mediated caspase-1 cleavage was completely dependent

186 on NLRP3 and ASC, but did not require NLRC4 (**Fig. 3A**). As expected, the positive controls  
187 nigericin and *Salmonella enterica serovar* Typhimurium (*S. Typhimurium*) strain SL1344 acted  
188 via NLRP3 and NLRC4, respectively (**Fig. 3A**). Analysis of LDH release confirmed that  
189 NLRP3, ASC and Caspase-1/11 were required for CFT073-mediated cell death (**Fig. 3B**). These  
190 inflammasome components were also indispensable for CFT073-triggered IL-1 $\beta$  release from  
191 LPS-primed BMM (**Fig. 3C**). As with caspase-1 cleavage, NLRC4-deficiency did not affect  
192 LDH or IL-1 $\beta$  release upon infection with CFT073. Again, the positive controls for NLRP3,  
193 ASC and caspase-1 involvement (nigericin), and NLRC4 and caspase-1 involvement (*S.*  
194 *Typhimurium*), behaved as expected. ASC was dispensable for *S. Typhimurium*-mediated cell  
195 death (**Fig. 3B**) as previously reported<sup>24,22</sup>, and NLRP3, ASC and caspase-1/11 were  
196 indispensable for nigericin-triggered IL-1 $\beta$  release (**Fig. 3C**). *S. Typhimurium*-triggered IL-1 $\beta$   
197 release was partially dependent on NLRP3 and ASC, and completely dependent on NLRC4 and  
198 caspase-1/11. Hence, rapid cell death and IL-1 $\beta$  secretion triggered by CFT073 in mouse  
199 macrophages is dependent on NLRP3, ASC and caspase-1 and/or -11.

200

201 **In human macrophages, UPEC-mediated IL-1 $\beta$  secretion is ~~partially~~ dependent on NLRP3,**  
202 **whereas cell death is primarily NLRP3-independent**

203 We next investigated NLRP3 involvement in human macrophage responses to UPEC, ~~using a~~  
204 ~~recently described NLRP3 inhibitor, MCC950<sup>22</sup>, using MCC950, a recently described NLRP3~~  
205 ~~inhibitor that does not affect AIM2, NLRP1 or NLRC4-mediated inflammasome activation<sup>23</sup>.~~

206 Surprisingly, in LPS-primed HMDM, MCC950 blocked nigericin-triggered cell death, but had  
207 little effect on CFT073-triggered cell death in cells from most donors examined (**Fig. 4A, Table**  
208 **S1**). In contrast, MCC950 significantly reduced both nigericin- and CFT073-triggered IL-1 $\beta$

10

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

232

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

245

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

12

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

258

259  **$\alpha$ -hemolysin is the main factor in CFT073 triggering cell death and IL-1 $\beta$  release in mouse**  
260 **but not human macrophages**

261 The capacity for CFT073 and UTI89 to trigger human macrophage cell death was also conferred  
262 by culture supernatants (Fig. S4). Crude biochemical analysis indicated that the factor(s)  
263 responsible were heat- and protease-sensitive, and with a likely molecular weight of greater than  
264 30 kDa. Candidate proteins mediating cell death and/or IL-1 $\beta$  secretion included the pore  
265 forming toxin  $\alpha$ -hemolysin, as well as serine-protease autotransporter toxins (Sat and Vat).  
266 Genes encoding all three toxins are present in CFT073, while UTI89 contains the *hlyCABD* ( $\alpha$ -  
267 hemolysin operon) and *vat* genes. To test the involvement of these three toxins in triggering the  
268 inflammasome response, we generated a series of CFT073 mutants deleted for genes encoding  
269 each individual toxin and a triple-mutant deficient in the ability to produce all three toxins.

270 Compared to wild type CFT073, the  $\alpha$ -hemolysin (*hlyA*)-mutant (CFT073 $\Delta$ hlyA) was greatly  
271 impaired in its ability to trigger cell death of mouse macrophages (~~(-70%)~~(~30% of wild type))  
272 and, to a lesser extent, of human macrophages (~~(-40%)~~(~60% of wild type) when macrophages  
273 were exposed to an MOI of 10 (Fig. 7A)). CFT073 *sat*- and *vat*- mutants were very modestly  
274 compromised for their ability to trigger mouse and human macrophage cell death. However, no  
275 additive effect was observed, as the triple-mutant showed no difference to the CFT073 $\Delta$ hlyA  
276 single mutant in this assay. Similar observations were made at an MOI of 100 with both CFT073  
277 and UTI89 (Fig. 7B). ~~*hlyA* single mutant in this assay. Intriguingly, analysis of IL-1 $\beta$  secretion In~~

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

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## 302 Discussion

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

318  
319 It remains unclear as to what roles UPEC-mediated inflammasome activation has in different  
320 pathophysiological contexts. Two out of the four strains that did not elicit inflammasome  
321 activation are associated with ABU<sup>17, 18, 17, 18</sup>, whilst the remaining two belong to the globally  
322 disseminated fluoroquinolone-resistant *fimH30*/clade C ST131 lineage that is frequently  
323 associated with symptomatic infection<sup>16, 30, 16, 31</sup>. The two inflammasome-activating strains are



324 also associated with UTI pathology. Hence, the capacity for inflammasome activation is variable,  
325 further highlighting the genetic diversity that exists amongst different UPEC isolates. Since the  
326 capacity to trigger inflammasome activation and macrophage cell death was not common to all  
327 UPEC strains, some UPEC strains may have gained inflammasome-activating factors as a  
328 component of their virulence ~~mechanisms~~armoury or lost these to avoid host detection. In the  
329 case of the former, candidate virulence factors included the pore forming toxin  $\alpha$ -hemolysin<sup>34,32</sup>,  
330 as well as serine-protease autotransporter toxins (Sat and Vat) that are known to elicit cytotoxic  
331 effects on epithelial cells<sup>32</sup>, ~~and for which the genes are present in CFT073 and UTI89 but not~~  
332 ~~83972, VR50<sup>33</sup> or EC958<sup>15,33</sup>, and for which the genes are present in CFT073 (*hlyA, sat, vat*) and~~  
333 ~~UTI89 (*hlyA, vat*), but not 83972, VR50<sup>34</sup> or EC958<sup>15</sup>.~~ Mutation of all three factors in CFT073  
334 revealed that only the absence of  $\alpha$ -hemolysin substantially reduced inflammasome responses in  
335 mouse macrophages. Intriguingly, however, CFT073-triggered IL-1 $\beta$  release and cleavage was  
336 completely independent of  $\alpha$ -hemolysin in human macrophages, and there was also a  
337 pronounced  $\alpha$ -hemolysin-independent cell death pathway. This finding points towards  
338 fundamentally different recognition mechanisms for these UPEC strains in human versus mouse  
339 macrophages, yet conservation in the overall outcomes. Whether this extends to other cell types  
340 needs to be further examined, given ~~an earlier~~a recent report on *E. coli*  $\alpha$ -hemolysin triggering  
341 IL-1 $\beta$  secretion in human urothelial cells<sup>35</sup>, an earlier study showing a similar phenomenon in  
342 human monocytes<sup>36</sup> and well documented cell type-specific effects of  $\alpha$ -hemolysin<sup>34,32</sup>

343  
344 Our aim to characterize the specific inflammasome involved in UPEC recognition led us to study  
345 the response of mouse macrophages as a more tractable genetic system, as compared to human  
346 macrophages. Initial experiments revealed that mouse and human macrophages seemed to

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

359  
360 The conclusion that the acute CFT073-mediated inflammasome response leading to cell death  
361 and IL-1 $\beta$  secretion in BMM was absolutely dependent on NLRP3 was also supported by  
362 experiments using small molecule inhibitors of NLRP3 (MCC950) and caspase-1 (VX-765).  
363 However, primary human macrophages that were analysed in parallel showed a remarkably  
364 different response. The NLRP3 and caspase-1 inhibitors substantially reduced or blocked IL-1 $\beta$   
365 ~~secretion~~release from HMDM in response to UPEC infection or the NLRP3 agonist nigericin,  
366 respectively. In contrast, UPEC-triggered cell death was largely unaffected by either inhibitor.  
367 Although analysis of HMDM generated from several donors revealed some variation in the level  
368 of NLRP3-dependence, the overall conclusion is that in human macrophages cell death is  
369 NLRP3-independent, whereas IL-1 $\beta$  ~~release always shows at least some NLRP3-~~

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

383

384 ~~Divergence in the repertoire of NLR family members between human macrophages. Human~~  
385 ~~versus~~ and mouse can contribute to differences in UPEC-triggered inflammasome responses  
386 could potentially be explained by divergence in the repertoire of NLR family members between  
387 these species<sup>10, 3440</sup>. However, differences in the recognition of pathogens by orthologous human  
388 and mouse NLRs have also been reported. For example, *F. tularensis* activates only the AIM2  
389 inflammasome in mouse macrophages, but triggers NLRP3- and AIM2-dependent responses in  
390 human macrophages<sup>35, 41</sup>. Conversely, *L. monocytogenes* was reportedly recognized by AIM2,  
391 NLRP3 and NLRC4 in mouse cells<sup>36-38, 42-44</sup>, but exclusively by NLRP3 in human cells<sup>39, 45</sup>. The  
392 causes for these differences are not fully understood, but may be related to species differences in

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

404 |  
405 | ~~The selective role of NLRP3 in promoting IL-1 $\beta$  release but not cell death in human~~  
406 | ~~macrophages responding to UPEC suggests~~ Emerging evidence indicates that cytokine processing  
407 | and pyroptosis can be uncoupled. ~~This is reminiscent of recent studies of mouse neutrophils,~~  
408 | ~~where in some systems. For example, *Salmonella*-mediated NLRC4 activation promoted IL-1 $\beta$~~   
409 | ~~maturation but not pyroptosis<sup>40</sup> in mouse neutrophils<sup>46</sup>. Other studies have also reported distinct~~  
410 | roles for individual inflammasomes in cytokine processing versus pyroptosis. For example,  
411 | NLRP3 was shown to mediate cell death and IL-1 $\beta$  release in *S. aureus*-infected HMDM, whilst  
412 | a novel NLRP7 inflammasome was shown to selectively promote IL-1 $\beta$  secretion<sup>44,47</sup>. Similarly,  
413 | NLRP3 and NLRC4 were shown to mediate *B. pseudomallei*-induced IL-1 $\beta$  and IL-18 release in  
414 | the mouse, whilst pyroptotic cell death was attributed only to NLRC4<sup>42,48</sup>. At present, there is no  
415 | unifying model explaining why similar recognition systems lead to cytokine maturation in one

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

426  
427 A protective role for inflammasome activation and IL-1 $\beta$  production has been shown in many *in*  
428  *vivo* infection models including *S. Typhimurium*, *L. monocytogenesis* and *Burkholderia* species<sup>11</sup>  
429 On the other hand, the role of pathogen-induced cell death is ambiguous, having either protective  
430 or detrimental effects by either eradicating intracellular niches or promoting dissemination,  
431 respectively<sup>43</sup>. ~~Since UPEC can occupy both extracellular and intracellular niches, it is difficult~~  
432 ~~to predict what role pyroptosis plays during UTI. Approaches for genetically and/or~~  
433 ~~pharmacologically uncoupling pyroptosis from other inflammasome responses will be required~~  
434 ~~to address this question. In the case of cytokine processing, previous studies have associated IL-~~  
435 ~~1 $\beta$  release with renal pathology of UTI in patients<sup>44,45</sup> and in a mouse model<sup>46</sup>. Whether NLRP3~~  
436 ~~has a causal role in host defence or pathology is unknown at this stage. Given differences~~  
437 ~~between human and mouse macrophages in NLRP3 dependence for UPEC responses, this is not~~  
438 ~~a trivial task.~~<sup>49</sup> Since UPEC can occupy both extracellular and intracellular niches, it is difficult

439 to predict what role pyroptosis plays during UTI. Activation of caspase-1/11 was shown to  
440 facilitate clearance of UPEC in a mouse model, presumably by inducing pyroptosis and  
441 subsequent exfoliation of bladder epithelial cells<sup>35</sup>. In another study however, activity of caspase-  
442 1/11 was associated with chronicity and higher bacterial loads in the bladder in a model of  
443 recurrent UTI<sup>50</sup>. In the case of cytokine processing, previous studies have associated IL-1 $\beta$   
444 release with renal pathology of UTI in patients<sup>51, 52</sup>, and also in a mouse model<sup>53</sup>. Only very  
445 recently was IL-1 $\beta$  release in *atg1611*<sup>-/-</sup> mice shown to be associated with protection from UTI<sup>37</sup>.  
446 While the effects of IL-1 $\beta$  can be studied simply by knockout or by blocking its interaction with  
447 receptors, new approaches for genetically and/or pharmacologically uncoupling pyroptosis from  
448 other inflammasome responses will be required to address the role of cell death in pathology.  
449 Whether NLRP3-dependent responses have a causal role in host defence or pathology remains to  
450 be elucidated.

451  
452 In conclusion, our study highlights the complexity of interactions between UPEC and the innate  
453 immune system. Some UPEC strains trigger inflammasome activation and rapid, lytic cell death  
454 in macrophages, whereas others, including two strains from the globally disseminated multidrug  
455 resistant ST131 lineage, do not. Further, whereas This again highlights the genetic complexity  
456 that exists amongst different UPEC-mediated cell death strains and IL-1 $\beta$  secretion are mediated  
457 by that host response pathways engaged, as well as host colonization strategies employed, will  
458 vary depending on the specific UPEC strain encountered. For inflammasome-activating strains  
459 such as CFT073, NLRP3 ~~is~~ drives IL-1 $\beta$  maturation in both human and mouse macrophages.  
460 However, this pathway only partly marginally contributes to IL-1 $\beta$  maturation cell death in human  
461 macrophages, despite its causal role in cell death in mouse macrophages. Finally,  $\alpha$ -hemolysin is

462 the primary trigger for cell death and IL-1 $\beta$  release in mouse macrophages, whereas these  
463 cellular responses are either primarily or completely independent of this toxin in human  
464 macrophages. The yet-to-be-identified death pathway in human monocyte-derived macrophages  
465 highlights a keypotential difference between human and mouse innate immune UPEC  
466 recognition pathways: and needs to be considered in future studies using macrophages and other  
467 cell types from a variety of sources, as well as in *in vivo* studies. Given the importance of mouse  
468 UTI models for understanding host colonization and pathology, and especially in the light of  
469 recent studies showing detrimental and beneficial effects of inflammasome activation in mouse  
470 models<sup>37, 50</sup>, as well as a prominent role for  $\alpha$ -hemolysin<sup>35</sup>, our findings are likely to have broad  
471 significance for understanding susceptibility and severity of UTI in humans.

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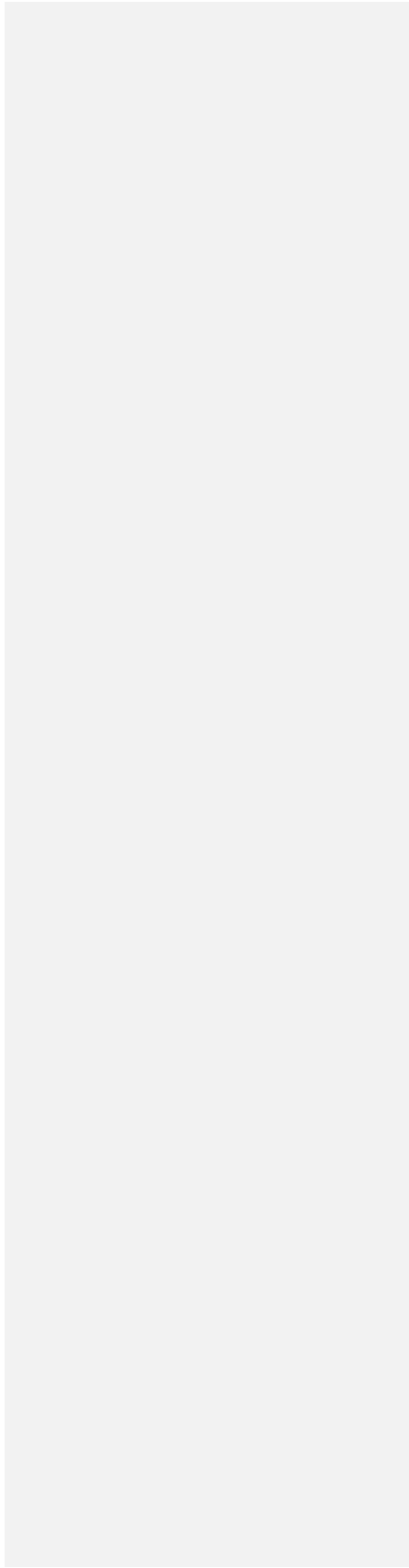
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## 491 **Methods**

492

### 493 **Bacterial strains and growth conditions**

494 UPEC strains CFT073<sup>13</sup>, UTI89<sup>14</sup>, 83972<sup>17</sup>, VR50<sup>18</sup> and EC958<sup>15,15</sup> have been described  
495 previously. MS3179 is an ST131 strain isolated from a patient presenting with UTI at the Royal  
496 Brisbane and Women's Hospital, Brisbane, Australia. *S. Typhimurium* strain SL1344<sup>47,54</sup> was  
497 used as a control for NLRP3-independent inflammasome activation in some experiments. All  
498 strains were routinely grown at 37°C on solid or in liquid Luria-Bertani (LB) medium.

499

### 500 **Genetic manipulation procedures and generation of mutants**

501 Mutation of the *hlyA*, *sat* and *vat* genes in CFT073, and the *hlyA* gene in UTI89, was performed  
502 using the  $\lambda$ -Red recombinase gene replacement system<sup>48,55</sup>. The primers used for amplification of  
503 the kanamycin resistance gene (*hlyA*) or chloramphenicol resistance gene (*vat*, *sat*), and  
504 subsequent insertion into the chromosome of CFT073 (or UTI89) were as follows: *vat* (3353: 5'-  
505 tcgtaatgaacacagttcatctgatctccacaccaagacttgataagctcacgtcttgagcgattgtgtagg and 3354: 5'-  
506 gaaaccaccacccatgattttgtttaccgctgtacaggcctgctgacgcgacatgggaattagccatggtcc), *sat* (3351: 5'-  
507 aagaaattccaatgattttgagattcagaggttaataaattgtgtggacacgcttgagcgattgtgtagg and 3352: 5'-  
508 ccaggagtgggagctgtagtctctggtccaaggccggcgaaagttcggtgacatgggaattagccatggtcc), *hlyA* (2049: 5'-  
509 aaattaaagcacactacagtctgcaaagcaatcctctgcaaataaattgtgtaggctggagctgcttc and 2050: 5'-  
510 tgetctgctgcttttttaatgcatctttcgtgctttgctctgctgagtgcatatgaatctccttag). CFT073 *hlyA*  
511 (*CFT073* $\Delta$ *hlyA*), *sat* (*CFT073* $\Delta$ *sat*) and *vat* (*CFT073* $\Delta$ *vat*) mutants, as well as the UTI89 *hlyA*  
512 mutant (*UTI89* $\Delta$ *hlyA*), were confirmed by PCR and DNA sequencing. The *CFT073hly-sat-vat*

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

515

#### 516 **Mammalian cell culture**

517 Approval for all experiments using primary human and mouse cells was obtained from the  
518 University of Queensland Medical Research Ethics Committee -or the Animal Ethics Committee.

519 Human monocytes were isolated from buffy coats of healthy donors (kindly provided by the  
520 Australian Red Cross) by positive selection for CD14 using MACS technology (Miltenyi Biotec,

521 Bergisch Gladbach, Germany), as previously described<sup>49,56</sup>. HMDM were differentiated for 7  
522 days with CSF-1 (10,000 U/ml, Chiron Emeryville, CA, USA) from CD14<sup>+</sup> cells, as previously

523 described<sup>49,56</sup>, but in the absence of antibiotics. Cells from a single donor were used in every  
524 experiment. Murine BMM were differentiated using 10,000 U/ml CSF-1 (Chiron) from bone

525 marrow of C57BL/6 wild type, *Nlrp3*<sup>-/-</sup>, *Nlr4*<sup>-/-</sup>, *Asc*<sup>-/-</sup>, and *Caspase-1/11*<sup>-/-</sup> mice (all described  
526 in<sup>49,56</sup>), in the absence of antibiotics as previously described<sup>6</sup>. PCC were flushed from the

527 peritoneal cavity of C57BL/6 mice by injection of 5 ml PBS. THP-1 (TIB-202, ATCC,  
528 Manassas, VA, USA), THP-1 Null and THP-1 defNLRP3 (InvivoGen, San Diego, CA, USA)

529 cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 1  
530 mM Na-Pyruvate and 10 mM HEPES (all Life Technologies, Carlsbad, CA, USA). THP-1 cells

531 were differentiated into macrophage-like cells by culture for 48 h in medium containing 30 ng/ml  
532 phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA). PMA or CSF-1

533 was removed 4 h prior to infection with UPEC strains. Human bladder epithelial cell lines  
534 56345637 (HTB-9, ATCC) and T24 (HTB-4, ATCC) were cultured in FBS (10%) and 2 mM L-

535 glutamine supplemented RPMI-1640 or McCoy's 5A medium (Life Technologies), respectively.

536

537 **Bacterial culture and macrophage infection assays**

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

554

555 **Cytotoxicity assays**

556 Cell culture supernatants were collected at 2 or 24 h post-infection, centrifuged for 5 min at 500  
557 g, and analysed for LDH release using the *In Vitro* Toxicology Assay Kit (Sigma-Aldrich).  
558 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 =  $(100/LDH_{total}-LDH_{spontaneous}) \times (LDH_{treatment}-LDH_{spontaneous})$ . ~~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<sup>6</sup>. 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<sup>54,57</sup>. ASC specks were counted  
572 manually in a blinded fashion (5 fields at 40x magnification per condition per replicate).

573

### 574 **Immunoblotting**

575  $4 \times 10^5$  cells were lysed in 100  $\mu$ l 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<sup>49,56</sup>. Membranes were stained with  
581 cleaved IL-1 $\beta$  (Asp116) rabbit mAb, IL-1 $\beta$  (3A6) mouse mAb, cleaved caspase-1 (ASP297)

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

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## 592 ELISA

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

600

## 601 Statistical Analysis

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

605 with  $N > 4$ , two-sided Wilcoxon matched-pairs signed-rank tests were performed using GraphPad  
606 Prism Version 6 (GraphPad software, Inc., La Jolla, CA, USA). For these datasets, the  
607 differences between pairs were plotted and were generally distributed approximately  
608 symmetrically around the median.  
609  
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624

## 625 Disclosure

626 The author declared no conflict of interest.

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628

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## 862 | **Figure legends**

863 |

864 | **Figure 1: The UPEC strains CFT073 and UTI89 induce rapid cell death in macrophages.**

865 | **A-C)** HMDM or BMM were infected with the indicated UPEC strains (MOI 1, 10 and 100).

866 | Supernatants were analyzed by LDH release assays at the indicated times- post-infection (p.i.).

867 | Data represent the mean+SEM of three independent experiments. **D)** Human macrophage-like

868 | cells (THP-1) and mouse peritoneal cavity cells (PCC), as well as ~~the~~ human T24 and 5637

869 | bladder epithelial ~~cell lines (T24 and 5637)~~ cells, were infected with the indicated UPEC strains

870 | (MOI 10) for 2 h. Supernatants were analyzed by LDH release assay. Data represent the

871 | mean+range of two independent experiments.

872 |

873 | **Figure 2: The UPEC strains CFT073 and UTI89 promote IL-1 $\beta$  secretion, caspase-1**

874 | **cleavage and ASC-speck formation. A)** HMDM and BMM were primed with 100 ng/ml LPS

875 | for 4 h, then infected with the indicated UPEC strains (MOI 10) for 2 h. Supernatants were

876 | analyzed by ELISA. Data represent the mean+SEM of three independent experiments. **B)**

877 | HMDM and BMM were infected with the indicated UPEC strains for 1 h. Whole cell lysates and

878 | concentrated supernatants were analyzed by western blot with antibodies detecting human or

879 | mouse caspase-1 p20. GAPDH is shown as a loading control. Similar findings were apparent in

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

883

884 **Figure 3: In murine macrophages, CFT073-induced caspase-1 cleavage and cell death is**  
885 **dependent on the NLRP3 inflammasome.** BMM derived from wild type, *Nlrp3*<sup>-/-</sup>, *Nlr4*<sup>-/-</sup>, *Asc*<sup>-/-</sup>  
886 and *Casp1/11*-deficient mice were infected with the UPEC strain CFT073 or the *S. Typhimurium*  
887 strain SL1344 (MOI 10), or were stimulated with nigericin (10 μM, pre-stimulated with 100  
888 ng/ml LPS for 4 h). A) After 1 h, whole cell lysates and concentrated supernatants were analyzed  
889 by western blot with antibodies detecting murine full-length caspase-1, as well as multiple  
890 caspase-1 cleavage products. GAPDH was used as loading control. Data is representative of two  
891 independent experiments. B) Supernatants were analyzed by LDH assay at 2 h p.i. Data is the  
892 mean+range of two independent experiments. C) LPS-primed BMM (100 ng/ml, 4 h) were  
893 treated as above, after which secreted IL-1β was measured by ELISA after 2 h. Data are  
894 mean+SEM of three independent experiments.

895

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

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

909  
910 **Figure 5: UPEC-mediated cell death is blocked by caspase-1 inhibition in mouse but not**  
911 **human macrophages. A-B)** HMDM and BMM were primed with LPS (100 ng/ml, 4 h), or left  
912 untreated, then pretreated for 1 h with the caspase-1 inhibitor VX-765 (50  $\mu$ M), and  
913 subsequently infected with the UPEC strain CFT073 (MOI 10) or ~~were~~ stimulated with nigericin  
914 (10  $\mu$ M). Supernatants were collected at 2 h p.i., and were analyzed for cell death by LDH  
915 release assay **(A)** and IL-1 $\beta$  release by ELISA **(B)**. Data represent the mean+SEM of three  
916 independent experiments. **(C-D)** HMDM and BMM were treated as above for 1 h. Cell lysates,  
917 as well as cell culture supernatants, were analyzed by western blot for cleavage of caspase-1.  
918 GAPDH served as loading control. Similar results were obtained in two independent  
919 experiments.

920  
921 **Figure 6: UPEC triggers NLRP3-independent cell death in human macrophages.** Stable  
922 NLRP3 knockdown THP-1 cells (defNLRP3), as well as THP-1 control cells (Null) were  
923 infected with the indicated UPEC strains or *S. Typhimurium* (MOI 10), or were treated with  
924 nigericin (10  $\mu$ M) for 1 h **(A)** or 2 h **(B)**. **(A)** Cell lysates were analyzed by western blot for  
925 expression of NLRP3 and GAPDH as a loading control. Caspase-1 p20 was detected in cell

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

929

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

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