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

K Schaale^{1,2}, KM Peters^{2,3}, AM Murthy¹, AK Fritzsche¹, M-D Phan^{2,3}, M Totsika^{2,4}, AAB Robertson^{1,2}, KB Nichols^{2,3}, MA Cooper^{1,2}, KJ Stacey^{2,3}, GC Ulett⁵, K Schroder^{1,2}, MA Schembri^{2,3} and MJ Sweet^{1,2}

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

INTRODUCTION

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

Mouse UTI models, as well as genetic associations within patient cohort studies, have helped to pinpoint the roles of specific innate immune pathways in defense against

uropathogens.² Such studies have highlighted the importance of members of the Toll-like receptor family in controlling bacterial growth and dissemination, as well as causing symptoms and pathology. At the cellular level, the roles of the urothelium and neutrophils in innate defense and host subversion during UTI are well established. However, surprisingly little is known about the roles of monocytes and macrophages in these processes.⁴ A recent study by Schiwon *et al.*⁵ dissected the role of different macrophage populations in a mouse UTI model and unraveled complex interactions of sentinel and helper macrophages governing antimicrobial actions of neutrophils. We previously demonstrated that some

¹Institute for Molecular Bioscience, University of Queensland, St Lucia, Queensland, Australia. ²Australian Infectious Diseases Research Centre, University of Queensland, St Lucia, Queensland, Australia. ³School of Chemistry and Molecular Biosciences, University of Queensland, St Lucia, Queensland, Australia. ⁴Institute of Health and Biomedical Innovation, School of Biomedical Sciences, Faculty of Health, Queensland University of Technology, Kelvin Grove, Queensland, Australia and ⁵School of Medical Sciences, Griffith Health Institute, Griffith University, Gold Coast, Queensland, Australia. Correspondence: MJ Sweet (m.sweet@imb.uq.edu.au)

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UPEC strains can survive for up to 24 h in murine bone marrow-derived macrophages (BMMs) within LAMP1⁺ compartments,⁶ reminiscent of quiescent intracellular reservoirs observed in epithelial cells that may facilitate recurrent infection. Thus, the role of myeloid cells in UPEC infection may not always be protective.

Upon detection of cellular stress and/or microbial products, some Nod-like-receptor (NLR) family members, as well as the PYHIN-family member AIM2, form large cytoplasmic multi-protein complexes known as inflammasomes. Inflammasomes have important functions in many bacterial infections,⁷ as well as in chronic diseases.^{8,9} The NLR family comprises 22 genes in humans and > 30 in mice, and can be phylogenetically grouped into the NLRP, IPAF, and NOD subfamilies.¹⁰ The NLRP family members NLRP1 and NLRP3, as well as the IPAF family member NLRC4, can all initiate inflammasome formation. Most NLRP family members contain a C-terminal leucine-rich repeat that is involved in danger sensing, a central nucleotide-binding and oligomerization domain, and an N-terminal pyrin domain that relays downstream signaling. NLRC4 has a similar domain structure, but contains an N-terminal caspase recruitment domain (CARD), rather than a pyrin domain. Upon activation, NLRPs oligomerize and cluster into a cytoplasmic complex with the adapter protein ASC and the protease caspase-1, facilitating its autocatalytic cleavage and activation. Active caspase-1 is required for maturation and secretion of the proinflammatory interleukin (IL)-1 family cytokines, IL-1 β and IL-18. One of the many functions of IL-1 β is to facilitate neutrophil and macrophage recruitment to sites of infection. In addition to mediating cytokine processing, inflammasome activation also initiates a programmed, proinflammatory form of cell death called pyroptosis. Pyroptotic cell death is thought to eliminate the intracellular replication niche of pathogens that infect macrophages (e.g., *Shigella*, *Salmonella*, *Legionella*, and *Listeria*) and to reexpose them to antimicrobial effector functions.^{7,11}

Among the different pathogenic *E. coli* subtypes, enterohemorrhagic *E. coli* O157:H7, which causes severe enteritis, triggers inflammasome activation.¹² However, until very recently, no studies had investigated inflammasome involvement in UPEC recognition or UTI. In this study, we show that the genome-sequenced UPEC reference strains CFT073 and UTI89 trigger inflammasome activation and rapid cell death in macrophages, whereas others do not. Moreover, we define key mechanistic differences between human and mouse macrophages in the host recognition pathways and bacterial factors that initiate these responses. Our findings of fundamental differences between different UPEC strains in inflammasome engagement, as well as between human and mouse innate immune recognition pathways for UPEC, have major implications for understanding and modeling UTI pathogenesis.

RESULTS

UPEC strains CFT073 and UTI89 cause rapid cell death in macrophages

Given the paucity of information on interactions between UPEC and macrophages, we investigated whether the survival

of human monocyte-derived macrophages (HMDMs) was affected by different UPEC strains. We analyzed strains associated with different UTI severity, including the reference strains CFT073 (a blood culture isolate from a patient with pyelonephritis)¹³ and UTI89 (a urine isolate from a patient with recurrent cystitis),¹⁴ the sequence type (ST) 131 strains EC958^{15,16} and MS3179 (urine isolates from patients with UTI), and the asymptomatic bacteriuria strains 83972 and VR50.^{17,18} These experiments revealed that only CFT073 and UTI89 caused rapid, lytic cell death by 2 h after infection as assessed by lactate dehydrogenase (LDH) release (**Figure 1a**). Cell death was further increased by 24 h after infection (**Figure 1b**). A direct comparison of HMDMs with murine BMMs over a multiplicity of infection (MOI) range confirmed that mouse macrophages were also susceptible to CFT073-induced cell death (**Figure 1c**). In BMMs, UTI89 did not trigger cell death, except at the highest MOI used (MOI 100, **Figure 1c**). This is consistent with our previous findings that UTI89 can survive for up to 24 h within BMMs.^{6,19} To investigate whether UPEC-mediated cell death is a macrophage-specific phenomenon, the response to CFT073, UTI89, and MS3179 was also analyzed in phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells (a human macrophage-like cell line), murine peritoneal cavity cells (predominantly comprising resident peritoneal macrophages²⁰), and two human bladder epithelial cell lines (5637 and T24) commonly used to study UPEC infection *in vitro* (**Figure 1d**). PMA-differentiated THP-1 cells showed a similar response to HMDMs. CFT073 also triggered cell death in peritoneal cavity cells, whereas the effect of UTI89 was much weaker, similar to the findings with mouse BMMs (compare **Figure 1c**). The ST131 isolate MS3179 did not trigger cell death in any of the cell types tested, and the two epithelial cell lines were not killed efficiently by any of the UPEC strains at an MOI of 10 at 2 h after infection (**Figure 1d**). However, 24 h exposure of epithelial cell lines to a very high MOI (MOI 1,000) of all UPEC strains did result in some cell death (**Supplementary Figure S1** online). Collectively, these data demonstrate substantial variability in the capacity of different UPEC strains to elicit macrophage cell death.

UPEC-mediated macrophage cell death correlates with inflammasome activation

To investigate potential involvement of the inflammasome pathway in cell death, we first examined the capacity of UPEC strains to trigger IL-1 β release from lipopolysaccharide (LPS)-primed macrophages. LPS priming was performed to boost pro-IL-1 β levels, allowing the use of IL-1 β release as a marker for inflammasome activation with minimal interference by rapid cell death or by other confounding processes such as suppression of cytokine production by some UPEC strains.²¹ Indeed, we found that whereas CFT073, UTI89, and MS3179 all elicited similar levels of secreted tumor necrosis factor- α (TNF- α) from BMMs, this response was greatly reduced in HMDMs infected with strains triggering rapid cell death (CFT073 and UTI89) as compared with MS3179 that did not

Figure 1 The uropathogenic *Escherichia coli* (UPEC) strains CFT073 and UTI89 induce rapid cell death in macrophages. (**a–c**) Human monocyte-derived macrophages (HMDMs) or bone marrow-derived macrophages (BMMs) were infected with the indicated UPEC strains (multiplicity of infection (MOI) 1, 10, and 100). Supernatants were analyzed by lactate dehydrogenase (LDH) release assays at the indicated times postinfection (p.i.). Data represent the mean $\pm$ s.e.m. of three independent experiments. (**d**) Human macrophage-like cells (THP-1) and mouse peritoneal cavity cells (PCCs), as well as human T24 and 5637 bladder epithelial cells, were infected with the indicated UPEC strains (MOI 10) for 2 h. Supernatants were analyzed by LDH release assay. Data represent the mean $\pm$ range of two independent experiments.

cause cell death (**Supplementary Figure S2A**). Thus, TNF- α release inversely correlated with UPEC-induced rapid cell death in HMDMs, as might be expected given that this cytokine must be synthesized before its release. In the case of IL-1 β release from LPS-primed cells that already express pro-IL-1 β , there was a clear correlation with induction of cell death in all cases. In human macrophages (HMDMs and PMA-differentiated THP-1 cells), both CFT073 and UTI89 (MOI 10) triggered IL-1 β release (**Figure 2a** and **Supplementary Figure S2B**). In contrast, CFT073 but not UTI89 (MOI 10) elicited IL-1 β release from LPS-primed mouse macrophages (BMMs and peritoneal cavity cells) (**Figure 2a** and **Supplementary Figure S2C**), consistent with the failure of UTI89 to trigger robust cell death in mouse macrophages at low MOI (compare **Figure 1c,d**). In addition, consistent with the cell death data, the ST131 strain MS3179 did not trigger IL-1 β release from LPS-primed macrophages of either human or murine origin. LPS-primed epithelial cell lines did not release IL-1 β in response to any of the UPEC strains tested (**Supplementary Figure S2B**). Similar patterns were observed for caspase-1 cleavage: both CFT073 and UTI89 triggered comparable caspase-1 cleavage in human macrophages (**Figure 2b**), whereas in mouse macrophages the response to CFT073 was much more pronounced than for UTI89 (**Figure 2b**). Another hallmark of inflammasome activation, the formation of ASC specks, was also apparent in CFT073- and UTI89-infected human macrophages, whereas the ST131 strain MS3179 did not elicit this effect (**Figure 2c**). Furthermore, infection with the two ASC speck-inducing

UPEC strains (i.e. CFT073 and UTI89) appeared to induce morphological changes and loss of nuclear integrity in HMDMs, as visualized by actin and DNA staining in the same samples (**Figure 2c**).

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

Causality of inflammasome activation and cell death in BMMs was analyzed using macrophages deficient for NLRP3 and NLRC4 (two NLRs most commonly activated by bacterial infection⁷), the inflammasome adaptor protein ASC, or the inflammatory caspases, caspase-1 and -11. As UTI89 did not trigger pronounced inflammasome activation in mouse macrophages at an MOI of 10, only CFT073 was assessed. CFT073-mediated caspase-1 cleavage was completely dependent on NLRP3 and ASC, but did not require NLRC4 (**Figure 3a**). As expected, the positive controls nigericin and *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) strain SL1344 acted via NLRP3 and NLRC4, respectively (**Figure 3a**). Analysis of LDH release confirmed that NLRP3, ASC, and caspase-1/11 were required for CFT073-mediated cell death (**Figure 3b**). These inflammasome components were also indispensable for CFT073-triggered IL-1 β release from LPS-primed BMMs (**Figure 3c**). As with caspase-1 cleavage, NLRC4 deficiency did not affect LDH or IL-1 β release upon infection with CFT073. Again, the positive controls for NLRP3, ASC, and caspase-1 involvement (nigericin), and NLRC4 and caspase-1 involvement (*S. Typhimurium*), behaved as expected. ASC was dispensable

Figure 2 The uropathogenic *Escherichia coli* (UPEC) strains CFT073 and UTI89 promote interleukin-1 β (IL-1 β) secretion, caspase-1 cleavage, and ASC speck formation. **(a)** Human monocyte-derived macrophages (HMDMs) and bone marrow-derived macrophages (BMMs) were primed with 100 ng ml⁻¹ lipopolysaccharide (LPS) for 4 h, and then infected with the indicated UPEC strains (multiplicity of infection (MOI) 10) for 2 h. Supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA). Data represent the mean $\pm$ s.e.m. of three independent experiments. **(b)** HMDMs and BMMs were infected with the indicated UPEC strains for 1 h or were left untreated (Ctrl). Whole-cell lysates and concentrated supernatants were analyzed by western blot with antibodies detecting human or mouse caspase-1 p20. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is shown as a loading control. Similar findings were apparent in three independent experiments. **(c)** HMDMs were infected with the indicated UPEC strains (MOI 10) for 2 h, after which they were fixed and analyzed by confocal microscopy. Arrowheads indicate ASC specks. Similar results were apparent in two independent experiments.

for *S. Typhimurium*-mediated cell death (**Figure 3b**) as previously reported,²² and NLRP3, ASC, and caspase-1/11 were indispensable for nigericin-triggered IL-1 β release (**Figure 3c**). *S.*

Typhimurium-triggered IL-1 β release was partially dependent on NLRP3 and ASC, and completely dependent on NLRC4 and caspase-1/11. Hence, rapid cell death and IL-1 β secretion

Figure 3 In murine macrophages, CFT073-induced caspase-1 cleavage and cell death is dependent on the NLRP3 inflammasome. Bone marrow-derived macrophages (BMMs) derived from wild-type (WT), *Nlrp3*^{-/-}, *Nlrc4*^{-/-}, *Asc*^{-/-}, and *Casp1/11*^{-/-} mice were infected with the uropathogenic *Escherichia coli* (UPEC) strain CFT073 or the *S. Typhimurium* strain SL1344 (multiplicity of infection (MOI) 10), or were stimulated with nigericin (10 μ M, prestimulated with 100 ng ml⁻¹ lipopolysaccharide (LPS) for 4 h). (a) After 1 h, whole-cell lysates and concentrated supernatants were analyzed by western blot with antibodies detecting murine full-length caspase-1, as well as multiple caspase-1 cleavage products. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. Data are representative of two independent experiments. (b) Supernatants were analyzed by lactate dehydrogenase (LDH) assay at 2 h postinfection (p.i.). Data are the mean $\pm$ range of two independent experiments. (c) LPS-primed BMMs (100 ng ml⁻¹, 4 h) were treated as above, after which secreted interleukin-1 β (IL-1 β) was measured by enzyme-linked immunosorbent assay (ELISA) after 2 h. Data are mean $\pm$ s.e.m. of three independent experiments. Sal, *Salmonella*; Ctrl or Unst., unstimulated.

triggered by CFT073 in mouse macrophages is dependent on NLRP3, ASC, and caspase-1 and/or caspase-11.

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

We next investigated NLRP3 involvement in human macrophage responses to UPEC using MCC950, a recently described NLRP3 inhibitor that does not affect AIM2, NLRP1, or NLRC4-mediated inflammasome activation.²³ Surprisingly, in LPS-primed HMDMs, MCC950 blocked nigericin-triggered cell death, but had little effect on CFT073-triggered cell death in cells from most donors examined (Figure 4a and Supplementary Table S1). In contrast, MCC950 significantly reduced both nigericin- and CFT073-triggered IL-1 β release from LPS-primed HMDMs (Figure 4b). Similar observations were apparent for UTI89, where MCC950 had only modest effects in reducing UTI89-triggered cell death for HMDMs from three out of four donors (Supplementary Table S1). This suggests that there are differences between human and mouse macrophages in NLRP3 responses to UPEC. Indeed, a direct comparison revealed that whereas MCC950 completely inhibited responses to nigericin in both HMDMs and BMMs, CFT073-mediated cell death was only blocked in mouse macrophages (Supplementary Figure S3A). Moreover, LPS priming had no apparent effect on NLRP3-dependency of CFT073-mediated cell death in human or mouse macrophages. In contrast to differential effects on cell death, MCC950

inhibited CFT073-triggered IL-1 β release in both LPS-primed HMDMs and BMMs, although more effectively in BMMs (Supplementary Figure S3B). The level of NLRP3-dependence for CFT073-triggered IL-1 β release varied between cells from different donors (Supplementary Table S1), and MCC950 never completely abolished IL-1 β release, as was the case for nigericin. We therefore investigated the possibility that the residual IL-1 β response detected by enzyme-linked immunosorbent assay (ELISA) might be because of the release of unprocessed IL-1 β , as a consequence of cell death. Indeed, analysis of concentrated cell culture supernatants by immunoblotting revealed that treatment with MCC950 completely blocked release of mature IL-1 β from CFT073-infected HMDMs, whereas pro-IL-1 β was still present in culture supernatants (Figure 4c). We also monitored ASC speck formation upon NLRP3 inhibition in HMDM. In these experiments, the NLRP3 inhibitor MCC950 completely blocked nigericin- but not *S. Typhimurium*-triggered ASC speck formation (Figure 4d). MCC950 substantially reduced, but did not ablate, CFT073- and UTI89-induced ASC speck formation in all experiments (Figure 4d).

To further investigate inflammasome involvement in human macrophages, the effect of the caspase-1 specific inhibitor VX-765²⁴ on CFT073-triggered cell death in human and mouse macrophages was examined. VX-765 effectively blocked CFT073- and LPS/nigericin-triggered cell death and IL-1 β release by LPS-primed BMMs at 2 h after infection (Figure 5a,b). However, similar to the observations with

Figure 4 NLRP3 contributes to interleukin-1 β (IL-1 β) release and maturation but does not mediate cell death in human macrophages responding to CFT073. (a,b) Human monocyte-derived macrophages (HMDMs) were primed with lipopolysaccharide (LPS; 100 ng ml⁻¹, 4 h) or left untreated, pretreated for 1 h with the NLRP3 inhibitor MCC950 (10 μ M), and subsequently infected with the uropathogenic *Escherichia coli* (UPEC) strain CFT073 or *S. Typhimurium* SL1344 (multiplicity of infection (MOI) 10), or stimulated with nigericin (Nig; 10 μ M). Supernatants were collected at 2 h postinfection (p.i.), and analyzed by (a) lactate dehydrogenase (LDH) release assay and (b) enzyme-linked immunosorbent assay (ELISA). Data are from nine (CFT073, LPS/nigericin) and six (*S. Typhimurium*) independent experiments (different donors), respectively. Statistical analysis 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 western blot with antibodies detecting full-length or cleaved human IL-1 β . (d) HMDMs were treated as above, but in the presence of 12 mM glycine, and analyzed by confocal microscopy. ASC specks and nuclei were counted manually in a blinded manner. Data are mean $\pm$ s.e.m. of three independent experiments. Ctrl, control; DMSO, dimethyl sulfoxide; Sal, *Salmonella*.

MCC950, VX-765 reduced CFT073-mediated IL-1 β release from LPS-primed HMDMs, but did not affect cell death. Control experiments confirmed that both inhibitors blocked cleavage and release of caspase-1 in response to CFT073 infection and LPS/nigericin stimulation in HMDMs and BMMs (Figure 5c,d). Together, these findings indicate that (i) the NLRP3 inflammasome drives UPEC-triggered IL-1 β maturation in human macrophages; and (ii) an NLRP3-independent pathway is the primary mediator of UPEC-triggered cell death in human macrophages.

To independently verify that an NLRP3-independent pathway mediates UPEC-triggered cell death, we used THP-1 defNLRP3 cells that stably express an NLRP3 short hairpin RNA and have reduced NLRP3 expression compared with a control cell line (THP-1 Null) transfected with an “empty” construct (Figure 6a). Caspase-1 processing in THP-1 defNLRP3 cells responding to CFT073, UTI89, or nigericin was greatly reduced, whereas caspase-1 p20 was still detectable at high levels after infection with *S. Typhimurium*. In these

cells, CFT073 and UTI89 still triggered substantial cell death, whereas nigericin did not (Figure 6b). Again, LPS priming had no effect on the degree of NLRP3-dependency of UPEC-mediated cell death, although it did appear to reduce the effect of NLRP3 knockdown in the nigericin control. As expected, no reduction in cell death was observed in defNLRP3 cells when using *S. Typhimurium* as an NLRP3-independent trigger for cell death. Collectively, these data suggest the involvement of another NLRP3-independent cell death pathway triggered by UPEC.

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

The capacity for CFT073 and UTI89 to trigger human macrophage cell death was also conferred by culture supernatants (Supplementary Figure S4). Crude biochemical analysis indicated that the factor(s) responsible were heat and protease sensitive, and with a likely molecular weight of > 30 kDa.

Figure 5 Uropathogenic *Escherichia coli* (UPEC)-mediated cell death is blocked by caspase-1 inhibition in mouse but not human macrophages. (a,b) Human monocyte-derived macrophages (HMDMs) and bone marrow-derived macrophages (BMMs) were primed with lipopolysaccharide LPS; 100 ng ml⁻¹, 4 h) or left untreated, pretreated for 1 h with the caspase-1 inhibitor VX-765 (50 μM), and subsequently infected with the UPEC strain CFT073 (multiplicity of infection (MOI) 10) or stimulated with nigericin (Nig; 10 μM). Supernatants were collected at 2 h postinfection (p.i.), and were analyzed for cell death by (a) lactate dehydrogenase (LDH) release assay and (b) interleukin-1β (IL-1β) release by enzyme-linked immunosorbent assay (ELISA). Data represent the mean ± s.e.m. of three independent experiments. (c,d) HMDMs and BMMs were treated as above for 1 h. Cell lysates, as well as cell culture supernatants, were analyzed by western blot for cleavage of caspase-1. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as loading control. Similar results were obtained in two independent experiments. Ctrl, control; DMSO, dimethyl sulfoxide.

Figure 6 Uropathogenic *Escherichia coli* (UPEC) triggers NLRP3-independent cell death in human macrophages. Stable NLRP3 knockdown THP-1 cells (defNLRP3), as well as THP-1 control cells (Null), were infected with the indicated UPEC strains or *S. Typhimurium* (multiplicity of infection (MOI) 10), or were treated with nigericin (10 μM) for (a) 1 h or (b) 2 h. (a) Cell lysates were analyzed by western blot for expression of NLRP3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control. Caspase-1 p20 was detected in cell lysates. Similar findings were apparent in two independent experiments. (b) Supernatants of unprimed or lipopolysaccharide (LPS)-primed (100 ng ml⁻¹ LPS, 4 h) THP-1 cells were analyzed by lactate dehydrogenase (LDH) assay. Data are the mean ± s.e.m. of four independent experiments. Ctrl, control.

Candidate proteins mediating cell death and/or IL-1β secretion included the pore-forming toxin α-hemolysin, as well as serine-protease autotransporter toxins (Sat and Vat). Genes encoding all three toxins are present in CFT073, whereas UTI89 contains the *hlyCABD* (α-hemolysin operon) and *vat* genes. To test the involvement of these three toxins in triggering the

inflammasome response, we generated a series of CFT073 mutants deleted for genes encoding each individual toxin and a triple-mutant deficient in the ability to produce all three toxins. Compared with wild-type CFT073, the α-hemolysin mutant (CFT073Δ*hlyA*) was greatly impaired in its ability to trigger cell death of mouse macrophages (~30% of wild type) and, to a

Figure 7 Differences between human and mouse macrophages in cellular responses to α -hemolysin. (a,b) Human monocyte-derived macrophages (HMDMs) and bone marrow-derived macrophages (BMMs) were infected with wild-type uropathogenic *Escherichia coli* (UPEC) strains or mutants deficient for α -hemolysin ($\Delta hlyA$), *sat* (Δsat), *vat* (Δvat), or all three genes ($\Delta hlyA; \Delta sat; \Delta vat$) at the indicated multiplicity of infection (MOI) for 2 h. Supernatants were analyzed by lactate dehydrogenase (LDH) assay. (c) Lipopolysaccharide (LPS)-primed (100 ng ml⁻¹, 4 h) cells were treated as above (MOI 10) and analyzed by interleukin-1 β (IL-1 β) enzyme-linked immunosorbent assay (ELISA). Data are the mean $\pm$ s.e.m. of (a,c) three or (b) four independent experiments. (d) Concentrated supernatants from cells treated as described above were analyzed by western blot with antibodies detecting cleaved human or mouse IL-1 β . Similar observations were made in two independent experiments.

lesser extent, of human macrophages ($\sim 60\%$ of wild type) when macrophages were exposed to an MOI of 10 (Figure 7a). CFT073 *sat* and *vat* mutants were very modestly compromised for their ability to trigger mouse and human macrophage cell death. However, no additive effect was observed, as the triple mutant showed no difference to the CFT073 $\Delta hlyA$ single mutant in this assay. Similar observations were made at an MOI of 100 with both CFT073 and UTI89 (Figure 7b). In this case, cell death induced by CFT073 and UTI89 was almost completely *hlyA*-independent in HMDMs (cell death for *hlyA* mutants was $\sim 85\%$ of the wild-type strains), whereas in BMMs the response was largely *hlyA*-dependent (~ 30 and 40% of wild-type strains). Intriguingly, analysis of IL-1 β release revealed a striking difference between human and mouse macrophages with respect to triggering by α -hemolysin. Whereas IL-1 β levels were substantially reduced in BMMs responding to CFT073 $\Delta hlyA$ compared with wild-type CFT073, deletion of *hlyA* had no effect on HMDM responses (Figure 7c). Again, we analyzed cleavage of released IL-1 β by western blot and found that in HMDMs, levels of cleaved IL-1 β were similar in samples infected with CFT073 and CFT073 $\Delta hlyA$ (Figure 7d). In contrast, CFT073 $\Delta hlyA$ was drastically impaired in its ability to induce the release of cleaved IL-1 β in BMMs. Deletion of *sat* or *vat* had no effect on IL-1 β release from either human or mouse macrophages, and the

response to the triple mutant was again identical to that of the *hlyA* single mutant. Thus, α -hemolysin is the primary, but not only, mediator of cell death and IL-1 β release in mouse macrophages. Moreover, generation of mature IL-1 β was completely dependent on α -hemolysin. In contrast, in the human macrophage response to UPEC, α -hemolysin does not contribute to IL-1 β release or cleavage, and plays a lesser role in rapid cell death. These differential effects of α -hemolysin further highlight the divergent UPEC recognition pathways of human and mouse macrophages. In summary, UPEC triggers both NLRP3-independent cell death and α -hemolysin-independent IL-1 β processing in human macrophages, thus indicating that additional host and pathogen-derived factors are likely to be important in the macrophage response to UPEC.

DISCUSSION

In this study, we demonstrate that some UPEC strains can trigger both NLRP3-dependent inflammasome activation and rapid cell death in macrophages. We also provide important insights into these processes in the context of similarities and differences between human and mouse macrophage responses to UPEC. Other *E. coli*, including enterohemorrhagic *E. coli* isolates¹² as well as nonpathogenic or commensal strains,^{25,26} have been reported to activate inflammasomes by a variety of different mechanisms involving several bacterial

factors. These include nucleic acids²⁷ and protein toxins (enterohemolysin¹² and heat-labile enterotoxin²⁸) acting via NLRP3, the T3SS rod protein EprJ²⁹ and flagellin²⁵ acting via NLRC4, and (intracellular) LPS³⁰ acting via noncanonical inflammasomes. Our study adds to this literature by identifying UPEC α -hemolysin-dependent and -independent mechanisms of inflammasome activation in macrophages.

It remains unclear as to what roles UPEC-mediated inflammasome activation has in different pathophysiological contexts. Two out of the four strains that did not elicit inflammasome activation are associated with asymptomatic bacteriuria,^{17,18} whereas the remaining two belong to the globally disseminated fluoroquinolone-resistant *fimH30*/clade C ST131 lineage that is frequently associated with symptomatic infection.^{16,31} The two inflammasome-activating strains are also associated with UTI pathology. Hence, the capacity for inflammasome activation is variable, further highlighting the genetic diversity that exists among different UPEC isolates. As the capacity to trigger inflammasome activation and macrophage cell death was not common to all UPEC strains, some UPEC strains may have gained inflammasome-activating factors as a component of their virulence armoury or lost these to avoid host detection. In the case of the former, candidate virulence factors included the pore-forming toxin α -hemolysin,³² as well as serine–protease autotransporter toxins (Sat and Vat) that are known to elicit cytotoxic effects on epithelial cells,³³ and for which the genes are present in CFT073 (*hlyA*, *sat*, *vat*) and UTI89 (*hlyA*, *vat*), but not 83972 and VR50³⁴ or EC958.¹⁵ Mutation of all three factors in CFT073 revealed that only the absence of α -hemolysin substantially reduced inflammasome responses in mouse macrophages. Intriguingly, however, CFT073-triggered IL-1 β release and cleavage was completely independent of α -hemolysin in human macrophages, and there was also a pronounced α -hemolysin-independent cell death pathway. This finding points toward fundamentally different recognition mechanisms for these UPEC strains in human vs. mouse macrophages, yet conservation in the overall outcomes. Whether this extends to other cell types needs to be further examined, given a recent report on *E. coli* α -hemolysin triggering IL-1 β secretion in human urothelial cells,³⁵ an earlier study showing a similar phenomenon in human monocytes,³⁶ and well-documented cell type-specific effects of α -hemolysin.³²

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

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

The conclusion that the acute CFT073-mediated inflammasome response leading to cell death and IL-1 β secretion in BMMs was absolutely dependent on NLRP3 was also supported by experiments using small-molecule inhibitors of NLRP3 (MCC950) and caspase-1 (VX-765). However, primary human macrophages that were analyzed in parallel showed a remarkably different response. The NLRP3 and caspase-1 inhibitors substantially reduced or blocked IL-1 β release from HMDMs in response to UPEC infection or the NLRP3 agonist nigericin, respectively. In contrast, UPEC-triggered cell death was largely unaffected by either inhibitor. Although analysis of HMDMs generated from several donors revealed some variation in the level of NLRP3-dependence, the overall conclusion is that in human macrophages cell death is NLRP3 independent, whereas IL-1 β cleavage was shown to be completely NLRP3-dependent. Interestingly, LPS-primed HMDMs released unprocessed IL-1 β upon UPEC infection, even when the NLRP3 inflammasome was blocked. Biologically, this may be of significance as it was shown that uncleaved IL-1 β can be processed in the extracellular space by inflammasome complexes³⁸ or by enzymes such as cathepsin-G and elastase.³⁹ Consistent with the existence of an NLRP3-independent death pathway in human macrophages, stable knockdown of NLRP3 in THP-1 cells blocked nigericin-induced cell death, but only marginally reduced CFT073- and UTI89-mediated cell death. Whether NLRP3-independent cell death involves activation of another inflammasome is unknown at this stage. However, given that NLRP3 inhibition ablated both UPEC-induced IL-1 β maturation and caspase-1 cleavage in HMDMs, other modes of cell death such as necroptosis would appear to be more likely.

Divergence in the repertoire of NLR family members between humans and mice can contribute to differences in inflammasome responses between these species.^{10,40} However, differences in the recognition of pathogens by orthologous human and mouse NLRs have also been reported. For example, *Francisella tularensis* activates only the AIM2 inflammasome in mouse macrophages, but triggers NLRP3- and AIM2-dependent responses in human macrophages.⁴¹ Conversely, *Listeria monocytogenes* was reportedly recognized by AIM2, NLRP3, and NLRC4 in mouse cells,^{42–44} but exclusively by NLRP3 in human cells.⁴⁵ The causes for these differences are not fully understood, but may be related to species differences in ligand recognition. Our study highlights that one pathogen can activate NLRP3 in both human and mouse macrophages, but through distinct mechanisms. Our demonstration of α -hemolysin-dependent IL-1 β cleavage and cell death in

mouse macrophages is consistent with a recent study showing α -hemolysin-mediated inflammasome activation in UTI89-infected mice.³⁵ In stark contrast however, our studies with human macrophages identified an α -hemolysin-independent pathway to IL-1 β maturation. This suggests that another UPEC factor selectively promotes NLRP3 activation in human but not mouse macrophages, or that its relative potency in triggering inflammasome responses differs between these species or between different cell types.

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

A protective role for inflammasome activation and IL-1 β production has been shown in many *in vivo* infection models including *S. Typhimurium*, *Listeria monocytogenes*, and *Burkholderia* species.¹¹ On the other hand, the role of pathogen-induced cell death is ambiguous, having either protective or detrimental effects by either eradicating intracellular niches or promoting dissemination, respectively.⁴⁹ As UPEC can occupy both extracellular and intracellular niches, it is difficult to predict what role pyroptosis plays during UTI. Activation of caspase-1/11 was shown to facilitate clearance of UPEC in a mouse model, presumably by inducing pyroptosis and subsequent exfoliation of bladder epithelial cells.³⁵ In another study however, activity of caspase-1/11 was associated with chronicity and higher bacterial loads in the bladder in a model of recurrent UTI.⁵⁰ In the case of cytokine processing, previous studies have associated IL-1 β release with renal pathology of UTI in patients,^{51,52} and also in a mouse model.⁵³ Only very recently was IL-1 β release in *atg16l1*^{-/-} mice shown to be associated with protection from UTI.³⁷ Although the

effects of IL-1 β can be studied simply by knockout or by blocking its interaction with receptors, new approaches for genetically and/or pharmacologically uncoupling pyroptosis from other inflammasome responses will be required to address the role of cell death in pathology. Whether NLRP3-dependent responses have a causal role in host defense or pathology remains to be elucidated.

In conclusion, our study highlights the complexity of interactions between UPEC and the innate immune system. Some UPEC strains trigger inflammasome activation and rapid, lytic cell death in macrophages, whereas others, including two strains from the multidrug-resistant ST131 lineage, do not. This again highlights the genetic complexity that exists among different UPEC strains and that host response pathways engaged, as well as host colonization strategies employed, will vary depending on the specific UPEC strain encountered. For inflammasome-activating strains such as CFT073, NLRP3 drives IL-1 β maturation in both human and mouse macrophages. However, this pathway only marginally contributes to cell death in human macrophages, despite its causal role in cell death in mouse macrophages. Finally, α -hemolysin is the primary trigger for cell death and IL-1 β release in mouse macrophages, whereas these cellular responses are either primarily or completely independent of this toxin in human macrophages. The yet-to-be-identified death pathway in human monocyte-derived macrophages highlights a potential difference between human and mouse innate immune UPEC recognition pathways and needs to be considered in future studies using macrophages and other cell types from a variety of sources, as well as in *in vivo* studies. Given the importance of mouse UTI models for understanding host colonization and pathology, and especially in the light of recent studies showing detrimental and beneficial effects of inflammasome activation in mouse models,^{37,50} as well as a prominent role for α -hemolysin,³⁵ our findings are likely to have broad significance for understanding susceptibility and severity of UTI in humans.

METHODS

Bacterial strains and growth conditions. UPEC strains CFT073,¹³ UTI89,¹⁴ 83972,¹⁷ VR50,¹⁸ and EC958¹⁵ have been described previously. MS3179 is an ST131 strain isolated from a patient presenting with UTI at the Royal Brisbane and Women's Hospital, Brisbane, Australia. *S. Typhimurium* strain SL1344⁵⁴ was used as a control for NLRP3-independent inflammasome activation in some experiments. All strains were routinely grown at 37 °C on solid or in liquid Luria-Bertani (LB) medium.

Genetic manipulation procedures and generation of mutants.

Mutation of the *hlyA*, *sat*, and *vat* genes in CFT073, and the *hlyA* gene in UTI89, was performed using the λ -Red recombinase gene replacement system.⁵⁵ The primers used for amplification of the kanamycin resistance gene (*hlyA*) or chloramphenicol resistance gene (*vat*, *sat*), and subsequent insertion into the chromosome of CFT073 (or UTI89), were as follows: *vat* (3353: 5'-TCGTAATGAACACAGTTCATCTGATCTCCACACACCAAGACTTGATAAGCTCAGGTC TTGAGCGATTGTGTAGG-3' and 3354: 5'-GAAACCACCAACCCATGATTTTGTGTTTACCGCTGTACAGGCCTGCTGACGCGACA TGGGAATTAGCCATGGTCC-3'), *sat* (3351: 5'-AAGAAATTCCA

ATGATTTTGGAGATTGAGAGGTTAAATAAATTTGTTGTGGAC ACGTCTTGAGCGATTGTGTAGG-3' and 3352: 5'-CCAGGAGTG GGAGCTGTAGTCTCTGGTGCCAGGCCGCGCAAAGTTGCG GTGACATGGGAATTAGCCATGGTCC-3'), and *hlyA* (2,049: 5'-A AATTAAGACACACTACAGTCTGCAAAGCAATCCTCTGCA AATAAATTTGTGTAGGCTGGAGCTGCTTC-3' and 2050: 5'-TGCT CTGCTGCTTTTAAATGCATCTTTCGTGCTTTGCTCTGCTGAG TGCATATGAATATCCTCCTTAG-3'). CFT073 *hlyA* (CFT073Δ*hlyA*), *sat* (CFT073Δ*sat*), and *vat* (CFT073Δ*vat*) mutants, as well as the UT189 *hlyA* mutant (UT189Δ*hlyA*), were confirmed by PCR and DNA sequencing. The CFT073 *hlyA-sat-vat* triple mutant was constructed by sequential deletion of each gene, as described above, and was confirmed by PCR and DNA sequencing.

Mammalian cell culture. Approval for all experiments using primary human and mouse cells was obtained from the University of Queensland Medical Research Ethics Committee or the Animal Ethics Committee. Human monocytes were isolated from buffy coats of healthy donors (kindly provided by the Australian Red Cross) by positive selection for CD14 using MACS technology (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described.⁵⁶ HMDMs were differentiated for 7 days with colony stimulating factor-1 (10,000 U ml⁻¹, Chiron, Emeryville, CA) from CD14⁺ cells, as previously described,⁵⁶ but in the absence of antibiotics. Cells from a single donor were used in every experiment. Murine BMs were differentiated using 10,000 U ml⁻¹ colony stimulating factor 1 (Chiron) from bone marrow of C57BL/6 wild-type, *Nlrp3*^{-/-}, *Nlrp4*^{-/-}, *Asc*^{-/-}, and *Caspase-1/11*^{-/-} mice (all described in Chen *et al.*⁴⁶), in the absence of antibiotics as previously described.⁶ Peritoneal cavity cells were flushed from the peritoneal cavity of C57BL/6 mice by injection of 5 ml PBS. THP-1 (TIB-202, ATCC, Manassas, VA), THP-1 Null, and THP-1 defNLRP3 (InvivoGen, San Diego, CA) cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM Na-Pyruvate, and 10 mM HEPES (all from Life Technologies, Carlsbad, CA). THP-1 cells were differentiated into macrophage-like cells by culture for 48 h in medium containing 30 ng ml⁻¹ PMA (Sigma-Aldrich, St Louis, MO). PMA or colony stimulating factor-1 was removed 4 h before infection with UPEC strains. Human bladder epithelial cell lines 5637 (HTB-9, ATCC) and T24 (HTB-4, ATCC) were cultured in fetal bovine serum (10%) and 2 mM L-glutamine supplemented with RPMI-1640 or McCoy's 5A medium (Life Technologies), respectively.

Bacterial culture and macrophage infection assays. UPEC strains were grown statically at 37 °C overnight in LB broth. *S. Typhimurium* strain SL1344 was grown overnight in LB broth (200 r.p.m., 37 °C), diluted 1:33, and grown for another 3 h (to ensure logarithmic growth). Cells were pelleted, washed, and adjusted to the same optical density at 600 nm. A MOI of ~10 was used (unless indicated otherwise) and confirmed by enumeration of colony-forming units following serial dilution. Mammalian cells were seeded at a density of 4–8 × 10⁴/0.2 ml in 96-well plates or 2–4 × 10⁵ per ml in 24-well plates (Nunc, Roskilde, Denmark). Medium was changed for all cell types to RPMI-1640 supplemented with fetal bovine serum (10%) and 2 mM L-glutamine (all from Life Technologies) 4 h before infection. LPS priming was performed by addition of 100 ng ml⁻¹ Ultrapure LPS from *Salmonella minnesota* R595 (InvivoGen). Nigericin sodium salt (Sigma-Aldrich) at a concentration of 10 μM was used as a positive control for NLRP3-dependent responses. In some experiments, cells were preincubated for 1 h with the caspase-1 inhibitor VX-765 (Selleck Chemicals, Houston, TX) or the NLRP3 inhibitor MCC950,²³ before performing infections. At 1 h after infection, 200 μg ml⁻¹ gentamicin (Life Technologies) was added for 1 h to inhibit growth of extracellular bacteria. For infections over a 24-h time course, medium was replaced with fresh medium containing 20 μg ml⁻¹ gentamicin for the remaining 22 h.

Cytotoxicity assays. Cell culture supernatants were collected at 2 or 24 h after infection, centrifuged for 5 min at 500 g, and analyzed for LDH

release using the In Vitro Toxicology Assay Kit (Sigma-Aldrich). Cytotoxicity (%) was calculated by quantification of LDH in culture supernatants vs. total cellular LDH (present in supernatant after cell lysis with 0.1% Triton X-100) according to the formula: % cell death = (100/LDH_{total} – LDH_{spontaneous}) × (LDH_{treatment} – LDH_{spontaneous}). The 24 h values represent summed measurements of the same well at 2 and 24 h after infection, as medium was changed at 2 h after infection as part of the gentamicin exclusion protocol (see the section “Bacterial culture and macrophage infection assays”).

Confocal microscopy. Confocal microscopy was performed as previously described.⁶ Cells were stained with 200 ng ml⁻¹ Alexa Fluor 594 Phalloidin (Life Technologies) to visualize cell morphology, and ASC was detected with a rabbit anti-ASC Antibody (N-15)-R (Santa Cruz Biotechnology, Santa Cruz, CA) (1:300) and Alexa Fluor 647- or 688-conjugated chicken anti-rabbit IgG (Life Technologies) as a secondary antibody (1:150). For quantifying ASC speck formation, HMDMs were cultured in 12 mM glycine to reduce loss of cells due to lytic cell death.⁵⁷ ASC specks were counted manually in a blinded manner (5 fields at ×40 magnification per condition per replicate).

Immunoblotting. 4 × 10⁵ cells were lysed in 100 μl 2 × SDS loading buffer (125 mM Tris-HCl, 20% glycerol (v/v), 4% SDS (w/v), pH 6.8). For analysis of secreted caspase-1 in cell culture supernatants, medium was replaced with OptiMEM medium (Life Technologies) 4 h before infection. Cell culture supernatants were precipitated by incubation with 4 volumes of acetone at –20 °C overnight and centrifugation at 5,300 g and –10 °C for 30 min. Pellets were taken up in 2 × SDS loading buffer. Western blotting was performed as previously described.⁵⁶ Membranes were stained with cleaved IL-1β (Asp116) rabbit monoclonal antibody (mAb), IL-1β (3A6) mouse mAb, cleaved caspase-1 (ASP297) (D57A2) rabbit mAb (all from Cell Signalling Technology, Danvers, MA), mouse IL-1β/IL-1F2 affinity-purified polyclonal Ab, Goat IgG (R&D Systems, Minneapolis, MN), anti-caspase-1 (p20) (mouse) mAb (Adipogen, San Diego, CA), anti-NLRP3/NALP3 mAb (Cryo-2) (Adipogen), or human anti-G3PDH antibody (Trevigen, Gaithersburg, MD). All primary antibodies were diluted 1:1,000 except for human anti-G3PDH antibody, which was used at 1:10,000. As secondary antibodies, anti-mouse and anti-rabbit IgG, horseradish peroxidase-linked antibodies (Cell Signalling Technology) (1:2,500), and anti-goat IgG-peroxidase antibody (Sigma-Aldrich) (1:5,000) were used. Horseradish peroxidase was detected using ECL Plus substrate (GE Healthcare, Buckinghamshire, UK) and Super RX film (Fujifilm, Tokyo, Japan).

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

Statistical analysis. All LDH, ELISA, and MTT assays were performed using duplicate or triplicate cell culture wells for individual experiments. Presented data are typically mean values combined from three or more independent experiments, unless otherwise indicated. For statistical analysis of data sets with *N* > 4, two-sided Wilcoxon matched-pairs signed-rank tests were performed using GraphPad Prism Version 6 (GraphPad software, La Jolla, CA). For these data sets, the differences between pairs were plotted and were generally distributed approximately symmetrically around the median.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declared no conflict of interest.

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