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SHORT COMMUNICATION

NLRP3 inflammasome activation downstream of cytoplasmic LPS recognition by both caspase-4 and caspase-5

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Humans encode two inflammatory caspases that detect cytoplasmic LPS, caspase-4 and caspase-5. When activated, these trigger pyroptotic cell death and caspase-1-dependent IL-1β production; however the mechanism underlying this process is not yet confirmed. We now show that a specific NLRP3 inhibitor, MCC950, prevents caspase-4/5-dependent IL-1β production elicited by transfected LPS. Given that both caspase-4 and caspase-5 can detect cytoplasmic LPS, it is possible that these proteins exhibit some degree of redundancy. Therefore, we generated human monocytic cell lines in which caspase-4 and caspase-5 were genetically deleted either individually or together. We found that the deletion of caspase-4 suppressed cell death and IL-1β production following transfection of LPS into the cytoplasm, or in response to infection with *Salmonella typhimurium*. Although deletion of caspase-5 did not confer protection against transfected LPS, cell death and IL-1β production were reduced after infection with *Salmonella*. Furthermore, double deletion of caspase-4 and caspase-5 had a synergistic effect in the context of *Salmonella* infection. Our results identify the NLRP3 inflammasome as the specific platform for IL-1β maturation, downstream of cytoplasmic LPS detection by caspase-4/5. We also show that both caspase-4 and caspase-5 are functionally important for appropriate responses to intracellular Gram-negative bacteria.

Keywords: Caspase-4 · Caspase-5 · LPS · NLRP3 inflammasome · Pyroptosis


See accompanying article by Sebastian Rühl and Petr Broz and Schmid-Burgk et al.


See accompanying Commentary by Rivers-Auty and Brough


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Introduction

For almost a decade, it was assumed that lipopolysaccharide (LPS) simply acted as a priming signal in inflammasome cascades. This assumption was overturned when Kayagaki et al. demonstrated that caspase-1 is not the only caspase capable of inducing pyroptotic death [1]. The *Casp1*$^{-/-}$ mice that have historically been used to study inflammasome activity are in fact deficient in both caspase-1 and caspase-11 due to a passenger mutation carried over during backcrossing of the 129 stem cell background into C57BL/6 mice. When mice ectopically expressing *Casp11* were created on the same background, it was demonstrated that caspase-11 induces cell death and IL-1α release independently of caspase-1 in LPS-primed BM-derived macrophages (BMDMs) treated with cholera toxin B. In response to the same stimuli, NOD-like receptor family, pyrin domain containing 3 (NLRP3), Apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD) (ASC), and caspase-1 were also required for caspase-11-dependent IL-1β and IL-18 maturation and secretion. The exact mechanism by which cholera toxin B triggered caspase-1-independent death was unknown until 2013, when it was revealed that the toxin carried LPS into the macrophage cytoplasm. Other artificial means of transfecting LPS into macrophages produced a similar phenomenon, as did infections with invasive Gram-negative bacteria such as *Escherichia coli*, *Citrobacter rodentium*, and *Shigella flexneri* [2, 3]. Further, caspase-11 deficiency reduced the incidence of mortality in murine in vivo LPS-induced endotoxic shock models, independently of caspase-1 [4, 5].

Casp11 has two human orthologs, *CASP4* and *CASP5* (encoding caspase-4 and caspase-5, respectively) [6]. The overriding paradigm in the pyroptosis field is that an inflammasome complex activates caspase-1; therefore much effort was invested to uncover the equivalent receptor and adaptors responsible for caspase-11, caspase-4, and caspase-5 activation. Shi et al. recently reported that this much-anticipated signaling cascade may actually not exist. Instead LPS directly interacted with the CARD domains of caspase-11, caspase-4, and caspase-5 to trigger caspase oligomerisation and activation, but did not interact with other CARD-containing caspases [7]. This suggests that pyroptosis induced by cytoplasmic LPS is not initiated by a traditional inflammasome, but merely through a single caspase that acts as both the receptor and pyroptotic initiator.

The study by Shi et al. further showed that overexpression of either caspase-4 or caspase-5 could restore cell death responses in caspase-11-deficient immortalized murine BMDMs upon electroporation with LPS. Additionally, human macrophage (U937), epithelial (HeLa, HT29), and keratinocyte (HaCaT) cell lines with *CASP4* silenced by siRNA were protected from death following LPS electroporation and *S. flexneri* infection. HeLa cells with *CASP4* knocked out by CRISPR/Cas9 were also protected following LPS electroporation or infection with *S. typhimurium*. Susceptibility to cytoplasmic LPS was also dependent on the catalytic activity of caspase-4 in human cells and caspase-11 in murine cells. *CASP4* knockout (KO) HeLa cells overexpressing a caspase-4 variant in which the active site cysteine was mutated (C258A) were unable to induce pyroptosis in response to cytoplasmic LPS, as were *Casp11*$^{-/-}$ BMDMs complemented with the equivalent (C254A) mutation [7].

A role for either caspase-4 or caspase-5 in inflammatory cytokine production in human myeloid cells has not been conclusively shown. Murine BMDMs transgenically overexpressing *CASP4* were augmented in their ability to produce IL-1β and IL-18, however this utilizes the mouse inflammasome machinery [8]. We now show that indeed human myeloid cells that are genetically deficient for caspase-4 produce reduced amounts of IL-1β upon cytosolic LPS exposure. Further, NLRP3-inhibition prevents caspase-4-dependent IL-1β production independently of cell death. We also show that caspase-4 and caspase-5 together regulate both cell death and cytokine production during infection with *S. typhimurium*.

Results and discussion

Genetic deletion of caspase-4 and caspase-5 in THP-1 cells

A lentiviral CRISPR system was used to target *CASP4*, *CASP5*, or *CASP1* in THP-1 monocytes. These cells stably expressed the *Streptococcus pyogenes* Cas9 endonuclease, which was directed by a doxycycline-inducible small guide RNA (sgRNA) to create ds breaks, inducing nonhomologous end joining (NHEJ) and frameshift mutations within the open reading frame (ORF) of the targeted genes. Using the CRISPR design tool created by the Zhang lab at MIT [9], two sgRNAs were designed homologous to regions within *CASP4*. Two sgRNAs specific to the *CASP5* and one targeting *CASP1* were likewise generated (Supporting Information Table 1). sgRNAs were designed against the regions of these genes encoding the CARD domains, as this is the most divergent region between *CASP4* and *CASP5*, which share 73% sequence homology at the amino acid level. Upon doxycycline induction, the cells carrying *CASP4*-specific sgRNAs produced a truncated version of caspase-4, corresponding to deletion of exons 2 and 3 (~35 kDa, Fig. 1A). Skipping of these exons should result in ablation of the CARD domain and translation of a nonfunctional caspase-4 variant that is unlikely to be able to bind LPS as previously reported [7]. Reduction in caspase-5 was undetectable by Western blotting using four different commercially available α-caspase-5 antibodies, suggesting that they are not specific; in fact, two of these antibodies detected caspase-4 (MBL 4F7, abcam ab10448; Supporting Information Fig. 1). To confirm that caspase-5 was correctly targeted, we performed semiquantitative PCR of mRNA isolated from *CASP5* KO or *CASP4/5* double KO (dKO) pools, using primers that fall inside, or flank the CRISPR targeted region (Fig. 1B). This showed a reduced ability to produce mature transcript in cells targeted with the *CASP5*-specific sgRNAs. In cells additionally targeted with the *CASP4* sgRNAs, the mutated *CASP4* transcript was seen to persist, supporting the idea that a truncated caspase-4 variant is still translated following CRISPR targeting (Fig 1B, Supporting Information Fig. 2). As has been previously published,

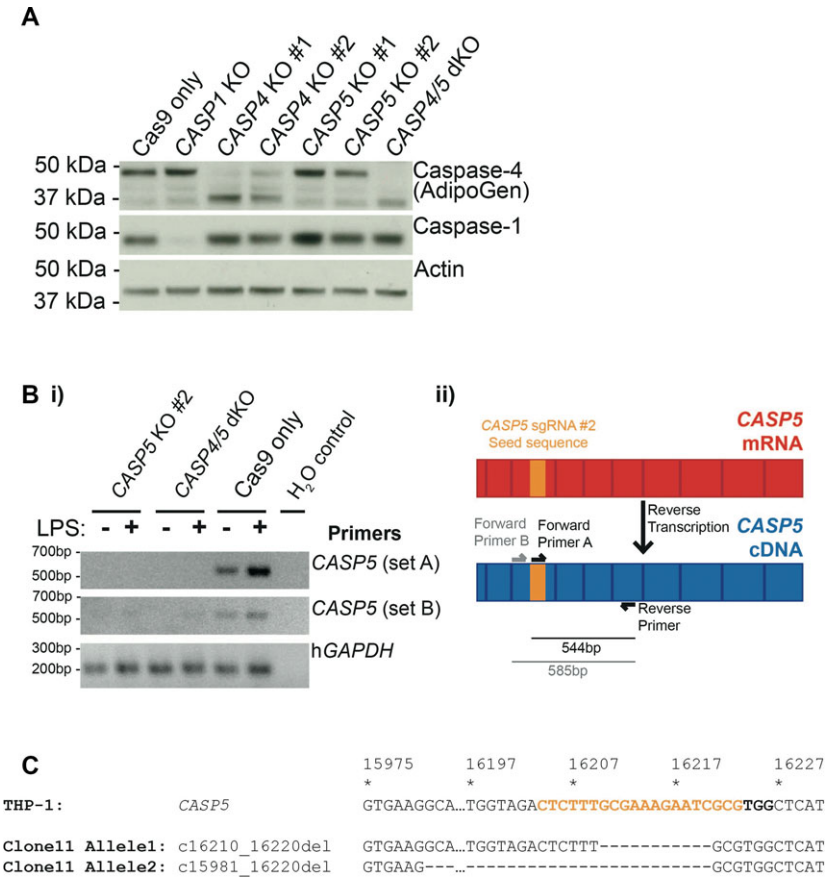

Figure 1. Genetic deletion of caspase-4 and caspase-5 in human monocytic THP-1 cells. (A) Protein lysates from THP-1 monocytes that had been targeted by CRISPR (doxycycline-inducible) for *CASP4*, *CASP5*, or *CASP1* KO were immunoblotted with the indicated antibodies and protein loading was confirmed by blotting for actin. *CASP4* and *CASP5* were each targeted using two independent sgRNAs. The ~37-kDa band detected in lysates from cells targeted for *CASP4* KO corresponds to a truncated form of the protein that likely lacks exons 2 and 3. (B) RNA was prepared from *CASP5*-targeted THP-1 pools both with and without LPS stimulation (200 ng/mL for 4 h), reverse transcribed, and amplified using the primer sets described (i). Schematic showing semiquantitative PCR strategy for *CASP5* mRNA (ii). Primers were designed to fall inside (Set A) or upstream (Set B) of the sgRNA targeted region. (C) Genomic DNA was prepared from single-cell clones of *CASP5*-targeted cells, then individual alleles were purified by agarose gel electrophoresis and subjected to Sanger sequencing. Clone 11 reveals large biallelic deletions in the *CASP5* locus. The *CASP5* 1 sgRNA seed sequence is highlighted in bold orange and the protospacer adjacent motif (PAM) is highlighted in bold black. Data shown are representative of at least three independent experiments.

LPS priming of the THP-1 monocytes resulted in increased *CASP5* expression [10]. Different groups have reported conflicting evidence regarding the expression of caspase-5 in THP-1 monocytes, indicating a degree of variability between THP-1 lines cultured in separate facilities [7, 10, 11].

Clonal analysis of CASP5-targeted THP-1 cells

In order to further probe the ability of the KO cells to produce caspase-5, single-cell clones were sorted and cultured. A band approximately the predicted size of caspase-5 (49 kDa) was still detected upon Western blotting of lysate from *CASP5*-targeted clones (Supporting Information Fig. 3). We therefore sequenced the DNA of *CASP5* KO THP-1 single-cell clones and confirmed genetically that indeed these cells are appropriately targeted in both alleles (Fig. 1C). Based on the loss of mRNA and the disruption of *CASP5* at the genomic level, it appears that the α-caspase-5 antibodies used do not exclusively detect caspase-5 but may recognize another protein of similar size, frequently caspase-4.

Caspase-4 is required for pyroptosis and IL-1β secretion in response to cytoplasmic LPS

Murine cells require a priming signal to induce *Casp-11* expression [12]. To investigate pyroptosis and inflammasome activation downstream of cytoplasmic LPS, we therefore treated *CASP4, CASP5, CASP4/5 or CASP1* KO THP-1 monocytes with the TLR1/2 agonist Pam3CSK4 before transfecting cells with LPS. TLR4 stimulation by extracellular LPS prior to transfection produced identical results to priming with Pam3CSK4 (data not shown). As expected, the *CASP4* KO pool was significantly protected from cell death induced by cytoplasmic LPS (Fig. 2A). Deletion of caspase-5 afforded no protection, and *CASP4/5* dKO cells showed no additional resistance to death compared to the *CASP4* single KO (Fig. 2A). As human monocytic cells were used for this analysis, we were able to interrogate production of inflammasome-dependent cytokines such as IL-1β. In keeping with the observations for cell death, deletion of caspase-4 prevented IL-1β production; however this was not enhanced by the deletion of caspase-5. *CASP1* KO monocytes were also resistant to IL-1β release, but not death induced by cytoplasmic LPS (Fig. 2B).

These results indicate that caspase-4 is a key contributor to pyroptosis and caspase-1-mediated IL-1β production in human monocytes transfected with LPS. Our data agree with the previously published observation that *CASP4* gene silencing protected both THP-1 and U937 monocyte cell lines from pyroptosis induced by cytoplasmic LPS [7]. The orthologous murine system requires caspase-1 in addition to caspase-11 for IL-1β production in response to transfected LPS, and the data presented here suggest that caspase-4 shares this dependence in the human pathway.

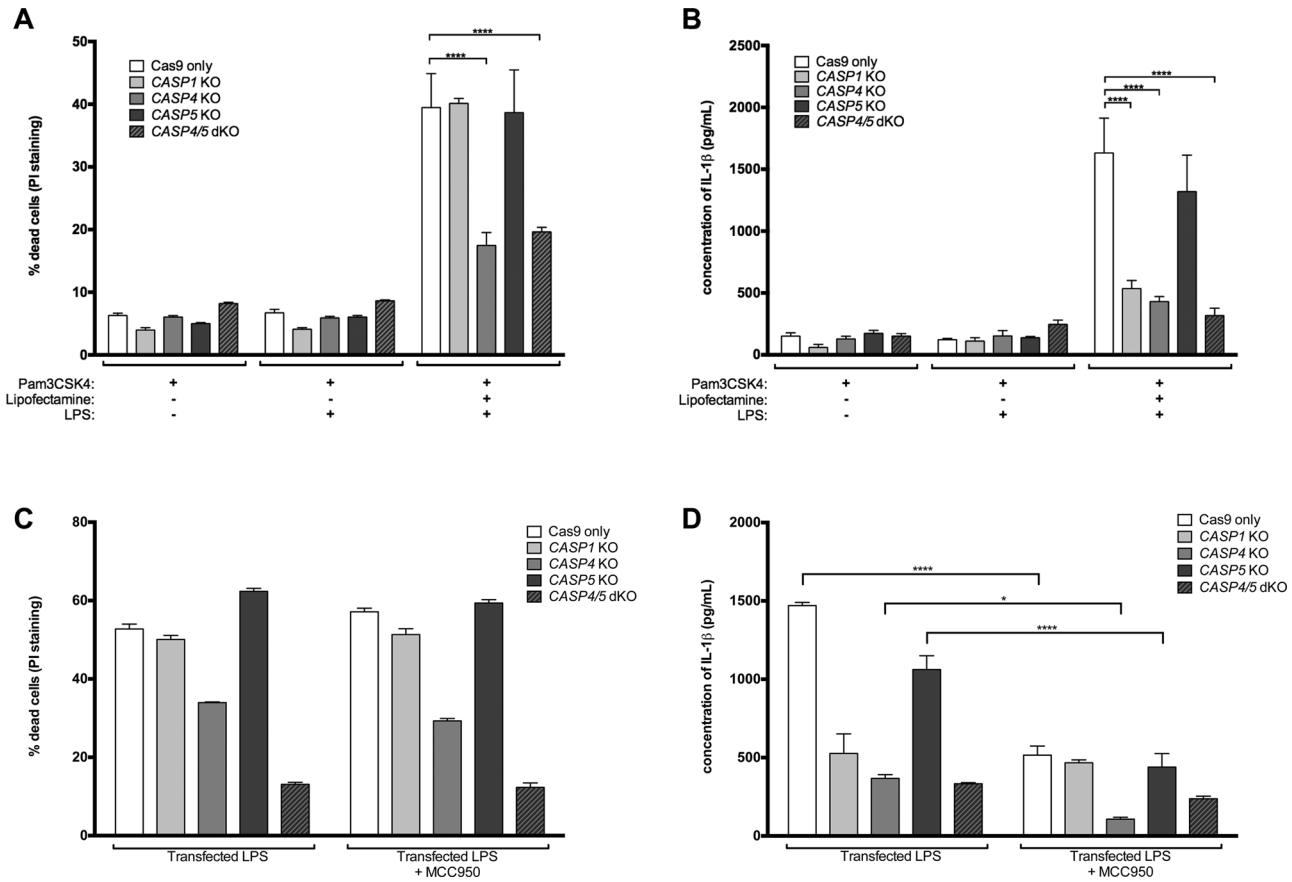

Figure 2. Caspase-4 regulates cell death and IL-1β production in response to transfected LPS. Pools of *CASP-1*, *CASP-4*, *CASP-5*, or *CASP-4/5* KO THP-1 monocytes were primed with Pam3CSK4 and then transfected with LPS. (A) Cell death was measured by PI staining and flow cytometry. (B) IL-1β production was measured by ELISA. (C and D) Cells transfected with LPS were also treated with or without the NLRP3 inhibitor MCC950 and cell death (C) and IL-1β production (D) were measured as in (A) and (B). Data are shown as mean + SEM of $n = 3$ samples/replicates from one experiment representative of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ by two-way ANOVA.

Similar to caspase-11, caspase-4 was sufficient for cytoplasmic LPS-induced pyroptosis in the absence of caspase-1 [1–3].

NLRP3 is required for caspase-4-dependent production of IL-1β

MCC950 is a potent, specific NLRP3 inhibitor that prevents cell death and IL-1β production in response to a range of traditional NLRP3 activators [13]. Pretreatment of THP-1 monocytes for 30 min with this compound resulted in a significant reduction in IL-1β release, but not pyroptosis upon transfection with LPS (Fig. 2C and D). Likewise, preventing NLRP3 activation by pretreating the cells with glyburide or culture media containing high extracellular potassium [14, 15] also blocked IL-1β release but not pyroptosis (Supporting Information Fig. 4A) These inhibitors also suppressed both IL-1β release and pyroptosis in response to nigericin, a robust NLRP3 activator (Supporting Information Fig. 4B). Overall, this suggests that, similar to caspase-11 in murine myeloid cells, caspase-4 activates NLRP3 to induce caspase-1-dependent IL-1β processing and secretion. This adds to an emerging body of literature suggesting specific caspases can activate NLRP3; for example, caspase-8 was recently proposed to induce NLRP3 activity [16, 17].

Caspase-4 and caspase-5 are required for IL-1β release and cell death in response to *Salmonella*

KO THP-1 monocytes were primed with LPS and infected with a mid-log phase culture of *S. typhimurium* at MOI 5 for 20 h. *CASP4* KOs, *CASP5* KOs, and the *CASP4/5* dKOs produced less IL-1β than Cas9-only THP-1 monocytes upon infection with WT (SL1344) *S. typhimurium* (Fig. 3A). Remarkably, double deletion of caspase-4 and caspase-5 conferred the greatest reduction in IL-1β production (Fig. 3A). Caspase-1 deficiency suppressed IL-1β release to a similar extent as the dKO, in agreement with NLRP3 and caspase-1 activation downstream of caspase-4 and caspase-5. Protection from pyroptosis, as measured by viability staining, was also observed in *CASP4* and *CASP5* KO cells (Fig. 3B). The greatest protection from death was also afforded by the *CASP4/5* dKO, suggesting that caspase-4/5-dependent pathways dominate over

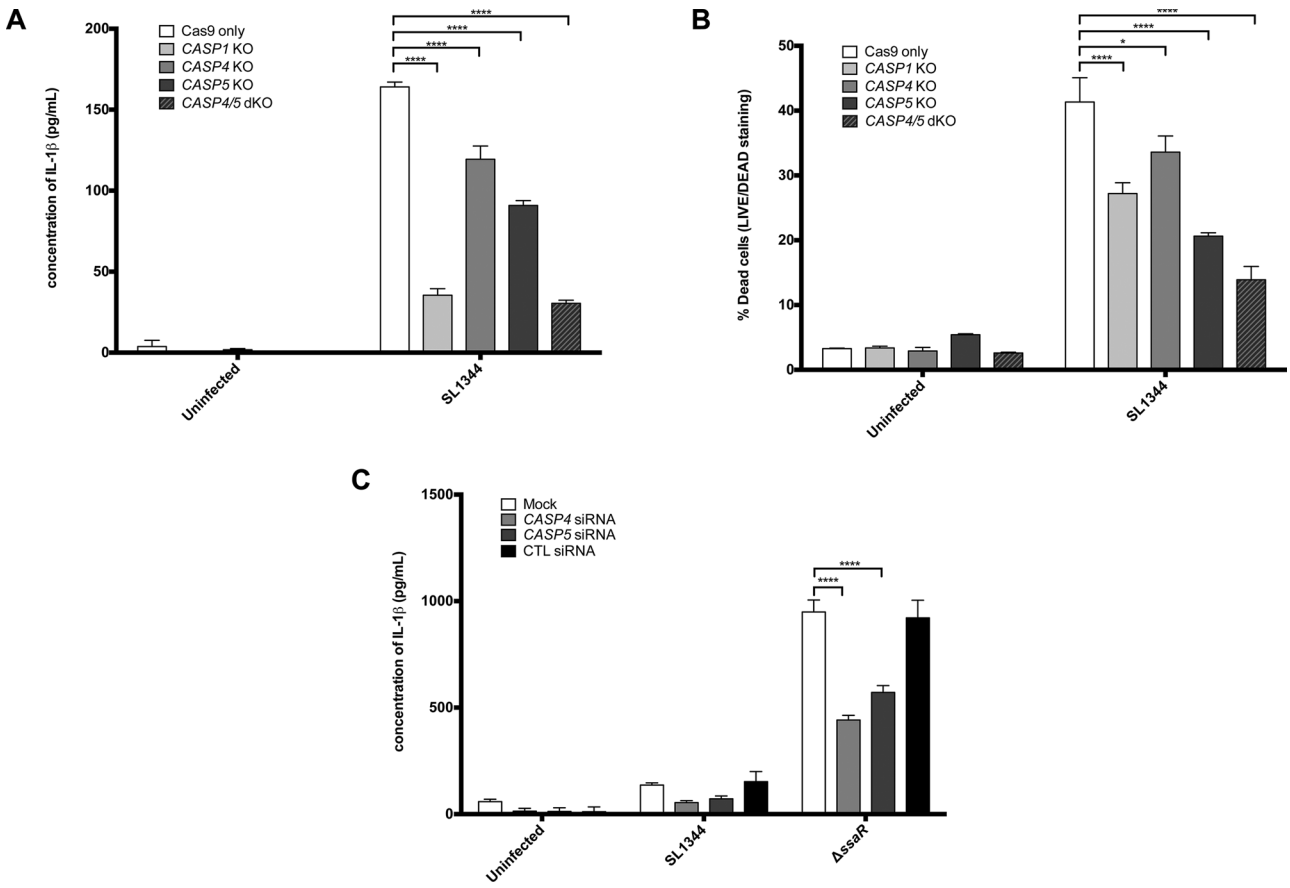

Figure 3. Caspase-4 and caspase-5 regulate cell death and IL-1β production in response to *Salmonella typhimurium*. Pools of CASP-1, CASP-4, CASP-5, or CASP-4/5 KO THP-1 monocytes were primed with LPS, then infected with log-phase WT *Salmonella typhimurium* (SL1344) at MOI 5 for 20 h. (A) IL-1β production was measured by ELISA. (B) Cell death was measured by flow cytometry of cells stained with a fixable viability dye (LIVE/DEAD staining). (C) Primary human macrophages were treated with siRNA specific for either caspase-4 or caspase-5 or a nontargeting control, primed with LPS and then infected with SL1344 or a SPI2 mutant (Δ*ssaR*) at MOI 10. IL-1β production was measured by ELISA. Data are shown as mean + SEM of $n = 3$ samples/replicates from one experiment representative of at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ by two-way ANOVA.

caspase-1-induced pyroptosis (Fig. 3B). Although complementation of caspase-11-deficient murine BMDMs with caspase-5 has been shown to restore sensitivity to cytoplasmic LPS [7], this is the first pattern recognition function to be attributed to caspase-5 in an endogenous human setting.

Caspase-4, caspase-5, and NLRP3 are required for IL-1β production from primary human macrophages

To support our observations from THP-1 cells infected with *S. typhimurium,* human monocyte derived macrophages (HMDMs) from healthy donors were transfected with siRNA complementary to *CASP4* or *CASP5* mRNA or a nontargeting control (Supporting Information Table 1). Cells were then infected with log-phase SL1344 at MOI 10 for 20 h. IL-1β processing in response to WT SL1344 tended to be decreased when caspase-4 or caspase-5 were targeted; however this was not significant (Fig. 3C). Effector proteins secreted by the type III secretion system encoded within *Salmonella* pathogenicity island 2 (SPI2) modify the intramacrophage environment to facilitate bacterial growth and evade the host immune system [18]. *S. tphimurium* deficient in SsaR (Δ*ssaR*), a component of the SPI2 secretion apparatus, show a defect in survival and proliferation in murine macrophages [19]. To investigate the possibility that SPI2 effectors subvert the caspase-4/5 pathway, we also infected HMDMs with an SL1344 Δ*ssaR* mutant. IL-1β release was enhanced in response to Δ*ssaR S. typhimurium* and *CASP4* or *CASP5* silencing significantly reduced IL-1β production. Although preliminary, this suggests that *S. typhimurium* evades caspase-4/5-mediated immune responses through an SPI2-dependent mechanism. One possible explanation is that an altered host response to Δ*ssaR Salmonella* somehow facilitates LPS translocation into the HMDM cytosol, thereby enhancing caspase-4/5 activation. Alternatively, as the SPI2 secretion apparatus is required for inhibition of macrophage NAPDH oxidase dependent bacterial killing [20, 21] and ROS have been implicated in NLRP3 priming and activation [22, 23], suppression of SPI2 activity by deleting SsaR may augment IL-1β release through NLRP3-dependent pathways, such as the caspase-4/5 system.

We have shown that caspase-5 responds to intracellular LPS in the context of live bacterial infection, but not when LPS alone is transfected into the cell. The inducible nature of *CASP5* expression in response to LPS has previously been reported [10, 24], and although this tended to be increased further by *Salmonella* infection it was not significant and therefore is unlikely to account for this effect (Supporting Information Fig. 5). Another explanation may lie in the context in which LPS is detected. Shi et al. [7] showed that caspase-5 can bind transfected LPS, but did not observe activation in an endogenous setting. Bacteria often modify their cell wall structures in order to evade host immunity or withstand environmental stresses. Two-component regulatory systems such as PhoPQ or PmrAB monitor the intramacrophage environment and induce expression of enzymes required for a wide variety of chemical modifications to LPS [25, 26]. These modifications, which are unlikely to be present on commercial LPS used for the transfection experiments, may be required for activation of caspase-5. Alternatively, a physiological caspase-5:LPS interaction may require other Gram-negative cell wall structures such as glycerophospholipids or transmembrane proteins, or other host-cell cofactors that are induced by exposure to bacterial pathogens but not LPS alone.

Concluding remarks

This work demonstrates that caspase-4 is orthologous to murine caspase-11 in human myeloid cells, as it not only induces caspase-1-independent pyroptosis but is also required for IL-1β production in response to cytoplasmic LPS or invasive Gram-negative bacteria. Our inhibitor studies demonstrate that the mechanism behind this IL-1β production is NLRP3 dependent, similar to the caspase-11 pathway. We have also shown a function for caspase-5 in the context of live bacterial infection, presenting for the first time an endogenous role for this protein in a pattern recognition cascade and the inflammatory response, a role for which other members of the same caspase family are renowned. As mice deficient in caspase-11 are protected in a model of i.p. LPS-induced endotoxic shock [1, 2], the functions of its human orthologs are likely to be significant in clinical Gram-negative sepsis. Therefore, these caspases warrant further investigation to determine their potential as therapeutic targets for the treatment of septic infection.

Materials and methods

Tissue culture

THP-1 human monocytes were cultured in RPMI (Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich), 23.8 mM sodium bicarbonate, and 1 mM sodium pyruvate. 293T human embryonic kidney cells were cultured in DMEM (Gibco) supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin, and 40 mM sodium bicarbonate. All cell types were cultured at 37°C and 10% CO_2. Prior to infection or transfection, THP-1 cells were cultured in RPMI without antibiotics supplemented with 10% FBS. Isolation and culture of HMDMs has been previously described [24].

Lentiviral constructs

The parental sgRNA and Cas9 plasmids were described previously [27]. Specific sgRNAs were designed and ligated into the *Bsm*BI restriction site on the inducible sgRNA vector (FgH1t_UTG) as detailed in Supporting Information Table 1. The gene encoding enhanced green fluorescent protein (eGFP) on the FgH1t_UTG vector was modified by site-directed mutagenesis (Y67H; QuikChange Lightning Site-Directed Mutagenesis kit, Agilent) to shift peak fluorescence from ~510 nm (green) to ~450 nm (blue) as has been previously observed [28], thereby creating eGFP_blue (FgH1t_UTG_blue).

Lentivirus production and transduction of THP-1 cells

Lentiviral particles were produced by transient transfection of 293T cells in 10 cm Petri dishes with passenger DNA (10 μg), packaging vectors pMDL (5 μg) and pRSV-rev (2.5 μg), and the envelope vector pVSV-G (3 μg) using Lipofectamine 2000 (Life Technologies). The media was changed to RPMI + 10% FBS at 24 h and virus-containing supernatants were collected at 48–72 h. A total of 5×10^5 THP-1 cells per well in 6-well plates were infected with 2–3 mL viral supernatant supplemented with 8 μg/mL polybrene and centrifuged at 840× *g* for 3 h at 32°C, before incubation at 37°C overnight. This process was repeated on the following day; however, viral supernatant was replaced with fresh media following centrifugation. dKOs were created by infection of *CASP5* (sgRNA 2) KO cells with FgH1t_UTG_blue:*CASP4* 1 lentiviral supernatant that had been concentrated 10× by ultracentrifugation using only one spin infection. Cells positive for mCherry, eGFP, and eGFP_blue were sorted and CRISPR targeting was induced as previously described [27].

Antibodies and Western blotting

Cultured cells were lysed in Radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor (complete protease inhibitor cocktail, Roche) and protein samples were prepared in SDS-reducing sample buffer. Proteins were separated by SDS-PAGE (Novex) and transferred to nitrocellulose membranes. Successful transfer was determined by staining membranes in Ponceau S solution (Sigma-Aldrich) and washing two times with water. Membranes were washed two times in PBS with 0.1% Tween 20 (PBS-T) and blocked in 5% skim milk in PBS-T for 1 h at room temperature before being incubated in primary Ab overnight at 4°C.

Caspase-4 was detected with AdipoGen monoclonal mouse α-caspase-4/11 (Flamy-1) or MBL monoclonal mouse α-caspase-4 (clone 4B9). Human caspase-1 was detected with Santa Cruz polyclonal rabbit α-caspase-1 p10 (sc-515) Actin was detected with Santa Cruz polyclonal goat α-actin (sc-1616). Unsuccessful attempts were made to detect caspase-5 with MBL monoclonal mouse α-caspase-5 (clone 4F7) Abbexa α-caspase-4/5 p20 (abx15558), abcam monoclonal mouse α-caspase-5 (ab10448), and Cell Signaling polyclonal rabbit α-caspase-5 (#4429). Membranes were washed five times in PBS-T, incubated in secondary Ab for 1 h at room temperature, washed another five times, and coated in Millipore Immobilon Western Chemiluminescent HRP Substrate (ECL). Membranes were exposed to Amersham Hyperfilm and developed using a Kodak X-OMAT 3000.

Sequencing of genomic DNA from single-cell clones

A total of 2×10^6 *CASP5* KO 1 clones or pooled Cas-9-only THP-1 monocytes were counted and lysed in 200 μL tail lysis buffer (Viagen Biotech) supplemented with 8 μL proteinase K (Roche) at 55°C with agitation overnight. Two microliters of sample was amplified in a 50 μL PCR using Phusion polymerase and primers that span the CRISPR sgRNA seed sequence (Supporting Information Table 1, allowing enough extension time for at least a 1-kb insertion mutation). Twenty microliters of each sample was run on a 3% agarose gel containing ethidium bromide and clones with alleles that could be differentiated by size were identified, bands were excised and gel extracted using a column-based kit (BioBasic Canada). Purified DNA was sent for Sanger sequencing (AGRF).

Semiquantitative and quantitative PCRs

A total of 2×10^6 THP-1 monocytes were primed with 200 ng/mL ultrapure *E. coli* EH100 (Ra) LPS (Enzo Life Sciences) for 4 h, before lysis and purification of RNA. cDNA was reverse transcribed from 2 μg of RNA and 1 μL was amplified in a 50 μL reaction with Taq polymerase for 20 (h*GAPDH*, *CASP4* primer sets A and B), 30 (*CASP5* primer set A), or 35 cycles (*CASP5* primer set B). Ten microliters of amplified product was loaded onto a 2% agarose gel with ethidium bromide and then imaged (Gel Doc EZ, Bio-Rad).

qPCR was performed as described previously [24]. Briefly, RNA from 5×10^5 HMDM was extracted using a column-based kit (RNAeasy, Qiagen) following treatment with LPS (100 ng/mL for 12 h) or *Salmonella typhimurium* infection (MOI 10 for 8 h, either with or without 4 h of LPS priming at 100 ng/mL).

LPS transfection

A total of 5×10^4 THP-1 cells per well in 96-well plates were primed with 500 ng/mL Pam3CSK4 (Invivogen) for 3 h in optiMEM (Gibco). Inhibitors were added 30 min prior to transfection at the following concentrations: 1 μM MCC950 [13], 50 μM Z-VAD-FMK (R&D Systems), 100 μM glyburide (Invivogen), 10 mM KCl, 10 mM NaCl. Lipofectamine 2000 (Life Technologies) was added to optiMEM at 25 μL/mL and incubated at room temperature for 5 min before being added to an equal volume of optiMEM containing 20 μg/mL of ultrapure *E. coli* EH100 (Ra) LPS. This mixture was incubated at room temperature for 25 min before being added directly to cells at a 1/10 dilution (final concentration 2 μg/mL LPS in 50 μL/well). Transfected cells were incubated at 37°C for 6 h before diluting out the transfection mixture with antibiotic-free RPMI supplemented with 20% FBS to a final volume of 100 μL/well (final concentration 10% FBS). Inhibitors were added as appropriate to maintain the concentrations detailed above and cells were incubated at 37°C, 10% CO_2 for a further 14 h. Cells were treated with 10 μM nigericin (Invivogen) for 1 h as a positive control for NLRP3 activation.

Bacterial culture and infections

Salmonella enterica subsp. *enterica* serovar Typhimurium SL1344 and its isogenic Δ*ssaR* mutant were cultured overnight at 37°C with agitation in Luria broth supplemented with 100 μg/mL streptomycin. Ten milliliter subcultures were prepared and grown to mid-log phase (OD_{600}: 0.6–0.8). 1.5 mL aliquots of log-phase bacteria were prepared by addition of 1 mL 80% glycerol to 10 mL of culture and stored at –80°C. Prior to infection, aliquots were defrosted and washed two times in 1 mL PBS before dilution to the appropriate concentration in antibiotic-free RPMI (the concentration of viable bacteria was determined previously by thawing and plating aliquots from the same batch). It is important to note that these growth conditions result in infection during mid-log phase growth rather than late-log-phase as is used in most studies using *S. typhimurium*. A total of 5×10^4 THP-1 monocytes were plated in 50 μL antibiotic-free RPMI per well in 96-well plates and primed with 200 ng/mL ultrapure LPS for 3–4 h prior to transfection. Bacteria were added to cells at MOI 5 and plates were centrifuged for 5 min (400× g). Infections were allowed to proceed at 37°C + 5% CO_2 for 1 h before addition of gentamycin at a final concentration of 100 μg/mL to kill any extracellular *S. typhimurium*. Infected cells were incubated for a further 20 h. HMDMs were infected similarly; however bacteria were propagated to log phase on the day of infection from an overnight preculture, cells were primed with 100 ng/mL ultrapure LPS for 4 h and infected at MOI 10, and gentamycin was added at 20 μg/mL.

Cell death and cytokine release assays

Supernatants were collected from transfected or infected cells and analyzed for IL-1β production by ELISA (R&D Systems (THP-1) or Affymetrix eBiocience (HMDM)). Transfected THP-1 cells were stained with PI (Sigma-Aldrich) at 1 μg/mL. *Salmonella*-infected THP-1 monocytes were stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain (Molecular Probes) at a 1/10 000 dilution, washed

three times in PBS, fixed with 4% paraformaldehyde, and washed once more in PBS. Stained cells were detected by flow cytometry using a BD LSRFortessa X-20 and data were analyzed using FlowJo X 10.0.7r2.

RNA interference

A total of 7×10^4 HMDM per well were plated in 96-well plates on day 6 of differentiation as previously described [24]. The next day media were replaced with 40 μL of transfection mix containing 500 nM siRNA (Stealth siRNA, Life Technologies) and 0.4% of Lipofectamine LTX, according to the manufacturer's instructions. Twenty hours post transfection, supernatant was replaced with fresh culture media (RPMI 1640, 10% FCS, L-GLU) and the cells were incubated for an additional 24 h prior to use in infection assays.

Statistical analysis

All data were plotted and statistics generated using Microsoft Excel for Mac 2011 and Graphpad Prism 6 for Mac OS X. Data are presented as mean + SEM. Comparisons were performed using two-way ANOVA with a Tukey multiple comparison test.

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Abbreviations: **ASC**: Apoptosis-associated speck-like protein containing a CARD · **BMDM**: BM-derived macrophage · **CARD**: Caspase activation and recruitment domain · **dKO**: double KO · **eGFP**: enhanced GFP · **HMDM**: human monocyte derived macrophage · **NLRP3**: NOD-like receptor family, pyrin domain containing 3 · **PBS-T**: PBS with 0.1% Tween 20 · **sgRNA**: small guide RNA · **SPI2**: *Salmonella* pathogenicity island 2 · **THP-1**: Human peripheral blood monocytic leukaemia cell line

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