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**Salmonella** Infection Induces Recruitment of Caspase-8 to the Inflammasome To Modulate IL-1β Production

Si Ming Man, Panagiotis Tourlomousis, Lee Hopkins, Tom P. Monie, Katherine A. Fitzgerald, Si Ming Man,* Panagiotis Tourlomousis,* Lee Hopkins,* Tom P. Monie,† Katherine A. Fitzgerald,‡ and Clare E. Bryant*

Nucleotide-binding oligomerization domain–like receptors (NLRs) detect pathogens and danger-associated signals within the cell. *Salmonella enterica* serovar Typhimurium, an intracellular pathogen, activates caspase-1 required for the processing of the proinflammatory cytokines, pro–IL-1β and pro–IL-18, and pyroptosis. In this study, we show that *Salmonella* infection induces the formation of an apoptosis-associated specklike protein containing a CARD (ASC)–Caspase-8–Caspase-1 inflammasome in macrophages. Caspase-8 and caspase-1 are recruited to the ASC focus independently of one another. *Salmonella* infection initiates caspase-8 proteolysis in a manner dependent on NLRC4 and ASC, but not NLRP3, caspase-1 or caspase-11. Caspase-8 primarily mediates the synthesis of pro–IL-1β, but is dispensable for *Salmonella*-induced cell death. Overall, our findings highlight that the ASC inflammasome can recruit different members of the caspase family to induce distinct effector functions in response to *Salmonella* infection. *The Journal of Immunology*, 2013, 191: 5239–5246.

Members of the nucleotide-binding oligomerization domain–like receptor (NLR) family detect pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) and initiate the formation of a multimeric complex known as the inflammasome. NAIPs and NLRC4, for example, have been shown to recognize bacterial flagellin and certain type III secretion system–associated rod or needle proteins (1–4), whereas NLRP3 is activated by a large repertoire of PAMPs and DAMPs, including ATP, uric acid crystals, silica, aluminum hydroxide, asbestos, and bacterial or viral RNA (5–10). Formation of the inflammasome facilitates processing of the proinflammatory cytokines pro–IL-1β and pro–IL-18 into their mature forms, which is critical for host defense during microbial infection (11). Conversely, sterile inflammation induced by dysregulated inflammasome activation in response to endogenous DAMPs could lead to autoinflammatory disorders (12).

The inflammasome consists of an NLR, such as NLRC4 or NLRP3, the adaptor protein apoptosis-associated specklike protein containing a CARD (ASC) and caspase-1. *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium) activates caspase-1 via both NLRC4 and NLRP3 (13). Emerging evidence suggests that caspases other than caspase-1 play an important role in inflammasome signaling (14–18). Human caspase-4 or the mouse equivalent, caspase-11, activates caspase-1 in response to a subset of NLRP3 activators (14–18). During infection with Gram-negative bacteria including *S*. Typhimurium, LPS from these microorganisms induces TLR4-TRIF–mediated type I IFN production, which upregulates caspase-11 (16–18). Caspase-11 does not process pro–IL-1β directly (19), but serves as an activator of the NLRP3 inflammasome (16, 18, 20). Caspase-11, however, is not required when NLRP3 is activated independently of Gram-negative bacteria, for example by ATP, nigericin, silica, and Gram-positive bacteria (15, 16). Caspase-11 is also dispensable for NLRC4 inflammasome activity induced by *S*. Typhimurium or *Pseudomonas aeruginosa* (16, 17). Other pathogens including *Candida* and *Mycobacterium* species trigger caspase-8 activation via dectin-1, where caspase-8 processes pro–IL-1β independently of the classical inflammasome (21). Evidence now suggests a central role for caspase-8 in the AIM2 and NLRP3 inflammasomes in driving cell death (22, 23). *Francisella tularensis* activates AIM2 to drive caspase-8–dependent cell death in the absence of caspase-1 (23). The role of caspase-8 during *Salmonella* infection is unclear.

Caspase-8 is an initiator caspase that can activate another initiator caspase, caspase-9, as well as downstream effector caspases, caspase-3 and caspase-7 (24). Caspase-8 is synthesized as a single-chain zymogen, procaspase-8, which consists of two death effector domains and two active domains, p18 and p10 (25). Caspase-8 can be activated by death receptors, CD95 (also known as FAS or Apo1), or TNF receptor 1 (TNFR1), both requiring the adaptor protein FAS-associated death domain protein (FADD) (26, 27). Binding of TNF-α to TNFR1 induces the formation of a RIPK1–FADD–procaspase-8 complex that mediates apoptosis (27). In response to CD95 signaling, the death-inducing signaling complex (DISC) is formed, which results in the recruitment and activation of procaspase-8 by FADD (27). CD95 activation of caspase-8 leads to pro–IL-1β and pro–IL-18 maturation independently of NLRP3 in TLR-stimulated macrophages and dendritic cells (28). A later report, in contrast, suggests that caspase-8 deficiency results in LPS-induced pro–IL-1β maturation in an RIPK3- and RIPK1-dependent manner.
and that caspase-8 has an inhibitory role in the NLRP3 inflammasome when dendritic cells are stimulated with LPS (29).

In this study, we show that Salmonella infection activates a caspase-8–dependent pathway via NLRC4 that induces an ASC-caspase-8–caspase-1 inflammasome complex. Caspase-8 primarily contributes to pro–IL-1β synthesis, but not to cell death driven by NLRC4 and caspase-1. These results highlight a novel effector function of caspase-8 within the inflammasome, which contributes to the host response against Salmonella infection.

**Materials and Methods**

**Mice**

Wild type C57BL/6 mice (Harlan, Loughborough, U.K.), Nlrp3−/− mice, Nltnp3−/− mice, Asc−/− mice, and caspase-1−/− (caspase-1−/−) mice on the C57BL/6 background were housed in a specific pathogen-free facility according to the Animals Scientific Procedures outlined by the U.K. Home Office regulations. Caspase-8−/− ripk3−/−, Caspase-8−/− ripk3−/−, and caspase-8−/− ripk3−/− mice were from D.R. Green (St. Jude Children’s Research Hospital, Memphis, TN).

**Cell stimulation and analysis**

Primary bone marrow–derived macrophages (BMMs) were infected with log-phase S. Typhimurium strain SL1344 using the indicated multiplicities of infection (MOI). For 2-h infections, supernatant was removed after 1 h and replaced with media containing 50 μg/ml gentamicin (Sigma) for 1 h to kill extracellular bacteria. For 6- and 24-h infections after 1 h of incubation in media containing 50 μg/ml gentamicin, supernatant was removed and replaced with media containing 10 μg/ml gentamicin. In experiments that required LPS priming, BMMs were stimulated with 200 ng/ml of ultrapure LPS from *Escherichia coli* (Invivogen) for 3 h. Ultrapure flagellin (60 ng) from *S. Typhimurium* (InvivoGen) was incubated with Prefect-P1 reagent (Targetting Systems) for 20 min to promote complex formation, added to BMMs (in 40 μl volume per well in a 96-well plate) and centrifuged at 11 × g for 10 min. For inhibitor experiments, cells were incubated with 30 or 50 μM Z-ITED-FMK, a caspase-8 inhibitor (21, 30) (Merck) at the same time as bacterial infection or ligand stimulation. Host cell viability was determined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Cytokines secreted into cell culture supernatants were measured using the OptEIA Mouse IL-1β Set (BD Biosciences), the mouse TNF-α DuoSet ELISA kit (R&D Systems) or the mouse IL-18 ELISA kit (MBL International).

**Immunofluorescence staining**

BMMs were washed twice with PBS, fixed in −30°C methanol for 5 min or 4% (w/v) paraformaldehyde for 15 min. Blocking was performed using 10% normal goat serum or rabbit serum (Dako) in 0.1% saponin (Sigma) for 1 h. Cells were stained with primary and secondary Abs for 50 min or overnight and 40 min, respectively. The primary Abs used were rabbit anti-ASC Ab (AL177; Enzo), mouse anti-NLRP3 Ab (ALX-804-881-C100; Enzo), rabbit anti-ASC Ab (AL177; Enzo), goat anti-mouse IL-1β (AF-401-NA; R&D Systems) and mouse anti–β-actin mAb (ab3280; Abcam). The secondary Abs were goat anti-rabbit IgG-HRP, rabbit anti-goat IgG-HRP (sc-2004 and sc-2922, respectively; Santa Cruz Biotechnology) or polyclonal goat anti-mouse IgG-HRP (PA0474, Dako). Blots were developed using American Hyperfilm ECL (GE Healthcare) and Curix 60 Tabletop processor (AGFA Healthcare).

**Real-time PCR**

RNA from BMMs was extracted using TRIzol (Life Technologies) according to the manufacturers’ instructions. Synthesis of cDNA was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Levels of transcripts were quantified using SYBR-Green with an Applied Biosystems 7500 real-time PCR instrument. The primers used were: miL-1βF 5′-GAT CCA CAC TCT GCA GTC 3′; miL-1βR 5′-CACA ACA AGT GAT ATT CCT CAT G 3′; mgAPDH-RF 5′-TTG ATG GTA CCA ACA ATC TCC AC 3′; and mgAPDH-RF-T 5′-CGT CCC OTA GAC AAA ATG GT-3′. Relative expression of pro–IL-1β was calculated using the ΔΔCt standardization method.

**Statistical analysis**

Statistical significance between values from two groups was determined using unpaired Student *t* test, and values between three or more groups was determined using the Kruskal–Wallis one-way ANOVA with all values corrected using a Dunnett’s multiple comparisons test; *p* < 0.05 was considered significant.

**Results**

Salmonella infection of macrophages induces caspase-8 recruitment to the ASC inflammasome

Recent work suggests that inflammasomes, in addition to recruiting caspase-1, can also recruit caspase-11 (15–18). Caspase-8 processes pro–IL-1β in response to dectin-1 stimulation independently of classical inflammasome formation (21). We wondered whether caspase-8 might be recruited to the *Salmonella*-induced inflammasome. Inflammasome activation results in ASC and caspase-1 redistribution in the host cytosol to form a single cytoplasmic focus (speck), which can be visualized using immunolabeling and microscopy techniques (13, 31, 32). We infected unprimed primary bone marrow–derived macrophages (BMMs) with *Salmonella* pathogenicity Island-1 (SPI-1) competent *S. Typhimurium* to activate the NLRC4 inflammasome (1, 3, 33, 34) and used immunolabeling and microscopy techniques to determine whether caspase-8 forms a distinct specklike focus that colocalizes with ASC. We found caspase-8 colocalized with the ASC foci formed in wild type and *caspase-8−/−* ripk3−/− control BMMs infected with *S. Typhimurium*, but not in the ASC foci of *caspase-8−/− * ripk3−/− BMMs (Fig. 1A, 1B). *Caspase-8−/− * ripk3−/− BMMs were used because genetic ablation of caspase-8 in mice results in embryonic lethality, which can be rescued by ablation of RIPK3 (35, 36).

Both caspase-8 and caspase-1 redistributed to the same ASC focus in wild type BMMs stimulated with *S. Typhimurium* (Fig. 1A, 1B). We quantified the percentage of ASC focus containing no caspase, caspase-1, caspase-8 or caspase-1, and caspase-8 in wild type, caspase-8−/− * ripk3−/−, and caspase-8−/− * ripk3−/− BMMs infected *S. Typhimurium*. A large proportion of ASC foci found in wild type and caspase-8−/− * ripk3−/− BMMs contained both caspase-1 and caspase-8 (57% in wild type and 45% in caspase-
Salmonella infection induces recruitment of caspase-8 to the ASC inflammasome. (A) Wild type (Caspase-8+/+ ripk3+/+), Caspase-8+/− ripk3+/−, and Caspase-8−/− ripk3−/− primary BMMs were infected with S. Typhimurium (STM; MOI 10) for 30 min and stained for ASC (magenta), active caspase-1 (green), caspase-8 (red), and DNA (blue). Scale bar, 7.5 μm. (B) Percentage of ASC foci that harbored a caspase-1 focus, caspase-8 focus, or both caspase-1 and caspase-8 foci in (A). (C) Wild type, caspase-1−/− (caspase-11−/−), and Asc−/− BMMs were infected with S. Typhimurium for 30 min and stained for ASC (red), active caspase-8 (green), and DNA (blue). Scale bar, 10 μm. (D) Percentage of ASC foci containing a caspase-8 focus in (C). (E) Wild type BMMs were stimulated with flagellin from S. Typhimurium for 1 h and stained for ASC (magenta), active caspase-1 (green), caspase-8 (red), and DNA (blue). Scale bar, 7.5 μm. Original magnification ×100 for (A), (C), and (E). (F) Percentage of ASC foci that harbored a caspase-1 focus, caspase-8 focus, or both caspase-1 and caspase-8 foci in (E). Cells were fixed and stained with a rabbit anti-ASC Ab and a rat anti-caspase-8 Ab (A, E) or caspase-8 FLICA stain (C). At least 100 (B, F) or 200 (D) ASC-focus–containing BMMs were counted in each independent experiment. Data are from three independent experiments.

8+/− ripk3−/− BMMs), suggesting that the formation of the ASC–caspase-8–caspase-1 inflammasome in these cells is a common event (Fig. 1B). Caspase-1 was still recruited to the ASC focus of caspase-8−/− ripk3−/− BMMs, indicating that caspase-8 deficiency does not impair the recruitment of caspase-1 (Fig. 1A, 1B). Caspase-8 within the ASC focus was active, as shown by staining with FAM-IETD-FMK, a fluorescent compound that binds irreversibly to active caspase-8 (Fig. 1C, 1D). We observed active caspase-8 in ASC foci of caspase-1−/− (caspase-11−/−) BMMs infected with S. Typhimurium, suggesting that caspase-8 is recruited independently of caspase-1 or caspase-11 (Fig. 1C, 1D). Caspase-8 foci failed to form in Asc−/− BMMs (Fig. 1C, 1D).
Previous studies have shown that S. Typhimurium activates caspase-1 via the inflammasome receptor NLRC4 (1, 3, 13, 37). To investigate whether NLRC4 is involved in Salmonella-induced ASC–caspase-8 focus formation, we stimulated caspase-8 \textsuperscript{+/+} ripk3 \textsuperscript{+/+} (wild type) BMMs with ultrapure flagellin from S. Typhimurium to activate NLRC4 and used immunofluorescence staining techniques to visualize the distribution of ASC, caspase-8, and caspase-1. We found that caspase-8 and caspase-1 were recruited to the ASC focus of BMMs stimulated with flagellin (Fig. 1E). Quantification of the prevalence of caspase-8 and caspase-1 in the ASC focus revealed that 38% of the ASC foci were colocalized with both caspases (Fig. 1F). We also infected unprimed wild type or Nlr4 \textsuperscript{−/−} BMMs with S. Typhimurium and found that wild type, but not Nlr4 \textsuperscript{−/−}, BMMs induced ASC focus formation (Supplemental Fig. 1), indicating that Salmonella-induced ASC focus formation is NLRC4 dependent. These results collectively suggest that Salmonella-induced formation of the ASC–Caspase-8–Caspase-1 inflammasome is dependent on NLRC4.

Finally, we used coinmunoprecipitation techniques to confirm that caspase-8 and ASC are part of the same complex. We found that endogenous caspase-8 coinmunoprecipitated with ASC in BMMs infected with S. Typhimurium (MOI 10) after 30 min, but not in uninfected BMMs (Fig. 2A).

In cells primed with LPS or at 6 h after infection, Salmonella also activates the NLRP3 inflammasome (13). We wondered whether caspase 8 would also associate with this second Salmonella-driven inflammasome. Kang et al. (29) have shown previously that caspase-8 inhibits the NLRP3 inflammasome following LPS treatment in dendritic cells. In LPS-primed BMMs, we did not observe the formation of an ASC–caspase-8 focus in the absence of Salmonella infection. When we pulled down caspase-8 in LPS-primed BMMs, we saw caspase-8 associated with NLRP3, but not with ASC (Fig. 2A). Caspase-8 coinmunoprecipitated with both ASC and NLRP3 in LPS-primed BMMs infected with S. Typhimurium (Fig. 2A). When unprimed BMMs were infected with S. Typhimurium, caspase-8 coinmunoprecipitated only with ASC, which could be due to insufficient levels of NLRP3 proteins in unprimed cells (Fig. 2A). These results suggest that caspase-8 has distinct roles depending on the contextual cue received by the cell. In cells stimulated with LPS alone (signal 1, priming), caspase-8 may interact with NLRP3 and inhibit IL-1β production as shown by Kang et al. (29), whereas Salmonella infection (signal 2, priming and inflammasome activation) induces the formation of the ASC inflammasome that contains caspase-8 and caspase-1, where these caspases can potentially undergo proteolysis.

Salmonella infection induces caspase-8 proteolysis via NLRC4 and ASC

To investigate whether Salmonella infection induces caspase-8 proteolysis, we infected BMMs with S. Typhimurium for 30 min and immunoblotted for the presence of the caspase-8 p18 subunits, which are yielded upon proteolysis of procaspase-8 (38). We found that Salmonella infection induced caspase-8 proteolysis in wild type BMMs (Fig. 2B). To investigate whether NLRC4 and NLRP3 (two NLRs involved in the recognition of S. Typhimurium) are involved in Salmonella-induced caspase-8 proteolysis, we compared caspase-8 proteolysis in wild type, Nlr4 \textsuperscript{−/−}, and Nlrp3 \textsuperscript{−/−} BMMs infected with S. Typhimurium for 30 min and only found NLRC4-dependent caspase-8 proteolysis (Fig. 2B). Previous studies have shown that LPS priming is required to induce NLRP3 expression (39), but even in LPS-primed BMMs, we could only observe NLRC4-induced caspase-8 proteolysis in response to Salmonella infection (Fig. 2B).

Asc \textsuperscript{−/−} BMMs stimulated with S. Typhimurium failed to induce caspase-8 proteolysis, indicating that ASC was essential in this process (Fig. 2B). Caspase-1 \textsuperscript{−/−} (caspase-11 \textsuperscript{−/−}) BMMs retained the capacity to undergo caspase-8 proteolysis (Fig. 2B). These results support the observation that recruitment of caspase-8 into a single focus requires ASC but not caspase-1 and caspase-11 (Fig. 1). These results demonstrate that NLRC4 initiates ASC-dependent, caspase-1– and caspase-11–independent proteolysis of caspase-8 in response to Salmonella infection.

Caspase-8 contributes to Salmonella-induced IL-1β production

To investigate the role of caspase-8 in the inflammasome in response to Salmonella infection, we infected caspase-8 \textsuperscript{−/−} ripk3 \textsuperscript{−/−} BMMs with S. Typhimurium and examined IL-1β production, pro–IL-1β processing, caspase-1 proteolysis and cell death. IL-1β production was significantly impaired in caspase-8 \textsuperscript{−/−} ripk3 \textsuperscript{−/−} BMMs compared with caspase-8 \textsuperscript{−/−} ripk3 \textsuperscript{−/−} BMMs or wild type BMMs (caspase-8 \textsuperscript{−/−} ripk3 \textsuperscript{−/+} or caspase-8 \textsuperscript{+/+} ripk3 \textsuperscript{−/+}) infected with S. Typhimurium (Fig. 3A; \(p < 0.001\) for 2, 6, and 24 h). Reduced levels of cleaved IL-1β were also found in the supernatant of caspase-8 \textsuperscript{−/−} ripk3 \textsuperscript{−/−} BMMs compared with the corresponding controls (Fig. 3B). Caspase-8 \textsuperscript{−/−} ripk3 \textsuperscript{−/−} BMMs stimulated with S. Typhimurium maintained their capacity to induce caspase-1 proteolysis, suggesting that caspase-1 proteolysis occurred independently of caspase-8 (Fig. 3B) and confirmed our observation that caspase-8 deficiency did not impair recruitment of caspase-1 into the ASC focus (Fig. 1A and 1B). Conversely,
caspase-8 proteolysis was observed in caspase-11−/− (caspase-11−/−) BMMs (Fig. 3B), supporting our findings that recruitment of caspase-8 into the ASC focus is independent of caspase-1 and caspase-11 (Fig. 1C, 1D). These results indicate that caspase-8 plays a role in modulating IL-1β production during Salmonella infection.

Our data demonstrating pro–IL-1β cleavage also showed a reduced level of total pro–IL-1β in the infected caspase-8−/−/ripk3−/− BMMs, suggesting that pathways leading to the generation of the inflammasome signal 1 may be affected in these cells (Fig. 3B). In agreement, quantitative PCR analysis showed that caspase-8−/−/ripk3−/− BMMs infected with S. Typhimurium produced a substantially lower level of pro–IL-1β transcripts than caspase-8+/+ or caspase-8+/−/ripk3−/− BMMs and infected with S. Typhimurium (MOI 1) for 1, 2, 6, and 24 h and levels of IL-1β were measured. (B) Western blot analysis of the cleaved IL-1β p17 subunit (processed IL-1β), cleaved caspase-1 p10 subunit (active caspase-1), cleaved caspase-8 p18 subunit (active caspase-8), procaspase-1, NLRP3, pro–IL-1β, and β-actin in the supernatant or cell lysate of LPS-primed BMMs. (C) BMMs were infected with S. Typhimurium (MOI 1) for 2, 6, and 24 h and levels of TNF-α were measured. (D) LPS-primed wild type and caspase-1−/− (caspase-11−/−) BMMs were infected with S. Typhimurium in the presence or absence of a caspase-8 inhibitor (30 μM) for 2 or 6 h. Western blot analysis of the cleaved IL-1β p17 subunit (processed IL-1β), pro–IL-1β, and β-actin in the supernatant or cell lysate. Data are representative of two (C, D) or three (A, B) independent experiments and error bars represent SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 3. Salmonella infection drives caspase-8-dependent IL-1β production. (A) Unprimed wild type (Caspase-8+/+ ripk3+/+), caspase-8−/−/ripk3−/−, caspase-8−/−/ripk3−/−, and caspase-8−/−/ripk3−/− primary BMMs were infected with S. Typhimurium (STm; MOI 1) for 1, 2, 6, and 24 h and levels of IL-1β were measured. (B) Western blot analysis of the cleaved IL-1β p17 subunit (processed IL-1β), cleaved caspase-1 p10 subunit (active caspase-1), cleaved caspase-8 p18 subunit (active caspase-8), procaspase-1, NLRP3, pro–IL-1β, and β-actin in the supernatant or cell lysate of LPS-primed BMMs. (C) BMMs were infected with S. Typhimurium (MOI 1) for 2, 6, and 24 h and levels of TNF-α were measured. (D) LPS-primed wild type and caspase-1−/− (caspase-11−/−) BMMs were infected with S. Typhimurium for 2 and 24 h and measured...
IL-18 from the supernatant of these cells. Caspase-8−/−ripk3−/− BMMs did not produce less IL-18 compared with any of the controls after 2 h of infection, which confirms that caspase-8 may not be involved in early processing of IL-18 at this time point (Supplemental Fig. 3). The levels of IL-18 released by caspase-8+/+ripk3+/+ and caspase-8−/−ripk3−/− BMMs were similar; however, both were higher than the levels observed in caspase-8+/−ripk3−/− and caspase-8+/−ripk3+− BMMs (not statically significant; p > 0.05). We were unable to detect any IL-18 from BMMs infected with S. Typhimurium for 24 h (data not shown); therefore, it is unclear whether caspase-8 has a role in delayed processing of IL-18.

Recruitment of pro–IL-1β to the ASC focus is an important event that mediates processing of this cytokine. We have shown that caspase-8 inhibition in wild type BMMs did not prevent recruitment of pro–IL-1β to the inflammasome in response to Salmonella infection (Fig. 4A, 4B). Caspase-1, however, was required for efficient recruitment of pro–IL-1β to the ASC inflammasome, because 52% of the ASC foci in wild type BMMs contained pro–IL-1β compared with only 15% in BMMs deficient in caspase-1 (Fig. 4B). Taken together, these results demonstrate that caspase-1 and caspase-8 orchestrate distinct roles in the inflammasome. Caspase-1 recruits and processes pro–IL-1β, whereas caspase-8 primarily controls the synthesis of pro–IL-1β.

Recruitment of pro–IL-1β to the ASC focus is dependent on caspase-1 and unaffected by inhibition of caspase-8. (A and B) LPS-primed wild type or caspase-1−/− (caspase-11−/−) BMMs were infected with S. Typhimurium for 30 min in the presence of DMSO (vehicle control) or a caspase-8 inhibitor (50 μM) and were immunostained for active caspase-1 (green), ASC (magenta), pro–IL-1β/IL-1β (red), and DNA (blue). Scale bar, 10 μm. Original magnification ×100. (B) Percentage of ASC foci that harbored caspase-1, pro–IL-1β/IL-1β, or both. At least 100 ASC-focus–containing BMMs were counted for each treatment in each independent experiment. Data are representative of three independent experiments, and error bars represent SEM.

Salmonella infection does not induce early cell death via Caspase-8. (A) Wild type (Caspase-8+/−ripk3+/+), Caspase-8+/−ripk3−/−, and Caspase-8−/−ripk3−/− primary BMMs were infected with S. Typhimurium (STM) for 2, 6, and 24 h, and levels of lactate dehydrogenase were measured. (B) LPS-primed wild type (caspase-8+/−ripk3+/+), caspase-8+/−ripk3−/−, caspase-8−/−ripk3−/−, and caspase-8+/−ripk3+/− BMMs were infected with S. Typhimurium (MOI 10) for 30 min or 1 h, and levels of lactate dehydrogenase were measured in the lysate. Data are representative of three independent experiments, and error bars represent SEM.
Salmonella activation of NLRC4, but not NLRP3 results in a rapid, caspase-1–dependent cell death, but this is independent of ASC (Supplemental Fig. 4) (37). The proteolysis of caspase-8 dependent on ASC in response to Salmonella infection suggested that caspase-8 might not, therefore, be involved in the NLRC4–induced cell death pathway. We have shown that the levels of cell death in wild type, caspase-8–/– ripk3–/–, and caspase-8–/– ripk3–/– BMMs were similar after 2, 6, and 24 h of infection with S. Typhimurium, suggesting that caspase-8 had no effect on cell death induced by Salmonella infection (Fig. 5A). LPS priming of BMMs to induce NLRP3 levels prior to Salmonella infection also show a lack of a role for caspase-8 in driving Salmonella-induced cell death (Fig. 5B). These data confirm that caspase-8 does not contribute to Salmonella-induced cell death in the presence of caspase-1.

**Discussion**

Caspase-8 is a multifunctional effector protein that is recruited to different complexes according to the stimulus received by the cell. In this study, we have shown that Salmonella infection induces caspase-8 proteolysis in macrophages infected with S. Typhimurium. Caspase-8 is clearly associated with ASC and caspase-1 within the inflammasome. It is likely that caspase-8 underlies proteolysis in the assembled inflammasome complex, because the lack of ASC prevented caspase-8 proteolysis in response to Salmonella infection. Our observation showing that distinct members of the caspase family (caspase-1 and caspase-8) and pro–IL-1β are colocalized in the ASC structure is interesting and supports the notion that only certain substrates specific for inflammasome processing, such as pro–IL-1β and pro–IL-18, would gain access into the ASC inflammasome. These results highlight that the inflammasome is a dynamic complex with the ability to recruit distinct members of the caspase family. It will be interesting to investigate the spatial orientation of NLR proteins in the ASC–Caspase-8–Caspase-1 inflammasome in future studies to understand whether NLR proteins reside in the same ASC complex.

We and others have shown that caspase-8 has the capacity to induce pro–IL-1β processing in host cells infected with pathogenic bacteria (21). It is possible that the effect of caspase-8–mediated processing of pro–IL-1β is more apparent in the absence of caspase-1 or caspase-11. Pharmacologic inhibition affects the catalytic activity of caspase-8; however, it does not substantially affect caspase-8–mediated processing of pro–IL-1β synthesis. This suggests that caspase-8 itself, rather than its proteolytic activity, has a role in driving NF-kB signaling, possibly acting as a scaffolding protein. Caspase-8 has been shown to assemble a multimeric protein complex in response to dectin-1 activation, which is distinct from the NLRP3–caspase-1 inflammasome (21). It is possible that caspase-8 is preferentially recruited to other structures in a stimulus-dependent manner, such as after dectin-1 activation by Candida and mycobacterial species. The tyrosine kinase Syk is downstream of dectin-1. A previous study has shown that inhibition of Syk does not affect IL-1β production in dendritic cells infected with S. Typhimurium (40), which suggests a lack of a role for Syk in the Salmonella–induced inflammasome. It is, therefore, likely that caspase-8 could be recruited to the ASC inflammasome during Salmonella infection in the absence of a competing complex induced by Dectin-1–Syk activation. Caspase-8 has been shown to assemble the DISC after CD95 activation, indicating that it is entirely possible for this effector protein to be recruited to a different complex in a stimulus-dependent manner (27).

Kang et al. (29) have shown that caspase-8 deficiency in bone marrow dendritic cells results in LPS-induced pro–IL-1β maturation and suggest that caspase-8 has an inhibitory role in the NLRP3 inflammasome. Caspase-8, however, could have distinct roles during LPS stimulation and Salmonella infection. Coimmunoprecipitation of endogenous proteins in macrophages show that caspase-8 interacts with NLRP3 in cells stimulated with LPS alone (signal 1), which could contribute to inhibition of NLRP3-mediated IL-1β production (29). Salmonella infection (signal 2), however, activates NLRC4 and induces the formation of the ASC inflammasome that contains caspase-8 and caspase-1, where these caspases undergo proteolysis. These results suggest that caspase-8 has an effector, rather than an inhibitory, function during microbial infection.

We did not observe IL-1β production in BMMs deficient in caspase-8 when they were primed with LPS alone. Instead, we found reduced pro–IL-1β levels in these BMMs. The differences in findings may be due to differences in cell type and the type of mouse used. We used BMMs from caspase-8–/– ripk3–/– mice, whereas Kang et al. (29) used bone marrow–derived dendritic cells from caspase-8–/–;Itgax-Cre conditional knockout mice. Kang et al. (29) did not observe a decrease in the levels of IL-1β and TNF-α in the serum of LPS-stimulated caspase-8–/–;Itgax-Cre conditional knockout mice. These mice, however, lack caspase-8 specifically in the dendritic cell population, and it is possible that other cell types that still express functional caspase-8, including macrophages, neutrophils, and keratinocytes, release IL-1β and TNF-α in a caspase-8–dependent manner. It is, therefore, difficult to compare results from our study to the study by Kang et al. (29). We have observed that caspase-8 mediates NF-kB signaling following stimulation of TLRs in T and B cells (41, 42). B cells from caspase-8 conditional knockout mice have delayed ability to translocate NF-kB–p65 into the nucleus, and therefore, display defective transcriptional activation of NF-kB genes, including IL-6, TNF-α, and IFN-β (42). Caspase-8 deficiency in human T cells and NK cells reduces NF-kB transcription (41). These results firmly support our observation that caspase-8 deficiency results in reduced pro–IL-1β and TNF-α production. We have shown previously that TLR4 is the major TLR involved in the recognition of S. Typhimurium in macrophages (43, 44). It is, therefore, likely that caspase-8 has a role in mediating TLR4–dependent NF-kB activation. It is also possible that caspase-8 has cell type–specific functions in response to TLR activation.

Although caspase-8 itself is important for pro–IL-1β synthesis, we hypothesize that the process of caspase-8 recruitment to the inflammasome is not critical for pro–IL-1β synthesis. This hypothesis is based on the observations that ASC-deficient BMMs infected with S. Typhimurium retain the capacity to synthesize pro–IL-1β (45) despite their inability to form the ASC and caspase-8 inflammasome. We propose that caspase-8 has distinct roles depending on its localization within the cell. In conclusion, we have shown that Salmonella infection induces a dynamic inflammasome unit that comprises an ASC platform that recruits caspase-8 and caspase-1 for proteolysis.

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**Disclosures**

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References