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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,‡ 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 and 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-chainzymogen, 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 RIPK3 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 NLRC4 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.), Nlrc4−/− mice, Nlrp3−/− 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 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. Ulp5rap flagellin (60 ng) from S. Typhimurium (InvivoGen) was incubated with Percof-P1 reagent (Targeting 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-LETD-FMK, a caspase-8 inhibitor (21, 30) (Merck) at the same time as bacterial infection or ligand plex formation, added to BMMs (in 40 µl 3°C). Caspase-8 was assayed according to the Animals Scientific Procedures outlined by the U.K. Home Office regulations. Host cell viability was determined using the CytoTox 96 Non-Hibitor (21, 30) (Merck) at the same time as bacterial infection or ligand plex formation, added to BMMs (in 40 µl 3°C).

Western blotting

Proteins from cell culture supernatants were precipitated using the methanol-chloroform method as described previously (7). Cell lysates were prepared by adding cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8], 1% Triton X-100, 1 mM PMSE, 10 µg/ml leupeptin, 1 µg/ml aprotinin) to cells and incubated for 10 min on ice. Cell lysates were collected after centrifugation. Samples were separated using 4–20% gradient SDS-PAGE and transferred onto PVDF membranes. PVDF membranes were probed with primary Abs overnight and with secondary Abs for 2 h. The primary Abs used were rabbit anti-mouse caspase-1 p10 (sc-514; Santa Cruz Biotechnology), rabbit anti-mouse cleaved caspase-8 (no. 8592; Cell Signaling Technology), rabbit anti-caspase-8 Ab (IG12, Enzo), mouse anti-Nlrip3 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 (P0447, 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 CCA GCT GCA-3′; miL-1βR 5′-CAA ACC ACA AGT GAT ATT CTC CAT G-3′; and GAPDH-RT-F 5′-TTG ATG GCA ACA ATC TTC AC-3′ and GAPDH-RT-R 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 were 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 death–in stimulation independently of classical inflammasome formation (21). We wondered whether caspase-8 might be recruited to the Salmonella-induced inflammasome. Infammasome 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 8, 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-8−/− ripk3−/− BMMs).
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−/− ripk3+/− (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. 2A). 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−/− BMNs with S. Typhimurium and found that wild type, but not Nlr4−/− BMNs 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 coimmunoprecipitation techniques to confirm that caspase-8 and ASC are part of the same complex. We found that endogenous caspase-8 coimmunoprecipitated with ASC in BMMs infected with S. Typhimurium (MOI 10) after 30 min, but not in uninfected BMNs (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 BMNs, 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 BMNs, we saw caspase-8 associated with NLRP3, but not with ASC (Fig. 2A). Caspase-8 coimmunoprecipitated with both ASC and NLRP3 in LPS-primed BMNs infected with S. Typhimurium (Fig. 2A). When unprimed BMNs were infected with S. Typhimurium, caspase-8 coimmunoprecipitated 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 BMNs with S. Typhimurium for 30 min and immunoblotted for the presence of 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 BMNs (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−/−, and Nlrp3−/− BMNs 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 BMNs, we could only observe NLRC4-induced caspase-8 proteolysis in response to Salmonella infection (Fig. 2B). Asc−/− BMNs stimulated with S. Typhimurium failed to induce caspase-8 proteolysis, indicating that ASC was essential in this process (Fig. 2B). Caspase-1−/− (caspase-11−/−) BMNs 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−/− ripk3−/− BMNs 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−/− ripk3−/− BMNs compared with caspase-8−/− ripk3−/− BMNs or wild type BMNs (caspase-8−/− ripk3+/− or caspase-8+/+ ripk3+/−) 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−/− ripk3−/− BMNs compared with the corresponding controls (Fig. 3B). Caspase-8−/− ripk3−/− BMNs 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,

FIGURE 2. Salmonella infection induces recruitment of caspase-8 into the inflammasome where it undergoes proteolysis. (A) Unprimed or LPS-primed wild type primary BMNs were infected with S. Typhimurium (STM; MOI 10) for 30 min. Endogenous caspase-8 was immunoprecipitated using an anti-caspase-8 Ab. Western blotting was used to detect caspase-8, ASC, and NLRP3. (B) Western blot analysis of caspase-8 proteolysis in wild type, Nlr4−/−, Nlrp3−/−, Asc−/−, and caspase-1−/− (caspase-11−/−) BMNs infected with STM for 30 min. Data are representative of two (B) or three (A) independent experiments. *H chain of the IP Ab.
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 caspase-8−/−/ripk3−/− BMMs infected with S. Typhimurium produced a substantially lower level of pro–IL-1β transcripts than the corresponding controls (Supplemental Fig. 2). The expression of NLRP3 and caspase-1, however, did not appear to be impaired in the absence of caspase-8 (Fig. 3B). We investigated whether TLR signaling was intact in caspase-8−/−/ripk3−/− BMMs infected with S. Typhimurium by measuring TNF-α production. The levels of TNF-α released by caspase-8−/−/ripk3−/− BMMs stimulated with S. Typhimurium were significantly lower than the corresponding controls (Fig. 3C; p = 0.01 for 2 h; p < 0.01 for 6 and 24 h). These results suggest that caspase-8 operates as a checkpoint at the level of pro–IL-1β synthesis to regulate the amount of pro–IL-1β available for caspase-1–dependent processing.

We then investigated whether caspase-8 has a role in processing of pro–IL-1β in BMMs infected for 2 and 6 h. Processing of pro–IL-1β after 2 h of infection with S. Typhimurium was abolished in caspase-1−/− (caspase-11−/−) BMMs, confirming that caspase-1 and -11 were essential for early processing of pro–IL-1β (Fig. 3D). At 6 h after infection, a low level of cleaved IL-1β (p17 subunit) was detected in the supernatant of these cells (Fig. 3D). Inhibition of caspase-8 using Z-IETD-FMK in caspase-1−/− (caspase-11−/−) BMMs almost abolished caspase-1– and caspase-11–independent pro–IL-1β processing, but additional experiments are required to confirm the role of caspase-8 in delayed processing of pro–IL-1β (Fig. 3D). We then investigated whether caspase-8 is involved in the processing of IL-1β, a constitutively expressed inflammasome–dependent cytokine whose expression is not under the influence of TLRs. We infected BMMs from caspase-8−/−/ripk3−−, caspase-8−/−/ripk3−−, caspase-8−/−/ripk3−−, and caspase-8−/−/ripk3−− mice 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.

**FIGURE 4.** 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.

**FIGURE 5.** *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 undergoes 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 pro–IL-1β synthesis. This suggests that caspase-8 itself, rather than its proteolytic activity, has a role in driving NF-κB 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−/−:Igαx-Cre conditional knockout mice. Kang et al. (29) used bone marrow–derived dendritic cells from caspase-8+/−:Igαx-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). Previous studies have shown that caspase-8 mediates NF-κB 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-κB–p65 into the nucleus, and therefore, display defective transcriptional activation of NF-κB genes, including IL-6, TNF-α, and IFN-β (42). Caspase-8 deficiency in human T cells and NK cells reduces NF-κB 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-κB 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
The authors have no financial conflicts of interest.
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