Caspase-8 as an Effector and Regulator of NLRP3 Inflammasome Signaling*

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Background: NLRP3 inflammasomes regulate caspase-1-dependent IL-1β release and pyroptotic death in dendritic cells (DC) and macrophages.

Results: Caspase-8 mediates IL-1β production and apoptosis by NLRP3 inflammasomes in caspase-1-deficient DC and facilitates pyroptosis in wild type DC.

Conclusion: Caspase-8 is activated within NLRP3 inflammasome signaling platforms.

Significance: In addition to caspase-1, NLRP3 inflammasomes engage caspase-8 as an important effector of innate immune signaling responses.

We recently described the induction of noncanonical IL-1β processing via caspase-8 recruited to ripoptosome signaling platforms in myeloid leukocytes. Here, we demonstrate that activated NLRP3:ASC inflammasomes recruit caspase-8 to drive IL-1β processing in murine bone marrow-derived dendritic cells (BMDC) independent of caspase-1 and -11. Sustained stimulation (>2 h) of LPS-primed caspase-1-deficient (Casp1/11−/−) BMDC with the canonical NLRP3 inflammasome agonist nigericin results in release of bioactive IL-1β in conjunction with robust caspase-8 activation. This IL-1β processing and caspase-8 activation do not proceed in Nlrp3−/− or Asc−/− BMDC and are suppressed by pharmacological inhibition of caspase-8, indicating that caspase-8 can act as a direct IL-1β-converting enzyme during NLRP3 inflammasome activation. In contrast to the rapid caspase-1-mediated death of wild type (WT) BMDC via NLRP3-dependent pyroptosis, nigericin-stimulated Casp1/11−/− BMDC exhibit markedly delayed cell death via NLRP3-dependent apoptosis. Biochemical analyses of WT and Casp1/11−/− BMDC indicated that caspase-8 is proteolytically processed within detergent-insoluble ASC-enriched protein complexes prior to extracellular export during nigericin treatment. Although nigericin-stimulated caspase-1 activation and activity are only modestly attenuated in caspase-8-deficient (Casp8−/−Rip3−/−) BMDC, these cells do not exhibit the rapid loss of viability of WT cells. These results support a contribution of caspase-8 to both IL-1β production and regulated death signaling via NLRP3 inflammasomes. In the absence of caspase-1, NLRP3 inflammasomes directly utilize caspase-8 as both a pro-apoptotic initiator and major IL-1β-converting protease. In the presence of caspase-1, caspase-8 acts as a positive modulator of the NLRP3-dependent caspase-1 signaling cascades that drive both IL-1β production and pyroptotic death.

IL-1β is a pro-inflammatory cytokine that requires NFκB-dependent expression of its pro-cytokine form, proteolytic processing into its mature, biologically active form, and non-canonical release mechanisms for initiation of IL-1 receptor-driven inflammatory responses (1, 2). Regulation of IL-1β production in macrophages and dendritic cells has focused on caspase-1, the predominant interleukin-1-converting enzyme (ICE), downstream of the NLRP3 inflammasome signaling complex, the best characterized sentinel for proteolytic maturation of IL-1β (3, 4). In addition to IL-1β processing, active caspase-1 initiates pyroptosis, a pro-inflammatory mode of regulated lytic cell death that is mechanistically distinct from apoptosis (5).

Caspase-8 is best characterized as an initiator caspase involved in death receptor-mediated apoptosis stimulated by FasL, TNFα, or tumor necrosis factor-related apoptosis-inducing ligand and as a suppressor of RIP1/Rip3-dependent necroptosis (6). This latter role for caspase-8 in limiting RIP3-mediated necroptosis underscores the pleiotropic functions of caspase-8 (7). However, recent studies have also implicated...
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cated caspase-8 as either a direct executioner caspase for generating mature 17-kDa IL-1β or an initiator caspase for the activation of caspase-1 in response to infection by diverse microbial pathogens (8–12). Gurung and Kangnategi (13) and Monie and Bryant (14) have recently reviewed the growing literature describing diverse roles for caspase-8 in the regulation of different inflammatory signaling platforms, including inflammasomes and IL-1β production. The assembly of RIP1-RIP3-caspase-8 complexes triggers the activation of caspase-1 and subsequent caspase-1-dependent death of murine macrophages infected with *Yersinia pestis* (15, 16). In the *Salmonella* infection model, an NLRC4-ASC-caspase-8-caspase-1 pathway is engaged whereby active caspase-1 and caspase-8 are both recruited to the inflammasome complex as observed by fluorescence microscopy in murine bone marrow-derived macrophages (BMDM) (17, 18). *Candida* and mycobacterial infections can trigger Syk kinase-dependent caspase-8 activation in macrophages or dendritic cells via dectin-1, an extracellular sensor that detects microbial carbohydrate ligands (19, 20). Thus, findings from several models of microbial infection indicate that caspase-8 is recruited to diverse signaling platforms that regulate the proteolytic maturation of caspase-1 and/or IL-1β. Additionally, we have described a TLR4/TRIF-dependent RIP1-FADD-caspase-8-dependent signaling platform for noncanonical caspase-8-mediated IL-1β processing in BMDM cotreated with LPS and pro-apoptotic chemotherapeutic drugs; the induced IL-1β maturation was correlated with decreased cIAP1 expression and apoptotic DC death (21).

Other reports indicate that caspase-8 contributes to canonical NLRP3 inflammasome signaling (22, 23). Gurung et al. (23) showed that genetic deletion of FADD or caspase-8 markedly attenuated the ability of LPS-primed BMDM to produce mature IL-1β in response to extracellular ATP or nigericin, two canonical NLRP3 agonists. We and others found that the absence of caspase-8 reduces the sustained accumulation of pro-IL-1β and NLRP3 protein in LPS-primed murine macrophages and dendritic cells (21, 22). With respect to cell death signaling, Sagulenko et al. (24) reported pyroptotic cell death in control BMDM versus apoptotic cell death in *Casp1/11−/−* BMDM upon nigericin stimulation of NLRP3 inflammasomes. However, IL-1β processing and release responses were not fully characterized in that study. Overall, these studies indicate multiple types of cross-talk between caspase-1 and caspase-8 in various innate immune responses triggered by NLRP3 inflammasomes.

In this study, we compared canonical NLRP3 inflammasome signaling, IL-1β production, and cell death in wild type (WT) versus *Casp1/11−/−* versus *Casp8/Rip3−/−* BMDM. The experiments uncovered an NLRP3-ASC-caspase-8-dependent IL-1β processing pathway that required prolonged (>2 h) nigericin stimulation in caspase-1-deficient DC in contrast to the very rapid (<0.5 h) engagement of the NLRP3-ASC-caspase-1 pathway for IL-1β maturation in WT cells. The data indicate that caspase-8 acts as an alternative ICE, but also a pro-apoptotic initiator, in response to NLRP3 inflammasome assembly in *Casp1/11−/−* BMDM. However, in the presence of caspase-1, the recruited caspase-8 acts to facilitate caspase-1-mediated pyroptotic cell death. This study emphasizes the ability of either caspase-1 or caspase-8 to mediate the multiple responses to NLRP3 inflammasome signaling platforms that drive IL-1β production as well as cell death to shape innate immune responses at sites of tissue injury or damage.

**Experimental Procedures**

*Reagents—*Reagents and their sources were as follows: *Escherichia coli* LPS serotype O1101:B4 (List Biological Laboratories); Pam3CSK4 (Invivogen); Ac-YVAD-chloromethyl ketone (Bachem); Z-IETD-fmk (R&D Systems); Z-VAD-fmk (APExBio); recombinant murine GM-CSF (PeproTech); nigericin sodium salt (Tocris or APExBio); suberic acid bis(N-hydroxysuccinimide ester) (Sigma); Inject Alum (Thermo Scientific); and alamarBlue® cell viability reagent (Life Technologies, Inc./Invitrogen). Anti-ASC rabbit polyclonal antibody (sc-22514-R) and all HRP-conjugated secondary antibodies (Abs) were from Santa Cruz Biotechnology. Anti-caspase-1 (p20) mouse monoclonal antibody (AG-20B-0042) was from Adipogen. The monoclonal 3ZD anti-IL-1β Ab, which recognizes both 31–/29-kDa pro-IL-1β and 17-kDa mature IL-1β in Western blot analysis, was provided by the Biological Resources Branch, NCI, Frederick Cancer Research and Development Center (Frederick, MD). Other antibodies included anti-caspase-8 mouse monoclonal antibody (ALX-804-448) from Enzo and anti-NLRP3 mouse monoclonal antibody (MAB7578) from R&D Systems. Murine IL-1β DuoSet ELISA kit was from R&D Systems. HEK-Blue-IL-1R™ reporter cells and QUANTI-Blue alkaline phosphatase assay reagent were from Invivogen. Anakinra (recombinant IL-1R antagonist, IL-1ra)/Kineret® was from Amgen. The cytotoxicity detection kit (LDH release) was from Roche Applied Science.

*Murine Models—*Wild type C57BL/6 mice were purchased from Taconic and The Jackson Laboratory. Mice lacking both caspase-1 and caspase-11 on the C57BL/6 background (*Casp1−/−* *Casp11−/−*) have been previously described (25–27). Bones from *Asc−/−* and *Nlrp3−/−* (C57BL/6 background) mice were provided by Eric Pearlman and Amy Hise (Case Western Reserve University). Femoral and tibial bones for BMDC cultures were also isolated from *Casp8−/−* *Rip3−/−* and *Casp8−/−* *Rip3−/−* mice as described previously (7). All experiments and procedures involving mice were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University.

*Isolation, Culture, and Experimental Testing of BMDC—*BMDC from 9- to 12-week-old mice were isolated as described previously (21, 28). For experimental tests, plated BMDC were centrifuged at 300 × g for 5 min, and the differentiation medium was removed and replaced with low serum DMEM (0.1% bovine calf serum plus penicillin, streptomycin, and L-glutamine). BMDC were routinely primed with 100 ng/ml LPS for 4 h to activate TLR4 signaling prior to treatment with 10 µM nigericin (30 min to 6 h) or 240–480 µg/ml alum (6 h). Stimulation of NLRP3/caspase-1 inflammasome signaling with 30 min of nigericin treatment was routinely used as a positive control. In some experiments, the cells were primed with 2 µg/ml Pam3CSK4 to induce TLR2, rather than TLR4, signaling cascades. Where indicated, BMDC cultures were treated with...
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pharmacological inhibitors, such as Z-IETD-fmk, Ac-YVAD-chloromethyl ketone, and Z-VAD-fmk, 3.5–4 h after LPS treatment and 25–30 min prior to nigericin stimulation.

ELISA of IL-1β—BMDC were seeded in 24-well plates. Extracellular media samples were removed and centrifuged at 10,000 × g for 15 s to pellet floating BMDC. The supernatants were then assayed for murine IL-1β by standard ELISA (R&D Systems) according to the manufacturer’s protocol.

**Bioassay of IL-1β—HEK™-Blue-IL-1R reporter cells were used to measure production of biologically active IL-1β by wild type or Casp1−/−/Casp11−/− BMDC stimulated with nigericin.** The reporter cells stably express the murine IL-1 receptor and a secreted alkaline phosphatase (SEAP) reporter gene under the control of a minimal IFN-β promoter fused to NFκB- and AP-1-binding sites. These cells were cultured as described by the vendor in 24-well plates (2–3 × 10^5 cells/500 μl/well) and then stimulated with either 3–5 μl of diluted (1:50) conditioned medium from stimulated BMDC or recombinant murine IL-1β standards. In some wells, the BMDC-conditioned medium aliquots were co-added with 10 μg/ml anakinra (IL-1Ra). After 15–18 h, 20-μl aliquots of conditioned medium from the stimulated HEK™-Blue-IL-1R cells were transferred to 96-well plates containing 180 μl of QUANTI-Blue SEAP assay reagent per well. Time-dependent production of blue SEAP product was measured by absorbance (620 nm) in a BioTek Synergy HT plate reader.

**Preparation of Detergent-soluble and Detergent-insoluble Cell Lysate Fractions, Processing of Extracellular Media, and Western Blot Protocols—BMDC were seeded in 6-well plates and treated as described above prior to processing of the extracellular medium and separation of the detergent-soluble lysate supernatant fraction from the detergent-insoluble lysate pellets. Incubations were terminated by removal of supernatant medium for sedimentation and isolation of detached cells.** Detached cells were centrifuged at 400 × g for 5 min and washed with 1 ml of ice-cold PBS. Whole cell detergent lysates were prepared by addition of 56 μl of RIPA lysis buffer (0.5% sodium deoxycholate, 0.1% SDS, 1% IgePal CA630 in PBS, pH 7.4, plus protease inhibitor mixture) to the adherent cells on the dish and incubated on ice for 5 min. lysed adherent cells were scraped with a rubber policeman, pooled with detached cells, and extracted for an additional 10 min on ice. The whole cell lysates were separated into detergent-soluble and detergent-insoluble fractions by centrifugation at 15,000 × g for 15 min at 4 °C. SDS sample buffer (18 μl) was added to the detergent-soluble fractions, and 56 μl of RIPA lysis buffer (supplemented with 5 mM MgCl₂) was added to the detergent-insoluble lysate pellet. The insoluble lysate pellet was vigorously vortexed and DNase-treated (2 units/sample) by incubation on ice for 10 min prior to addition of SDS sample buffer (12 μl) and extraction at 100 °C for 5 min.

Extracellular medium samples were concentrated by trichloroacetic acid precipitation/aceton washing; detergent-soluble cell lysates were prepared by detergent-based extractions as described previously (25) prior to standard processing by SDS-PAGE, transfer to PVDF membranes, and Western blot analysis. Primary Abs were used at the following concentrations: 5 μg/ml for IL-1β; 1 μg/ml for caspase-1; 1 μg/ml for caspase-8; 2 μg/ml for NLRP3; and 0.4 μg/ml for ASC. HRP-conjugated secondary Abs were used at a final concentration of 0.13 μg/ml. Chemiluminescent images of the developed blots were detected, stored, and quantified using a FluorChemE processor (Cell Biosciences).

**ASC Oligomerization Assay—BMDC were seeded in 6-well plates (2 × 10^5 cells/well). After stimulation, culture supernatants were collected and separated from the pelleted cells.** Cell lysates were prepared using RIPA lysis buffer and centrifuged at 6,000 rpm at 4 °C for 10 min. The sample pellets were washed 1–2 times with PBS and then resuspended to a final concentration of 2 mg DSS in PBS followed by brief vortexing and incubation for 30 min at room temperature. The cross-linked pellets were centrifuged at 6,000 rpm for 10 min followed by a brief 5-s high speed centrifugation to pellet the cross-linked material. The DSS solution was decanted, and the cross-linked pellets were resuspended in 30 μl of SDS sample buffer followed by extraction at 100 °C for 5 min. A 12% SDS-polyacrylamide gel was used to run the samples, and Western blots were performed and probed for ASC to determine ASC oligomer formation for monomeric (24 kDa), dimeric (48 kDa), and oligomeric (>48 kDa) ASC.

**Assay of Cell Death by LDH Release—Control or LPS-primed BMDC in 24-well plates (5 × 10^5 cells/0.5 ml BSS/well) were stimulated for 30–300 min in the absence of presence of 10 μM nigericin at 37 °C. Extracellular medium was removed from each well and centrifuged at 10,000 × g for 15 s to pellet detached cells.** The cell-free supernatants were then assayed for LDH enzyme activity using the cytotoxicity detection kit according to the protocol of Roche Applied Science. The released LDH was normalized to total LDH content measured in 1% Triton X-100-permeabilized samples of BMDC.

**Cell Viability—BMDC were seeded in 96-well plates (1 × 10^5 cells/well). Viability of BMDC was assayed using the metabolic redox sensor dye, alamarBlue® dye reagent to monitor the time-dependent conversion of resazurin to resorufin at the excitation wavelength of 540 nm and emission wavelength of 620 nm. Experiments were timed such that all stimulation ended simultaneously, and the alamarBlue® dye reagent (10 μl/well) was added thereafter and allowed to incubate for 1 h at 37 °C. Fluorescence was quantified in relative light units using a BioTek Synergy HT plate reader and normalized to the values measured in control, untreated BMDC.

**Propidium Influx Assay—BMDC were seeded in 24-well plates (5 × 10^5 cells/well). After LPS priming, the culture medium was removed from each well, washed once with PBS, and replaced with 0.5 ml/well of a balanced salt solution (130 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, 25 mM HEPES, 0.1% bovine serum albumin and 5 mM glucose, pH 7.4) containing 1 μg/ml propidium iodide. The plate was placed into a Synergy HT plate reader (BioTek) preheated to 37 °C. Baseline fluorescence (540 nm excitation → 620 nm emission at 1-min intervals) was recorded for 10 min. Cells were then stimulated with 10 μM nigericin for another 4 h, and the changes in fluorescence were recorded at 1-min intervals. Assays were terminated by permeabilization of the BMDC with 1% Triton X-100 to quantify maximum fluorescence of the propidium²⁺-DNA complexes. An increase in propidium...
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fluorescence correlates with pyroptosis driven by the NLRP3 inflammasome and caspase-1 activation. Propidium binding to DNA occurs when there is either gating of a propidium-permeable plasma membrane channel/pore or frank cell lysis that releases cellular DNA. The time-dependent increases in fluorescence induced by nigericin were normalized to the maximum fluorescence measured in Triton X-100-permeabilized cells after subtraction of basal intrinsic fluorescence.

Data Processing and Analysis—All experiments were repeated 2–16 times with separate BMDC preparations. Figures illustrating Western blot results are from representative experiments. As indicated, figures illustrating IL-1 ELISA or cell viability results represent either the mean (±S.E.) of three or more identical experiments or the means (±S.E.) where each condition was performed in triplicate, quadruplicate, or more within a single experiment. Experiments with three or more repeats were analyzed by one-way ANOVA with Bonferroni post test. For some experimental series, the IL-1 release measured in different conditions or genotypes was normalized to the maximal release for each BMDC preparation, and the normalized values from several identical experiments were used to generate means (±S.E.) for evaluation by ANOVA.

Results

Sustained Nigericin Stimulation Induces Delayed Processing and Release of Mature, Bioactive IL-1β in LPS-primed Casp1/11−/− Murine BMDC—Consistent with previous reports (29–31), nigericin-induced caspase-1 activation and IL-1β release were maximal within 30 min in WT BMDC, and this rapid response to nigericin was completely suppressed in Casp1/11−/− BMDC (Fig. 1, A, B, and F). However, a delayed phase of IL-1β processing and release was observed during sustained (>2 h) stimulation of these knock-out cells with nigericin. This slower accumulation of mature IL-1β by Casp1/11−/− BMDC reached a plateau within 4–6 h, and the peak magnitude was ~33% that observed in WT cells; the absolute amount of caspase-1-independent IL-1β secretion from 5 × 10⁵ BMDC was ~20 ng/ml/6 h as measured by ELISA (Fig. 1A). Western blot analysis of the intracellular compartments and extracellular compartments from Casp1/11−/− BMDC indicated that the slower accumulation of IL-1β induced by ELISA was temporally correlated with decreases in intracellular 31-kDa pro-IL-1β and increases in extracellular 17-kDa IL-1β (Fig. 1, B and F). We also observed intracellular accumulation of an ~29-kDa cleavage product of pro-IL-1β during acute stimulation with nigericin (0.5–1 h) in Casp1/11−/− BMDC (Fig. 1, B and F). At later (>1 h) time points, the 29-kDa IL-1β cleavage product was co-released with the 17-kDa product. Little accumulation or release of this 29-kDa IL-1β product was observed in WT BMDC.

The ability of IL-1β to activate IL-1R signaling is critically dependent on proteolytic maturation of pro-IL-1β to the 17-kDa IL-1β product (1, 2). Western blot analysis verified that nigericin stimulated the production and release of a 17-kDa IL-1β protein in Casp1/11−/− BMDC. However, the observed intermediate accumulation of the 29-kDa product in these cells suggested possible engagement of a proteolytic cascade that generates an alternatively cleaved 17-kDa IL-1β product that lacks significant biological activity. We used HEK-Blue-IL-1RTM reporter cells to measure production of biologically active IL-1β by WT or Casp1/11−/− BMDC stimulated with nigericin. These cells stably express the murine receptor for IL-1 and a reporter gene encoding SEAP under the control of NFκB and AP-1 regulatory elements. Recombinant murine IL-1β triggered concentration-dependent (EC₅₀ = 4 pg/ml) increases in SEAP expression that were completely suppressed in the presence of anakinra/IL-1Ra (Fig. 1C). Treatment of the reporter cells with conditioned medium from cultures of WT BMDC stimulated with nigericin for 30 min or 6 h also induced robust and IL1Ra-inhibitable SEAP production. Importantly, IL1Ra-sensitive SEAP expression was also elicited by conditioned medium from Casp1/11−/− BMDC stimulated with nigericin for 6 h but not 30 min. Quantification of the reporter cell assays indicated that nigericin stimulated the WT BMDC to secrete ~5 ng/ml bioactive IL-1 within 30 min with peak production of ~15 ng/ml by 2 h (Fig. 1D). Consistent with the ELISA measurements, the reporter cell assay indicated a delayed production of bioactive IL-1 by the Casp1/11−/− cells with a plateau level (5 ng/ml) at 33% that observed in WT cells. The modestly different time courses for the IL-1β release indicated by ELISA (Fig. 1A) versus the reporter cells (Fig. 1D) may reflect the additional release of IL-1α that will be detected by the latter but not the former assay method (2).

ASC-based inflammasomes form large protein aggregates that sediment following low speed centrifugation of detergent-solubilized cell extracts, particularly when stabilized by crosslinking to detect ASC oligomerization (32). Nigericin induced a similarly rapid rate and extent of ASC oligomerization in WT or Casp1/11−/− BMDC (Fig. 1E), consistent with previous studies (32).

Notably, the delayed export of mature IL-1β from Casp1/11−/− BMDC during sustained nigericin treatment temporally correlated with enhanced proteolytic processing of 55-kDa procaspase-8 and release of the mature 18-kDa caspase-8 subunit (Fig. 1F). In contrast, WT BMDC produced significantly lower amounts of the mature caspase-8 subunit. This temporal correlation in Casp1/11−/− cells between production of active caspase-8 and release of processed IL-1β is consistent with the seminal observations of Maelfait et al. (8) who demonstrated that recombinant active caspase-8 cleaves recombinant pro-IL-1β at the canonical caspase-1 cleavage site (Cys-116↓Asp-117) for generation of 17-kDa mature IL-1β. There is also a potential cleavage site in murine pro-IL-1β at Ser-16↓Asp-17, which may be targeted by caspase-8 to generate the 29-kDa fragment prior to cleavage at the canonical Cys-116/Asp-117 locus. Taken together, the data indicate that nigericin stimulation of Casp1/11−/− BMDC induces a delayed release of biologically active IL-1β that correlates with robust activation of caspase-8.

NLRP3 Inflammasomes Mediate the Delayed IL-1β Release and Caspase-8 Activation Responses to Sustained Nigericin Stimulation of Casp1/11−/− BMDC—Fig. 1 indicated robust activation of caspase-8 during sustained nigericin stimulation of the Casp1/11−/− BMDC and equivalent ASC oligomerization in the two DC genotypes. Thus, we hypothesized that procaspase-8 would be recruited into the NLRP3-ASC inflam-
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Nigerin-induced IL-1β processing and release at all time points (0.5, 2, 4, and 6 h) was completely suppressed in Nlrp3−/− and Asc−/− BMDC as indicated by ELISA quantification (Fig. 2A). This was confirmed by Western blot analysis comparing WT, Nlrp3−/− (Fig. 2C), and Asc−/− (data not shown) BMDC. Notably, caspase-8 activation was also suppressed at all time points during nigerin stimulation of Nlrp3−/− (Fig. 2C) and Asc−/− (data not shown) BMDC, suggesting that NLRP3 and ASC are both essential for association of caspase-8 with the inflammasome platform. The gradual
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Thus, as with sustained nigericin stimulation (Fig. 1A), we also observed a significant release of IL-1β in WT BMDC with Nlrp3−/− BMDC stimulated with LPS alone or LPS plus a 30-min and 2-, 4-, or 6-h nigericin stimulus. C, WT and Nlrp3−/− BMDC were primed with LPS in low serum-containing medium (0.1% calf serum) followed by stimulation with nigericin either in a NaCl-based buffered saline solution or in a high KCl-containing (130 mM KCl) buffered saline solution, and the amount of IL-1β released was quantified by ELISA. Results are from a single experiment with each condition performed in triplicate.

Figure 2. NLRP3 inflammasomes mediate the delayed IL-1β release and caspase-8 activation responses to sustained nigericin stimulation of Casp1/11−/− BMDC. A, WT, Asc−/−, and Nlrp3−/− BMDC were primed with LPS (100 ng/ml) for 4 h before stimulation with nigericin (Nig) for 30 min and 2, 4, or 6 h. The extracellular medium was collected and assayed for IL-1β by ELISA. BMDC were primed with LPS for 5.5 h prior to nigericin stimulation for 30 min. Results are the mean ± S.E. of three experiments. B, detergent-insoluble lysate fractions were cross-linked with DSS, and ASC oligomerization was assayed by Western blot analysis in WT and Nlrp3−/− BMDC stimulated with LPS alone or LPS plus a 30-min and 2-, 4-, or 6-h nigericin stimulus. C, WT and Nlrp3−/− BMDC were primed and stimulated as in A, and the extracellular medium and cell lysates were collected and processed for Western blot analysis of IL-1β, caspase-1, and caspase-8. The data are representative of results from three separate experiments. D, BMDC were primed with LPS in low serum-containing medium (0.1% calf serum) followed by stimulation with nigericin either in a NaCl-based buffered saline solution or in a high KCl-containing (130 mM KCl) buffered saline solution, and the amount of IL-1β released was quantified by ELISA. Results are from a single experiment with each condition performed in triplicate.

Decrease in pro-IL-1β levels in Nlrp3−/− cells likely reflects the pro-IL-1β ubiquitination and proteosomal clearance pathway recently described by Ainscough et al. (33). We also verified the absence of ASC oligomerization in response to both acute and sustained nigericin stimulation in Nlrp3−/− (Fig. 2B) and Asc−/− (data not shown) BMDC.

Because decreased cytosolic [K+] is a well characterized signal for NLRP3 inflammasome assembly, we assayed the caspase-1-independent IL-1β production response to nigericin in a test medium containing increased extracellular K+ (130 mM KCl versus the physiological 5 mM KCl) to disrupt the normal gradient for K+ efflux. The ability of high K+ medium to completely suppress IL-1β release in both WT and Casp1/11−/− BMDC during acute or sustained nigericin stimulation supported the involvement of a common NLRP3 inflammasome platform in both cell types (Fig. 2D). We also observed significant release of IL-1β when Casp1/11−/− BMDC were treated for 6 h with 240–480 ng/ml α-lum, a well characterized particulate stimulus for NLRP3 inflammasome activation (10 ± 2 ng of IL-1β/ml/6 h in Casp1/11−/− BMDC versus 28 ± 6 ng of IL-1β/ml/6 h in WT BMDC; n = 4 experiments, data not shown). Thus, as with sustained nigericin stimulation (Fig. 1A), the magnitude of the lum-induced IL-1β release from Casp1/11−/− BMDC was ~33% that observed in WT cells. These data indicate that caspase-1-independent IL-1β processing can be triggered by multiple signal 2 stimuli that activate NLRP3 inflammasomes.

Nigericin-induced Assembly of NLRP3 Inflammasomes Mediates Rapid Pyroptosis in WT BMDC but Delayed Apoptosis in Casp1/11−/− BMDC—In addition to maturation and release of IL-1β, NLRP3-dependent activation of caspase-1 triggers rapid pyroptotic death of DC and macrophages. We compared the time courses of LDH release as an indicator of nigericin-regulated cell death responses in WT, Casp1/11−/−, and Nlrp3−/− BMDC in the absence or presence of LPS priming. In LPS-primed WT cells, nigericin induced a rapidly developing LDH release with a plateau response of ~75% cell death within 2 h (Fig. 3A). Nigericin-induced BMDC death was critically dependent on both LPS priming and NLRP3 expression with only minor LDH release from nonprimed WT cells or LPS-primed Nlrp3−/− cells incubated with the ionophore for up to 6 h. Notably, the kinetic profile of nigericin-stimulated LDH release from LPS-primed Casp1/11−/− cells was intermediate between the profiles observed in WT and Nlrp3−/− cells; no release was observed during the initial 30 min but progressively increased over the next 5.5 h to reach the same plateau level (75% cell...
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A. WT, Casp1/11−/−, and Nlrp3−/− BMDC were LPS-primed (or left unprimed “w/o LPS” as indicated) for 4 h and then stimulated with nigericin (Nig) (10 μM). The indicated times, extracellular medium was collected and assayed for lactate dehydrogenase (LDH) activity. Parallel samples of unstimulated cells were permeabilized with Triton X-100 to induce maximal LDH release for normalization of the LDH released from nigericin-stimulated cells (% of Max). Data points represent the mean ± S.E. of 6–12 replicates from four (WT) or two (Casp1/11−/−) identical experiments. B. WT and Casp1/11−/− BMDC were primed and stimulated as in Fig. 1F, and the extracellular media and cell lysates were collected and processed for Western blot analysis of caspase-1, caspase-8, and caspase-3; this same membrane was used for the Western blot analysis shown in Fig. 1F. C. WT, Casp1/11−/−, and Nlrp3−/− BMDC were LPS primed for 4 h and then stimulated with or without nigericin (10 μM) for 240 min (arrow indicates the addition of nigericin), and the accumulation of fluorescent propidium2+ DNA complexes was quantified every 5 min. Propidium fluorescence is expressed as a percentage of maximal dye accumulation after Triton X-100 permeabilization. Data points represent the mean ± S.E. of 2–8 replicates from four (WT) or two (Casp1/11−/− and Nlrp3−/−) identical experiments. D, viability of WT, Asc−/−, and Nlrp3−/− BMDC stimulated with LPS (4 h) or LPS (4 h) followed by a 3–4-h nigericin stimulus was assayed using the redox potential indicator dye, alamarBlue®. Viability is expressed as a percentage relative to untreated BMDC. Results are the mean ± S.E. of seven independent wells per condition from two experiments. *** p < 0.001 by ANOVA. E and F, viability of WT and Casp1/11−/− BMDC stimulated as described in B was measured using the redox potential indicator dye, alamarBlue®. Results are the mean ± S.E. of 2–3 experiments.

FIGURE 3. Nigericin-induced assembly of NLRP3 inflammasomes mediates rapid pyroptosis in WT BMDC but delayed apoptosis in Casp1/11−/− BMDC. From 34.6 ± 8.1% (n = 3) of WT cells in the absence of IL-1Ra versus 57.1 ± 9.9% of WT cells in its presence. Treatment of the caspase-1-deficient cells with nigericin for 30 min induced 7.4 ± 2% versus 11.8 ± 1.2% LDH release in the respective absence or presence of IL-1Ra. Thus, autocrine signaling by death) as in WT cells (Fig. 3A). Other experiments tested whether the more rapid LDH release observed in WT versus Casp1/11−/− BMDC might reflect autocrine actions of the rapidly secreted IL-1β by inclusion of IL-1Ra during nigericin stimulation. A 30-min nigericin stimulus induced LDH release
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Sustained Nigericin Stimulation Induces Caspase-1-independent Release of Mature IL-1β in DC Primed by TRIF-independent TLR2 Signaling—The preceding experiments suggest that caspase-8 can efficiently catalyze IL-1β processing downstream of NLRP/ASC inflammasome platforms in caspase-1-deficient BMDC primed with LPS as a signal 1 stimulus. LPS binding to TLR4 engages both the MyD88 and TRIF adapter proteins to activate distinct but overlapping arrays of pro-inflammatory responses. TRIF contains an RHIM (RIP homotypic interaction motif) domain that facilitates direct binding to the RHIMs of RIP1 and RIP3. Duong et al. (35) recently reported that the A20 ubiquitin editing enzyme supports assembly of a RIP3-dependent complex of Lys-63-ubiquitinated pro-IL-1β and unanchored polyubiquitin that restricts access to caspase-1 and caspase-8 within NLRP3 inflammasomes. These IL-1β-based ubiquitin complexes were induced in BMDM primed with pathogen-associated molecular patterns (such as LPS) that engage TRIF signaling but not with pathogen-associated molecular patterns (such as TLR2 agonists) that signal only via MyD88. We tested whether nigericin can also stimulate caspase-8-mediated IL-1β processing in Casp1/11−/− BMDC primed with the TLR2 lipopeptide, Pam3CysK4 or for 4 h. Wild type and Casp1/11−/− BMDC accumulated similar levels of intracellular pro-IL-1β in response to either LPS or Pam3CysK4 (Fig. 4B). However, the extent of IL-1β processing and release during either acute (30 min) or sustained (6 h) nigericin treatment was markedly lower in the TLR2-primed WT cells relative to the TLR4-primed WT DC (Fig. 4, A and B). As with LPS treatment, Pam3CysK4 primed Casp1/11−/− cells did not release mature IL-1β during a 30-min exposure to nigericin (Fig. 4A). In contrast, sustained nigericin stimulation of Casp1/11−/− BMDC resulted in significant (~2 ng/ml/6 h) accumulation of extracellular IL-1β (Fig. 4A). This magnitude of the nigericin-induced IL-1β release from TRL2-primed Casp1/11−/− BMDC was ~33% of that measured in the TLR2-primed WT cells.

IL-1β Release during Sustained Nigericin Stimulation Is Similarly Reduced by Caspase-1 or Caspase-8 Ablation and Pharmacological Inhibition of Either Caspase—We assessed the relative contributions of caspase-1 versus caspase-8 during sustained (6 h) nigericin stimulation by comparing IL-1β release in LPS-primed WT, Casp1/11−/−, and Casp8−/−/Rip3−/− BMDC in the absence or presence of pharmacological inhibitors assumed to selectively target caspase-1 (YVAD-fmk) or caspase-8 (IETD-fmk). Caspase-8-knock-out mice are embryonic lethal due to unrestrained necroptosis and require co-deletion of RIP3 to rescue viability (7, 36). In Fig. 5, A and B, the absolute magnitudes of IL-1β release in the three DC genotypes are normalized to the maximal response in WT cells in the absence of YVAD or IETD. In Fig. 5, C and D, the same data are plotted with each genotype’s response normalized to the maximal response for that genotype in the absence of YVAD or IETD. This additional mode of analysis is important because we (21) and others (13, 17, 20, 22, 23) have reported that Casp8−/−/Rip3−/− BMDC and BMDM are characterized by decreased accumulation of intracellular pro-IL-1β. Thus, reduced accumulation of extracellular IL-1β by nigericin-
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stimulated Casp8<sup>−/−</sup>Rip3<sup>−/−</sup> BMDC can reflect both attenuation of NLRP3-ASC-caspase-1 inflammasome assembly and lower levels of the pro-IL-1β substrate for active caspase-1. The genetic absence of either caspase-1 or caspase-8 produced similar reductions (60–70%) in the absolute amount of IL-1β released in response to sustained (6 h) nigericin stimulation (Fig. 5A), indicating significant contributions of both caspases to the overall IL-1β production process. Notably, the potency of YVAD was markedly greater in WT and Casp8<sup>−/−</sup>Rip3<sup>−/−</sup> BMDC (IC<sub>50</sub> < 1 μM) than in Casp1/11<sup>−/−</sup> BMDC (IC<sub>50</sub> ~ 10 μM) (Fig. 5A and C). The ability of higher YVAD concentrations to further suppress IL-1β release in the Casp1/11<sup>−/−</sup> cells indicated cross-inhibition of caspase-8 activity with increasing [YVAD] (Fig. 5C). In contrast to the differential potency of YVAD in WT and Casp8<sup>−/−</sup>Rip3<sup>−/−</sup> cells versus Casp1/11<sup>−/−</sup> cells, the potency and efficacy of IETD in suppressing nigericin-stimulated IL-1β release (IC<sub>50</sub> ~ 5 μM) were similar in all three genotypes of BMDC (Fig. 5, B and D).

As with the inhibitory actions of YVAD in caspase-1-null DC, the ability of IETD to further suppress IL-1β release in the caspase-8-null DC indicated cross-inhibition of caspase-1 activity with increasing [IETD] (Fig. 5D). However, IETD was modestly more potent and efficacious than YVAD in inhibiting the IL-1β release response in the Casp1/11<sup>−/−</sup> BMDC (compare Fig. 5, C and D).

The Fig. 5 concentration-response data with Casp8<sup>−/−</sup>Rip3<sup>−/−</sup> and Casp1/11<sup>−/−</sup> BMDC allowed us to select concentrations of YVAD (10 μM) and IETD (20 μM) that, respectively, produced at least 80% suppression of the IL-1β release regulated by caspase-1 (in the absence of caspase-8) or by caspase-8 (in the absence of caspase-1). We used YVAD and IETD at these concentrations to further characterize the roles of caspase-8 in regulating nigericin-stimulated NLRP3 inflammasome signaling in the presence or absence of caspase-1. Multiple indices of NLRP3 inflammasome activity stimulated by acute (30 min) or sustained (6 h) nigericin treatment in WT BMDC were markedly and similarly attenuated in the presence of 20 μM IETD or 10 μM YVAD; these included release of total IL-1β (by ELISA, Fig. 6, A and B), release of 17-kDa mature IL-1β (by Western blot, Fig. 6D), and extracellular accumulation of caspase-1 p20 subunit (Fig. 6D). The ability of 20 μM IETD to attenuate the rapidly induced phase of NLRP3-dependent IL-1β processing and release in WT cells was not due to suppression of proximal ASC oligomer formation (Fig. 5C). Indeed, similarly rapid accumulation of ASC oligomers in response to nigericin was observed in WT and Casp1/11<sup>−/−</sup> BMDC in the absence or presence of 20 μM IETD, 10 μM YVAD, or 50 μM Z-VAD fmk caspase inhibitor. This indicates that treatment of WT BMDC with IETD attenuates release of mature IL-1β at the level of caspase-1 activation/activity rather than NLRP3 inflammasome assembly. Notably, treatment of Casp1/11<sup>−/−</sup> BMDC with 20 μM IETD (Fig. 6, A and D), but not 10 μM YVAD (Fig. 6, B and D), produced complete suppression of nigericin-stimulated IL-1β release as measured by ELISA or Western blot. IETD, but not YVAD, also suppressed the early intracellular generation of the 29-kDa IL-1β cleavage product in nigericin-stimulated Casp1/11<sup>−/−</sup> cells (Fig. 6D). Intracellular accumulation of mature p17 IL-1β was observed at 1 h post-nigericin stimulation in Casp1/11<sup>−/−</sup> BMDC, but release of p17 IL-1β to the extracellular compartment required an additional 60 min of incubation (see Fig. 4B). Caspase-8 Is Proteolytically Processed within Detergent-insoluble Protein Complexes Prior to Extracellular Export from Nericin-stimulated Dendritic Cells—The Western blot analyses of intracellular caspase-8 levels during nigericin stimulation illustrated in Figs. 1F, 2C, 3B, and 6D utilized standard whole cell lysates prepared by detergent extraction followed by brief centrifugation to sediment the nuclei and insoluble cytoskeleton. Because oligomerized ASC complexes in nigericin-stimulated BMDC distribute into this detergent-insoluble fraction (Fig. 4), we hypothesized that procaspase-8 recruited to ASC inflammasomes or other signaling complexes (21) would also be present in this compartment. Fig. 7A shows that procaspase-8 is
localized in both detergent-soluble and detergent-insoluble lysate fractions of LPS-primed WT and Casp1/11−/− BMDC. Nigericin stimulation induced significant redistribution of procaspase-8 from these intracellular compartments to the extracellular medium, and this correlated with accumulation of the p18 subunit of cleaved caspase-8. The extracellular accumulation of caspase-8/p18 was enhanced in the nigericin-stimulated Casp1/11−/− cells and temporally correlated with near-complete loss of procaspase-8/p55 from the detergent-insoluble compartment and accumulation of 43-kDa caspase-8 cleavage product in that fraction. 20 μM IETD did not recapitulate these latter effects of Z-VAD indicating that IETD at this concentration does not license caspase-8-regulated necroptosis in the LPS-primed DC (Fig. 7, A, C and D) and that its inhibitory effects on IL-1β processing and release reflect actions on inflammasome signaling. Taken together, these analyses based on genetic, pharmacological, and biochemical approaches support a role for caspase-8 in the regulation of nigericin-stimulated IL-1β release in either the presence or absence of caspase-1.

Caspase-8 Ablation Preserves BMDC Viability during Nigericin Stimulation of NLRP3 Inflammasome Signaling Despite Robust Caspase-1 Activation—Two parallel arms of inflammatory signaling are regulated by nigericin-stimulated NLRP3 inflammasomes in WT DC or macrophages as follows: 1) processing and release of IL-1β (and IL-18); and 2) initiation of pyroptotic cell death. The studies described above indicate that caspase-8 contributes to the IL-1β production arm given the similar attenuation of nigericin-stimulated IL-1β release by either pharmacological inhibition or genetic ablation of caspase-8 (Fig. 5). However, mechanistic interpretation of these results is not...
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straightforward. The similar potency of IETD as an inhibitor of IL-1β secretion in Casp1/11−/− and Casp8−/−Rip3−/− BMDC (Fig. 5D) limits its utility as a selective reagent for interrogating how caspase-8 may regulate the activation or activity of canonical NLRP3-ASC/caspase-1 inflammasomes in wild type cells. Studies of IL-1β processing using caspase-8-knock-out cells are similarly complicated by the additional role of caspase-8 as a positive modulator of NFκB transcriptional signaling, including induction of NLRP3 or pro-IL-1β, independently of its protease activity (21–23, 38, 39). This will result in reduced processing and release of IL-1β at the level of signal 1 priming independent of other possible roles for caspase-8 as an upstream regulator or downstream effector of NLRP3 inflammasome signaling. Thus, we compared readouts of NLRP3 inflammasome signaling, other than mature IL-1β production, in caspase-8 deficient versus WT BMDC in response to both acute (30 min) and sustained (6 h) nigericin stimulation. The readouts included ASC oligomerization, proteolytic processing of caspase-1, induction of caspase-1-dependent propidium²⁺ influx, and loss of viability; each experiment directly compared the responses in BMDC from WT (Casp8+/+Rip3+/+), Casp8−/−Rip3−/−, and Casp8+/−Rip3−/− BMDC.

We observed attenuated mature 17-kDa IL-1β release in Casp8−/−Rip3−/− BMDC compared with WT and Rip3−/− BMDC in response to both acute and sustained nigericin stimulation (Figs. 5, A and B and 8A ). There was also modest reduction of caspase-1 activation (indicated by release of p20 caspase-1 subunit, Fig. 8A) in the Casp8−/−Rip3−/− cells at 30 min, but not 6 h, post-stimulation. It is important to note that Casp8−/−Rip3−/− BMDC express normal levels of caspase-1, which will be used as the predominant ICE in this genotype. Western blot analysis revealed similar magnitude of nigericin-stimulated ASC relocalization from the detergent-soluble to the detergent-insoluble fractions in BMDC lysates regardless of genotype (Fig. 8B). However, the extent of ASC oligomerization and procaspase-1 recruitment within the insoluble fraction of
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FIGURE 7. **Caspase-8 is proteolytically processed within detergent-insoluble protein complexes prior to extracellular export from nigerin-stimulated dendritic cells.** WT and Casp1/11-/- BMDC were treated with LPS (4 h) and nigericin (Nig) (6 h) in the presence or absence of Z-VAD (50 μM) or IETD (20 μM). The extracellular media, detergent-soluble lysate fractions, and detergent-insoluble lysate fractions were collected and processed for Western blot analysis of caspase-8 (A), ASC (B), caspase-1 (C), and IL-1β (D). A and D, membrane with extracellular media samples was simultaneously probed with anti-IL-1β and anti-caspase-8 antibodies. Results are from one experiment representative of two similar studies.

the Casp8-/-Rip3-/- cells was reduced with 30 min, but not 6 h, of nigericin stimulation (Fig. 8B). Relative to the WT and Rip3-/- cells, the nigericin-stimulated Casp8-/-Rip3-/- BMDC were additionally characterized by only a modest decrease in the rate of induction of enhanced propidium²⁺ influx as an alternative (to IL-1β release) readout of active caspase-1 accumulation (Fig. 8C). Thus, nigericin induction of canonical NLRP3-ASC-caspase-1 inflammasome signaling, upstream of IL-1β processing per se, remains intact in LPS-primed Casp8-/-Rip3-/- BMDC but becomes activated at a modestly reduced rate. This further supports the interpretation that the marked decrease in nigerin-stimulated accumulation of extracellular IL-1β release from Casp8-/-Rip3-/- BMDC (Fig. 5) predominantly reflects reduced levels of the intracellular pro-IL-1β substrate for caspase-1.

Despite the similar caspase-1 activation and propidium²⁺ influx responses to sustained nigericin stimulation in the three genotypes, Casp8-/-Rip3-/- BMDC exhibited sustained metabolic viability, whereas the Rip3-/- cells were characterized by a slower progression to compromised cell viability as compared with WT cells (Fig. 8D). This suggests a role for caspase-8 (and RIP3) in facilitating the pyroptotic death signaling program initiated by caspase-1. Caspase-8 also negatively regulates RIP3-dependent necroptosis. TLR4 activation in WT (and Casp1/11-/-) BMDC drives the TRIF-dependent assembly of RIP1/RIP3/FADD/caspase-8 ripoptosome platforms (21). When combined with high [Z-VAD] to suppress caspase-8-mediated inactivation of RIP1 and RIP3, the resulting RIP1-RIP3-MLKL phosphorylation cascade leads to MLKL oligomerization and recruitment of MLKL oligomeric pores to the plasma membrane (40, 41). As a consequence, necroptotic cytolysis will release intracellular contents, including unprocessed 31-kDa pro-IL-1β (Fig. 8A). To verify functional deletion of RIP3 in the Casp8-/-Rip3-/- and Rip3-/- BMDC, we stimulated the three DC genotypes with LPS + Z-VAD (for 6 h) as a pro-necroptotic stimulus; this also permitted direct comparison with the inflammasome responses to sustained LPS + nigericin in the same DC populations. The LPS + Z-VAD-induced release of pro-IL-1β was absent in Casp8-/-Rip3-/- and Rip3-/- BMDC but not WT BMDC consistent with complete suppression of necroptosis in cells lacking RIP3 (Fig. 8A). Notably, activation of necroptosis in WT but not Rip3-deficient BMDC induced the following: 1) redistribution of ASC from the detergent-soluble to the detergent-insoluble compartment; and 2) the accumulation of ASC oligomers (Fig. 8B). This suggests that assembly of NLRP3-ASC inflammasome complexes may be a secondary consequence of necroptotic signaling in inflammatory DCs and macrophages. Consistent with this possibility, ASC oligomers were absent in Nlrp3-/- BMDC treated with LPS + Z-VAD (data not shown).

**Discussion**

This study provides five findings that extend the growing literature on the involvement of caspase-8 in inflammasome-based signaling responses in macrophages and dendritic cells. First, caspase-8 was identified as a downstream ICE of NLRP3 inflammasomes by demonstrating the robust, but delayed, processing and release of IL-1β in caspase-1-deficient DC stimu-
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Related with nigericin. This caspase-1-independent, but NLRP3-dependent, release of mature IL-1β is temporally correlated with strong caspase-8 activation and suppressed by IETD, a widely used pharmacological inhibitor of caspase-8. Second, we confirmed and extended recent reports that genetic ablation of caspase-8 limits overall production of mature IL-1β in response to NLRP3 stimulation. Notably, this marked attenuation of IL-1β release from caspase-8-deficient DC was observed in the context of near-normal caspase-1 activation/activity and thus indicated limitation predominantly at the level of pro-IL-1β substrate availability. Third, we determined that the IETD inhibitor lacks sufficient selectivity for caspase-8 over caspase-1 to interrogate specific roles for caspase-8 in NLRP3 inflammasome signaling in wild type DC that express both proteases. Fourth, we used primary murine dendritic cells to extend previous observations with murine macrophages (24) that NLRP3 inflammasomes can drive either rapid pyroptosis via caspase-1 in WT cells or slower apoptotic death mediated by serial caspase-8 → caspase-3 activation in caspase-11-deficient cells. Finally, we identified a role for caspase-8 in facilitating progression of the pyroptotic death signaling cascade initiated by active caspase-1 by demonstrating more prolonged preservation of cell viability in Casp8−/−Rip3−/− DC stimulated with nigericin. This marked attenuation of cell death in caspase-8-deficient DC was observed in the context of near-normal caspase-1 activation/activity and thus supports an action of caspase-8 in coupling caspase-1 to dysregulation of the osmotic and metabolic homeostasis that drives cell death in pyroptosis.

Multiple studies have described a role for caspase-8 as a major IL-1β-processing enzyme downstream of ASC/ inflammasome-independent signaling complexes assembled when DCs or macrophages are costimulated with TLR4/TLR3 agonists and a variety of extrinsic or intrinsic cell stressors (8, 12, 15, 17, 21, 42). These noninflammasome platforms for caspase-8 variously include TRIF, RIP1, FADD, and in some cases RIP3 (42). It is important to note that caspase-8 physiologically acts as the predominant IL-1β-processing enzyme in these noninflammasome stimulus models despite the expression of normal procaspase-1 levels. However, the findings described in this study add to the recent reports which indicate that caspase-8 also interacts with NLRP3 inflammasomes or other ASC-based inflammasomes. These previous reports have...
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used wild type or caspase-8-deficient/inhibited macrophages to define how caspase-8 acts as an upstream positive modulator of NLRP3 inflammasome activation or activity (20, 23, 35, 43). In contrast, we used caspase-1-deficient cells to define a role for caspase-8 as an effector protease downstream of assembled NLRP3-ASC complexes. Although caspase-8 was modestly activated during the initial 30 min of nigericin treatment in both wild type and caspase-1 knock-out cells, it did not contribute to IL-1β processing as indicated by the complete suppression of IL-1β release in Casp1/11−/− cells at this time point. However, with sustained nigericin stimulation (>2 h), there was a marked increase in caspase-8 activation in the absence, but not the presence of caspase-1, and this temporally correlated with the significant increase in IL-1β maturation and release. Notably, these delayed increases in caspase-8 activation and IL-1β processing were not observed in Nlrp3−/− or Asc−/− BMDC. Recent biophysical studies have indicated that both the ASC-CARD and the ASC-PYD protein interaction domains can be displayed on the surfaces of oligomerized ASC filaments or specks (44, 45). Although the exposed CARDs on these ASC filaments/specks provide binding sites for the CARDs of procaspase-1 monomers, the exposed PYDs can facilitate interaction with the DEDs of procaspase-8 monomers. Indeed, biochemical interaction between the PYD of ASC and the DED of caspase-8 (24) has been described in support of the ability of ASC inflammasomes to recruit and activate caspase-8. Both ASC and caspase-8 were recruited into detergent-insoluble compartments during acute (0.5 h) and sustained (6 h) nigericin stimulation in our BMDC model system.

We found that nigericin-stimulated caspase-8 activation was markedly enhanced in the absence of caspase-1 expression. This suggests that recruitment of procaspase-1 to assembled ASC filaments/specks may sterically limit the association of procaspase-8. However, even in the absence of caspase-1, there was a significant delay (~2 h) in the robust accumulation of active caspase-8 and consequent processing of IL-1β. We previously described the TLR4- and TRIF-dependent assembly of RIP1-FADD-caspase-8 complexes in LPS-primed DC (21). LPS will also up-regulate the noncatalytic caspase-8 paralog, cFLIP, which can dimerize with procaspase-8 in RIP1-FADD platforms. Indeed, a recent study indicated that cFLIP expression modulates the efficacy of IL-1β maturation and release induced by NLRP3 or AIM2 inflammasomes (46). Sequestration within RIP1-FADD-cFLIP complexes may include the predominant pool of procaspase-8 in LPS-primed DC prior to accumulation of oligomerized NLRP3-ASC platforms. The NLRP3 inflammasome complexes accumulate rapidly and maximally within 30 min of nigericin exposure to generate an additional pool of DED interaction sites (on ASC-PYD) that presumably compete with the FADD sites for procaspase-8. Thus, the delayed activation of caspase-8 in nigericin-stimulated Casp1/11−/− DC may reflect kinetic constraints inherent in generation of a new steady distribution of procaspase-8 between the NLRP3-ASC and RIP1-FADD complexes.

The experiments with Casp1/11−/− DC unequivocally indicate that caspase-8 can be recruited to, and activated within, NLRP3 inflammasomes. However, an equally critical question is how caspase-8 participates in NLRP3 inflammasome signaling in wild type macrophages and DC that express caspase-1. Recent studies in several inflammasome models have supported two distinct, but not mutually exclusive, roles for caspase-8 in this context as follows: 1) that caspase-8 acts in parallel with caspase-1 as a direct IL-1β/IL-18 processing enzymes; and 2) that caspase-8 acts as an initiator caspase to cleave procaspase-1 and thereby facilitate accumulation of active caspase-1 as the principal executioner caspase for cytokine processing (15, 16, 23, 35, 47–49). Most of these studies have used caspase-8-deficient cells and/or the IETD caspase-8 inhibitor to define these possible roles of caspase-8. We also used utilized these latter reagents but found that both presented limitations with regard to mechanistic interpretation. Although IETD markedly suppressed both caspase-1 processing and release of mature IL-1β during acute or sustained nigericin stimulation of wild type DC, it also strongly suppressed the IL-1β processing/release induced by nigericin in caspase-8 knock-out BMDC. This indicates that IETD can directly and potently inhibit caspase-1 in intact cells with an IC50 similar to that describing its action on caspase-8 per se. This reduces its utility as a tool for discriminating between roles for caspase-8 as a proximal activator of caspase-1 versus a parallel (to caspase-1) IL-1β-processing enzyme. Importantly, several noncaspase-family proteases, including granzyme A, cathepsin G, and elastase, can also cleave pro-IL-1β to produce bioactive IL-1β (50). For example, in a Pseudomonas aeruginosa-infected neutrophil model, Karmakar et al. (51) found that serine esterases, including neutrophil elastase, mediated IL-1β maturation and release independent of caspase-1 expression. However, our observation that IETD almost completely suppressed nigericin-stimulated IL-1β release in Casp1/11−/− DC argues against a significant role for serine esterases in this inflammasome model.

Although caspase-8 ablation significantly reduced the magnitude of IL-1β release elicited by sustained nigericin stimulation, it only modestly delayed the production of active caspase-1 as indicated by either Western blot analysis or induction of the caspase-1-dependent plasma membrane pores/channels that initiate the pyroptotic cell death response. These observations argue against a major role for caspase-8 as an initiator caspase for caspase-1 activation in this particular model of NLRP3 inflammasome signaling driven by a rapidly acting K+ efflux agonist. The delayed caspase-1 activation in Casp8−/− Rip3−/− BMDC was as follows: 1) characterized by reduced accumulation of the active p20 caspase-1 subunit during acute (30 min) nigericin treatment but not longer term exposure to this K+ ionophore; and 2) correlated with a delayed accumulation of ASC oligomers. This delay in ASC oligomerization (and consequent caspase-1 activation) is consistent with the reduction in LPS-induced accumulation of NLRP3 we previously described in Casp8−/− Rip3−/− BMDC (21) and the similar attenuation in NLRP3 expression observed in Casp8−/− Rip3−/− macrophages (23). Likewise, the marked decrease in nigericin-stimulated IL-1β release observed in the Casp8−/− Rip3−/− BMDC is consistent with the significant reduction in pro-IL-1β accumulation that has been described in multiple models of caspase-8-deficient myeloid leukocytes (17, 21–23, 43). As recently reviewed by Gurung and Kanneganti (13), these findings indicate an important role for caspase-8 in the transcrip-
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may involve a canonical caspase-8-driven apoptotic cascade that facilitates caspase-3-mediated proteolytic gating of pan-
nexin-1 channels that are also expressed in macrophages and DCs (72–74).

Caspase-1-induced pores/channels include an early and crit-
ical component of the pyroptotic cascade, but the reactions that
link these channels to execution of cell lysis remain poorly
defined (69, 70). Although necessary for initiation of pyroptosis,
activation of the channels is not sufficient for the ultimate lytic
collapse of cell integrity, which also involves cell swelling, pro-
trusion and dilation of plasma membrane blebs, and final rupt-
ture of the diluted blebs (70, 71, 75). We (76, 77) and others (37,
70, 71, 78) have demonstrated that inclusion of millimolar gly-
cine in culture media stabilizes dilation of membrane blebs and
attenuates the rate at which macrophages with active caspase-1 or
other osmotically stressed cells progress to cell lysis. Although
nigericin stimulated a near-normal rate of propidium
++-permeant channel/pore induction in Casp8−/−/Rip3−/−/BMDC, these cells
were also characterized by a much slower loss of cell viability.
RIP3-deficient cells also exhibited a slower loss of viability,
albeit markedly less than in the double knock-out DC. This
effect of caspase-8 (and RIP3) ablation is similar to the protec-
tive action of glycine whereby the defining proximal step of the
pyroptotic cascade, caspase-1-induced channels/pores, does
not drive efficient cell lysis in the absence of one or more
distal signaling reactions. Additional experiments are required to
identify the caspase-8-sensitive reaction(s) in the distal pyro-
ptotic cascade. Taken together, our current observations indi-
cate that caspase-8 can participate in the two major arms of
NLRP3 inflammasome signaling. IL-1β processing and
pyroptosis.

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nated the study and wrote the paper. C. A. performed and analyzed
the experiments shown in Figs. 1 (A, B, E, and F), 2 and 3 (B–F), and
4–8. H. M. R. performed and analyzed the experiments shown in
Fig. 3A. G. R. D. performed and analyzed the experiments shown in
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