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J Immunol 2010; 184:5287-5297; Prepublished online 26 March 2010;
doi: 10.4049/jimmunol.0903536
http://www.jimmunol.org/content/184/9/5287

Supplementary Material **http://www.jimmunol.org/content/suppl/2010/03/25/jimmunol.0903536.DC1**

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Pathogenic *Vibrio* Activate NLRP3 Inflammasome via Cytotoxins and TLR/Nucleotide-Binding Oligomerization Domain-Mediated NF-κB Signaling

Claudia Toma,* Naomi Higa,* Yukiko Koizumi,* Noboru Nakasone,* Yasunori Ogura,* Andrea J. McCoy,*[,1] Luigi Franchi,[†] Satoshi Uematsu,[‡] Junji Sagara,[§] Shun'ichiro Taniguchi,[¶] Hiroko Tsutsui,[‖] Shizuo Akira,[‡] Jürg Tschopp,[#] Gabriel Núñez,[†] and Toshihiko Suzuki*

***Vibrio vulnificus* and *Vibrio cholerae* are Gram-negative pathogens that cause serious infectious disease in humans. The β form of pro–IL-1 is thought to be involved in inflammatory responses and disease development during infection with these pathogens, but the mechanism of β form of pro–IL-1 production remains poorly defined. In this study, we demonstrate that infection of mouse macrophages with two pathogenic *Vibrio* triggers the activation of caspase-1 via the NLRP3 inflammasome. Activation of the NLRP3 inflammasome was mediated by hemolysins and multifunctional repeat-in-toxins produced by the pathogenic bacteria. NLRP3 activation in response to *V. vulnificus* infection required NF-κB activation, which was mediated via TLR signaling. *V. cholerae*-induced NLRP3 activation also required NF-κB activation but was independent of TLR stimulation. Studies with purified *V. cholerae* hemolysin revealed that toxin-stimulated NLRP3 activation was induced by TLR and nucleotide-binding oligomerization domain 1/2 ligand-mediated NF-κB activation. Our results identify the NLRP3 inflammasome as a sensor of *Vibrio* infections through the action of bacterial cytotoxins and differential activation of innate signaling pathways acting upstream of NF-κB.** *The Journal of Immunology*, **2010, 184: 5287–5297.**

The innate immune system recognizes a vast array of pathogen-associated molecular patterns (PAMPs) via membrane-associated TLRs and cytosolic nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs). All TLRs and some NLRs, including Nod1 and Nod2, activate the transcription of numerous genes, including cytokines and chemokines (1). Some cytokines, such as the β form of pro–IL-1 (IL-1β), are expressed as proforms and require proteolytic cleavage for maturation. The cysteine protease caspase-1 is responsible for the proteolytic processing and secretion of IL-1β and IL-18, as well as for the induction of pyroptosis, a form of proinflammatory necrotic cell death induced by infection with certain pathogenic bacteria (2).

Critical to the activation of caspase-1 and induction of inflammatory responses is the formation of a multiprotein complex termed the inflammasome (3–5). Inflammasome complexes are comprised of at least one NLR and the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which links NLRs to caspase-1. Depending on the activating stimuli, distinct inflammasomes are formed. For example, anthrax lethal toxin (6) triggers caspase-1 activation via Nlrp1b in the mouse, whereas muramyl dipeptide (MDP) (7) induces the formation of the human NLRP1 (also called NALP1) inflammasome (3–5). Likewise, flagellin is sensed by NLRC4 (also called IPAF) postinfection with *Salmonella enterica* serovar Typhimurium and *Legionella pneumophila,* a process that is mediated by cytosolic delivery of flagellin via the respective bacterial type III (T3SS) and type IV (T4SS) secretion system (8–11). Recently, another NLR, Naip5, was shown to be required for NLRC4 inflammasome activation in response to *L. pneumophila* flagellin (12). In contrast to infection by these pathogens, *Shigella flexneri* induces caspase-1 activation via NLRC4 independently of flagellin (13), whereas the role of flagellin in the activation of the NLRC4 inflammasome during *Pseudomonas aeruginosa* infection remains controversial (14–16). These studies suggest that like TLRs, different PAMPs may be recognized by the same NLR to activate the inflammasome.

The activation and regulation of the NLRP3 (also called NALP3 and cryopyrin) inflammasome is complex. Activation occurs in response to many PAMPs (17–21) poststimulation with ATP or in response to non–pathogen-associated signals, such as aluminum salts (alum) (22–26), crystals of monosodium uric acid (27),

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Received for publication October 30, 2009. Accepted for publication February 26, 2010.

This work was supported by a Grant-in-Aid for Scientific Research (to C.T. and Y.O.) and a Grant-in-Aid for Scientific Research on Priority Areas (to T.S.) from the Japanese Ministry of Education, Culture, Sports, and Technology in Japan, the Takeda Science Foundation (to T.S. and C.T.), the Uehara Memorial Foundation and Ohyama Health Foundation (to T.S.), and Grants AI063331 and AR051790 from the National Institutes of Health (to G.N.).

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The online version of this article contains supplemental material.

Abbreviations used in this paper: alum, aluminum salts; ASC, apoptosis-associated speck-like protein containing a caspase recruitment domain; BMM, bone marrow-derived macrophage; cLPS, conventional LPS; hpi, h postinfection; iE-DAP, γ-D-glutamyl-meso-diaminopimelic acid; IL-1β, β form of pro–IL-1; LDH, lactate dehydrogenase; MARTX, multifunctional repeat-in-toxin; MDP, muramyl dipeptide; n.d., none detected; NLR, nucleotide-binding oligomerization domain-like receptor; Nod, nucleotide-binding oligomerization domain; PAMP, pathogen-associated molecular pattern; TRIF, Toll/IL-1 resistance domain-containing adaptor-inducing IFN-β; T3SS, type III secretion system; T4SS, type IV secretion system; WT, wild-type.

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amyloid-β (28), and other irritants in LPS-stimulated macrophages (24, 29, 30). NLRP3 inflammasome is also activated by bacterial pore-forming toxins (21, 31–34), inorganic crystalline metabolite from the infection of malaria parasite (35–37), or the infection by other bacteria (38, 39), viruses (40–44), or fungi (45, 46). Despite the growing list of NLRP3 inflammasome agonists, the molecular mechanisms by which NLRP3 senses and responds to these stimulators are poorly understood. The sterile NLRP3 stimulators listed above, but not bacterial infection, require LPS priming for activating NLRP3 inflammasome. The PAMPs, including LPS, were initially thought to enter the cytosol via pore-forming toxins or ATP-mediated membrane pore; alternatively, phagocytosed PAMPs leak into the cytosol from phagosomes damaged by crystalline particles (5, 47). However, very recent papers have shown that NF-κB activation via pattern-recognition receptors as well as by TNF-α, IL-1α, or IL-1β is important for supporting NLRP3 activation in response to ATP or silica (48, 49). Also, NF-κB–mediated upregulation of NLRP3 is thought to be a limited factor for NLRP3 activation by LPS plus ATP (48).

Vibrio vulnificus and *Vibrio cholerae* are Gram-negative pathogens that cause significant infectious disease in humans. *V. vulnificus* is a highly virulent pathogen that causes gastroenteritis, wound infection, and often primary septicemia in immune-compromised individuals. *V. cholerae* causes severe and fatal diarrhea via the action of cholera toxin. To date, several bacterial factors that play a role in *V. vulnificus* and *V. cholerae* virulence have been characterized, yet little is known about the host factors contributing to the disease process and susceptibility to these pathogens. IL-1β, in addition to TNF-α and IL-6, are thought to be involved in inflammatory responses and disease development during infection with these pathogens (50, 51), but the relationship between bacterial and host factors contributing to IL-1β production remains poorly defined.

In this study, we demonstrate that the infection by *V. vulnificus* and *V. cholerae* in macrophages triggers the activation of caspase-1 and IL-1β secretion. We investigated the role of NLR inflammasomes during infection of macrophages with two *Vibrio* spp. and demonstrate that hemolysin and multifunctional repeat-in-toxin (MARTX) secreted from each species are required to elicit caspase-1 activation via the NLRP3 inflammasome and subsequent IL-1β secretion. We further show that *V. vulnificus*-inducing NLRP3 activation requires NF-κB activation via TLRs, whereas NLRP3 activation by *V. cholerae* is supported by NF-κB activation independent of TLR signaling. Finally, we provide evidence that purified *V. cholerae* hemolysin-stimulated NLRP3 activation is supported by both TLR and Nod1/Nod2 ligand-mediated NF-κB activation. Our results suggested that TLR-dependent and Nod1/2-dependent NF-κB activation signals are differentially used for NLRP3 inflammasome activation, and these events may be influenced, at least in part, by the type of NLRP3 stimulator produced by *Vibrio* spp.

Materials and Methods

Bacterial strains

The wild-type (WT) *V. vulnificus* MO6-24/O strain was provided by Dr. Anita C. Wright (University of Florida, Gainesville, FL). The WT *V. cholerae* El Tor O1 N86 strain has been described previously (52). This strain does not have a T3SS. The isogenic *V. vulnificus* mutants—Δ*vvhA*, Δ*rtxA*, Δ*rtxA*Δ*vvhA*, Δ*vasK*, and Δ*flgE*—were constructed by allele replacement strategies by using suicide vector pYAK1 provided from Dr. T. Iida (Osaka University, Japan). The isogenic *V. cholerae* mutants—Δ*hlyA*, Δ*rtxA*, Δ*hlyA*Δ*rtxA*, and Δ*ctxA*—were constructed using same vector. The bacterial strains were grown in Luria-Bertani broth containing 1% NaCl.

Mice and preparation of macrophages

C57BL/6 mice were from CREA Japan (Tokyo, Japan) as WT mice. C57BL/6 background caspase-1–deficient mice were provided by Dr. R. Flavell (Yale University School of Medicine, New Haven, CT). NALP3-deficient (*Nlrp3*$^{-/-}$) (17, 27), NLRC4-deficient (*Nlrc4*$^{-/-}$) (9), and ASC-deficient (*Asc*$^{-/-}$ or *Pycard*$^{-/-}$) mice (53) as well as mice doubly deficient in MyD88 and Toll/IL-1 resistance domain-containing adaptor-inducing IFN-β (TRIF) (*Myd88*$^{-/-}$*Trif*$^{-/-}$) were described previously (54). MyD88- or TRIF-deficient mice were generated from MyD88/TRIF-deficient mice. P2X7R-deficient (*P2X7r*$^{-/-}$) mice were from Jackson laboratories. Bone marrow-derived macrophages (BMMs) were prepared from the femurs and tibias of mice cultured for 5 d in 10% FCS-RPMI 1640 supplemented with 30% L-cell supernatant. Mice were housed in a pathogen-free facility. Animal-studies used protocols approved by the Animal Care and Use Committee of University of the Ryukyus (Okinawa, Japan) and the University of Michigan Committee on Use and Care of Animals (Ann Arbor, MI).

Reagents

Conventional LPS (cLPS; O55:B5) was purchased from Sigma-Aldrich (St. Louis, MO). Ultra-pure LPS (uLPS), γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), and MDP were from Invivogen (San Diego, CA). NF-κB inhibitor BAY 11-7082 was from Calbiochem (San Diego, CA). The cathepsin B inhibitor CA-074-Me was from Calbiochem, and E64d was from Peptide Institute (Osaka, Japan). Abs specific for IκBα and p38MAPK were from Cell Signaling Technology (Beverly, MA). The following Abs were obtained commercially: rabbit anti-mouse caspase-1 and rabbit anti-NLRP3 (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-mouse IL-1β (R&D Systems, Minneapolis, MN), and rabbit anti-mouse IL-18 Ab (BioVision, Mountain View, CA).

Bacterial infection, lactate dehydrogenase assay, and ELISA

LPS-primed (1 μg/ml) or untreated BMMs were seeded at 5×10^5 cells in 24-well plates containing 10% FCS-RPMI 1640. The cells were infected with *Vibrio* sp. at multiplicity of infection of ∼10 per cell. The plates were centrifuged at $600 \times g$ for 10 min to synchronize the stage of infection and incubated. At the times indicated postinfection, the lactate dehydrogenase (LDH) activity of the culture supernatants of infected cells was measured by using a CytoTox 96 assay kit (Promega, Madison, WI) according to the manufacturer's protocol. The cytokines released in culture supernatants were quantified by ELISA (R&D Systems). The end point of time-course experiments was limited at 3 h postinfection (hpi), because *Vibrio* are extracellular pathogens and multiply fast in the cell culture media.

Immunoblot

BMMs seeded at 2×10^6 cells in six-well plates were infected with bacteria. Cells were lysed and combined with supernatants precipitated with 10% TCA. The samples were loaded onto 15% SDS-PAGE, and the cleaved forms of caspase-1, IL-1β, or IL-18 were detected with anti–caspase-1, anti–IL-1β Ab, or anti–IL-18 Ab, respectively.

Protein purification

V. cholerae hemolysin was purified from bacterial culture supernatants according to a previous report (52) with modification. Briefly, the culture supernatants of Δ*rtxA* mutant were precipitated with ammonium sulfate, and an active fraction was obtained from two gel filtration columns: Sephadex G-100 and Sephadex G-100 superfine (GE Healthcare, Piscataway, NJ). The hemolysin was purified by using affinity chromatography HiTrap NHS-activated HP column (GE Healthcare) coupled with anti-hemolysin Ab.

Statistical analyses

Statistical analyses were performed by the Mann-Whitney *U* test (ELISA assays) and log-rank test (survival assays). Differences were considered significant at $p < 0.05$.

Results

Vibrio *infection induces caspase-1 activation, IL-1β secretion, and pyroptosis*

It has been reported that *V. vulnificus* and *V. cholerae* induce cell death in infected macrophages (55, 56). We observed that primary mouse BMMs infected with WT strains of either *Vibrio* sp. underwent rapid cell death, which was associated with swelling of the infected cells (Fig. 1*A*), a morphological feature of necrotic cell death, and the release of LDH (Fig. 1*E*). Furthermore, infection with *V. vulnificus* or *V. cholerae* induced caspase-1 activation (Fig. 1*B*), processing of pro–IL-1β (Fig. 1*C*) and secretion of mature

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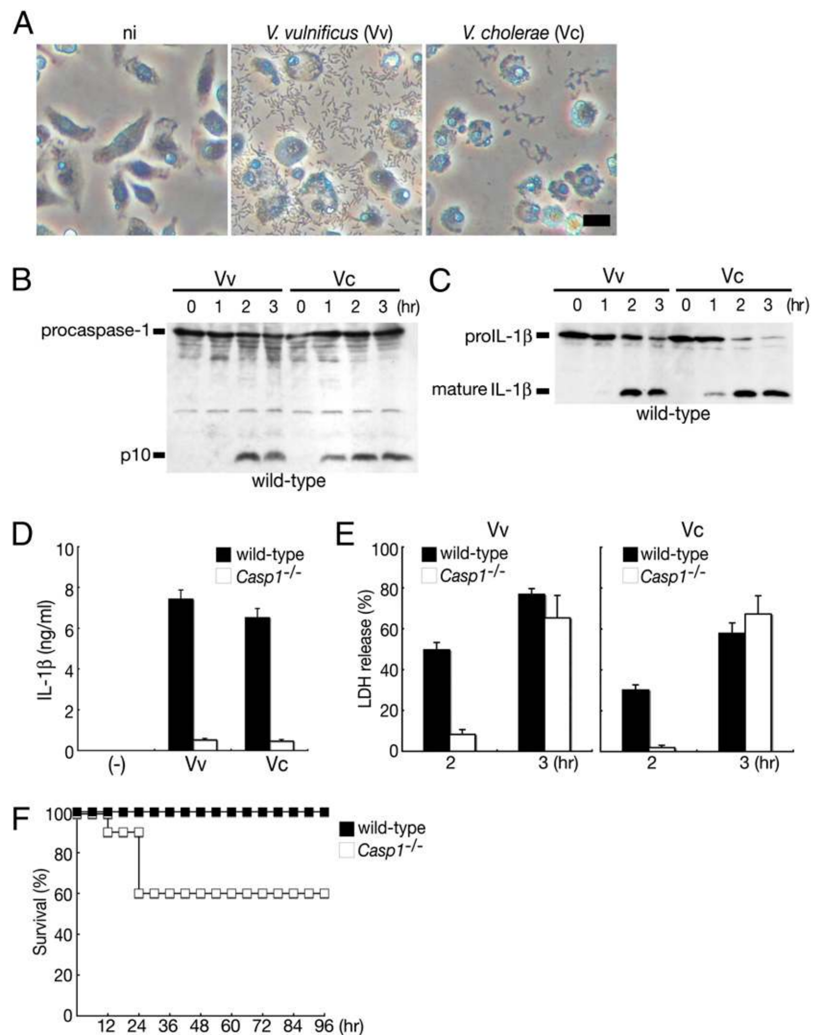

FIGURE 1. *V. vulnificus* (Vv) and *V. cholerae* (Vc) induce caspase-1 activation, IL-1β secretion, and pyroptosis. BMMs from WT or caspase-1–deficient mice were primed with cLPS (1 μg/ml; 3 h) and infected with WT Vv or Vc at a multiplicity of infection of 10 for indicated times. The samples were collected at indicated times. *A*, Phase contrast images of WT BMMs at 1 hpi. The representative damaged macrophages are shown. Scale bar, 10 μm. *B*, Immunoblot analysis of the cleavage of caspase-1 to its active p10 subunit in infected WT BMMs. *C*, Immunoblot analysis of the IL-1β processing in infected WT BMMs. *D*, ELISA for IL-1β secretion into culture supernatants from infected WT or caspase-1–deficient BMMs at 3 hpi. The values are means ± SD of triplicate samples. *E*, Release of macrophage LDH into culture supernatants from infected WT or caspase-1–deficient BMMs. The values are means ± SD of triplicate samples. *F*, Survival curve for WT or caspase-1–deficient mice after i.p. infection with Vv (1.5×10^4 cfu/mouse; $n = 10$/group). Results are representative of two independent experiments.

IL-1β (Fig. 1*D*). These results indicated that the infections of two *Vibrio* spp. induce pyroptosis of macrophages, a form of cell death associated with bacterial infection. Secretion of mature IL-1β induced by *V. vulnificus* or *V. cholerae* was caspase-1–dependent (Fig. 1*D*). Compared to WT macrophages, LDH release was almost abrogated in caspase-1–deficient macrophages at early times of infection, but was indistinguishable by 3 hpi (Fig. 1*E*), indicating that the effect of caspase-1 on LDH release is partial, and *Vibrio* spp. also induce caspase-1–independent cell death. We also tested the role of caspase-1 in vivo during *V. vulnificus* infection. *V. vulnificus* challenge resulted in higher mortality of caspase-1–deficient mice compared with WT mice ($p = 0.0297$ by log-rank test; Fig. 1*F*), indicating that caspase-1 contributes to the susceptibility of mice to *V. vulnificus*.

Bacterial hemolysin and MARTX are required for inducing pyroptosis

Hemolysins and MARTX produced by *V. vulnificus* and *V. cholerae* have been implicated in disease pathogenesis (57–60), yet the mechanism by which these cytotoxins induce host immune responses remains largely unknown. We hypothesized that *Vibrio* cytotoxins play a direct role in caspase-1 activation and pyroptosis and assessed this role by constructing *Vibrio* mutants. In *V. vulnificus*, two major cytotoxins (VvhA, a hemolysin/cytolysin, and $MARTX_{Vv}$, a multifunctional cytotoxin encoded by *rtxA* gene) have been identified. Infection of macrophages with *V. vulnificus* lacking VvhA (VvΔ*vvhA*) induced caspase-1 activation, IL-1β processing/secretion, and LDH release which was comparable to that induced by infection with the isogenic WT strain, although the activation of caspase-1 induced by the mutant bacteria was slightly delayed (Fig. 2*A*–*C*). Furthermore, infection of macrophages with the VvΔ*rtxA* mutant induced comparable IL-1β secretion assessed by 3 hpi, but delayed LDH release and caspase-1 activation when compared with the WT strain (Fig. 2*A*–*C*), suggesting that $MARTX_{Vv}$ plays a role during the early induction of caspase-1 activation and LDH release. Notably, caspase-1 activation, IL-1β processing/secretion, and LDH release were completely abrogated when the macrophages were infected with a VvΔ*rtxA*Δ*vvhA* double mutant (Fig. 2*A*–*C*). These results indicated that both VvhA and $MARTX_{Vv}$ play a critical and redundant role in triggering caspase-1 activation and pyroptosis in response to *V. vulnificus* and suggested that these cytotoxins are capable of inducing not only caspase-1–dependent pyroptosis but also caspase-1–independent cell death.

Several bacteria including *Salmonella* and *Legionella* induce caspase-1 activation and pyroptosis by a mechanism involving the delivery of flagellin to the cytosol by T3SS and T4SS, respectively (8–11). Although *V. vulnificus* does not encode a T3SS or T4SS, the bacterium contains a putative type VI secretion system that might serve as a conduit for flagellin delivery by this pathogen. However, neither a type VI secretion system-deficient (Δ*vasK*) nor a flagella-null (Δ*flgE*) mutant of *V. vulnificus* was impaired in the ability to induce caspase-1 activation and processing/secretion of IL-1β (Supplemental Fig. 1).

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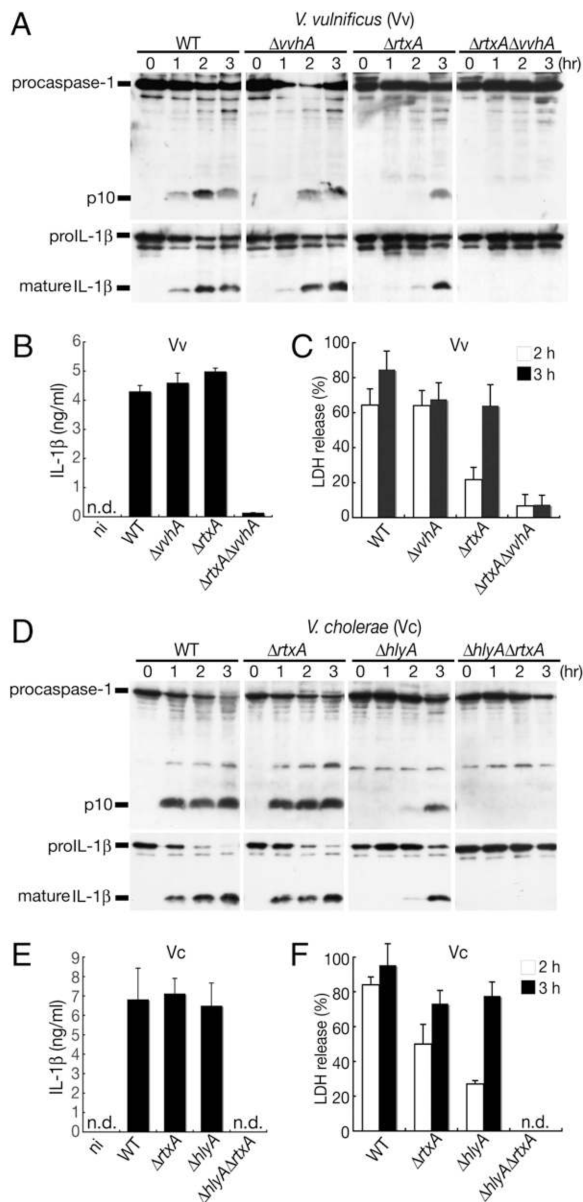

FIGURE 2. Two cytotoxins of *V. vulnificus* (Vv) and *V. cholerae* (Vc) are essential to induce caspase-1 activation and cell death. BMMs from WT mice were primed with cLPS and infected with WT Vv, WT Vc, or their respective isogenic cytotoxin mutants. The samples were collected at indicated times. *A*, Immunoblot for caspase-1 activation and IL-1β processing during infection with WTVv, Δ*vvhA* (VvhA mutant), Δ*rtxA* ($MARTX_{Vv}$ mutant), or Δ*rtxA*Δ*vvhA* (double mutant). *B*, ELISA for IL-1β secretion at 3 hpi with Vv strains. The values are means ± SD of triplicate samples. *C*, LDH release into culture supernatants during the infection of Vv strains at 2 and 3 hpi. The values are means ± SD of triplicate samples. *D*, Immunoblot for caspase-1 activation and IL-1β processing during infection with WT Vc, Δ*rtxA* ($MARTX_{Vc}$ mutant), Δ*hlyA* (HlyA mutant), or Δ*hlyA*Δ*rtxA* (double mutant). *E*, ELISA for IL-1β secretion at 3 hpi with Vc strains. The values are means ± SD of triplicate samples. *F*, LDH release into culture supernatants during the infection of Vc strains infection at 2 and 3 hpi. The values are means ± SD of triplicate samples. n.d., none detected.

Similar to *V. vulnificus*, two cytotoxins (HlyA, known as El Tor hemolysin, and $MARTX_{Vc}$) have been identified in *V. cholerae* (58, 59, 61). We found that caspase-1 activation and LDH release induced by *V. cholerae* required HlyA and $MARTX_{Vc}$ as determined by the analyses of macrophages infected with mutant VcΔ*hlyA*, VcΔ*rtxA*, or VcΔ*hlyA*Δ*rtxA* and WT bacteria (Fig. 2*D*–*F*). Notably, it has been reported that $MARTX_{Vv}$ can form pores on host cell membranes (62), whereas $MARTX_{Vc}$ lacks pore-forming activity but can induce cross-linking of cellular actin (61). Our results indicate that these different types of MARTXs can mediate induction of caspase-1 activation and LDH release. Caspase-1 activation was independent of cholera toxin activity, as the infection with *V. cholerae* deficient in cholera toxin induced caspase-1 activation and IL-1β processing comparably to WT bacteria (Supplemental Fig. 2). Also, HlyA or $MARTX_{Vc}$ does not affect the internalization of cholera toxin in macrophages, because immunofluorescence analysis using Abs to cholera toxin A subunit and LAMP1 (a marker of late endosome and lysosome) revealed that the internalization of the secreted toxin and association with LAMP1 were similar in WT and Δ*hlyA*Δ*rtxA* mutant bacteria (Supplemental Fig. 3). Collectively, these results suggested that two cytotoxins of *V. vulnificus* and *V. cholerae* are critical factors to induce caspase-1 activation, pyroptosis, and caspase-1–independent cell death during infection.

The NLRP3-ASC inflammasome is essential for the induction of caspase-1 during Vibrio *infection*

Recent studies have suggested that potassium efflux is essential for activation of NLRP1 and NLRP3 inflammasomes (63, 64). During infection with either *Vibrio* sp., we observed a complete inhibition of caspase-1 activation and IL-1β processing when infection was performed in medium containing a high concentration of potassium (Supplemental Fig. 4). These results, in conjunction with the identification of cytotoxins as activators of caspase-1 activation, led us to predict that NLRP3 and the ASC adaptor protein would be essential for inflammasome activation and IL-1β processing/secretion after *Vibrio* infection. Indeed, caspase-1 activation and IL-1β processing/secretion induced by infection with *V. vulnificus* or *V. cholerae* were abrogated in macrophages deficient in NLRP3 or ASC, but not NLRC4 (Fig. 3*A*–*D*). Furthermore, LDH release from infected NLRP3- and ASC-deficient macrophages was slightly reduced at 2 hpi similar to that observed in caspase-1–deficient macrophages, but by 3 hpi, the amount of LDH release in culture supernatants was comparable in mutant and WT macrophages (Fig. 3*E*, 3*F*). These results indicate that the NLRP3-ASC inflammasome is critical for caspase-1 activation and IL-1β secretion but plays only a partial and transient role in mediating LDH release in response to *Vibrio* infection, consistent with the role of caspase-1 on pyroptosis (Fig. 1*E*). We further tested the role of NLRP3 or ASC in vivo during *V. vulnificus* infection. Although there is significant difference in the survival between ASC-deficient mice and WT mice ($p = 0.0081$ by log-rank test), *V. vulnificus* challenge resulted in no significant differences in mortality between NLRP3-deficient mice and WT mice ($p = 0.2019$) (Fig. 3*G*). These results indicated that ASC contributes to the susceptibility of mice to *V. vulnificus*, but NLRP3 does not play an important role in the susceptibility to infection in vivo.

ATP-mediated potassium efflux and NLRP3 activation is induced via P2X7R (21). We considered the possibility that *Vibrio* hemolysin and MARTX may indirectly trigger NLRP3 inflammasome by inducing the release of ATP from infected cells. However, because caspase-1 activation was not impaired in P2X7R-deficient BMMs upon *V. vulnificus* and *V. cholerae* (Supplemental Fig. 5), we excluded cellular ATP release-mediated NLRP3 activation in *Vibrio*-infected macrophages.

NLRP3 inflammasome activation by V. cholerae *is supported by NF-κB activation via the MyD88/TRIF-independent pathway*

We examined the role of NF-κB in NLRP3 activation by *V. vulnificus* or *V. cholerae*. Macrophages were infected with bacteria in the absence of previous stimulation with LPS that was used to

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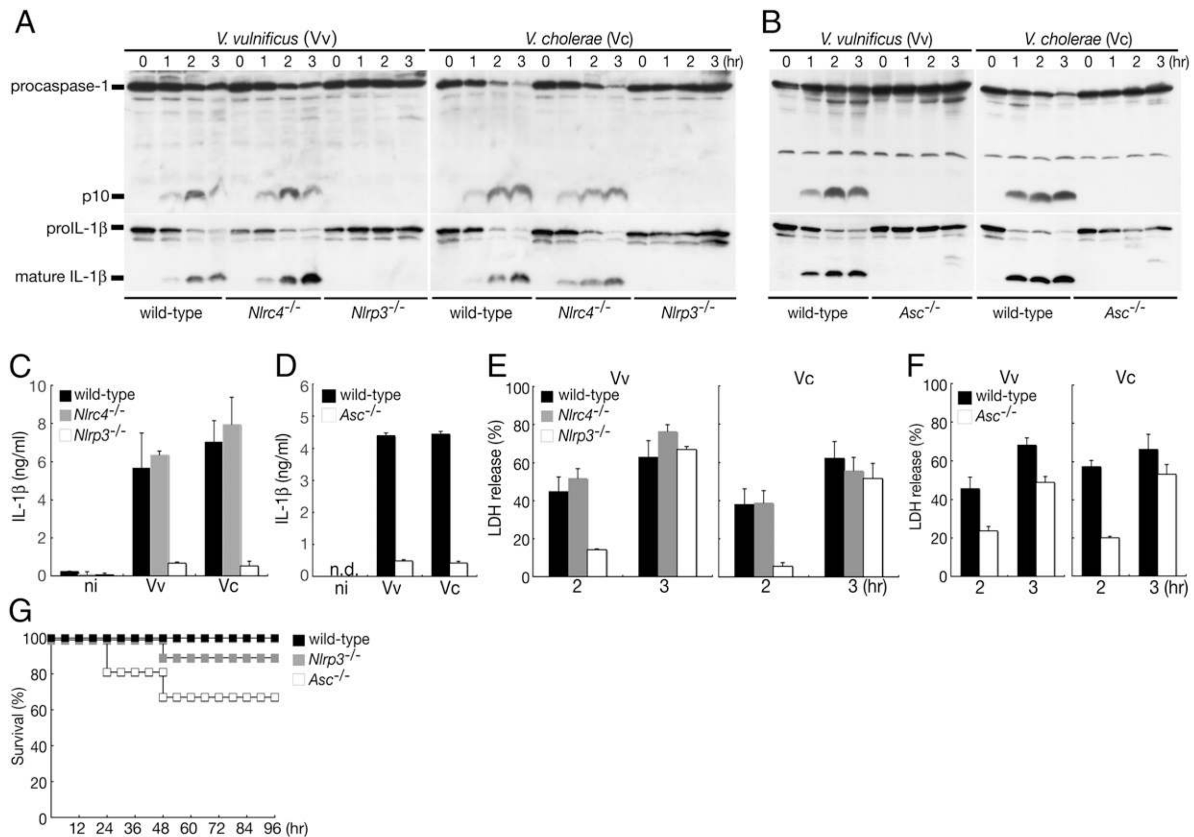

FIGURE 3. *V. vulnificus* (Vv)- and *V. cholerae* (Vc)-induced caspase-1 activation and IL-1β secretion are mediated by the NLRP3 inflammasome. BMMs from WT, NLRC4- (*Nlrc4*$^{-/-}$), NLRP3- (*Nlrp3*$^{-/-}$), or ASC-deficient (*Asc*$^{-/-}$) mice were primed with cLPS and infected with WT Vv or WT Vc. The samples were collected at indicated times. *A*, Immunoblot for caspase-1 activation or IL-1β processing during the infection of Vv or Vc in WT, NLRC4-, or NLRP3-deficient BMMs. *B*, Immunoblot for caspase-1 activation or IL-1β processing during the infection of Vv or Vc in WT or ASC-deficient BMMs. *C*, ELISA for IL-1β secretion into culture supernatants from infected WT, NLRC4-, or NLRP3-deficient BMMs at 3 hpi. The values are means ± SD of triplicate samples. *D*, ELISA for IL-1β secretion into culture supernatants from infected WT or ASC-deficient BMMs at 3 hpi. The values are means ± SD of triplicate samples. *E*, LDH release into culture supernatants from infected WT, NLRC4-, or NLRP3-deficient BMMs at 2 or 3 hpi. The values are means ± SD of triplicate samples. *F*, LDH release into culture supernatants from infected WT or ASC-deficient BMMs at 3 hpi. The values are means ± SD of triplicate samples. *G*, Survival curve for WT, NLRP3-, or ASC-deficient mice after i.p. infection with Vv (2.5×10^4 cfu/mouse; n = 15 for WT; n = 19 for NLRP3-deficient; n = 21 for ASC-deficient mice). n.d., none detected.

induce the expression of pro–IL-1β in Figs. 1–3. The expression of pro–IL-1β and its cleavage were induced by infection with both *Vibrio* pathogens in unprimed macrophages (Fig. 4*A*). We also examined the processing of IL-18 that is expressed constitutively and is processed by activated caspase-1. The processing of caspase-1 and IL-18 was induced in unprimed macrophages by infection with both *Vibrio* pathogens (Fig. 4*A*), indicating that the priming by LPS is not necessary for inducing caspase-1 activation. Likewise, IκBα degradation, a marker of NF-κB activation, was readily detected 10 min postinfection with the two *Vibrio* spp. (Fig. 4*B*). Caspase-1 activation was inhibited by BAY 11-7082, a drug that inhibits NF-κB activation by targeting the IκB kinase complex (Fig. 4*C*, *left panel*) (65), thus NF-κB activation is induced soon after bacterial infection and required for caspase-1 activation.

We next wished to examine the relationship of TLR signaling and the NLRP3 inflammasome during *Vibrio* infection, because TLR engagement induces NF-κB activation upon bacterial infection. To clarify whether TLR-mediated signaling is necessary for caspase-1 induction, we used MyD88/TRIF double-deficient macrophages, which are impaired in downstream signaling and cytokine production by all TLRs (54). As expected, pro–IL-1β was not induced in response to *Vibrio* infection in MyD88/TRIF double-deficient macrophages (Fig. 4*A*). The lack of TLR signaling in MyD88/TRIF-deficient macrophages inhibited caspase-1 activation as well as IL-18 processing and secretion after *V. vulnificus* infection (Fig. 4*A*, 4*D*). Thus, *V. vulnificus*-induced NLRP3 activation occurs predominantly in a MyD88/TRIF-dependent manner similar to that observed with LPS plus ATP (48, 49).

In contrast to *V. vulnificus*, infection with *V. cholerae*-induced caspase-1 activation and IL-18 secretion was comparable in MyD88/TRIF-deficient macrophages and WT macrophages (Fig. 4*A*, 4*D*). However, the induction of IκBα degradation was still observed during infection (Fig. 4*B*), and the NF-κB inhibitor BAY 11-7082 abrogated caspase-1 activation and IL-18 processing in MyD88/TRIF-deficient cells (Fig. 4*C*, *right panel)*. These results suggested that, in the *V. cholerae* infection, non-TLR ligands produced by the bacteria can induce NF-κB activation to support the activation of the NLRP3 inflammasome. Moreover, infection of MyD88/TRIF double-deficient macrophages with either VvΔ*rtxA* or VvΔ*vvhA* resulted in markedly reduced caspase-1 activation and IL-18 processing (Fig. 4*E*, *left panel*), whereas no significant differences were observed between WT and MyD88/TRIF-deficient macrophages postinfection with either VcΔ*hlyA* or VcΔ*rtxA* (Fig. 4*E*, *right panel*). These results suggest that the dependency on TLR signaling is related to the cytotoxins produced by *V. vulnificus*.

Recent studies have shown that NF-κB activation by LPS leads the upregulation of NLRP3 expression, which appears critical for

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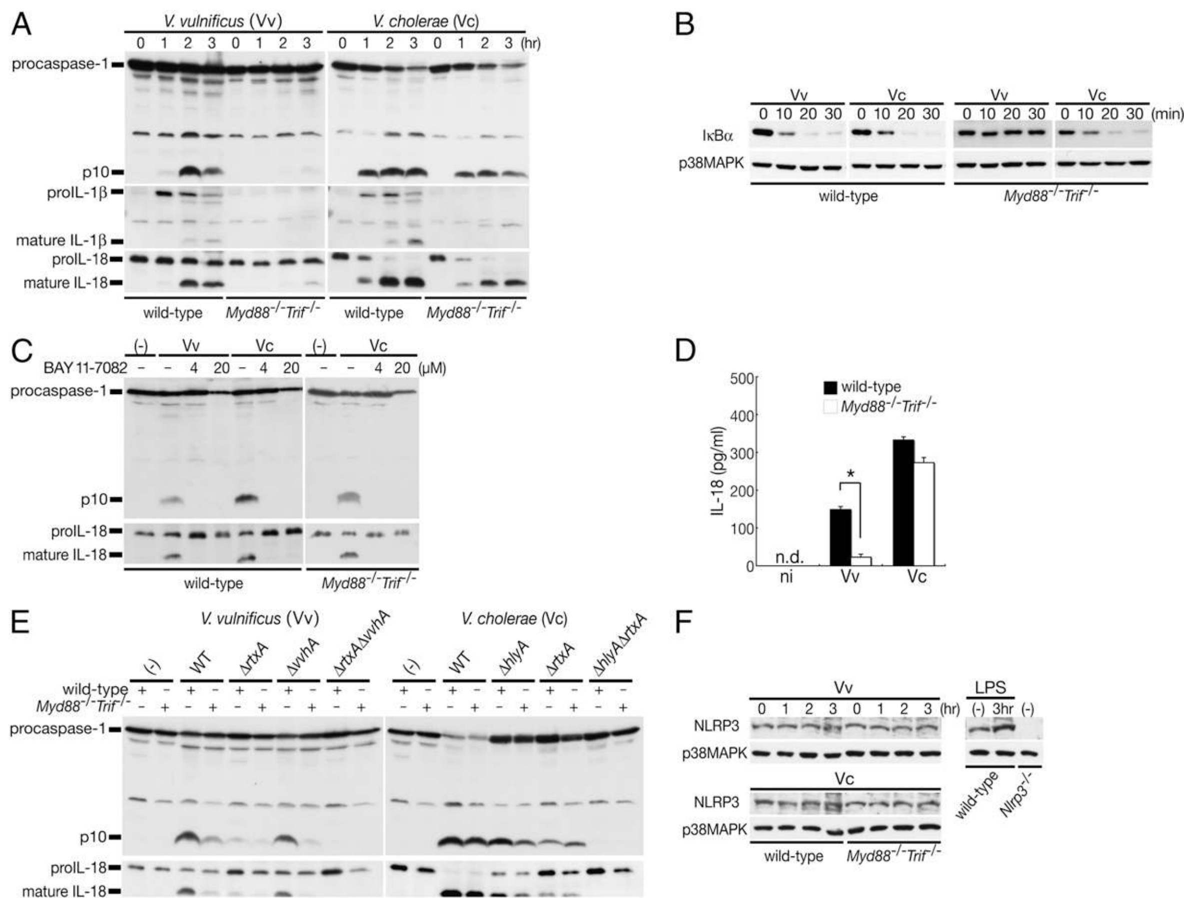

FIGURE 4. The NLRP3 inflammasome activated by *V. vulnificus* (Vv) and *V. cholerae* (Vc) is mediated by NF-κB activation and differentially regulated by TLR signaling. Unprimed WT or MyD88/TRIF double-deficient BMMs were infected with WT Vv, WT Vc, or their respective isogenic mutants. *A*, Immunoblot for caspase-1 activation or processing of IL-1β or IL-18 postinfection with WT strains of Vv or Vc. *B*, Immunoblot for IκBα degradation and p38MAPK as a loading control. The samples were collected at indicated time. *C*, The effect of BAY 11-7082, the inhibitor of NF-κB activation, on caspase-1 activation and IL-18 processing induced by WT Vv or WT Vc. The inhibitor was added to the cells before 1 h of infection, and samples were collected at 2 hpi. *D*, ELISA for IL-18 secretion at 3 hpi with WT Vv or WT Vc strains. The values are means ± SD of triplicate samples. *E*, Immunoblot for caspase-1 activation or IL-18 processing in BMMs infected with WT or isogenic mutants (Vv, *left panel*; Vc, *right panel*) at 3 hpi. *F*, Immunoblot for NLRP3 and p38MAPK as a loading control postinfection with WT strains of Vv or Vc. As controls, unprimed, and uLPS-primed (3 h) WT BMMs and NLRP3-deficient BMMs are also shown. The samples were collected at indicated time. Significant differences (*$p < 0.05$). n.d., none detected.

caspase-1 activation (48). Therefore, we evaluated the NLRP3 levels upon infection with two *Vibrio* spp. in unprimed WT or MyD88/TRIF-deficient macrophages. Consistent with previous studies (48), LPS induced the expression of NLRP3 in WT but not NLRP3-deficient macrophages as determined by immunoblotting (Fig. 4*F*). However, we found no significant change in NLRP3 expression postinfection with *Vibrio* spp. (Fig. 4*F*), suggesting that upregulation of NLRP3 is dispensable for caspase-1 activation in *Vibrio* infection.

Because potassium efflux is a limiting factor to induce NLRP3 inflammasome activation, we examined the efflux of potassium after bacterial infection of WT, NLRP3-deficient, and MyD88/TRIF double-deficient macrophages. The lack of MyD88 and TRIF or NLRP3 did not affect potassium efflux induced by ATP or either *Vibrio* sp. (Supplemental Fig. 6), indicating that potassium efflux itself is triggered independently of NLRP3 or MyD88/TRIF.

Heat-stable bacterial component released from V. cholerae *and* Escherichia coli *can mediate MyD88/TRIF-independent caspase-1 activation in the presence of hemolysin*

To begin to investigate the mechanisms of MyD88/TRIF-independent NLRP3 activation by cytotoxins secreted by *V. cholerae*, we assessed the ability of culture supernatants from WT and mutant of *V. cholerae* to induce caspase-1 activation. Culture supernatants from WT *V. cholerae*, VcΔ*hlyA* (secreting MARTX), and VcΔ*rtxA* (secreting HlyA), but not VcΔ*hlyA*Δ*rtxA* (neither toxin produced), induced comparable levels of caspase-1 activation and IL-18 processing in WT and MyD88/TRIF-deficient macrophages (Fig. 5*A*). These results suggest that either secreted toxin is sufficient to activate caspase-1 in the absence of TLR signaling. We next sought to confirm these results using purified *V. cholerae* cytotoxin. We could not perform experiments with *V. cholerae* MARTX because this toxin is of very high m.w. and unstable, thus we encountered technical difficulties during purification. However, *V. cholerae* hemolysin could be purified from bacterial culture supernatants (Supplemental Fig. 7). Caspase-1 activation induced by purified hemolysin was greatly reduced as well as IL-18 processing and secretion in MyD88/TRIF double-deficient macrophages compared with that observed in WT macrophages (Fig. 5*B*, 5*C*). These results suggest that *V. cholerae* hemolysin activates caspase-1 in a TLR-dependent manner. However, the latter finding was inconsistent with data revealing MyD88/TRIF-independent caspase-1 activation in response to infection with whole bacteria and stimulation with bacterial culture supernatants (Fig. 4*A*, 5*A*). Both active and heat-inactivated hemolysin induced IκBα degradation in WT macrophages, but not in MyD88/TRIF double-deficient macrophages (Fig. 5*D*), suggesting that TLR signaling is involved in caspase-1 activation by hemolysin and that the purified hemolysin preparation is contaminated with TLR ligands to activate NF-κB. We therefore hypothesized that, as with other NLRP3

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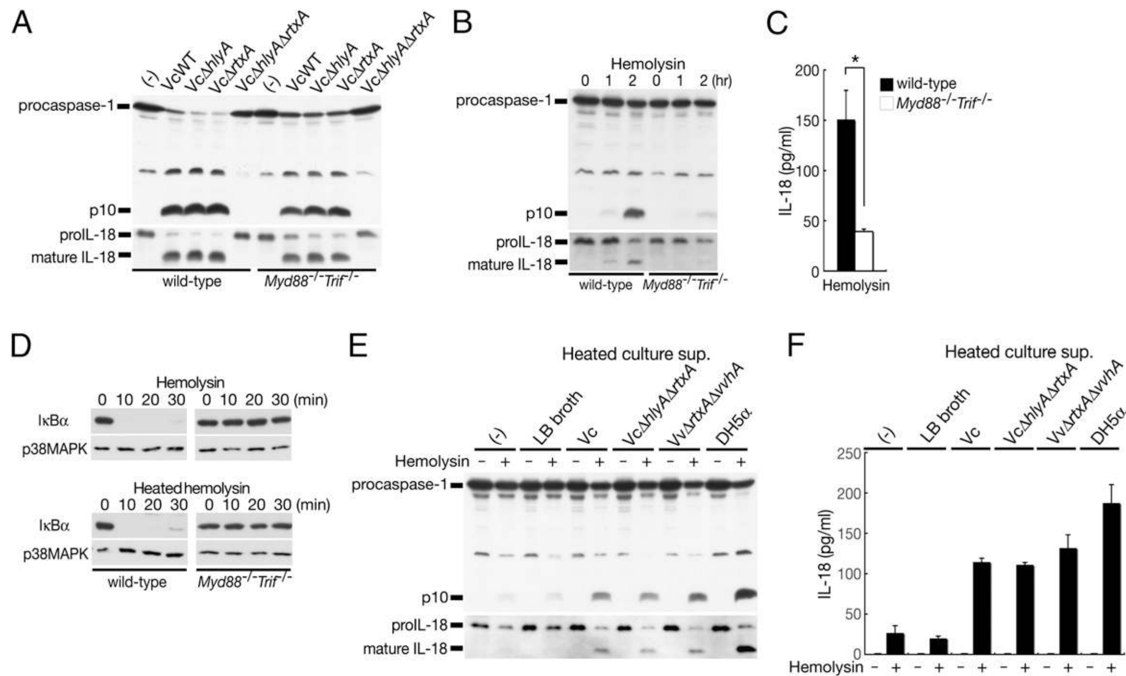

FIGURE 5. Activation of the NLRP3 inflammasome by purified *V. cholerae* hemolysin is dependent upon MyD88/TRIF but is independent of MyD88/TRIF in the presence of culture supernatants from Gram-negative bacteria. WT or MyD88/TRIF double-deficient BMMs were treated with purified *V. cholerae* (Vc) hemolysin (5 μg/ml) and/or culture supernatants from Gram-negative bacteria. Immunoblot and ELISA samples were collected at 2 h postincubation unless indicated. *A*, Immunoblot for caspase-1 activation or IL-18 processing in WT or MyD88/TRIF double-deficient BMMs postincubation with culture supernatants from WT Vc, Δ*hlyA* (HlyA mutant), Δ*rtxA* ($MARTX_{Vc}$ mutant), or Δ*hlyA*Δ*rtxA* (double mutant). *B*, Immunoblot for caspase-1 or IL-18 in WT or MyD88/TRIF double-deficient BMMs posttreatment with purified Vc hemolysin. *C*, ELISA for IL-18 in WT or MyD88/TRIF double-deficient BMMs posttreatment with purified Vc hemolysin. The values are means ± SD of triplicate samples. *D*, Immunoblot for IκBα degradation and p38MAPK in WT or MyD88/TRIF double-deficient BMMs poststimulation with purified or heat-treated (100°C for 10 min) Vc hemolysin. *E*, Immunoblot for caspase-1 or IL-18 in MyD88/TRIF double-deficient BMMs postincubation with bacterial culture supernatants in the presence or absence of purified Vc hemolysin. *F*, ELISA for IL-18 secretion from MyD88/TRIF double-deficient BMMs postincubation with bacterial culture supernatants in the presence or absence of purified Vc hemolysin. The values are means ± SD of triplicate samples. Results are representative of at least two independent experiments. Significant differences ($*p < 0.05$).

activators, such as ATP (48, 49), *V. cholerae* hemolysin-induced caspase-1 activation can be also supported by TLR-independent NF-κB activation pathways, and such NF-κB activators might be removed during hemolysin purification. To further analyze the mechanism of MyD88/TRIF-independent caspase-1 activation induced via hemolysin, purified hemolysin was mixed with heat-treated culture supernatants from WT *V. cholerae* and VcΔ*hlyA*Δ*rtxA*. Heat-treated culture supernatants enhanced caspase-1 activation in the presence of hemolysin when compared with that observed in macrophages incubated with hemolysin or culture supernatant alone (Fig. 5*E*). Moreover, heat-treated culture supernatants of VvΔ*rtxA*Δ*vvhA* mutant and *E. coli* DH5α also facilitated hemolysin-induced caspase-1 activation and IL-18 maturation/secretion in MyD88/TRIF double-deficient macrophages (Fig. 5*E*, 5*F*). These results suggest that non-TLR–stimulating heat-stable bacterial component(s) released from *Vibrio* spp. and *E. coli* can support hemolysin-mediated MyD88/TRIF-independent caspase-1 activation.

Nod1/Nod2 ligands promote caspase-1 activation triggered by V. cholerae *hemolysin in MyD88/TRIF double-deficient macrophages*

Peptidoglycan fragments are released by growing Gram-negative bacteria (66–68). To examine the release of peptidoglycan moieties in the culture supernatant of *Vibrio*, we assessed the presence of Nod1 and Nod2 stimulatory activity by an NF-κB reporter assay using 293T cells transfected with Nod1 or Nod2 as described previously (69, 70). The heat-treated culture supernatants of *V. cholerae* contained at least Nod1 ligand but little or no Nod2 stimulatory activity (Supplemental Fig. 8). To determine whether the peptidoglycan-related molecules iE-DAP (Nod1 ligand) and MDP (Nod2 ligand) can support caspase-1 activation induced by hemolysin, we stimulated macrophages with Nod1 or Nod2 ligand or LPS as a control with or without purified hemolysin. Both iE-DAP and MDP, but not LPS, enhanced hemolysin-mediated caspase-1 activation and IL-18 processing/secretion in MyD88/TRIF double-deficient macrophages (Fig. 6*A*, 6*B*). The addition of iE-DAP, MDP, iE-DAP plus MDP, or LPS alone did not affect caspase-1 activation. Caspase-1 activation triggered with hemolysin plus Nod1/Nod2 ligands was inhibited by the addition of BAY11-7082 (Fig. 6*C*), suggesting NF-κB activation is involved. Indeed, IκBα degradation was observed only when the cells were stimulated with active hemolysin plus iE-DAP and MDP, but not with heat-inactivated hemolysin plus ligands or ligands alone (Fig. 6*D*). Furthermore, we ruled out the alternative caspase-1 activation pathway by MDP-stimulated NLRP1 (7), because caspase-1 activation by hemolysin plus Nod1/Nod2 ligands was abolished in NLRP3-deficient macrophages (Fig. 6*C*). Together, these data suggest that Nod1/Nod2 ligands facilitate hemolysin-stimulated NLRP3 inflammasome activation by inducing NF-κB activation.

Cathepsin B activity is not involved in caspase-1 activation induced by Vibrio *infection*

We examined whether the caspase-1 activation by *V. vulnificus* or *V. cholerae* is mediated by lysosome damage-induced NLRP3 activation. The cytotoxin-mediated caspase-1 activation induced by *V. vulnificus* and *V. cholerae* as well as stimulation with LPS plus ATP was not affected by the cathepsin B inhibitor CA-074-Me or E64d

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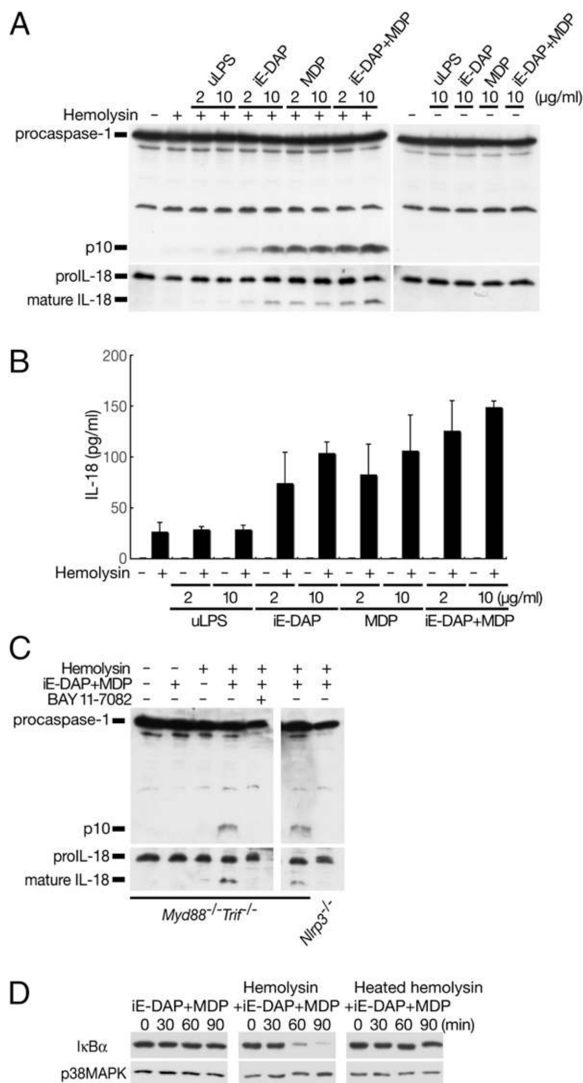

FIGURE 6. TLR-independent activation of the NLRP3 inflammasome by purified Vc hemolysin is enhanced by addition of Nod1 or Nod2 ligands. MyD88/TRIF double-deficient BMMs were incubated with or without purified Vc hemolysin (5 μg/ml) in the presence of TLR or Nod1/Nod2 ligands. Immunoblot and ELISA samples were collected at 2 h postincubation unless indicated. *A*, Immunoblot for caspase-1 activation and IL-18 processing in MyD88/TRIF double-deficient BMMs poststimulation with or without purified Vc hemolysin (5 μg/ml) in the presence or absence of 2 or 10 μg/ml uLPS (TLR4 ligand), iE-DAP (Nod1 ligand), MDP (Nod2 ligand), or iE-DAP plus MDP. *B*, ELISA for IL-18 secretion from MyD88/TRIF double-deficient BMMs poststimulation as shown in *A*. The values are means ± SD of triplicate samples. *C*, Immunoblot for caspase-1 activation and IL-18 processing in MyD88/TRIF double-deficient or NLRP3-deficient BMMs poststimulation with or without purified Vc hemolysin (5 μg/ml) in the presence or absence of 10 μg/ml iE-DAP and MDP or BAY 11-7082 (20 μM). *D*, MyD88/TRIF-deficient BMMs were incubated with iE-DAP plus MDP with either purified or heat-treated Vc hemolysin followed by immunoblot for IκBα degradation and p38 MAPK. Results are representative of at least two independent experiments.

(Fig. 7), indicating that different signals acting downstream of lysosomal damage and cytotoxins induce caspase-1 activation via NLRP3.

Discussion

V. vulnificus and *V. cholerae* are Gram-negative pathogens that cause significant infectious disease in humans. The production of cytokines, such as IL-1β, is thought to be involved in inflammatory responses and disease development during infection with these

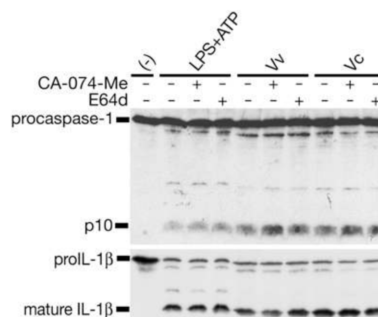

FIGURE 7. The cathepsin B inhibitor CA-074-Me or E64d in macrophages does not affect caspase-1 activation and IL-1β processing in response to *Vibrio* infection. The uLPS-primed WT BMMs were treated with ATP or infected with either *V. vulnificus* (Vv) or *V. cholerae* (Vc) for 1 h in the presence or absence of CA-074-Me (10 μM) or E64d (10 μM) followed by immunoblot for caspase-1 activation and IL-1β processing.

pathogens (50, 51). Although a number of virulence factors of *V. vulnificus* and *V. cholerae* have been characterized, little is known about the pathogen and host factors contributing to the inflammatory response observed during *Vibrio* infection. In this study, we show that infection with *V. vulnificus* or *V. cholerae* induces secretion of IL-1β and IL-18 from macrophages, and these host responses are mediated by caspase-1 activation via the NLRP3 inflammasome.

Growing evidence suggests that a variety of structurally different molecules activate the NLRP3 inflammasome (47). We undertook a genetic approach to demonstrate that hemolysins and MARTXs produced by *V. vulnificus* and *V. cholerae* mediate NLRP3-dependent caspase-1 activation. Recent studies showed certain bacterial pore-forming toxins induce caspase-1 activation via NLRP3 (21, 31–34). Because *V. vulnificus* VvhA and *V. cholerae* HlyA are known as pore-forming toxins (71, 72), it is possible that these hemolysins can trigger NLRP3 inflammasome activation by forming pores on host cell membranes. We also identified MARTXs produced by *V. vulnificus* and *V. cholerae* as other bacterial factors capable of mediating caspase-1 activation. Notably, $MARTX_{Vv}$ can form pores on host cell membranes (62), whereas $MARTX_{Vc}$ possesses no pore-forming activity but can induce cross-linking of cellular actin (61). Although the precise function of $MARTX_{Vv}$ is not fully understood, it is possible that its pore-forming activity plays a major role in promoting the activation of the NLRP3 inflammasome. Unlike $MARTX_{Vc}$, $MARTX_{Vv}$ does not possess an actin cross-linking domain, but contains a putative Rho GTPase inactivation domain and C-terminal autocatalytic cysteine protease domain whose activity is essential for the toxic action of $MARTX_{Vc}$ (60). Besides pore-forming activity, it is possible that intracellular cleaved fragments of $MARTX_{Vv}$ induce NLRP3 inflammasome activation by a mechanism that remains unclear. Further work is also needed to understand whether the actin cross-linking function of $MARTX_{Vc}$ is involved in NLRP3 inflammasome activation. A common property of all bacterial MARTXs is their large size; molecular masses range from 100–177 kDa, and and they have conserved regions and putative functional domains. Although the MARTX family of toxins is of biochemical interest because of their unusual structural properties and cytopathic effects, the function of each MARTX is still unknown. Our results provide a new biological function of MARTXs through their ability to induce NLRP3 inflammasome activation for eliciting proinflammatory responses upon infection.

Recent studies showed that NF-κB activation is necessary for the activation of NLRP3 inflammasome (48, 49). We confirmed that NF-κB activation is also necessary for inducing the NLRP3 inflammasome by two *Vibrio* infections using specific inhibitor for

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NF-κB activation. NF-κB activation is induced by MyD88/TRIF-mediated signaling in *V. vulnificus* infection, but unexpectedly, we discovered that NF-κB activation by *V. cholerae* infection is independent of MyD88/TRIF-mediated signaling. TLR signaling-independent NLRP3 activation was also shown in the infection of *Streptococcus pyogenes* (33) or *Staphylococcus aureus* (34). It has been reported that NF-κB activation-mediated upregulation of NLRP3 facilitates caspase-1 activation in LPS-primed and ATP-stimulated macrophages (48). Although the discrepancy may be attributable to different experimental conditions and cells used, our results suggest that, in addition to NLRP3 induction, other factors mediated by NF-κB activation are likely involved in NLRP3 inflammasome activation in *Vibrio* infection.

Culture supernatants from *V. cholerae* mutants expressing either hemolysin or $MARTX_{Vc}$ triggered caspase-1 activation and IL-18 maturation in MyD88/TRIF-deficient macrophages, yet macrophages treated with partially purified hemolysin displayed a significant reduction in caspase-1 activity in MyD88/TRIF-deficient macrophages, which could be compensated for by the addition of Nod1/Nod2 ligands. These data suggest that, in the absence of TLR signaling pathway, Nod1/Nod2 ligands released by bacteria into the extracellular milieu may be introduced into the host cell cytosol via hemolysin pores to induce NF-κB activation for caspase-1 activation. This notion is similar to the notion that *S. aureus* α-toxin facilitates Nod2-dependent recognition of MDP to induce inflammatory cytokine responses (73). Although the precise role of Nod1/Nod2 in NLRP3 inflammasome activation must await further analysis, this hypothesis is supported by our observation that IκBα degradation is induced only in the presence of biologically active hemolysin and Nod1/Nod2 ligands. The mechanism that accounts for the differential role of TLR signaling in the activation of the NLRP3 inflammasome in response to pathogenic *Vibrio* is unclear. One possibility is that hemolysin produced by *V. cholera* is more capable than that produced by *V. vulnificus* in allowing internalization of Nod1/Nod2 ligands into the host cytosol to induce NF-κB activation. It is also possible that damage of the cells by the actin-depolymerization activity of MARTXVc may allow Nod1/Nod2 ligand internalization. It is tempting to speculate that the different toxins exert differential efficiency to promote the transport of microbial ligands across the membrane pores or damaged membranes. Therefore, TLR- and Nod1/2-mediated signaling can induce NF-κB activation in *V. cholerae* infection, and this redundant signaling may promote caspase-1 activation. The biological significance of TLR-independent NLRP3 inflammasome activation induced by *V. cholerae* remains unclear. It is possible that it may be beneficial to the host by triggering an inflammatory response against *V. cholerae* infection in the small intestine, where lamina propria macrophages are hyporesponsive to TLR ligands (74). Alternatively, *V. cholerae* may induce inflammatory responses through the infection of intestinal cells, which do not express TLRs or are insensitive to TLR signals to evoke tissue damage that may promote bacterial colonization.

Based on recent findings and our data presented in this study, the NLRP3 inflammasome appears to be activated by two classes of stimulators; one class is represented by pore-forming inducers, such as ATP and bacterial pore-forming toxins, and the second class by lysosomal destabilizing agents including alum, monosodium uric acid, asbestos, and silica. In the case of alum and silica, phagocytosed particles induce NLRP3 inflammasomes by an unidentified mechanism brought about, at least in part, as a result of lysosomal membrane damage and cathepsin B activation (24). We propose that actin cross-linking $MARTX_{Vc}$ of *V. cholerae* belongs to a third class of NLRP3 activator. Cellular signaling via MyD88/TRIF or presumably via Nod1/Nod2 is required for NF-κB activation that promotes NLRP3 inflammasome in *Vibrio* infection. Although the underlying principles of molecular signaling supporting NLRP3 activation are still not fully understood, our findings indicate that *V. vulnificus* hemolysin and $MARTX_{Vv}$ activate NLRP3 in a TLR-dependent manner, whereas *V. cholerae* hemolysin and $MARTX_{Vc}$ can induce NLRP3 activation via TLR-independent signals. Our results indicate that V. *cholerae* hemolysin can promote NLRP3 activation through both TLR- and Nod1/Nod2-dependent stimulation. Further work is needed to fully understand how NLRP3 recognizes bacterial infection and danger signals to ultimately activate innate inflammatory responses.

Acknowledgments

We thank R. Flavell and K. Kuida for providing caspase-1–deficient mice and Anthony Coyle, Ethan Grant, and John Bertin (Millennium Pharmaceuticals, Cambridge, MA) for the generous supply of mutant mice. We also thank Dr. T. Iida for materials and Dr. Anita C. Wright for bacterial strain.

Disclosures

The authors have no financial conflicts of interest.

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