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A Critical Role for Hemolysins and Bacterial Lipoproteins in Staphylococcus aureus-Induced Activation of the Nlrp3 Inflammasome†

Raúl Muñoz-Planillo,* Luigi Franchi,* Lloyd S. Miller, † and Gabriel Núñez2*

The mechanism by which bacterial pathogens activate caspase-1 via Nlrp3 remains poorly understood. In this study, we show that the ability of Staphylococcus aureus, a leading cause of infection in humans, to activate caspase-1 and induce IL-1β secretion resides in culture supernatants of growing bacteria. Caspase-1 activation induced by S. aureus required α-, β-, and γ-hemolysins and the host Nlrp3 inflammasome. Mechanistically, α- and β-hemolysins alone did not trigger caspase-1 activation, but they did so in the presence of bacterial lipoproteins released by S. aureus. Notably, caspase-1 activation induced by S. aureus supernatant was independent of the P2X7 receptor and the essential TLR adaptors MyD88 and TIR domain-containing adapter-inducing IFN-β, but was inhibited by extracellular K⁺. These results indicate that S. aureus hemolysins circumvent the requirement of ATP and the P2X7 receptor to induce caspase-1 activation via Nlrp3. Furthermore, these studies revealed that hemolysins promote in the presence of lipoproteins the activation of the Nlrp3 inflammasome. The Journal of Immunology, 2009, 183: 3942–3948.

Staphylococcus aureus is a Gram-positive bacterium responsible for a wide variety of superficial and life-threatening systemic infections (1). It produces an array of virulence factors that allow the bacteria to colonize epithelial surfaces, invade host tissues, and evade the immune response (2). The bacterium also produces a number of disease-relevant exotoxins, such as toxic shock syndrome toxin-1, enterotoxins, and membrane-damaging toxins (cytolysins), which include hemolysins (α, β, γ, and δ) and Panton-Valentine Leucocidin (3, 4). α-Hemolysin is secreted as a 33-kDa monomer and oligomerizes, forming heptamer transmembrane pores (5). β-Hemolysin is a neutral sphingomyelinase (6). Both α- and β-hemolysins have the ability to damage the membrane of human monocytes and promote the secretion of IL-1β, IL-6, and TNF-α (7–10). Furthermore, α-hemolysin can induce K⁺ efflux in host cells (11, 12). However, the mechanism by which S. aureus hemolysins promote host immune responses remains poorly understood.

Host recognition of bacterial pathogens, including S. aureus, is mediated in part by pattern recognition receptors, including membrane-bound TLRs and intracellular nucleotide-binding oligomerization domain receptors (NLRs) (13). TLRs recognize conserved bacterial components such as LPS, flagellin, lipoproteins, lipoteichoic acid, and unmethylated CpG DNA (14). There is evidence that TLR2 is critical for S. aureus recognition and host defense (15). More recently, nucleotide-binding oligomerization domain-containing 2, a NLR protein that senses muramyl dipeptide produced during the synthesis and/or degradation of peptidoglycan, has been implicated in the host response to S. aureus (16). Although TLR2 and nucleotide-binding oligomerization domain-containing 2 induce immune responses via the activation of the transcription factor NF-κB and MAPKs (17), another group of NLRs that include Nlrp3 and Nlrc4 is critical for the activation of caspase-1 and IL-1β secretion in response to bacterial and endogenous stimuli (18). Nlrp3 and Nlrc4 link specific microbial stimuli to caspase-1 activation through the formation of a multiprotein platform called the inflammasome, which includes the adaptor apoptosis-associated speck-like (Asc) protein (18). Upon microbial stimulation, the IL-1β precursor is processed into mature IL-1β by the enzymatically active heterodimer composed of a 10- and a 20-kDa chain of caspase-1 (20). Nlrp3 is essential for caspase-1 activation in response to a variety of microbial molecules in combination with ATP and particulate matter, including urate crystals, silica particles, and aluminum salts (21–23). Stimulation of the purinergic P2X7 receptor (P2X7R) with ATP induces K⁺ efflux via the rapid opening of a nonselective cation channel and the gradual opening of a large pore possibly mediated by pannexin-1, both of which are important for Nlrp3-mediated caspase-1 activation in response to ATP and bacterial products (21, 24). In addition to ATP, activation of the Nlrp3 inflammasome is induced in LPS-stimulated macrophages by pore-forming toxin maitotoxin and the ionophore nigericin, which induce changes in cellular K⁺ concentration (25). However, the mechanism by which bacterial pathogens activate the Nlrp3 inflammasome is poorly understood.

Recent studies showed that the adaptor protein Asc and IL-1β can play a critical role in neutrophil recruitment elicited by S. aureus skin infection and elimination of the bacterium in vivo (26, 27). Furthermore, infection of macrophages with S. aureus induced low-level, but significant, caspase-1 activation via Nlrp3 in vitro (25). However, the latter finding remains controversial because...
some authors did not observe caspase-1 activation and IL-1β secretion in response to S. aureus infection in the absence of exogenous ATP (28). Furthermore, the S. aureus molecules that induce caspase-1 activation and IL-1β secretion remain unknown. The objective of this study was to clarify the mechanism by which S. aureus induces IL-1β and triggers the activation of caspase-1.

Materials and Methods

Mice and macrophages

Nlrp3−/−, Asc−/−, caspase-1−/− mice have been described (29). P2X7R−/− mice were obtained from G. Dubyak, Case Western Reserve University (Cleveland, OH). MyD88−/− and MyD88/TIR domain-containing adapter-inducing IFN-β (Trif)−/− mice were obtained from S. Akira, Osaka University (Osaka, Japan). All mice were backcrossed onto the C57BL/6 background at least eight times, except for the Nlrp3−/− mice, which were in mixed 129/C57BL/6 background. Wild-type (WT) C57BL/6 and 129/C57BL/6 mice were maintained in our animal facility. All animal studies were approved by the University of Michigan Committee on Use and Care of Animals.

Bacteria

S. aureus WT strain 8325-4 and its isogenic hemolysin mutants have been previously described (4). S. aureus WT strain SA113 and its isogenic mutant deficient in lipoprotein diacylglycerol transferase (Δgro) have also been previously described (30). Clinical isolates of S. aureus were obtained from the laboratory of Clinical Microbiology at the University of Michigan University Hospital with the approval of the Institutional Review Board of the University of Michigan Medical School. Tryptic soy broth was used for the growth of the bacteria.

Purified toxins

Purified α-hemolysin was purchased from Sigma-Aldrich (H9395), and β-hemolysin from Toxin Technology (BHT1). For caspase-1 activation experiments with purified toxins, macrophages were stimulated for 3 h with a 1/10 dilution of S. aureus supernatant collected after 20 h of subculture, washed three times with PBS, and subsequently incubated with 10 μg/ml α-hemolysin or β-hemolysin in IMDM.

In vitro S. aureus stimulation

Bacteria in midlogarithmic phase were obtained after subculturing bacteria at a 1/100 dilution of an overnight culture for 3 h. Bone marrow-derived macrophages were infected with S. aureus for 3 h or indicated time at the bacterial-macrophage ratio indicated in figures and either pulsed for 30 min, with 5 mM ATP or left untreated. S. aureus supernatants were prepared by subculturing 1/100 dilutions of overnight S. aureus cultures for different times (0–20 h). The cultures were centrifuged at 4000 × g for 10 min, and the supernatants were collected and sterilized by filtration. All in vitro stimulation of macrophages with S. aureus supernatants was performed for 3 h at 1/10 dilution of supernatants collected after 20 h of subculture, unless otherwise specified.

Cytotoxicity assay

The percentage of macrophage death was determined by measurement of the release of lactate dehydrogenase after incubation with the Cytotox 96 nonradioactive cytotoxicity assay (Promega). The absorbance at 490 nm was measured, and the percentage of cell death was calculated as follows: (experimental release — spontaneous release)/(maximum release — spontaneous release) × 100, in which spontaneous release is that found in untreated macrophages, and maximum release is the value obtained after lysis of macrophages with a solution of 0.1% Triton X-100.

Cutaneous S. aureus infection model

The s.c. abscess model has been previously described (26). Briefly, the mice were inoculated s.c. with 10⁶ CFUs of midlogarithmic growth phase S. aureus strain 8325-4 in 50 μl of PBS. Lesional skin biopsies were collected 24 h later and homogenized in the presence of proteinase inhibitors (Roche).

Immunoblotting and cytokine measurements

Membranes were probed with Abs against caspase-1 (a gift of P. Vandenbergbeele, Ghent University, Ghent, Belgium), mature IL-1β (p17, Asp116; Cell Signaling Technology), pro-IL-1β (pIL-1β; R&D Systems) (27), and α-hemolysin antiserum (Sigma-Aldrich). As a positive control for caspase-1 activation, WT macrophages were incubated with 1 μg/ml LPS for 3 h and stimulated for 30 min with 5 mM ATP. Levels of IL-1β, mature IL-18, and MIP-2α were determined by ELISA (MBL International).

Statistical analysis

Statistical significance between groups was determined by two-tailed Student’s t test. Animals studies were analyzed by one-way ANOVA. Differences were considered significant when p < 0.05.

Results

Culture supernatant of S. aureus is a potent activator of caspase-1

The ability of S. aureus to active caspase-1 in macrophages remains controversial (25, 28). Consistent with our previous results (28), infection of macrophages with S. aureus did not induce detectable activation of caspase-1 at different bacterial-macrophage ratio for up to 24 h of culture (Fig. 1A). However, incubation of macrophages with the highest multiplicity of infection of S. aureus tested and for extended periods of time (12–24 h) led to significant release of IL-1β (Fig. 1B), which was associated with elevated cytotoxicity (Fig. 1C). To further assess the ability of S. aureus to activate caspase-1, we incubated macrophages with S. aureus supernatants collected after bacterial growth in culture for different times. Notably, stimulation of macrophages for 3 h with culture supernatants from S. aureus collected after 2 h of growth elicited significant caspase-1 activation, which increased by 6 h and peaked by 12 h of bacterial culture (Fig. 1D). Consistently, a parallel increase in IL-1β secretion was elicited by culture supernatants of growing S. aureus (Fig. 1E). Caspase-1 activation and IL-1β secretion triggered by culture supernatant from S. aureus were comparable to that observed with LPS, but unlike that induced with the TLR4 agonist, that triggered by the bacterium supernatant did not require exogenous ATP stimulation (Fig. 1, D and E). These results indicate that S. aureus releases factor(s) during bacterial growth that activates caspase-1 in the absence of exogenous ATP.

α-, β-, and γ-hemolysins are required for the induction of caspase-1 activation by S. aureus supernatant

S. aureus secretes several toxins, including hemolysins, that function as pores and/or membrane-damaging factors (4, 7, 8). To begin to assess a role for hemolysis in caspase-1 activation, we determined the production of α-hemolysin by S. aureus during bacterial growth culture. Immunoblotting analysis revealed that α-hemolysin was first detectable in the culture supernatants by 6 h and increased by 12 h of bacterial culture (Fig. 2A). To assess whether α-hemolysin was induced during infection, we determined the production of α-hemolysin during culture of S. aureus with macrophages under conditions similar to those shown in Fig. 1A in which caspase-1 was not activated. Under these conditions, we did not observe detectable production of α-hemolysin (Fig. 2B). To assess the role of hemolysins in caspase-1 activation, culture supernatants from WT S. aureus or its isogenic mutants lacking α-, β-, or γ-hemolysins and all possible combinations were tested for their ability to induce caspase-1 activation in macrophages. The culture supernatants from single hemolysin mutant strains, particularly α-hemolysin, induced significant, although reduced, levels of caspase-1 activation when compared with WT S. aureus supernatant (Fig. 2C). Notably, combined deficiency of α-, β-, and γ-hemolysins abolished the ability of the S. aureus culture supernatant to activate caspase-1 (Fig. 2C) and to induce IL-1β secretion (Fig. 2D).

Thus, α-, β-, and γ-hemolysins released by S. aureus play an essential, although redundant, role in the activation of caspase-1 and the secretion of IL-1β in macrophages.
S. aureus supernatant activates caspase-1 via the Nlrp3 inflammasome independently of P2X7R and TLR signaling

To study the host recognition system involved in hemolysin-mediated caspase-1 activation, we analyzed macrophages deficient in several inflammasome components as well as critical TLR adapters and the P2X7R. Caspase-1 activation and IL-1β secretion induced by S. aureus supernatant were abolished in macrophages deficient in Nlrp3 or Asc (Fig. 3, A and B). In contrast, Nlrc4, a NLR family member required for caspase-1 activation in response to LPS and ATP, was not detectable in macrophages deficient in Nlrp3 or Asc (Fig. 3, A and B).

FIGURE 1. Factors released during S. aureus growth activate caspase-1. A–C, S. aureus (S.a.) does not activate caspase-1 in macrophages in vitro. Macrophages were infected with a S. aureus strain (S.a.) at MOI 1:10, 1:30, and 1:100 for 3, 6, 12, and 24 h. Caspase-1 activation was measured by immunoblotting with anti-caspase-1 Ab (A), IL-1β in culture supernatant by ELISA (B), and macrophage viability by lactate dehydrogenase assay (C). D and E, S. aureus supernatant activates caspase-1. S. aureus culture supernatants were prepared by subculturing S. aureus for the indicated amount of time. Macrophages were incubated for 3 h with a 1/10 dilution of each S. aureus supernatant and IL-1β and caspase-1 processing was determined. Stimulation with LPS plus ATP is shown as a control. ND, not detectable. Values represent mean ± SEM (n = 3). Results are representative of at least three separate experiments.

FIGURE 2. α-, β-, and γ-hemolysins secreted during S. aureus growth activate caspase-1. A, S. aureus secretes α-hemolysin during in vitro growth. S. aureus (S.a.) was subcultured for the indicated amount of time, and supernatants were immunoblotted with an anti-α-hemolysin antiserum. An isogenic strain deficient in α-hemolysin was used as a specificity control. The arrow indicates the 33-kDa monomer. B, Production of α-hemolysin during infection of macrophages with S. aureus under conditions similar to those used for Fig. 1A. Cell extracts were immunoblotted with anti-α-hemolysin antiserum. Culture supernatant from growing S. aureus (WT) was used as a positive control. C and D, α-, β-, and γ-hemolysins mediate caspase-1 activation induced by S. aureus supernatant. Macrophages were stimulated for 3 h with supernatant from WT S. aureus or its isogenic hemolysin mutants. Cell extracts were immunoblotted with anti-caspase-1 Ab and IL-1β measured in the cell supernatants. Values represent mean ± SEM (n = 3). ND, not detectable. *, Indicates a nonspecific band. Results are representative of at least three separate experiments.

**S. aureus supernatant activates caspase-1 via the Nlrp3 inflammasome independently of P2X7R and TLR signaling**

To study the host recognition system involved in hemolysin-mediated caspase-1 activation, we analyzed macrophages deficient in several inflammasome components as well as critical TLR adapters and the P2X7R. Caspase-1 activation and IL-1β secretion induced by S. aureus supernatant were abolished in macrophages deficient in Nlrp3 or Asc (Fig. 3, A and B). In contrast, Nlrc4, a NLR family member required for caspase-1 activation in response to LPS and ATP, was not detectable in macrophages deficient in Nlrp3 or Asc (Fig. 3, A and B).

FIGURE 3. S. aureus activates the Nlrp3 inflammasome independently of TLR signaling. A and B, Macrophages from WT, Asc−/−, Nlrp3−/−, p2X7R−/−, MyD88−/−, and MyD88/Trif−/− were stimulated for 3 h with supernatant from WT S. aureus. Stimulation with LPS and ATP is shown as a control. C and D, Caspase-1 activation and IL-1β secretion by S. aureus supernatant require K+ efflux. Macrophages were pretreated with 130 mM NaCl or KCl for 30 min and subsequently left unstimulated, or WT S. aureus supernatant (S.a. SN) was added to a final dilution of 1/10. Three hours later, supernatants were collected and analyzed for IL-1β, and cell extracts were immunoblotted with anti-caspase-1 Ab. ND, not detectable. Values represent mean ± SEM (n = 3). Results are representative of at least three separate experiments.
to Salmonella, was dispensable for caspase-1 activation and IL-1β secretion induced by S. aureus supernatant (data not shown). Nlrp3-mediated caspase-1 activation induced by bacterial molecules, including LPS, requires ATP-mediated stimulation of the P2X7R (25). However, processing of procaspase-1 and IL-1β secretion induced by S. aureus supernatant was unimpaired in macrophages deficient in P2X7 (Fig. 3, A and B). Furthermore, caspase-1 activation proceeded normally in macrophages deficient in MyD88 or both MyD88 and Trif, the adaptors that are essential for TLR signaling (Fig. 3A). In contrast, IL-1β secretion induced by S. aureus supernatant was abrogated in macrophages deficient in MyD88 or MyD88 and Trif (Fig. 3B). Thus, TLR signaling is required for IL-1β secretion, but not caspase-1 activation induced by S. aureus supernatant.

Activation of the Nlrp3 inflammasome in response to ATP or particulate matter in combination with TLR agonists is inhibited by high concentration of extracellular K+ (31, 32). Furthermore, the K+ efflux elicited by α- and β-hemolysin is necessary for IL-1β secretion (8, 33). Consistently, incubation of macrophages in medium rich in K+, but not Na+, impaired caspase-1 activation and IL-1β secretion induced by S. aureus supernatant (Fig. 3C and D). These results suggest that the K+ efflux triggered by hemolysins released during S. aureus growth regulates Nlrp3 inflammasome activation independently of the P2X7R.

Hemolysins and the Nlrp3 inflammasome are required for the activation of caspase-1 by S. aureus in vivo

To study the role of hemolysins in caspase-1 activation in vivo, we used a previously described model of S. aureus s.c. infection (26, 27). WT mice were injected s.c. with 1 × 10⁶ CFUs of WT S. aureus (S.a.) or its isogenic hemolysin mutants, and tissue extracts of the lesions were prepared 24 h postinfection. Immunoblotting of the skin tissue revealed that infection with S. aureus induced the production of pro-IL-1β (pIL-1β) and the cleavage of pro-IL-1β into its mature form (p17) (A, C, and E) and for the production of IL-18 and MIP-2α (B, D, and F). ND, not detectable. Values represent mean ± SEM. Results are representative of at least three separate experiments. *p > 0.05 (one-way ANOVA).

FIGURE 4. α-, β-, and γ-hemolysins activate the Nlrp3 inflammasome in vivo. WT, caspase-1−/−, Asc−/−, and Nlrp3−/− mice (n = 6) were injected s.c. with 10⁶ CFUs of WT S. aureus (S.a.) or its isogenic hemolysin mutants. Tissue biopsies were collected 24 h after injection and analyzed by immunoblotting for the expression of pro-IL-1β (pIL-1β) and the cleavage of pro-IL-1β into its mature form (p17) (A, C, and E) for the production of IL-18 and MIP-2α (B, D, and F). ND, not detectable. Values represent mean ± SEM. Results are representative of at least three separate experiments. *p > 0.05 (one-way ANOVA).

We next determined the role of the Nlrp3 inflammasome in the processing of pro-IL-1β and secretion of IL-18 in vivo. Immunoblotting of skin tissue extracts prepared after infection with S. aureus showed that production of the mature IL-1β (p17) was reduced in tissue from mice deficient in Nlrp3, Asc, and caspase-1 (Fig. 4E). Furthermore, the production of IL-18 was abrogated in mice deficient in Nlrp3, Asc, and caspase-1 (Fig. 4F). Taken together, these results indicate that S. aureus α-, β-, and γ-hemolysins play a critical role in the production of mature IL-1β and IL-18 through the Nlrp3 inflammasome in vivo.

Clinical samples of S. aureus induce caspase-1 activation via Nlrp3 independently of TLR signaling

To validate the results obtained with the S. aureus laboratory strain 8325-4, we next determined whether bacterial strains isolated from patients suffering from S. aureus infection induce caspase-1 activation via Nlrp3. In line with our previous results, α-hemolysin was released to the culture medium during in vitro growth of clinical S. aureus clinical isolates (Fig. 5A). Importantly, caspase-1 activation induced by the culture supernatant of the clinical S. aureus strains required Nlrp3, but was independent of MyD88 and Trif (Fig. 5B). These results indicate that the culture supernatant from S. aureus strains isolated from infected patients can induce caspase-1 activation via Nlrp3 independently of TLR signaling.

α- and β-hemolysins and another factor(s) released by S. aureus activate the Nlrp3 inflammasome

To further assess the ability of hemolysins to activate the Nlrp3 inflammasome, we tested whether purified α- and β-hemolysins could restore the ability to activate caspase-1 of the supernatant from S. aureus deficient in α-, β-, and γ-hemolysins. Notably, incubation of macrophages with purified hemolysins did not activate caspase-1 (Fig. 6A). However, addition of purified α- or β-hemolysin to supernatant from α-, β-, and γ-deficient S. aureus...
molsins cooperate with other factor(s) released by the bacteria to activate the Nlrp3 inflammasome.

**Lipoproteins released during S. aureus growth induce expression of pro-IL-1β and promote caspase-1 activation**

The bacterial cell wall of *S. aureus* contains several immunostimulatory molecules that are released during bacterial growth (30, 34). To identify the factor(s) present in the *S. aureus* supernatant that is required with hemolysins to activate caspase-1, we next investigated the role of the lipoproteins released by *S. aureus* in the secretion of IL-1β. The enzyme lipoprotein diacylglycerol transferase, encoded in the lgt locus, catalyzes the first step of lipoprotein biosynthesis (30). To determine whether lipoproteins are required for IL-1β secretion, we incubated WT and TLR2−/− macrophages with culture supernatants from the WT *S. aureus* strain SA113 or its isogenic lgt deletion mutant (Δlgt) and assessed the induction of pro-IL-1β. Immunoblotting analysis revealed that the mutant Δlgt strain was impaired in its ability to induce pro-IL-1β when compared with the WT SA113 strain (Fig. 7A). Consistently, TLR2-deficient macrophages failed to induce pro-IL-1β in response to WT supernatant (Fig. 7A). In line with previous studies (30, 34), we found that IL-6 secretion was abrogated and TNF-α secretion greatly reduced in macrophages stimulated with lipoprotein-deficient bacterial supernatants (Fig. 7, B and C).

We next tested whether bacterial lipoproteins present in the *S. aureus* supernatant contribute to caspase-1 activation. Unlike the 8325-4 strain, the culture supernatant from the WT SA113 *S. aureus* did not induce detectable caspase-1 activation (Fig. 7D), and this was associated with undetectable levels of α-hemolysin (Fig. 7E). Therefore, we tested whether purified α-hemolysin could restore the ability of strain SA113 supernatant to activate caspase-1. We found that the addition of α-hemolysin to macrophages previously stimulated with culture supernatant from the WT strain SA113 induced caspase-1 activation and IL-1β secretion, but each factor alone did not (Fig. 7, F and G). Importantly, culture supernatant from Δlgt mutant strain exhibited impaired caspase-1 activation and IL-1β secretion in the presence of purified α-hemolysin, when compared with that induced by supernatant from the WT parental strain (Fig. 7, F and G). These results indicate that lipoproteins released by *S. aureus* are important in inducing caspase-1 activation and IL-1β secretion.

**Discussion**

Previous studies have shown that IL-1β signaling and the adaptor protein Asc play a critical role in the clearance of *S. aureus* infection in the skin (26, 27). However, the mechanism by which the bacterium induces such host responses remained poorly understood. In this study, we provide evidence both in vitro and in vivo for a critical, but redundant function of *S. aureus* α-, β-, and γ-hemolysins in eliciting caspase-1 activation via the Nlrp3 inflammasome. This redundancy may explain, at least in part, why a previous study analyzing single hemolysin *S. aureus* mutants (25) did not reveal any significant role for these exotoxins in caspase-1 activation. Unlike the present work, these authors observed weak, but significant caspase-1 activation after *S. aureus* infection in LPS-stimulated macrophages (25). These differences in results could be due to the use of different culture conditions or the priming step with LPS before the stimulation with the bacteria. We also show that the main caspase-1-activating activity resides in the supernatants of growing *S. aureus*, which is consistent with the fact that

*FIGURE 5. S. aureus clinical isolates activate the Nlrp3 inflammasome independently of TLR signaling. A, S. aureus clinical isolates were subcultured for the indicated amounts of time, and supernatants were prepared and immunoblotted with anti-serum against α-hemolysin. S. aureus strain 8325-4 and its isogenic α-hemolysin-deficient mutant (α−) were used as specificity controls. B, WT, MyD88/Trif−/−, and Nlrp3−/− macrophages were stimulated for 3 h with supernatants of *S. aureus* clinical isolates and strain 8325-4. Stimulation with LPS and ATP is shown as a control. Cell extracts were immunoblotted with caspase-1 Ab. Results are representative of at least three different experiments.*

*FIGURE 6. α- and β-hemolysins and another factor(s) released by *S. aureus* activate the Nlrp3 inflammasome. A and B, Purified α- or β-hemolysin restores the ability of α−, β−, and γ-hemolysin-deficient *S. aureus* supernatant (αβγ− SN) to activate caspase-1 (A) and to induce IL-1β secretion (B). Macrophages from WT, Asc−/−, and Nlrp3−/− mice were stimulated with purified α- or β-hemolysin alone (−) or in the presence of αβγ− SN (+). Lysate prepared from WT macrophages stimulated with LPS and ATP (LPS + ATP (WT)) is shown as a reference control in all blots. Results are representative of at least three different experiments.*
hemolysins are exotoxins secreted by the bacteria (2). Experiments with purified α- and β-hemolysins showed that these toxins are necessary, but not sufficient to activate the Nlrp3 inflammasome, because they required the presence of other bacterial products to activate caspase-1. Specifically, we provide evidence that lipoproteins released by the bacterium are important for the induction of pro-IL-1β and the activation of the Nlrp3 inflammasome. Although S. aureus supernatant did not require P2X7R signaling to activate the Nlrp3 inflammasome, caspase-1 activation was blocked by incubation of macrophages in medium containing high concentration of K+. These results suggest that S. aureus hemolysins released by S. aureus elicit K+ efflux independently of P2X7R, which is important for the activation of caspase-1. In line with these results, purified α-hemolysin can induce K+ efflux (11), and this activity is thought to be necessary for IL-1β maturation (8, 35).

TLR2 plays an important role in the innate immune response to S. aureus and mediates the production of proinflammatory cytokines in macrophages and monocytes (36, 37). In this study, we examined the role of TLRs and bacterial lipoproteins in caspase-1 activation and IL-1β secretion induced by S. aureus in macrophages. Using macrophages that are deficient in the adaptor protein MyD88 and Trif, and that therefore cannot signal via TLRs, we found that caspase-1 activation induced by S. aureus supernatant is independent of TLR signaling. The results suggest that products released by S. aureus induce TLR-independent caspase-1 activation in combination with pore-forming toxins. In contrast, lipoproteins in the bacterial supernatant are important for pro-IL-1β induction through TLR2 signaling. Collectively, these results reveal distinct, but critical roles for lipoproteins and hemolysins, as well as the host TLRs and the Nlrp3 inflammasome in IL-1β secretion in response to S. aureus in macrophages.

Several bacteria activate caspase-1 through the formation of membrane pores. Salmonella, for instance, requires the presence of a functional type III secretion system to activate the Nlrp4 inflammasome (29). Similarly, Listeria monocytogenes requires the expression of the pore-forming protein listerolysin O to activate caspase-1 (38). In the current work, we show that α-, β-, and γ-hemolysins produced by S. aureus are critical for activating the Nlrp3 inflammasome both in vitro and in vivo. A common feature of these observations is the formation of pores that alter the permeability of host membranes and may allow the cytosolic delivery of microbial molecules. Consistent with this hypothesis, we have previously shown that ATP triggers through pannexin-1 the intracellular delivery of muramyl dipeptide, which induces caspase-1 activation via Nlrp3 (39). Alternatively, lipoproteins and other microbial molecules released by S. aureus may provide a priming signal, at least in part, independently of TLRs for inflammasome activation, and hemolysins may mimic ATP and P2X7R signaling to activate caspase-1. Because a direct association between microbial molecules and Nlrp3 proteins has not yet been identified, a likely model is that the activation of the inflammasome by microbial ligands is indirectly induced via an endogenous mediator. Consistently, there is evidence that reactive oxygen species, calcium-independent phospholipase A2, and cathepsin B may contribute to the activation of the Nlrp3 inflammasome at least in response to some stimuli (23, 32, 40, 41). Further work is needed to understand the mechanism whereby bacterial pathogens and host factors interact to induce inflammasome activation.

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### Disclosures

The authors have no financial conflict of interest.

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