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Activation of the Nlrp3 Inflammasome by Streptococcus pyogenes Requires Streptolysin O and NF-κB Activation but Proceeds Independently of TLR Signaling and P2X7 Receptor

Jürgen Harder, Luigi Franchi, Raúl Muñoz-Planillo, Jong-Hwan Park, Thornik Reimer, and Gabriel Núñez

Macrophages play a crucial role in the innate immune response against the human pathogen Streptococcus pyogenes, yet the innate immune response against the bacterium is poorly characterized. In the present study, we show that caspase-1 activation and IL-1β secretion were induced by live, but not killed, S. pyogenes, and required expression of the pore-forming toxin streptolysin O. Using macrophages deficient in inflammasome components, we found that both NLR family pyrin domain-containing 3 (Nlrp3) and apoptosis-associated speck-like protein (Asc) were crucial for caspase-1 activation and IL-1β secretion, but dispensable for pro-IL-1β induction, in response to S. pyogenes infection. Conversely, macrophages deficient in the essential TLR adaptors Myd88 and Trif showed normal activation of caspase-1, but impaired induction of pro-IL-1β and secretion of IL-1β. Notably, activation of caspase-1 by TLR2 and TLR4 ligands in the presence of streptolysin O required Myd88/Trif, whereas that induced by S. pyogenes was blocked by inhibition of NF-κB. Unlike activation of the Nlrp3 inflammasome by TLR ligands, the induction of caspase-1 activation by S. pyogenes did not require exogenous ATP or the P2X7R. In vivo experiments revealed that Nlrp3 was critical for the production of IL-1β but was not important for survival in a mouse model of S. pyogenes peritoneal infection. These results indicate that caspase-1 activation in response to S. pyogenes infection requires NF-κB and the virulence factor streptolysin O, but proceeds independently of P2X7R and TLR signaling.

Recognition of invading microorganisms by multicellular organisms is pivotal for the induction of a rapid and effective immune response. Initial recognition of microorganisms is mediated by pattern recognition receptors, ancient molecules of the innate immune system that can be found in plants, invertebrates, and vertebrates (1). The most extensively studied pattern recognition receptors are the TLRs, which comprise transmembrane proteins that recognize conserved bacterial components such as LPS, flagellin, lipoproteins, lipoteichoic acid, and unmethylated CpG DNA (2). More recently, another class of pattern recognition receptors called intracellular nucleotide-binding oligomerization domain containing (Nod)-like receptors (NLRs) have been identified. Whereas TLRs sense bacterial products present at the outer cell surface or in endosomes, NLRs mediate cytoplasmic recognition of bacterial products (3). The two best studied NLRs, nucleotide-binding oligomerization domain-containing 1 and 2 (Nod1 and Nod2), sense bacterial molecules produced during the synthesis and/or degradation of peptidoglycan and mediate the activation of the transcription factor NF-κB and MAPKs (4).

Another group of NLRs participates in the formation of a protein complex called the inflammasome, which mediates the induction of caspase-1 activation in response to microbial and endogenous stimuli. The inflammasome includes a NLR protein and an adaptor protein called apoptosis associated speck-like protein (Asc), which can link the NLR to the pro-caspase-1. Several NLRs can form inflammasomes, including NLR family pyrin domain-containing 3 (Nlrp3) (also known as cryopyrin or Nalp3), NLR family CARD domain-containing 4 (Nlrc4) (also known as Ipaf), and Nlrp1 (also known as Nalp1) (5). Activation of the inflammasome results in self-cleavage and activation of pro-caspase-1 into the active protease. Caspase-1, in turn, mediates the processing of several targets, including pro-IL-1β, into its biological active form (6–8). IL-1β plays an important role in the induction of immune responses and the development of inflammatory conditions such as fever and septic shock (9). Additionally, gain-of-function Nlrp3 mutations are associated with several autoinflammatory syndromes that are characterized by aberrant IL-1β processing and elevated IL-1β levels (10).

The inflammasomes are activated by different stimuli (8, 11). For example, mouse Nlrp1b is a sensor of lethal toxin produced by Bacillus anthracis (12); Nlrc4 detects cytosolic flagellin in cells infected with Salmonella (13, 14), Legionella (15), and Pseudomonas (16, 17). Additionally, Nlrc4 is activated by cytosolic Shigella in a flagellin-independent manner (18). The Nlrp3 inflammasome is activated by a variety of microbial ligands, including muramyl dipeptide, bacterial and viral RNA (19), as well as endogenous signals such as urate crystals (20). In cells exposed to...
TLR ligands, the activation of the Nlrp3 inflammasome requires a second signal that includes stimulation of the purinergic receptor P2X, ligand-gated ion channel 7 (P2X7R) by ATP (21), or addition of certain pore-forming toxins (22). Recent studies showed that TLR agonists promote activation of the Nlrp3 inflammasome via a priming effect that is mediated through TLR signaling and NF-κB activation (23, 24). In human monocytes, stimulation with TLR ligands induces the release of endogenous ATP that is thought to contribute to the production of IL-1β (25, 26). Additionally, in macrophages exposed to TLR ligands both the P2X7R agonist ATP (27, 28) and certain bacterial pore-forming toxins (29) potentiate IL-1β production. However, the role of TLR, the P2X7R, and pore-forming toxins in the regulation of IL-1β secretion in the context of more physiological conditions, including bacterial infection, remains largely unknown.

The Gram-positive bacterium Streptococcus pyogenes is an important human pathogen that causes various infections ranging from mild superficial skin and respiratory tract infections to life-threatening systemic diseases (30, 31). Recent studies showed an important role of macrophages in the host defense against S. pyogenes infection (32). However, the molecular interactions between S. pyogenes and macrophages are poorly understood. In the present study, we demonstrate that the production of IL-1β in macrophages infected with S. pyogenes is dependent on both TLR and Nlrp3 signaling. We further show that TLR signaling is required for induction of pro-IL-1β, whereas activation of caspase-1 is mediated by the pore-forming toxin streptolysin O (SLO) and the host Nlrp3 inflammasome but proceeds independently of TLR signaling and the P2X7R.

Materials and Methods

Mice

Mice deficient in caspase-1, Nlrc4, Asc, Nlrp3, Myd88/Trif, and P2X7R in C57BL/6j background have been previously described (13, 19, 24, 33, 34). All mice were backcrossed onto a C57BL/6 background at least six times. Wild-type (WT) C57BL/6j mice were originally purchased from The Jackson Laboratory and were maintained in our Animal Facility. The animal studies were conducted under approved protocols by the University of Michigan Committee on Use and Care of Animals. Mice were housed in a specific pathogen-free facility.

Bacteria

The S. pyogenes WT strain 950771 and its isogenic SLO-deficient mutant, a gift of Dr. M. A. Wessels (Boston, MA), have been described (35, 36). Bacteria were grown at 37°C in Todd-Hewitt broth (Difco) supplemented with 5% yeast extract (Difco). For stimulation, an overnight culture of the bacteria was diluted 1/10 in fresh broth and grown to late log phase for 4 h. After centrifugation at 3000 × g for 5 min, bacteria were washed twice in PBS and resuspended in IMDM without FCS and antibiotics.

Stimulation of macrophages with bacteria or recombinant toxin

Mouse bone marrow-derived macrophages were obtained from femurs and tibia as described (37). For stimulation, macrophages were cultured in 48-well plates (2.5 × 10⁵/well) or 6-well plates (2 × 10⁶/well) and infected with S. pyogenes for 3.5 h and harvested for immunoblotting. For measurement of secreted cytokines, cells were washed twice with PBS and incubated for additional 16 h in IMDM containing 10% heat-inactivated FBS and 300 μg/ml gentamicin to limit the growth of extracellular bacteria. Unless otherwise stated, experiments for cytokine secretion were performed at a bacterial-macrophage ratio of 4:1, and experiments for the evaluation of caspase-1 activation were performed at a bacterial-macrophage ratio of 10:1. For experiments with recombinant SLO, macrophages were stimulated with 10 μg/ml SLO (Sigma-Aldrich) and 10 mM DTT for 30–60 min in PBS without Ca²⁺ and Mg²⁺ in the absence or presence of ligands, then rinsed and incubated for 5 h in IMDM medium supplemented with heat-inactivated serum and antibiotics. Ultrapure LPS from Escherichia coli (10 μg/ml) and Pam3CSK (bacterial lipopeptide) at 10 μg/ml were from InvivoGen.

RESULTS

Live, but not heat-killed S. pyogenes induces IL-1β production

We first tested the secretion of IL-1β in bone-marrow derived macrophages infected with S. pyogenes. Live, but not heat-inactivated S. pyogenes induced IL-1β production (Fig. 1A). Macrophage responses to heat-killed S. pyogenes were not generally impaired because heat-killed bacteria induced higher amounts of TNF-α than did live bacteria (Fig. 1B). These results indicate that live bacteria specifically trigger IL-1β secretion.

S. pyogenes induces IL-1β production through caspase-1

We next investigated the role of caspase-1 in S. pyogenes-induced IL-1β secretion using YVAD-cmk, a specific caspase-1 inhibitory peptide. Incubation of S. pyogenes-infected macrophages with YVAD-cmk reduced the release of IL-1β by 70–80% (Fig. 2A).

FIGURE 1. Live, but not heat-killed S. pyogenes induces IL-1β production in macrophages. Macrophages were infected with viable S. pyogenes or heat-killed S. pyogenes and the release of IL-1β (A) and TNF-α (B) was determined by ELISA. Data shown are means ± SD of triplicate samples of one experiment representative of three independent experiments; n.d. indicates not detectable.

Immunoblotting

Cells were lysed together with the cell supernatant by the addition of 1% Nonidet P-40, complete protease inhibitor cocktail (Roche), and 2 mM DTT. After centrifugation at 20,000 × g for 15 min, the supernatant was mixed with 5 × SDS buffer and boiled for 5 min, and samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting was performed with Abs against caspase-1 (a gift of P. Van denabeele, University of Ghent, Ghent, Belgium) or anti IL-1β Ab (R&D Systems) as described (37).

Mouse infection

Mice were injected i.p. with 5 × 10⁵ CFU of S. pyogenes in PBS. After 20 h, serum was harvested and levels of IL-1β were determined by ELISA. Results were analyzed with Student’s t test and differences in data values were considered significant at a p value of <0.05. For the animal survival experiments, comparisons between animal groups were performed by the Kaplan-Meier method using Prism software. Differences in data values were considered significant at a p value of <0.05.

Measurement of cytokines

Mouse cytokines were measured in culture supernatants or serum by ELISA kits from R&D Systems. Assays were performed in triplicate for each independent experiment. Student’s t test was used to determine statistical significance. A p value of <0.05 was considered significant.
To determine more conclusively the role of caspase-1 in *S. pyogenes*-induced IL-1β production, we infected macrophages deficient in caspase-1. IL-1β induced by *S. pyogenes* was undetectable in macrophages deficient in caspase-1 (Fig. 2B). In contrast, the production of IL-1α was comparable in WT and caspase-1-null macrophages (Fig. 2C). These results indicate that caspase-1 is essential for the secretion of IL-1β, but not IL-1α, in macrophages infected with *S. pyogenes*.

The production of IL-1β induced by *S. pyogenes* infection is independent of the P2X7R

Human monocytes stimulated with microbial ligands secrete IL-1β, which is in part mediated by the release of endogenous ATP and stimulation of the P2X7R (25, 26). In contrast, human and mouse macrophages stimulated with TLR ligands or certain extracellular bacteria do not secrete IL-1β unless ATP is added exogenously (33). To investigate the role of P2X7R in the secretion of IL-1β induced by *S. pyogenes*, we infected macrophages deficient in the P2X7R and assessed the production of IL-1β. WT and P2X7R-deficient macrophages secreted comparable amounts of IL-1β (Fig. 3A) and TNF-α (Fig. 3C) in response to *S. pyogenes* infection. In contrast, stimulation of WT macrophages with TLR ligands and ATP induced the production of IL-1β, which was abrogated in macrophages deficient in the P2X7R (Fig. 3B). These results indicate that IL-1β production in macrophages infected with *S. pyogenes* is independent of the P2X7R.

S. pyogenes-induced caspase-1 activation and IL-1β release is dependent on SLO

The addition of recombinant SLO to cells exposed to bacterial molecules such as muramyl dipeptide or flagellin can induce the activation of caspase-1 (21). To test the role of SLO under the more physiological context of bacterial infection, macrophages were incubated with WT bacteria and an isogenic mutant *S. pyogenes* strain deficient in SLO. Notably, the *S. pyogenes* lacking SLO did not induce detectable IL-1β secretion (Fig. 4A) but elicited even higher amounts of TNF-α than did the WT bacterium (Fig. 4B). Consistently, *S. pyogenes* induced the activation of caspase-1, as determined by the induction of the p20 subunit, which is generated by autoproteolytic processing of pro-caspase-1 into the active enzyme (Fig. 4C). In contrast, the mutant *S. pyogenes* strain deficient in SLO did not activate caspase-1 (Fig. 4C). Taken together, these results indicate that infection of macrophages with *S. pyogenes* induces caspase-1-dependent release of IL-1β, a process that requires the expression of SLO.

*S. pyogenes* infection activates the Nlrp3 inflammasome

We assessed next the role of the different inflammasomes in *S. pyogenes*-induced caspase-1 activation. To this end, we infected WT or macrophages deficient in Nlrp3, Nlrc4, and Asc and analyzed the activation of caspase-1. Infection of WT and Nlrc4-deficient macrophages, but not macrophages deficient in Nlrp3 or Asc, with WT bacterium induced caspase-1 activation as revealed
by the detection of the p20 subunit of caspase-1 (Fig. 5A). In agreement with these results, the secretion of IL-1β in S. pyogenes-infected macrophages was abrogated in macrophages deficient in Nlrp3 or Asc, but not Nlrc4 (Fig. 5B). In contrast, Nlrp3 and Asc as well as Nlrc4 were dispensable for the production of TNF-α in response to S. pyogenes (Fig. 5C). To further assess the role of the Nlrp3 inflammasome in S. pyogenes infection, we investigated the production of IL-1β in mice infected i.p. with S. pyogenes. Infection of WT mice with S. pyogenes induced the production of IL-1β in serum, which was impaired in mice deficient in Nlrp3 (Fig. 5D). However, we found that WT and Nlrp3-deficient mice were equally susceptible to S. pyogenes infection as assessed by i.p. administration of 10^2 and 10^4 CFU (supplemental Fig. 1). These results indicate that the Nlrp3 inflammasome is critical for caspase-1 activation and IL-1β secretion in response to S. pyogenes but does not play an important role in the susceptibility to infection.

**TLR signaling, but not Nlrp3, is required for pro-IL-1β induction in response to S. pyogenes infection**

Induction of pro-IL-1β is required for IL-1β secretion in response to inflammatory stimuli. We examined next whether TLR signal-

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### FIGURE 4. S. pyogenes-induced caspase-1 activation and IL-1β secretion is dependent on SLO.

Macrophages were infected with WT S. pyogenes (SP-WT) or S. pyogenes deficient in SLO (SP-SLO). The release of IL-1β (A) and TNF-α (B) was determined by ELISA, and activation of caspase-1 was analyzed by immunoblotting (C). For A and B, data shown are means ± SD of triplicate samples of one experiment representative of three independent experiments; n.d. indicates not detectable.

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**TLR signaling is required for caspase-1 activation induced by SLO and LPS or synthetic lipopeptide but not S. pyogenes infection**

Stimulation of macrophages with ATP and TLR agonists, including LPS (TLR4 agonist) and synthetic lipopeptide (TLR2 agonist), induces caspase-1 activation via the Nlrp3 inflammasome (23, 24). Furthermore, caspase-1 activation via Nlrp3 in response to TLR agonists and ATP requires a priming effect mediated through TLR signaling and NF-kB activation (23, 24). Consistently, activation of caspase-1 induced by LPS or synthetic lipopeptide was impaired in macrophages deficient in Myd88 and Trif (Fig. 7A). Notably, the activation of caspase-1 induced by infection with S. pyogenes was independent of Myd88 and Trif (Fig. 7B). However, caspase-1 activation triggered by infection with S. pyogenes was abrogated by pretreatment of macrophages with BAY 11-7082 (Fig. 7C), a drug that inhibits NF-kB activation by targeting the Ik-B kinase complex (38). These results suggest that TLR signaling is dispensable for S. pyogenes-mediated caspase-1 activation but this process relies on NF-kB activation.

### Discussion

*S. pyogenes* is a highly pathogenic bacterium, but its interaction with the innate immune system remains poorly characterized. In this study, we show that infection with *S. pyogenes* induces the secretion of IL-1β, and this response is mediated by the coordinated interaction between TLR signaling and the Nlrp3 inflammasome. Additionally, the results indicate that active production of the pore-forming toxin SLO is required for caspase-1 and IL-1β secretion induced by *S. pyogenes*, as heat-inactivated bacteria and bacteria lacking SLO were impaired in stimulating the Nlrp3 inflammasome.

*S. pyogenes* is known to secrete several virulence factors, among them the cytotoxic SLO, a member of a conserved family of cholesterol-dependent pore-forming cytolysins (39). It has been demonstrated that various cytolysins such as nigericin (40) and maityotoxin (29) can induce caspase-1-dependent release of IL-1β in macrophages prestimulated with TLR ligands (22). In the present study, we provide evidence that SLO is critical for activation of the Nlrp3 inflammasome in response to *S. pyogenes* infection. Unlike TLR ligands that require exogenous ATP stimulation for activation of caspase-1 via Nlrp3, *S. pyogenes* triggered caspase-1 activation via Nlrp3 independently of the P2X7R. These results indicate that the Nlrp3 inflammasome can be activated via P2X7R-dependent and P2X7R-independent mechanisms in response to microbial stimuli. One possibility is that SLO acts by mimicking the function induced by ATP activation, thereby bypassing the requirement for P2X7R stimulation. Because the pore-forming SLO can mediate the delivery of microbial molecules to the host cytosol and cytosolic escape of *S. pyogenes* (41), it is possible that infection with *S. pyogenes* results in cytosolic internalization of bacterial molecules or the bacterium via SLO to trigger Nlrp3-dependent caspase-1 activation. Because activation of caspase-1 induced by LPS or lipopeptide and SLO requires TLR signaling, the results

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*The online version of this article contains supplemental material.*
suggested that Myd88/Trif-independent activation of the Nlrp3 inflammasome in response to *S. pyogenes* infection cannot be explained by SLO-mediated internalization of TLR ligands. However, SLO could promote caspase-1 activation by mediating internalization of microbial molecules distinct from TLR ligands.

Recent studies showed that *S. pyogenes* induces macrophage cell death, a process that requires SLO, but the cellular demise was largely independent of caspase-1 (42). Failure to induce cell death was associated with higher survival rates of the internalized bacteria inside macrophages (42). Consistent with these results, we found that the mutant strain SLO induced higher amounts of TNF-α than WT bacteria, which may be explained by the presence of a higher number of mutant *S. pyogenes* inside macrophages. Additionally, SLO can induce TLR-independent production of type-I IFNs (43). Collectively, these results indicate that SLO can induce different processes in host cells including cell death, caspase-1 activation, IL-1β, and IFN production, which are likely to contribute to bacterial virulence and host defense.

Activation of the Nlr4 inflammasome is dependent on the presence of a functional type III/IV secretion system, a feature of pathogenic bacteria (11). Here we show that the activation of the Nlrp3 inflammasome by *S. pyogenes* is similarly dependent on the expression of a virulence factor, the pore-forming toxin SLO. A common feature of these virulence factors is the formation of pores in host membranes or alteration of membrane permeability, which may allow the cytosolic localization of microbial molecules. Consistent with this hypothesis, costimulation of macrophages with recombinant SLO and certain TLR ligands, but not each stimulus alone, triggered caspase-1 activation through the Nlrp3 inflammasome (21). We found that activation of caspase-1 with TLR ligands and SLO required TLR signaling, as it was abolished in Myd88/Trif-deficient macrophages. These results are consistent with recent findings showing that TLR ligands promote activation of the Nlrp3 inflammasome, at least in part, through a priming effect mediated via TLR signaling and NF-κB activation (23, 24). Using macrophages that are deficient in the adaptor proteins Myd88 and Trif, and that therefore cannot signal via TLRs, we showed that *S. pyogenes*-induced caspase-1 activation is independent of TLR signaling. One possible model to explain these results is that *S. pyogenes* induces TLR-independent priming of the Nlrp3 inflammasome. This priming effect may be mediated via NF-κB, as caspase-1 activation induced by *S. pyogenes* infection was blocked by treatment with a NF-κB inhibitor. In a nonexcluding model, SLO may mediate internalization of bacterial molecules that are important for priming and/or activation of the Nlrp3 inflammasome independently of TLR signaling. Activation of Nlrp3 through SLO is likely to be indirect, as a physical interaction between...
microbial molecules and NLR proteins has not yet been identified. Such an indirect mechanism has been proposed for the NLRp3 inflammasome by TLR agonists and ATP or particulate matter such as silica or urate crystals (44). Indeed, there is evidence that reactive oxygen species and cathepsin B may contribute to the activation of the Nlrp3 inflammasome by a mechanism that remains undefined (45–48). Irrespective of the mechanism involved, the activation of inflammasomes is triggered by pathogenic bacteria via virulence factors such as pore-forming toxins or bacterial secretion systems. Further work is needed to understand the role of inflammasomes in host defense against bacterial pathogens.

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Disclosures

The authors have no financial conflicts of interest.

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