Differential Requirement of P2X7 Receptor and Intracellular K\(^+\) for Caspase-1 Activation Induced by Intracellular and Extracellular Bacteria*  

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Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that plays an important role in host defense and inflammatory diseases. The maturation and secretion of IL-1β are mediated by caspase-1, a protease that processes pro-IL-1β into biologically active IL-1β. The activity of caspase-1 is controlled by the inflammasome, a multiprotein complex formed by NLR proteins and the adaptor ASC, that induces the activation of caspase-1. The current model proposes that changes in the intracellular concentration of K\(^+\) potentiate caspase-1 activation induced by the recognition of bacterial products. However, the roles of P2X7 receptor and intracellular K\(^+\) in IL-1β secretion induced by bacterial infection remain unknown. Here we show that, in response to Toll-like receptor agonists such as lipopolysaccharide or infection with extracellular bacteria Staphylococcus aureus and Escherichia coli, efficient caspase-1 activation is only triggered by addition of ATP, a signal that promotes caspase-1 activation through depletion of intracellular K\(^+\) caused by stimulation of the purinergic P2X7 receptor. In contrast, activation of caspase-1 that relies on cytosolic sensing of flagellin or intracellular bacteria did not require ATP stimulation or depletion of cytoplasmic K\(^+\). Consistently, caspase-1 activation induced by intracellular Salmonella or Listeria was unimpaired in macrophages deficient in P2X7 receptor. These results indicate that, unlike caspase-1 induced by Toll-like receptor agonists and ATP, activation of the inflammasome by intracellular bacteria and cytosolic flagellin proceeds normally in the absence of P2X7 receptor-mediated cytoplasmic K\(^+\) perturbations.

Interleukin-1β (IL-1β)\(^3\) is a key mediator of host immune responses and plays an important role in inflammatory disease, fever, and septic shock (1). The production of IL-1β is tightly controlled to prevent harmful effects induced by aberrant activation of IL-1 receptor signaling pathways (2, 3). In response to various pro-inflammatory stimuli, IL-1β is produced by monocytes and macrophages as an inactive cytoplasmic precursor (pro-IL-1β) that is processed into the biologically active IL-1β molecule by caspase-1 (4–7). The protease caspase-1 is constitutively expressed as an inactive zymogen that becomes activated by self-cleavage at specific aspartic residues to generate an enzymatically active heterodimer composed of two p20 and p10 chains (8). Recent studies have implicated members of the NOD-like receptor (NLR) family of proteins in the regulation of caspase-1 activation (9, 10). For example, Ipaf, an NLR family member, and the adaptor ASC have been implicated in activation of caspase-1 in response to Salmonella (11). ASC is critical for caspase-1 activation and secretion of IL-1β and IL-18 in response to several purified microbial components, including LPS as well as multiple intracellular bacteria (11–13). In contrast, Ipaf was found to be essential for the control of caspase-1 activation in response to Salmonella or Legionella infection, but dispensable for that induced by the intracellular pathogen Francisella tularensis and several TLR agonists, including LPS, bacterial lipopeptide, and peptidoglycan (14–17). Mutant analyses of intracellular bacteria including Salmonella, Listeria, and Francisella revealed that the presence of bacteria or bacterial molecules in the host cytosol is critical for the activation caspase-1 (14–16, 18). Furthermore, a nonpathogenic Escherichia coli strain engineered to escape the phagosome by expressing Listeria LLO (19, 20) could induce IL-1β secretion (15). In addition, caspase-1 activation in response to Salmonella occurs in macrophages that are insensitive to TLR signaling (15). Recent studies have revealed that intracellular flagellin is sensed by Ipaf independently of TLR5 and MyD88 to mediate Ipaf-mediated caspase-1 activation to Salmonella and Legionella (14–16). These studies indicate that TLR-independent cytosolic recognition of bacteria is critical for caspase-1 activation in response to intracellular bacteria.

In mouse macrophages, induction of IL-1β secretion by TLR agonists, including LPS, is very inefficient in the absence of a secondary signal that is provided by extracellular ATP (21–25). In vitro stimulation of macrophages with millimolar ATP activates the P2X7 receptor (P2X7R), an ATP-gated cation channel that upon activation induces rapid collapse of ionic gradients by promoting efflux of K\(^+\) (26–28). Several studies have suggested
that depletion of intracellular K\(^+\) is essential for caspase-1 activation and IL-1\(\beta\) secretion in response to several TLR agonists, including LPS (2, 28). Consistent with this, stimuli other than ATP such as the K\(^+\)/H\(^+\) ionophore nigericin, hypotonicity, staphylococcal α-toxin, and protegrins that cause rapid lowering of cytoplasmic K\(^+\) concentration also induce potent IL-1\(\beta\) secretion in LPS-primed macrophages (21, 25, 28–31). In LPS-stimulated macrophages, low K\(^+\) concentration promotes the formation of the inflammasome and caspase-1 activation in cell-free systems (32). Furthermore, incubation of tumor cell lines with purified aerolysin, a pore-forming toxin secreted by Aeromonas species, induces a decrease in cytoplasmic K\(^+\) and formation of the inflammasome (33). However, the importance of intracellular K\(^+\) in caspase-1 activation and IL-1\(\beta\) secretion induced by infection with bacteria remains unknown. In addition, the relevance of ATP-mediated activity remains controversial because non-physiological concentrations of ATP are required for caspase-1 activation and enhanced IL-1\(\beta\) secretion (34, 35). In the present report, we have studied the role of cytoplasmic K\(^+\) and the P2X7R in the induction of caspase-1 activation in macrophages infected with intracellular and extracellular bacteria.

**EXPERIMENTAL PROCEDURES**

**Mice and Cells**—Mice deficient in Ipaf, ASC, cryopyrin, or P2X7R have been previously described (13, 15, 27, 36). Mice were housed in a pathogen-free facility. Bone marrow-derived macrophages (BMDMs) were isolated as previously described (37). Briefly, femurs and tibia were removed from the euthanized mouse and briefly sterilized in 70% ethanol. IMDM was used to wash out the marrow cavity plugs, and bone marrow cells were resuspended in L-cell-conditioned medium containing macrophage-colony stimulating factor to stimulate proliferation and differentiation of the marrow progenitors into macrophages. After 5–6 days, the resulting BMDMs were replated and used within 2 days. The animal studies were conducted under approved protocols by the University of Michigan Committee on Use and Care of Animals.

**Reagents and Bacterial Infection**—ATP was from Sigma. Ultrapure *E. coli* LPS was from InvivoGen. In an experiment with ligands, LPS from *Salmonella typhimurium* (Sigma) was used with superimposable results. *Salmonella enterica* serovar *typhimurium* strain SL1344 and the isogenic orgA mutant strain BJ66 (SipB-) were a gift of D. Monack, Stanford University, and the isogenic clicC-βββ- mutant was a gift of A. Aderem, University of Washington. The bacteria were grown as previously described (15). Bacteria were diluted to the desired concentration in IMDM plus 10% heat-inactivated fetal bovine serum and used to infect macrophages at different bacterial/macrophage ratios. After 30-min infection at 37 °C, the macrophages were washed twice with phosphate-buffered saline, and IMDM containing 100 \(\mu\)g/ml gentamycin was added to limit the growth of extracellular bacteria.

**Intracellular K\(^+\) Concentration**—Macrophages were seeded at 1 \( \times \) 10\(^6\) cells per well of a 12-well dish the day before the experiment. The day of the experiment cells were washed once with phosphate-buffered saline before stimulation or infection. At the indicated time point, the medium was removed, and the cells were lysed in 1 ml of 10% nitric acid. The intracellular K\(^+\) content was quantified using atomic absorbance spectroscopy and compared with standards.

**Immunoblotting**—Cells were lysed together with the cell supernatant by the addition of 1% Nonidet P-40, complete protease inhibitor mixture (Roche Applied Science, Mannheim, Germany) and 2 mM dithiothreitol. Clarified lysates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes by electro-blotting. The rabbit anti-mouse caspase-1 was a kind gift from Dr. Vandanaeele (Ghent University, Ghent, Belgium). Anti-IL-1\(\beta\) was from R & D Systems, Minneapolis, MN.

**Stimulation of Macrophages with Liposome**—DOTAP cationic lipid was purchased from Roche Applied Science and used according to the manufacturer’s instructions. Briefly, 50 \(\mu\)l of DOTAP cationic lipid was incubated for 30 min in serum-free media with 4 \(\mu\)g of purified flagellin (InvivoGen) in a final volume of 500 \(\mu\)l. After the incubation, 1.5 ml of serum-free media was added, and 1 ml was used to stimulate 2 \( \times \) 10\(^6\) macrophages seeded in 6-well plates for 3–5 h.

**Measurements of Cytokine**—Mouse cytokines were measured in culture supernatants with enzyme-linked immunosorbent assay (ELISA) kits (R & D Systems, Minneapolis, MN).

**RESULTS**

**Cytosolic Flagellin, but Not LPS, Induces Activation of Caspase-1 in the Absence of ATP Stimulation**—To determine the role of ATP in activation of the Ipaf inflammasome, we assessed the requirement of ATP stimulation for activation of caspase-1 induced by cytosolic flagellin, a signal that mediates Ipaf-dependent caspase-1 activation and compared the response to that induced by LPS. In these experiments bone marrow-derived macrophages were incubated with various concentrations of LPS, followed by a short stimulation with 5 mM ATP or left untreated, and caspase-1 processing was determined by immunoblotting. In control experiments, treatment with ATP was required for effective caspase-1 activation in macrophages pre-stimulated with LPS (Fig. 1A). To assess if stimulation with cytosolic flagellin requires ATP for caspase-1 activation, we used macrophages deficient in TLR5 to avoid activation of TLR5 by flagellin. Notably, incubation of macrophages with purified flagellin, in the presence of cationic lipid to deliver flagellin to the cytosol, induced caspase-1 activation in

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**FIGURE 1.** Cytosolic flagellin, but not LPS, induces activation of caspase-1 in the absence of ATP stimulation. a, wild-type macrophages were primed for 4 h with LPS and stimulated with ATP (5 mM) at the indicated macrophage/bacterial ratio. b, TLR5-KO macrophages were stimulated with flagellin (1 \( \mu \)g/ml), DOTAP, or with DOTAP (25 \( \mu \)g/ml) plus flagellin (1 \( \mu \)g/ml) for 4 h, and then the macrophages were stimulated or not with ATP (5 mM). c, wild-type macrophages were primed, or not, for 4 h with LPS and infected with Salmonella or the fli-C-fjB- Salmonella mutant for the indicated time points. a–c, extracts were prepared from cells together with culture supernatants and immunoblotted with caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. Results are representative of at least three separate experiments.

**FIGURE 2.** Extracellular bacteria do not induce caspase-1 activation in the absence of ATP stimulation. Wild-type macrophages were infected with \( S. \) aureus or \( E. \) coli at a macrophage/bacterial ratio of 1:10 at the indicated time points. After infection macrophages were stimulated or not with ATP (5 mM). Extracts were prepared from cells together with culture supernatants and immunoblotted with caspase-1 antibody (a) or IL-1\( \beta \) antibody (b). Arrows denote procaspase-1 and its processed p20 subunit (a) and pro-IL-1\( \beta \) and mature IL-1\( \beta \) (b). Results are representative of at least three separate experiments.

the activation of caspase-1 in naïve macrophages or macrophages pre-stimulated with LPS. We found similar levels and kinetics of caspase-1 activation in naïve and LPS-stimulated macrophages in response to Salmonella infection (Fig. 1, C and D). Furthermore, the processing of caspase-1 was abolished by infection with a Salmonella mutant lacking flagellin in both naïve and LPS-stimulated macrophages (Fig. 1C), indicating that flagellin was required for caspase-1 activation for both non- and LPS-stimulated macrophages. Extracellular bacteria such as \( E. \) coli and \( S. \) aureus express several pathogen-associated molecular patterns that like LPS stimulate macrophages via TLRs to promote caspase-1 activation. We therefore determined the ability of these extracellular bacteria to activate caspase-1 in the presence and absence of ATP stimulation. Unlike Salmonella, pre-stimulation of macrophages with \( E. \) coli or \( S. \) aureus did not induce the processing of caspase-1 in the absence of ATP stimulation (Fig. 2A). Notably, as was observed with LPS, pre-stimulation with \( E. \) coli or \( S. \) aureus induced caspase-1 processing only after stimulation with ATP (Fig. 2A). The processing of caspase-1 was associated with cleavage of pro-IL-1\( \beta \) into mature IL-1\( \beta \) in macrophage extracts stimulated with both extracellular bacteria and then ATP (Fig. 2B). These results indicate that Salmonella, but not extracellular bacteria, induces caspase-1 activation in the absence of ATP stimulation.

Flagellin Stimulation in the Cytosol of Ipaf-induced Caspase-1 Activation Does Not Require Low Intracellular \( K^+ \) Concentration—The expression of flagellin is required for Salmonella to induce caspase-1 activation through Ipaf (15, 16). We tested next if caspase-1 activation induced by flagellin requires depletion of the intracellular \( K^+ \), an event that appears to be important for LPS-induced caspase-1 activation (11, 12, 22, 23, 38). To test directly this possibility, we delivered purified flagellin, the Salmonella component recognized by Ipaf, to the macrophage cytosol to activate Ipaf and measured the concentration of \( K^+ \) inside the cell as well as caspase-1 activation. Fig. 3A shows that flagellin, in the presence or
absence of the cationic lipid, did not significantly reduce the intracellular K⁺/H⁺ levels in both wild-type and Ipaf-deficient macrophages. Analysis of cell extracts revealed that flagellin in the presence of the cationic lipid induced caspase-1 activation as effectively as ATP in LPS-stimulated macrophages (Fig. 3B). The activation of caspase-1 triggered by ATP was abolished when the macrophages were incubated in high K⁺ medium in both wild-type and Ipaf-deficient cells (Fig. 3, B and C). In contrast, the activation of caspase-1 induced by cytosolic flagellin was dependent on Ipaf but was unaffected by the presence of high K⁺ concentration in the culture medium (Fig. 3, B and C). The different results obtained with LPS and flagellin were unrelated to the source of LPS in that identical results were obtained with LPS from E. coli and S. typhimurium (data not shown). These results indicate that caspase-1 activation induced through intracellular flagellin and Ipaf, unlike that mediated by ATP in LPS-primed macrophages, is independent of K⁺ depletion in the host cytosol.

ASC Functions Independently of Intracellular K⁺ Depletion to Regulate Caspase-1 Activation—ASC is an adaptor protein that is required for caspase-1 activation induced by Salmonella and a variety of TLR agonists, including LPS (11–13, 17, 39). However, it remains unclear whether ASC controls caspase-1 activation induced by cytoplasmic flagellin or regulates this process by regulating intracellular K⁺. To determine whether ASC controls intracellular K⁺, we measured the intracellular K⁺ levels in wild-type or ASC-KO macrophages primed with LPS for 4 h, stimulated or not with ATP (5 mM) for 30 min, and then intracellular K⁺ was analyzed. Wild-type (b and d) or ASC-KO macrophages (c) were cultured in medium with normal or high K⁺ concentration and stimulated with DOTAP plus flagellin for 3 h or primed with LPS for 3 h and stimulated with ATP for the last 30 min (LPS + ATP). Extracts were prepared from cells together with culture supernatants at the indicated time points and immunoblotted with a caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. Results are representative of three separate experiments.

FIGURE 3. Cytosolic flagellin-induced caspase-1 activation does not require low intracellular K⁺ concentration. a, wild-type macrophages were stimulated with flagellin, DOTAP, or flagellin plus DOTAP for the indicated time points, and intracellular K⁺ was analyzed. Values represent mean ± S.D. of triplicate cultures. Results are representative of two separate experiments. b and c, wild-type (b) or Ipaf-KO (c) macrophages were cultured in medium with normal or high K⁺ concentration and stimulated with DOTAP plus flagellin for 3 h or primed with LPS for 3 h and stimulated with ATP for the last 30 min (LPS + ATP). Extracts were prepared from cells together with culture supernatants at the indicated time points and immunoblotted with a caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. Results are representative of three separate experiments.

FIGURE 4. ASC functions independently of intracellular K⁺ to regulate caspase-1 activation. a, wild-type or ASC-KO macrophages were primed with LPS for 4 h, stimulated or not with ATP (5 mM) for 30 min, and then intracellular K⁺ was analyzed. Wild-type (b and d) or ASC-KO macrophages (c) were cultured in medium with normal (b and c) or high K⁺ concentration media (d). Macrophages were stimulated with flagellin (1 µg/ml), DOTAP, DOTAP (25 µg/ml) plus flagellin (1 µg/ml), or LPS for 5 h with ATP alone for the last 30 min (LPS + ATP) or ATP alone for 30 min. Extracts were prepared from cells together with culture supernatants at the indicated time points and immunoblotted with caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. Results are representative of three separate experiments.
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![Graphs and Images]

**FIGURE 5.** Loss of intracellular K⁺ concentration is not required for Salmonella-induced caspase-1 activation. (a) Wild-type and Ipaf-KO macrophages were infected with wild-type Salmonella. (b) Wild-type macrophages were infected with wild-type Salmonella, flic-fliB-Salmonella, or SipB-Salmonella mutant at 1:10 macrophage/bacterial ratio or stimulated with ATP 5 mM. At the indicated time points, intracellular K⁺ was analyzed. (c) Wild-type macrophages were cultured in medium with normal or high K⁺ concentration and infected with Salmonella at a macrophage/bacterial ratio of 1:5. Extracts were prepared from cells together with culture supernatants at the indicated time points and immunoblotted with a caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. (d) Wild-type macrophages were primed with LPS for 4 h. Macrophages cultured in medium with normal or high K⁺ concentration were infected with Salmonella at a bacterial/macrophone ratio of 1:10 for 3 h. Cell-free supernatants were analyzed by ELISA for production of IL-1β. Values represent mean ± S.D. of triplicate cultures. a–d, results are representative of at least three separate experiments.

experiments shown in Fig. 4C, caspase-1 processing induced by ATP in LPS-primed was abolished in ASC-deficient macrophages, which is consistent with previous results (11). Notably, the activation of caspase-1 induced by stimulation with cytosolic flagellin was also dependent on ASC (Fig. 4, B and C). Furthermore, caspase-1 activation elicited by intracellular flagellin was unimpaired when macrophages were incubated in high K⁺ medium, a setting in which caspase-1 activation mediated by LPS and ATP was blocked (Fig. 4D). Collectively, these results indicate that ASC is required for flagellin-induced Ipaf-mediated caspase-1 activation and that the function of ASC in the regulation of caspase-1 activation can proceed independently of low intracellular K⁺ concentration.

**Loss of Intracellular K⁺ Concentration Is Not Required for Salmonella-induced Caspase-1 Activation**—In LPS-primed macrophages, stimulation of the P2X7R with ATP induces a rapid K⁺ efflux and depletion of cytoplasmic K⁺ that has been shown to be important for caspase-1 activation and enhancement of IL-1β secretion (28, 40). Furthermore, there is evidence that depletion of intracellular K⁺ concentration is important for caspase-1 activation induced by the inflammasome (32). To determine whether infection with Salmonella induces lowering of cytoplasmic K⁺, we measured the intracellular K⁺ levels in uninfected macrophages and at several times following Salmonella infection. There was no depletion of cytoplasmic K⁺ levels in wild-type and Ipaf-deficient macrophages after Salmonella infection (Fig. 5A). To verify these results, we compared cytoplasmic K⁺ levels in macrophages stimulated with wild-type Salmonella and mutant Salmonella lacking SipB or flagellin that are deficient in the induction of caspase-1 activation (15) or with LPS followed by short pulse with ATP. There was ~70% loss of intracellular K⁺ levels after stimulation with ATP, but the levels of intracellular K⁺ did not decrease after infection of macrophages with wild-type or mutant Salmonella (Fig. 5B). The depletion of cytoplasmic K⁺ levels was detected within 20 min and was maintained 60 min after ATP stimulation (Fig. 3B and data not depicted). Previous studies showed that incubation of macrophages in medium containing high K⁺ concentration prevents lowering of cytoplasmic K⁺ and IL-1β secretion as well as caspase-1 activation induced by ATP in LPS-stimulated macrophages (28, 40). To further assess the requirement for intracellular K⁺ loss in caspase-1 activation induced by Salmonella, macrophages were infected with Salmonella in physiological medium or medium containing 20 mM KCl. Immunoblotting revealed that caspase-1 activation was unimpaired in macrophages infected in high K⁺ medium when compared with macrophages cultured in “low K⁺ medium” (Fig. 5C). Furthermore, IL-1β secretion induced by Salmonella was similar in macrophages cultured in normal medium or medium containing high concentration of K⁺ (Fig. 5D). Collectively, these results indicate that caspase-1 activation and IL-1β secretion induced by Salmonella are independent of alterations in intracellular K⁺ concentrations.

**Caspase-1 Activation Induced by Listeria Does Not Require Depletion of Intracellular K⁺**—To determine whether the results obtained with Salmonella could be extended to other intracellular bacteria, we studied the role of intracellular K⁺ in caspase-1 activation induced by Listeria monocytogenes. The activation of caspase-1 triggered by infection of macrophages with Listeria has been shown to be attenuated in the absence of cryopyrin (12). We therefore tested first if cryopyrin was required for caspase-1 activation under our experimental conditions. Unlike previous results, we found in multiple experiments that caspase-1 activation was unimpaired in cryopyrin-deficient macrophages when compared with wild-type macrophages (Fig. 6A). Consistent with these results, IL-1β secretion induced by Listeria was similar in wild-type and cryopyrin-null macrophages (Fig. 6B). In addition, Listeria-induced caspase-1 activation was independent of Ipaf (Fig. 6C). To assess if Listeria induces changes in intracellular K⁺, we measured the intracellular K⁺ levels in macrophages before and
after infection with the bacterium. As shown in Fig. 6C, there was no depletion of cytoplasmic K⁺ levels in macrophages infected with wild-type *Listeria* or a *Listeria* mutant lacking LLO, a factor required for cytosol invasion and caspase-1 activation (41). Furthermore, the levels of intracellular K⁺ were not altered by the absence of ASC in macrophages after *Listeria* infection (Fig. 6D). Previous studies showed that incubation of macrophages in medium containing high K⁺ concentration prevents lowering of cytoplasmic K⁺ and IL-1β secretion as well as caspase-1 activation induced by ATP in LPS-stimulated macrophages (28, 40). To further assess the requirement for intracellular K⁺ loss in caspase-1 activation induced by *Listeria*, macrophages were infected with *Listeria* in physiological medium or medium containing high concentration of K⁺. Immunoblotting revealed that culture in high K⁺ medium abrogated caspase-1 activation induced by LPS and ATP stimulation, but it did not affect that elicited by *Listeria* infection (Fig. 6, E and F). These results indicate that caspase-1 activation induced by *Listeria* does not require lowering of intracellular K⁺ concentrations.

**P2X7R Is Not Required for Caspase-1 Activation Induced by Salmonella or Listeria**—To determine the requirement for the P2X7R in IL-1β secretion induced by *Salmonella*, we infected macrophages from wild-type and P2X7-deficient mice with *S. typhimurium* at different macrophage-bacterial ratio and measured IL-1β secretion 16 h after infection. We included in these experiments macrophages lacking Ipaf, an NLR protein that is required for *Salmonella*-induced caspase-1 activation, as controls. As expected, IL-1β production in response to *Salmonella* was abolished in Ipaf-deficient macrophages (Fig. 7A).
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Notably, the secretion of IL-1β was unimpaired in P2X7R-null macrophages when compared with wild-type macrophages (Fig. 7A). To determine if caspase-1 activation required the P2X7R, extracts from uninfected and *Salmonella*-infected macrophages were immunoblotted with an antibody that recognizes the p20 subunit of caspase-1. The analysis revealed that activation of caspase-1 induced by *Salmonella* infection was unaffected by P2X7R deficiency as determined by the processing of pro-caspase-1 (Fig. 7B and C). In contrast, P2X7R was required for IL-1β secretion induced by a short pulse with ATP in LPS-primed macrophages (Fig. 7D), which is consistent with previous results (28, 40, 42). Reciprocally, Ipaf was required for caspase-1 activation induced by cytosolic flagellin but not by that mediated by ATP in LPS-primed macrophages (Fig. 7E). These results demonstrate that the P2X7R is essential for ATP-mediated IL-1β secretion in LPS-stimulated macrophages but dispensable for Ipaf-dependent IL-1β production and caspase-1 activation induced by flagellin in the cytosol.

**DISCUSSION**

Our studies demonstrate a differential requirement for intracellular $K^+$ in caspase-1 activation induced by intracellular bacteria *versus* stimulation via the endogenous P2X7R channel in macrophages primed with LPS. Efflux of intracellular $K^+$ mediated by the P2X7R appears to be critical for secretion of high levels of IL-1β and caspase-1 activation in LPS-primed macrophages in agreement with previous reports (28, 40, 42). In contrast, we found that both activation of caspase-1 and IL-1β secretion induced by infection of macrophages with intracellular bacteria, including *Salmonella* and *Listeria*, are independent of the P2X7R and depletion of cytoplasmic $K^+$ levels. Thus, the mechanism of caspase-1 activation triggered by intracellular bacteria is different from that induced via P2X7R. The latter functions in TLR-primed macrophages, including those stimulated with LPS and extracellular bacteria. Thus, caspase-1 activation mediated by extracellular ATP in macrophages stimulated with TLR agonists can be dissociated from those induced by intracellular bacteria based on the requirement of intracellular $K^+$ and P2X7R activation.

We found that intracellular bacteria induce robust activation of caspase-1, whereas under our experimental conditions that induced by extracellular bacterial is undetectable in the absence of exogenous ATP. Unlike extracellular bacteria, intracellular bacteria have evolved mechanisms to invade and survive inside infected cells. In the case of *Salmonella*, the bacterium relies on a type III secretion system that delivers effector proteins into the host cytosol for invasion and survival. Similarly, *Listeria* uses the pore-forming toxin LLO to escape from the vacuole into the host cytosol, an environment that is critical for survival and replication of the bacterium (43). Notably, functional type III secretion system and LLO are required for the activation of caspase-1 in macrophages infected with *Salmonella* and *Listeria*, respectively (13, 15). The lack of requirement of intracytoplasmic P2X7R and $K^+$ depletion for caspase-1 activation induced by intracellular bacteria suggests that cytosolic sensing of these microbial agents is sufficient for NLR-mediated inflammasome activation. Consistent with this, delivery of purified flagellin to the macrophage cytosol induced Ipaf-dependent caspase-1 activation, which was independent of intracytoplasmic $K^+$ efflux and unaffected by incubation in medium containing high concentration $K^+$.

Thus, the activation of caspase-1 by purified flagellin in the cytosol resembles that induced by live *Salmonella*. Gurcel *et al.* (33) found that incubation of HeLa cells with purified *Aeromonas* aerolysin, a pore-forming toxin that induces cytoplasmic $K^+$ efflux, mediates caspase-1 activation, a process that required Ipaf or Nalp3 in combination with ASC. These authors suggested that efflux of cytoplasmic $K^{2+}$ mediated by aerolysin is important for the activation of caspase-1 via the inflammasome (33). It is difficult to compare the results from the two studies given that the experimental systems used to study caspase-1 activation are different; i.e., we used primary macrophages infected with infectious bacteria, whereas Gurcel *et al.* employed tumor epithelial cells stimulated with purified bacterial toxin. Together, our results indicate that microbial proteins such as flagellin can...
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“substitute” for the inflammasome assembly signal provided by the loss of K⁺ in the cytosol. Although we found no role for depletion of K⁺ in caspase-1 activation induced by intracellular bacteria, our results do not rule out that K⁺ could regulate inflammasome-dependent caspase-1 activation. There is evidence that K⁺ inhibits the activity of the apoptosome, an inflammasome-like platform that controls Apaf-1-dependent caspase-9 activation (43). Given the striking molecular homology between the inflammasome and apoptosome, it is possible that intracytoplasmic K⁺ could play a role in preventing inappropriate caspase-1 activation in the absence of pro-inflammatory stimuli. Consistent with this hypothesis, LPS-induced IL-1β production in human monocytes is blocked by high concentration of K⁺ in the absence of ATP stimulation (38).

There are several mechanisms by which exposure of microbial components to the host cytosol may lead to the activation of caspase-1 through NLR proteins. In the case of Salmonella, flagellin might enter the cytosol through the needle complex formed by the Salmonella Type III secretion apparatus, through pores formed in the phagosome membrane or by escape of some bacteria into the cytosol (44, 45). A role for the secretion apparatus is suggested by the observation that Salmonella requires SipB, a translocase of the Type III secretion system, for the induction of caspase-1 and IL-1β secretion (15, 16). Similarly, delivery of peptidoglycan-derived molecules to the host cytosol by the Helicobacter pylori type IV secretion system activates Nod1, another NLR family member (46). Thus, Ipaf may sense flagellin directly or through another host factor in the cytosol to promote the activation of caspase-1. This proposed mechanism is supported by the finding that purified flagellin delivered to the cytosol is sufficient for the induction of caspase-1 activation in wild-type, but not in Ipaf-KO macrophages (15, 16). Another possibility is that flagellin itself is not recognized, but the presence of flagellin induces a cellular response that is sensed by Ipaf. The NLR protein involved in caspase-1 induced by Listeria infection remains controversial. A previous study showed that the absence of cryopyrin reduced, but did not abolish, caspase-1 activation (12). In contrast, our studies have shown that both caspase-1 activation and IL-1β secretion in response to Listeria infection are unimpaired in cryopyrin-deficient macrophages. Although both studies revealed that cryopyrin is not essential for Listeria-induced caspase-1 activation, there is not a clear explanation that may account for the differential contribution of cryopyrin to caspase-1 in both studies. It is possible that subtle differences in the experimental protocol and/or genetic background of the mice could account at least in part for the difference in results. Caspase-1 activation induced by Listeria requires cytosolic invasion of the bacterium as LLO- Listeria mutants are unable to trigger caspase-1 activation (13). Furthermore, the adaptor ASC is required for Listeria-induced caspase-1 activation. In contrast Ipaf is not required for Listeria-induced caspase-1 activation. Together these results suggest that Listeria activates caspase-1 through a mechanism that is independent of K⁺ efflux and ATP and relies on the adaptor ASC and a cytosolic NLR protein distinct from cryopyrin and Ipaf.

The physiological relevance of ATP-mediated caspase-1 activation is controversial given that the concentrations of ATP required for the induction of caspase-1 activation are in the millimolar range (34, 35). These high concentrations of ATP that are required for in vitro P2X7R activation and production of high levels of IL-1β secretion are not found normally in the in vivo extracellular milieu, although they could perhaps be reached under certain situations in the context of cell lysis or injury (35). P2X7R knock-out mice are resistant to the development of anti-collagen-induced arthritis (27) and accumulate markedly reduced levels of IL-1β within inflamed foot pads induced by local injection of Freund’s complete adjuvant (47). This suggests that P2X7R can be activated by endogenous factors within in situ inflammatory loci. Other studies have reported that P2X7R in murine T cells can be activated in vitro (48) and in vivo (49) by an ATP-independent mechanism involving ADP ribosylation of the receptor in the presence of micromolar extracellular NAD. Activation of the P2X7R with ATP leads to the rapid opening of the ion channel selective for small ions, which is followed by gradual opening of a larger pore that allows passage of larger molecules up to 900 Da (34, 50). Recent studies have shown that the larger pore is mediated by pannexin-1, which is recruited upon P2X7R activation (51). Notably, pannexin-1 was found to be critical for caspase-1 activation and IL-1β secretion in response to ATP stimulation and P2X7R activation (52). Further studies are needed to determine the mechanism and physiological role of the P2X7R via pannexin-1 in caspase-1 activation.

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