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Highlights

FRONTLINE:

Critical role for Ipaf in *Pseudomonas aeruginosa*-induced caspase-1 activation

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Pseudomonas aeruginosa is an opportunistic Gram-negative human pathogen that is responsible for a broad range of infections in individuals with a variety of predisposing conditions. After infection, *P. aeruginosa* induces a marked inflammatory response in the host. However the mechanisms involved in bacterium recognition and induction of immune responses are poorly understood. Here we report that the Nod-like receptor family member Ipaf is required for optimal bacterial clearance in an *in vivo* model of *P. aeruginosa* lung infection. Further analysis showed that bacterial flagellin was essential for caspase-1 and IL-1β and this activity depended on Ipaf and the adaptor ASC but not TLR5. Notably, *P. aeruginosa* induced macrophage cell death and this event relied on flagellin and Ipaf but not on ASC. Analysis of *Pseudomonas* mutants revealed that different amino acid residues of flagellin were critical for sensing by Ipaf and TLR5. Finally, activation of caspase-1 and IL-1β secretion by *P. aeruginosa* required a functional type III secretion system, but not the effector molecules ExoS, ExoT and ExoY. These results provide new insight into the interaction of *P. aeruginosa* with host macrophages and suggest that distinct regions of flagellin are sensed by Ipaf and TLR5.

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Introduction

IL-1β plays an important role in the induction of immune responses and in the development of inflammatory disease, fever and septic shock [1]. In response to proinflammatory stimuli including pathogenic bacteria, the IL-1β precursor is induced in monocytes and macrophages and processed into the biologically active IL-1β molecule by caspase-1 [2–5]. The protease caspase-1 is expressed in monocytes/macrophages as an inactive zymogen that is activated by self cleavage in large multi-protein complexes named 'inflammasomes' [6].

The mechanism responsible for activation of caspase-1 in response to microbial stimuli has remained poorly understood. Recent studies have revealed members of the Nod-like receptor (NLR) family as critical components of the inflammasomes by linking microbial sensing to

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Abbreviations: LDH: lactate dehydrogenase · **NLR:** Nod-like receptor · **TRIF:** Toll/IL-1R domain-containing adaptor inducing IFN-β · **TTSS:** type III secretion system

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caspase-1 activation [7, 8]. For example, Ipaf, an NLR family member and the adaptor ASC have been implicated in activation of caspase-1 in response to *Salmonella* and *Legionella* through the cytosolic sensing of flagellin [7, 9, 10]. Notably, flagellin is also recognized by TLR5 [11]. However, it is unclear whether Ipaf and TLR5 sense identical or distinct regions of flagellin. Similarly, Cryopyrin/Nalp3 is critical for caspase-1 activation and secretion of IL-1β and IL-18 in response to microbial RNA, synthetic purine-like compounds and endogenous urate crystals [12–14]. In addition, Cryopyrin regulates caspase-1 activation triggered by exogenous ATP or pore-forming toxins in macrophages stimulated with several TLR agonists [15, 16].

Pseudomonas aeruginosa is a flagellated opportunistic Gram-negative human pathogen that is responsible for a broad range of infections in individuals with a variety of predisposing conditions including cystic fibrosis, immunodefiency, impaired pulmonary ventilation and loss of skin integrity [17]. A component of *P. aeruginosa* that is critical for virulence is the type III secretion system (TTSS) that allows the bacterium to invade hosts and to overcome host defense mechanisms [18–20]. *P. aeruginosa* uses the TTSS to directly inject effector proteins into the cytosol of the host cell [21]. Four type III-secreted effectors, ExoS, ExoT, ExoY and ExoU, have been identified in *P. aeruginosa*. However, the expression of *exoS* and that of *exoU* appear to be mutually exclusive [22, 23]. Once inside the host cell, these effector molecules promote cellular invasion by modulating host functions important in cytoskeletal organization and signal transduction [24, 25]. In addition to the TTSS, other *P. aeruginosa* factors haven implicated in virulence including flagellin [26, 27], but the mechanism by which these factors contribute to host infection remain poorly understood.

P. aeruginosa infections are usually associated with marked inflammatory responses in host tissues [28]. Immune recognition of bacterial pathogens is mediated by specific pattern recognition molecules, such as the TLR and NLR that sense microbial structures at the cell surface/endosomes and the cytosol, respectively [29, 30]. Recent studies have implicated several TLR and their adaptors MyD88 and Toll/IL-1R domain-containing adaptor inducing IFN-β (TRIF) as well as Nod1 in the cytokine/chemokine response elicited upon recognition of *P. aeruginosa* by host cells [31–37]. Caspase-1 and IL-1β are known to contribute to the inflammatory response induced by *P. aeruginosa* infection [38–40]. However, the machinery whereby *P. aeruginosa* is sensed by innate immune cells to induce the activation of caspase-1 and secretion of IL-1β is unknown. In the present report, we have identified Ipaf as a critical NLR protein that is required for the activation of caspase-1 and secretion of IL-1β in response to *P. aeruginosa*.

Results

P. aeruginosa induces IL-1β secretion in alveolar macrophages through Ipaf

Ipaf has been implicated in the regulation of IL-1β secretion [41, 42]. Therefore, we first tested whether Ipaf is critical for IL-1β secretion induced by *P. aeruginosa* infection by comparing the response of WT and Ipaf-deficient macrophages isolated from mouse lungs. Exposure of alveolar macrophages to *P. aeruginosa* elicited production of IL-1β in WT macrophages, but this response was almost abolished in Ipaf-null macrophages (Fig. 1A).

To assess the role of Ipaf *in vivo*, WT and mutant mice were infected intratracheally with 5×10^5 colony-forming units (CFU) of *P. aeruginosa* and the production of IL-18 in serum was determined by ELISA. We assessed IL-18 as readout of caspase-1 activation because the levels of IL-1β induced by the bacteria *in vivo* were small and difficult to evaluate with reliability. At 5 h post-infection, there were reduced levels of IL-18 in Ipaf-deficient mice but not of TNF-α when compared to WT mice (Fig. 1B, C). At 6 h post-infection, there were similar numbers of *P. aeruginosa* CFU in the lung tissue of WT and mutant mice. By 18 h post-infection, however, there was a significant reduction of *P. aeruginosa* in the tissue of WT mice but not in Ipaf-deficient mice (Fig. 1D). By 48 h post-infection, *P. aeruginosa* were almost undetected in the lungs of both WT and Ipaf-deficient mice (Fig. 1D). Nonetheless, there was a modest but significant increase in the number of bacteria in the liver, but not spleen, of Ipaf-deficient mice when compared to WT mice 48 h after infection (Fig. 1E).

Consistently, mouse survival was not reduced in WT and Ipaf-deficient animals after intratracheal infection with 5×10^5 organisms (Fig. 1F). In addition, we did not observe a significant difference in mortality between mutant and WT mice when infected with a higher number of *P. aeruginosa* organisms (data not shown). These results indicate that Ipaf is important for IL-1β production by alveolar macrophages but it has a transient and modest role in the host response against *P. aeruginosa in vivo*.

P. aeruginosa flagellin induces caspase-1 activation and IL-1β secretion

Given that flagellin has been implicated in IL-1β production and macrophage cell death induced by *Salmonella* and *Legionella* [7, 9], we tested the ability of a *P. aeruginosa* mutant deficient in flagellin to induce IL-1β secretion and cell death. To ensure similar contact of WT and non-motile *P. aeruginosa* with macrophages,

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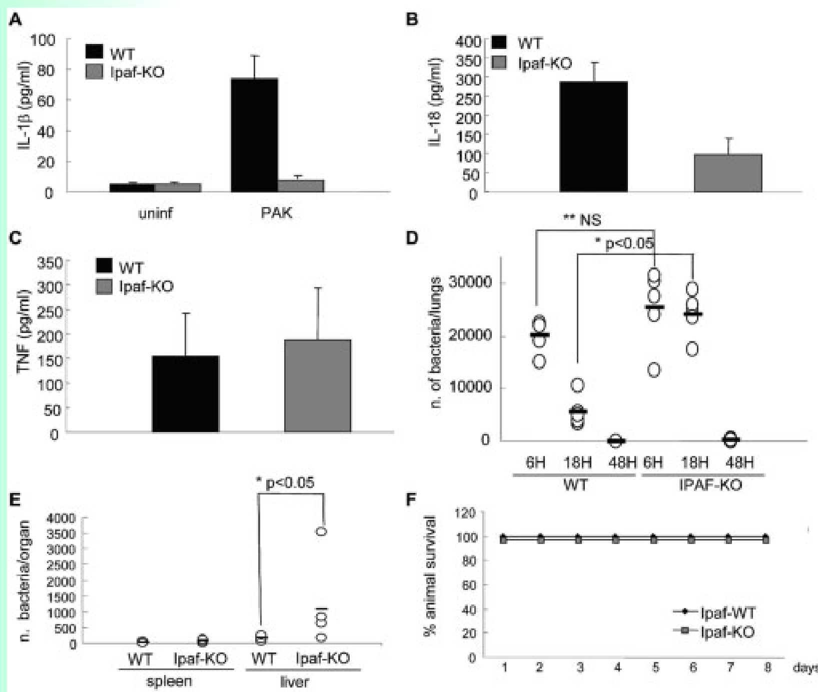

Figure 1. Ipaf is required for IL-1β secretion in alveolar macrophages and early elimination of *P. aeruginosa in vivo*. (A) Alveolar macrophages were infected with *Pseudomonas* at a macrophage/bacterial ratio of 1/40. Cell-free supernatants were analyzed by ELISA for production of IL-1β 4 h after infection. Values represent mean ± SD of triplicate cultures. (B, C) WT and Ipaf-KO mice were infected intratracheally with 5×10^5 *P. aeruginosa*. Levels of IL-18 and TNF were analyzed in the serum 5 h after the infection by ELISA. (D) WT and $Ipaf^{-/-}$ mice were infected intratracheally with 5×10^5 *P. aeruginosa*. Lungs were homogenized and plated for CFU at 6, 18 and 48 h post-infection. (E) WT and $Ipaf^{-/-}$ mice were infected intratracheally with 5×10^5 *P. aeruginosa*. Tissue was homogenized and plated for CFU at 48 h post-infection. (F) Mice were infected intratracheally with 5×10^5 *P. aeruginosa* and monitored for survival over time. Results are representative of three separate experiments with five mice per group per time; NS; not significant.

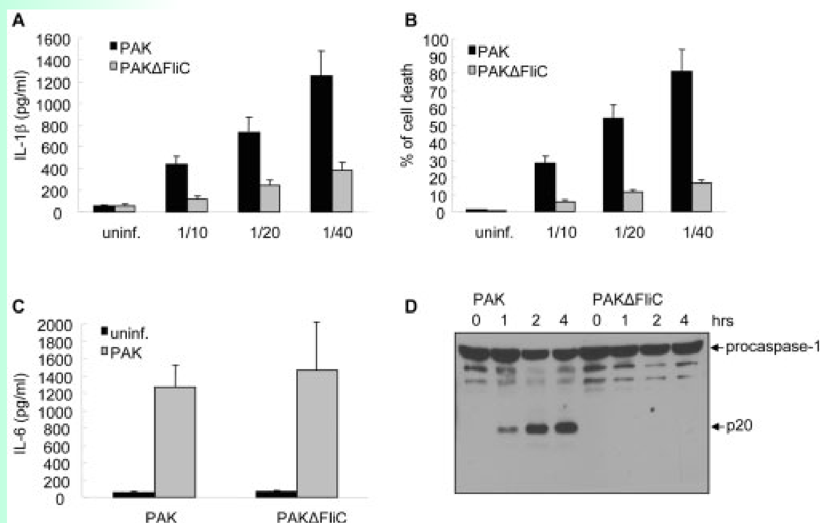

Figure 2. Flagellin is important in the induction of IL-1β secretion, cell death and caspase-1 activation in response to *P. aeruginosa*. (A) BMDM were primed for 4 h with LPS and infected with *P. aeruginosa* or Δ*fliC P. aeruginosa* mutant at the indicated macrophage/bacterial ratio. Cell-free supernatants were analyzed by ELISA for production of IL-1β 4 h after infection. (B) BMDM were infected with *P. aeruginosa* or Δ*fliC P. aeruginosa* mutant at the indicated macrophage/bacterial ratio. The induction of cell death was evaluated by the release of macrophage lactate dehydrogenase (LDH) 4 h after infection. (C) BMDM were infected with *P. aeruginosa* or Δ*fliC P. aeruginosa* mutant at a macrophage/bacterial ratio of 1/10. Cell-free supernatants were analyzed by ELISA for production of IL-6 4 h after infection. (D) BMDM were infected with *P. aeruginosa* or Δ*fliC P. aeruginosa* mutant at a macrophage/bacterial ratio of 1/10. Extracts were prepared from cell and culture supernatants and immunoblotted with caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. (A–D) Results are representative of at least three separate experiments; (A–C) values represent mean ± SD of triplicate cultures.

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infections were followed by mild centrifugation as described [7, 9]. Under these conditions, the secretion of IL-1β and cytotoxicity induced by *P. aeruginosa* lacking flagellin (Δ*fliC*) were greatly reduced when compared to that observed with WT bacteria (Fig. 2A, B). In contrast, secretion of IL-6 (Fig. 2C) and TNF-α (data not shown) was comparable after infection of macrophages with WT and Δ*fliC P. aeruginosa*.

To test whether expression of flagellin is important for caspase-1 activation, extracts were prepared from macrophages infected with WT and flagellin-deficient *P. aeruginosa* at different times post-infection and immunoblotted with an antibody that recognizes the p20 subunit of caspase-1. Infection with *P. aeruginosa* induced activation of caspase-1, but bacteria lacking flagellin did not (Fig. 2D). Thus, flagellin is important for caspase-1 activation, IL-1β secretion and cell death in response to *P. aeruginosa*.

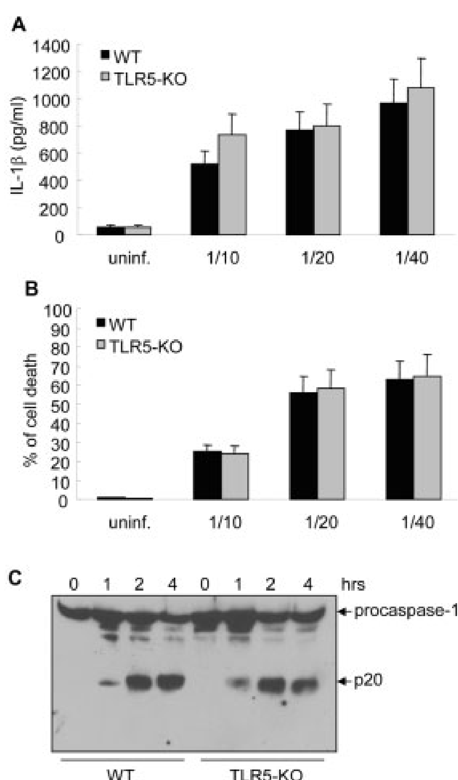

Figure 3. TLR5 is not required for caspase-1 activation, IL-1β secretion and macrophage cell death after *P. aeruginosa* infection. (A) WT and TLR5-KO macrophages were primed for 4 h with LPS and infected with *P. aeruginosa* at the indicated macrophage/bacterial ratio. Cell-free supernatants were analyzed by ELISA for production of IL-1β 4 h after infection. Values represent mean ± SD of triplicate cultures. (B) WT and TLR5-KO macrophages were infected with *P. aeruginosa* at the indicated macrophage/bacterial ratio. The induction of cell death was evaluated by the release of macrophage LDH 4 h after infection. Values represent mean ± SD of triplicate cultures. (C) WT and TLR5-KO macrophages were infected with *P. aeruginosa* at a macrophage/bacterial ratio of 1/10. Extracts were prepared from cell and culture supernatants and immunoblotted with caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. (A–C) Results are representative of at least three separate experiments.

TLR5 is not required for caspase-1 activation after *P. aeruginosa* infection

Flagellin is recognized by TLR5 [11]. We tested next whether TLR5 was required for IL-1β secretion, cell death and caspase-1 activation in response to *P. aeruginosa*. Both secretion of IL-1β and cell death were unimpaired in macrophages deficient in TLR5 when compared to WT macrophages (Fig. 3A, B). Similarly, the activation of caspase-1 was unaffected by the absence of TLR5 (Fig. 3C). These results indicate that the induction of caspase-1 activation and cell death is independent of TLR5 in *P. aeruginosa*-infected macrophages.

Ipaf and TLR5 sense different residues of flagellin

Flagellin is sensed by both Ipaf and TLR5. To determine whether the sensing of flagellin by these two host factors involves the same or different amino acid residues, we infected macrophages with *P. aeruginosa* mutants which express amino acid substitutions in a conserved region of flagellin predicted to interact with TLR5 [43]. Two flagellin *P. aeruginosa* mutants, L94A and Q83A, retained normal motility and the mutations in purified flagellin elicited slightly reduced and normal IL-8 production, respectively [43]. Notably, both *P. aeruginosa* mutants, Q83A and L94A, were defective in the induction of IL-1β secretion and cell death when compared to WT bacteria (Fig. 4A, B). Consistently, the activation of caspase-1 triggered by infection with both mutants was reduced when compared to that observed with WT *P. aeruginosa* (Fig. 4C). These results indicate that Q83 and L94 of flagellin are critical for Ipaf-mediated caspase-1 activation, IL-1β production and cell death, but not for bacterial motility or TLR5 recognition.

P. aeruginosa activation of caspase-1 requires a functional TTSS

We determined next the role of the TTSS, a factor that is critical for virulence, in caspase-1 activation, IL-1β secretion and cell death induced by *P. aeruginosa*. To address this question, we infected macrophages with a *P. aeruginosa* mutant that lacks PscC, an essential component of the TTSS apparatus [25]. These experiments showed that caspase-1 activation, IL-1β secretion and cell death were greatly reduced in macrophages infected with mutant *P. aeruginosa* (Fig. 5A–C). We infected next macrophages with *P. aeruginosa* mutants in which the genes encoding the TTSS effectors ExoS,

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ExoT and ExoY have been deleted individually or in combination [25]. The analyses revealed that the TTSS effectors were dispensable for the induction of IL-1β secretion, cytoxicity and caspase-1 activation in *P. aeruginosa*-infected macrophages (Fig. 6A–F). These results indicate that a functional TTSS is critical for the induction of caspase-1 activity, IL-1β secretion and cell death, whereas the effectors ExoS, ExoT and ExoY are dispensable.

P. aeruginosa-induced caspase-1 activation requires ASC, but not Cryopyrin

We examined next the requirement for NLR proteins and the adaptor ASC in IL-1β production and caspase-1 activation triggered by *P. aeruginosa*. Analysis of WT and mutant macrophages lacking Ipaf, Cryopyrin or ASC revealed that *P. aeruginosa*-induced IL-1β secretion was greatly reduced in Ipaf- or ASC-null macrophages, but not in Cryopyrin-deficient macrophages (Fig. 7A). Consistent with the latter observations, both Ipaf and ASC, but not Cryopyrin, were required for caspase-1 activation induced by *P. aeruginosa* (Fig. 7B–E). In agreement with the results shown in Fig. 2, a *P. aeruginosa* mutant lacking flagellin (Δ*fliC*) was unable to induce caspase-1 activation (Fig. 7B–E). These results indicate that caspase-1 activation in response to *P. aeruginosa* relies on the Ipaf/ASC inflammasome.

Differential role for Ipaf and ASC in *P. aeruginosa*-induced macrophage cell death

We examined next the role of Ipaf, Cryopyrin and ASC in induction of cell death by *P. aeruginosa*. At 4 h post-infection, macrophage cell death was significantly reduced in Ipaf-deficient macrophages but not in macrophages lacking ASC or Cryopyrin when compared to WT macrophages (Fig. 8A–C). Further studies revealed similar kinetics of cell death in WT, ASC- and Cryopyrin-deficient macrophages after *P. aeruginosa* infection (Fig. 8D). In contrast, Ipaf-deficient macrophages were greatly protected against cell death at all times examined (Fig. 8D).

Discussion

In this study, we demonstrate a critical role for Ipaf and its adaptor protein ASC in the activation of caspase-1 and IL-1β secretion in *P. aeruginosa*-infected macrophages. The activation of caspase-1 induced through Ipaf required expression of the bacterium flagellin but was independent of the TTSS effector molecules ExoS, ExoT and ExoY. The sensing of flagellin by Ipaf appears to involve different amino acid residues compared to those required for TLR5 recognition [43].

Remarkably, both caspase-1 activation and IL-1β secretion were abolished or greatly reduced in response to

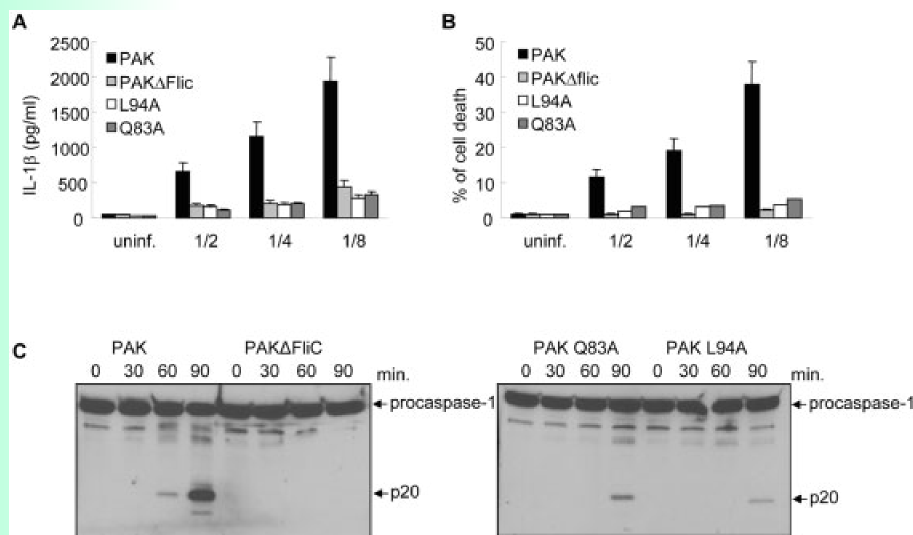

Figure 4. Different amino acid residues of flagellin are critical for sensing through Ipaf and TLR5. BMDM were primed for 4 h with LPS and infected with WT *P. aeruginosa or* Δ*fliC or* Q83A *fliC* or L94A *fliC P. aeruginosa* mutant at the indicated macrophage/bacterial ratio. Cell-free supernatants were analyzed by ELISA for production of IL-1β 4 h after infection. Values represent mean ± SD of triplicate cultures. (B) BMDM were infected with WT *P. aeruginosa or* Δ*fliC or* Q83A *fliC* or L94A *fliC P. aeruginosa* mutant at the indicated macrophage/bacterial ratio. The induction of cell death was evaluated by the release of macrophage LDH 4 h after infection. Values represent mean ± SD of triplicate cultures. (C) BMDM were infected with WT *P. aeruginosa or* Δ*fliC or* Q83A *fliC* or L94A *fliC* at a macrophage/bacterial ratio of 1/5. Extracts were prepared from cell and culture supernatants and immunoblotted with caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. (A–D) Results are representative of at least three separate experiments.

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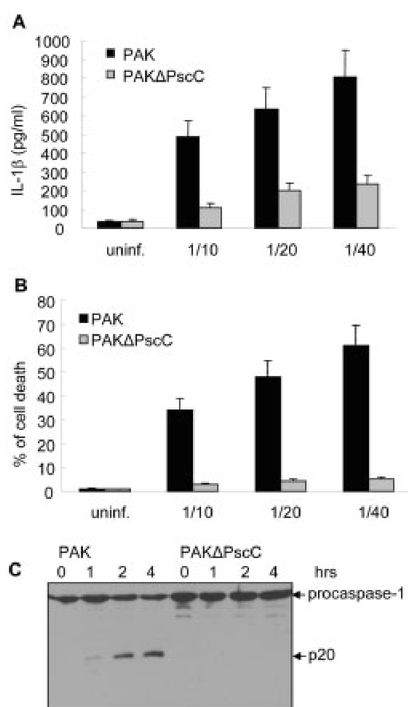

Figure 5. A functional TTSS is important for induction of caspase-1, IL-1β secretion and cell death in *P. aeruginosa*-infected macrophages. (A) BMDM were primed for 4 h with LPS and infected with *P. aeruginosa* or *PscC⁻ P. aeruginosa* mutant at the indicated macrophage/bacterial ratio. Cell-free supernatants were analyzed by ELISA for production of IL-1β 4 h after infection. Values represent mean ± SD of triplicate cultures. (B) BMDM were infected with *P. aeruginosa* or *PscC⁻ P. aeruginosa* mutant at the indicated macrophage/bacterial ratio. The induction of cell death was evaluated by the release of macrophage LDH 4 h after infection. Values represent mean ± SD of triplicate cultures. (C) BMDM were infected with *P. aeruginosa* or *PscC⁻ P. aeruginosa* mutant at a macrophage/bacterial ratio of 1/10. Extracts were prepared from cell and culture supernatants and immunoblotted with caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. (A–C) Results are representative of at least three separate experiments.

a *P. aeruginosa* mutant lacking an essential component of the TTSS machinery. A critical role for the TTSS apparatus in caspase-1 activation is also suggested by the observation that *Salmonella* requires SipB, a translocase of the TTSS, for the induction of caspase-1 and IL-1β secretion [10, 41]. Similarly, the *Legionella* type IV secretion system has been shown to be essential for the induction of Ipaf-mediated caspase-1 in macrophages [9].

Although the precise mechanism by which the TTSS contributes to caspase-1 through Ipaf requires further investigation, a reasonable possibility is that small amounts of soluble flagellin might enter the cytosol during the assembly of the TTSS across the macrophage membrane or through the needle complex formed by the *P. aeruginosa* TTSS apparatus. Thus, Ipaf may sense flagellin directly or through another host factor in the cytosol to promote the activation of caspase-1. Such a mechanism has been proposed to explain the requirement of both flagellin and the TTSS for caspase-1 activation in response to *Salmonella* [10, 41]. Similarly, peptidoglycan-derived molecules are delivered to the host cytosol by the *Helicobacter pylori* type IV secretion system for the activation of Nod1, another NLR family member [44].

Alternatively, the TTSS might induce an activity at the membrane of infected macrophages independent of ExoS, ExoT and ExoY that is critical cofactor for the activation of the Ipaf inflammasome. Because the TTSS forms a pore in the membrane of the contacted host cell, it may induce changes in cytosolic ion concentrations or another event across macrophage membranes that promote caspase-1 activation. However it should be noted that the Ipaf-inflammosome is not modulated by intracellular K^+ concentration [42]. Finally, there is the possibility that there may be undiscovered TTSS effectors that regulate caspase-1 activation. Further studies are needed to understand the contribution of the *P. aeruginosa* TTSS and flagellin to the activation of the Ipaf inflammasome.

We found a critical role for the TTSS, flagellin, Ipaf and caspase-1 in the induction of rapid cell death in macrophages infected with *P. aeruginosa*. This mode of bacteria-induced macrophage cell death that relies on caspase-1 is triggered by several bacterial pathogens including *Salmonella* and *Shigella* and refereed as pyroptosis [45]. The induction of pyroptosis by *P. aeruginosa* proceeded normally in ASC-deficient macrophages despite the absence of caspase-1 activation. These results indicate that the function of Ipaf and ASC differ in a subtle manner and that the absence of caspase-1 activation is not sufficient to inhibit pyroptosis.

One possibility is that both caspase-1 activation and the failure to induce pro-survival signals are required for pyroptosis. In this model, ASC promotes survival signals and in the absence of ASC, but not caspase-1 or Ipaf, these ASC-mediated pro-survival signals will not be induced leading to pyroptosis. Consistent with this hypothesis, ASC mediates NF-κB activation [46–48] and thus NF-κB or another activity induced *via* ASC independently of caspase-1 might counter the induction of pyroptosis in *P. aeruginosa*-infected macrophages. An alternative possibility is that the inflammasome formed in the absence of Ipaf and ASC might vary in a subtle manner. For example, they may differ in the recruitment of host molecules that regulate the induction of pyroptosis in infected macrophages. Further studies are needed to understand the differential role of Ipaf and ASC in pyroptosis induced by bacterial infection.

Several studies have assessed the host mechanisms that mediate the immune response to *P. aeruginosa*. These experiments revealed a role for TLR2, TLR4 and

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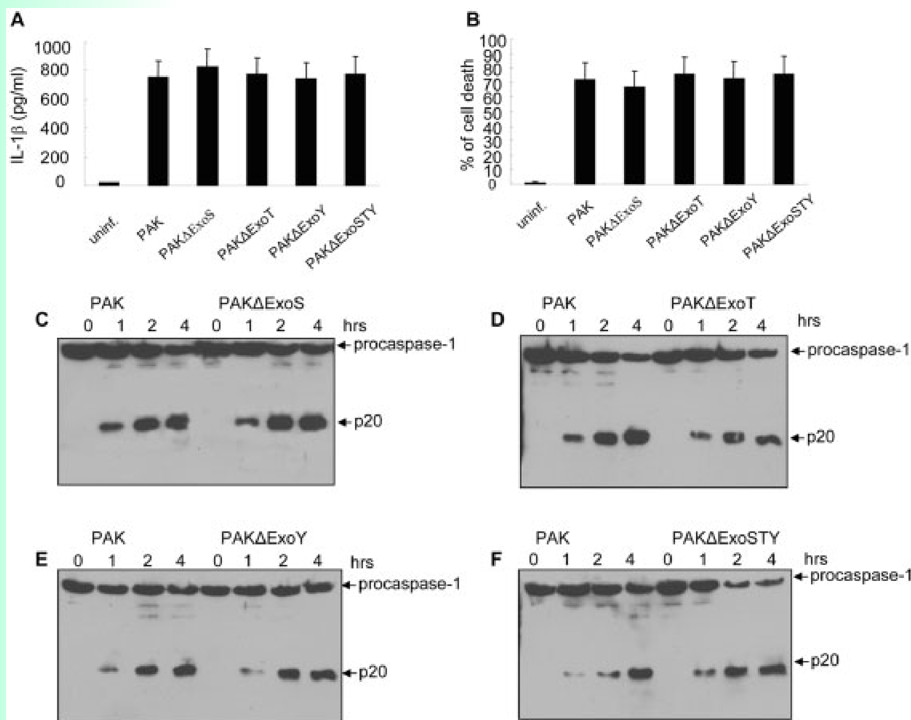

Figure 6. The effector proteins *ExoS*, *ExoT* and *ExoY* are not important for induction of caspase-1, IL-1β secretion and cell death in *P. aeruginosa*-infected macrophages (A) BMDM were primed for 4 h with LPS and infected with *P. aeruginosa* or ExoS, *ExoT*, *ExoY* or *ExoSTY P. aeruginosa* mutant at the indicated macrophage/bacterial ratio. Cell-free supernatants were analyzed by ELISA for production of IL-1β 4 h after infection. Values represent mean ± SD of triplicate cultures. (B) BMDM were infected with *P. aeruginosa* or *ExoS*, *ExoT*, *ExoY* or *ExoSTY P. aeruginosa* mutant at the indicated macrophage/bacterial ratio. The induction of cell death was evaluated by the release of macrophage LDH 4 h after infection. Values represent mean ± SD of triplicate cultures. (C–F) BMDM were infected with *P. aeruginosa* or *ExoS* (C), *ExoT* (D), *ExoY* (E) or *ExoSTY* (F) *P. aeruginosa* mutant. Extracts were prepared from cell and culture supernatants and immunoblotted with caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. (A–F) Results are representative of at least three separate experiments.

TLR5 in the cytokine/chemokine response of epithelial cells and macrophages to the bacterium *in vitro* and *in vivo* [32–36]. However, while analyses of MyD88-null and TRIF-null mice have shown a critical role for this adaptor in bacterial resistance, there is no or little evidence that individual TLR are critical for susceptibility to *P. aeruginosa* and bacterial clearance *in vivo* [32–36]. The high susceptibility of mice deficient in the adaptor MyD88 to *P. aeruginosa* in contrast to individual TLR suggests redundancy of TLR in the host response to *P. aeruginosa* and/or involvement of IL-1R in that this pathway also uses MyD88 for signaling [49].

A role for IL-1 signaling in controlling *P. aeruginosa* lung infection in mice is controversial. Recent studies have suggested an important role for IL-1R using a chronic colonization model whereas no significant role was found in acute pulmonary infection with *P. aeruginosa* [40]. Similarly, some authors have reported that pre-treatment with IL-1β protected the mice against bacterial infection, but other studies showed that IL-1β-neutralizing antibody administered after *P. aeruginosa* protected mice from sepsis and acute pneumonia. These seemingly contradictory results are likely to reflect differences in the experimental models including the timing of administration and the dose of bacterial inocolum. In a model of corneal infection induced by *P. aeruginosa,* both caspase-1 and IL-1β were found to be important in eliciting acute inflammatory responses and tissue damage [39].

Our studies have revealed that the absence of Ipaf is associated with a transient defect in the clearance of *P. aeruginosa* in the lung tissue after intratracheal infection. This modest effect is comparable with results obtained in the *Salmonella* system in which no or minimal effects in bacterial clearance were observed after oral infection [50]. These findings suggest redundancy between different NLR family members and other pattern recognition receptors in host defense against *P. aeruginosa*. Therefore, it will be important in future studies to assess the role of Ipaf in the presence and absence of other NLR and TLR in mouse models of *P. aeruginosa* infection.

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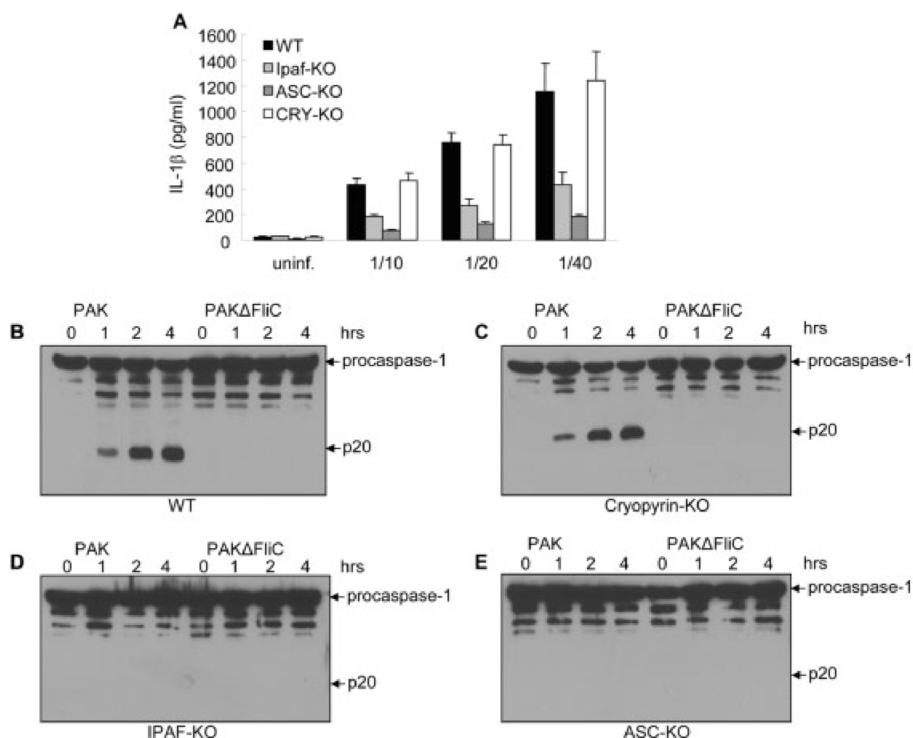

Figure 7. Ipaf and ASC, but not Cryopyrin, are critical for the induction of caspase-1 and IL-1β secretion in response to *P. aeruginosa* (A) WT, Ipaf-KO, ASC-KO and Cryopyrin-KO macrophages were primed for 4 h with LPS and infected with *P. aeruginosa* at the indicated macrophage/bacterial ratio. Cell-free supernatants were analyzed by ELISA for production of IL-1β 4 h after infection. Values represent mean ± SD of triplicate cultures. (B–E) WT (B), Cryopyrin-KO (C), Ipaf-KO (D) and ASC-KO (E) macrophages were infected with *P. aeruginosa* or Δ*fliC P. aeruginosa* mutant at a macrophage/bacterial ratio of 1/10. Extracts were prepared from cell and culture supernatants and immunoblotted with caspase-1 antibody. Arrows denote procaspase-1 and its processed p20 subunit. (A–E) Results are representative of at least three separate experiments.

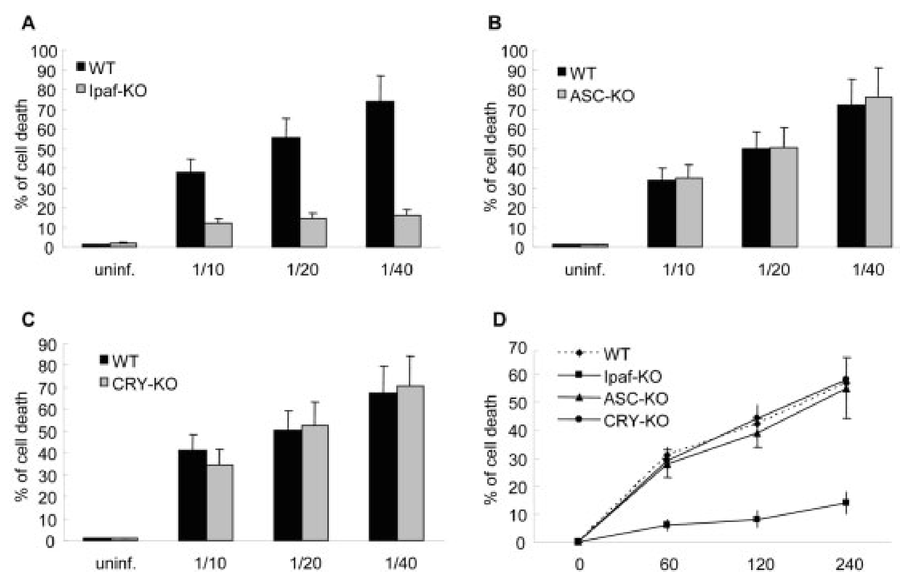

Figure 8. Differential role for Ipaf and ASC in *P. aeruginosa*-induced macrophage cell death. (A–C) WT, Ipaf-KO (A), ASC-KO (B) and Cryopyrin-KO (C) macrophages were infected with *P. aeruginosa* at the indicated macrophage/bacterial ratio. The induction of cell death was evaluated by the release of macrophage LDH 4 h after infection. Values represent mean ± SD of triplicate cultures. (D) WT, Ipaf-KO, ASC-KO and Cryopyrin-KO macrophages were infected with *P. aeruginosa* at a macrophage/bacterial ratio of 1/20. The induction of cell death at the indicated time point was evaluated by the release of macrophage LDH. (A–D) Results are representative of at least three separate experiments.

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Materials and methods

Mice and cells

Mice deficient in Ipaf, ASC, Cryopyrin, caspase-1, TLR5 have been previously described [12, 41, 51, 52]. For *in vivo* experiments Ipaf-KO mice were backcrossed five times on a Balb/c background. Mice were housed in a pathogen-free facility.

Bone marrow-derived macrophages (BMDM) were isolated as previously described [53]. 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 M-CSF to stimulate proliferation and differentiation of the marrow progenitors into macrophages. After 5–6 days, the resulting BMDM were replated and used within 2 days. Alveolar macrophages were prepared as previously described [54]. The animal studies were conducted under approved protocols by the University of Michigan Committee on Use and Care of Animals.

Reagents and bacterial infection

Ultrapure *Escherichia coli* LPS was from Invivogen. All the bacterial strains used in this study were derived from the WT *P. aeruginosa* strain PAK. *P. aeruginosa* deletion mutants pscC, fliC, exoS, exoT, exoY, exoSTY used in this study were described elsewere. The bacteria were propagated in liquid Luria–Bertani broth or on Luria–Bertani agar plates. The bacteria were grown at 30°C overnight. The next day cultures were diluted 10^{-1} and grown for 4 h at 37°C to late exponential/early stationary phase before macrophage infection. Bacteria were diluted to the desired concentration in IMDM + 10% heat-inactivated FBS and used to infect macrophages at different bacterial/macrophage ratios. After 1 h, gentamycin (100 μg/mL) was added to limit the growth of extracellular bacteria. In all the experiments immediately after infection *P. aeruginosa* were spun onto the cells at 1500 rpm to synchronize the infection.

Immunoblotting

Cells were lysed together with the cell supernatant by the addition of 1% NP-40, complete protease inhibitor cocktail (Roche, Mannheim, Germany) and 2 mM dithiothreitol. Clarified lysates were resolved by SDS-PAGE and transferred to PVDF membranes by electro-blotting. The rabbit anti-mouse caspase-1 was a kind gift from Dr. Vandanabeele (Ghent University, Ghent, Belgium). Anti IL-1β was from R&D Systems, Minneapolis, MN.

Measurements of cytokines

Mouse cytokines were measured in culture supernatants, or serum, with ELISA kits (R&D Systems, Minneapolis, MN). Assays were performed in triplicate for each independent experiment.

Mouse infection and statistical analysis

WT and Ipa-KO mice were infected intratracheally with 5×10^5 *Pseudomonas*, and the number of bacteria in the lungs was determined at 6, 18 and 48 h post-infection by serial dilution plating. Comparisons between two experimental groups were performed with Student's *t*-test. Differences in data values were considered significant at a p value of less than 0.05.

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