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Asc and Ipaf Inflammasomes Direct Distinct Pathways for Caspase-1 Activation in Response to *Legionella pneumophila*▽

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Caspase-1 activation is a key feature of the innate immune response of macrophages elicited by pathogens and a variety of toxins. Here, we determined the requirement for different adapter proteins involved in regulating host processes mediated by caspase-1 after macrophage infection by *Legionella pneumophila*. The adapter protein Asc was found to be important for caspase-1 activation during *L. pneumophila* infection. Activation of caspase-1 through Asc did not require the flagellin-sensing pathway involving the host nucleotide-binding domain and leucine-rich repeat-containing protein Ipaf (NLRC4). Asc-dependent caspase-1 activation was inhibited by high extracellular potassium levels, whereas Ipaf-dependent activation was unaffected by potassium treatment. Activation of caspase-1 in macrophages occurred independently of Nalp3 and proteasome activity, suggesting that a previously uncharacterized mechanism for caspase-1 activation through Asc may be triggered by *L. pneumophila*. Rapid pore formation and pyroptosis induced by *L. pneumophila* required caspase-1, Ipaf, and bacterial flagellin but occurred independently of Asc. Equivalent levels of active interleukin-18 (IL-18) were detected in the lungs of mice infected with a flagellin-deficient strain of *L. pneumophila* and Asc-deficient mice infected with wild-type *L. pneumophila*. Active IL-18 was undetectable in the lungs of Asc-deficient mice infected with an *L. pneumophila* flagellin mutant, indicating independent roles for Ipaf and Asc in caspase-1-mediated processing and release of IL-18 in vivo. Ipaf-dependent activation of caspase-1 restricted bacterial replication in vivo, whereas Asc was dispensable for restriction of *L. pneumophila* replication in mice. Thus, *L. pneumophila*-mediated caspase-1 activation involves the coordinate activities of inflammasomes differentially regulated by Ipaf and Asc.

Caspase-1 activation is a key feature of the innate immune response of macrophages elicited by pathogens and a variety of toxins (44). Activation of this cysteine protease enables the cleavage and subsequent secretion of the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 (4). In addition, caspase-1 activation can lead to the formation of pores in the host cell membrane, eventually resulting in osmotic lysis of the cell (9). This form of proinflammatory cell death has been termed pyroptosis (8).

Caspase-1 activation occurs following stimulation of a group of cytosolic sensor proteins known as the nucleotide-binding domain and leucine-rich repeat proteins, called NLRs (44). Similar to Toll-like receptors, the NLRs represent a group of pattern recognition molecules capable of sensing pathogen-associated molecular patterns as well as endogenous signals indicating host damage. The NLRs known to be involved in caspase-1 activation include Ipaf (NLRC4, CARD12), pyrin domain-containing NLRs (including the Nalps), and the Naip proteins (NLRB and BIRC1). Although the roles for many NLRs remain unclear, it is evident that different NLRs enable the host to respond to a variety of different stimuli upon infection by microbial pathogens. For instance, *Salmonella enterica* serovar Typhimurium (21), *Shigella flexneri* (41), and *Pseudomonas aeruginosa* (12, 29, 38) activate caspase-1 by a pathway involving Ipaf, an NLR capable of directly interacting with caspase-1 through homotypic association of the CARD domains of these proteins (33). Bacterial flagellin has been shown to be important in the sensing of *S. enterica* serovar Typhimurium by Ipaf (10, 28); however, Ipaf-mediated caspase-1 activation in response to *Shigella flexneri* (41) and at least one strain of *P. aeruginosa* (38) is flagellin independent. *Francisella tularensis* activation of caspase-1 is Ipaf independent and involves stimulation of one or more Nalps (22). Caspase-1 activation by most Nalp proteins requires Asc, an adapter protein capable of interactions with Nalps and caspase-1 through pyrin-pyrin and CARD-CARD interactions, respectively (25). Nalp3 activation of caspase-1 has been studied extensively, and there is evidence that Nalp3 responds to various triggers of both endogenous and bacterial origin (14, 16, 17, 23, 24, 26, 32, 39, 40, 42). Interestingly, activation of caspase-1 through Nalp3 is inhibited in the presence of high extracellular potassium levels (32). In vitro data suggest that potassium inhibition of caspase-1 activation may be common among all of the Asc-dependent Nalp pathways due to the ability of high potassium levels to prevent Asc oligomerization, a key step for caspase-1 activation (5).

The facultative intracellular bacterium *Legionella pneumophila* induces caspase-1 activation in macrophages (1, 45). Normally found in freshwater environments where it parasitizes several protozoan organisms (6), *L. pneumophila* can invade and replicate within alveolar macrophages upon inhalation of water droplets from a contaminated source, potentially leading to a severe pneumonia known as Legionnaires' disease (13, 15, 27). Activation of caspase-1 by *L. pneumophila* requires the type IV secretion system called Dot/Icm (45), an apparatus that facilitates translocation of *L. pneumophila* proteins from the bacterial cytoplasm into the host cell cytosol (31). Trans-

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location of bacterial proteins upon entry into macrophages allows *L. pneumophila* to inhibit fusion of the vacuole in which it resides with early and late endocytic organelles and to recruit vesicles exiting the endoplasmic reticulum (ER), resulting in the formation of an ER-derived compartment that supports replication of *L. pneumophila* (35). Caspase-1 activation occurs independently of correct trafficking of the *Legionella*-containing vacuole because *Legionella* mutants that fail to efficiently create an ER-derived vacuole but produce a functional Dot/Icm apparatus will activate caspase-1 to levels that are similar to those in wild-type bacteria (45). These data indicate that caspase-1 activation by *L. pneumophila* requires the type IV secretion machinery, which likely facilitates perturbations in the macrophage membrane and allows access of bacterial products to the host cytosol.

The NLR proteins Naip5 and Ipaf are important for caspase-1 activation by *L. pneumophila* (1, 20, 45). A C-terminal region of the *L. pneumophila* flagellin protein was recently shown to be sufficient to activate caspase-1 through Ipaf and Naip5 (20). Flagellin or peptide fragments of the flagellin protein presumably gain access to the macrophage cytosol during infection by subverting the translocation functions provided by the Dot/Icm system, but this has not been demonstrated. In vitro data suggest that Naip5 and Ipaf interact directly (3, 45). These data indicate that Naip5, Ipaf, and bacterial flagellin comprise a pathway for caspase-1 activation in response to *L. pneumophila*.

In addition to Ipaf and Naip5, it has been shown that the adapter protein Asc has a role in IL-1β secretion by macrophages in response to *L. pneumophila* (45), but the role that Asc plays in this process is unclear. Here, we investigated whether Asc is a component of the caspase-1 activation pathway regulated by Naip5 and Ipaf in response to flagellin or whether Asc defines an alternate pathway used for caspase-1 activation.

MATERIALS AND METHODS

Bacterial strains. *L. pneumophila* serogroup 1 strains were used. For ex vivo macrophage infections, *L. pneumophila thyA* (Lp02) (2), a thymidine auxotroph derived from the *L. pneumophila* serogroup 1 strain Lp01, was used, along with the *dotA* (2) and *flaA* (34) isogenic mutants. For in vivo mouse infections, the JR32 strain (36) was used along with *dotA* and *flaA* isogenic mutants. Bacteria were cultured for 48 h on charcoal-yeast extract agar plates [1% yeast extract, 1% *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) (pH 6.9), 3.3 mM L-cysteine, 0.33 mM $Fe(NO_3)_3$, 1.5% Bacto agar, and 0.2% activated charcoal] supplemented with thymidine (100 μg/ml) where necessary. For in vivo mouse infections, bacteria were cultured to an optical density at 600 nm of approximately 1.0 in ACES-buffered yeast extract medium [1% ACES, (pH 6.9), 3.3 mM L-cysteine, 0.33 mM $Fe(NO_3)_3$].

Mice. $Ipaf^{-/-}$, $Asc^{-/-}$, $Nalp3^{-/-}$, and $Casp1^{-/-}$ mice have been described previously (19, 21, 26, 39). $Ipaf^{-/-}$, $Asc^{-/-}$, $Nalp3^{-/-}$, and $Casp1^{-/-}$ mice were backcrossed to the C57BL/6 background for 5, 9, 10, and 10 generations, respectively. C57BL/6 mice were purchased from Jackson Laboratories. For the generation of $Ipaf^{-/-}$ $Asc^{-/-}$ mice, $Ipaf^{-/-}$ and $Asc^{-/-}$ mice were crossed, and the resulting heterozygous progeny (F_1) were crossed. The progeny resulting from this secondary cross (F_2) were screened for the absence of Ipaf and Asc by PCR using tail DNA and the following primer sets: for Ipaf, Mill29-28 (GCAGGAA TCAATCCAGAGTCTGAG), Mill29-37 (GAAGCCTCAACGGCAACGAGC ACTC), and Neo3A GCAGCGCATCGCCTTCTATC; for Asc, Card5-018-F2 (GTGGACGGAGTGCTGGATG), Card5-018-F2 (GTCCATCACCAAGTAG GGATG), and NeoF (GCTGACCGCTTCCTCGTGCTTTAC). All animals were maintained in accordance with the guidelines of the Yale Institutional Animal Use and Care Committee (protocol 07847).

Ex vivo macrophage infections. Bone marrow cells were collected from the femurs and tibiae of mice and cultured for 7 days in RPMI 1640 containing 20% fetal bovine serum (FBS), 25% macrophage colony-stimulating factor (M-CSF), and penicillin-streptomycin (100 units/ml). Macrophages were replated 1 day prior to infection in RPMI 1640 containing 10% FBS and 10% M-CSF for all experiments except where specified. Supernatants from L-929 fibroblast cells (ATCC) served as the source of M-CSF. All infections were carried out by centrifuging bacteria onto macrophage monolayers for 5 min at 400 × *g*. For experiments involving potassium inhibition, KCl or NaCl was added to the medium immediately following infection to a level of 50 mM above the basal concentration of these salts. For proteasome inhibition experiments, cells were pretreated with 1 μg/ml lipopolysaccharide (LPS) (Sigma) for 3 hours, washed to remove LPS, and infected with *L. pneumophila* for 6 h in the presence of 10 μM MG132 (Calbiochem).

Cytokine assays. Macrophages were added to 24-well plates (2×10^5 cells per well) and infected with *L. pneumophila* at a multiplicity of infection (MOI) of 10. Unless otherwise indicated, supernatants were harvested at 8 h postinfection and cleared by centrifugation prior to cytokine measurements. IL-1β was measured using a mouse IL-1β enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences). IL-18 was captured using an antibody specific to mature mouse IL-18 (R&D Systems) and detected with a biotinylated antibody to mouse IL-18 (R&D Systems). Tumor necrosis factor alpha (TNF-α) was measured using antibodies against mouse TNF-α (R&D Systems).

Immunoblot analysis. Macrophages (5×10^5) were added to 12-well plates and infected with *L. pneumophila* at an MOI of 10. Supernatants were treated with 10% trichloroacetic acid in the presence of protease inhibitor cocktail (Roche), and precipitated proteins were pelleted by centrifugation at 16,000 × *g* for 20 min at 4°C. The resulting pellets were washed with cold acetone, dried, and resuspended in sample buffer. Trichloroacetic acid-precipitated supernatants were combined with cell lysates prior to gel electrophoresis. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to Immobilon-P membranes (Millipore), and blocked overnight at 4°C in Tris-buffered saline with 0.1% Tween 20 and 5% nonfat dry milk, followed by incubation in Tris-buffered saline with 0.1% Tween 20 and 5% bovine serum albumin at 25°C for 1 h. Caspase-1 cleavage was detected using a rabbit polyclonal antibody against the p10 subunit (Santa Cruz Biotechnology) and goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Zymed).

Cell death and pore formation assays. Cell death was measured by lactate dehydrogenase (LDH) release assay. Macrophages (8×10^4) were added to 48-well plates and infected with *L. pneumophila* at an MOI of 50. Supernatants were harvested at various time points after infection for analysis of LDH release. Release of LDH by cells was quantified using the Cytotox 96 kit (Promega).

Pore formation in macrophages was determined by quantification of propidium iodide (PI) uptake. Macrophages (5×10^5) were added to non-tissue-culture-treated 12-well plates in RPMI 1640 containing 20% FBS and 25% M-CSF. Macrophages were infected at an MOI of 50 for 2 h, followed by gentle washing in cold phosphate-buffered saline (PBS), and then resuspended in 400 μl cold PBS containing 2 mM EDTA. PI was added to a final concentration of 62.5 μg/ml, and cells were analyzed by fluorescence-activated cell sorting.

In vivo mouse infections. Seven- to 11-week-old mice were used for in vivo infections. Mice were anesthetized by subcutaneous injection of a ketamine (100 mg/kg)-xylazine (10 mg/kg) solution and then intranasally inoculated with 5×10^6 CFU of *L. pneumophila*. For bacterial growth studies, mice were euthanized with CO_2 at 4 h postinfection or by subcutaneous injection of a ketamine (250 mg/kg)-xylazine (25 mg/kg) solution at 24 or 48 h postinfection. Lungs were harvested and homogenized in sterile distilled water for 30 s using a PowerGen 125 hand-held homogenizer (Fisher Scientific). Lung homogenates were plated on charcoal-yeast extract agar plates and incubated for 72 h at 37°C prior to counting colonies. For cytokine measurements, mice were euthanized by injection of a ketamine (250 mg/kg)-xylazine (25 mg/kg) solution at 24 h postinfection. Bronchoalveolar lavage was performed using 400 μl of PBS, and the resulting fluid was assayed for IL-1β and IL-18 by ELISA as described above.

Statistical analysis. Statistical significance for cytokine assays and bacterial growth assays was calculated using the unpaired Student *t* test. Differences were considered statistically significant if the *P* value was <0.05.

RESULTS

Caspase-1 activation and IL-1β/IL-18 release by macrophages infected with *L. pneumophila* involve both Asc and Ipaf. To determine the roles of Ipaf and Asc in caspase-1 activation in response to *L. pneumophila*, mouse bone marrow-derived

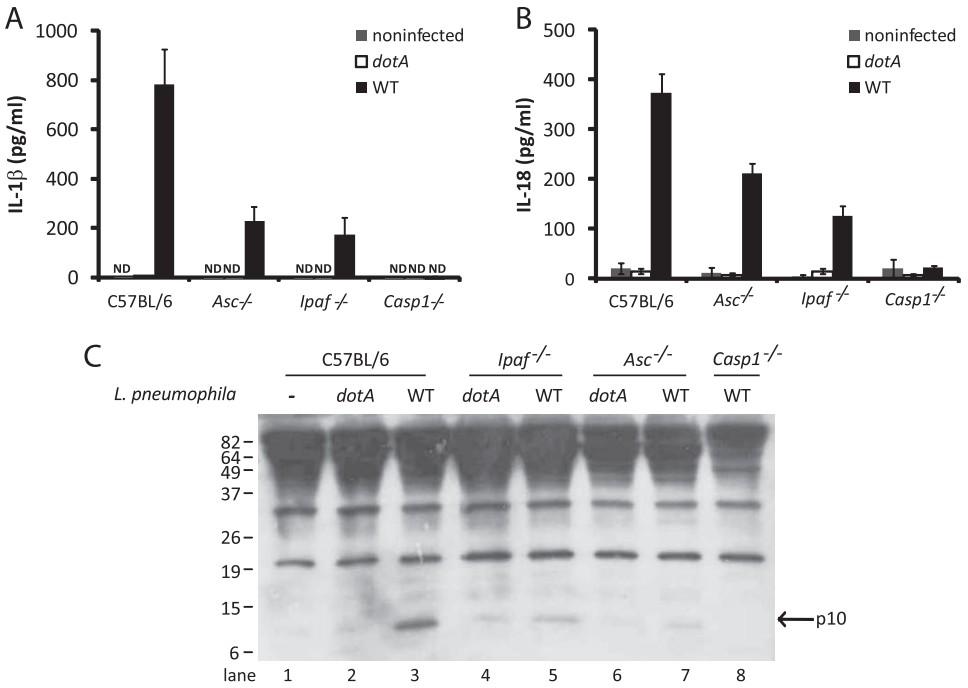

FIG. 1. Caspase-1 activation by macrophages in response to *L. pneumophila* involves both Ipaf and Asc. BMMs from wild-type (WT), Asc$^{-/-}$, Ipaf$^{-/-}$, or Casp1$^{-/-}$ mice were either noninfected or infected with wild-type or *dotA L. pneumophila* at an MOI of 10. (A and B) IL-1β (A) and IL-18 (B) levels in culture supernatants at 8 h postinfection were quantified by ELISA. Data are represented as averages ± standard deviations. (C) Immunoblot of the p10 fragment of caspase-1 from combined supernatants and pellets of BMMs obtained at 4 h postinfection. Data are representative of four (A) or three (B and C) experiments. ND, measurable quantities not detected.

macrophages (BMMs) were infected and analyzed. Macrophages derived from wild-type mice were compared to macrophages from mice deficient in either caspase-1, Ipaf, or Asc. Macrophages were incubated with wild-type or type IV secretion-deficient (*dotA*) *L. pneumophila* for 8 h, and the supernatants resulting from these infections were assayed for the presence of IL-1β and IL-18 by ELISA (Fig. 1A and B). Macrophages deficient in Ipaf or Asc secreted significantly less IL-1β and IL-18 ($P < 0.005$) than macrophages from wild-type mice in response to wild-type *L. pneumophila*. Higher levels of IL-1β and IL-18 ($P < 0.05$) were detected in supernatants from Ipaf- or Asc-deficient macrophages than in those from macrophages deficient in caspase-1 after infection with wild-type *L. pneumophila* (Fig. 1A and B). These results indicate that neither Asc nor Ipaf is completely required for caspase-1-dependent IL-1β/IL-18 release, suggesting that there might be two different pathways for caspase-1 activation in response to *L. pneumophila*. Consistent with this hypothesis, caspase-1 activation in response to *L. pneumophila* infection was detected in macrophages deficient in Asc or Ipaf as determined by the presence of the p10 fragment of active caspase-1 (Fig. 1C, lanes 5 and 7).

***L. pneumophila* can activate caspase-1 by an Asc-dependent pathway that does not require Ipaf or flagellin.** Flagellin has been shown previously to be important for caspase-1 activation and IL-1β release in response to *L. pneumophila* by a pathway that requires both Ipaf and Naip5 (1, 20, 30). To determine whether Asc is required for the Naip5/Ipaf/flagellin pathway of caspase-1 activation during *L. pneumophila* infection, macrophages were infected with the wild type or the flagellin-deficient *L. pneumophila flaA* mutant, and IL-1β and IL-18 levels in the supernatant were measured after 8 h by ELISA (Fig. 2A and B). Wild-type macrophages secreted significantly less IL-1β and IL-18 ($P < 0.01$) in response to flagellin-deficient *L. pneumophila* than did wild-type bacteria. IL-1β and IL-18 levels secreted by the Ipaf$^{-/-}$ macrophages were similar in response to wild-type and flagellin-deficient *L. pneumophila*. IL-1β and IL-18 levels secreted by Asc$^{-/-}$ macrophages infected with wild-type *L. pneumophila* were significantly higher ($P < 0.001$) than the IL-1β and IL-18 levels secreted after infection by the *L. pneumophila flaA* mutant. Importantly, the defect in IL-1β and IL-18 secretion observed for Asc$^{-/-}$ macrophages after infection with the *L. pneumophila flaA* mutant was similar to the defect observed for caspase-1$^{-/-}$ macrophages. These results indicate that caspase-1-mediated IL-1β and IL-18 release in response to *L. pneumophila* flagellin occurs through an Ipaf-dependent pathway and that there is also an Ipaf/flagellin-independent mechanism for *L. pneumophila* activation of caspase-1 that involves the adapter protein Asc. In support of these findings, active caspase-1 was detected in Ipaf$^{-/-}$ and wild-type macrophages infected with the *L. pneumophila flaA* mutant (Fig. 2C, lane 6) but not in Asc$^{-/-}$ macrophages infected with flagellin-deficient *L. pneumophila* (Fig. 2C, lane 8).

High extracellular potassium levels inhibit Asc-mediated IL-1β and IL-18 release and caspase-1 activation by macrophages in response to *L. pneumophila*. To further demonstrate that *L. pneumophila* is able to induce caspase-1-mediated IL-1β and IL-18 release through the adapter protein Asc independently of Ipaf, the ability to preferentially inhibit the

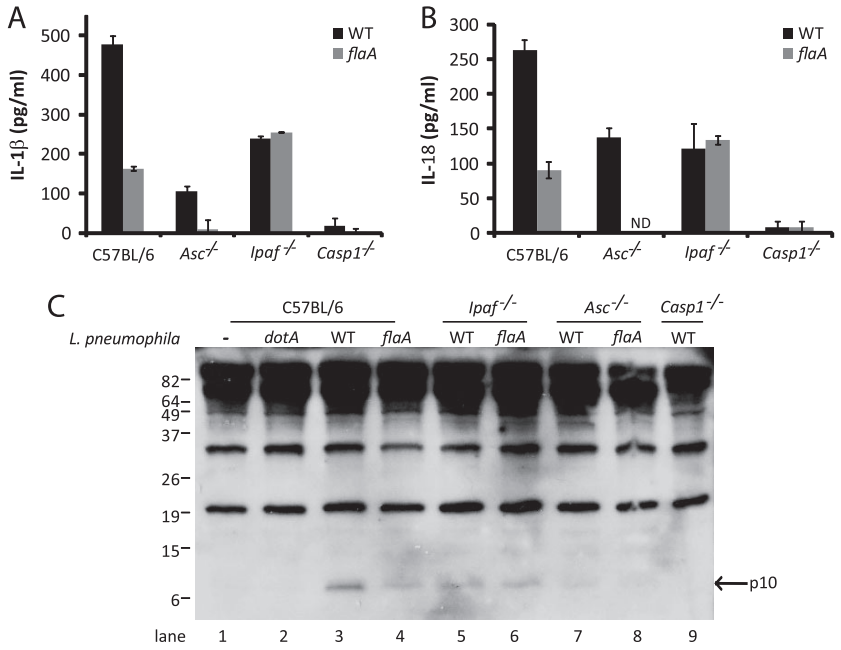

FIG. 2. *L. pneumophila* activates caspase-1 through Asc, independent of the Ipaf/flagellin pathway. BMMs from wild-type (WT), Asc$^{-/-}$, Ipaf$^{-/-}$, or Casp1$^{-/-}$ mice were infected with the indicated *L. pneumophila* variants at an MOI of 10. (A and B) IL-1β (A) and IL-18 (B) levels in culture supernatants at 8 h postinfection were quantified by ELISA. Data are represented as averages ± standard deviations. (C) Immunoblot of the p10 fragment of caspase-1 from combined supernatants and pellets of BMMs obtained at 4 h postinfection. Data are representative of four (A) or three (B and C) experiments. ND, measurable quantities not detected.

Asc-dependent pathway was tested. It has been shown that activation of the Nalp3 inflammasome by LPS and ATP can be inhibited by the addition of extracellular potassium to macrophage cultures (32). In addition, in vitro data suggest that formation of a functional Asc/caspase-1 complex is inhibited by increased potassium levels (5). To test whether *L. pneumophila*-induced IL-1β and IL-18 release from macrophages is inhibited by extracellular potassium, infections were carried out in the presence of high extracellular potassium levels. Supernatants from macrophages infected in the presence or absence of high potassium were harvested at 8 h postinfection and assayed for IL-1β and IL-18 by ELISA (Fig. 3A and B). Wild-type and Ipaf$^{-/-}$ macrophages showed significant decreases in IL-1β and IL-18 release ($P < 0.05$) in response to wild-type *L. pneumophila* in the presence of high extracellular potassium levels; however, there was no further decrease in cytokine release by Asc$^{-/-}$ macrophages in the presence of high potassium. The observed inhibition resulting from potassium treatment appeared to be specific for caspase-1-mediated cytokine release, as there was no apparent defect in release of TNF-α (Fig. 3C). These data indicate that the caspase-1-mediated IL-1β and IL-18 release inhibited by the presence of high extracellular potassium levels was dependent on Asc. Additionally, Ipaf$^{-/-}$ macrophages infected with wild-type *L. pneumophila* in the presence of high extracellular potassium secreted IL-1β and IL-18 to levels similar to those observed with caspase-1$^{-/-}$ macrophages and phenotypically resembled Asc-deficient cells infected with the *L. pneumophila flaA* mutant.

To investigate whether high levels of extracellular potassium specifically inhibited Asc-mediated caspase-1 activation, cleavage of caspase-1 was monitored by immunoblot analysis after macrophages were infected with *L. pneumophila* (Fig. 4). Wild-type macrophages infected with wild-type *L. pneumophila* in the presence of high extracellular potassium levels still induced substantial caspase-1 activation (Fig. 4, lane 4). However, infection with flagellin-deficient *L. pneumophila* or infection of Ipaf$^{-/-}$ macrophages resulted in the apparent abrogation of caspase-1 cleavage in the presence of high extracellular potassium levels, as indicated by the loss of detection of the p10 fragment under these conditions (Fig. 4, lanes 6 and 8). There were no apparent differences in p10 levels when Asc$^{-/-}$ macrophages infected with wild-type *L. pneumophila* in the presence of high extracellular potassium levels were compared to similarly infected Asc$^{-/-}$ macrophages without potassium added (Fig. 4, lanes 9 and 10). These data indicate that Asc-dependent caspase-1 activation is specifically inhibited by high levels of extracellular potassium, whereas the Ipaf-mediated pathway for caspase-1 activation is relatively unaffected.

Macrophages deficient in Ipaf and Asc fail to activate caspase-1 in response to *L. pneumophila*. *L. pneumophila* induced an Ipaf-dependent and an Asc-dependent pathway for caspase-1 activation. In addition, macrophages deficient in Asc and infected with *L. pneumophila flaA* or macrophages deficient in Ipaf and infected in the presence of high extracellular potassium were similar phenotypically to caspase-1-deficient macrophages. These data suggest that caspase-1 is no longer activated in the absence of both Ipaf- and Asc-mediated signaling in response to *L. pneumophila*. To further test this hypothesis, BMMs from Asc$^{-/-}$ Ipaf$^{-/-}$ double-knockout mice were examined for caspase-1 activation following *L. pneumophila* infection. Macrophages deficient in both Ipaf and Asc

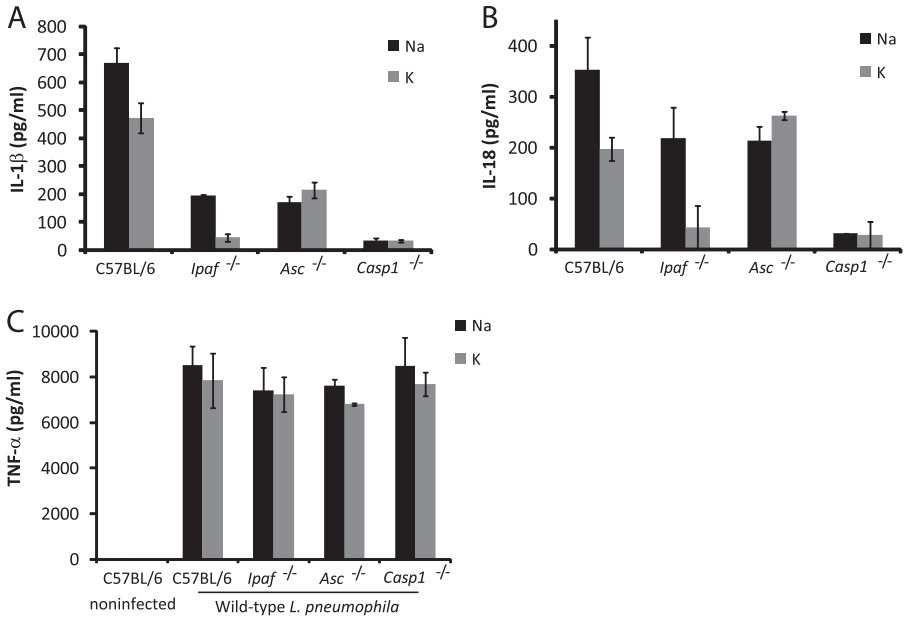

FIG. 3. Asc-dependent IL-1β and IL-18 release by macrophages in response to *L. pneumophila* is inhibited by high extracellular potassium. BMMs from wild-type, Asc$^{-/-}$, Ipaf$^{-/-}$, or Casp1$^{-/-}$ mice were infected with wild-type or *flaA L. pneumophila* at an MOI of 10. During infection, cells were cultured in the presence of an additional 50 mM KCl; control cells were incubated with an additional 50 mM NaCl. IL-1β (A), IL-18 (B), and TNF-α (C) levels in culture supernatants at 8 h postinfection were quantified by ELISA. Data are represented as averages ± standard deviations. Data are representative of four (A) or three (B and C) experiments.

displayed a defect in IL-1β and IL-18 secretion that was indistinguishable from that of caspase-1-deficient macrophages in response to *L. pneumophila* (Fig. 5A and B). Thus, in the absence of both Ipaf- and Asc-mediated signaling, *L. pneumophila* no longer activated caspase-1. In agreement with this hypothesis, active caspase-1 was not detected by immunoblot analysis in macrophages deficient in both Ipaf and Asc after *L. pneumophila* infection (Fig. 5C, lane 8).

***L. pneumophila*-induced caspase-1 activation occurs independently of Nalp3 and proteasome activity.** Nalp3-dependent caspase-1 activation in response to microbial pathogens and toxins has been shown to require Asc (16, 17, 23, 24, 32, 39, 40, 42). In addition, activation of the Nalp3 inflammasome by LPS and ATP is blocked by high extracellular potassium levels (32). Thus, *L. pneumophila*-induced caspase-1 activation might involve Nalp3. Previous data have indicated that wild-type *L. pneumophila* induced IL-1β release by a Nalp3-independent pathway (40). To investigate the role of Nalp3 further, Nalp3$^{-/-}$ macrophages were infected with either wild-type or flagellin-deficient *L. pneumophila* for 8 h, and the resulting culture supernatants were assayed for the presence of IL-1β by ELISA (Fig. 6A). Cytokine levels from Nalp3$^{-/-}$ macrophages were compared to those from wild-type and Asc-deficient macrophages. Nalp3$^{-/-}$ macrophages behaved similarly to wild-type macrophages in response to both wild-type and flagellin-deficient *L. pneumophila*. In contrast, Asc$^{-/-}$ macrophages were significantly impaired for IL-1β secretion in response to wild-type *L. pneumophila*, and secreted IL-1β levels were comparable to those for caspase-1$^{-/-}$ macrophages when infected with *L. pneumophila flaA*. Thus, Asc-dependent activation of caspase-1 by *L. pneumophila* does not require Nalp3.

Anthrax lethal toxin induces caspase-1 activation through Nalp1 by a process that requires proteasome activity (7, 37, 43). To investigate whether *L. pneumophila* activates caspase-1 through Asc by a proteasome-dependent process, wild-type and Asc$^{-/-}$ macrophages were infected with *L. pneumophila* in the presence of the proteasome inhibitor MG132. Culture supernatants were harvested at 6 h postinfection and assayed for the presence of IL-18 and IL-1β by ELISA (Fig. 6B and C). Prior to IL-1β release in macrophages, pro-IL-1β expression is induced by NF-κB, and NF-κβ activation requires proteasome activity. To overcome pro-IL-1β inhibition at the transcrip-

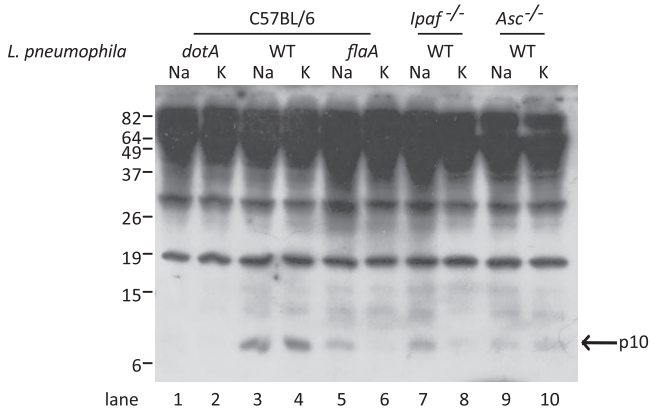

FIG. 4. High extracellular potassium levels inhibit Asc-dependent caspase-1 activation. An immunoblot of the p10 fragment of caspase-1 from combined supernatants and pellets of BMMs from wild-type (WT), Asc$^{-/-}$, or Ipaf$^{-/-}$ mice is shown. Cells were infected for 4 h with wild-type, *dotA*, or *flaA L. pneumophila* at an MOI of 10 in the presence of an additional 50 mM NaCl or 50 mM KCl. Data are representative of three experiments.

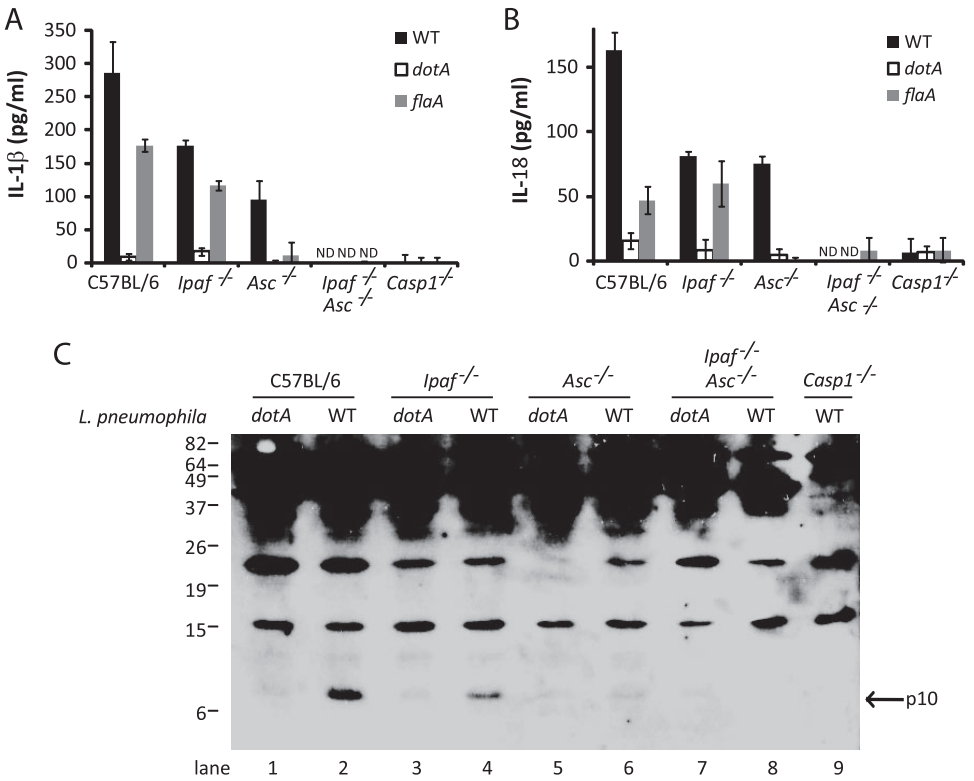

FIG. 5. Macrophages deficient in both Ipaf and Asc fail to activate caspase-1 in response to *L. pneumophila.* BMMs from wild-type (WT), $Asc^{-/-}$, $Ipaf^{-/-}$, $Ipaf^{-/-}$ $Asc^{-/-}$, or $Casp1^{-/-}$ mice were infected with the indicated *L. pneumophila* variants at an MOI of 10. (A and B) IL-1β (A) and IL-18 (B) levels in culture supernatants at 8 h postinfection were quantified by ELISA. Data are represented as averages ± standard deviations. (C) Immunoblot of the p10 fragment of caspase-1 from combined supernatants and pellets of BMMs obtained at 4 h postinfection. Data are representative of four (A and C) or two (B) experiments. ND, measurable quantities not detected.

tional level, macrophages were treated with LPS for 3 hours prior to infection and proteasome treatment. To confirm that proteasome treatment did not significantly affect cell viability, LDH release assays were performed on culture supernatants prior to cytokine measurements (Fig. 6D). Secreted IL-1β and IL-18 levels were relatively unaffected when wild-type and Asc-deficient macrophages were infected with *L. pneumophila* in the presence of proteasome inhibitor. Thus, caspase-1-mediated processing and release of IL-1β and IL-18 in response to *L. pneumophila* do not require proteasome activity.

Pore formation induced by *L. pneumophila* requires Ipaf/flagellin-mediated caspase-1 activation and is independent of Asc-mediated caspase-1 activation. Because Ipaf- and Asc-deficient macrophages appear to define two different pathways for activation of caspase-1 in response to *L. pneumophila*, we investigated whether distinct responses were associated with the different inflammasome activities. Activation of caspase-1 can lead to pyroptotic cell death, a process by which macrophages lyse and release their intracellular contents. It has been demonstrated that cell death results from osmotic lysis due to formation of pores in cell membranes by a caspase-1-mediated process (9). It has been shown previously that *L. pneumophila* induces pore formation in macrophages by a type IV-dependent process (18). In addition, *L. pneumophila* has been shown to induce cell lysis through a caspase-1-dependent process involving Ipaf, Naip5, and bacterial flagellin (20, 30). Thus, it is possible that *L. pneumophila*-induced cell lysis results from caspase-1-mediated pore formation in the host cell membrane. To examine this possibility, the role of caspase-1 in *L. pneumophila*-induced pore formation was investigated. Pore formation was measured at 2 hours postinfection by flow cytometry following PI staining (Fig. 7A). *L. pneumophila* induced the formation of pores in macrophages at 2 hours by a Dot/Icm-dependent process. Although wild-type macrophages showed significant PI uptake, there was no detectable PI uptake by caspase-1-deficient macrophages. Because the Ipaf/flagellin pathway of caspase-1 activation by *L. pneumophila* induces cell death, we investigated whether this pathway was required for pore formation. Indeed, PI uptake was diminished in $Ipaf^{-/-}$ macrophages or macrophages infected with flagellin-deficient *L. pneumophila*, suggesting that pore formation requires Ipaf and bacterial flagellin. In contrast, macrophages deficient in Asc exhibited PI uptake similar to that of wild-type macrophages, indicating that Asc is dispensable for caspase-1-mediated pore formation during *L. pneumophila* infection. In addition to pore formation, LDH release was used to measure cell lysis following *L. pneumophila* infection of macrophages (Fig. 7B and C). These data show that rapid caspase-1-mediated lysis of macrophages following *L. pneumophila* infection requires Ipaf and flagellin but is independent of Asc. If Asc is dispensable for caspase-1-mediated pore formation and cell lysis, specific inhibition of Asc-dependent caspase-1 activation by high extracellular potassium levels should have no significant impact on pore formation or cell lysis. Indeed, high ex-

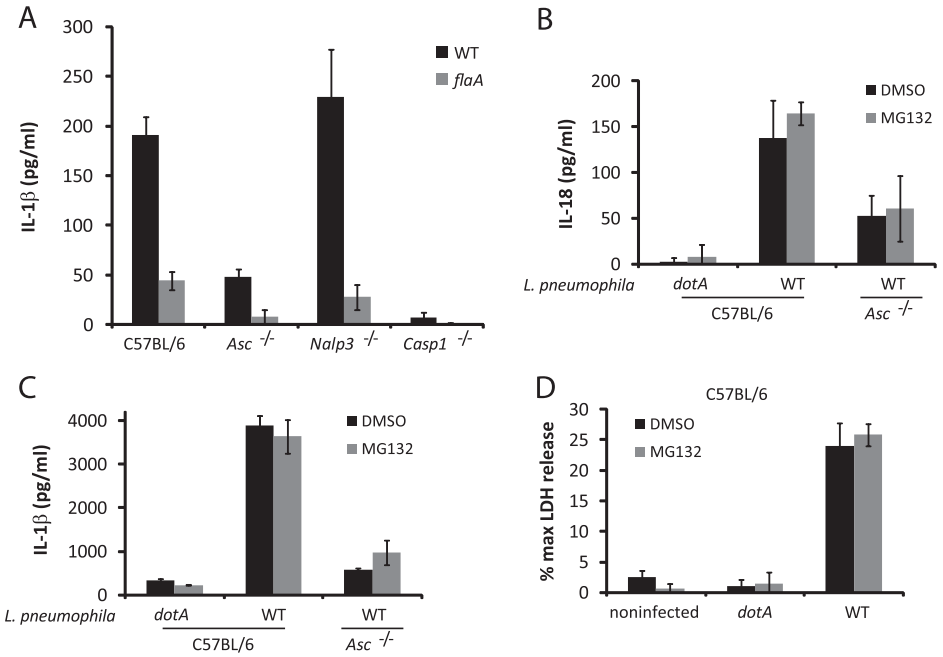

FIG. 6. *L. pneumophila* pathways for caspase-1 activation do not involve Nalp3 or the proteasome. (A) BMMs from wild-type (WT), Asc$^{-/-}$, Nalp3$^{-/-}$, or Casp1$^{-/-}$ mice were infected with wild-type or *flaA L. pneumophila* at an MOI of 10 and assayed for IL-1β in supernatants at 8 h postinfection. (B and C) Wild-type or Asc-deficient (Asc$^{-/-}$) BMMs were pretreated with LPS (1 μg/ml) for 3 hours, followed by infection with wild-type or *dotA L. pneumophila* in the presence of dimethyl sulfoxide (DMSO) (mock treated) or MG132 and assayed for IL-1β or IL-18 in culture supernatants at 6 h postinfection. Data are represented as averages ± standard deviations. (D) LDH release assays were performed at 6 h postinfection for wild-type macrophages infected with wild-type or *dotA L. pneumophila* in the presence or absence of MG132. Values represent the percentage of LDH released compared to that for cells lysed with Triton X-100. Data are represented as averages ± standard deviations. Data are representative of two experiments.

tracellular potassium, which inhibits Asc-dependent caspase-1 activation, did not significantly affect pore formation and cell lysis in *L. pneumophila*-infected macrophages as measured by PI uptake and LDH release, respectively (Fig. 7D and E). Thus, Ipaf and Asc control different downstream functions of caspase-1 in response to *L. pneumophila*.

Ipaf- and Asc-mediated caspase-1 activation by *L. pneumophila* occurs during pulmonary infection. *L. pneumophila* activates caspase-1 through Ipaf- and Asc-dependent pathways in BMMs cultured ex vivo. To determine if similar events take place during the course of pulmonary infection, wild-type mice and mice deficient in Asc, Ipaf, or caspase-1 were infected intranasally with *L. pneumophila.* Active IL-18 was measured in bronchoalveolar lavage fluid by ELISA at 1 day postinfection using an antibody specific for the caspase-1-cleaved form of the secreted cytokine (Fig. 8A). Mice deficient in Ipaf or Asc produced significantly less active IL-18 ($P < 0.05$) in the lung in response to wild-type *L. pneumophila* than did wild-type mice; however, IL-18 levels in the lungs of these mice were significantly higher ($P < 0.005$) than those in similarly infected caspase-1$^{-/-}$ mice. In addition, wild-type and Asc-deficient mice infected with *L. pneumophila flaA* produced significantly less IL-18 ($P < 0.05$) in the lung than wild-type and Asc-deficient mice infected with wild-type *L. pneumophila*, whereas IL-18 levels were similar when Ipaf-deficient mice infected with wild-type *L. pneumophila* were compared to Ipaf-deficient mice infected with *L. pneumophila flaA*. Importantly, the severe defect in IL-18 levels in the lungs of Asc-deficient mice infected with *L. pneumophila flaA* was similar to the defect observed for infected caspase-1$^{-/-}$ mice. Taken together, these data agree with the ex vivo studies with macrophages and indicate that Asc and Ipaf can function independently in response to *L. pneumophila* to activate caspase-1 during infection of the lung.

Lastly, we examined whether Ipaf or Asc influences the ability of mice to restrict the growth of *L. pneumophila* in the lung. Mice were infected intranasally, and CFU in the lung were measured at 1 and 2 days postinfection (Fig. 8B and C). Growth of *L. pneumophila* was significantly enhanced ($P < 0.05$ at 48 h) in the lungs of mice deficient in Ipaf and infected with wild-type *L. pneumophila* and in those of wild-type mice infected with flagellin-deficient *L. pneumophila* (Fig. 8B and C). Replication of wild-type *L. pneumophila* in the lungs of Asc-deficient mice was limited and not significantly different from the limited replication observed in wild-type mice (Fig. 8C). Because IL-18 levels in the lungs of Asc-deficient mice were similar to those in Ipaf-deficient mice, these data indicate that restriction of *L. pneumophila* replication in the mouse lung is independent of IL-18 levels produced and that Asc and Ipaf control two distinct caspase-1 activation pathways that differ in functional outcomes.

DISCUSSION

Activation of caspase-1 by the NLRs in response to pathogens and toxins is an important feature of the innate immune

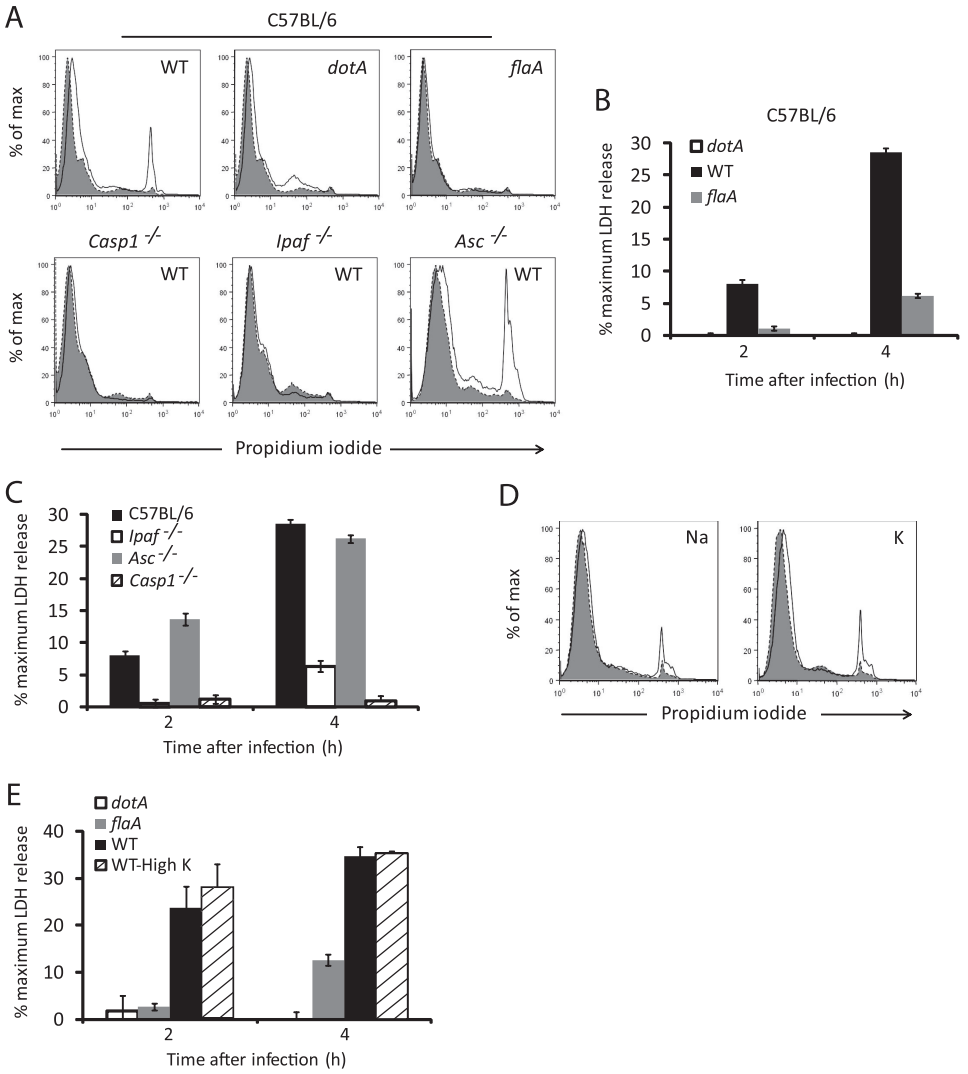

FIG. 7. Pore formation and cell lysis induced by *L. pneumophila* requires caspase-1, Ipaf, and bacterial flagellin but not Asc. BMMs from wild-type (WT), Asc$^{-/-}$, Ipaf$^{-/-}$, or Casp1$^{-/-}$ mice were either noninfected or infected with the indicated *L. pneumophila* variants at an MOI of 50. (A and D) Macrophages were assayed for PI uptake at 2 h postinfection by flow cytometry. Noninfected controls are represented in gray with a dashed outline, and the overlaid samples are represented by a solid black line. (B, C, and E) LDH release assays were performed at the indicated times for wild-type BMMs infected with indicated *L. pneumophila* variants (B and E) or wild-type, Asc$^{-/-}$, Ipaf$^{-/-}$, or Casp1$^{-/-}$ BMMs infected with wild-type *L. pneumophila* (C). Values represent the percentage of LDH released compared to that for cells lysed with Triton X-100. Data points are averages ± standard deviations. Data for wild-type macrophages infected with wild-type *L. pneumophila* are the same in panels B and C. Macrophages were incubated in the presence of an additional 50 mM KCl or 50 mM NaCl where indicated (D and E). Data are representative of five (A), four (B and C), or three (D and E) experiments.

response. The mammalian genome encodes a large repertoire of NLRs. The presence of multiple NLRs confers upon the host the potential to respond independently and distinctly to a multitude of signals that can be generated by a single pathogenic organism. The intracellular pathogen *L. pneumophila* has been shown to activate caspase-1 through the host NLRs Ipaf and Naip5 (1, 45). Recent data indicate that these proteins are critical for the induction of caspase-1 activation and cell death in response to a region of *L. pneumophila* flagellin (20). It remained likely, however, that other NLRs would be capable of responding to *Legionella* to activate caspase-1 by pathways independent of Ipaf and Naip5. In fact, previous studies had demonstrated a role for Asc in IL-1β release by macrophages after infection (45). However, an indirect role for Asc in IL-1β secretion could not be ruled out from previous data because Asc was not critical for caspase-1-dependent restriction of *L. pneumophila* growth in macrophages by the Ipaf/Naip5-dependent pathway. For this reason, it was important to determine whether Asc has a direct impact on caspase-1 activation in response to *L. pneumophila* or only on IL-1β secretion.

Our data demonstrate a pathway that requires Asc and is independent of Ipaf that resulted in the activation of caspase-1 by *L. pneumophila*. This pathway was revealed by showing the activation of caspase-1 in macrophages deficient in Ipaf and infected with wild-type *L. pneumophila* and in wild-type macrophages infected with an *L. pneumophila* mutant deficient in

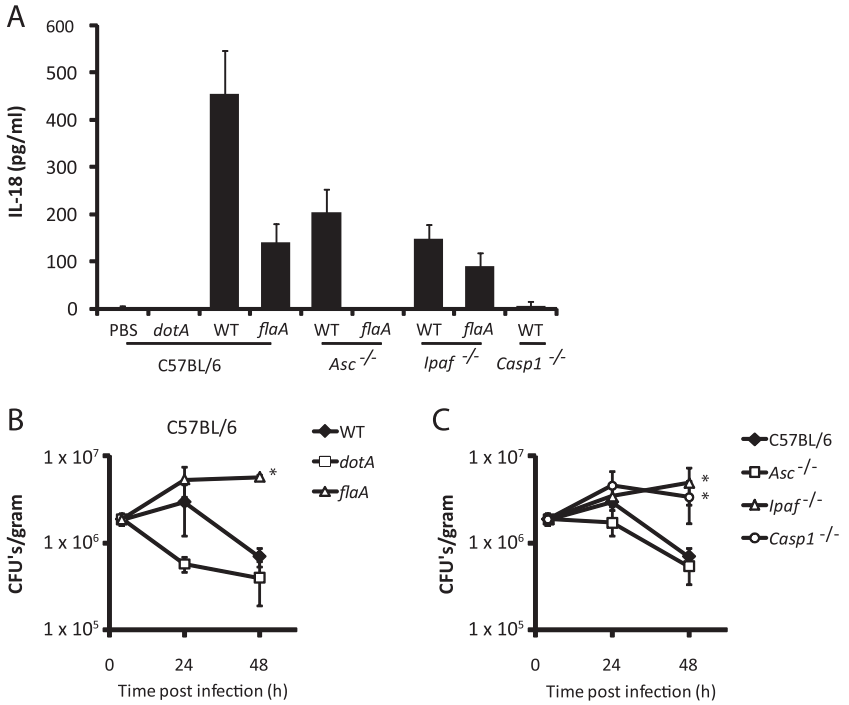

FIG. 8. *L. pneumophila* induces IL-18 release through Ipaf/flagellin- and Asc-dependent pathways in the mouse lung. Wild-type (WT), Ipaf$^{-/-}$, Asc$^{-/-}$, and Casp1$^{-/-}$ mice were infected with wild-type, *dotA*, or *flaA L. pneumophila* through intranasal inoculation. (A) At 24 h postinfection, bronchoalveolar lavage fluid was assayed for IL-18 by ELISA as described above. IL-18 levels indicated are the averages ± standard errors for five mice in all groups except that of C57BL/6 mice infected with PBS or *dotA L. pneumophila*, which contained four mice. (B and C) For assaying *L. pneumophila* growth, wild-type mice infected with the indicated *L. pneumophila* variants (B) and wild-type, Ipaf$^{-/-}$, Asc$^{-/-}$, or Casp1$^{-/-}$ mice infected with wild-type *L. pneumophila* (C) were sacrificed at various time points, and the lungs were harvested for quantifying CFU. Each point is displayed as the average ± standard error for four mice. Data for C57BL/6 mice infected with wild-type *L. pneumophila* are the same in panels B and C. An asterisk indicates a significant difference ($P < 0.05$) when comparing the indicated sample to a wild-type mouse infected with wild-type *L. pneumophila.* Data are representative of three experiments.

flagellin. Caspase-1 activation under these infection conditions resulted in detectable levels of IL-1β and IL-18 release in response to *L. pneumophila* (Fig. 1 and 2). The ability of high levels of extracellular potassium to block Asc-dependent caspase-1 activation was examined because a number of Asc-mediated pathways of caspase-1 activation can be blocked by potassium without having an effect on Ipaf-dependent pathways (11, 32). We found that potassium inhibited *L. pneumophila*-induced caspase-1 activation and IL-1β/IL-18 release only for the Asc-dependent pathway (Fig. 3 and 4). Additionally, macrophages deficient in Ipaf or infected with flagellin-deficient *L. pneumophila* no longer activated caspase-1 or induced IL-1β/IL-18 release when cultured in the presence of high extracellular potassium levels (Fig. 3 and 4), which provides further support that Asc and Ipaf function independently to activate caspase-1 in response to *L. pneumophila*. Furthermore, it is likely that Ipaf- and Asc-mediated pathways represent the primary mechanisms for caspase-1 activation by macrophages in response to *L. pneumophila*, because macrophages deficient in both Ipaf and Asc no longer activated caspase-1 during infection with this pathogen (Fig. 5).

The mechanism by which extracellular potassium inhibits caspase-1 activation in response to microbial pathogens and toxins remains unclear. One hypothesis is that some NLRs respond directly to changes in potassium concentration (5, 32). It is possible that insertion of the type IV apparatus into the host cell membrane induces a potassium efflux due to a localized loss of membrane integrity. An alternate possibility is that addition of extracellular potassium prevents NLR signals from being "sensed" by inhibiting the formation of an active inflammasome complex. In support of the latter hypothesis, formation of an active Asc-containing inflammasome was shown to be inhibited by increasing potassium concentrations in vitro independent of the upstream signals (5, 32). Thus, it is equally plausible that bacterial molecules enter the host cell cytosol by a translocation process dependent on the type IV secretion apparatus and that detection of these molecules by a cytosolic receptor(s) coupled with an efflux of potassium results in caspase-1 activation through an Asc-dependent pathway.

It is likely that *L. pneumophila* stimulates activation of one or more Nalps during infection to activate caspase-1 by the Asc-dependent pathway. There are 14 Nalp proteins in mice, and the agonists that activate most of these remain unknown. Stimuli that can activate Nalp3 and Nalp1 have been reported (14, 16, 17, 23, 24, 26, 32, 39, 40, 42), and the presence of high extracellular potassium levels can prevent the activation of caspase-1 through Nalp3 or Nalp1 (32). Investigations on the roles of individual Nalp proteins in the activation of caspase-1 by *L. pneumophila* revealed no detectable defect in IL-1β release in response to *L. pneumophila* when Nalp3$^{-/-}$ macrophages were compared to wild-type macrophages (Fig. 6A), suggesting either that Nalp3 is not the sole Nalp activated or

that Asc-mediated caspase-1 activation by *L. pneumophila* is independent of Nalp3. Additionally, the mechanism by which *L. pneumophila* induced Asc-dependent caspase-1 activation did not appear to require Nalp1, as inhibiting proteasome activity did not prevent activation of caspase-1 (Fig. 6B and C). Thus, it is possible that the Asc-mediated pathway for caspase-1 activation by *L. pneumophila* represents an uncharacterized mechanism that is distinct from the Nalp3 pathway and the mechanism for Nalp1 activation mediated by anthrax lethal toxin.

A number of studies have established that caspase-1, Ipaf, and bacterial flagellin are required for induction of pyroptotic cell death by *L. pneumophila* (20, 30, 34). Here, we show clearly that caspase-1 activity alone is not sufficient for cell death, because similar levels of caspase-1 activation were observed in the Ipaf-deficient macrophages and the Asc-deficient macrophages; however, in the absence of Ipaf or bacterial flagellin, formation of pores in the macrophage membrane, pyroptotic cell lysis, and restriction of *L. pneumophila* replication were all prevented. Importantly, by infecting Asc- and Ipaf-deficient mice, we were able to show that caspase-1-dependent cytokine responses to *L. pneumophila* in the lung were similar, validating ex vivo experiments with macrophages suggesting that Ipaf and Asc control independent pathways for caspase-1 activation. Additionally, these data indicate that Ipaf specifically, and not simply cytokine production in general, is important for the caspase-1-dependent restriction of *L. pneumophila* replication. These data support the hypothesis that pyroptosis is a physiologically important cell-autonomous mechanism for restricting *L. pneumophila* replication.

In conclusion, these data indicate that *L. pneumophila* stimulates at least two pathways for caspase-1 activation in macrophages. One pathway involves flagellin-mediated activation of Ipaf and Naip5. Formation of the Ipaf inflammasome appears to involve the correct assembly of molecules necessary for downstream events such as formation of pores in the host cell membrane, cell lysis, and restriction of *L. pneumophila* growth. A second pathway that involves the adapter protein Asc and most likely one or more Nalp proteins promotes caspase-1 activation and release of proinflammatory cytokines but does not appear to activate downstream events that can elicit pore formation, cell lysis, and restriction of *L. pneumophila* growth. Future studies are required to uncover how these pathways differentially regulate downstream events in response to *L. pneumophila*, as well as to identify possible upstream molecules in the Asc-mediated pathway of caspase-1 activation.

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