Regulation of *Legionella* Phagosome Maturation and Infection through Flagellin and Host Ipaf

Received for publication, May 23, 2006, and in revised form, July 31, 2006. Published, JBC Papers in Press, September 19, 2006, DOI 10.1074/jbc.M604933200

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*Legionella pneumophila* is an intracellular bacterium that causes an acute form of pneumonia called Legionnaires’ disease. After infection of human macrophages, the *Legionella*-containing phagosome (LCP) avoids fusion with the lysosome allowing intracellular replication of the bacterium. In macrophages derived from most mouse strains, the LCP is delivered to the lysosome resulting in *Legionella* degradation and restricted bacterial growth. Mouse macrophages lacking the NLR protein Ipaf or its downstream effector caspase-1 are permissive to intracellular *Legionella* replication. However, the mechanism by which Ipaf restricts *Legionella* replication is not well understood. Here we demonstrate that the presence of flagellin and a competent type IV secretion system are critical for *Legionella* to activate caspase-1 in macrophages. Activation of caspase-1 in response to *Legionella* infection also required host Ipaf, but not TLR5. In the absence of Ipaf or caspase-1 activation, the LCP acquired endoplasmic reticulum-derived vesicles, avoided fusion with the lysosome, and allowed *Legionella* replication. Accordingly a *Legionella* mutant lacking flagellin did not activate caspase-1, avoided degradation, and replicated in wild-type macrophages. The regulation of phagosome maturation by Ipaf occurred within 2 h after infection and was independent of macrophage cell death. *In vivo* studies confirmed that flagellin and Ipaf play an important role in the control of *Legionella* clearance. These results reveal that Ipaf restricts *Legionella* replication through the regulation of phagosome maturation, providing a novel function for NLR proteins in host defense against an intracellular bacterium.

*Legionella pneumophila* (*Legionella*) is a Gram-negative, intracellular bacterium and the causative agent of Legionnaires pneumonia (1). *Legionella* is able to infect and replicate inside human monocytes and macrophages (2–5). Replication within macrophages is critical for the disease, as mutants defective for intracellular growth *in vitro* are unable to cause pneumonia. Once phagocytosed, instead of getting degraded inside lysosomes of phagocytic cells, the bacteria resides in a specialized vacuole and starts to multiply within 8–10 h after internalization. Biogenesis of this replicative phagosome requires a functional bacterial type IV secretion system. The *Legionella*-containing phagosome (LCP) avoids fusion with the lysosome and acquires vesicles from the endoplasmic reticulum (ER) (2, 3, 5). In this compartment, *Legionella* multiply exponentially, then exit the infected cell and infect new neighboring cells. However, the host factors that regulate maturation of the LCP are largely unknown.

Most cells from inbred mouse strains are nonpermissive for *Legionella* intracellular replication with the exception of a few strains such as A/J (2, 8, 9). The mouse strain-specific variation that is responsible for permissiveness to *Legionella* replication is controlled in part by *Lgn1*, an autosomal, recessive locus (10). Recent studies have revealed that *Lgn1* maps to the *Birc1e* gene (11, 12). The product of *Birc1e*, Naip5, is a member of the NOD-like receptor (NLR) family (also called NOD-LRR, NODs, or Caterpillar proteins) that is involved in innate immune signaling in response to microbial stimulation (13–15). The NLR protein family contains more than 20 members including nod1, nod2, cryopyrin/Naip3, Naip1, Naip, and Ipaf. NLRs contain an N-terminal effector domain, a centrally located nucleotide-binding oligomerization domain (NOD) and C-terminal leucine-rich repeats (LRRs). Recent studies have shown that Naip5 and caspase-1 restrict *Legionella* growth by activating cell death when macrophages are infected at high multiplicity of infection (MOI) (16). Another NLR protein, Ipaf, has been implicated in the proteolytic activation of pro-caspase-1 into an enzymatically active heterodimer composed of 10- and 20-kDa...
subunits in response to Salmonella (17). However, the role of Ipaf in restricting Legionella replication is not well understood. In the present studies, we demonstrate an important function for Ipaf in the host response to Legionella through the regulation of flagellin-induced caspase-1 activation and phagosome maturation.

**MATERIALS AND METHODS**

*Bacterial Strains—*Legionella pneumophila* strain Lp02, is a thymine auxotrophic derivative of Philadelphia (1). The dotA mutant strain Lp03 is defective in the Dot/Icm Type IV secretion system (18). The flaA mutant *Legionella* was previously described (19). Bacterial strains were supplemented with a plasmid that complements thymine auxotrophy and expresses green fluorescent protein (GFP) at the post-exponential phase (PE) (4, 20). *Legionella pneumophila* was cultured as described previously (4, 20) in ACES-yeast extract broth supplemented with ferric nitrate and L-cysteine. To examine bacterial intracellular multiplication, infections were performed at low MOI of 0.5 followed by centrifugation and rinsing of the wells after 1 h of infection. Experiments were performed in the absence of ferric nitrate and L-cysteine from the macrophage culture medium, to allow *Legionella* multiplication only intracellularly. To quantify viable bacteria per well, culture supernatants were collected, and monolayers were lysed by trituration in ice-cold PBS. Pooled supernatants and lysates were cultured for 3–4 days on CYET agar plates. The scoring of the colony-forming units (CFU) and *in vivo* was performed as described (20, 21).

*Mice—*The generation of Ipaf knock-out (KO) mice has been described (22). Primers 5'-ctgacgcagctacattgcg-3' and 5'-gggctcttctctctgc-3', were used for *Birc1e* genotyping. DNA sequencing of *birc1e* exons verified the presence of the C57BL/6 allele in both Ipaf WT and KO mice. Caspase-1 KO mice (a generous gift of R. Flavell) were in a C57BL/6 background and C57BL/6 mice (the Jackson laboratory) were used as wild-type. TLR4 and TLR5 KO mice in C57BL/6 background and C57BL/6 mice (a generous gift of R. Flavell) were in a C57BL/6 background.

*Macrophages—*Bone marrow was prepared from leg bones of 5–8-week-old mice as previously described (20, 21).

*Fluorescence Microscopy—*GFP-expressing *Legionella* was detected directly by fluorescence microscopy. Synchronized infections were performed at an MOI of 2 followed by centrifugation. Samples were washed within 15 min of infection giving a final MOI of ~0.3. Intact and degraded bacteria were detected with mouse anti-*Legionella* antibody (Abcam). Extracellular bacteria were identified by staining with anti-*Legionella* antibody and secondary Texas Red-conjugated antibody before permeabilization as previously described (4). To evaluate *Legionella* viability, the bacteria containing a plasmid from which expression of green fluorescent protein could be induced by isoprropyl-thio-β-D-galactopyranoside (IPTG) was used to infect WT and Ipaf−/− macrophages. After 2 h of infection, IPTG was added for 4 h, and the percent of metabolically active bacteria-expressing GFP was determined (20). Cell death in GFP- *Legionella*-infected macrophages was determined using the Live/dead cell viability kit (Invitrogen) according to the manufacturer, and the results were expressed as percent dead cells among 100 infected cells. Localization of markers on *Legionella* phagosomes was performed as previously described (4). Antbodies used were rat anti-lyso-sosomal-associated membrane protein 1 (LAMP1; 1D4B; Developmental Hybridoma Bank), rabbit anti-calcereulin (Stressgen Bioreagents) followed by fluorescent secondary antibodies (Molecular Probes). Nuclei were stained with nucleic acid dye 4',6'-diamino-2-phenylindole (DAPI; Molecular Probes). For membrane markers, vacuoles were defined as positive only when a ring of fluorescence was observed at the vacuole periphery; this stringent criterion likely underestimates the fraction of LAMP-1 and calcereulin-containing vacuoles. Samples were analyzed with Zeiss Axioplan 2 fluorescence microscope and Olympus Fluoview Confocal Laser scanning microscope. Pictures were captured with Olympus Fluoview Confocal Laser scanning microscope.

*Immunoblotting—*Cells were infected at an MOI of 2, centrifuged, incubated for 15 min, then rinsed three times to remove extracellular bacteria. Cell extracts were prepared and immunoblotted with an antibody that recognizes the large subunit of mouse caspase-1 (generous gift of P. Vandenabeele) followed by appropriate secondary anti-rabbit antibody as described (22). When indicated macrophages were permeabilized with 10 ng/ml streptolysin O for 5 min in the absence or presence of ligands as previously described (25), then rinsed, and incubated for 2 h. Purified bacterial ligands were purchased from InvivoGen. LPS followed by ATP treatment of macrophages was performed as previously described (23).

*Electron Microscopy—*Electron microscopy was performed as previously described (26).

*Mouse Infection and Statistical Analysis—*WT and Ipaf−/− mice were infected intratracheally with 1 × 10⁷ bacteria of WT or *flaA*− mutant *Legionella*, and the number of bacteria in the lungs was determined at 6 h and at day 4 post-infection. Data were analyzed with the Mann-Whitney test as appropriate. Differences in data values were considered significant at a *p* value of less than 0.05.

**RESULTS**

Ipaf−/− and Caspase-1−/− but Not Nod1−/− or Cryopyrin−/− Bone Marrow-derived Macrophages Allow Legionella Replication—Macrophages from the great majority of mouse strains are non-permissive to *Legionella* replication (8, 9). To identify host factors that restrict the growth of *Legionella*, we tested the ability of macrophages lacking several proteins implicated in the regulation of innate immunity including Toll-like receptor-4 (TLR4) and members of the NLR family of cytosolic receptors to support bacterial replication (13, 27). Bone marrow (BM)-derived macrophages from wild-type mice and mutant mice deficient in TLR4, Nod1, or Cryopyrin/Nalp3 (23) did not support significant bacterial replication as assessed over 72 h after infection with infectious *Legionella* at an MOI of 0.5 (Fig. 1A). In contrast, by 72 h of infection, the bacterial counts increased 150-fold in macrophages deficient in Ipaf (Fig. 1A), another NLR family member. *Legionella* infection of Ipaf−/− peritoneal macrophages supported bacterial growth around 1,000-fold (Fig. 1B). Ipaf interacts with caspase-1 through a homophilic
caspase-recruitment domain (CARD) and regulates the activation of caspase-1 (17). To test directly the role of caspase-1 in bacterial replication, wild-type and caspase-1-/- macrophages (28) were infected with Legionella, and the number of bacteria in the cultures was assessed over time. Caspase-1-/- macrophages supported the growth of the bacterium greater than 200-fold by 72 h of infection, comparable to the results obtained with Ipaf-/- macrophages under similar infection conditions (Fig. 1C). To visualize the pathogenic organism inside the cells, WT, Ipaf-/-, and caspase1-/--deficient macrophages were infected with Legionella that express green fluorescence protein (GFP), and the presence of bacteria was monitored by fluorescence microscopy. At 24-h postinfection, only a few individual bacteria were identified inside wild-type macrophages, whereas expanded compartments packed with Legionella were observed inside Ipaf-/- macrophages (Fig. 1D, arrows). The difference in intracellular bacterial replication was not due to differential uptake of Legionella because differential immunofluorescence staining showed that the number of internalized bacteria was similar in wild-type and mutant macrophages at 1-h postinfection (supplemental Fig. S1A). In contrast to pathogenic Legionella, Ipaf-/- macrophages did not allow the replication of a non-pathogenic Legionella mutant defective in Dot/Icm (supplemental Fig. S1B). Therefore, the bacterial replication is regulated by Ipaf and caspase-1, a process that requires a functional Dot/Icm system of pathogenic Legionella.

Ipaf Is Required for Caspase-1 Activation in Response to Legionella Infection—We next tested if Legionella infection activates caspase-1 and if this process requires Ipaf. By 1-h postinfection, the bacterium induced proteolytic activation of pro-caspase-1 as determined by the detection of the mature 20-kDa subunit in cell extracts alone or combined with culture supernatants (A) were immunoblotted with antibody against the p20 subunit of caspase-1. Gels are representative of three independent experiments. C, macrophages from WT and Ipaf-/- mice were infected with Legionella, Salmonella, or left untreated. The level of cell death in infected macrophages was determined in triplicate cultures at 2 h (white bars), 4 h (gray bars), and 6 h (black bars) after infection. Values represent the mean ± S.D. of three independent experiments.

Legionella Induces Caspase-1 Activation via Ipaf in Macrophages—Wild-type BM-derived macrophages were infected with Legionella, and cell lysates alone (A) or combined with culture supernatants (B) were immunoblotted with antibody against the p20 subunit of caspase-1. WT and Ipaf-/- BM-derived macrophages infected with infectious Legionella were examined 24 h after infection by fluorescence microscope. Wild-type panels, magnification ×600. Ipaf KO and caspase-1 KO panels, magnification ×1000.

Regulation of Legionella Phagosome Maturation via Ipaf—We previously showed that differential uptake of Legionella was similar in wild-type and mutant macrophages at 1-h postinfection (supplemental Fig. S1A). In contrast to pathogenic bacteria, the number of internalized bacteria was similar in wild-type and mutant macrophages at 1-h postinfection (supplemental Fig. S1A). In contrast to pathogenic Legionella, Ipaf-/- macrophages did not allow the replication of a non-pathogenic Legionella mutant defective in Dot/Icm (supplemental Fig. S1B). Therefore, the bacterial replication is regulated by Ipaf and caspase-1, a process that requires a functional Dot/Icm system of pathogenic Legionella.
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conditions, less than 3% of the *Legionella*-infected cells were dead at 2, 4, or 6 h after infection in both WT and Ipaf-null macrophages (Fig. 2C).

Upon activation, caspase-1 cleaves pro-interleukin-1β (IL-1β), pro-IL-18, and IL-33 to generate biologically active cytokines (29). To evaluate the role of IL-1β and IL-18 in *Legionella* infection, we tested whether supplementation of IL-1β or IL-18 could inhibit the growth of *Legionella* observed in Ipaf−/− macrophages. We found that addition of IL-1β, IL-18 or a combination of both cytokines to macrophages at the time of infection did not suppress *Legionella* replication in Ipaf−/− macrophages (supplemental Fig. S2). These results indicate that Ipaf mediates caspase-1 activation in response to *Legionella* and suggest that caspase-1 activation is critical for the clearance of the bacterium independently of the activity of IL-1β and IL-18.

**Ipaf and Caspase-1 Regulate the Maturation of the Legionella-containing Phagosome at Low Multiplicity of Infection**—The fusion of the LCP with lysosomes is the critical event that restricts the replication of *Legionella* in wild-type macrophages (30, 4). To determine if Ipaf controls *Legionella* replication by regulating LCP lysosome fusion, we examined the maturation of the Legionella containing phagosome using the same conditions that allowed replication of the bacterium in Ipaf−/− macrophages. In these experiments, WT and Ipaf−/− macrophages were infected with *Legionella* a MOI of ~0.3 and under conditions that allowed the internalization of a single bacterium in 20–30% of macrophages. To monitor the maturation of the LCP, we examined the rate of acquisition of the lysosomal-associated membrane protein-1 (LAMP-1) by phagosomes harboring *Legionella*. Whereas in wild-type macrophages, greater than 20% of the phagosomes containing the pathogen colocalized with LAMP-1, only 5% of the phagosomes in Ipaf−/− and caspase-1−/− macrophages acquired the lysosomal marker at 2, 4, or 6 h after infection (Fig. 3A and B, see also supplemental Fig. S3A). The active recruitment of LAMP-1 to the Legionella compartment implies the survival of the macrophages at this time of infection, consistent with results shown in Fig. 2C. The difference in LAMP-1 colocalization between WT and Ipaf−/− is likely to be underestimated, because *Legionella* is rapidly degraded within phagolysosomes of wild-type macrophages. Moreover, the decrease in *Legionella* colocalization with LAMP-1 in Ipaf−/− macrophages is not due to a general defect in lysosome fusion because non-pathogenic *Escherichia coli* were effectively delivered to the lysosomes within 2 h of infection in both WT and Ipaf−/− macrophages (supplemental Fig. S3B).

To assess the role of Ipaf and caspase-1 in conveying bacterial degradation, macrophages were infected with *Legionella*, and the intact and degraded bacteria were differentiated and scored by labeling with anti-*Legionella* antibody. In wild-type macrophages, by 2-h postinfection, about 40% of internalized bacteria lost their rod-shaped contour and were degraded into multiple small rounded particles as opposed to only ~10% in Ipaf− or caspase-1-deficient macrophages (Fig. 3, C and D). Accordingly, in Ipaf−/− macrophages, ~80% of internalized *Legionella* were metabolically active and expressed GFP in response to IPTG added within 2 h of infection, compared with only 20% in wild-type cells (supplemental Fig. S4). Therefore, in the absence of Ipaf or caspase-1 activation, the maturation of the LCP is stalled and *Legionella* escape degradation.

**The Legionella Vacuole Avoids Fusion with the Lysosome and Intracellular Degradation in Ipaf and Caspase-1−Deficient Macrophages**—Given that the LCP is remodeled into an ER-derived organelle in human monocytes and in A/J-derived macrophages (2, 3), we examined the recruitment of calreticulin, an ER protein, to phagosomes harboring *Legionella*. By 5-h postinfection, 20–30% of the bacteria colocalized with calreticulin in Ipaf−/− macrophages, whereas no bacteria associated with the ER marker in wild-type macrophages (Fig. 4, A and B). Similarly, in caspase-1−/− macrophages the ER marker calreticulin accumulated around 20% of the internalized bacteria (Fig. 4, A and B). To confirm the recruitment of ER-derived vesicles to the LCP, we examined WT and Ipaf−/− macrophages after 4 h of infection by electron microscopy. Indeed, ribosome-studded vesicles accumulated around the LCP in Ipaf−/− macrophages, but not in wild-type cells (Fig. 4, C and D). Furthermore, many intact *Legionella* were detected in Ipaf−/− macrophages (Fig. 4C), while only scarce number of bacteria were found in wild-type cells and most showed signs of degradation (Fig. 4D).

**Flagellin Is Required for Legionella-induced Caspase-1 Activation**—Ipaf is known to play a critical role in the activation of caspase-1 in response to *Salmonella* via the sensing of flagellin (22, 31). To assess the molecule that triggers caspase-1 activation in response to *Legionella*, we performed several experiments. Initially, macrophages were stimulated with various microbial molecules in the presence of streptolysin-O (SLO), a protein that allows delivery of exogenous molecules into the cytosol of living cells (25). Consistent with recent results (22), stimulation of wild-type macrophages with purified flagellin,
but not synthetic bacterial lipopeptide (BLP) or CpG oligodeoxynucleotide (CpG ODN), induced activation of caspase-1 when added with SLO (Fig. 5A). Notably, activation of caspase-1 induced by flagellin was abrogated in Ipaf−/− macrophages (Fig. 5A and B). The role of Ipaf was specific in that caspase-1 activation induced by LPS and ATP was unimpaired in Ipaf-null macrophages (Fig. 5B). The low amount of SLO used to deliver flagellin to the cytosol was necessary but not sufficient for caspase-1 activation as SLO alone did not induce caspase-1 activation (Fig. 4A). To verify the role of flagellin in inducing caspase-1 activation, we infected macrophages with wild-type and mutant Legionella lacking flagellin and measured the activation of caspase-1. To ensure equal internalization of wild-type and flagellin-deficient Legionella, infections were followed by mild centrifugation to enhance contact between macrophages and mutant bacteria. Under these conditions, the uptake of wild-type and flagellin-deficient bacteria was similar at 1-h postinfection (supplemental Fig. S5). Unlike wild-type bacteria, infection of macrophages with a Legionella (flaA−) mutant lacking flagellin did not induce caspase-1 activation in wild-type macrophages (Fig. 5C). These results indicate that sensing of flagellin through Ipaf is required for Legionella to induce caspase-1 activation.

**TLR5 Is Not Required for Flagellin-induced Caspase-1 Activation**—TLR5 mediates the sensing of extracellular bacteria, resulting in NF-κB activation through MyD88 (32). We tested if Ipaf, or TLR5 modulate the activation of caspase-1 by flagellin. Analysis of mutant macrophages revealed that caspase-1 activation induced by flagellated Legionella was independent of TLR5 (Fig. 5D).

The Presence of Bacterial Flagellin Restricts Legionella Replication in Macrophages and in Mice—To further test the role of flagellin in restricting Legionella replication inside macrophages, we infected macrophages with WT and flaA− mutant Legionella and assessed bacterial growth over time. In contrast to wild-type Legionella, mutant bacteria lacking flagellin replicated equally in both WT and Ipaf−/− macrophages (Fig. 6A). Thus, both flagellin and Ipaf are required for restricting the intracellular replication of Legionella in macrophages. To verify the mechanism by which flagellin regulates Legionella growth, we assessed the recruitment of LAMP-1 and calreticulin on phagosomes harboring mutant Legionella. Less than 5% of flaA− mutant Legionella acquired LAMP-1 in WT and Ipaf−/− macrophages, whereas greater than 20% of the phagosomes containing the wild-type pathogen colocalized with the lysosomal marker in wild-type macrophages (Fig. 6B). Accordingly, flaA− mutant Legionella did not show signs of degradation either in WT or Ipaf−/− macrophages (Fig. 6C). By 5-h postinfection, greater than 40% of the bacteria lacking flagellin colocalized with calreticulin in both WT and Ipaf−/− macrophages, whereas practically no wild-type Legionella associated with the ER marker in wild-type macrophages (Fig. 6, D and E). Thus, flagellin modulates phagosome maturation in Legionella-infected macrophages, and this process is mediated through host Ipaf. We then tested if flagellin can regulate Legionella growth in vivo. WT and Ipaf−/− mice were infected intratracheally with 1 × 10⁶ CFU of WT or flaA− mutant Legionella and the number of bacteria in the lungs was determined at 6 h and at day-4 postinfection. At day-4 postinfection, there were ∼10-fold...
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**FIGURE 6. Legionella FlA mutants replicate in vitro and in vivo in WT and Ipaf−/− mice.** A, wild-type BM-derived macrophages were infected with wild-type (Leg) or flaA− mutant ( flaA) Legionella and bacterial replication was compared with Ipaf−/− macrophages infected with wild-type or flaA− mutant Legionella over 72 h. Samples were centrifuged after infection. B, WT and Ipaf−/− macrophages were infected with wild-type or flaA− mutant Legionella, and scored for colocalization with LAMP-1. C, percent degraded bacteria was scored using anti-Legionella antibody. D and E, colocalization of flaA− mutant Legionella with ER vesicles was scored using anti-calreticulin antibody. A total of 100 internalized bacteria were analyzed for colocalization with the ER and lysosomal markers in three independent experiments. F, WT and Ipaf−/− mice received 106 wild-type or flaA− mutant Legionella intratracheally. Lungs were homogenized and plated for CFUs at 96 h postinfection. Statistical analysis was performed with Mann-Whitney U test.

more Legionella CFUs in the lung tissue of Ipaf−/− mice, when compared with their wild-type counterparts (Fig. 6F) although initial bacterial counts in the lungs were identical (supplemental Fig. S5). Notably, the number of flaA− Legionella was similar in the lungs of both WT and Ipaf−/− mice (Fig. 5F), indicating flagellin is required for restricting the intracellular replication of Legionella in the animal.

**DISCUSSION**

Recent results that showed that flagellin is critical for caspase-1 activation in Legionella-infected macrophages and can restrict the growth of the bacterium (33, 34). However, the host factor that mediates flagellin-induced caspase-1 activation during Legionella infection remained unclear. Our studies show that the host factor Ipaf is required for caspase-1 activation in macrophages upon infection with Legionella. These studies are consistent with recent observations demonstrating that Ipaf is required for flagellin-induced caspase-1 activation in response to Salmonella (22, 31). Thus, both Salmonella and Legionella induce caspase-1 through the sensing of flagellin by Ipaf. The most significant finding of the present studies is that Ipaf-mediated caspase-1 activation induced by flagellin restricts Legionella growth by promoting LCP maturation and intracellular degradation, thus restricting bacterial replication. Upon Ipaf-dependent caspase-1 activation, Legionella is efficiently delivered to the lysosome for degradation. In the absence of flagellin or Ipaf-mediated caspase-1 activation, the fusion of the pathogen-enclosing compartment with the lysosome was drastically reduced, which was associated with a robust increase in Legionella growth. Under our culture conditions, we did not observe significant cell death of WT, Ipaf−/−, or caspase-1−/− macrophages during the first 6 h of Legionella infection using low MOIs. Because the fusion of the LCP to lysosomes is largely completed by 2 h after infection, the regulation of the LCP maturation mediated by Ipaf appears to be an important event that dictates the fate of the bacteria preceding the induction of cell death. However, our studies do not rule out that cell death contributes to the restriction of Legionella growth particularly at conditions that resemble high MOI of infection. For example, recent studies showed that Naip5, another NLR protein distinct from Ipaf, restricts Legionella replication through the induction of caspase-1-dependent cell death (16). There are several possible mechanisms by which Ipaf and caspase-1 could control phagosome maturation in response to Legionella infection. Active caspase-1 cleaves pro-IL-1β and pro-IL-18 to generate biologically active cytokines (29). However, addition of IL-1β and IL-18 to Ipaf-deficient macrophages did not affect Legionella replication, suggesting that production of these cytokines is not important for restriction of Legionella growth. Therefore, it is possible that caspase-1 acts by targeting and processing host proteins involved in the formation and/or transport of the LCP (35). Alternatively, caspase-1 could process bacterial factors that are required for formation of the specialized Legionella vacuole. Regardless of the mechanism, these results establish Ipaf and caspase-1 as important host factors for the control of the maturation of the LCP. Unlike Legionella, non-pathogenic bacteria such as E. coli were promptly delivered to the lysosome for degradation in the presence or absence of Ipaf or caspase-1. Thus, the regulation of phagosome maturation by Ipaf signaling may be restricted to Legionella or to pathogenic bacteria that employ similar molecular strategies to avoid lysosomal degradation in host macrophages. Thus, the role of Ipaf in the regulation of LCP maturation identifies a novel function for microbial pattern recognition receptors in the context of an intracellular bacterium.

There are several mechanisms by which flagellin could induce the activation of caspase-1 through Ipaf in Legionella-infected macrophages. Flagellin could enter the host cytosol through the pore formed in the macrophage membrane by the Legionella Type IV secretion system (36). Consistent with this is the observation that Legionella requires the Dot-Icm system for the induction of caspase-1 and phagosome maturation.
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 (37). The sensing of flagellin by Ipaf is most likely to be transient and/or indirect because we have been unable to detect an interaction between Ipaf and flagellin in cell extracts by immunoprecipitation (data not shown). The lack of direct interaction between flagellin and Ipaf is consistent with the mechanism of recognition of microbial components by plant Ipaf homologs (6, 7). Our results have shown a novel role for Ipaf in regulating *Legionella* growth in the presence of bacterial flagellin. These findings provide important insight into the role of NLR proteins in the host response to intracellular pathogens.

Acknowledgments—We thank Anthony Coyle, Ethan Grant, and John Bertin (Millennium Pharmaceuticals) for generous supply of mutant mice, R. Flavell for providing caspase-1 KO mice, P. Vandenabeele for anti-caspase-1 antibody, M. Swanson for bacterial strains, Christine McDonald for critical review of the manuscript, and Jolie Hoffman for technical help.

**REFERENCES**

1. McDade, J. E., Shepard, C. C., Fraser, D. W., Tsai, T. R., Redus, M. A., and Dowdle, W. R. (1977) *N. Engl. J. Med.* **297**, 1197–1203
2. Horwitz, M. A. (1983) *J. Exp. Med.* **158**, 1319–1331
3. Kagan, J. C., and Roy, C. R. (2002) *Nat. Cell Biol.* **4**, 945–954
4. Amer, A. O., and Swanson, M. S. (2005) *Cell Microbiol.* **7**, 765–778
5. Tilney, L. G., Harb, O. S., Connelly, P. S., Robinson, C. G., and Roy, C. R. (2001) *J. Cell Science* **114**, 4637–4650
6. Mackey, D., Belkhdar, Y., Alonso, J. M., Ecker, J. R., and Dangl, J. L. (2003) *Cell* **112**, 379–389
7. Mackey, D., Holt, B. F., 3rd, Wiig, A., and Dangl, J. L. (2002) *Cell* **108**, 743–754
8. Yamamoto, Y., Klein, T. W., Newton, C. A., Widen, R., and Friedman, H. (1998) *Infect. Immun.* **66**, 370–375
9. Fortier, A., Diez, E., and Gros, P. (2005) *Trends Microbiol.* **13**, 328–335
10. Dietrich, W. F., Damron, D. M., Isberg, R. R., Lander, E. S., and Swanson, M. S. (1995) *Genomics* **26**, 443–450
11. Diez, E., Lee, S. H., Gauthier, S., Yaraghi, Z., Tremblay, M., Vidal, S., and Gros, P. (2003) *Nat. Genetics* **33**, 55–60
12. Wright, E. K., Goodart, S. A., Grownwy, J. D., Hadian, V., Endrizzi, M. G., Long, E. M., Sadig, A., Abney, A. L., Bernstein-Hanley, I., and Dietrich, W. F. (2003) *Curr. Biol.* **13**, 27–36
13. Inohara, N., Chamaillard, M., McDonald, C., and Nunez, G. (2005) *Annu. Rev. Biochem.* **74**, 355–383
14. Ting, J. P., and Davis, B. K. (2005) *Annu. Rev. Immunol.* **23**, 387–414
15. Philpott, D. J., and Girardin, S. E. (2004) *Mol. Immunol.* **41**, 1099–1108
16. Zamboni, D. S., Kobayashi, K. S., Kohlsdorf, T., Ogura, Y., Long, E. M., Vance, R. E., Kuida, K., Mariathasan, S., Dixit, V. M., Flavell, R. A., Dietrich, W. F., and Roy, C. R. (2006) *Nature Immunol.* **7**, 318–325
17. Mariathasan, S., Newton, K., Monack, D. M., Uy, J. L., and Roy, C. R. (2006) *Nature Immunol.* **7**, 572–582
18. Kagan, J. C., Stein, M. P., Pypaert, M., and Roy, C. R. (2002) *Mol. Microbiol.* **40**, 755–769
19. Kagan, J. C., Stein, M. P., Pypaert, M., and Roy, C. R. (2006) *Nat. Immunol.* **7**, 569–575
20. Hayashi, F., Smith, K. D., Ozinsky, A., Hawn, T. R., Yi, E. C., Goodlett, D. R., Eng, J. K., Akira, S., Underhill, D. M., and Aderem, A. (2001) *Nature* **410**, 1099–1103
21. Molofsky, A. B., Byrne, B. G., Whitfield, N. M., Magican, C. A., Fus, E. T., Tateda, K., and Swanson, M. S. (2006) *J. Exp. Med.* **203**, 1093–1104
22. Ren, T., Zamboni, D. S., Roy, C. R., Dietrich, W. F., and Vance, R. E. (2006) *PloS Pathog.* **2**, e18
23. Kagan, J. C., Stein, M. P., Pypaert, M., and Roy, C. R. (2004) *J. Exp. Med.* **199**, 1201–1211
24. Coers, J., Kagan, J. C., Matthews, M., Nagai, H., Zuckman, D. M., and Roy, C. R. (2000) *Mol. Microbiol.* **38**, 719–736
25. Viala, J., Chapat, C., Boneca, I. G., Cardona, A., Girardin, S. E., Moran, A. P., Athman, R., Memet, S., Huerre, M. R., Coyle, A. J., DiSfistano, P. S., Sansonetti, P. J., Labigne, A., Bertin, J., Philpott, D. J., and Ferrero, R. L. (2004) *Nature Immunol.* **5**, 1166–1174