Caspase-11 stimulates rapid flagellin-independent pyroptosis in response to *Legionella pneumophila*

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A flagellin-independent caspase-1 activation pathway that does not require NAIP5 or NRLC4 is induced by the intracellular pathogen *Legionella pneumophila*. Here we demonstrate that this pathway requires caspase-11. Treatment of macrophages with LPS up-regulated the host components required for this caspase-11 activation pathway. Activation by *Legionella* differed from caspase-11 activation using previously described agonists in that *Legionella* caspase-11 activation was rapid and required bacteria with a functional type IV secretion system called Dot/Icm. *Legionella* activation of caspase-11 induced pyroptosis by a mechanism independent of the NAIP/NLRC4 and caspase-1 axis. *Legionella* activation of caspase-11 stimulated activation of caspase-1 through NLRP3 and ASC. Induction of caspase-11–dependent responses occurred in macrophages deficient in the adapter proteins TRIF or MyD88 but not in macrophages deficient in both signaling factors. Although caspase-11 was produced in macrophages deficient in the type-I IFN receptor, there was a severe defect in caspase-11–dependent pyroptosis in these cells. These data indicate that macrophages respond to microbial signatures to produce proteins that mediate a capsase-11 response and that the caspase-11 system provides an alternative pathway for rapid detection of an intracellular pathogen capable of evading the canonical caspase-1 activation system that responds to bacterial flagellin.

innate immunity | cell death | inflammasome

An intracellular pathogen, *Legionella pneumophila* activates multiple innate immune sensing pathways upon macrophage infection (1, 2). Caspase-1 activation is a process that for many pathogens is highly dependent on the detection of bacterial products by cytosolic sensors, and there are multiple examples in which bacterial secretion systems play an important role in mediating the delivery of the agonists that stimulate these sensors. During *Legionella* infection of mouse macrophages the nucleotide-binding domain, leucine-rich repeat containing protein (NLR) neuronal apoptosis inhibitory protein 5 (NAIP5) has been shown to detect flagellin and activate NLRC4, which results in activation of caspase-1 (3–6, 7, 8). Caspase-1 activation by the NAIP/NLR family CARD domain-containing protein 4 (NLRC4) pathway leads to macrophage cell death within 1 h of *Legionella* infection by a process called pyroptosis. The rapid cell death response, and possibly other pathways downstream of NAIP/NLRC4 activation, restricts the ability of macrophages to support intracellular replication of *Legionella* (3, 4, 6, 9). Importantly, pyroptosis and *Legionella* growth restriction induced by the NAIP/NLRC4 pathway occurs independently of a scaffolding factor apoptosis associated speck-like protein containing a CARD (ASC) (6, 9), which assembles a macromolecular structure required for caspase-1 cleavage and efficient processing and secretion of the cytokines IL-1β and IL-18 by caspase-1 (10).

The *Legionella* Dot/Icm system can activate caspase-1 by a NAIP/NLRC4-independent pathway (9, 11). A noncanonical pathway of caspase-1 activation that is mediated by a related protein called caspase-11 has recently been described (12, 13). Several Gram-negative bacteria activate caspase-11 by a process that requires signaling through toll/interleukin-1 receptor domain-containing adaptor inducing IFN-β (TRIF) and type I IFN (13). Unlike stimulation of the NAIP/NLRC4 pathway by bacteria with specialized secretion systems, which results in rapid caspase-1 activation and cell death, the kinetics of caspase-11 activation demonstrated by cholera toxin B-subunit (CTB) or Gram-negative bacteria is slower and can take up to 24 h to detect (12). Here we show that the *Legionella* type IV secretion system rapidly activates caspase-11 by a mechanism that does not involve flagellin sensing by NAIP5/NLRC4.

Results

Macrophage Priming Up-Regulates a Flagellin-Independent Pathway of Caspase-1 Activation. Previous data examining the kinetics of caspase-1 activation by *Legionella* indicated that in the absence of NLRC4 or bacterial flagellin, caspase-1 activation was significantly delayed, and robust activation was not observed until 4 h after infection (11). The delay in caspase-1 activation could indicate that essential host factors required for caspase-1 activation need to be up-regulated upon infection, as was shown previously for caspase-1 activation by NLR family PYRIN domain-containing protein 3 (NLRP3) (14). To test this hypothesis, caspase-1 cleavage at 1 h after infection was used to assess activation in primed and unprimed macrophages. Cleavage of caspase-1 was detected in macrophages primed with LPS or TNF-α for 4 h and then infected with wild-type *Legionella*. Caspase-1 cleavage was not detected in unprimed macrophages infected with the flagellin-deficient *Legionella* strain (Δ*flaA*), which does not activate the NAIP/NLRC4 pathway (Fig. 1*A*). Priming of macrophages with LPS or TNF-α, however, resulted in robust caspase-1 activation by the Δ*flaA* strain (Fig. 1*A*). As shown previously (11), ASC was essential for caspase-1 cleavage under all conditions (Fig. 1*A*). Thus, priming up-regulates a critical factor that promotes NAIP/NLRC4-independent activation of caspase-1 by *Legionella*.

Macrophage Priming Stimulates NLRC4-Independent Pyroptosis. Caspase-1 activation through the NAIP/NLRC4 axis results in macrophage pyroptosis, which results in formation of pores in the plasma membrane and cell lysis (15). Infection of unprimed macrophages with wild-type *Legionella* resulted in rapid pore formation in both the propidium iodide uptake assay (Fig. 1*B*) and the lactate dehydrogenase (LDH) release assay (Fig. 1*C*). Pore formation was not detected using

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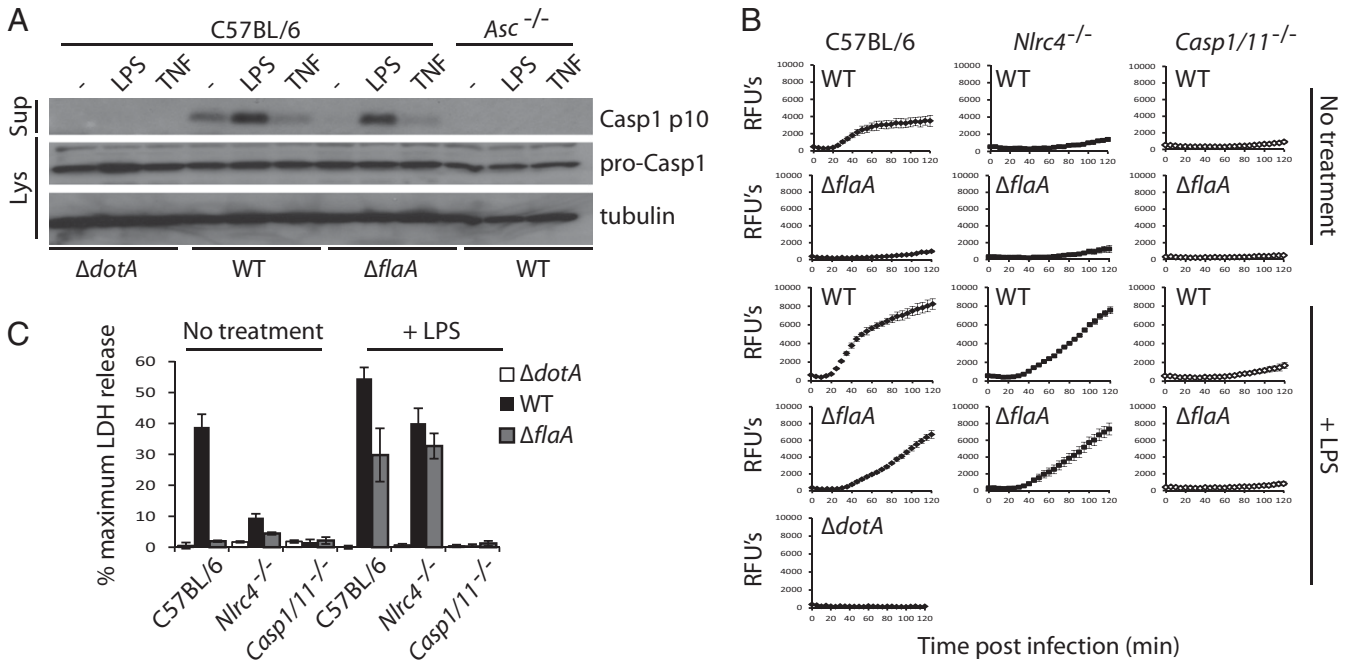

Fig. 1. Rapid caspase-1 activation is induced by flagellin-deficient *Legionella* in primed macrophages. (*A*) As indicated, immunoblots show levels of processed caspase-1 (Casp1 p10), unprocessed caspase-1 (pro-Casp1), and tubulin in lysates and supernatants from C57BL/6 or ASC-deficient (*Asc*$^{-/-}$) BMMs infected with wild-type *Legionella*, Dot/Icm-deficient *Legionella* (Δ*dotA*), or flagellin-deficient *Legionella* (Δ*flaA*) for 2 h. BMMs were left untreated (-) or pretreated with LPS (0.1 μg/mL) or TNF-α (0.01 μg/mL) for 3 h before infection, as indicated above each lane. (*B*) C57BL/6, NLRC4-deficient (*Nlrc4*$^{-/-}$), or caspase-1/11–deficient (*Casp1/11*$^{-/-}$) BMMs with no pretreatment (No treatment) or pretreated with LPS (+LPS) were infected with the *Legionella* strains indicated on the right. Fluorometric plots show propidium iodide uptake [relative fluorescence units (RFUs)] over time to reveal the kinetics of pore formation induced upon infection of BMMs with *Legionella*. Data are shown as averages ± SD of three independent wells after subtraction of values for noninfected samples. (*C*) Cell death was measured in an LDH release assay 2 h after infection of BMMs from the mouse strains indicated on the *x* axis with *Legionella* wild type (black bars), Δ*dotA* mutant (white bars), or a Δ*flaA* mutant (gray bars). Values represent the percentage of LDH released compared with cells lysed with Triton X-100.

macrophages from the NLRC4-deficient mouse, macrophages from the caspase-1/11–deficient mouse, or in macrophages infected with the *Legionella* Δ*flaA* strain (Fig. 1 *B* and *C*). Using primed macrophages, rapid pyroptosis was observed after

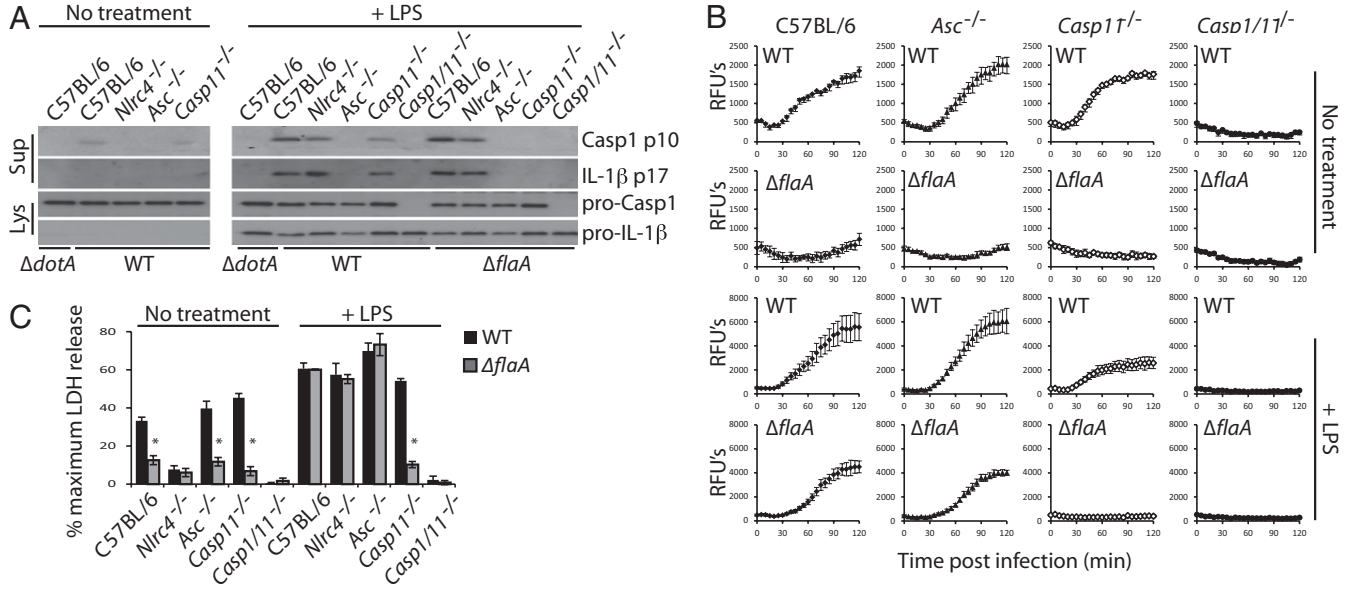

Fig. 2. Caspase-11 is required for flagellin-independent caspase-1 activation and cell death during *Legionella* infection. (*A*) BMMs from the indicated mouse strains were either treated with LPS (*Right*) or untreated (*Left*) and then infected with the *Legionella* strains indicated above the panels for 2 h. Immunoblot analysis of the lysates and supernatants from the infected BMMs was conducted using antibodies specific for the proteins indicated on the right side of each panel set. (*B*) Fluorometric plots show propidium iodide uptake (RFUs) over time to reveal the kinetics of pore formation induced upon infection of BMMs with *Legionella*. Data are shown as averages ± SD of three independent wells after subtraction of values for noninfected samples. (*C*) Cell death of BMMs after 2 h of infection. Values represent the percentage of LDH released compared with cells lysed with Triton X-100.

infection by wild-type *Legionella* in the wild-type macrophages (C57B/L/6) and in the NLRC4-deficient macrophages, but not in the caspase-1/11–deficient macrophages. Additionally, pyroptosis was observed in the primed C57BL/6 macrophages infected with the Δ*flaA* strain. In all cases, induction of pyroptosis was dependent on the Dot/Icm system, because pore formation was not detected after infection with the Δ*dotA* strain. Thus, the *Legionella* Dot/Icm system induces pyroptosis in primed macrophages by a process that is independent of flagellin sensing through the NAIP/NLRC4 system.

Caspase-11 Is Required for NAIP/NLRC4-Independent Pyroptosis Induced by *Legionella*. Because caspase-11 expression is induced by LPS treatment (16), whether caspase-11 was important for the NLRC4-independent pyroptosis induced by *Legionella* in primed macrophages was investigated. As expected, caspase-1 activation and pyroptosis were all detected after primed or unprimed caspase-11–deficient macrophages were infected with wild-type *Legionella* (Fig. 2 *A*–*C*), which is consistent with the NAIP/NLRC4 pathway of activation being caspase-11 independent. By contrast, caspase-1 activation was not detected in primed caspase-11–deficient macrophages infected with the *Legionella* Δ*flaA* strain (Fig. 2*A*). The elimination of caspase-11 also abrogated the pyroptotic response in the primed macrophages infected with *Legionella* Δ*flaA*, as indicated by a defect in both the pore formation assay (Fig. 2*B*) and in the LDH release assay (Fig. 2*C*). Thus, caspase-11 is an essential component of the NAIP/NLRC4-independent response to *Legionella* that activates caspase-1 in primed macrophages. Pretreatment of cells with LPS increased overall pore-formation and cell death by WT *Legionella* in wild-type macrophages by two- to threefold, and this increase was not observed in the absence of caspase-11 (Fig. 2 *B* and *C*). These data indicate that LPS priming of cells specifically up-regulates components involved in the caspase-11–mediated pyroptosis pathway and that this pathway works in parallel to the NAIP/NLRC4 pathway to enhance overall pyroptosis in response to WT *Legionella.*

Caspase-1 Is Dispensable for Caspase-11–Dependent Pyroptosis Induced by *Legionella*. To determine whether caspase-1 was required for caspase-11–dependent pyroptosis induced by flagellin-deficient *Legionella*, we used macrophages derived from mice deficient for caspase-1 only. Rapid pyroptosis was not detected when naïve caspase-1–deficient macrophages or NLRC4-deficient macrophages were infected with *Legionella*; however, rapid pyroptosis was observed in caspase-1–deficient macrophages and NLRC4-deficient macrophages primed with LPS (Fig. 3). Importantly, pyroptosis was still observed when primed caspase-1–deficient macrophages or NLRC4-deficient macrophages were infected with the *Legionella* Δ*flaA* strain, which failed to induce pyroptosis in primed macrophages from the caspase-11–deficient mice (Fig. 3). Thus, caspase-11–dependent pyroptosis is induced by *Legionella* that evade the canonical caspase-1–dependent pyroptotic pathway stimulated by NAIP/NLRC4.

NLRP3 Is Required for NAIP/NLRC4-Independent Activation of Caspase-1 in Primed Macrophages. Previous data showed that caspase-11–dependent activation of caspase-1 involves the cytosolic sensor NLRP3 (12). NLRP3 was not required for caspase-1 activation by the NAIP/NLRC4-dependent pathway induced by wild-type *Legionella* (Fig. S1*A*). When macrophages were infected with *Legionella* Δ*flaA*, however, caspase-1 activation was greatly diminished in the NLRP3-deficient cells (Fig. S1*A*). Although NLRP3 was important for caspase-1 activation under these conditions, NLRP3 was dispensable for the rapid pyroptotic cell death induced by *Legionella* Δ*flaA* in primed macrophages (Fig. S1 *B* and *C*). These data suggest that NLRP3 is not an upstream component involved in caspase-11 activation and that caspase-11 activation generates a signal that converges on the NLRP3 pathway to activate caspase-1. Indeed, caspase-11–dependent activation of caspase-1 by *Legionella* Δ*flaA* was inhibited when signaling through

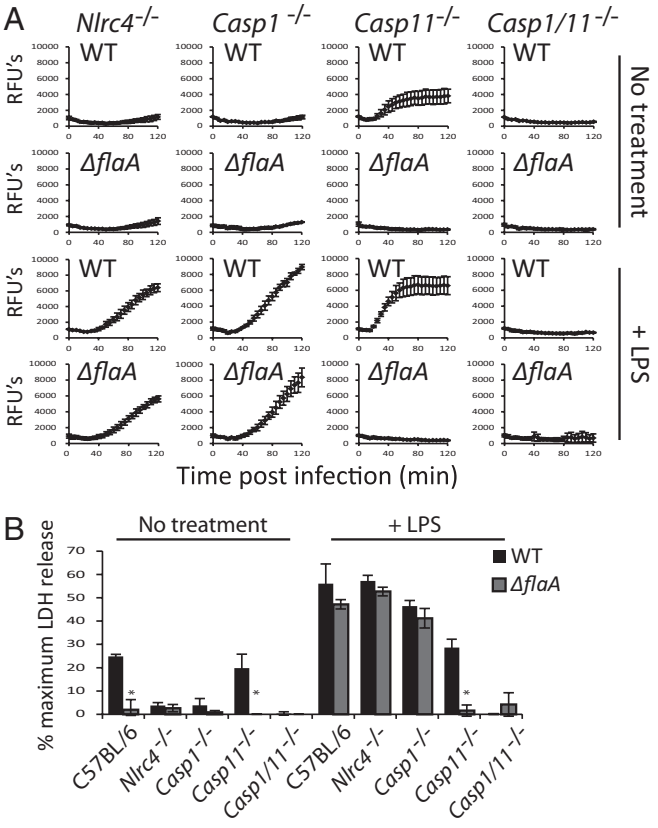

Fig. 3. Caspase-1 is dispensable for NLRC4/flagellin-independent pyroptosis during *Legionella* infection. (*A*) BMMs from the indicated mouse strains were infected with *Legionella* with or without LPS pretreatment. Fluorometric plots show propidium iodide uptake (RFUs) over time to reveal the kinetics of pore formation induced upon infection of BMMs with *Legionella*. Data are shown as averages ± SD of three independent wells after subtraction of values for noninfected samples. (*B*) Cell death of BMMs after 2 h of infection. Values represent the percentage of LDH released compared with cells lysed with Triton X-100. **P* < 0.05 compared with wild-type *Legionella* infections for each cell type.

the NLRP3 inflammasome was prevented by the addition of extracellular potassium, whereas the caspase-11–dependent cell death response remained unaffected (Fig. S1 *D*–*F*). Thus, caspase-11 activation by *Legionella* induces pyroptosis by an NLRP3-independent mechanism and promotes caspase-1 activation by a pathway that requires NLRP3.

Pathway for Caspase-11 Activation Is Primed upon Macrophage Infection by *Legionella*. *Legionella* infection of naïve macrophages results in NAIP/NLRC4-independent caspase-1 activation, which is observed roughly 4 h after infection (11). To determine whether this pathway requires caspase-11, which would suggest that the delay in activation reflects the time it may take for *Legionella* to prime the macrophages, naïve caspase-11–deficient macrophages were infected with the *Legionella* Δ*flaA* strain, and caspase-1 activation was assessed. These data show that activation of caspase-1 by the *Legionella* Δ*flaA* strain required caspase-11 (Fig. 4*A*). Secretion of IL-1α, IL-1β, and IL-18 were all below the threshold of detection when caspase-11–deficient macrophages were infected with *Legionella* Δ*flaA* (Fig. 4*B*). Additionally, macrophage cell death detected 4 h after infection of naïve macrophages by the *Legionella* Δ*flaA* strain was found to be dependent on caspase-11, suggesting that *Legionella* primes macrophages upon infection to up-regulate the caspase-11–mediated pathway of caspase-1 activation (Fig. 4*C*).

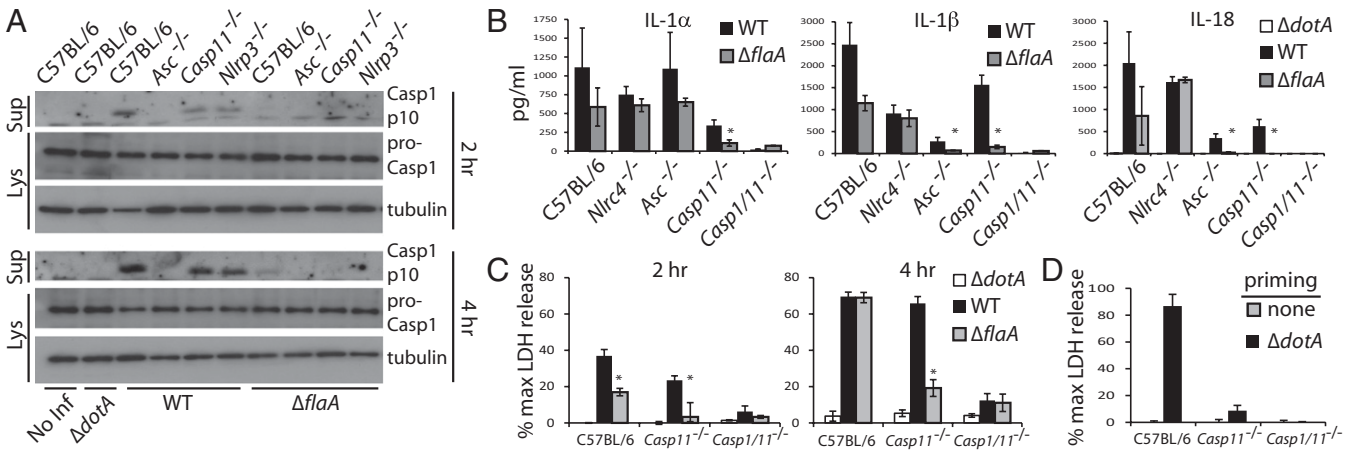

Fig. 4. Pathway for caspase-11 activation is primed upon macrophage infection by *Legionella*. (*A*) Naïve BMMs from the indicated mouse strains were infected with *Legionella* strains for either 2 h or 4 h. Cleaved caspase-1 in the supernatant fraction from the infected BMMs (Casp1 p10) was measured by immunoblot analysis and compared with the levels of procaspase-1 and tubulin in the BMM lysates. (*B*) IL-1α, IL-1β, and IL-18 levels from indicated naïve BMM culture supernatants after infection by the indicated strains of *Legionella* for 8 h. (*C*) Naïve BMM's were derived from the mice indicated (*x* axis) and infected for either 2 h or 4 h with wild-type *Legionella* (black bars), Δ*dotA* (white bars), or Δ*flaA* (gray bars). Cell death of BMMs was determined as the percentage of LDH released compared with cells lysed with Triton X-100 (*y* axis). (*D*) BMMs derived from C57BL/6 mice, caspase-11–deficient mice (Casp1$^{-/-}$), and caspase-1/11–deficient mice (Casp1/11$^{-/-}$) mice were either left untreated (none, gray bars) or primed for 3 h by infection with a Δ*dotA* mutant (black bars), as indicated. Cell death of BMMs was determined as the percentage of LDH released compared with cells lysed with Triton X-100 (*y* axis). Data are represented as averages ± SD. *$P < 0.05$ compared with WT *Legionella* infections.

To determine whether the priming of naïve macrophage by *Legionella* requires type IV secretion system functions, cells were treated with the *Legionella* Δ*dotA* mutant for 4 h to assess priming, then infected for 1 h with the *Legionella* Δ*flaA* strain to stimulate caspase-11 activation. Pyroptosis was not observed in the untreated macrophages infected with *Legionella* Δ*flaA*; however, caspase-11–dependent cell death was observed in the macrophages pretreated with *Legionella* Δ*dotA* mutant bacteria (Fig. 4*D*). Thus, macrophage priming by a Dot/Icm-independent mechanism is sufficient to up-regulate the machinery required to detect virulent *Legionella* and activate caspase-11.

Caspase-11 Activation by *Legionella* Has Distinct Kinetic and Molecular Parameters. To determine whether there were any molecular or kinetic differences in caspase-11 activation by an intracellular pathogen compared with a nonpathogenic bacterium, the *Legionella* type IV-dependent pathway of caspase-11 activation was compared with the *E. coli* pathway reported previously (12, 13). These data show that the caspase-11–dependent pathway induced in macrophages is quick and robust, as determined by a measurable increase in caspase-1 cleavage and pyroptosis detected within 4 h of infection (Fig. 5 *A* and *B*). By contrast, caspase-1 cleavage and signs of pyroptosis were not detected in response to *E. coli* at 4 h but were observed 18 h after infection (Fig. 5 *A* and *B*). Thus, there are clear kinetic differences observed in the two pathways of caspase-11 activation. Previous data suggested that *E. coli*-mediated caspase-1 activation and cell death required the toll-like receptor (TLR)-adaptor molecule TRIF (17), which was confirmed by data indicating that caspase-1 cleavage and cell death observed 18 h after macrophage infection by *E. coli* required TRIF (Fig. 5 *A* and *B*). By contrast, TRIF was not required for the caspase-11–dependent response to *L. pneumophila* (Fig. 5 *A* and *B*). Macrophages deficient in other signaling factors were used to further define the caspase-11–dependent response to *Legionella.* Similar to TRIF-deficient macrophages, caspase-11–dependent pyroptosis was detected using primed MyD88-deficient macrophages infected with flagellin-deficient *Legionella* but not in macrophages deficient in both MyD88 and TRIF (Fig. 5 *C*, *E*, and *F*). Thus, eliminating either TRIF or MyD88 reduced caspase-11 expression to levels that remained sufficient for signaling, whereas eliminating both TRIF and MyD88 had an additive effect that further diminished caspase-11 expression to nonfunctional levels (Fig. 5*D*). To determine whether type I IFN signaling was important for caspase-11 activation in response to flagellin-deficient *Legionella*, pore formation was measured using macrophages deficient in the type-I IFN receptor (IFNAR1). Caspase-11–dependent pore-formation and cell death were not observed when IFNAR1-deficient macrophages were infected with flagellin-deficient *Legionella*, but NAIP/NLRC4-dependent pore formation and cell death induced by wild-type *Legionella* were unaffected in these macrophages (Fig. 5 *C* and *E*). Importantly, immunoblot analysis showed similar levels of caspase-11 protein in macrophages deficient in IFNAR1 or MyD88 or TRIF (Fig. 5*D*). Thus, the defect in caspase-11 activation observed in the IFNAR1-deficient macrophages could result from the absence of a factor required to activate caspase-11 in response to *Legionella* rather than a defect in production of the caspase-11 protein.

Discussion

We previously defined a caspase-1 activation pathway induced by virulent *Legionella* that was independent of NAIP/NLRC4 sensing of bacterial flagellin (9). This pathway differed from the canonical pathway of caspase-1 activation in that caspase-1 activation occurred with slower kinetics compared with the NAIP/NLRC4 pathway, and ASC was required for caspase-1 activation (11). Data presented here demonstrate that this pathway is controlled by caspase-11. Pretreating cells with LPS or avirulent *Legionella* was sufficient to prime caspase-11 production and eliminated the kinetic delay in activation of the NAIP/NLRC4-independent pathway stimulate by virulent *Legionella*. Thus, the caspase-11–dependent pathway of caspase-1 activation represents an alternative pathway that is induced by virulent bacteria capable of evading the canonical caspase-1 activation pathway controlled by NAIP/NLRC4.

Importantly, this work demonstrates that NAIP/NLRC4-independent activation of caspase-1 required both caspase-11 production and Dot/Icm signaling by *Legionella*, which means caspase-11 is activated by a cytosolic sensing mechanism that detects bacterial-derived signals delivered into the host cytosol through the type IV secretion apparatus. Thus, there are important differences between the caspase-11 activation pathway induced by *Legionella* described here and the general caspase-11 activation pathway triggered in response to a large class of Gram-negative bacteria (12, 13). In recent studies, activation of the caspase-11 pathway by Gram-negative bacteria was not observed until macrophages were infected for at least 8 h (12,

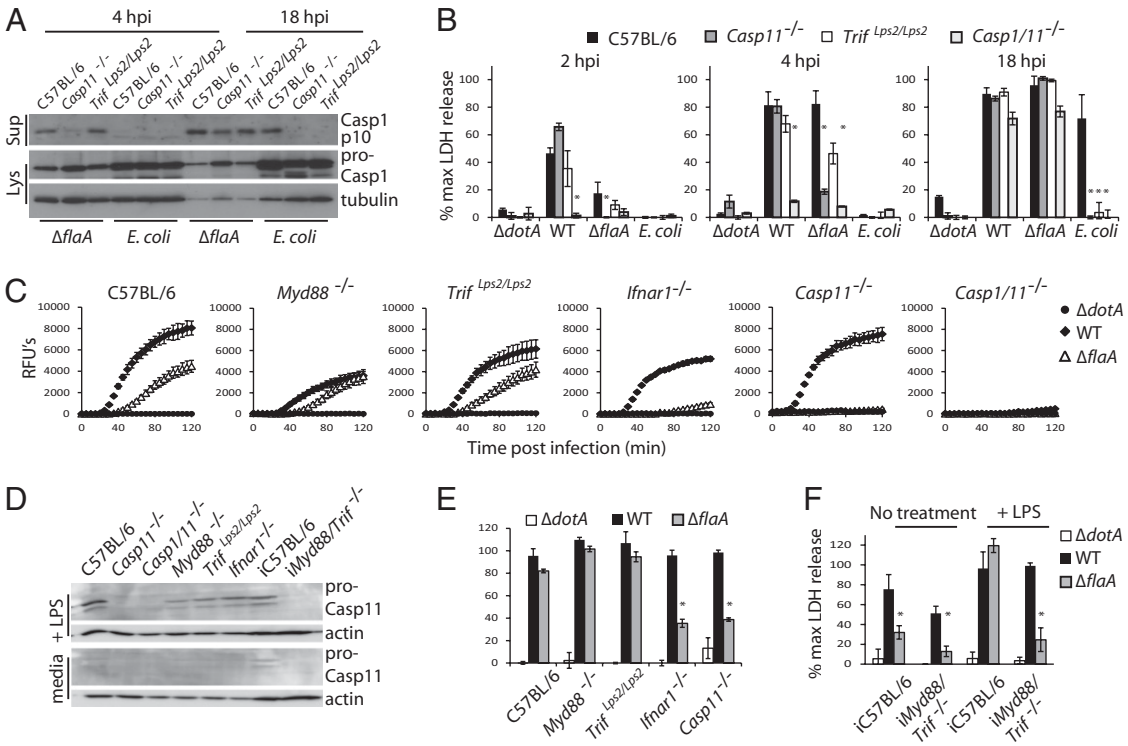

Fig. 5. Caspase-11 activation by *Legionella* has distinct kinetic and molecular parameters. (*A*) BMMs from the indicated mouse strains were pretreated with LPS and infected with flagellin-deficient *Legionella* (Δ*flaA*) or *E. coli* for 4 h or 18 h, as indicated. Cleaved caspase-1 in the supernatant fraction (Casp1 p10) was measured by immunoblot analysis and compared with the levels of procaspase-1 and tubulin in the lysates. (*B*) Cell death of C57BL/6 (black bars), TRIF-deficient (*Trif*$^{Lps2/Lps2}$; white bars), caspase-11–deficient (*Casp11*$^{-/-}$; dark gray bars), or caspase-1/11–deficient (*Casp1/11*$^{-/-}$; light gray bars) BMMs pretreated with LPS and infected with the indicated *Legionella* strains or *E. coli* for 2 h, 4 h, or 18 h, as indicated. (*C*) Fluorometric plots show propidium iodide uptake (RFUs) over time to reveal the kinetics of pore formation after infection of LPS-treated BMMs derived from the indicated mouse strains and infected with *Legionella* strains. (D) BMMs derived from indicated mouse strains or immortalized BMMs from wild-type (iC57BL/6) or Myd88/TRIF double-knockout (i*Myd88/Trif*$^{-/-}$) mice were either left untreated or treated with LPS (0.1 μg/mL) for 4 h, and levels of procaspase11 and actin were measured by immunoblot analysis. (*E* and *F*) Cell death of primed BMMs derived from indicated mouse strains (*E*) or immortalized BMMs from wild-type (iC57BL/6) or Myd88/TRIF double-knockout (i*Myd88/Trif*$^{-/-}$) mice (*F*) infected for 2 h was measured. Cell death was determined as the percentage of LDH released compared with cells lysed with Triton X-100 (*y* axis). *$P < 0.01$ compared with the corresponding values from wild-type infections.

13, 18, 19). By contrast, *Legionella* activated the caspase-11–dependent pathway within 4 h of infection, which suggested that activation of caspase-11 could occur by different pathways depending on the bacterial agonist. Priming alone was not sufficient to activate caspase-11 but provided a mechanism that allowed macrophages to rapidly activate capsase-11 upon sensing *Legionella* with a functional type IV system. Response pathways induced by other bacteria may require different or additional host factors to transduce microbial-derived signals from the vacuolar compartment into the host cytosol. A separate study has shown a noncanonical caspase-1 activation pathway stimulated by nonpathogenic *E. coli* may be triggered by the release of RNA from viable bacteria as they are destroyed in a phagolysosome (17). Consistent with *Legionella* avoiding phagosome–lysosome fusion, it is possible that *Legionella* expressing a functional type IV secretion system bypass this requirement and stimulate an alternate response pathway that is rapid.

Noncanonical caspase-1 activation through caspase-11 was shown recently to require type I IFN production mediated by TRIF-dependent TLR signaling (13). Current models suggest that type I IFN production as a result of TRIF-mediated signaling leads to caspase-11 up-regulation and that production of caspase-11 is sufficient for activation in the presence of an undefined microbial-derived stimulus. *Legionella* activation of caspase-11–dependent responses was independent of TRIF but required signaling through IFNAR1. Importantly, these data indicated that caspase-11 up-regulation alone was not sufficient to induce the caspase-11–dependent responses to *Legionella* infection because caspase-11 was up-regulated to similar levels in both TRIF-deficient and IFNAR1-defecient macrophages after LPS priming for 4 h. Although IFNAR1 was not required for up-regulation of caspase-11, the loss of both MyD88 and TRIF resulted in macrophages that were unable to up-regulate caspase-11 in response to LPS priming and were defective in the caspase-11 response to flagellin-deficient *Legionella*. Thus, IFNAR1 signaling is likely required for expression of a factor in addition to caspase-11 that is needed for activation, and signaling through either MyD88 or TRIF is sufficient for up-regulation of caspase-11 to levels that will promote a rapid response when primed macrophages are infected with virulent *Legionella*.

NLRP3 and ASC were both required for caspase-1 activation through the caspase-11–dependent pathway induced by *Legionella*. Thus, all of the noncanonical caspase-1 activation pathways described so far converge at the point of NLRP3 activation (12, 13, 18, 19), which is consistent with NLRP3 being capable of integrating signals from different microbial agonists. We predict that, similar to the canonical caspase-1 signaling pathways, caspase-11 activation requires an upstream protein(s) capable of sensing a microbial or endogenous danger signal.

These data suggest that caspase-11 and caspase-1 have functionally redundant roles in stimulating macrophage pyroptosis in response to intracellular pathogens. Both pathways promote inflammation although the secretion of cytokines IL-1 and IL-18 by integrating activated caspase-1 into the ASC platform needed for cytokine processing. Flagellin sensing through the NAIP/NLRC4 pathway is sufficient to induce pyroptosis by a mechanism that

requires caspase-1 but is independent of caspase-11. Here we show that the caspase-11 system can respond to intracellular pathogens that evade the flagellin-detection system and induce pyroptosis by a process that is independent of caspase-1. Thus, caspase-11 further enhances the ability of cells to respond to microbes by responding to microbial signatures that are not detected by sensors used for caspase-1 activation.

Materials and Methods

Bacterial Strains. *L. pneumophila thyA* (Lp02) (20), a thymidine auxotroph derived from the *L. pneumophila* serogroup 1 strain Lp01 was used, along with the *dotA* (20) and *flaA* (5) isogenic mutants.

Mice and Cell Culturing. All mice were on the C57BL/6 background. All animals were maintained in accordance with the guidelines of the FMRP/USP and Yale Institutional Animal Use and Care Committee (protocol 07847). Bone marrow was collected from femurs and tibiae of mice. Details of mice used in this study and macrophage differentiation procedures are found in *SI Materials and Methods*.

Western Blot Analysis and Cytokine Assays. Macrophages were seeded in 48-well plates (2×10^5 cells per well) and infected with *Legionella* at a multiplicity of infection (MOI) of 10. Cytokine levels in culture supernatants were determined by ELISA and culture lysates, and supernatants were analyzed by Western blot. Full details are found in *SI Materials and Methods*.

Pore-Formation and Cell Death Assays. Bone marrow-derived macrophages (BMMs) were seeded in 96-well plates (1×10^5 per well) and infected with bacterial strains at an MOI of 20. Pore formation was determined by quantification of propidium iodide (PI) uptake. Cell death was monitored by measuring LDH released by dying cells. Full details are found in *SI Materials and Methods*.

Statistical Analysis. Statistical significance for LDH assays and ELISA was calculated using the unpaired Student *t* test.

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