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Flagellin-Deficient Legionella Mutants Evade Caspase-1- and Naip5-Mediated Macrophage Immunity

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Macrophages from C57BL/6J (B6) mice restrict growth of the intracellular bacterial pathogen Legionella pneumophila. Restriction of bacterial growth requires caspase-1 and the leucine-rich repeat-containing protein Naip5 (Birc1e). We identified mutants of L. pneumophila that evade macrophage innate immunity. All mutants were deficient in expression of flagellin, the primary flagellar subunit, and failed to induce caspase-1-mediated macrophage death. Interestingly, a previously isolated flagellar mutant (lli) that expresses, but does not assemble, flagellin did not replicate in macrophages, and induced macrophage death. Thus, flagellin itself, not flagella or motility, is required to initiate macrophage innate immunity. Immunity to Legionella did not require MyD88, an essential adaptor for toll-like receptor 5 (TLR5) signaling. Moreover, flagellin of Legionella and Salmonella induced cytotoxicity when delivered to the macrophage cytosol using Escherichia coli as a heterologous host. It thus appears that macrophages sense cytosolic flagellin via a TLR5-independent pathway that leads to rapid caspase-1-dependent cell death and provides defense against intracellular bacterial pathogens.

Introduction

Legionella pneumophila is a motile gram-negative bacterium that is the cause of a severe form of pneumonia called Legionnaires’ disease [1]. Virulence of Legionella depends on its capacity to replicate inside host macrophages. Legionella utilizes a type IV secretion system (encoded by the dot/icm genes) to inject effector proteins into the cytosol of host cells [2,3]. The secreted effector proteins (more than 30 of which have now been identified [4,5]) direct the host cell to create a unique intracellular vacuole in which Legionella can thrive and multiply [6,7].

Early studies found that macrophages from A/J mice support more than three logs of Legionella growth [8], whereas C57BL/6J (B6) macrophages are almost entirely restrictive. Remarkably, this large phenotypic difference is controlled by a single gene on mouse Chromosome 13 called Naip5 (Neuronal apoptosis inhibitory protein 5, also called Birc1e [haculoviral IAP repeat-containing 1e]) [9,10]. Transgenic complementation experiments indicate that B6 mice encode a functional Naip5 allele that confers resistance to Legionella [9,10]. A/J mice also express a Naip5 gene; however, the A/J allele appears to be expressed at lower levels and encodes 14 amino acid polymorphisms as compared to the B6 allele. The precise polymorphism(s) within Naip5 that is responsible for differential permissiveness to Legionella has yet to be identified.

Naip5 is a member of a superfamily of proteins that contain C-terminal leucine rich repeats (LRR) and a central nucleotide-binding domain (NBD) [11,12]. Evidence exists that many “NBD–LRR” proteins can function as microbe-detector proteins. For example, Nod1 and Nod2 confer recognition of bacterial cell wall–derived molecules [13–16] and Nod2 is required for resistance to orally delivered Listeria monocytogenes [17]. Human NALP1 is also reported to recognize muramyl dipeptide [18], and mouse Naip1 is required for susceptibility to anthrax lethal toxin [19]. Ipaf, the closest homolog of Naip, has been shown to be required for caspase-1 activation in response to Salmonella [20]. Although many NBD–LRR proteins appear to function in pathogen detection, there is no evidence that they bind directly to microbial products.

We hypothesized that B6 macrophages might restrict Legionella growth upon cytosolic detection of a Legionella-derived molecule. Here we identify flagellin in a genetic selection to identify essential elicitors of macrophage innate immunity. Our results suggest that flagellin initiates a caspase-1-dependent cell-death pathway that functions to restrict bacterial growth.

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Synopsis

*Legionella pneumophila* is a bacterial pathogen that is the cause of a severe form of pneumonia known as Legionnaires’ disease. A crucial aspect of the propensity of *Legionella* to cause disease lies in its ability to survive and multiply inside host immune cells known as macrophages. The intracellular survival and replication of *Legionella* can be studied using isolated macrophages grown in culture. Macrophages isolated from different laboratory mouse strains are differentially permissive for intracellular *Legionella* growth. This difference in permissiveness is genetic, and is conferred by differences in a mouse protein known as Naip5. The authors determined that *Legionella* strains that are unable to produce a protein called flagellin are able to grow inside normally resistant mouse macrophages. In addition, these flagellin strains are defective in initiating a cell-death response on the part of infected macrophages. Based on these data, the authors suggest that there is an intracellular mechanism for detecting the presence of bacterial flagellin protein, and that a cell-death response is initiated upon the detection of flagellin.

Results/Discussion

*Legionella* Flagellin Mutants Evade Macrophage Immunity

We adopted a genetic approach to identify *Legionella* gene products that might be recognized by host macrophages. We reasoned that *Legionella* strains lacking such a gene product might be able to evade macrophage immunity and replicate in normally restrictive B6 macrophages. A mariner transposon, which inserts with low bias into frequently occurring TA dinucleotides, was used to generate 30 independent pools of *L. pneumophila* transposon mutants. Each pool, containing approximately 2,500 mutants, was used to infect an independent well of B6 macrophages. After 6 d, we observed numerous bacteria in all the wells infected with transposon mutants, whereas virtually no bacteria were observed in wells infected with unmutagenized *Legionella*. Sixty-four transposon mutants obtained from one round of selection in B6 macrophages were retested and, of these, 60 mutants (93.8%) were found to be capable of robust growth in B6 macrophages. Thus, growth in B6 macrophages represents a powerful and highly efficient selection procedure.

The gene disrupted by the transposon was determined for 29 independent mutants. Twenty-four mutants were found to have insertions within the **flaA** open reading frame, four mutants were found to harbor insertions within the **flaA** promoter, and one mutant (with a weaker growth phenotype, **flaA** promoter::Tn) was generated and then probed with an anti-FlaA monoclonal antibody. **flaA** promoter::Tn is the **flaA** mutant which has been complemented with a copy of **flaA** inserted on the *Legionella* chromosome just after the **ahpC** locus.

Figure 1. Characterization of *Legionella* Mutants Harboring Transposon Insertions in Flagellin (**flaA**)

(A) Nucleotide positions of transposon insertions in **flaA**.

(B) Western blotting for flagellin (**FlaA**). Proteins from pelleted bacteria (top) or TCA-precipitated culture supernatants (bottom) obtained from stationary-phase cultures were separated by SDS-PAGE, blotted, and probed with an anti-FlaA monoclonal antibody. Delta**flaA** ahpc:C::flaA is the **flaA** mutant which has been complemented with a copy of **flaA** inserted on the *Legionella* chromosome just after the **ahpC** locus.

(C) Electron microscopy showing the presence or absence of flagella on various *Legionella* strains. Representative bacteria (of >100 surveyed) are shown. No flagellated bacteria were seen for any of the strains shown lacking flagella. **Tn** transposon.

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nucleotides 891, 1,365, and 1,418 of the open reading frame, respectively. The latter strain is of particular interest since the transposon inserted only 10 bp from the stop codon. Translation read-through into the transposon is predicted to result in the last two amino acids of FlaA being replaced with a 37 amino-acid peptide.

All Mutants Evading Macrophage Defenses Are Nonflagellated and Nonmotile

In order to confirm the results of the transposon mutagenesis, an unmarked deletion of **flaA** was generated and then complemented by a chromosomal copy of **flaA**. For comparison, we also obtained a previously characterized **fliI** mutant [22]. **FliI** encodes an ATPase required for flagellar secretion and assembly. The **flaA** promoter::Tn, **flaA**418::Tn, and the **DeltaflaA** strains did not detectably express FlaA protein (Figure 1B), whereas the **deltaflaA** ahpc:C::**flaA** strains expressed **flaA** but were defective in the secretion of FlaA to the culture supernatant (Figure 1B). Owing to its extended open reading frame, the FlaA protein expressed by LP02 **flaA**418::Tn exhibited a higher molecular weight (Figure 1B). As expected, LP02 and the **DeltaflaA** ahpc:C::**flaA** (complemented) strains were motile (unpublished data). The four transposon mutants examined were all nonmotile, as were the unmarked **DeltaflaA** mutant and the **fliI** mutant (unpublished data). Each mutant was also examined by electron microscopy (Figure 1C). LP02...
and the ΔflaA ahpC::flaA (complemented) strains were found to exhibit normal flagella, whereas the fliI mutant and all the flaA mutants were completely nonflagellated.

Flagellin Itself Is Required for Restriction of Bacterial Growth

We tested the ability of various mutants to grow in bone marrow-derived macrophages. In addition to using B6 macrophages, which express a fully functional allele of Naip5 and therefore normally restrict Legionella growth, we also used a consomic mouse strain, B6.A-Chr13, which is B6 at all loci (~95%), except for those that lie on Chromosome 13, which is derived from the A/J strain of mouse. Since Chromosome 13 harbors the Naip5 locus, both A/J and B6.A-Chr13 mice carry the A/J allele of Naip5 and are permissive for Legionella growth.

As expected, the ΔflaA strain recapitulated the phenotype of the transposon mutants and grew robustly in B6 macrophages, indicating that it is indeed loss of flaA, and not polar or unlinked mutations, which results in the growth phenotype (Figure 2). This observation confirms identical results obtained independently by the Swanson group at the University of Michigan (Ari Molofsky and Michele Swanson, personal communication). In contrast, the ΔflaA ahpC::flaA (complemented) strain was restricted in growth in B6 macrophages, but grew well in permissive B6.A-Chr13 macrophages. The fliI mutant (which expresses but does not secrete or assemble flagellin) failed to grow in B6 macrophages (Figure 2A), but grew in B6.A-Chr13 macrophages (Figure 2B), suggesting that innate immunity to Legionella is triggered by FlaA itself, and not by motility or flagella. Interestingly, although the fliI, flaA^K65T, and flaA^{1418:76} strains expressed similar intracellular levels of FlaA protein (Figure 1), only the flaA mutant was growth-restricted. We conclude that transposon insertions at the very C-terminus of flaA must affect secretion of flagellin into host cells or subsequent flagellin recognition by host cells.

Macrophage Immunity Is Independent of Toll-Like Receptor 5

Flagellin stimulates immune signaling through toll-like receptor 5 (TLR5) [23], and there is evidence that TLR5 plays an important role in controlling Legionella infections in vivo [24]. However, mouse macrophages do not express TLR5 [25]. Thus, it is unlikely that TLR5 plays a role in mouse-macroage restriction of Legionella growth in our in vitro system. This is difficult to prove directly since TLR5 knockouts have not yet been reported. Nevertheless, we sought to confirm that macrophage recognition of flagellin does not require TLR5 by examining the phenotype of macrophages deficient in MyD88, a signaling adaptor required for all TLR5 signaling [23]. After 48 h of growth, B6 MyD88^−/− macrophages restricted Legionella growth as well as wild-type macrophages (Figure 2A), implying that TLR5 is not required for the initial restriction of flagellin^+ bacteria. Interestingly, by 96 h, MyD88^−/− macrophages exhibited more growth of wild-type Legionella than did B6 macrophages, and this is likely a consequence of reduced MyD88-dependent signaling (e.g., NF-κB activation), downstream of other toll-like receptors that may play a role in the later phases of infections.

Figure 2. Growth of Legionella in Bone Marrow-Derived Macrophages

Growth of Legionella strains in bone marrow-derived macrophages from (A) B6 mice or (B) B6.A-Chr13 mice (which carry the permissive A/J allele of Naip5). A confluent layer of macrophages were grown in 24-well plates and were infected with the indicated Legionella strains at an MOI of 0.02. Growth of wild-type Legionella (LP02) in B6-backcrossed MyD88^−/− macrophages is also depicted in (A). After addition of bacteria, the plate was spun at 400 g for 10 min. One hour after infection, the media was changed. At daily intervals (starting with 1 h post-infection), a timepoint was taken. Macrophages were lysed with sterile water and the lysate combined with supernatant from the same well. Colony-forming units per well were determined by plating dilutions onto BCYE plates.

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Flagellin Mediates Caspase-1-Dependent Cytotoxicity of B6 Macrophages

The mechanism by which flagellin-mediated signaling restricts Legionella growth in B6 macrophages is unknown. However, a recent study demonstrated that wild-type Legionella activates caspase-1, and that caspase-1 is required for restriction of Legionella growth [26]. Caspase-1 does not mediate classical apoptosis but is instead required for processing of pro-IL-1 and pro-IL-18 into their mature (secreted) forms [27,28]. Since we found that addition of exogenous IL-1 and/or IL-18 did not render macrophages resistant to Legionella (unpublished data), we concluded that caspase-1 is required for host defense via a mechanism that is
compared to the wild-type LP02 strain (Figure 3A). Remarkably, we observed that the death of B6 macrophages in a 4-h LDH-release assay. transposon mutants for the ability to initiate macrophage pinocytosis, or by their increased permeability to ethidium (LDH), by their failure to take up neutral red by micro-plasma membrane integrity and can be detected by their death. flagellin promoted host defense by activating rapid host cell characteristics [32]. We therefore considered the possibility that Salmonella to several stimuli, including Shigella [31], and anthrax lethal toxin [19], caspase-1 has also been shown independent of IL-1 and/or IL-18. Interestingly, in response to several stimuli, including Salmonella [29,30], Shigella [31], and anthrax lethal toxin [19], caspase-1 has also been shown to be required for an unusual form of macrophage cell death that is distinguishable from apoptosis by its extremely rapid time-course (<4 h) and its inflammatory/necrosis-like characteristics [32]. We therefore considered the possibility that flagellin promoted host defense by activating rapid host cell death.

Macrophages dying due to caspase-1 activation rapidly lose plasma membrane integrity and can be detected by their release of the intracellular enzyme lactate dehydrogenase (LDH), by their failure to take up neutral red by micro-pinocytosis, or by their increased permeability to ethidium bromide. Initially, we tested the ΔflaA mutant and our transposon mutants for the ability to initiate macrophage death of B6 macrophages in a 4-h LDH-release assay. Remarkably, we observed that the ΔflaA mutant and the strains with transposon insertions in flaA were all noncytotoxic, as compared to the wild-type LP02 strain (Figure 3A). MyD88−/− macrophages behaved similarly to wild-type macrophages (Figure 3A), implying that flagellin-induced cell death, like restriction of bacterial growth, does not depend on TLR5. In contrast, caspase-1-deficient macrophages, which are permissive for Legionella growth [26], exhibited marked resistance to Legionella-induced death (Figure 3B and 3C). The ΔflaA mutant was confirmed to be noncytotoxic by two other assays of rapid cell death: failure to take up neutral red (Figure 3C) and inability to exclude ethidium bromide (Figure 3D). Our results reveal a striking correlation between the induction of cytotoxicity (seen only with flaA+ bacteria infecting wild-type or MyD88-deficient macrophages) and restriction of bacterial replication.

Naip5 Genotype Is Associated with Resistance to Flagellin-Induced Death

If rapid macrophage death is required for resistance to Legionella growth, a simple prediction is that macrophages permissive for Legionella growth (e.g., from A/J or B6.A-Chr13 mice) should be resistant to flagellin-mediated cytotoxicity. As predicted, flagellin+ bacteria killed a larger percentage of B6 macrophages (as assessed by failure to take up neutral red; Figure 3C), as compared to macrophages carrying the AJ allele of Naip5. A significant difference between B6 and B6.A-Chr13 macrophages in susceptibility to Legionella-induced cell death was also observed when cell death was measured by permeability to ethidium bromide (Figure 3D). B6 macrophages also showed greater release of LDH as compared to susceptible B6.A-Chr13 macrophages (Figures 3B and 4), though the difference was not always dramatic, possibly
because the LDH-release assay is not a single-cell assay and does not distinguish between generalized nonlethal leaking of cells and individual cell death. Thus, although B6A-Chr13 mice were significantly resistant to flagellin-mediated killing, resistance was not complete, suggesting that the A/J-derived Naip5 allele in these mice is likely not a complete null. Consistent with these results, Molofsky et al. [33] also observed significant flaA-dependent killing of A/J macrophages.

Motility Is Not Required to Induce Cytotoxicity

In the cytotoxicity experiments, we usually promoted contact between Legionella and macrophages by centrifugation (400 g for 10 min). This was done because we and others [33,34] have observed that nonmotile bacteria exhibit reduced cytotoxicity simply because they fail to contact macrophages in sufficient numbers. For example, the nonmotile but flagellin-positive flaA mutant appeared noncytotoxic when infection was initiated without a spin (Figure 4A). Importantly, however, centrifugation restored complete cytotoxicity to the flaA mutant (Figure 4B), but had virtually no effect on the cytotoxicity of the ΔflaA mutant. Thus, consistent with the growth assays (Figure 2), flagellin itself, and not motility or flagella, appears to be key for initiating macrophage innate immunity.

Flagellin-Induced Cytotoxicity Requires Type IV Secretion

It has been previously reported that bacteria harboring mutations in the type IV (dot/icm) secretion apparatus are nontoxic [7,35]. It has been proposed that type IV-dependent toxicity, usually seen at high multiplicities of infection (MOIs), is due to pores induced in the host membrane by the dot/icm secretion apparatus [36]. We found that even at relatively low MOI, flagellin-induced killing of macrophages was dot/icm–dependent (Figures 3B and 4B).

A trivial explanation of our data is that flagellin is required for expression or function of the dot/icm apparatus, but this model cannot easily reconcile with the fact that the dot/icm apparatus is strictly required for intracellular growth and the observation that flagellin mutants exhibit robust intracellular growth. Thus we considered two other nonmutually exclusive models: (1) a “secretion model” in which flagellin is secreted into host cells via the type IV (dot/icm) apparatus, or leaks into the cytosol via pores in the phagosome generated by the dot/icm apparatus; or (2) a “two signal” model, in which puncture of the cell membrane by the dot/icm apparatus provides a signal that is independent of that provided by flagellin, and both signals are required to initiate cell death. In the two-signal model, the signal provided by the dot/icm apparatus might be the pores themselves, a pore-induced ion flux, or a pore-induced leakage of microbial products, such as peptidoglycan fragments [37], into the cytosol.

We tested the “two signal” model in several ways. First, to test whether the dot apparatus induces a calcium flux that (in combination with flagellin) induces cell death, we infected macrophages with dotA mutant (but flagellin+) bacteria in the presence of A29187, a calcium ionophore. A29187 did not restore cytotoxicity to the dotA mutant (Figure 4A and 4B) and, moreover, pretreatment of cells with thapsigargin (to deplete intracellular calcium stores [38]) failed to protect macrophages from cytotoxicity of wild-type bacteria (unpublished data). To test whether a dot/icm–dependent, flagellin-independent but non–calcium-mediated signal was required for cell death, we coinfected macrophages with dotA and flaA mutant bacteria. If the dot/icm apparatus and flagellin provide two independent signals required for cell death, we predicted that coinfection with both mutants should restore cytotoxicity, but this was not the case (Figure 4A and 4B). Even at high MOIs (Figure 4C), where each macrophage was infected with multiple bacteria of each genotype, there was no synergy between flaA and dotA mutants in induction of cell death. Thus, the “two signal” model is not sufficient to account for our observations, and it appears likely that one role of the dot/icm apparatus in induction of cytotoxicity is to permit flagellin access to the cytosol. It was interesting that at very high MOIs, the flaA mutant exhibited significant apparent toxicity. The basis for this toxicity is unclear, but it appears to depend on the dot apparatus and may represent a “pore-forming” cytotoxicity [36] that is revealed at high MOIs.

In the above coinfection experiments, the dotA and flaA mutants were not frequently present in the same phagosome (unpublished data), even using specialized infection procedures [39] (Figure 4C) and high MOIs. Therefore we could not distinguish whether flagellin is directly transported into the cytosol via the dot/icm secretion apparatus (in cis), or merely leaks into the cytosol via dot/icm–induced pores in the phagosome (in trans). Nevertheless, we favor the idea that the dot/icm apparatus translocates flagellin into the host cell cytosol, since we observed that flaA mutants retain cytotoxicity, despite the fact that flaA is required for flagellar secretion (Figure 1) [22,33]. Although another secretion system might be responsible for secretion of flagellin into the host cell cytosol, the type IV secretion system seems the most likely candidate.

Salmonella Flagellin Also Induces Death of B6 Macrophages

We complemented the Legionella ΔflaA mutant with flagellin (fliC) from Salmonella, Shigella, and Escherichia coli, as well as with flagellin (fliA) from Legionella. Remarkably, we found that Salmonella flagellin was able to complement the Legionella ΔflaA mutation for cytotoxicity of B6 macrophages (Figure 5A), despite only 59% similarity at the amino acid level. Interestingly, both Salmonella and Legionella flagellins contain two leucines near their C-termini, a potential signal for recognition by the Legionella type IV secretion system [40]. Complementation of the ΔflaA mutant with a Legionella FlaA protein lacking its final 48 amino acids failed to restore cytotoxicity, as did complementation with E. coli or Shigella flagellin (Figure 5A). We do not know why these other flagellins failed to complement cytotoxicity, but it is possible that the flagellin proteins from these species are not recognized, or that expression and delivery of these heterologous flagellin proteins are low. However, our findings suggest a partial molecular explanation for the ability of Salmonella to induce rapid, caspase-1-dependent death of macrophages (see below).

An E. coli strain (CM735) carrying an in-frame deletion of flagellin (ΔfliC) was used as a heterologous host to express Legionella and Salmonella flagellin. CM735 does not express a type III or type IV secretion system capable of delivering proteins to the host cytosol and, as expected, Legionella or Salmonella flagellin did not confer cytotoxicity to E. coli (Figure 5B). This result suggests that flagellin must be delivered to the cytosol in order to induce cytotoxicity. To
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Figure 3. An MOI of 2 was used in this experiment. Asterisks denote cytosol. Infection and LDH release of B6 macrophages was assayed as in pores in the phagosome, giving phagosomal contents access to the degraded in the phagolysosome, releasing cytoplasmic LLO. LLO forms secreted form of the pore-forming toxin LLO. In this system, Legionella (B) Flagellin from when delivered to the cytosol by

The ability to swim through soft agar (motility) plates. Nonmotile strains remain at the point of inoculation, whereas motile strains show a halo of bacteria swimming outward from the point of inoculation. DOI: 10.1371/journal.ppat.0020018.g005

Figure 5. Salmonella and Legionella Flagellin Induce Macrophage Death

(A) Salmonella flagellin complements a ΔflaA mutant of Legionella. The LP02 ΔflaA mutant was complemented with plasmids expressing flagellin of L. pneumophila (Lp flaA), S. typhimurium (St fliC), S. flexneri (Sf fliC), or E. coli (Ec fliC). A 48-amino acid C-terminal truncation mutant of FlaA (LP flaAΔ48) was also expressed as a control. Infection and LDH release of B6 macrophages were assayed as in Figure 3. An MOI of 2 was used in this experiment. Asterisks denote p < 0.02 (two-tailed student’s t-test) versus ΔflaA + Lp flaAΔ48.

(B) Flagellin from Legionella and Salmonella induce macrophage death when delivered to the cytosol by E. coli. A flagellin-deficient strain of E. coli (CM735ΔfliC) was transformed with the same plasmids as in (A), or an empty vector as a control. In some cases, the E. coli also expressed a non-secreted form of the pore-forming toxin LLO. In this system, E. coli is degraded in the phagolysosome, releasing cytoplasmic LLO. LLO forms pores in the phagosome, giving phagosomal contents access to the cytosol. Infection and LDH release of B6 macrophages were assayed as in Figure 3. An MOI of 2 was used in this experiment. Asterisks denote p < 0.02 (two-tailed student’s t-test) versus LLO + empty vector (gray bar). The p-value for LLO + Sf fliC was 0.05; the p-value for LLO + Ec fliC was 0.05; and the p-value for LLO + Lp flaAΔ48 was 0.155.

(C) The E. coli CM735ΔfliC-derived strains from (B) were tested for the ability to swim through soft agar (motility) plates. Nonmotile strains remain at the point of inoculation, whereas motile strains show a halo of bacteria swimming outward from the point of inoculation.

Conclusions

Our data are consistent with a model in which rapid caspase-1-dependent macrophage death, induced in B6 macrophages by flagellin, leads to restriction of Legionella growth. Caspase-1 activation has been proposed to promote immune defense by initiating rapid release of inflammatory cytokines (IL-1 and IL-18). Although such inflammation may sometimes [42] (but not always [43]) provide immune defense in vivo, our results suggest that caspase-1-mediated death can also play a more direct role in restricting bacterial growth.

How might rapid cell death restrict Legionella growth? We propose that two effects may be in play. First, it is well established that after infection of a macrophage, Legionella delays replication for several hours until a proper replicative vacuole can be established [44]. Second, during the late phases of replication in susceptible cells, Legionella is known to upregulate gene products (e.g., the dot/icm apparatus) that help promote infectivity for new host cells [45]. Thus, a macrophage that undergoes rapid cell death will not only release fewer bacteria, but each released bacterium may also exhibit reduced infectivity for a new host cell. These two effects, in combination with other potential mechanisms [46], may lead to the significant growth restriction observed in B6 macrophages.

If our model is correct, and Legionella flagellin is sensed by host cells, an obvious question is: what host protein is responsible for the sensing? We have provided evidence that sensing of flagellin, rapid macrophage death, and growth restriction of Legionella appear to be independent of TLR5. We consequently favor the idea that a novel intracellular sensor protein may be responsible for sensing flagellin. An obvious candidate is Naip5, since this protein is known to be required for restriction of Legionella growth, and the Naip5 genotype correlates with the ability to sense flagellin (Figure 3). However, a recent study [26] demonstrated that the Naip5 homolog, Ipaf, is also required for restriction of Legionella growth, and moreover, Ipaf has been shown to be critical for activation of caspase-1 in response to Salmonella [20]. Caspase-1 is also regulated by the “inflammasome”, a complex of proteins including Nalp family members [47]. Higher-order complexes containing Naip, Nalp, Ipaf, and/or caspase-1 may exist and, thus, it is possible that Ipaf, Naip, or another host protein is the sensor of flagellin, and that Naip5 plays an important role as a subsequent regulator of caspase-1 activation. For the future, analyzing a null allele of Naip5 may provide more specific information about its role in the test this, E. coli CM735ΔfliC was transformed with a plasmid encoding listeriolysin O (LLO), a cholesterol-dependent pore-forming toxin that Listeria utilizes to disrupt phagosomal (but not plasma) membrane [41]. Expression of LLO itself caused some toxicity of macrophages (Figure 5B), but this was markedly increased when the E. coli strain also expressed Legionella or Salmonella flagellin (p < 0.02; Figure 5B). Delivery of Shigella flexneri or E. coli flagellin induced weak but statistically insignificant cytotoxicity (p = 0.05), whereas a truncated form of Legionella flagellin (lacking the C-terminal 48 amino acids) was noncytotoxic. The results imply that Legionella (or Salmonella) flagellin does not require other Legionella (or Salmonella) proteins in order to induce cytotoxicity of macrophages. In addition, since the E. coli strain expressing Legionella flagellin was entirely nonmotile, as assessed by movement through soft agar (Figure 5C), the results reconfirmed that bacterial motility is not required for induction of macrophage death.
caspase-1-mediated cell-death pathway than studying the naturally occurring A/J variant.

We do not anticipate that it will be easy to prove direct recognition of flagellin by a host receptor. Indeed, extensive characterization of the much more intensively studied toll-like or Nod-like receptors has yet to provide uncontestable direct evidence of binding to a bacterial ligand. We believe that it is likely that other bacterial products, such as peptidoglycan, will also induce macrophage death when delivered to the cytosol. Therefore, conclusive evidence that flagellin itself is sensed, as opposed to a flagellin-associated contaminant, will be difficult to obtain until direct binding assays with purified proteins are developed, and a source of recombinant flagellin, expressed in the absence of bacteria, is utilized.

In addition, there is the distinct possibility that flagellin itself is not sensed, but that the presence of flagellin imparts some physiological change on host macrophages, and it is this physiological alteration that is sensed. It is also possible that flagellin imparts a physiological change to Legionella that renders it more cytotoxic to macrophages. This latter possibility seems unlikely given the ability of Legionella flagellin to induce cell death when delivered to the macrophage cytosol by E. coli.

Finally, irrespective of mechanism, we believe our findings may be relevant not only to Legionella, but also for understanding immune defense against a variety of flagellated pathogens. Along these lines, we found that Salmonella flagellin is also capable of inducing rapid macrophage cell death (Figure 5). It is well established that Salmonella induces caspase-1-dependent death of macrophages [48]. It has been proposed that the Salmonella type III-secreted effector SipB binds to caspase-1 [30], leading to caspase-1 activation, but this conclusion has been challenged for a variety of reasons [48]. In fact, Salmonella-induced macrophage death may be caused by several redundant mechanisms. Thus, it seems plausible that flagellin, perhaps delivered to the cytosol by the Salmonella type III secretion system, may be responsible, at least in part, for caspase-1-dependent toxicity induced by Salmonella.

Consistent with this model, we found that a Salmonella strain unable to express either of two flagellin genes (fflB fliC) had a reduced cytotoxicity similar to a type III mva secretion mutant (Figure S1A). This experiment must be interpreted with caution, however, because the invasiveness of the flagellin mutants is visibly less than that of the wild-type, higher MOIs tend to increase the cytotoxicity of the flagellin mutants (Figure S1B), and we did not perform experiments with a control strain equivalent to the Legionella fliI mutant (flagellin+, but flagellum−). However, we do note that one report demonstrated that mutations in flaA (required for flagellin expression) but not fliK (required for flagellar assembly) resulted in a motility-independent defect in induction of rapid macrophage cytotoxicity [34].

Interestingly, we did not observe differences between B6 and B6.A-Chr13 macrophages in their cytotoxicity response to Salmonella infection (Figure S1). This is not very surprising, since variations in mouse Salmonella susceptibility have been mapped in several instances, and have never been linked to the Lgn1 locus. Based on this information, we conclude that the Naip5 allelic variations found in B6 and A/J may not cause differential responses to intracellular flagellins from all flagellated pathogens.

Although many questions remain, our unbiased genetic mutagenesis and selection has identified Legionella flagellin (but not the flagellum) as a key determinant in initiating innate immune-defense mechanisms, including caspase-1-dependent macrophage cell death and restriction of bacterial growth. We believe our results provide insights for understanding macrophage resistance to intracellular bacterial pathogens.

Materials and Methods

Mice. B6 and consomic B6.A-Chr13 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, United States). Two independently derived caspase-1-deficient mouse lines [27,28] were used and gave similar results. MyD88− mice, backcrossed at least five times to B6, were obtained from the Medzhitov Laboratory (Yale University School of Medicine). All knockout mice were confirmed to be homozygous for the B6 Naip5 locus.

Bacterial strains. LP02 is a streptomycin-resistant thymidine auxotroph derived from L. pneumophila LP01. An unmarked deletion of flaA (corresponding to nucleotides 1478105–1479574 of the LP01 genome [49]) was generated in LP02 by use of the allelic exchange vector pSR47S [22]. The fliA strain was also complemented by inserting the fliC gene and its own promoter (corresponding to nucleotides 1478156–1479915 of the LP01 genome) on the chromosome just after the ahpC gene (at position 3354877 of the LP01 genome). The ahpC locus is highly expressed but is not essential for Legionella virulence [50]. The fliK null strain LP02 fliK−Cm [22] was the kind gift of R. Isberg (Tufts University School of Medicine). The broad-host-range plasmid pBBR1-MCS2 was used to express flagellin from Legionella, E. coli MG1655 (fliC, b1923), S. flexneri 2457T (fliC, S2062), and Salmonella typhimurium LT2 (fliC, STM1959). The various flagellin open reading frames were first cloned into pET28a (Nov, XhoI) and then transferred to pBBR1-MCS2 as pBBR flagellin constructs were also expressed in E. coli CM753ΔfliC [51], along with LLO (pACYC184-LLOc) [41]. Salmonella strains were on the LT2 background and were the kind gift of the Starnbach Laboratory.

Transposon mutagenesis. A mariner transposon (pSCL123, kind gift of S. Chiang and J. Mekalanos) was mated from E. coli strain SM102pir into L. pneumophila strain LP02. Eight separate mating plates were plated on a total of 30 buffered charcoal yeast extract (BCYE) plates containing streptomycin (50 μg/ml) and kanamycin (25 μg/ml). Approximately 2,500 colonies from each independent plate were pooled and used to infect bone marrow–derived macrophages from B6 mice at an MOI of 0.05. Unmutagenized LP02 was used to infect macrophages as a control. After 6 d, bacteria were visible in each well that had been infected with mutagenized LP02, but virtually none were evident in wells that had been infected with unmutagenized LP02. Bacteria were harvested from each well, plated on BCYE plates, and individual colonies were tested for the ability to grow in B6 macrophages. The transposon insertion site for selected strains was determined by arbitrary PCR.

Arbitrary PCR. Crude DNA from a single Legionella colony was used in a first round of PCR with primers JZ320–1 (GCCGGGGGAT CATTTGGA, ARB1 (GCCGCCGCTGCTAGTACATCAANNNNNNN NNAGATG), and ARB6 (GCCGCCGCTGCTAGTACATCAANNNNN NNAGATG)). The first-round PCR product was subcloned to a second round of PCR with primers JZ320–2 (GCCGGGCCCATCTAG TGT) and ARB2 (GCCGCCGCCCTGCTAGTAC). The resulting PCR product was sequenced with primer ARB2. Various insertions were apparent in the fliA gene. Mutants for which arbitrary PCR failed were tested for insertions in fliC by PCR (e.g., using fliA primers ACGCGTGGCAGTCGGACAGTTTCATGTTTCAAGTACCCATG [upstream] and CCGCTGCTGTCGTACATCAAAAGAGATAATACA GATG [downstream] in combination with JZ320–2 [transposon-specific]).

Motility. For Legionella, bacterial cultures (optical density at 600 nm > 4.0) were diluted 10-fold, placed on a hemocytometer, and observed under high power by a neutral observer blinded with respect to bacterial genotype. Bacteria were considered motile (as opposed to merely subject to Brownian motion) if multiple bacteria could be observed crossing the gridlines on the hemocytometer. For E. coli, bacteria were inoculated at a single point in soft (motility) agar (0.35%), incubated at 37°C, and observed after ~16 h.
**Electron microscopy.** A stationary-phase culture (5 μl) of *Legionella* was adsorbed onto a carbon-coated grid that had been made hydrophilic by a 30-s exposure to a glow discharge in an Edwards Auto 306 vacuum evaporator (http://www.bocedwards.com). Excess liquid was removed with a filter paper (Whatman number 1), and the samples were stained with 1% uranyl acetate for 1 min. The grids were examined using a Tecnai 12 Bio TWIN (FEL, Hillsboro, Oregon, United States) transmission electron microscope.

**Western blotting.** Stationary-phase bacteria (2.5 x 10^6, or trichloroacetic acid (TCA) precipitates of supernatant from 0.5 ml of stationary-phase culture) were pelleted and resuspended in SDS-containing loading buffer. Proteins were separated on a 10% precast SDS-PAGE gel (Bio Rad, Hercules, California, United States), and transferred to PVDF membrane (Amersham Biosciences), and flagellin was visualized using enhanced chemiluminescence (PerkinElmer, Wellesley, California, United States).

**Legionella growth curves.** *Legionella* growth in bone marrow–derived macrophages was assayed as described previously [10].

**Cytotoxicity assay.** The Cytotox assay (Promega, Madison, Wisconsin, United States) was used to measure LDH release. In this assay, 1 x 10^6 macrophages were plated per well of a 96-well plate. Two-fold dilutions of *Legionella* bacteria were grown overnight in liquid buffered-yeast-extract culture and, at the time of infection, samples were matched as closely as possible for optical density (600 nm). In experiments using *Salmonella* or *E. coli*, bacteria were grown to midlog phase in Luria-Bertani media before being used to infect macrophages. Bacteria (*Legionella, Salmonella, or E. coli*) were added to wells at the indicated MOI and the plate was then spun at 400 g (1,400 rpm) for 10 min, except where indicated. After 30 min of incubation at 37 °C, the media was removed from the infected cells and replaced with fresh media containing 50 μg/ml of gentamicin. After an additional 3.5 h of incubation at 37 °C, LDH release was calculated as a percentage of detergent-lysed macrophages for each strain of bacteria. Alternatively, neutral red (Sigma, St. Louis, Missouri, United States) was added to macrophages 2 h after infection at a final concentration of 15 μg/ml. Two hours later, viable macrophages (positive for neutral red) and dead macrophages (unstained) were enumerated by light microscopy. Permeability to ethidium bromide–homodimer, indicative of cell death, was assessed using the Live/Dead kit (Invitrogen, Carlsbad, California, United States) according to the manufacturer’s instructions.

**Supporting Information**

**Figure S1.** *Salmonella* Induction of Cell Death in Infected Macrophages

The indicated strains were used to infect B6 or B6.A-Chr13 macrophages. (Infection and LDH release of macrophages was assayed as shown in Figure 3 and as described in Materials and Methods). The βfβCβC mutant strain does not express either βfβ or βfC (the two *Salmonella* flagellin genes). The insA mutant is defective in SPI-1 type III secretion. The βfB gene is required for expression of the entire flagellar regulon. (A) MOI of 10. Found at DOI: 10.1371/journal.ppat.0020018.sg001 (52 KB PPT).

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