

Listeria monocytogenes engineered to activate the Nlrc4 inflammasome are severely attenuated and are poor inducers of protective immunity

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Inflammasomes are intracellular multiprotein signaling complexes that activate Caspase-1, leading to the cleavage and secretion of IL-1 β and IL-18, and ultimately host cell death. Inflammasome activation is a common cellular response to infection; however, the consequences of inflammasome activation during acute infection and in the development of long-term protective immunity is not well understood. To investigate the role of the inflammasome in vivo, we engineered a strain of *Listeria monocytogenes* that ectopically expresses *Legionella pneumophila* flagellin, a potent activator of the Nlrc4 inflammasome. Compared with wild-type *L. monocytogenes*, strains that ectopically secreted flagellin induced robust host cell death and IL-1 β secretion. These strains were highly attenuated both in bone marrow-derived macrophages and in vivo compared with wild-type *L. monocytogenes*. Attenuation in vivo was dependent on Nlrc4, but independent of IL-1 β /IL-18 or neutrophil activity. *L. monocytogenes* strains that activated the inflammasome generated significantly less protective immunity, a phenotype that correlated with decreased induction of antigen-specific T cells. Our data suggest that avoidance of inflammasome activation is a critical virulence strategy for intracellular pathogens, and that activation of the inflammasome leads to decreased long-term protective immunity and diminished T-cell responses.

cell-mediated immunity | innate immunity | pathogenesis | CD8⁺ T cells | vaccine

The innate immune system functions to detect invading microbes, eliminate or contain infections, and orchestrate the development of adaptive immune responses (1). One innate immune pathway triggered by infection is inflammasome activation. Inflammasomes are multiprotein complexes that activate proinflammatory caspases and subsequently lead to cytokine secretion. Detection of pathogen-associated molecular patterns (PAMPs) by cytosolic pattern recognition receptors (PRRs) leads to inflammasome complex formation and activation of Caspase-1. Active Caspase-1 cleaves and activates the proinflammatory cytokines IL-1 β and IL-18, leading to their secretion. Concomitant with IL-1 β and IL-18 secretion is Caspase-1–dependent cell death, known as pyroptosis (2).

Multiple cytoplasmic PRRs can trigger inflammasome activation, each responding to a different ligand or stimulus (2). One of the most well-characterized PRRs leading to inflammasome activation is Nlrc4 (Ipaf) (3–5). Nlrc4 activates Caspase-1 in response to contamination of the cytosol with either bacterial flagellin or type III secretion inner-rod proteins (3, 4, 6). For example, infection with *Legionella pneumophila* robustly activates the Nlrc4/Naip5 inflammasome in a process that is dependent on both bacterial flagellin and a type IV secretion system thought to mediate delivery of the flagellin to the cytosol (5, 7).

Numerous microbes trigger Caspase-1 activation in vitro, and in a few cases Caspase-1–deficient mice are more susceptible to infection, implying that pyroptosis can be a host innate immune

defense mechanism (8, 9). Not surprisingly, pathogens have evolved mechanisms to avoid inflammasome activation, either by direct inhibition of Caspase-1 activation or by regulating PAMPs expression (10). In addition to its potential role in innate immune defense, inflammasome activation has been implicated in the development of adaptive immunity to influenza virus, fungal β -glucan, and that mediated by the adjuvant alum (11–13).

Listeria monocytogenes is a Gram-positive, facultative intracellular pathogen that has been extensively used as a model to study cell biology, bacterial pathogenesis, and innate and adaptive immunity. Following internalization by a host cell, *L. monocytogenes* uses a cholesterol-dependent cytolysin, listeriolysin O (LLO encoded by the gene *hly*), to break out of a phagosome and enter the host cytosol (14). Once in the cytosol, *L. monocytogenes* synthesizes and secretes ActA to hijack the host actin machinery and spread to neighboring cells (15). Maintenance of its intracellular replication niche is essential to *L. monocytogenes* virulence as strains that fail to compartmentalize LLO activity to the phagosome are cytotoxic and highly attenuated (16). Nevertheless, there have been numerous reports that *L. monocytogenes* infection leads to activation of multiple inflammasomes in vitro, including the Nlrp3, Nlrc4, and AIM2 inflammasomes (17–23). Although these responses can be detected in vitro, the role of inflammasome activation and pyroptosis during in vivo infections is not appreciated. Therefore, to address the role of the inflammasome in vivo, we used *L. monocytogenes* as a model pathogen and compared wild-type bacteria to a strain engineered to activate the Nlrc4 inflammasome. We found that activation of the inflammasome not only attenuated virulence, but also inhibited the development of long-term protective immunity.

Results

***L. monocytogenes* Infection Triggers Negligible Inflammasome Activation.** *L. monocytogenes* has evolved multiple mechanisms to maintain its intracellular niche (16). Nevertheless, there are numerous reports that *L. monocytogenes* infection triggers inflammasome activation in vitro (17–23). To reexamine the degree of inflammasome activation upon *L. monocytogenes* infection, we measured cell death and IL-1 β secretion induced by *L. monocytogenes* compared with *L. pneumophila*, a robust activator of the Nlrc4 inflammasome. Following infection of bone

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marrow-derived macrophages at low multiplicities of infection (MOI = 5), *L. monocytogenes* induced significantly less lactate dehydrogenase release (10% vs. 89%) and IL-1 β secretion (~60-fold less) than *L. pneumophila* (Fig. S1). Consistent with previous reports (20), the low levels of cell death and IL-1 β secretion induced by *L. monocytogenes* were dependent on bacterial access to the cytosol, as infection with Δ *hly* *L. monocytogenes* led to almost no cell death or IL-1 β secretion (Fig. S1).

Because Caspase-1 is required for inflammasome-mediated cell death and IL-1 β secretion, we used Caspase-1 $^{-/-}$ mice to evaluate the role of the inflammasome during primary listeriosis. Wild-type and Caspase-1 $^{-/-}$ mice were equally susceptible to infection as monitored by bacterial load in the liver and spleen 48 h postinfection (Fig. 1). In fact, Caspase-1 $^{-/-}$ mice were slightly more resistant to *L. monocytogenes* infection at both 2 and 5 d postinfection (Fig. 1 and Fig. S2). The observation that wild-type *L. monocytogenes* induced only low levels of pyroptosis, as well as the observation that Caspase-1 $^{-/-}$ mice are not hypersusceptible to infection, suggested that the low levels of inflammasome activation observed in vitro play at most a minor role in host defense during primary listeriosis.

***L. monocytogenes* Ectopically Expressing Flagellin Hyperactivate Pyroptosis.** To further evaluate the potential impact of inflammasome activation in vivo, we engineered a *L. monocytogenes* strain, referred to as *L. monocytogenes*-L.p.FlA, that ectopically secreted *L. pneumophila* flagellin, a potent activator of the Nlr4/Naip5 inflammasome (3–5). Flagellin was fused to the N-terminal 100 amino acids of ActA to facilitate secretion, and was expressed under control of the *actA* promoter to restrict expression to cytosolic bacteria. *L. monocytogenes*-L.p.FlA induced host cell death 6 h postinfection, killing 70% of infected cells compared with 10% killing by wild-type *L. monocytogenes* (Fig. 2A). As expected, *L. monocytogenes*-L.p.FlA-induced host cell death was largely dependent on Nlr4 (Fig. 2A). The majority of cell death induced by *L. monocytogenes*-L.p.FlA was also independent of ASC (apoptosis-associated speck-like protein containing a CARD). The small but statistically significant portion of cell death that was dependent on ASC was likely caused by background induction of AIM2-dependent cell death normally induced by wild-type *L. monocytogenes* (Fig. 2A) (18).

Concomitant with cell death, *L. monocytogenes*-L.p.FlA induced ~35-fold more IL-1 β secretion than wild type *L. monocytogenes* infection (Fig. 2B). As expected, the secretion of IL-1 β by *L. monocytogenes*-L.p.FlA was dependent on both Nlr4 and the adaptor ASC. Hyper-induction of host cell death and IL-1 β secretion was not unique to expression of *L. pneumophila* flagellin as ectopic expression of another Nlr4 agonist, *Salmonella typhimurium* PrgJ (6), also resulted in hyperinduction of pyroptosis (Fig. S3).

***L. monocytogenes*-L.p.FlA Are Highly Attenuated in Vitro and in Vivo.** To determine if inflammasome activation affects the virulence of intracellular pathogens, we analyzed intracellular growth of

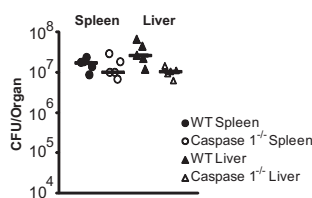


Fig. 1. *L. monocytogenes* minimally activates the inflammasome. Wild-type (closed symbols) or Caspase-1 $^{-/-}$ (open symbols) mice were infected with 1×10^5 wild-type *L. monocytogenes* and CFU per organ were determined 48 h postinfection. Data are representative of at least two independent experiments.

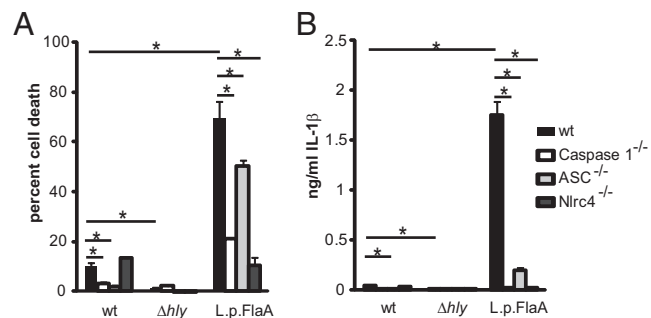


Fig. 2. *L. monocytogenes* strains that ectopically secrete flagellin hyperinduce host cell death and IL-1 β secretion. Cell death (A) and IL-1 β (B) secretion were measured following 6 h of infection at an MOI of 5 in wild-type, Caspase-1 $^{-/-}$, ASC $^{-/-}$, or Nlr4 $^{-/-}$ bone marrow-derived macrophages. Data are representative of at least three independent experiments. * $P < 0.05$ by Student's *t*-test.

wild-type bacteria compared with *L. monocytogenes*-L.p.FlA. Growth of *L. monocytogenes*-L.p.FlA in wild-type bone marrow-derived macrophages was severely attenuated compared with growth of wild-type *L. monocytogenes* (Fig. S4A). The growth defect was rescued in Nlr4 $^{-/-}$ bone marrow-derived macrophages, suggesting that the defect was a result of inflammasome activation (Fig. S4B).

To examine the effect of inflammasome activation on virulence in vivo, we infected wild-type mice intravenously with wild-type or *L. monocytogenes*-L.p.FlA. *L. monocytogenes*-L.p.FlA were severely attenuated compared with wild-type *L. monocytogenes*, as indicated by fewer colony forming units (CFU) in both the spleen (~900-fold) and the liver (~400-fold) of *L. monocytogenes*-L.p.FlA-infected mice 48 h postinfection (Fig. 3A). The severe virulence defect was largely rescued (>99% in the spleen and 100% in the liver) in mice deficient for the cytosolic flagellin detection system (Naip5 $^{-/-}$ /Nlr4 $^{-/-}$) (Fig. 3B). In addition, the LD₅₀ of the *L. monocytogenes*-L.p.FlA strain was 7.5×10^6 , ~75-fold higher than the LD₅₀ of the wild-type *L. monocytogenes* strain in wild-type mice. Taken together, these data suggested that robust inflammasome activation can help control infections by intracellular pathogens in vitro and in vivo.

***L. monocytogenes*-L.p.FlA Is Attenuated in Vivo Independent of Neutrophil Activity and the Adaptor ASC.** *L. monocytogenes* strains (e.g., LLO_{S44A}) that induce inflammasome-independent cell death are highly attenuated in vivo. However, depletion of neu-

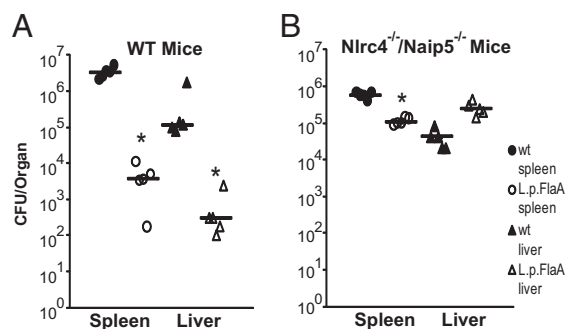


Fig. 3. Inflammasome activation attenuates *L. monocytogenes* virulence. Wild-type (A) or Naip5 $^{-/-}$ /Nlr4 $^{-/-}$ (B) mice were infected with 1×10^4 wild type (closed symbols) or *L. monocytogenes*-L.p.FlA (open symbols) and CFU was determined 48 h postinfection in the spleen (circles) and liver (triangles). Data are representative of at least two independent experiments. * $P < 0.05$ by Mann-Whitney test.

trophils rescues the virulence defect, suggesting that neutrophils kill bacteria released by dying cells (16). To determine if neutrophils were responsible for controlling infection of *L. monocytogenes*-L.p.FlaA strains, we performed competitive index assays in wild-type mice and mice rendered neutropenic by depletion of neutrophils using an anti-Gr1 antibody (24). As previously shown, virulence of *L. monocytogenes*-LLO_{S44A} was rescued by depletion of neutrophils (>99% spleen and liver) (Fig. 4A). In contrast, the virulence defect of *L. monocytogenes*-L.p.FlaA was not rescued by depletion of neutrophils as *L. monocytogenes*-L.p.FlaA was still outcompeted by wild-type bacteria more than 500-fold in both the spleen and liver (Fig. 4A).

It was previously shown that inflammasome-mediated control of infection by influenza virus is partially mediated by IL-1 β (25). Additionally, IL-18 has been shown to mediate resistance to variety of pathogens, including *Mycobacterium tuberculosis* and *Shigella flexneri* (26, 27). Although ASC is required for activation and secretion of IL-1 β and IL-18 downstream of the Nlr4 inflammasome, it is dispensable for activation of host cell death (Fig. 2B) (28). Therefore, to determine if IL-1 β and IL-18 were required for attenuation of *L. monocytogenes*-L.p.FlaA, we assayed virulence in ASC^{-/-} mice. *L. monocytogenes*-L.p.FlaA was still highly attenuated, greater than 500-fold in both the spleen and liver, in ASC-deficient mice (Fig. 4B), indicating that activation of IL-1 β and IL-18 did not mediate attenuation. Taken together, these data suggested that attenuation of *L. monocytogenes*-L.p.FlaA infection was not caused by clearance by neutrophils or inflammatory cytokine activation.

Immunization with *L. monocytogenes*-L.p.FlaA Results in Decreased Protection to Subsequent *L. monocytogenes* Challenge. In addition to its role in host defense, inflammasome activation may play

a role in the development of adaptive immunity (11, 13). To determine if inflammasome activation, in the context of a *L. monocytogenes* infection, had an effect on the development of adaptive immunity, we immunized mice with 0.1LD₅₀s of either $\Delta actA/\Delta inlB$ bacteria or $\Delta actA/\Delta inlB$ bacteria expressing *L. pneumophila* flagellin. The $\Delta actA/\Delta inlB$ background was used to minimize differences in CFUs as the LD₅₀s of $\Delta actA/\Delta inlB$ and $\Delta actA/\Delta inlB$ *L. monocytogenes*-L.p.FlaA were identical. Thirty days postimmunization, we challenged mice with 2 LD₅₀s (2×10^5) of wild-type *L. monocytogenes*. Seventy-two hours following challenge, $\Delta actA/\Delta inlB$ *L. monocytogenes*-L.p.FlaA-immunized mice had slightly higher bacterial burdens in both the spleen (10-fold) and the liver (twofold) compared with $\Delta actA/\Delta inlB$ -immunized mice (Fig. 5). Similarly, mice challenged 90 d postimmunization with $\Delta actA/inlB$ were more protected from subsequent lethal challenge than mice immunized with $\Delta actA/\Delta inlB$ *L. monocytogenes*-L.p.FlaA (Fig. S5).

$\Delta actA/\Delta inlB$ *L. monocytogenes* induce potent protective immunity even at low doses, therefore, to further investigate the role of the inflammasome on the development of cell-mediated immunity, we immunized mice with 0.00001 LD₅₀ (10^3 CFU) and returned 30 d later with a $2 \times$ LD₅₀ challenge. At an immunizing dose of 10^3 , there were identical CFU of $\Delta actA/\Delta inlB$ and $\Delta actA/\Delta inlB$ *L. monocytogenes*-L.p.FlaA 24 and 48 h postimmunization (Fig. S6). Mice immunized with 10^3 $\Delta actA/\Delta inlB$ displayed ~ 3 to 4 logs of protection in both the spleen and the liver following challenge with 2 LD₅₀s of wild-type *L. monocytogenes* (Fig. 5). In contrast, mice immunized with $\Delta actA/\Delta inlB$ *L. monocytogenes*-L.p.FlaA were completely unprotected from challenge, harboring bacterial loads similar to naive mice (Fig. 5). Importantly, $\Delta actA/\Delta inlB$ *L. monocytogenes*-L.p.FlaA were ca-

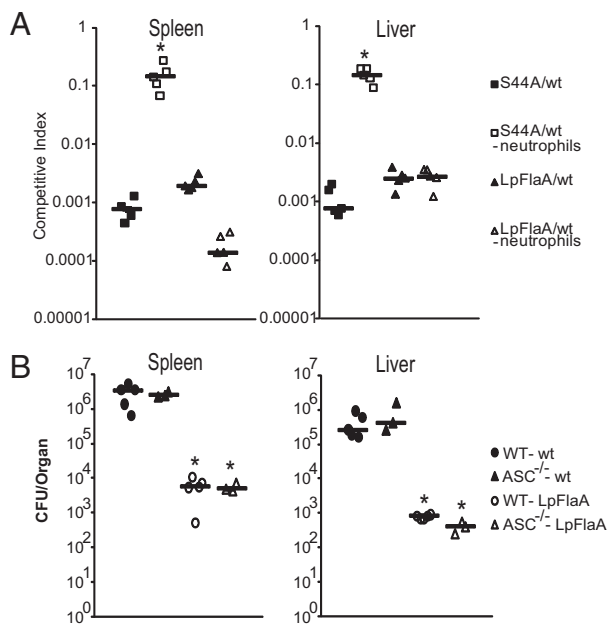


Fig. 4. Neither neutrophil depletion or ASC deficiency rescue the *L. monocytogenes*-L.p.FlaA virulence defect. (A) Wild-type mice were mock treated (closed symbols) or treated with 10 μ g/mL of RB6-8C5 anti-GR1 antibody (open symbols) and infected with either 5×10^3 wild-type + 5×10^3 LLO_{S44A} mutants (squares) or 5×10^3 wild-type + 5×10^3 *L. monocytogenes*-L.p.FlaA (triangles) and competitive index was determined 48 h postinfection. Data are representative of at least two experiments. (B) Wild-type (circles) or ASC^{-/-} (triangles) mice were infected with 1×10^4 wild-type (closed symbols) or *L. monocytogenes*-L.p.FlaA (open symbols) and CFU were determined 48 h postinfection. Data are representative of at least two independent experiments. * $P < 0.05$ by Mann-Whitney test.

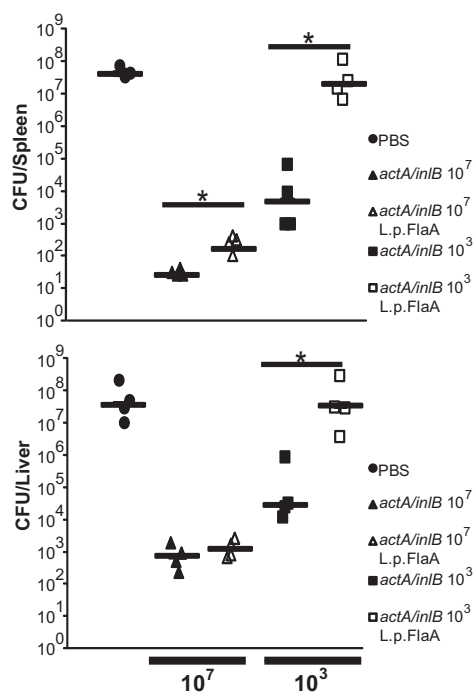


Fig. 5. Immunization with inflammasome activating *L. monocytogenes* results in a loss of protective immunity. Wild-type mice were immunized with 1×10^7 (triangles) or 1×10^3 (squares) $\Delta actA/inlB$ (closed symbols) or $\Delta actA/inlB$ *L. monocytogenes*-L.p.FlaA (open symbols) bacteria. Thirty days postimmunization, mice were challenged with 2×10^5 wild-type bacteria and CFU per organ were analyzed 68 to 72 h postchallenge. Data are representative of at least two independent experiments. * $P < 0.05$ by Mann-Whitney test.

pable of inducing protective immunity in Caspase-1^{-/-} mice (Fig. S5), suggesting that activation of the inflammasome during immunization inhibited the development of protective immunity.

L. monocytogenes-L.p.FlaA Induced Defective T-Cell Responses Following Immunization. Immune protection against *L. monocytogenes* is largely mediated by CD8⁺ T cells (29). *L. monocytogenes* stimulates only modest antibody responses and antibodies do not provide protection from subsequent challenge with *L. monocytogenes* (30). Our previous results demonstrated that activation of the inflammasome during immunization resulted in failure to develop long-term protective immunity to *L. monocytogenes*. Therefore, to address whether inflammasome activation specifically resulted in defects in T-cell development, we analyzed antigen specific CD8⁺ T-cell responses to *L. monocytogenes* strains that secreted ovalbumen (OVA) and B8R, two well-characterized CD8⁺ T-cell antigens. Mice immunized with $\Delta actA/\Delta inlB$ *L. monocytogenes*-L.p.FlaA had approximately half as many antigen-specific OVA and B8R CD8⁺ T cells 7 d postimmunization compared with $\Delta actA/\Delta inlB$ -immunized mice. This phenotype was observed by both ex vivo peptide stimulation of splenocytes (Fig. 6A) and by direct tetramer staining of OVA-specific CD8⁺ T cells (Fig. S7). Similarly, LLO_{190–201} specific CD4⁺, IFN- γ -producing T cells were reduced in $\Delta actA/inlB$ *L. monocytogenes*-L.p.FlaA-immunized mice compared with $\Delta actA/inlB$ -immunized mice (Fig. S8). Importantly, the defect in development of antigen-specific T cells following immunization with $\Delta actA/inlB$ *L. monocytogenes*-L.p.FlaA was rescued in

Caspase-1-deficient mice, suggesting that the T-cell development defect was correlated with inflammasome activation (Fig. 6B and Fig. S7).

In addition to abrogated primary T-cell responses, we saw fewer antigen-specific, CD8⁺ memory T cells 35 d postimmunization in mice immunized with $\Delta actA/\Delta inlB$ *L. monocytogenes*-L.p.FlaA compared with $\Delta actA/\Delta inlB$ (Fig. S9A). However, the smaller memory cell population did not result in a differential number of antigen-specific CD8⁺ T cells upon secondary challenge. There were equal numbers of IFN- γ -producing antigen-specific CD8⁺ T cells 5 d after challenge of mice immunized with either $\Delta actA/inlB$ or the isogenic strain *L. monocytogenes*-L.p.FlaA (Fig. S9B). Taken together, these data suggested that inflammasome activation in the context of *L. monocytogenes* immunization resulted in a defect in both the primary CD8⁺ T-cell response and in the maintenance of long-term memory CD8⁺ T cells.

Discussion

The goal of this study was to determine the role of inflammasome activation during infection and immunity using *L. monocytogenes* as a model pathogen. We determined that wild-type *L. monocytogenes* infection triggered low levels of inflammasome activation and that this response played a negligible role in the host defense against wild-type *L. monocytogenes* in vivo. *L. monocytogenes*-L.p.FlaA, engineered to activate the inflammasome via ectopic expression of flagellin, were severely attenuated in vitro and in vivo. In addition, inflammasome activation resulted in decreased long-term protection from subsequent lethal challenge and diminished antigen-specific T-cell responses.

Although many pathogens activate the inflammasome in vitro, the role of inflammasome activation in vivo is less appreciated. We propose that many intracellular pathogens either inhibit or avoid inflammasome activation to promote their virulence (10). For example, *L. monocytogenes* has a variety of regulatory mechanisms to avoid activation of host cell death, including regulation of LLO compartmentalization and lack of flagellin expression in vivo (3, 4, 17, 18, 22). Indeed, when LLO or flagellin regulation is disrupted, *L. monocytogenes* are rendered less virulent (16, 17, 31). As another example, Miao et al. recently reported that ectopic expression of flagellin by *S. typhimurium* attenuates virulence in an inflammasome-dependent manner (32), further supporting the hypothesis that some intracellular pathogens avoid activation of the inflammasome to promote their virulence.

We previously reported that wild-type *L. monocytogenes* activates low levels of AIM2-dependent inflammasome activation caused by infrequent bacteriolysis in the cytosol during infection (18). In the present work, we also observed very low levels of inflammasome activation by *L. monocytogenes* infection. In contrast, other studies have reported substantial activation of the inflammasome by *L. monocytogenes*. These studies frequently used nonphysiologic MOIs (20, 21), extended infection times (19, 22), or bacterial mutants (17, 18) to study inflammasome activation in vitro. Although these studies are useful for understanding mechanisms of inflammasome activation, they did not address the role of the inflammasome during *L. monocytogenes* infection in vivo. We propose that the level of inflammasome activation triggered by wild-type *L. monocytogenes* under normal physiological conditions is so low that it plays a negligible role in vivo. Our observation that Caspase-1 mice are not highly susceptible to infection supports this model, but contradicts previously published reports (33, 34). Differences in infectious dose (1 LD₅₀ vs. 5 LD₅₀), as well as bacterial and mouse genetic backgrounds, likely explain the differences between our results. However, the very low level of inflammasome activation that we see induced by wild-type *L. monocytogenes* in vitro is consistent with our finding that Caspase-1 mice are not highly susceptible to infection. Fur-

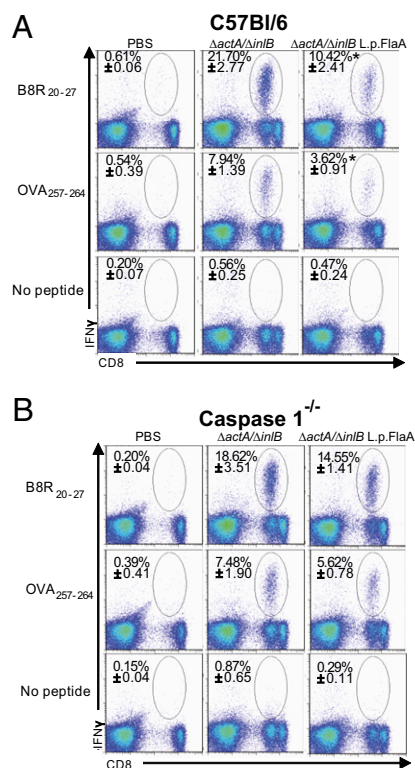


Fig. 6. *L. monocytogenes* that activates the Nlr4 inflammasome impairs the primary specific CD8⁺ T-cell response. C57BL/6 (A) or Caspase1^{-/-} (B) mice were injected with 1×10^7 CFU of $\Delta actA/inlB$ or $\Delta actA/inlB$ *L. monocytogenes*-L.p.FlaA-expressing OVA and B8R epitope. Seven days postimmunization, the percentage of antigen-specific IFN- γ ⁺ CD8⁺ T cells was determined using intracellular cytokine staining after in vitro restimulation with the indicated peptide. Values in each plot represent the mean \pm SD of antigen-specific cells within a CD8⁺ cell gate among splenocytes from four to five animals per groups. One representative experiment of two to four is shown.

thermore, the severe attenuation of *L. monocytogenes*-L.p.FlaA in vivo (Fig. 3) illustrates that the inflammasome can exert a strong selective pressure that pathogens, including *L. monocytogenes*, must avoid to promote their virulence.

Miao et al. recently demonstrated that inflammasome-mediated attenuation of *S. typhimurium* was independent of IL-1 β and IL-18 but dependent on the phagocyte oxidase component p47, suggesting that neutrophil oxidative burst was responsible for controlling infection in the context of inflammasome activation (32). In the present study, neutrophil depletion did not rescue the virulence of *L. monocytogenes*-L.p.FlaA. Both the *S. typhimurium* study and the present study find attenuation of intracellular bacteria caused by activation of the Nlr4 inflammasome; however, we find differences in the role that neutrophils play in this process. In agreement with our findings, Warren et al. recently reported that *L. monocytogenes* that hyperactivate the Nlr4 inflammasome were highly attenuated in vivo by a mechanism independent of IL-1 β and IL-18 (35). Understanding how inflammasome activation mediates host defense and how pathogens have overcome these defenses is central to our broader understanding of both mechanisms of pathogenesis and the function of the innate immune system.

The inflammasome may also play a role in the development of adaptive immune responses (11–13). *L. monocytogenes* has been used for almost 50 years as a model to study basic aspects of cell-mediated immunity. The hallmark of this model is the robust induction of antigen-specific CD8⁺ T cells, but the factors leading to the development of such potent cell-mediated immunity remain an important topic of investigation. In this study we examined the role of inflammasome activation in the development of protective immunity to *L. monocytogenes* infection. Warren et al. recently reported that *L. monocytogenes* strains engineered to activate the Nlr4 inflammasome were capable of inducing protective immunity at high immunizing doses (35). In agreement with these findings, we also found that immunization with high doses of *L. monocytogenes*-L.p.FlaA induced protection from subsequent challenge. However, when analyzing antigen-specific T-cell responses or analyzing protection at low immunizing doses, we found that there was an inverse correlation between inflammasome activation and induction of protective immunity: that is, strains engineered to activate the Nlr4-dependent inflammasome failed to induce wild-type levels of immunity.

The simplest explanation is that the failure of the attenuated strain to replicate in the tissues resulted in decreased antigen load. However, using a $\Delta actA/\Delta inlB$ background strain, the number of CFUs in the liver and spleen were identical between $\Delta actA/\Delta inlB$ *L. monocytogenes*-L.p.FlaA and the $\Delta actA/\Delta inlB$ strain (Fig. S6). Another possibility is that $\Delta actA/\Delta inlB$ *L. monocytogenes*-L.p.FlaA kills antigen-presenting cells. Indeed, the majority of splenic *L. monocytogenes* are found within dendritic cells (DCs) rapidly after infection (36, 37). However, we saw no significant difference in the total number of DCs or the number of dead DCs between $\Delta actA/\Delta inlB$ *L. monocytogenes*-L.p.FlaA and the $\Delta actA/\Delta inlB$ infections, with the exception being the slightly higher number of both total and dead DC in $\Delta actA/\Delta inlB$ *L. monocytogenes*-L.p.FlaA-infected mice 24 h postinfection (Fig. S10). Although there are not striking differences in the number of DC or the amount of DC death, because DCs are critical for the induction of acquired immunity to *L. monocytogenes* (38), even if the antigen load and number of DCs are the same, specific death of infected DCs could have a detrimental impact on antigen presentation and the development of protective immunity. A third possibility is that cytokines released by pyroptotic cells, IL-1 β and IL-18, alter the adaptive immune response. IL-1 β and IL-18 exert highly pleiotropic effects that can promote T_H1, T_H2, and T_H17 responses (39). Furthermore, acute induction of these cytokines likely alters levels of other cytokines, leading to changes

in the inflammatory milieu that may affect development of cell-mediated immunity. Thus, inflammasome activation results in a complex set of signals and dissecting which signal(s) affect the development of cell-mediated immunity, although complicated, is of central importance for understanding the development of long-term protective immunity.

We have shown that, for *L. monocytogenes*, avoiding activation of the inflammasome is critical not only to its pathogenesis, but also to its ability to stimulate adaptive immunity. How inflammasome activation attenuates virulence and how pathogens have evolved to circumvent this host response remain open questions. In addition, the surprising finding that inflammasome activation is detrimental to the development of cell-mediated immunity leaves many unanswered questions, especially as it pertains to the mechanism of adjuvant activity. This work highlights the importance of studying innate immune responses in the context of infection. Understanding the role of inflammasome activation during acute infections and in the development of immunity will be critical in the rational design of potent vaccines.

Materials and Methods

Bacterial Strains and Construction. All *L. monocytogenes* strains used in this study were in the 10403s background. *L. monocytogenes* were cultured in brain heart infusion (BHI) media, as indicated below for different infection models.

Mouse Strains and Macrophages. Six- to 8-wk-old C57BL/6 female were purchased from The Jackson Laboratory. Caspase-1^{-/-}, Nlr4^{-/-}, and Naip5/Nlr4^{-/-} mice were a kind gift from Russell Vance (University of California, Berkeley, CA) and ASC^{-/-} mice were a kind gift from Vishva Dixit (Genentech Inc., South San Francisco, CA).

Six- to 8-wk-old wild-type C57BL/6J, ASC^{-/-}, Caspase1^{-/-}, and Nlr4^{-/-} mice were used to make bone marrow-derived macrophages as previously described (40). All macrophages used in this study were cultured in the presence of 100 ng/mL Pam3CSK4 (Invivogen) for 12 to 16 h before infection. All protocols were reviewed and approved by the Animal Care and Use Committee at the University of California, Berkeley.

Cell Death and IL-1 β Release Assays. Bone marrow-derived macrophages were plated in 24-well plates at 5×10^5 macrophages per well in 500 μ L DMEM (Invitrogen) overnight before infection. *L. monocytogenes* cultures were grown to stationary phase overnight in BHI at 30 °C, nonshaking before infection, and macrophages were infected at an MOI of five bacteria per cell. Following 30 min of infection, media was aspirated from cells and replaced with 500 μ L containing 100 ng/mL fresh Pam3CSK4 and 50 μ g/mL gentamicin for an additional 5.5 h. Following 6 h total incubation, supernatants were collected and assayed for lactate dehydrogenase release, as previously described (18). Supernatants were also collected and assayed for IL-1 β using the ready-set-go mouse IL-1 β ELISA (eBioscience).

Intracellular Growth Curves. Pam3CSK4 treated bone marrow-derived macrophages were infected at an MOI of one bacterium per macrophage and CFU were enumerated as previously described (41).

In Vivo Infections. Six- to 8-wk-old female mice were infected intravenously with either 1 (1×10^5) or 0.1 LD₅₀s (1×10^4) of logarithmic phase *L. monocytogenes* (OD₆₀₀ 0.5) subcultured in BHI at 37 °C shaking for ~2 h. Forty-eight hours postinfection, livers and spleens from infected mice were harvested and homogenized in 0.1% Nonidet P-40 and plated on BHI plates to enumerate CFU.

T-Cell Analysis. For analysis of primary CD8⁺ T-cell responses, mice were infected with 0.1 LD₅₀ (1×10^7 CFU) of either $\Delta actA/inlB$ or $\Delta actA/inlB$ *L. monocytogenes*-L.p.FlaA (both expressing full length OVA and B8R₂₀₋₂₇ epitope) and spleens were harvested on day 7. Spleens were dissociated and red blood cells removed using red blood cell lysing buffer (Sigma). A total of 1.4×10^6 splenocytes were stimulated for 5 h with 2 μ M OVA₂₅₇₋₂₆₄ (SIINFELK), B8R₂₀₋₂₇ (TSYKFESV), or LLO₁₉₀₋₂₀₁ (NEKYAQAYPNVS) peptide in the presence of brefeldin A (GolgiPlug reagent; BD Biosciences). Stimulated cells were surface stained with anti-CD4 (clone GK1.5; eBioscience) and anti-CD8 α (clone 53-6.7; eBioscience), fixed and permeabilized using the Cytofix/

Cytoperm kit (BD Biosciences), then stained for intracellular IFN- γ with anti-IFN- γ (clone XMG1.2; eBioscience).

Samples were acquired using a LSRII flow cytometer (BD Biosciences) with DIVA 6 software (BD Biosciences). Data were analyzed using FlowJo software (Treestar).

Protection Assays. Six- to 8-wk-old female mice were immunized intravenously with either 0.1 (1×10^7) or 0.00001 (1×10^3) LD₅₀s of Δ actA/*inlB* or Δ actA/*inlB* *L. monocytogenes*-L.p.FlaA (both expressing OVA and B8R) grown as described above. Thirty or 90 d postimmunization, mice were challenged with $2 \times$ LD₅₀s (2×10^5) of wild type *L. monocytogenes* grown as described above. Sixty-eight to 72 h postchal-

lenge, livers and spleens were harvested and analyzed for CFU, as described above.

Statistics. Student's *t*-test or Mann-Whitney test statistical analysis was performed using Analyze-It software, as indicated in each figure legend. Asterisk indicates *P*-value less than 0.05.

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