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MEETING PRESENTATION

Potentiation of Cytokine-Mediated Restriction of *Legionella* Intracellular Replication by a Dot/Icm-Translocated Effector

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ABSTRACT *Legionella pneumophila* is ubiquitous in freshwater environments, where it replicates within unicellular protozoa. However, *L. pneumophila* is also an accidental human pathogen that can cause Legionnaires' disease in immunocompromised individuals by uncontrolled replication within alveolar macrophages. To replicate within eukaryotic phagocytes, *L. pneumophila* utilizes a Dot/Icm type IV secretion system to translocate a large arsenal of over 300 effector proteins directly into host cells. In mammals, translocated effectors contribute to innate immune restriction of *L. pneumophila*. We found previously that the effector LegC4 is important for *L. pneumophila* replication within a natural host protist but is deleterious to replication in a mouse model of Legionnaires' disease. In the present study, we used cultured mouse primary macrophages to investigate how LegC4 attenuates *L. pneumophila* replication. We found that LegC4 enhanced restriction of *L. pneumophila* replication within macrophages activated with tumor necrosis factor (TNF) or interferon gamma (IFN-γ). In addition, expression of *legC4* was sufficient to restrict *Legionella longbeachae* replication within TNF- or IFN-γ-activated macrophages. Thus, this study demonstrates that LegC4 contributes to *L. pneumophila* clearance from healthy hosts by potentiating cytokine-mediated host defense mechanisms.

IMPORTANCE *Legionella* spp. are natural pathogens of protozoa and accidental pathogens of humans. Innate immunity in healthy individuals effectively controls *Legionella* infection due in part to rapid and robust production of proinflammatory cytokines resulting from detection of Dot/Icm-translocated substrates, including effectors. Here, we demonstrate that the effector LegC4 enhances proinflammatory host restriction of *Legionella* by macrophages. These data suggest that LegC4 may augment proinflammatory signaling or antimicrobial activity of macrophages, a function that has not previously been observed for another bacterial effector. Further insight into LegC4 function will likely reveal novel mechanisms to enhance immunity against pathogens.

KEYWORDS LegC4, *Legionella*, effector functions, host-pathogen interactions, innate immunity, interferon gamma, tumor necrosis factor

Legionella spp. are natural pathogens of unicellular protozoa and accidental pathogens of humans that can cause a severe inflammatory pneumonia called Legionnaires' disease, which results from uncontrolled bacterial replication within alveolar macrophages. To replicate within eukaryotic phagocytes, *Legionella* spp. subvert normal endocytic signaling by establishing a specialized compartment called the *Legionella*-containing vacuole (LCV). To form the LCV and replicate intracellularly, *Legionella* spp. employ a Dot/Icm type IV secretion system (T4SS) to translocate virulence factors, called effector proteins, into host cells (1). Although >15 *Legionella* species are capable of causing human disease, the overwhelming majority of disease is caused by *L. pneumophila* (2, 3). In healthy individuals, *L. pneumophila* infection is

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efficiently controlled, and human-to-human transmission is incredibly rare (4). This is due to efficient detection and subsequent clearance of *L. pneumophila* by the mammalian innate immune system. Consequently, *L. pneumophila* is a well-established model pathogen used to characterize mechanisms of host defense against bacterial pathogens.

Innate immune detection of bacterial pathogens is facilitated by host pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). Surface Toll-like receptors (TLRs) are PRRs that are critical for host defense against *L. pneumophila*. The majority of TLRs signal through the adaptor MyD88 to activate proinflammatory gene expression. Mice lacking MyD88 are highly susceptible to *L. pneumophila* infection, which is mostly due to lack of interleukin-1 (IL-1)- and TLR2-mediated signaling (5–7). Intracellular PRRs such as Nod1, Nod2, and inflammasomes also contribute to innate immune restriction of *L. pneumophila* in macrophages (reviewed in references 8 and 9). Leakage of PAMPs, such as peptidoglycan, lipopolysaccharide, and flagellin (FlaA monomers), into the macrophage cytosol amplifies cell autonomous restriction of *L. pneumophila* within immune phagocytes. Specifically, recognition of *L. pneumophila* FlaA by the NAIP5/NLRC4 inflammasome is sufficient to restrict *L. pneumophila* replication within macrophages (10, 11). Engagement of both extracellular and intracellular PRRs results in a robust proinflammatory response mediated by secretion of cytokines by infected and bystander immune phagocytes (5, 12–16). In particular, tumor necrosis factor (TNF) and interferon gamma (IFN-γ) are critical for restriction of pulmonary *L. pneumophila* infection (17–21). TNF and IFN-γ both promote cell autonomous defense against *L. pneumophila* within macrophages and mediate bacterial killing by increasing phagolysosmal fusion (22, 23).

In addition to canonical PAMPs, translocated effectors can augment proinflammatory responses in *L. pneumophila*-infected macrophages. For example, effector-mediated inhibition of host protein translation results in increased expression of proinflammatory genes in macrophages (24–27). In addition, macrophage proinflammatory gene expression was decreased during infection with legionellae that possess a functional Dot/Icm T4SS but are unable to translocate a subset of effectors due to a mutation in the *icmS* effector chaperone gene (28). These studies elaborate the concept of effector-triggered immunity in animal cells (29) and provide further evidence for the contribution of effectors to innate immune restriction of *L. pneumophila*.

We recently demonstrated that the effector LegC4 attenuates *L. pneumophila* fitness in a mouse model of Legionnaires' disease (30). Loss-of-function mutation of the *legC4* gene conferred a fitness advantage on *L. pneumophila* in the mouse lung as evidenced by increased pulmonary bacterial burden and the ability to outcompete the wild-type strain (30). However, the *legC4* mutation had no effect on *L. pneumophila* replication in primary bone-marrow derived macrophages (BMDMs) and impaired replication within a natural amoeba host, *Acanthamoeba castellanii* (30). Moreover, expression of *legC4* from a plasmid further attenuated *L. pneumophila* fitness in the mouse lung compared to that of the wild-type strain. Thus, we hypothesized that LegC4 is deleterious to *L. pneumophila* in the presence of cell-mediated innate immunity.

The present study was designed to determine how LegC4 augments restriction of *L. pneumophila* in mammalian hosts. Expression of *legC4* from a plasmid was sufficient to attenuate *L. pneumophila* replication in BMDMs, which relied on TNF secretion and subsequent signaling. Moreover, a Δ*legC4* mutant exhibited increased replication in cytokine-activated BMDMs. Interestingly, expression of *legC4* was sufficient to attenuate *Legionella longbeachae* replication within TNF- and IFN-γ-activated BMDMs. These results suggest that LegC4 enhances macrophage cell autonomous defense against *Legionella* by potentiating cytokine-mediated restriction.

RESULTS

LegC4 confers a fitness disadvantage on nonflagellated *L. pneumophila* in wild-type mice. We found previously that the *L. pneumophila* effector LegC4 is

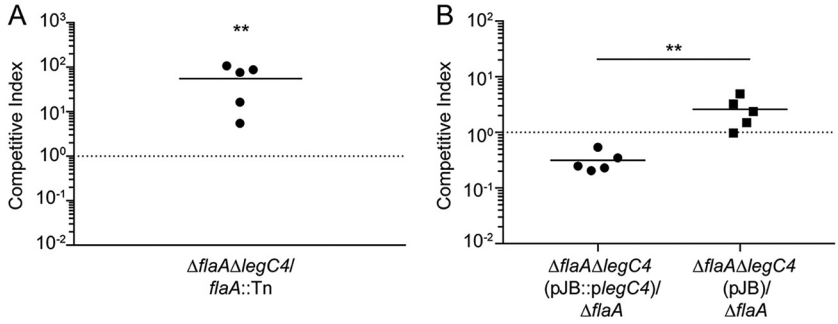

FIG 1 LegC4 attenuates *L. pneumophila* fitness in wild-type mice. (A) Competitive index (CI) of *L. pneumophila* Δ*flaA* Δ*legC4* versus *flaA*::Tn (chloramphenicol resistant [Cm^r]) from the lungs of wild-type mice. (B) CI of Δ*flaA* Δ*legC4*(pJB::p*legC4*) or Δ*flaA* Δ*legC4*(pJB) versus Δ*flaA L. pneumophila* from the lungs of wild-type mice. Each symbol represents an individual animal, and the line represents the mean CI values. Asterisks denote statistical significance by the Mann-Whitney U test (**, $P < 0.01$). Data are representative of at least two independent experiments.

detrimental to bacterial replication in the mouse lung (30). Those experiments were performed using mice and macrophages deficient for production of the NLRC4 inflammasome (*Nlrc4*$^{-/-}$) to prevent flagellin-mediated restriction of *L. pneumophila* replication (10, 30, 31). To confirm that LegC4-mediated phenotypes were not due to loss of NLRC4, we examined the fitness of a *legC4*-deficient (Δ*legC4*) *L. pneumophila* strain in the lungs of wild-type C57BL/6 mice using competitive index (CI) experiments. To prevent NLRC4-mediated restriction of bacterial replication, we generated flagellin (*flaA*) loss-of-function mutations in our wild-type (Δ*flaA*) and *legC4* mutant (Δ*flaA* Δ*legC4*) strains (see Materials and Methods). We also used a previously generated *flaA*::Tn mutant to facilitate selective plating, since the transposon confers resistance to chloramphenicol (30). Mice were infected intranasally with a 1:1 mixture of *L. pneumophila* Δ*flaA* Δ*legC4* and *flaA*::Tn for 48 h. Lung tissue was subsequently homogenized and plated on selective media for CFU enumeration and calculation of CI values (see Materials and Methods). The Δ*flaA* Δ*legC4* strain significantly outcompeted the *flaA*::Tn mutant in the lungs of wild-type mice, as evidenced by average CI values significantly greater than 1.0 ($P < 0.01$) (Fig. 1A). Our previous study also revealed that expression of *legC4* from a multicopy plasmid conferred a fitness disadvantage on *L. pneumophila* compared to the wild-type strain (30). To confirm these results in wild-type mice, we generated a strain of *L. pneumophila* Δ*flaA* Δ*legC4* harboring a plasmid carrying *legC4* under the control of its endogenous promoter (pJB::p*legC4*). Plasmid expression of *legC4*(pJB::p*legC4*) resulted in significantly impaired fitness compared with the Δ*flaA* parental strain, which was not observed for a Δ*flaA* Δ*legC4* strain harboring vector alone (pJB) ($P < 0.05$) (Fig. 1B). These data demonstrate that NLRC4 does not affect LegC4-mediated attenuation of *L. pneumophila* replication in the mouse lung. To fully evaluate LegC4-mediated phenotypes, the remainder of our study was performed using *flaA*-deficient *L. pneumophila* strains and bone marrow-derived macrophages (BMDMs) derived from wild-type mice.

Plasmid expression of *legC4* attenuates *L. pneumophila* replication in BMDMs. Since plasmid expression of *legC4* attenuated *L. pneumophila* fitness in the mouse lung, we examined whether this also occurred in macrophages *ex vivo* using BMDMs derived from wild-type mice. We quantified bacterial replication within BMDMs over 72 h. Consistent with our previous study, loss of endogenous *legC4* (Δ*flaA* Δ*legC4*) did not affect replication of *L. pneumophila* within primary mouse BMDMs compared to the parental strain (Δ*flaA*) (30) (Fig. 2A). However, *L. pneumophila* Δ*flaA* Δ*legC4*(pJB::p*legC4*) was significantly attenuated for replication in BMDMs at 48 and 72 h postinfection (p.i.) compared to the empty vector control strain (Fig. 2B). Furthermore, IPTG (isopropyl-β-D-1-thiogalactopyranoside)-induced expression of 3×FLAG epitope-tagged *legC4* (*3×FLAG-legC4*) from a plasmid (p*legC4*) also resulted in impaired *L. pneumophila* replication compared to that of the control strain (carrying pEV) ($P < 0.01$) (Fig. 2C).

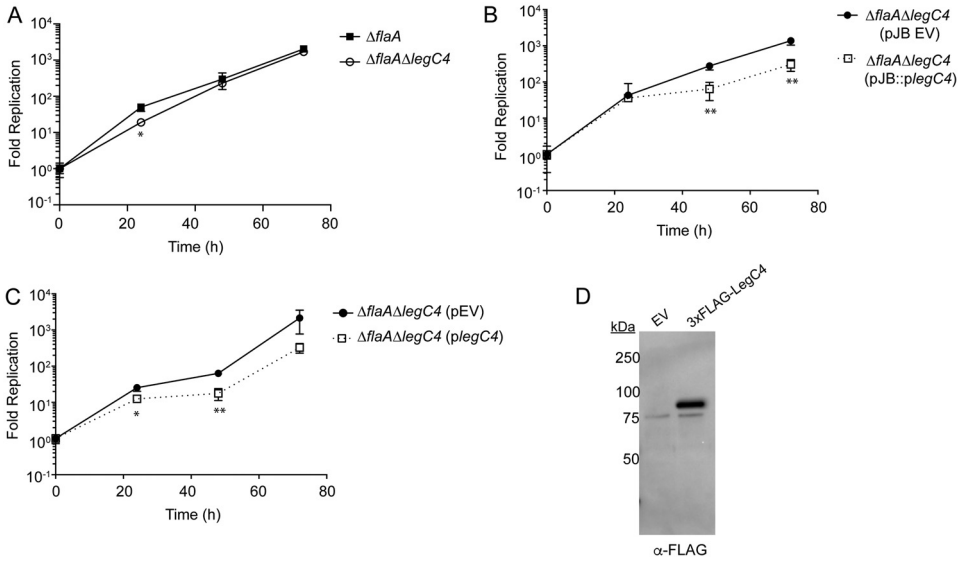

FIG 2 Plasmid expression of *legC4* impairs *L. pneumophila* replication within BMDMs. (A to C) Fold replication of *L. pneumophila* Δ*flaA* and Δ*flaA* Δ*legC4* (A), Δ*flaA* Δ*legC4*(pJB) and Δ*flaA* Δ*legC4*(pJB::p*legC4*) (B), or Δ*flaA* Δ*legC4*(pEV) and Δ*flaA* Δ*legC4*(p*legC4*) (C) within wild-type BMDMs over 72 h. (D) *L. pneumophila* Δ*flaA* Δ*legC4*(pEV) and Δ*flaA* Δ*legC4*(p*legC4*) were lysed, and 3×FLAG-LegC4 was visualized by Western blotting. Expression of *legC4* from p*legC4* was induced with 1 mM IPTG as described in Materials and Methods. Data are shown as mean ± SD for samples in triplicate. Asterisks denote statistical significance by Student's *t* test (**, $P < 0.01$; *, $P < 0.05$), and data are representative of at least two independent experiments.

Expression of *3×FLAG-legC4* was confirmed by Western blot analysis using an anti-FLAG antibody (Fig. 2D). Fitness defects associated with plasmid expression of *legC4* were specific to intracellular replication, since replication in rich medium *in vitro* was unaffected (see Fig. S1 in the supplemental material). To determine if the intracellular replication defect observed for IPTG-induced expression of *legC4* was specific, we cloned the effector gene *lpg2505* into the same plasmid used to overexpress *legC4*, which resulted in an IPTG-inducible 3×FLAG gene fusion (*3×FLAG-lpg2505*) (see Materials and Methods). The growth attenuation observed for plasmid-encoded *legC4* was not just due to overexpression of a Dot/Icm effector gene, since IPTG-induced expression of *3×FLAG-lpg2505* did not impair intracellular replication compared to that of the *L. pneumophila* Δ*flaA* strain (see Fig. S2A in the supplemental material). Expression of *3×FLAG-legC4* and *-lpg2505* was visualized by Western blotting using an anti-FLAG antibody (Fig. S2B). Together, these data demonstrate that overexpression of *legC4* is detrimental to *L. pneumophila* intracellular replication in macrophages.

LegC4-mediated restriction of *L. pneumophila* replication is dependent on cytokine production. We subsequently investigated the mechanism by which plasmid expression of *legC4* attenuates *L. pneumophila* replication. In BMDMs, *L. pneumophila* infection results in production of proinflammatory cytokines through engagement of TLRs by bacterial ligands. Indeed, we previously reported that BMDMs infected with *L. pneumophila* expressing *legC4* secreted increased levels of interleukin-12 (IL-12) (30). However, increased levels of IL-12 would likely not be sufficient to attenuate *L. pneumophila* intracellular replication within BMDMs. Like IL-12, tumor necrosis factor (TNF) is a proinflammatory cytokine expressed downstream of Toll-like receptors (TLRs) in macrophages. TNF is important for host defense against *L. pneumophila* in mice and humans, and TNF-mediated signaling is sufficient to restrict *L. pneumophila* intracellular replication within macrophages (17–19, 23, 32). Thus, increased TNF signaling could account for LegC4-mediated attenuation of *L. pneumophila* intracellular replication.

We hypothesized that plasmid expression of *legC4* would be sufficient to increase TNF secretion from *L. pneumophila*-infected BMDMs. Wild-type BMDMs were infected with *L. pneumophila* Δ*flaA* Δ*legC4*(pEV) or Δ*flaA* Δ*legC4*(p*legC4*) for 2 to 8 h, and

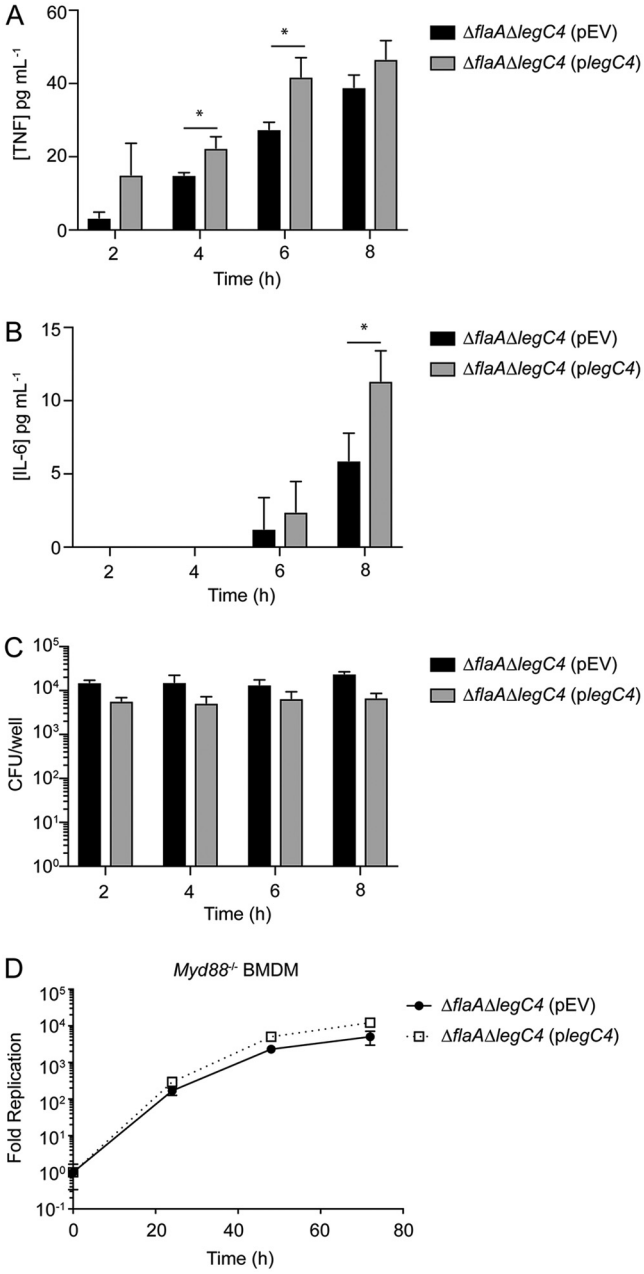

FIG 3 Role of TNF secretion in LegC4-mediated attenuation of *L. pneumophila* replication. (A and B) ELISA quantification of TNF (A) or IL-6 (B) secretion from wild-type BMDMs infected with *L. pneumophila* Δ*flaA* Δ*legC4*(pEV) and Δ*flaA* Δ*legC4*(p*legC4*) at the indicated time points. (C) Enumeration of *L. pneumophila* strains from BMDMs assayed for panels A and B. (D) Fold replication over 72 h of the indicated *L. pneumophila* strains within wild-type or *Myd88*$^{-/-}$ BMDMs. Expression of *legC4* was induced with 1 mM IPTG. Data shown are mean ± SD for samples in triplicate. Asterisks denote statistical significance (*, $P < 0.05$; **, $P < 0.01$) by Student's *t* test. Data are representative of at least two independent experiments.

secreted TNF was quantified by enzyme-linked immunosorbent assay (ELISA) (see Materials and Methods). Significantly greater concentrations of TNF were present in the supernatants of cells infected with *L. pneumophila* expressing *legC4* from a plasmid ($P < 0.05$) (Fig. 3A). We also observed a significant increase in IL-6 production from macrophages infected with *L. pneumophila*(p*legC4*) compared to those infected with the isogenic empty vector control strain at 8 h p.i. ($P < 0.05$) (Fig. 3B). Increased cytokine production was not correlated with increased bacterial replication, since

bacterial counts did not change during the course of infection (Fig. 3C). We also observed increased TNF secretion at 6 h p.i. in BMDMs infected with *L. pneumophila* strains constructed in the Lp02 background, which is metabolically active but does not replicate in the absence of exogenous thymidine (see Fig. S3 in the supplemental material). Consistent with our previous observations (30), these data demonstrate that LegC4 enhances cytokine secretion from *L. pneumophila*-infected macrophages.

To determine if TNF secretion contributed to LegC4-mediated attenuation of intracellular replication, intracellular replication of *L. pneumophila* within BMDMs deficient for production of MyD88 was quantified. We infected *Myd88*$^{-/-}$ BMDMs with *L. pneumophila* Δ*flaA* Δ*legC4*(pEV) or Δ*flaA* Δ*legC4*(p*legC4*) and quantified fold replication over 72 h. We found that loss of MyD88-mediated signaling abrogated LegC4-mediated attenuation of *L. pneumophila* intracellular replication in BMDMs (Fig. 2 and 3D). As expected, TNF was not secreted from *Myd88*$^{-/-}$ BMDMs under any of our experimental conditions (reference 33 and data not shown). Together, these data suggest that proinflammatory cytokine production is required for LegC4-mediated attenuation of *L. pneumophila* intracellular replication in BMDMs.

To further characterize LegC4-mediated restriction of *L. pneumophila* replication within BMDMs, *L. pneumophila* replication was evaluated in the absence of TNF signaling. To determine if TNF signaling contributed to *legC4*-mediated attenuation of *L. pneumophila* replication, we neutralized TNF in the supernatants of infected wild-type BMDMs using an anti-TNF antibody. Wild-type BMDMs were infected with *L. pneumophila* Δ*flaA* Δ*legC4* harboring p*legC4* or pEV in the presence of either anti-TNF, a rat IgG isotype control antibody, or neither, and fold replication at 48 h p.i. was quantified. Plasmid expression of *legC4* resulted in significantly attenuated *L. pneumophila* replication within untreated and rat IgG-treated BMDMs ($P < 0.05$); however, anti-TNF antibody neutralization of TNF restored replication of the *legC4*-overexpressing strain to wild-type levels (Fig. 4A). We subsequently examined replication of these strains in BMDMs deficient for signaling from TNF receptor 1 (TNFR1). *Tnfr1*$^{-/-}$ BMDMs were infected with *L. pneumophila* Δ*flaA* Δ*legC4* harboring either p*legC4* or pEV, and fold replication was quantified over 72 h of infection. Overexpression of *legC4* did not impair *L. pneumophila* intracellular replication within *Tnfr1*$^{-/-}$ BMDMs compared to that of the empty vector control strain (Fig. 4B). Interestingly, overexpression of *legC4* resulted in significantly increased *L. pneumophila* replication within *Tnfr1*$^{-/-}$ BMDMs ($P < 0.01$) (Fig. 4B). These data demonstrate that TNF signaling contributes to LegC4-mediated attenuation of *L. pneumophila* replication within BMDMs.

Endogenous LegC4 exacerbates TNF-mediated restriction of *L. pneumophila* from BMDMs. To further characterize LegC4-mediated restriction of *L. pneumophila* from BMDMs, we examined replication of *L. pneumophila* in BMDMs activated with recombinant mouse TNF (rTNF). Wild-type BMDMs were infected with *L. pneumophila* Δ*flaA* or Δ*flaA* Δ*legC4* in the presence or absence of rTNF, and fold replication was quantified at 48 h p.i. *L. pneumophila* Δ*flaA* Δ*legC4* replicated to significantly higher levels than the parental Δ*flaA* strain in rTNF-treated BMDMs ($P < 0.01$) (Fig. 4C). As reported above, loss of endogenous *legC4* does not affect *L. pneumophila* replication within untreated BMDMs (Fig. 4C). These data show that endogenous levels of LegC4 can augment TNF-mediated restriction of *L. pneumophila* replication.

Loss of TNFR1-mediated signaling is not sufficient for LegC4-mediated restriction of *L. pneumophila* replication *in vivo*. We subsequently investigated whether loss of TNFR1-mediated signaling would be sufficient to restore the fitness of *legC4*-expressing *L. pneumophila* strains *in vivo*. To test this hypothesis, we performed a CI experiment to quantify the fitness of *legC4*-deficient *L. pneumophila* strains in the lungs of mice deficient for production of *Tnfr1*. *Tnfr1*$^{-/-}$ mice were infected with a 1:1 mixture of *L. pneumophila* Δ*flaA* and Δ*flaA* Δ*legC4*(pJB) or *L. pneumophila* Δ*flaA* and Δ*flaA* Δ*legC4*(pJB::p*legC4*) for 48 h. Lung tissue was homogenized and plated on selective media for enumeration of CFU and calculation of CI values (see Materials and Methods). *L. pneumophila* Δ*flaA* Δ*legC4*(pJB) had a significant fitness advantage over

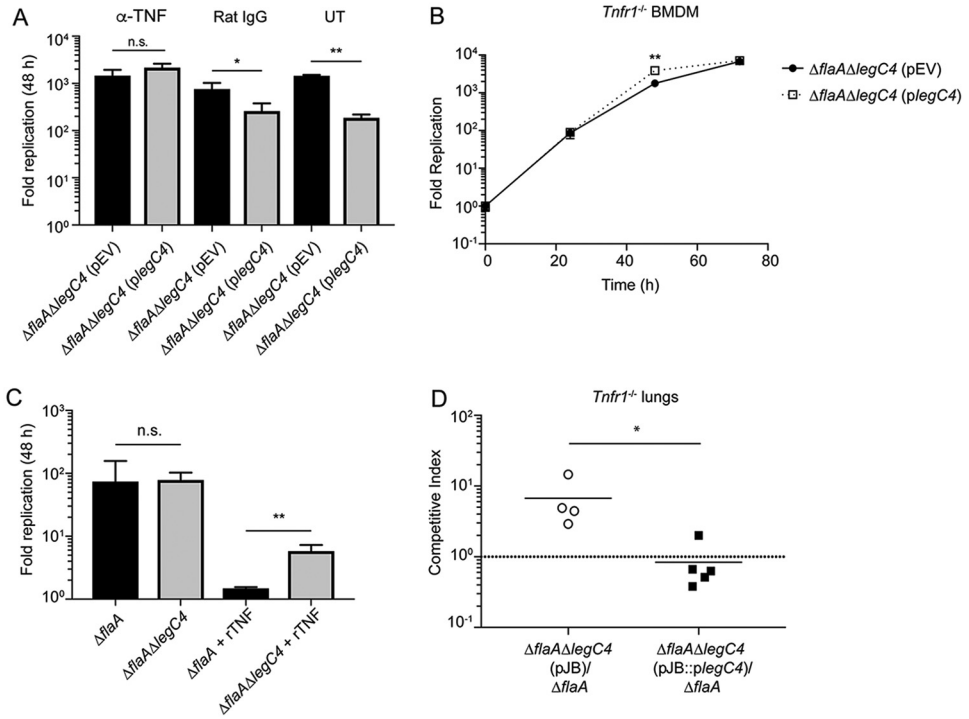

FIG 4 LegC4 augments TNF-mediated restriction of *L. pneumophila* replication. (A) Fold replication (48 h) of the indicated *L. pneumophila* strains within wild-type BMDMs treated with 50 ng ml^{-1} anti-TNF or an isotype control (rat IgG) or left untreated (UT) (see Materials and Methods). (B) Fold replication of the indicated *L. pneumophila* strains within *Tnfr1*$^{-/-}$ BMDMs over 72 h. Expression of *legC4* was induced with 1 mM IPTG. (C) Fold replication (48 h) of *L. pneumophila* Δ*flaA* and Δ*flaA* Δ*legC4* within wild-type BMDMs the presence or absence of 50 ng ml^{-1} recombinant mouse TNF (rTNF). Data shown are mean ± SD for samples in triplicate. Asterisks denote statistical significance (*, $P < 0.05$; **, $P < 0.01$; n.s., not significant) by Student's *t* test. Data are representative of at least two independent experiments. (D) CI of Δ*flaA* Δ*legC4*(pJB::p*legC4*) or Δ*flaA* Δ*legC4*(pJB) versus Δ*flaA L. pneumophila* from the lungs of wild-type mice. Each symbol represents an individual animal, and the lines represent the mean CI values. Asterisks denote statistical significance by the Mann-Whitney U test (*, $P < 0.05$). Data are representative of three independent experiments.

the parental Δ*flaA* strain compared to *L. pneumophila* Δ*flaA* Δ*legC4*(pJB::p*legC4*) ($P < 0.05$) (Fig. 4D). Thus, LegC4-mediated augmentation of TNFR1 signaling is not sufficient for attenuation of *L. pneumophila in vivo*.

LegC4 impairs *L. pneumophila* replication in IFN-γ-activated BMDMs. Since TNF signaling was not sufficient for LegC4-mediated restriction of *L. pneumophila* in the lungs of mice, we evaluated the contribution of LegC4 to interferon gamma (IFN-γ)-mediated restriction in BMDMs. IFN-γ plays a major role in host defense against *L. pneumophila* in the lung (20, 34). The fold replication of *L. pneumophila* Δ*flaA* Δ*legC4*(pEV) or Δ*flaA* Δ*legC4*(p*legC4*) within wild-type BMDMs activated with recombinant mouse IFN-γ (rIFN-γ) was quantified over 72 h. We found that overexpression of *legC4* significantly attenuated *L. pneumophila* replication within IFN-γ-activated BMDMs at all time points examined (Fig. 5A). We also determined that the *L. pneumophila* Δ*flaA* Δ*legC4* strain replicated to significantly higher levels than the Δ*flaA* parental strain in IFN-γ-activated, but not untreated, BMDMs ($P < 0.05$) (Fig. 5B). Together, these data demonstrate that LegC4 also augments IFN-γ-mediated restriction of *L. pneumophila* in macrophages.

IFN-γ-mediated signaling results in increased macrophage TNF production (35, 36). Thus, to determine if LegC4-mediated restriction of *L. pneumophila* within IFN-γ-activated macrophages was due to TNF signaling, we quantified *L. pneumophila* replication within *Tnfr1*$^{-/-}$ BMDMs treated with rIFN-γ. Overproduction of LegC4 resulted in significantly decreased *L. pneumophila* replication in IFN-γ-activated *Tnfr1*$^{-/-}$ BMDMs at 48 and 72 h p.i. compared to that of the control strain (Fig. 5C).

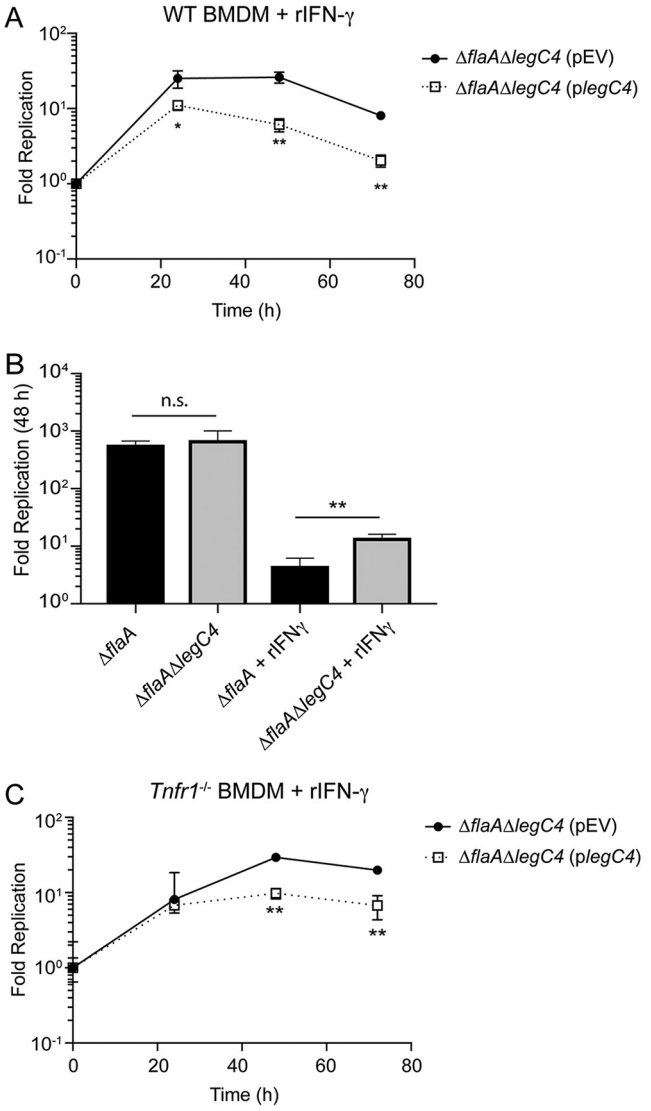

FIG 5 LegC4 enhances IFN-γ-mediated restriction of *L. pneumophila* replication. (A) Fold replication of the indicated *L. pneumophila* strains within wild-type (WT) BMDMs in the presence of 50 ng ml^{-1} rIFN-γ. (B) Fold replication (48 h) of the indicated strains within wild-type BMDMs in the presence or absence of 5 ng ml^{-1} rIFN-γ. (C) Fold replication of the indicated *L. pneumophila* strains within *Tnfr1*$^{-/-}$ BMDMs in the presence of 50 ng ml^{-1} rIFN-γ. Expression of *legC4* was induced with 1 mM IPTG. Data shown are mean ± SD for samples in triplicate. Asterisks denote statistical significance (**, $P < 0.01$; n.s., not significant) by Student's *t* test. Data are representative of at least two independent experiments.

Thus, LegC4 potentiates IFN-γ-mediated restriction of *L. pneumophila* intracellular replication in the absence of TNFR1-mediated signaling. Together, these data demonstrate that both IFN-γ- and TNF-mediated restriction of *L. pneumophila* are augmented by LegC4.

LegC4 impairs *L. longbeachae* replication within cytokine-activated BMDMs. We next aimed to determine if LegC4-mediated restriction in macrophages was specific to *L. pneumophila*. *Legionella longbeachae* is the second-leading cause of Legionnaires' disease and is dependent on Dot/Icm-mediated effector translocation for intracellular replication (37). The Dot/Icm secretion system is highly conserved between *L. longbeachae* and *L. pneumophila*; however, the effector repertoires are quite distinct, and *L. longbeachae* does not carry a homolog of *legC4* (38, 39). Importantly, *L. longbeachae* is more virulent than *L. pneumophila* and is lethal in a mouse model of infection (40). To determine if LegC4 attenuates bacterial replication in a non-*L. pneumophila Legionella*

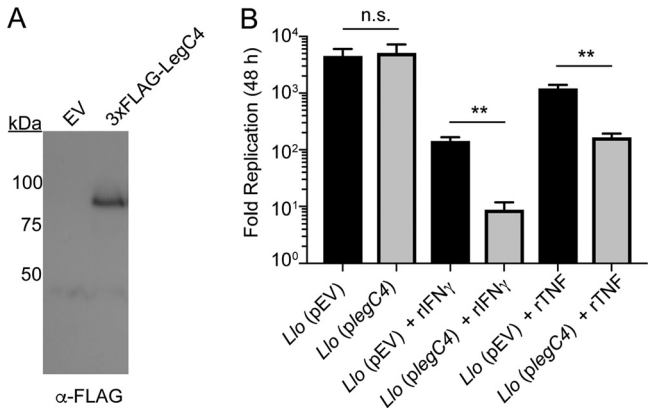

FIG 6 Replication of *L. longbeachae* producing LegC4 in cytokine-treated BMDMs. (A) Detection of 3×FLAG-LegC4 production by *L. longbeachae* (*Llo*) by Western blot analysis. (B) Fold replication (48 h) of *L. longbeachae* harboring the indicated plasmids within wild-type BMDMs the presence or absence of 50 ng ml^{-1} rTNF or rIFN-γ, as indicated. Expression of *legC4* was induced with 1 mM IPTG. Data shown are mean ± SD for samples in triplicate. Asterisks denote statistical significance (**, $P < 0.01$; n.s., not significant) by Student's *t* test. Data are representative of two independent experiments.

species, we generated *L. longbeachae* strains either expressing IPTG-inducible 3×FLAG-tagged *legC4* (p*legC4*) or harboring the empty vector (pEV). Expression of *3×FLAG-legC4* from *L. longbeachae* was confirmed by Western blot analysis (Fig. 6A). Wild-type BMDMs were infected with the indicated *L. longbeachae* strains in the presence or absence of rTNF or rIFN-γ as indicated, and fold replication at 48 h was quantified. Intracellular replication of the *legC4*-expressing strain of *L. longbeachae* was significantly attenuated within TNF- and IFN-γ-treated, but not untreated, BMDMs compared to that of the empty vector (pEV)-carrying control strain ($P < 0.001$) (Fig. 6B). These data demonstrate that LegC4 augments cytokine-mediated restriction of a non-*L. pneumophila Legionella* species within BMDMs.

DISCUSSION

The data presented in this study support the hypothesis that LegC4 potentiates cytokine-mediated host defense against *Legionella*. Our previous work (30) identifying LegC4 as contributing to *L. pneumophila* clearance from the lung was performed using flagellated *L. pneumophila* in an NLRC4-deficient (*Nlrc4*$^{-/-}$) mouse model. To fully evaluate the mechanisms of LegC4-mediated clearance, we utilized flagellin-deficient (Δ*flaA*) *L. pneumophila* and wild-type mice and BMDMs. Consistent with our previous study (30), we found that a loss-of-function mutation in the *legC4* gene (Δ*legC4*) conferred a fitness advantage on *L. pneumophila* Δ*flaA* within the wild-type mouse lung. Moreover, complementation of the Δ*legC4* mutation by a plasmid encoding *legC4* in *trans* conferred a fitness disadvantage on *L. pneumophila* compared to the parental strain. Also consistent with our previous report, *L. pneumophila* Δ*flaA* Δ*legC4* replication within BMDMs did not differ from replication of the Δ*flaA* strain. However, in the present study, we found that plasmid expression of *legC4* was sufficient to attenuate *L. pneumophila* replication within BMDMs. Although *legC4* was expressed downstream of its endogenous promoter, an exaggerated phenotype likely occurred due to expression from a multicopy plasmid, suggesting a potential dose response. Importantly, this strain provided us with a tool to increase the magnitude of LegC4-mediated fitness attenuation within cultured cells. These phenotypes were corroborated by the observation that endogenous LegC4 was deleterious in cytokine-activated BMDMs. We further found that the fitness disadvantage associated with plasmid expression of *legC4* was abolished in macrophages deficient for TNF-mediated signaling, suggesting that LegC4 is able to exacerbate cytokine-mediated antimicrobial responses. Finally, we determined that LegC4 could impair replication of *L. longbeachae* in cytokine-activated

macrophages. Together, these data suggest that LegC4 potentiates cytokine-mediated restriction of *L. pneumophila* within macrophages.

Inflammation is mediated primarily through cytokine secretion, which is critical for restriction of *L. pneumophila* replication *in vivo*. This has been evidenced by the inability of *Myd88*$^{-/-}$ mice to control *L. pneumophila* replication. Specifically, *Myd88*$^{-/-}$ BMDMs will not secrete TNF during infection. The inability of plasmid-expressed *legC4* to attenuate *L. pneumophila* replication in *Myd88*$^{-/-}$ BMDMs is likely due to lack of TNF signaling. In addition, LegC4-mediated increases in TNF secretion may amplify *Tnf* expression, which would further restrict *L. pneumophila* replication.

Proinflammatory cytokines contribute to host defense against *L. pneumophila in vivo* and in cultured macrophages (8, 41). Mice deficient for TNF-mediated signaling have increased pulmonary bacterial burdens and can succumb to infection (23, 32). TNF can signal through both TNFR1 and TNFR2; however, TNFR1-mediated signaling is primarily responsible for *L. pneumophila* restriction within alveolar macrophages *in vivo* (23) and is potentiated by LegC4. In the lung, multiple cell types contribute to TNF production, a consequence of which would be higher local TNF concentrations (16, 18, 23, 42). In addition, production of IFN-γ during *L. pneumophila* infection *in vivo* is mediated primarily by circulating natural killer (NK) cells (34, 43).

Our observation that the *L. pneumophila* Δ*legC4* mutant had a fitness advantage compared to the wild type in the mouse lung but not in cultured macrophages suggested that LegC4 was detrimental to replication under specific environmental conditions. This was supported by the observation that attenuated *L. pneumophila* replication was correlated with increased TNF secretion from BMDMs. Since the *L. pneumophila*-infected lung is an inflammatory environment, we examined whether cytokine-mediated restriction was exacerbated by LegC4. Abrogation of signaling from TNFR1 was sufficient to alleviate LegC4-mediated restriction of intracellular replication. Increased replication of the Δ*legC4* mutant within rTNF-treated BMDMs strongly suggests that proinflammatory responses are exacerbated by LegC4. This conclusion was corroborated by the observation that the Δ*legC4* mutant consistently replicated to higher levels within IFN-γ-activated macrophages. The observation that a *legC4*-deficient *L. pneumophila* strain still outcompeted the parental strain in *Tnfr1*$^{-/-}$ mice further suggests that LegC4-mediated growth attenuation occurs through multiple pathways. We are currently investigating whether additional factors are important for LegC4-mediated attenuation of *L. pneumophila* intracellular replication.

Similar to the case for *L. pneumophila*, *L. longbeachae* replicates within an LCV by employing a Dot/Icm secretion system and a repertoire of translocated effector proteins (37). Despite high levels of homology between the Dot/Icm secretion systems of these two organisms, the effector repertoires are quite diverse, and *L. longbeachae* does not carry a homolog of *legC4* (38, 39). In contrast to *L. pneumophila*, *L. longbeachae* is highly virulent in a mouse model of Legionnaires' disease (40, 44). Lethality in mice is likely due to *L. longbeachae* being poorly immunostimulatory and failing to induce substantial levels of proinflammatory cytokines during infection. However, proinflammatory cytokines contribute to the host defense against *L. longbeachae* in BMDMs and *in vivo* (40). Since interspecies translocation of Dot/Icm effectors by *Legionella* has been previously observed (37, 45), we introduced *legC4* into *L. longbeachae*. Production of LegC4 by *L. longbeachae* resulted in significantly attenuated replication within cytokine-treated, but not untreated, BMDMs. These data reinforce our previous observations and demonstrate that LegC4-mediated restriction is not specific to *L. pneumophila*. Since *L. longbeachae* infection does not induce appreciable TNF secretion from BMDMs (40), it is likely that the concentration of TNF secreted by these cells is too low to permit LegC4-mediated restriction. Together with relatively low levels of effector translocation by *L. longbeachae* compared to *L. pneumophila* (37), the amount of translocated LegC4 may be insufficient to restrict bacterial replication within untreated BMDMs. However, LegC4 is sufficient to attenuate *L. longbeachae* replication within BMDMs activated with either rTNF or rIFN-γ. Whether LegC4 can protect mice from *L. longbeachae*-mediated lethality will be the subject of a future study.

TABLE 1 Oligonucleotide primers used in this study

Name	Sequence[a]
legC4KO-up	TTGTGGACAATAGCTCTTGG
legC4KO-down	ATACGCTGGCTATAGCACC
flaAKO-up	CCAGTTCAGTACTGTAAAGC
flaAKO-down	TATTTCTGCCGTGACTATCG
Lpg2505BglII-F	TGG**AGATCT**TTATGATAAAAGGAAAACTTATGC
Lpg2505PstI-R	GG**CTGCAG**TTATAAAATAATTGGTCGAG
LegC4BglII-F	TGG**AGATCT**AATGAGTTAAGAAAGGATCCG
LegC4BamHI-F	TGG**GGATCC**TTTTGATTCATTATGTATCCTTG
LegC4XbaI-R	ATT**TCTAGA**TTATAGCTTAATATCAAAAG

[a]Restriction endonuclease cleavage sites are in bold.

Multiple effectors contribute to the innate immune response to *L. pneumophila* infection (reviewed in reference 46). Together with our data, these studies point to a complex interplay between effectors during *Legionella* infection of mammalian hosts. The effectors LnaB and LegK1 enhance NF-κB activation, which augments immune signaling (47, 48). Since mammals are a dead-end host for *Legionella*, the evolutionary basis for effector modulation of NF-κB is intriguing. Interestingly, the effector EnhC enhances *L. pneumophila* replication in TNF-activated macrophages (49), the opposite of what we have observed for LegC4. Thus, it is tempting to speculate that there may be interplay between EnhC and LegC4 within *L. pneumophila*-infected cells. Future investigations will reveal whether LegC4-mediated phenotypes are dependent on other Dot/Icm-translocated effectors.

In summary, we found that the Dot/Icm effector LegC4 can augment cytokine-mediated restriction of *Legionella* replication within macrophages. These data add to the growing body of literature on effector-triggered immunity in animal cells. As an accidental pathogen that did not coevolve under the selective pressure of an innate immune system, *L. pneumophila* continues to provide insight into novel mechanisms of innate immunity toward intracellular bacterial pathogens. Consequently, further understanding of LegC4 function will reveal strategies to augment proinflammatory signaling. Thus, this study has provided the foundation for future investigations into the molecular mechanism by which LegC4 enhances host defense against intracellular bacterial pathogens.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, and growth conditions. *Legionella pneumophila* Philadelphia-1 SRS43 (30), SRS43 *flaA*::Tn (30), Lp02 Δ*flaA* (10), and Lp03 (50) and *Escherichia coli* strains were gifts from Craig Roy (Yale University). *L. longbeachae* NSW 150 was a gift from Hayley Newton (University of Melbourne). *Escherichia coli* strains used for cloning (Top10; Invitrogen) and *L. pneumophila* mutagenesis (DH5α-λ*pir* [51]) were maintained in Luria-Bertani (LB) medium supplemented with 25 μg ml^{-1} chloramphenicol (pJB1806 and pSN85) or 50 μg ml^{-1} kanamycin (pSR47s). *Legionella* strains were cultured on supplemented charcoal–*N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)-buffered yeast extract (CYE) and grown at 37°C as described previously (52). *L. pneumophila* Lp02 strains were maintained on CYE supplemented with 100 μg ml^{-1} thymidine. Liquid cultures were grown at 37°C with aeration in supplemented ACES-buffered yeast extract (AYE) as described previously (52, 53). When necessary, media were supplemented with 10 μg ml^{-1} chloramphenicol (for plasmid maintenance), 10 μg ml^{-1} kanamycin (for allelic exchange), or 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG).

Unless otherwise indicated, recombinant mouse interferon-γ (rIFN-γ) (Thermo Fisher Scientific), recombinant mouse tumor necrosis factor (rTNF) (Gibco), rat anti-mouse TNF antibody (R&D Systems), or normal rat IgG control (R&D Systems) were used at a concentration of 50 ng ml^{-1}. In all cases, antibodies and recombinant cytokines were added at the time of infection.

A complete list of oligonucleotide primers used in this study is provided in Table 1.

Molecular cloning, plasmid construction, and generation of *Legionella* strains. In-frame deletions of *legC4* were generated by allelic exchange. Plasmids pSR47s::Δ*legC4* and pSR47s::Δ*flaA,* gifts from Craig Roy, were conjugated into SRS43 or Lp02, followed by selection for *L. pneumophila* deletions as described previously (54, 55). Sucrose-resistant, kanamycin-sensitive colonies were screened by PCR using the legC4KO-up/legC4KO-down and flaAKO-up/flaAKO-down primer pairs for Δ*legC4* and Δ*flaA* deletions, respectively.

To express *legC4* on a plasmid under control of its endogenous promoter, *legC4* plus the 300-bp upstream region was amplified from *L. pneumophila* genomic DNA (gDNA) with the LegC4BglII-F/LegC4XbaI-R primer pair and cloned as a BglII/XbaI fragment into BamHI/XbaI-digested pJB1806 (pJB) (56).

For IPTG-mediated expression of *legC4*, the *legC4* gene was amplified using the primer pair LegC4BamHI-F/LegC4XbaI-R and cloned as a BamHI/XbaI fragment into BamHI/XbaI-digested pSN85, a gift from Craig Roy (pEV; N-terminal 3×FLAG epitope tag fusion [57]). For IPTG-mediated expression of *lpg2505*, the *lpg2505* gene was amplified using the primer pair Lpg2505BglII-F/Lpg2505PstI-R and cloned as a BglII/PstI fragment into BamHI/PstI-digested pSN85. Sequence-confirmed pJB1806::p*legC4* (pJB::p*legC4*), pSN85::*legC4* (p*legC4*), and pSN85::*lpg2505* (p*lpg2505*) plasmids and empty vectors were transformed into *Legionella* strains as previous described (37).

Mice and BMDMs. C57BL/6 wild-type, *Myd88*$^{-/-}$, and *Tnfr1*$^{-/-}$ breeding pairs were purchased from the Jackson Laboratories (Bar Harbor, ME), and in-house colonies were maintained under specific-pathogen-free conditions at Kansas State University. All experiments involving animals were approved by the Kansas State University Institutional Animal Care and Use Committee (protocol number 4022) and performed in compliance with the Animal Welfare Act and NIH guidelines.

Bone marrow was harvested from mice as previous described (58). Bone marrow-derived macrophages were generated by differentiation in RPMI supplemented with 20% heat-inactivated fetal bovine serum (HI-FBS) (Gibco) and 15% L929 cell supernatant for 6 days prior to seeding for infection.

CI experiments in mice. Six- to 10-week-old age- and sex-matched C57BL/6 wild-type or *Tnfr1*$^{-/-}$ mice were infected for competitive index (CI) experiments as previously described (30). Mixed bacterial inoculums (1:1) were diluted and plated on selective medium (5 μg ml^{-1} chloramphenicol for *flaA*::Tn and 10 μg ml^{-1} chloramphenicol for plasmid selection). At 48 h p.i., mice were euthanized and whole lung tissue was harvested. Lung tissue was homogenized in 300 μl of sterile water using a Bullet Blender (Next Advance) as described previously (59). Dilutions were plated on selective medium as described above. CFU were enumerated and used to calculate CI values [$(CFUcm^{R}_{48h}/CFUwt_{48h})/(CFUcm^{R}_{IN}/CFUwt_{IN})$].

Quantification of *Legionella* replication within macrophages. Differentiated BMDMs were maintained in RPMI supplemented with 10% HI-FBS (Gibco) and 7.5% L929 cell supernatant. BMDMs were seeded at 2.5×10^5/well in 24-well plates 1 day prior to infection. BMDMs were infected with the indicated strains of *L. pneumophila* or *L. longbeachae* at a multiplicity of infection (MOI) of 1 in the presence or absence of 1 mM IPTG and/or recombinant cytokine as indicated. At 1 h p.i., cell monolayers were washed three times with phosphate-buffered saline (PBS), and fresh supplemented medium was added. Infections were allowed to proceed for up to 72 h or for 48 h, as indicated. To enumerate CFU, BMDMs were lysed in sterile water for 8 min, followed by repeat pipetting. Lysates were diluted as appropriate and plated on CYE agar plates, which were then incubated at 37°C for 4 days. For growth curve experiments, bacteria were enumerated after 1 h of infection and every 24 h thereafter for up to 72 h. To quantify fold replication, BMDMs were infected for 1 h and 48 h, and fold replication was enumerated by normalization of CFU counts at the indicated time points to the 1-h CFU counts.

Quantification of *L. pneumophila* replication in rich media *in vitro*. The *Legionella pneumophila* Δ*flaA*, Δ*flaA* Δ*legC4*, Δ*flaA* Δ*legC4*(pEV), and Δ*flaA* Δ*legC4*(p*legC4*) strains were streaked on CYE agar plates for single colonies, followed by generation of a 48-h heavy patch, which was harvested from plates and resuspended in supplemented AYE liquid medium (52, 53). Media for strains harboring plasmids were supplemented with 10 μg ml^{-1} chloramphenicol, and 1 mM IPTG was used to induce expression of plasmid-carried *legC4*. Liquid cultures for each strain in triplicate were started at an optical density at 600 nm (OD_{600}) of 0.3 and grown for 24 h. The OD_{600} was quantified at the indicated time points.

ELISA. BMDMs were seeded in a 24-well plate at 2.5×10^5/well 1 day prior to infection. The indicated Lp02 or SRS43 strains were used to infect the BMDMs ($n = 3$) at an MOI of 30 or 10, respectively, for the indicated times. For SRS43 strains, CFU were enumerated at each time point examined. Lp02 infections were performed in the absence of exogenous thymidine to prevent bacterial replication. At 1 h p.i., media were aspirated and cells were washed 3 times with PBS. Media were replaced, and supernatants were collected at the indicated time points. Expression of *legC4* was induced by addition of 1 mM IPTG to the culture medium. Supernatants were either used fresh or stored at −20°C for up to 1 week, followed by quantification of TNF or IL-6 using a mouse TNF or IL-6 enzyme-linked immunosorbent assay (ELISA) kit (BioLegend) following the manufacturer's instructions.

Western blot. To confirm production of 3×FLAG fusion proteins from *Legionella*, suspensions of strains harboring either pSN85 alone (pEV), pSN85::*legC4* (p*legC4*), or pSN85::*lpg2505* (p*lpg2505*) induced with IPTG were lysed by boiling in 3× Laemmli buffer. Proteins were separated by SDS-PAGE, followed by transfer to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher) using a wet transfer cell (Bio-Rad). Membranes were incubated in blocking buffer (5% nonfat milk dissolved in Tris-buffered saline–0.1% Tween 20 [TBST]). Anti-FLAG (clone M2; Sigma) was diluted at 1:1,000 in blocking buffer and incubated with membranes either overnight at 4°C or for 3 h at ambient temperature with rocking. Wash steps were performed 3 times for 10 min each in TBST. Horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Sigma) was diluted in blocking buffer at 1:5,000 and incubated with membranes for 1 to 2 h at room temperature with rocking. Membranes were washed, incubated with ECL substrate (Amersham), and imaged by chemiluminescence using a c300 Azure Biosystems Darkroom Replacer.

Statistical analysis. Statistical analysis was performed with GraphPad Prism software using either the Mann-Whitney U test or Student's *t* test, as indicated, with a 95% confidence interval. For all experiments, error bars denote ±standard deviations (SD) of samples in triplicate.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00755-18.

SUPPLEMENTAL FIGURES, PDF file, 0.3 MB.

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REFERENCES

1. Isberg RR, O'Connor TJ, Heidtman M. 2009. The *Legionella pneumophila* replication vacuole: making a cosy niche inside host cells. Nat Rev Microbiol 7:13–24. https://doi.org/10.1038/nrmicro1967.
2. Newton HJ, Ang DKY, van Driel IR, Hartland EL. 2010. Molecular pathogenesis of infections caused by *Legionella pneumophila*. Clin Microbiol Rev 23:274–298. https://doi.org/10.1128/CMR.00052-09.
3. Yu VL, Plouffe JF, Pastoris MC, Stout JE, Schousboe M, Widmer A, Summersgill J, File T, Heath CM, Paterson DL, Chereshsky A. 2002. Distribution of *Legionella* species and serogroups isolated by culture in patients with sporadic community-acquired legionellosis: an international collaborative survey. J Infect Dis 186:127–128. https://doi.org/10.1086/341087.
4. Borges V, Nunes A, Sampaio DA, Vieira L, Machado J, Simões MJ, Gonçalves P, Gomes JP. 2016. *Legionella pneumophila* strain associated with the first evidence of person-to-person transmission of Legionnaires' disease: a unique mosaic genetic backbone. Sci Rep 6:26261. https://doi.org/10.1038/srep26261.
5. Mascarenhas DPA, Pereira MSF, Manin GZ, Hori JI, Zamboni DS. 2015. Interleukin 1 receptor-driven neutrophil recruitment accounts to MyD88-dependent pulmonary clearance of *Legionella pneumophila* infection in vivo. J Infect Dis 211:322–330. https://doi.org/10.1093/infdis/jiu430.
6. Archer KA, Roy CR. 2006. MyD88-dependent responses involving Toll-like receptor 2 are important for protection and clearance of *Legionella pneumophila* in a mouse model of Legionnaires' disease. Infect Immun 74:3325–3333. https://doi.org/10.1128/IAI.02049-05.
7. Neild AL, Shin S, Roy CR. 2005. Activated macrophages infected with *Legionella* inhibit T cells by means of MyD88-dependent production of prostaglandins. J Immunol 175:8181–8190. https://doi.org/10.4049/jimmunol.175.12.8181.
8. Shin S. 2012. Innate immunity to intracellular pathogens: lessons learned from *Legionella pneumophila*. Adv Appl Microbiol 79:43–71. https://doi.org/10.1016/B978-0-12-394318-7.00003-6.
9. Mascarenhas DPA, Zamboni DS. 2017. Inflammasome biology taught by *Legionella pneumophila*. J Leukoc Biol 101:841–849. https://doi.org/10.1189/jlb.3MR0916-380R.
10. Ren T, Zamboni DS, Roy CR, Dietrich WF, Vance RE. 2006. Flagellin-deficient *Legionella* mutants evade caspase-1- and Naip5-mediated macrophage immunity. PLoS Pathog 2:e18. https://doi.org/10.1371/journal.ppat.0020018.
11. Molofsky AB, Shetron-Rama LM, Swanson MS. 2005. Components of the *Legionella pneumophila* flagellar regulon contribute to multiple virulence traits, including lysosome avoidance and macrophage death. Infect Immun 73:5720–5734. https://doi.org/10.1128/IAI.73.9.5720-5734.2005.
12. Case CL, Shin S, Roy CR. 2009. Asc and Ipaf Inflammasomes direct distinct pathways for caspase-1 activation in response to *Legionella pneumophila*. Infect Immun 77:1981–1991. https://doi.org/10.1128/IAI.01382-08.
13. Case CL, Kohler LJ, Lima JB, Strowig T, de Zoete MR, Flavell RA, Zamboni DS, Roy CR. 2013. Caspase-11 stimulates rapid flagellin-independent pyroptosis in response to *Legionella pneumophila*. Proc Natl Acad Sci U S A 110:1851–1856. https://doi.org/10.1073/pnas.1211521110.
14. Frutuoso MS, Hori JI, Pereira MSF, Junior DSL, Sônego F, Kobayashi KS, Flavell RA, Cunha FQ, Zamboni DS. 2010. The pattern recognition receptors Nod1 and Nod2 account for neutrophil recruitment to the lungs of mice infected with *Legionella pneumophila*. Microbes Infect 12:819–827. https://doi.org/10.1016/j.micinf.2010.05.006.
15. Opitz B, Vinzing M, van Laak V, Schmeck B, Heine G, Günther S, Preissner R, Slevogt H, N'Guessan PD, Eitel J, Goldmann T, Flieger A, Suttorp N, Hippenstiel S. 2006. *Legionella pneumophila* induces IFNbeta in lung epithelial cells via IPS-1 and IRF3, which also control bacterial replication. J Biol Chem 281:36173–36179. https://doi.org/10.1074/jbc.M604638200.
16. Copenhaver AM, Casson CN, Nguyen HT, Duda MM, Shin S. 2015. IL-1R signaling enables bystander cells to overcome bacterial blockade of host protein synthesis. Proc Natl Acad Sci U S A 112:7557–7562. https://doi.org/10.1073/pnas.1501289112.
17. Skerrett SJ, Bagby GJ, Schmidt RA, Nelson S. 1997. Antibody-mediated depletion of tumor necrosis factor-alpha impairs pulmonary host defenses to *Legionella pneumophila*. J Infect Dis 176:1019–1028. https://doi.org/10.1086/516530.
18. Brieland JK, Remick DG, Freeman PT, Hurley MC, Fantone JC, Engleberg NC. 1995. In vivo regulation of replicative *Legionella pneumophila* lung infection by endogenous tumor necrosis factor alpha and nitric oxide. Infect Immun 63:3253–3258.
19. Lanternier F, Tubach F, Ravaud P, Salmon D, Dellamonica P, Bretagne S, Couret M, Bouvard B, Debandt M, Gueit I, Gendre J-P, Leone J, Nicolas N, Che D, Mariette X, Lortholary O, Research Axed on Tolerance of Biotherapies Group. 2013. Incidence and risk factors of *Legionella pneumophila* pneumonia during anti-tumor necrosis factor therapy: a prospective French study. Chest 144:990–998. https://doi.org/10.1378/chest.12-2820.
20. Brieland J, Freeman P, Kunkel R, Chrisp C, Hurley M, Fantone J, Engleberg C. 1994. Replicative *Legionella pneumophila* lung infection in intratracheally inoculated A/J mice. A murine model of human Legionnaires' disease. Am J Pathol 145:1537–1546.
21. Shinozawa Y, Matsumoto T, Uchida K, Tsujimoto S, Iwakura Y, Yamaguchi K. 2002. Role of interferon-gamma in inflammatory responses in murine respiratory infection with *Legionella pneumophila*. J Med Microbiol 51:225–230. https://doi.org/10.1099/0022-1317-51-3-225.
22. Santic M, Molmeret M, Abu Kwaik Y. 2005. Maturation of the *Legionella pneumophila*-containing phagosome into a phagolysosome within gamma interferon-activated macrophages. Infect Immun 73:3166–3171. https://doi.org/10.1128/IAI.73.5.3166-3171.2005.
23. Ziltener P, Reinheckel T, Oxenius A. 2016. Neutrophil and alveolar macrophage-mediated innate immune control of *Legionella pneumophila* lung infection via TNF and ROS. PLoS Pathog 12:e1005591. https://doi.org/10.1371/journal.ppat.1005591.
24. Fontana MF, Shin S, Vance RE. 2012. Activation of host mitogen-activated protein kinases by secreted *Legionella pneumophila* effectors that inhibit host protein translation. Infect Immun 80:3570–3575. https://doi.org/10.1128/IAI.00557-12.
25. Barry KC, Ingolia NT, Vance RE. 2017. Global analysis of gene expression reveals mRNA superinduction is required for the inducible immune response to a bacterial pathogen. Elife 6:e1004229. https://doi.org/10.7554/eLife.22707.
26. Asrat S, Dugan AS, Isberg RR. 2014. The frustrated host response to *Legionella pneumophila* is bypassed by MyD88-dependent translation of pro-inflammatory cytokines. PLoS Pathog 10:e1004229. https://doi.org/10.1371/journal.ppat.1004229.
27. Hempstead AD, Isberg RR. 2013. Host signal transduction and protein kinases implicated in *Legionella* infection, p 249–269. *In* Hilbi H (ed),

Molecular mechanisms in Legionella pathogenesis. Springer, Berlin, Germany.
28. Shin S, Case CL, Archer KA, Nogueira CV, Kobayashi KS, Flavell RA, Roy CR, Zamboni DS. 2008. Type IV secretion-dependent activation of host MAP kinases induces an increased proinflammatory cytokine response to *Legionella pneumophila*. PLoS Pathog 4:e1000220. https://doi.org/10.1371/journal.ppat.1000220.
29. Stuart LM, Paquette N, Boyer L. 2013. Effector-triggered versus pattern-triggered immunity: how animals sense pathogens. Nat Rev Immunol 13:199–206. https://doi.org/10.1038/nri3398.
30. Shames SR, Liu L, Havey JC, Schofield WB, Goodman AL, Roy CR. 2017. Multiple *Legionella pneumophila* effector virulence phenotypes revealed through high-throughput analysis of targeted mutant libraries. Proc Natl Acad Sci U S A 114:E10446–E10454. https://doi.org/10.1073/pnas.1708553114.
31. Molofsky AB, Byrne BG, Whitfield NN, Madigan CA, Fuse ET, Tateda K, Swanson MS. 2006. Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection. J Exp Med 203:1093–1104. https://doi.org/10.1084/jem.20051659.
32. Fujita M, Ikegame S, Harada E, Ouchi H, Inoshima I, Watanabe K, Yoshida S-I, Nakanishi Y. 2008. TNF receptor 1 and 2 contribute in different ways to resistance to *Legionella pneumophila*-induced mortality in mice. Cytokine 44:298–303. https://doi.org/10.1016/j.cyto.2008.08.015.
33. Coers J, Vance RE, Fontana MF, Dietrich WF. 2007. Restriction of *Legionella pneumophila* growth in macrophages requires the concerted action of cytokine and Naip5/Ipaf signalling pathways. Cell Microbiol 9:2344–2357. https://doi.org/10.1111/j.1462-5822.2007.00963.x.
34. Archer KA, Alexopoulou L, Flavell RA, Roy CR. 2009. Multiple MyD88-dependent responses contribute to pulmonary clearance of *Legionella pneumophila*. Cell Microbiol 11:21–36. https://doi.org/10.1111/j.1462-5822.2008.01234.x.
35. Collart MA, Belin D, Vassalli JD, de Kossodo S, Vassalli P. 1986. Gamma interferon enhances macrophage transcription of the tumor necrosis factor/cachectin, interleukin 1, and urokinase genes, which are controlled by short-lived repressors. J Exp Med 164:2113–2118. https://doi.org/10.1084/jem.164.6.2113.
36. Vila-del Sol V, Punzón C, Fresno M. 2008. IFN-gamma-induced TNF-alpha expression is regulated by interferon regulatory factors 1 and 8 in mouse macrophages. J Immunol 181:4461–4470. https://doi.org/10.4049/jimmunol.181.7.4461.
37. Wood RE, Newton P, Latomanski EA, Newton HJ. 2015. Dot/Icm effector translocation by *Legionella longbeachae* creates a replicative vacuole similar to that of Legionella pneumophila despite translocation of distinct effector repertoires. Infect Immun 83:4081–4092. https://doi.org/10.1128/IAI.00461-15.
38. Cazalet C, Gomez-Valero L, Rusniok C, Lomma M, Dervins-Ravault D, Newton HJ, Sansom FM, Jarraud S, Zidane N, Ma L, Bouchier C, Etienne J, Hartland EL, Buchrieser C. 2010. Analysis of the *Legionella longbeachae* genome and transcriptome uncovers unique strategies to cause Legionnaires' disease. PLoS Genet 6:e1000851. https://doi.org/10.1371/journal.pgen.1000851.
39. Burstein D, Amaro F, Zusman T, Lifshitz Z, Cohen O, Gilbert JA, Pupko T, Shuman HA, Segal G. 2016. Genomic analysis of 38 *Legionella* species identifies large and diverse effector repertoires. Nat Genet 48:167–175. https://doi.org/10.1038/ng.3481.
40. Massis LM, Assis-Marques MA, Castanheira FVS, Capobianco YJ, Balestra AC, Escoll P, Wood RE, Manin GZ, Correa VMA, Alves-Filho JC, Cunha FQ, Buchrieser C, Borges MC, Newton HJ, Zamboni DS. 2016. *Legionella longbeachae* is immunologically silent and highly virulent in vivo. J Infect Dis 215:440–451. https://doi.org/10.1093/infdis/jiw560.
41. Massis LM, Zamboni DS. 2011. Innate immunity to *Legionella pneumophila*. Front Microbiol 2:109. https://doi.org/10.3389/fmicb.2011.00109.
42. Brown AS, Yang C, Hartland EL, van Driel IR. 2017. The regulation of acute immune responses to the bacterial lung pathogen *Legionella pneumophila*. J Leukoc Biol 101:875–886. https://doi.org/10.1189/jlb.4MR0816-340R.
43. Spörri R, Joller N, Albers U, Hilbi H, Oxenius A. 2006. MyD88-dependent IFN-gamma production by NK cells is key for control of *Legionella pneumophila* infection. J Immunol 176:6162–6171. https://doi.org/10.4049/jimmunol.176.10.6162.
44. Pereira MSF, Marques GG, Dellama JE, Zamboni DS. 2011. The Nlrc4 inflammasome contributes to restriction of pulmonary infection by flagellated *Legionella* spp. that trigger pyroptosis. Front Microbiol 2:33. https://doi.org/10.3389/fmicb.2011.00033.
45. Carey KL, Newton HJ, Lührmann A, Roy CR. 2011. The *Coxiella burnetii* Dot/Icm system delivers a unique repertoire of type IV effectors into host cells and is required for intracellular replication. PLoS Pathog 7:e1002056. https://doi.org/10.1371/journal.ppat.1002056.
46. Luo Z-Q. 2012. *Legionella* secreted effectors and innate immune responses. Cell Microbiol 14:19–27. https://doi.org/10.1111/j.1462-5822.2011.01713.x.
47. Losick VP, Haenssler E, Moy M-Y, Isberg RR. 2010. LnaB: a *Legionella pneumophila* activator of NF-kappaB. Cell Microbiol 12:1083–1097. https://doi.org/10.1111/j.1462-5822.2010.01452.x.
48. Ge J, Xu H, Li T, Zhou Y, Zhang Z, Li S, Liu L, Shao F. 2009. A *Legionella* type IV effector activates the NF-kappaB pathway by phosphorylating the IkappaB family of inhibitors. Proc Natl Acad Sci U S A 106:13725–13730. https://doi.org/10.1073/pnas.0907200106.
49. Liu M, Conover GM, Isberg RR. 2008. *Legionella pneumophila* EnhC is required for efficient replication in tumour necrosis factor alpha-stimulated macrophages. Cell Microbiol 10:1906–1923. https://doi.org/10.1111/j.1462-5822.2008.01180.x.
50. Berger KH, Isberg RR. 1993. Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. Mol Microbiol 7:7–19. https://doi.org/10.1111/j.1365-2958.1993.tb01092.x.
51. Zuckman DM, Hung JB, Roy CR. 1999. Pore-forming activity is not sufficient for *Legionella pneumophila* phagosome trafficking and intracellular growth. Mol Microbiol 32:990–1001. https://doi.org/10.1046/j.1365-2958.1999.01410.x.
52. Feeley JC, Gibson RJ, Gorman GW, Langford NC, Rasheed JK, Mackel DC, Baine WB. 1979. Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*. J Clin Microbiol 10:437–441.
53. Saito A, Rolfe RD, Edelstein PH, Finegold SM. 1981. Comparison of liquid growth media for *Legionella pneumophila*. J Clin Microbiol 14:623–627.
54. Luo Z-Q, Isberg RR. 2004. Multiple substrates of the *Legionella pneumophila* Dot/Icm system identified by interbacterial protein transfer. Proc Natl Acad Sci U S A 101:841–846. https://doi.org/10.1073/pnas.0304916101.
55. Nagai H, Roy CR. 2001. The DotA protein from Legionella pneumophila is secreted by a novel process that requires the Dot/Icm transporter. EMBO J 20:5962–5970. https://doi.org/10.1093/emboj/20.21.5962.
56. Bardill JP, Miller JL, Vogel JP. 2005. IcmS-dependent translocation of SdeA into macrophages by the *Legionella pneumophila* type IV secretion system. Mol Microbiol 56:90–103. https://doi.org/10.1111/j.1365-2958.2005.04539.x.
57. Folly-Klan M, Alix E, Stalder D, Ray P, Duarte LV, Delprato A, Zeghouf M, Antonny B, Campanacci V, Roy CR, Cherfils J. 2013. A novel membrane sensor controls the localization and ArfGEF activity of bacterial RalF. PLoS Pathog 9:e1003747. https://doi.org/10.1371/journal.ppat.1003747.
58. Case CL, Roy CR. 2013. Analyzing caspase-1 activation during *Legionella pneumophila* infection in macrophages. Methods Mol Biol 954:479–491. https://doi.org/10.1007/978-1-62703-161-5_29.
59. Ivanov SS, Roy CR. 2013. Pathogen signatures activate a ubiquitination pathway that modulates the function of the metabolic checkpoint kinase mTOR. Nat Immunol 14:1219–1228. https://doi.org/10.1038/ni.2740.