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Francisella tularensis Live Vaccine Strain Folate Metabolism and Pseudouridine Synthase Gene Mutants Modulate Macrophage Caspase-1 Activation

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Francisella tularensis **is a Gram-negative bacterium and the causative agent of the disease tularemia. Escape of *F. tularensis* from the phagosome into the cytosol of the macrophage triggers the activation of the AIM2 inflammasome through a mechanism that is not well understood. Activation of the AIM2 inflammasome results in autocatalytic cleavage of caspase-1, resulting in the processing and secretion of interleukin-1β (IL-1β) and IL-18, which play a crucial role in innate immune responses to *F. tularensis*. We have identified the *5-formyltetrahydrofolate cycloligase* gene (*FTL_0724*) as being important for *F. tularensis* live vaccine strain (LVS) virulence. Infection of mice *in vivo* with a *F. tularensis* LVS *FTL_0724* mutant resulted in diminished mortality compared to infection of mice with wild-type LVS. The *FTL_0724* mutant also induced increased inflammasome-dependent IL-1β and IL-18 secretion and cytotoxicity in macrophages *in vitro*. In contrast, infection of macrophages with a *F. tularensis* LVS *rluD pseudouridine synthase* (*FTL_0699*) mutant resulted in diminished IL-1β and IL-18 secretion from macrophages *in vitro* compared to infection of macrophages with wild-type LVS. In addition, the *FTL_0699* mutant was not attenuated *in vivo*. These findings further illustrate that *F. tularensis* LVS possesses numerous genes that influence its ability to activate the inflammasome, which is a key host strategy to control infection with this pathogen *in vivo*.**

Francisella tularensis is a virulent Gram-negative bacterium that infects phagocytic cells of the innate immune system (1). Both *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B) can cause disease in healthy humans (1). Cutaneous infection with *F. tularensis* results in an ulceroglandular form of tularemia following the bite of a vector, such as a tick or deer fly, carrying the bacteria or from direct contact with blood of an infected animal through an abrasion in the skin (1, 2). A pneumonic form of tularemia can be acquired through inhalation of as few as 10 type A organisms and is associated with high mortality if untreated (1, 3). The *F. tularensis* subsp. *holarctica* live vaccine strain (LVS) is attenuated in humans but still causes fatal disease in mice and retains many features of virulent type A and type B organisms *in vitro* (4); for this reason, *F. tularensis* LVS is widely studied as a model of the virulent type A and type B strains.

F. tularensis is recognized by pattern recognition receptors (PRRs), including Toll-like receptor 2 (TLR2) and the PYHIN family member absent in melanoma 2 (AIM2), both of which are critical for defense against infection (5–9). Sensing of *F. tularensis* by TLR2 at the macrophage surface leads to the elaboration of a number of proinflammatory cytokines (7). Following phagocytosis by the macrophage, *F. tularensis* escapes the phagosome into the cytosol of the cell, where it replicates (10, 11). Additionally, phagosomal escape of *F. tularensis* triggers the activation of the AIM2 inflammasome through the recognition of cytosolic bacterial double-stranded DNA (dsDNA) (6–9). AIM2 inflammasome activation in turn results in the processing and secretion of the proinflammatory cytokines interleukin-1β (IL-1β) and IL-18 as well as the induction of a pyroptotic death pathway in the infected macrophage (6–9).

Evasion or modulation of inflammasome activation is a survival strategy that is employed by a number of bacteria (12–16). We, and others, have previously described *F. tularensis* mutants that were capable of inducing increased activation of the AIM2 inflammasome, leading to enhanced production of IL-1β and IL-18 as well as increased macrophage cell death (12, 13, 16–18). In this report, we identify two novel *F. tularensis* LVS mutants that are capable of modulating inflammasome activation. Mutation of the *F. tularensis* LVS *FTL_0724* gene, which is annotated to encode 5-formyltetrahydrofolate cycloligase, results in enhanced inflammasome activation and cytotoxicity upon infection of macrophages. The *F. tularensis* LVS *FTL_0724* mutant is also markedly attenuated *in vivo*. In contrast, a mutation in the *FTL_0699* gene, which is annotated to encode the ribosomal large subunit pseudouridine synthase D (RluD), inhibits macrophage inflammasome activation without affecting bacterial virulence *in vivo*.

MATERIALS AND METHODS

Bacterial strains, plasmid construction, and growth conditions. *F. tularensis* subsp. *holarctica* LVS was obtained from ATCC (ATCC 29684). LVS containing a chromosomal insertion mutation in *FTL_0724* and *FTL_0699* was obtained using Tn*5* delivery plasmid pBB109 as previously

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described (19). To confirm that the Tn*5* insertion was affecting *FTL_0724* and *FTL_0699* expression, genomic DNA sequencing was performed as previously described (19). Complementation of the *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutant strains with wild-type *FTL_0699* and *FTL_0724* or catalytically inactive *FTL_0724* R127A containing the ribosomal binding site was performed as previously described (20). Briefly, the wild-type gene and the ribosomal binding site were amplified from LVS genomic DNA by PCR and ligated into the pBB103 plasmid (20). The plasmid containing the wild-type gene was incorporated into the bacteria via cryotransformation (19). The R127A mutation in *FTL_0724* was prepared by using a QuikChange II site-directed mutagenisis kit (Agilent Technologies). LVSs were grown on Difco cysteine heart agar supplemented with 9% sheep red blood cells (SRBC) for 48 h at 37°C; 25 μg/ml spectinomycin was added to plates for growth of the *FTL_0699*, *FTL_0724*, and *FTL_0724* R127A-complemented strains. For *in vivo* studies, bacteria were grown overnight in modified Mueller-Hinton (MMH) broth (Becton, Dickinson) supplemented with 1% (wt/vol) glucose, 0.025% ferric pyrophosphate, and 2% IsoVitaleX. For *in vitro* growth curve experiments, bacteria were grown over a time course in MMH or Chamberlain's defined media (CDM) (21).

Mice and *in vivo F. tularensis* LVS infections. The generation of ASC-, caspase-1-, NLRP3-, NLRC4-, AIM2-, IL-1RI-, and IL-18-deficient mice has been described previously (9, 22–26). Age- and sex-matched C57BL/6 mice purchased from NCI were used as wild-type controls. For *in vivo* infections, 6-to-8-week-old mice were injected intraperitoneally (i.p.) with the indicated dose of *F. tularensis* LVS or the *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants. Mice were monitored every 12 h for lethality; mice found to be in a moribund state for more than 4 h were considered terminal and euthanized. Bacterial burdens in the spleen and liver of infected mice were determined at day 3 postinfection by dilution plating of tissue homogenates onto Difco cysteine heart agar supplemented with 9% SRBC. The University of Iowa Institutional Animal Care and Use Committee approved all protocols used in this study.

Macrophage infections. Bone marrow-derived macrophages (BMMϕ) were generated as previously described (27). Unless otherwise indicated, BMMϕ were primed with 50 ng/ml lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 (Invivogen) for 4 h prior to infection. BMMϕ were infected with *F. tularensis* LVS, the *FTL_0724*::Tn5 mutant, and the *FTL_0699*::Tn5 mutant at a multiplicity of infection (MOI) of 50:1. At 9 h postinfection, or at the indicated time, supernatants were collected and assayed for IL-1β, IL-18, IL-12 p40, and keratinocyte-derived cytokine (KC) by enzyme-linked immunosorbent assay (ELISA). Antibody pairs for the IL-1β and IL-18 ELISAs were from R&D Systems and MBL, respectively. Antibody pairs for the IL-12 p40 and KC ELISAs were from eBiosciences. BMMϕ cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release using a cytotoxicity detection kit (Promega). Immunoblotting for caspase-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed as previously described (28).

Macrophage challenge with bacterial lysates and DNA. For challenge with crude cell lysates, LVS, *FTL_0699*::Tn5, or *FTL_0724*::Tn5 bacteria were sonicated and filtered through a 0.22-μm-pore-size cellulose acetate filter. Protein levels were determined by a bicinchoninic acid (BCA) protein assay. LPS-primed BMMϕ were transfected with 1 μg of protein using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Supernatants were collected 12 h posttransfection. To challenge cells with DNA from bacteria, DNA was first isolated using a Qiagen DNeasy blood and tissue kit according to the manufacturer's protocol; 750 ng was then transfected into cells using Lipofectamine 2000 according to the manufacturer's protocol. Supernatants were harvested 6 h posttransfection.

Statistical analysis. All results were analyzed using a two-tailed unpaired Student's *t* test, with the exception of bacterial burdens, which were analyzed by a two-tailed Mann-Whitney U test using Prism software. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Mutations in *F. tularensis* LVS *FTL_0699* and *FTL_0724* modulate *Francisella*-induced IL-1β and IL-18 secretion and cytotoxicity. We, and others, have previously identified *Francisella* mutants, in both *F. tularensis* LVS and *F. novicida*, that induce enhanced AIM2 inflammasome activation (12, 13, 16, 17). To identify the *F. tularensis* genes involved in AIM2 inflammasome activation, we screened a *F. tularensis* LVS transposon library for mutants that either enhanced or diminished IL-1β secretion from LPS-primed BMMϕ. We focused on two mutants (*FTL_0699* and *FTL_0724*) that had not previously been characterized as influencing inflammasome activation and that had no known defects in replication or survival.

FTL_0724 is annotated to encode a highly conserved enzyme, 5-formyltetrahydrofolate cycloligase (also known as 5,10-methenyltetrahydrofolate synthetase), involved in folate metabolism (29). Infection of LPS-primed BMMϕ with the *F. tularensis* LVS *FTL_0724*::Tn5 mutant resulted in increased secretion of both IL-1β and IL-18 in comparison to LPS-primed BMMϕ infected with wild-type LVS (Fig. 1A and B). In contrast, infection of LPS-primed BMMϕ with the *F. tularensis* LVS *FTL_0699*::Tn5 mutant resulted in diminished secretion of both IL-1β and IL-18 compared to infection with wild-type LVS (Fig. 1A and B). *FTL_0699* is annotated to encode the ribosomal large subunit pseudouridine synthase D (RluD). Consistent with the enhanced and diminished secretion of IL-1β and IL-18 induced by the *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants, respectively, infection of LPS-primed BMMϕ with the *FTL_0724*::Tn5 mutant resulted in increased cytotoxicity, whereas infection with the *FTL_0699*::Tn5 mutant caused a minimal but consistent decrease in cytotoxicity (Fig. 1C).

Complementation of both the *FTL_0699*::Tn5 mutant and the *FTL_0724*::Tn5 mutant with a wild-type copy of *FTL_0699* and *FTL_0724*, respectively, restored their induction of IL-1β and IL-18 production from infected macrophages to levels close to that of wild-type LVS (Fig. 2A and B). The function of 5-formyltetrahydrofolate cycloligase has previously been examined in *Mycoplasma pneumoniae*, and a conserved catalytic residue (R115) necessary for enzymatic function was identified (30). Hancock et al. also demonstrated that mutation of R115 to an alanine rendered the resulting protein catalytically inactive (30). The R115 catalytic residue in *M. pneumonia* is conserved in *F. tularensis* LVS (see Fig. S1 in the supplemental material). To determine if catalytic activity of 5-formyltetrahydrofolate cycloligase was required for limiting *F. tularensis* LVS-induced IL-1β and IL-18 secretion, we mutated the corresponding arginine in *FTL_0724* to an alanine (R127A). Whereas complementation of the *FTL_0724*::Tn5 mutant with a plasmid carrying the wild-type *FTL_0724* gene abrogated the enhanced IL-1β and IL-18 secretion induced by the *FTL_0724*::Tn5 mutant, complementation with *FTL_0724* R127A failed to restore the ability of the *FTL_0724*::Tn5 mutant to elicit normal levels of IL-1β or IL-18 relative to wild-type LVS (Fig. 2C and D).

Mutations in *F. tularensis* LVS *FTL_0699* and *FTL_0724* modulate *Francisella*-induced caspase-1 activation. The processing and secretion of IL-1β and IL-18 as well as pyroptotic cell death are dependent on the activation of the cysteine protease caspase-1. Activation of caspase-1 results in the autocatalytic processing of the 45-kDa pro-caspase-1 to generate two subunits, p20 and p10. This is a two-step process that requires both priming and

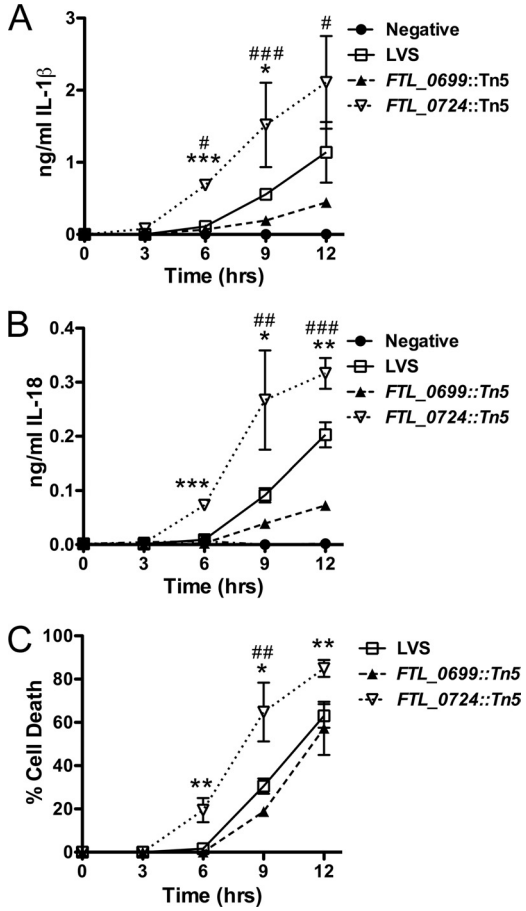

FIG 1 *F. tularensis* LVS *FTL_0699*::Tn5 and *FTL_0724*::Tn5 transposon mutants modulated *Francisella*-induced IL-1β and IL-18 secretion and cytotoxicity. (A to C) LPS-primed BMMϕ were infected with wild-type *F. tularensis* LVS or the indicated transposon mutant at an MOI of 50:1. Supernatants and cell lysates were collected at the indicated times postchallenge. Secretion of IL-1β (A) and IL-18 (B) into the supernatants was assessed by ELISA. (C) Cytotoxicity was assessed by measuring LDH release into the supernatant and expressed as a percentage of LDH release by Triton X-100 detergent. Determinations were performed in triplicate, and data are expressed as means ± standard deviations (SD). Data are representative of the results of three independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$ (for *FTL_0724*::Tn5 compared to LVS); #, $P \leq 0.05$; ##, $P \leq 0.01$; ###, $P \leq 0.001$ (for *FTL_0699*::Tn5 compared to LVS).

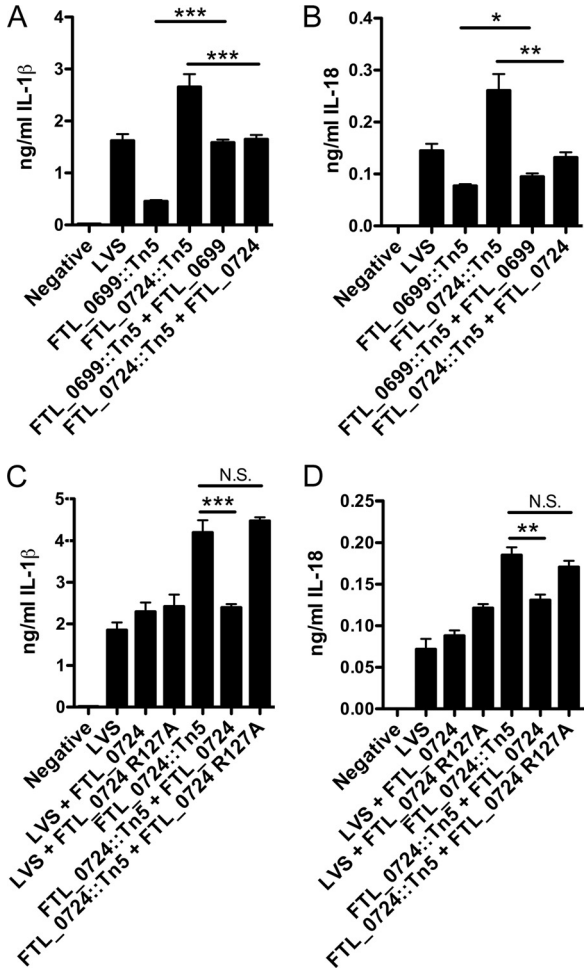

FIG 2 Complementation of *FTL_0699*::Tn5 and *FTL_0724*::Tn5 mutants with *FTL_0699* and *FTL_0724*, respectively, restored normal induction of IL-1β and IL-18 secretion. (A and B) LPS-primed BMMϕ were challenged at an MOI of 50:1 with wild-type LVS, the *FTL_0699*::Tn5 mutant, the *FTL_0724*::Tn5 mutant, or the complemented mutant bacterium (*FTL_0699*::Tn5 + *FTL_0699*, *FTL_0724*::Tn5 + *FTL_0724*). (C and D) LPS-primed BMMϕ were challenged at an MOI of 50:1 with wild-type LVS, the *FTL_0724*::Tn5 mutant, or bacteria complemented with either *FTL_0724* or catalytically inactive *FTL_0724* R127A. IL-1β and IL-18 release into the supernatant 9 h postinfection was determined by ELISA. Determinations were performed in triplicate, and the results are expressed as means ± SD. Data are representative of the results of three independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; N.S., nonsignificant.

activation steps. Priming with an agent such as LPS results in the generation of pro-IL-1β as well as in readying the inflammasome for subsequent activation through an as-yet-unidentified mechanism (31, 32). The phagosomal escape of *F. tularensis* into the macrophage cytoplasm, with the concurrent release of bacterial DNA into the cytosol, is thought to act as the second signal for the AIM2 inflammasome, leading to activation of caspase-1 (6, 8, 9).

LPS-primed BMMϕ infected with wild-type LVS activated caspase-1 by 9 h postinfection, as judged by immunoblot detection of the p10 cleavage product (Fig. 3A). In contrast, those infected with the *FTL_0724*::Tn5 mutant induced caspase-1 activation more rapidly (by 6 h postinfection) and to a greater extent than those infected with the wild-type LVS (Fig. 3A). Consistent with the diminished IL-1β and IL-18 secretion, the *FTL_0699*::Tn5 mutant induced diminished activation of caspase-1 compared to wild-type LVS (Fig. 3A).

Enhanced IL-1β secretion induced by the *FTL_0724*::Tn5 mutant was not secondary to it bypassing the need for a priming step, as neither wild-type LVS nor the *FTL_0724*::Tn5 mutant was capable of inducing IL-1β secretion from unprimed macrophages (Fig. 3B). In contrast to the changes in IL-1β and IL-18 release observed following infection of LPS-primed macrophages with either the *FTL_0724*::Tn5 mutant or the *FTL_0699*::Tn5 mutant, the secretion of IL-12 p40 and KC by unprimed BMMϕ infected with *F. tularensis* LVS was largely unaffected by the absence of *FTL_0724* or *FTL_0699* (Fig. 3C). Infection of BMMϕ with the *FTL_0724*::Tn5 mutant induced less IL-12 p40 production at 6 h postinfection than infection with the wild-type LVS; however, this reduction in cytokine production was not observed at later time points (Fig. 3C). Taken together, these data suggest that *F. tular-*

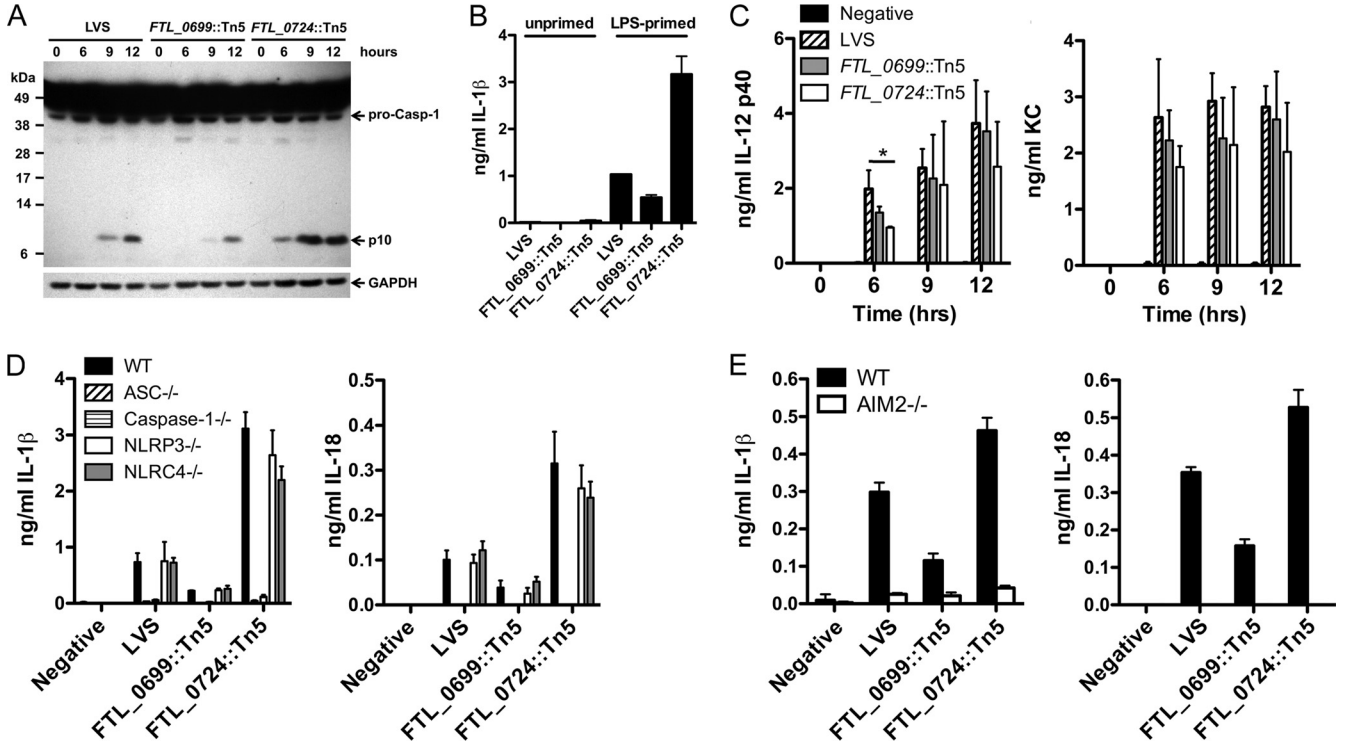

FIG 3 *F. tularensis* LVS *FTL_0699*::Tn5 and *FTL_0724*::Tn5 transposon mutants modulated *Francisella*-induced caspase-1 activation. (A) Lysates from LPS-primed BMMϕ infected with LVS, the *FTL_0699*::Tn5 mutant, or the *FTL_0724*::Tn5 mutant (50:1 MOI) for the indicated times were immunoblotted with antibodies against the p10 subunit of caspase-1 or GAPDH. Data are representative of the results of two separate experiments. (B) Unprimed or LPS-primed BMMϕ were infected as described for panel A. IL-1β and IL-18 release into the supernatant 12 h postinfection was determined by ELISA. (C) Unprimed BMMϕ were infected as described for panel A; supernatants were collected at the indicated times postinfection, and IL-12 p40 and KC levels were assessed by ELISA. (D and E) LPS-primed BMMϕ from wild-type (WT), ASC$^{-/-}$, caspase-1$^{-/-}$, NLRP3$^{-/-}$, NLRC4$^{-/-}$, and AIM2$^{-/-}$ mice were infected as described for panel A. IL-1β and IL-18 release into the supernatant 9 h postinfection was determined by ELISA. (B, D, and E) Determinations were performed in triplicate, and the results are expressed as means ± SD. Data are representative of the results of two (E) or three (B and D) independent experiments. (C) Data are expressed as means ± standard errors of the means (SEM) of the results of three independent experiments. *, $P \leq 0.05$.

ensis LVS expression of *FTL_0724* limits caspase-1 activation and the subsequent cytotoxicity and secretion of IL-1β and IL-18. In contrast, expression of *FTL_0699* by *F. tularensis* LVS is required for its efficient caspase-1 activation in LPS-primed macrophages.

In addition to AIM2, the NLR family members NLRP3 and NLRC4 can form functional inflammasomes in response to specific NLRP3 and NLRC4 agonists (33). To determine if NLRP3 or NLRC4 contributed to IL-1β and IL-18 secretion induced by the *FTL_0724*::Tn5 or *FTL_0699*::Tn5 mutant, LPS-primed BMMϕ from wild-type, caspase-1$^{-/-}$, ASC$^{-/-}$, NLRP3$^{-/-}$, and NLRC4$^{-/-}$ mice were infected with LVS, the *FTL_0724*::Tn5 mutant, or the *FTL_0699*::Tn5 mutant (Fig. 3D). Secretion of IL-1β and IL-18 in response to infection with wild-type LVS, the *FTL_0724*::Tn5 mutant, and the *FTL_0699*::Tn5 mutant was dependent on the presence of caspase-1 and ASC but independent of the presence of NLRP3 and NLRC4 (Fig. 3D). To directly test if AIM2 contributed to IL-1β and IL-18 secretion during challenge with *FTL_0724*::Tn5 or the *FTL_0699*::Tn5 mutants, LPS-primed BMMϕ from AIM2$^{-/-}$ and wild-type mice were challenged with LVS, the *FTL_0724*::Tn5 mutant, or the *FTL_0699*::Tn5 mutant. Secretion of both IL-1β and IL-18 by macrophages challenged with LVS and the *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants was dependent on the presence of AIM2 (Fig. 3E). These data suggest that the augmented caspase-1 activation induced by the *FTL_0724*::Tn5 mutant did not reflect activation of a different inflammasome pathway, such as NLRP3 or NLRC4, but was dependent on AIM2 inflammasome activation.

DNA from wild-type LVS and the *FTL_0699*::Tn5 and *FTL_0724*::Tn5 mutants induce comparable levels of IL-1β secretion. Activation of the AIM2 inflammasome occurs in response to recognition of cytosolic dsDNA (34–36). Given that both 5-formyltetrahydrofolate cycloligase and the RluD pseudouridine synthase, annotated to be encoded by *FTL_0724* and *FTL_0699*, respectively, indirectly play roles in nucleotide synthesis and modification, we investigated whether an intrinsic modification of bacterial components was responsible for the alteration in IL-1β secretion induced by the *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants (29, 37, 38). LPS-primed BMMϕ were transfected with equal amounts of bacterial crude lysates of wild-type LVS and the *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants (Fig. 4A). As expected, transfection with the synthetic dsDNA analogue poly(dA-dT) induced the secretion of IL-1β; however, no significant differences in IL-1β secretion between BMMϕ transfected with crude lysates of either wild-type LVS or the mutant were observed (Fig. 4A). Similarly, transfection of purified DNA from wild-type LVS or mutant bacteria resulted in comparable levels of IL-1β secretion (Fig. 4B). Taken together, these data suggest that modification of bacterial components or DNA in the *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants is not responsible for the observed alterations in macrophage IL-1β secretion.

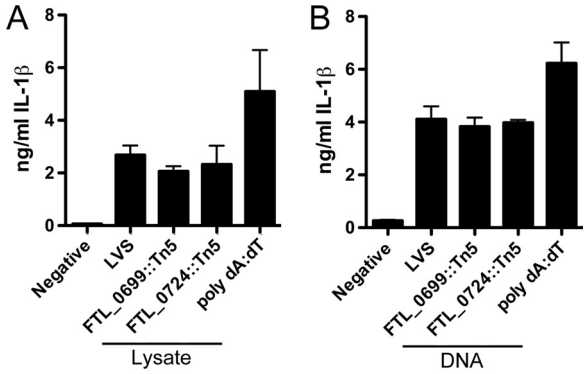

FIG 4 Intrinsic modification of a bacterial component was not responsible for the altered IL-1β secretion induced by the *FTL_0699*::Tn5 and *FTL_0724*::Tn5 mutants. LPS-primed BMMϕ were challenged with equivalent amounts of crude bacterial lysate (1 μg) (A) or purified bacterial DNA (750 ng) (B) from LVS, the *FTL_0699*::Tn5 mutant, or the *FTL_0724*::Tn5 mutant or the AIM2 agonist poly(dA-dT) in the presence of the Lipofectamine transfection agent. At 12 (A) or 6 (B) h postchallenge, IL-1β release into the supernatant was determined by ELISA. Determinations were performed in triplicate, and the results are expressed as means ± SD. Data are representative of the results of three independent experiments.

***FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants have decreased growth rates compared to wild-type LVS *in vitro*.** In order to assess whether the observed differences in the ability of the *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants to activate the inflammasome were due to different growth characteristics *in vitro*, we assessed the growth of the mutants and wild-type LVS within primed BMMϕ as well as growth in broth culture. The *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants displayed reduced intracellular replication within LPS-primed BMMϕ (Fig. 5A). Consistent with this, both mutants exhibited decreased growth in Chamberlain's defined media (CDM) (Fig. 5B). To determine if the *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants were more susceptible than LVS to various stressors, the bacteria were grown in modified Mueller-Hinton (MMH) broth at acidic pH. At pH 6.8, *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants grew with growth kinetics similar to those of LVS (Fig. 5C). However, at pH 5.2, the *FTL_0699*::Tn5 mutant had a decreased growth rate compared to LVS and the *FTL_0724*::Tn5 mutant had profoundly impaired growth (Fig. 5C). At pH 4.5, none of the bacterial strains were able to grow (Fig. 5C). To test if *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants were more susceptible than LVS to osmotic stress, the bacterial strains were grown in MMH broth supplemented with 3% or 4% NaCl. Although the bacterial strains grew more slowly than when no additional salt was provided, there were no differences between LVS and the two mutants in growth (data not shown). These data suggest that under optimal conditions, the *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants grow with kinetics similar to those of wild-type LVS; however, when nutrients are limited or under pH stress conditions, the *FTL_0724*::Tn5 and *FTL_0699*::Tn5 mutants exhibit decreased growth compared to wild-type LVS.

The *FTL_0724*::Tn5 mutant is attenuated *in vivo*, whereas the *FTL_0699*::Tn5 mutant retains its virulence. To determine if *FTL_0724* and *FTL_0699* were important for *F. tularensis* LVS virulence *in vivo*, wild-type mice were challenged i.p. with either LVS or the *FTL_0724*::Tn5 or *FTL_0699*::Tn5 mutant and monitored for survival. Infection with 3×10^4 CFU of LVS resulted in 88% mortality by day 10 postinfection (Fig. 6A). In contrast, all mice infected with 3×10^4 CFU of the *FTL_0724*::Tn5 mutant survived through day 14 postinfection (Fig. 6A). Infection with 3×10^4 CFU of the *FTL_0699*::Tn5 mutant, similar to infection with wild-type LVS, resulted in 70% mortality by day 5 (Fig. 6A). The increased survival of mice infected with the *FTL_0724*::Tn5 mutant relative to those infected with wild-type LVS was also reflected in significantly lower bacterial burdens in infected organs 3 days postinfection ($P < 0.01$; Fig. 6B). Collectively, these data demonstrate that the ability of *F. tularensis* LVS to limit inflammasome activation is critical for its virulence *in vivo*. In contrast, the *FTL_0699*::Tn5 mutant, which is inefficient in its ability to induce inflammasome activation, retained full virulence *in vivo*.

To further evaluate if enhanced inflammasome activation was responsible for the attenuation of the *FTL_0724*::Tn5 mutant *in vivo*, wild-type, ASC$^{-/-}$, and caspase-1$^{-/-}$ mice were challenged i.p. with either LVS or the *FTL_0724*::Tn5 mutant and survival was monitored. ASC$^{-/-}$ and caspase-1$^{-/-}$ mice infected with 3×10^4 CFU of the *FTL_0724*::Tn5 mutant had increased mortality (30% and 60% mortality at day 14 postinfection, respectively) compared to wild-type mice (0% and 10% mortality at day 14 postinfection, respectively; Fig. 6C and D). Infection with 5×10^5 CFU of the *FTL_0724*::Tn5 mutant increased mortality in the ASC$^{-/-}$ and caspase-1$^{-/-}$ mice to 60% and 70%, respectively, compared to that of wild-type mice (0% and 10% mortality at day 14 postinfection, respectively; Fig. 6C and D).

Inflammasome activation leads to processing and secretion of IL-1β and IL-18. To determine if cytokines IL-1β and IL-18 were important for survival following challenge with the *FTL_0724*::Tn5 mutant, we infected IL-1RI$^{-/-}$ and IL-18$^{-/-}$ mice with 5×10^5 CFU of LVS or the *FTL_0724*::Tn5 mutant. IL-1RI$^{-/-}$ and IL-18$^{-/-}$ mice infected with either LVS or the *FTL_0724*::Tn5 mutant succumbed to disease by day 7 postinfection, showing the importance of IL-1RI and IL-18 for protection against challenge with the *FTL_0724*::Tn5 mutant (Fig. 6E and F). Together, these data suggest that the attenuation of the *FTL_0724*::Tn5 mutant *in vivo* was due at least in part to increased inflammasome activation. However, the partial resistance of ASC$^{-/-}$ and caspase-1$^{-/-}$ mice to low-dose infection with the *FTL_0724*::Tn5 mutant suggests that additional factors may contribute to its attenuation *in vivo*.

DISCUSSION

Activation of the AIM2 inflammasome in response to infection with the facultative intracellular pathogen *F. tularensis* is critical for host defenses. As such, mice specifically deficient in components of the AIM2 inflammasome are particularly susceptible to infection with both *F. tularensis* LVS and *F. novicida* (6, 8). In this study, we demonstrated that the *F. tularensis* LVS gene *FTL_0724* is required for its *in vivo* virulence. The attenuation of infection with the *FTL_0724*::Tn5 mutant *in vivo* is in part due to the hyperactivation of the AIM2 inflammasome, as demonstrated by the susceptibility of ASC$^{-/-}$ and caspase-1$^{-/-}$ mice to infection with the *FTL_0724*::Tn5 mutant. In contrast, the *FTL_0699*::Tn5 mutant remained virulent *in vivo* despite having a defect in its ability *in vitro* to effectively activate caspase-1 in macrophages following infection. Taken together, these data highlight the critical function of caspase-1 in controlling *F. tularensis* LVS infection and demonstrate that mutant organisms that are unable to restrict their ability to activate this inflammatory pathway are markedly attenuated *in vivo*.

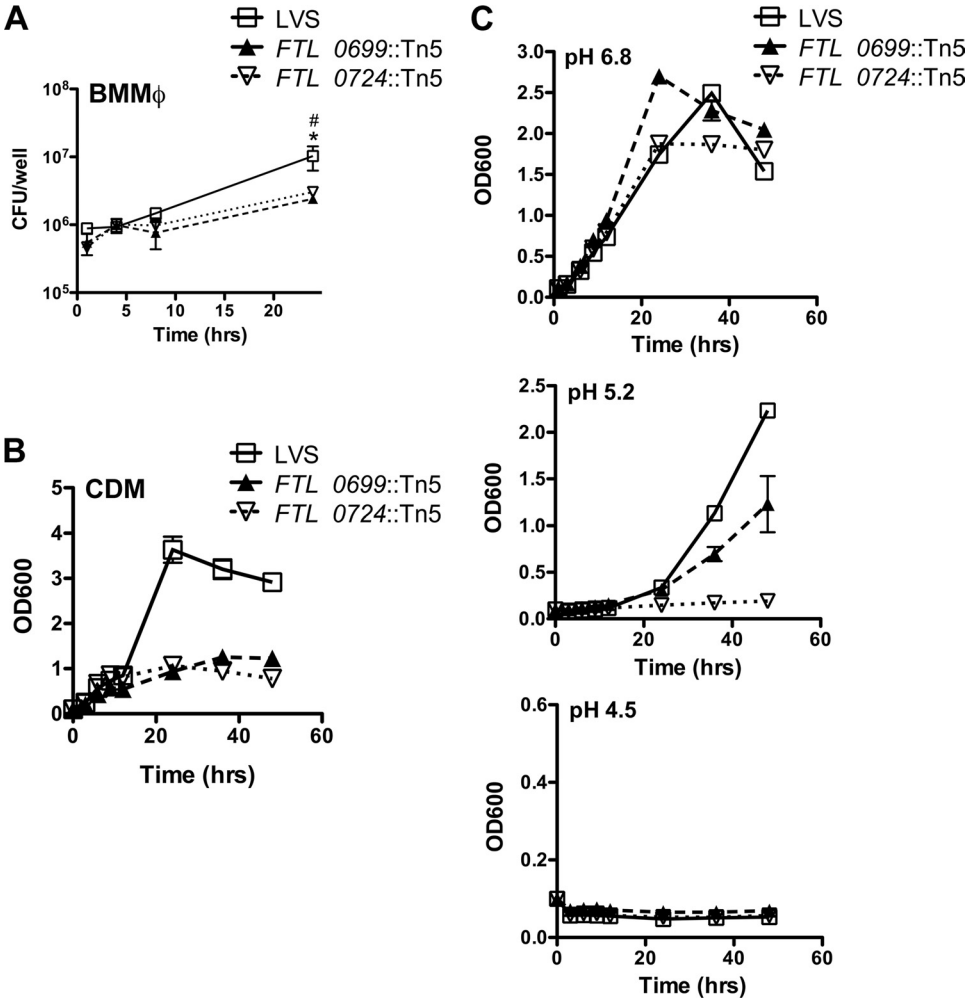

FIG 5 Growth characteristics of the *FTL_0699*::Tn5 and *FTL_0724*::Tn5 mutants. (A) LPS-primed BMMϕ were infected with LVS, the *FTL_0699*::Tn5 mutant, or the *FTL_0724*::Tn5 mutant (50:1 MOI); 1 h later, BMMϕ monolayers were washed to remove extracellular bacteria. At the indicated times postinfection, BMMϕ were lysed with 0.5% saponin and plated on bacterial media to determine numbers of CFU/well. (B and C) *F. tularensis* LVS and the *FTL_0699*::Tn5 and *FTL_0724*::Tn5 mutants were grown in Chamberlain's defined media (CDM; pH 6.8) (B) or modified Mueller-Hinton (MMH) broth at pH 4.5, 5.2, or 6.8 (C) at 37°C; at the indicated times, growth was determined by measuring absorbance at 600 nm. Data are representative of the results of three (A) or two (B and C) independent experiments. Determinations were performed in triplicate, and the results are expressed as means ± SD. *, $P \leq 0.05$ for *FTL_0724*::Tn5 compared to LVS; #, $P \leq 0.05$ for *FTL_0699*::Tn5 compared to LVS.

The AIM2 inflammasome is activated upon sensing of *Francisella* dsDNA within the cytosol of the macrophage (34–36, 39). Escape of *F. tularensis* from the phagosome into the macrophage cytosol precedes AIM2 inflammasome activation, as demonstrated by the finding that *F. tularensis* mutants that fail to escape the phagosome also do not induce caspase-1 activation (40–42). However, it remains unclear if bacterial damage and DNA leakage occur prior to or after phagosomal escape. Recently, we and others described a number of *F. tularensis* LVS and *F. novicida* mutants that induce increased caspase-1 activation and pyroptosis in infected macrophages (12, 13, 16–18). It appears that, rather than having direct inflammasome inhibitory properties, these genes play roles in the maintenance of bacterial membrane stability. All of the reported mutants display aberrant membrane morphologies and undergo increased lysis within the macrophage (13, 16). Consistent with these findings, we did not observe intrinsic differences in the ability of crude lysates or bacterial DNA from the *FTL_0724*::Tn5 mutant to induce IL-1β secretion when directly transfected into the macrophage.

As folate metabolism is important for numerous cellular functions, including the biosynthesis of nucleic acids and proteins, it is likely that mutations within the *5-formyltetrahydrofolate cycloligase* (*FTL_0724*) gene may influence bacterial membrane stability. The *FTL_0724*::Tn5 mutant exhibited reduced intracellular growth in macrophages compared to wild-type LVS. This reduced intracellular growth of the *FTL_0724*::Tn5 mutant might have been due to either increased pyroptosis of the macrophages or increased intracellular lysis of the *FTL_0724*::Tn5 mutant. The *FTL_0724*::Tn5 mutant exhibited a similar reduction in growth in Chamberlain's defined media, suggesting decreased fitness of the *FTL_0724*::Tn5 mutant compared to wild-type LVS. Interestingly, the *FTL_0724*::Tn5 mutant was unable to grow at an acidic pH of 5.2, suggesting that pH sensitivity led to increased bacterial damage within the phagosome.

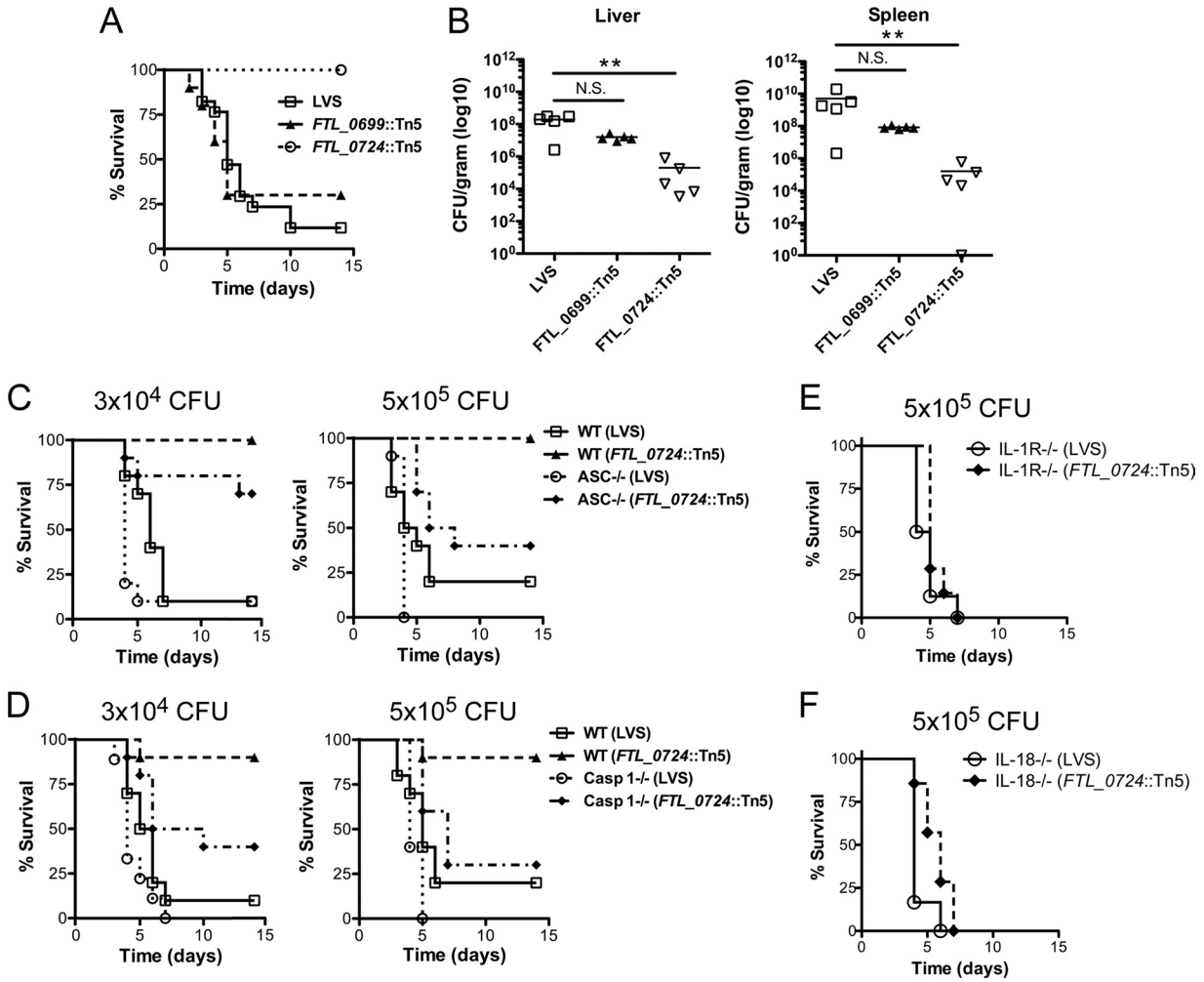

FIG 6 The *FTL_0724*::Tn5 mutant was attenuated *in vivo*, whereas the *FTL_0699*::Tn5 mutant remained virulent. (A and B) WT C57BL/6 mice were infected i.p. with 3×10^4 CFU of the indicated strain of bacteria. (A) Mice were monitored for survival ($n \geq 10$ mice per group). Results were pooled from two independent experiments. (B) At 3 days postinfection, bacterial burdens in liver and spleen were assessed ($n = 5$ mice per group). **, $P \leq 0.01$; N.S., nonsignificant. (C and D) WT, ASC$^{-/-}$, or caspase-1$^{-/-}$ mice ($n \geq 10$ mice per group) were infected i.p. with either 3×10^4 CFU or 5×10^5 CFU of LVS or the *FTL_0724*::Tn5 mutant and monitored for survival. Results were pooled from two independent experiments. (E and F) IL-1RI$^{-/-}$ or IL-18$^{-/-}$ mice were infected i.p. with 5×10^5 CFU of LVS or the *FTL_0724*::Tn5 mutant and monitored for survival ($n = 6$ to 8 mice per group).

In contrast to *FTL_0724*, mutation of *FTL_0699*, encoding RluD pseudouridine synthase, resulted in the diminished induction of caspase-1 in infected macrophages. Surprisingly, unlike other *Francisella* mutants that have been described to be defective in activating caspase-1, the *FTL_0699*::Tn5 mutant was capable of phagosomal escape and intracellular replication and, more importantly, was virulent *in vivo* (41, 42). Further studies are required to determine why the *FTL_0699*::Tn5 mutant does not effectively activate caspase-1; however, it does not appear to be due to a lack of functional AIM2 agonists, as crude lysates and DNA from the *FTL_0699*::Tn5 mutant directly transfected into macrophages were fully capable of inducing IL-1β secretion.

Through the use of *F. tularensis* mutant strains, we, and others, have identified several genes that are important for *F. tularensis* to evade recognition by specific PRRs. Identifying pathways that are required for *F. tularensis* to evade inflammasome activation would provide potential novel targets for therapeutic use. In addition, specific *F. tularensis* mutants that alter innate immune responses are also likely to affect the subsequent adaptive immune response that is generated. By integrating knowledge of both the innate and adaptive immune responses triggered by attenuated *F. tularensis* mutants, future studies may lead to the identification of improved vaccine strain candidates.

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