TolC-Dependent Modulation of Host Cell Death by the *Francisella tularensis* Live Vaccine Strain

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Francisella tularensis is a facultative intracellular, Gram-negative pathogen and the causative agent of tularemia. We previously identified TolC as a virulence factor of the *F. tularensis* live vaccine strain (LVS) and demonstrated that a $\Delta tolC$ mutant exhibits increased cytotoxicity toward host cells and elicits increased proinflammatory responses compared to those of the wild-type (WT) strain. TolC is the outer membrane channel component used by the type I secretion pathway to export toxins and other bacterial virulence factors. Here, we show that the LVS delays activation of the intrinsic apoptotic pathway in a TolC-dependent manner, both during infection of primary macrophages and during organ colonization in mice. The TolC-dependent delay in host cell death is required for *F. tularensis* to preserve its intracellular replicative niche. We demonstrate that TolC-mediated inhibition of apoptosis is an active process and not due to defects in the structural integrity of the $\Delta tolC$ mutant. These findings support a model wherein the immunomodulatory capacity of *F. tularensis* relies, at least in part, on TolC-secreted effectors. Finally, mice vaccinated with the $\Delta tolC$ LVS are protected from lethal challenge and clear challenge doses faster than WT-vaccinated mice, demonstrating that the altered host responses to primary infection with the $\Delta tolC$ mutant led to altered adaptive immune responses. Taken together, our data demonstrate that TolC is required for temporal modulation of host cell death during infection by *F. tularensis* and highlight how shifts in the magnitude and timing of host innate immune responses may lead to dramatic changes in the outcome of infection.

Francisella tularensis is a Gram-negative bacterial pathogen that causes the zoonotic disease tularemia (1, 2). *F. tularensis* is classified as a tier 1 select agent by the U.S. Centers for Disease Control and Prevention due to its low infectious dose, ease of aerosolization and dissemination, and the high morbidity and mortality rates associated with infection (3, 4). The most lethal form of tularemia occurs following pneumonic exposure; inhalation of as few as 10 bacteria can cause significant disease (3, 4). There are two clinically relevant subspecies, *F. tularensis* subsp. *tularensis* (type A) and *holarctica* (type B). *F. tularensis* subsp. *tularensis* is highly virulent and causes the most severe form of disease. *F. tularensis* subsp. *holarctica* is comparably less virulent and causes a milder form of disease. An attenuated live vaccine strain (LVS) was derived from an *F. tularensis* subsp. *holarctica* strain, but the basis for its attenuation is not fully understood and the LVS is not currently licensed for use in the United States (3). In addition, vaccination with the LVS does not fully protect against pneumonic challenge with type A *F. tularensis* (5, 6). The LVS causes a lethal infection in mice that mimics human tularemia, and the immune responses elicited resemble those seen during infection with virulent *F. tularensis* strains. Thus, the LVS has proved extremely useful as a model pathogen that can be manipulated under biosafety level 2 (BSL2) laboratory conditions. An additional species of *Francisella*, *F. novicida*, has low virulence in humans and has also proven useful as an experimental strain (7).

F. tularensis is a facultative intracellular pathogen that is able to invade and replicate within a variety of host cells, including macrophages, dendritic cells, neutrophils, hepatocytes, and pneumocytes (8–13). Following uptake by macrophages, *F. tularensis* prevents maturation of the phagosome and within ~1 to 4 h escapes into the host cell cytosol where bacterial replication occurs (14–16). Escape into the cytoplasm depends on the function of genes located in the *Francisella* pathogenicity island (FPI); FPI mutants that are defective for phagosomal escape are unable to replicate in

host cells and are avirulent (17–19). The bacteria replicate to large numbers in the cytoplasm, eventually triggering lysis of the host cell to release bacteria for additional rounds of infection and spread throughout the host.

A hallmark of *F. tularensis* is its ability to evade host defense mechanisms and subvert innate immune responses. This is evident in murine models of tularemia, where robust proinflammatory responses are not observed during the first 48 to 72 h of infection (20–24). *F. tularensis* also dampens host-programmed cell death responses during infection (20, 25–31). Death of *F. tularensis*-infected cells occurs via two primary mechanisms: caspase-1-mediated pyroptosis and caspase-3-mediated apoptosis (25, 32). During infection of activated macrophages, DNA from lysed intracellular *Francisella* bacteria is sensed in the cytoplasm by AIM2, caspase-1 is activated, and pyroptotic cell death occurs (33, 34). In addition, *F. tularensis* infection of a variety of host cell types, as well as in the mouse model of tularemia, results in caspase-3 activation and apoptotic cell death (32, 35–37). Notably, infection of mice with fully virulent type A *F. tularensis* results in extensive activation of caspase-3 but minimal activation of caspase-1, suggesting a central role for apoptosis in the pathogenesis of tularemia (35, 36). Although *F. tularensis* infection eventu-

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ally leads to activation of host-programmed cell death pathways, the bacteria interfere with this response. *F. tularensis* has been shown to delay induction of apoptosis during infection of both macrophages and neutrophils (38, 39). The ability of *F. tularensis* to dampen and delay host responses during infection likely provides time for the bacteria to gain an advantage within the host and cause disease. However, identification and characterization of the precise molecular mechanisms behind the immunomodulatory capacity of *F. tularensis* remain incomplete.

We previously identified the TolC protein as a virulence factor of the LVS that is important for the host-suppressive activities of *F. tularensis* (27, 40). TolC is the prototypical outer membrane channel component used by both multidrug efflux pumps and the type I protein secretion pathway. Multidrug efflux pumps confer resistance to a range of detergents, dyes, and antibiotics (41, 42), while type I secretion systems function in the export of a variety of proteins, including virulence factors such as α -hemolysin (*Escherichia coli*), alkaline protease (*Pseudomonas aeruginosa*), and adenylate cyclase toxin (*Bordetella pertussis*) (43). The *Francisella* genome encodes three TolC orthologs: TolC (Schu S4 locus tag FTT1724), FtlC (FTT1095), and SilC (FTT1258). Both FtlC and TolC have been shown to function in multidrug efflux, but only TolC is required for virulence (40). This suggests a distinct function for TolC, presumably in protein secretion. A Δ tolC mutant of the LVS is highly attenuated for virulence in mice, despite being able to disseminate from the site of infection and colonize the liver, spleen, and lungs (27). The LVS Δ tolC mutant maintains the ability to replicate intracellularly, although the bacteria reach levels in infected organs and cultured host cells that are consistently 1 to 2 logs lower than those for the wild-type (WT) LVS (27, 40). Compared to the WT strain, the LVS Δ tolC mutant causes 2- to 3-fold more cytotoxicity to both murine and human macrophages (27). This hypercytotoxicity is a result of increased apoptosis via a mechanism involving caspase-3 (27). In addition to causing hypercytotoxicity, the LVS Δ tolC mutant elicits increased release of proinflammatory chemokines from human macrophages compared to infection with the WT strain (27). Based on these data, we hypothesized that effector proteins secreted via TolC function to subvert innate immune pathways of the host and that the attenuation in virulence of the LVS Δ tolC mutant is due to a combination of hypercytotoxicity, leading to premature loss of the intracellular replication niche, and increased proinflammatory responses, leading to improved recruitment of immune cells to sites of infection and more effective bacterial clearance.

Here, we examined the kinetics of *F. tularensis*-induced inhibition of macrophage cell death and the specific host pathways involved. Our results demonstrate that TolC is required to delay activation of the intrinsic apoptotic pathway during the first ~24 to 36 h of infection and that this delay maximizes intracellular replication of the bacteria. We present evidence that the TolC-dependent delay of apoptosis is an active process and that the hypercytotoxicity of the LVS Δ tolC mutant is not due to compromised structural integrity of the mutant bacteria. Using the mouse model of tularemia, we show that TolC is required for *F. tularensis* to delay caspase-3-mediated apoptosis during infection of host tissues. In addition, we present evidence that the altered responses of the host to primary infection with the Δ tolC mutant lead to altered establishment of adaptive immunity. These findings suggest that *Francisella* Δ tolC mutants could function as safer and more effective live vaccine strains.

MATERIALS AND METHODS

Bacteria and growth conditions. The WT *F. tularensis* LVS (ATCC 29684) and Δ tolC mutant were grown on chocolate II agar plates (BD Biosciences) or in modified Mueller-Hinton broth (MHB; containing 1% glucose, 0.025% ferric pyrophosphate, and 0.05% L-cysteine HCl; BD Biosciences) (44), unless otherwise noted. The LVS Δ tolC strain was described previously (40).

Preparation of macrophages. Murine bone marrow-derived macrophages (BMDM) were isolated from the femurs of 6- to 8-week-old female C3H/HeN mice as previously described (27). Bone marrow-derived cells were cultured for 6 days in bone marrow medium (BMM; Dulbecco's modified Eagle's medium [DMEM] with GlutaMax [Gibco] supplemented with 30% L929 cell supernatant, 20% fetal bovine serum [FBS; HyClone], and 1 mM sodium pyruvate). For cytotoxicity and intracellular replication experiments, BMDM were seeded in 24-well plates at a concentration of 1.5×10^5 cells/well in 1 ml BMM. For immunoblotting experiments, BMDM were seeded in 6-well plates at a concentration of 1×10^6 cells/well in 3 ml BMM. BMDM were allowed to adhere to plates overnight and then were washed with phosphate-buffered saline (PBS) prior to infection with the indicated *F. tularensis* strains resuspended in bone marrow infection medium (BMIM; DMEM with GlutaMax [Gibco] supplemented with 15% L929 cell supernatant, 10% FBS, and 1 mM sodium pyruvate).

All protocols involving animals were approved by the Institutional Animal Care and Use Committee of Stony Brook University.

BMDM infections. For single-strain cytotoxicity, intracellular replication, and immunoblotting experiments, BMDM were seeded as described above and infected at a multiplicity of infection (MOI) of 50. Plates were centrifuged for 5 min at $500 \times g$ to facilitate bacterial contact with host cells. For coinfection cytotoxicity experiments, BMDM were seeded and infected as described above with the WT or Δ tolC LVS alone at an MOI of 50 or with a mixture of the Δ tolC mutant (MOI = 50) and WT LVS (MOI = 10, 20, 40, or 50). Plates were incubated with the bacteria for 2 h, washed with PBS, and incubated for an additional 1 h with gentamicin to kill extracellular bacteria. After gentamicin treatment, cells were washed with PBS and then incubated with fresh BMIM (lacking gentamicin) until the desired time points. For macrophage activation experiments, 50 ng/ml of purified lipopolysaccharide (LPS) was added to the BMDM 18 h prior to infection with bacteria.

Cytotoxicity assays. BMDM were cultured and infected as described above. For some experiments, 125 nM staurosporine (STS) in BMIM was added to cells after 1 h of gentamicin treatment. For some experiments, bacteria were grown in brain heart infusion broth (BHI; pH 6.8; BD Biosciences) instead of MHB. At the desired times postinfection, supernatants from infected BMDM were collected and analyzed for the presence of lactate dehydrogenase (LDH) using the CytoTox 96 nonradioactive cytotoxicity assay (Promega) according to the manufacturer's protocol. Background LDH release was quantified by examining supernatants from uninfected BMDM, and maximum LDH release was quantified from cells that were lysed via a single $-80^\circ\text{C}/54^\circ\text{C}$ freeze-thaw cycle. Percent LDH release was quantified by subtracting background LDH release from all sample values, dividing by the maximum LDH release minus background LDH release, and multiplying by 100.

Intracellular replication assays. BMDM were cultured and infected as described above. For some experiments, following 2 h of infection and 1 h of gentamicin treatment, the fresh BMIM added contained 10 $\mu\text{g}/\text{ml}$ gentamicin. At the desired times postinfection, cells were washed with PBS and lysed in DMEM-0.1% deoxycholate for 10 min at room temperature (45). Lysates were diluted, plated, and incubated for 3 days prior to enumeration of CFU.

Immunoblotting. BMDM were cultured and infected as described above. For positive apoptosis controls, cells were treated with 10 ng/ml of recombinant tumor necrosis factor alpha (TNF- α) (Invitrogen) for 12 h. At the desired times postinfection, cells were scraped and supernatant-cell mixtures were collected. Cells were pelleted, washed once with PBS, and

then lysed in RIPA buffer. Twenty micrograms of each protein sample was separated via 12% SDS-PAGE. After separation, proteins were transferred to nitrocellulose membranes at 110 V for 90 min. Membranes were blocked with 5% milk in TBS with 0.5% Tween 20 (TBST) at room temperature for 2 h. Membranes were then incubated with primary polyclonal antibodies recognizing poly-ADP ribose polymerase (PARP) (Cell Signaling catalog no. 9542; 1:1,000 dilution), tubulin (Sigma catalog no. T4026; 1:10,000 dilution), caspase-8 (R&D Systems catalog no. AF1650; 1:1,000 dilution), cleaved caspase-3 (Cell Signaling catalog no. 9661; 1:1,000 dilution), or caspase-9 (Cell Signaling catalog no. 9504; 1:1,000 dilution) overnight at 4°C. Membranes were washed with TBST four times and incubated with a horseradish peroxidase-conjugated mouse anti-rabbit IgG secondary antibody (Cell Signaling catalog no. 7074; 1:1,000 dilution) for 1 h at room temperature. Membranes were washed and bands were visualized by enhanced chemiluminescence (Pierce).

Disc diffusion assays. Lawns of the WT or $\Delta tolC$ LVS were plated from frozen stocks. Prior to incubation of the plates, solutions of the following drugs were diluted in sterile water: polymyxin B (stock concentration = 9.1 M), peroxyntirite (stock concentration = 85 mM), H_2O_2 (stock concentration = 8.8 M), and tBh (stock concentration = 7.8 M). A 15- μ l aliquot of each desired dilution was added to 6-mm paper discs (BD Biosciences), and saturated discs were placed onto the bacterial lawns. Following incubation for 72 h, zones of growth inhibition were measured and recorded as diameters (mm), including the 6-mm disc.

Serum sensitivity of bacteria. To obtain mouse serum, whole blood was collected from mice via cardiac puncture and then centrifuged in serum gel Z/1.1 microcentrifuge tubes (Sarstedt) for 10 min at 2,500 $\times$ g, 4°C, to remove red blood cells. Approximately 1×10^6 CFU of the WT or $\Delta tolC$ LVS was incubated at 37°C for 1 h in the presence of normal or heat-inactivated (54°C for 45 min) serum. *E. coli* strain DH5 α was used as a complement-sensitive control strain. Viable bacterial numbers were determined before and after serum incubation by dilution and plating for CFU.

Transmission electron microscopy. Overnight cultures of the WT and $\Delta tolC$ LVS grown in MHB, BHI, or Chamberlain's defined medium (CDM) (46) were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in the same medium and then grown to an OD_{600} of 0.2 to 0.3. Bacteria were pelleted, washed with PBS, and adsorbed onto polyvinyl formal-carbon-coated grids (Electron Microscopy Sciences) for 2 min. Bacteria were then fixed with 1% glutaraldehyde for 1 min, washed twice in PBS and twice in water, and then negatively stained with 0.5% phosphotungstic acid (Ted Pella) for 30 s. All samples were viewed with an FEI Tecnai 12 BioTwinG² electron microscope at an 80-kV accelerating voltage, and images were obtained using an AMT XR-60 charge-coupled-device digital camera system.

Outer membrane protein profiles. Outer membrane protein fractions were isolated as previously described (40). Fifty-milliliter cultures of the WT or $\Delta tolC$ LVS were grown to an OD_{600} of 0.2 to 0.4 in MHB. Bacteria were pelleted via centrifugation and resuspended in 100 μ l of 20 mM Tris HCl (pH 8) containing Complete protease inhibitor mixture (Roche) and lysed by sonication. Lysates were centrifuged for 10 min at 7,500 $\times$ g to pellet unbroken cells, and Sarkosyl was added to supernatants to a final concentration of 0.5% to solubilize inner membranes. After incubation for 5 min, supernatants were centrifuged at 100,000 $\times$ g for 1 h. Outer membrane pellets were then mixed with SDS sample buffer, boiled, and separated via SDS-PAGE before Coomassie blue staining.

Sytox green labeling. Overnight cultures of the WT and $\Delta tolC$ LVS grown in MHB were diluted to an OD_{600} of 0.05 in the same medium and then grown to an OD_{600} of 0.2 to 0.4. Bacteria were pelleted, washed twice with PBS, and resuspended in 50 μ l PBS. As a positive control for loss of membrane integrity, ethanol was added to a final concentration of 50%. Sytox green (Invitrogen) was added to a final concentration of 10%, bacteria were incubated for 30 min at 37°C, and then aliquots were mounted on glass slides. Phase-contrast and epifluorescence images were captured using a Spot camera (Diagnostic Instruments).

Infection of mice. For all LVS infections and vaccinations, bacterial inoculums were prepared by growing lawns of the strains for 60 h on solid medium. Bacteria were scraped from the plates, washed with PBS, and resuspended in MHB supplemented with 10% sucrose (MHB-sucrose). Inoculums were diluted in MHB-sucrose and frozen at -80°C until use. Intranasal infections of mice were performed by administering a 10- μ l inoculum into each naris (20 μ l total). Female C3H/HeN mice (6 to 8 weeks old; Charles River Labs) were used for all infections. Actual infectious doses were determined by retrospective CFU counts.

Immunohistochemistry. Groups of 6 mice were inoculated with PBS or infected as described above with 5×10^3 CFU of the WT or $\Delta tolC$ LVS. At days 2, 3, and 4 postinfection, 2 mice from each group were sacrificed via CO_2 asphyxiation, and spleens were harvested and bisected. One half of each spleen was fixed in 10% formalin and embedded in paraffin wax, while the remaining halves were examined for bacterial CFU as described below. Embedded spleens were cut into 5- μ m-thick sections and stained for the presence of cleaved caspase-3. Endogenous peroxidase activity was quenched by incubation in 0.5% H_2O_2 in methanol, and epitope retrieval was performed by heating in a decloaking chamber (Biocare Medical) for 1 h at 60°C in 10 mM sodium citrate buffer (pH 6.0). Slides were incubated for 1 h at room temperature with a primary mouse monoclonal antibody to cleaved caspase-3 (Cell Signaling catalog no. 9661) at a dilution of 1:1,500. After incubation with the primary antibody, slides were processed by an indirect avidin-biotin-based immunoperoxidase procedure using a biotinylated horse anti-mouse antibody (Vectastain Elite ABC kit; Vector Laboratories). Slides were incubated overnight at 4°C in TBS with 0.25% Triton X-100, developed with 3,3'-diaminobenzidine (DakoCytomation), and counterstained with hematoxylin. To quantitate apoptotic splenocytes, two separate sections per spleen per time point were stained, and 10 fields per section were examined for cells positive for cleaved caspase-3. Caspase-3-positive splenocytes in each field were classified as early apoptotic (cytoplasmic localization of cleaved caspase-3) or late apoptotic (cleaved caspase-3 localized to condensed, fragmented nuclei). Two independent experiments were performed for a total of 4 mice per infecting strain on day 2 postinfection, and three independent experiments were performed for a total of 6 mice per infecting strain on days 3 and 4 postinfection.

Vaccinations. For survival experiments, groups of 3 mice were inoculated with PBS or infected intranasally as described above with 1×10^3 CFU WT or $\Delta tolC$ LVS. Six weeks postvaccination, all mice were challenged with 1×10^8 CFU of the WT LVS and monitored for survival for 3 weeks. For organ burden analysis, groups of 3 or 4 mice were vaccinated and challenged as described above. The mice were sacrificed on days 3, 5, and 9 postchallenge and lungs, livers, and spleens were harvested. Bacterial organ burdens were determined as described below. Three independent experiments were performed for a total of 10 mice per vaccinating strain per time point. Mice that did not survive challenge infections were excluded from organ burden analysis.

Organ burden analysis. Organs from mice infected as described above were weighed in 1 ml PBS. Organs were manually homogenized in Whirl-Pak bags (Nasco), and undiluted and serial dilutions of the homogenates were plated to determine CFU. Bacterial burdens were calculated as CFU per gram of tissue.

Statistical analysis. *In vivo* caspase-3 quantitation and all organ burden CFU results were compared using the Mann-Whitney test for non-parametric data with one-tailed *P* values. For intracellular replication and LDH release time course experiments, one-tailed *P* values were calculated using the unpaired Student *t* test. For all other LDH release experiments, one-tailed *P* values were calculated by one-way analysis of variance (ANOVA) and Tukey's multiple-comparison posttest. Statistical calculations were performed using Prism 5 (GraphPad Software).

RESULTS

***F. tularensis* delays death of infected macrophages in a TolC-dependent manner.** Intracellular replication of *F. tularensis* leads

FIG 1 *F. tularensis* delays death of infected macrophages to facilitate intracellular replication in a TolC-dependent manner. (A and B) BMDM were infected with the WT or $\Delta tolC$ LVS at an MOI of 50 for 2 h, treated with gentamicin for 1 h to kill extracellular bacteria, and then incubated in gentamicin-free medium until the time points indicated. (A) Cytotoxicity was quantified by measuring LDH release. (B) Intracellular replication was quantified by lysing the BMDM and plating for CFU. (C) BMDM were infected as described for panels A and B (gentamicin $-$) or following this same protocol except that gentamicin was continuously maintained in the culture medium after the initial 2-h infection step (gentamicin $+$). Intracellular replication was quantified by plating for CFU at 24 h postinfection. Data represent means $\pm$ standard errors of the means (SEM) from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; calculated by one-way ANOVA and Tukey's posttest.

to induction of host cell death pathways (25, 32). We examined the kinetics of cell death during infection of murine bone marrow-derived macrophages (BMDM) with either the WT or $\Delta tolC$ LVS. Significantly increased cytotoxicity, measured by lactate dehydrogenase (LDH) release, was observed for the $\Delta tolC$ mutant compared to that of the WT strain as early as 7 h postinfection (Fig. 1A). The hypercytotoxicity of the LVS $\Delta tolC$ mutant persisted through 36 h postinfection. By 48 h postinfection, WT LVS-infected macrophages exhibited levels of cell death similar to those of $\Delta tolC$ -infected cells (Fig. 1A). Thus, *F. tularensis* delays host cell death in a TolC-dependent manner.

The LVS $\Delta tolC$ mutant is able to invade and replicate within various host cells similar to WT *F. tularensis*, but the $\Delta tolC$ mutant reaches CFU levels ~ 1 to 2 logs lower than the WT strain by 24 h postinfection (27, 40). This growth defect is observed during infection of mouse organs as well as in cell culture (27). To determine if the diminished replication of the $\Delta tolC$ mutant correlates

with its increased cytotoxicity, we quantified intracellular CFU over time in BMDM infected with either the WT or $\Delta tolC$ LVS. Matching the kinetics of cytotoxicity, we observed no significant differences in intracellular CFU between the WT and $\Delta tolC$ LVS at early time points postinfection (up to 8 h; Fig. 1B). However, at later times postinfection (16 to 24 h), the numbers of intracellular CFU recovered from macrophages infected with the $\Delta tolC$ mutant were significantly lower than CFU recovered from WT-infected cells (Fig. 1B). These results support the hypothesis that the growth defect observed for the LVS $\Delta tolC$ mutant is related to the inability of the mutant to delay host cell death.

In our standard *Francisella* infection assay, we used gentamicin to kill extracellular bacteria following the initial infection period but then performed the remainder of the assay in gentamicin-free medium (there is minimal *F. tularensis* replication in the macrophage infection medium). If the replication defect of the LVS $\Delta tolC$ mutant is indeed due to premature lysis of host cells and loss of the intracellular replication niche, then we should see an enhancement of this defect in the continuous presence of extracellular gentamicin, as the gentamicin will gain access to the intracellular bacteria upon host cell lysis. As shown in Fig. 1C, the number of CFU recovered at 24 h postinfection from BMDM infected with the $\Delta tolC$ mutant in the continuous presence of extracellular gentamicin was significantly lower than the number of CFU recovered under our standard infection conditions. In contrast, the continuous presence of extracellular gentamicin had no significant effect on CFU recovered following infection with the WT LVS (Fig. 1C). Taken together, these results demonstrate that *F. tularensis* prolongs viability of infected host cells in a TolC-dependent manner, allowing for greater bacterial replication in the protected intracellular niche.

TolC is required for *F. tularensis* to delay induction of the intrinsic apoptotic pathway. Previous work from our laboratory demonstrated that the hypercytotoxicity of the LVS $\Delta tolC$ mutant correlated with increased activation of caspase-3 and apoptosis (27). Cleavage and activation of caspase-3 is triggered during apoptosis by both extrinsic and intrinsic pathways that are dependent on caspase-8 and -9, respectively (47). Upon activation, caspase-3 translocates to the nucleus, where it facilitates many apoptotic processes, including poly-ADP ribose polymerase (PARP) cleavage, chromatin condensation, DNA fragmentation, and nuclear disruption (47–49). By immunoblot analysis, we detected proteolytic cleavage of caspase-3 and -9 in BMDM infected with the LVS $\Delta tolC$ mutant as early as 6 h postinfection, whereas macrophages infected with the WT LVS showed little to no cleavage of these caspases (Fig. 2). Apoptotic cleavage of PARP, an indication of caspase-3/7 activation, was observed by 6 h postinfection for $\Delta tolC$ mutant-infected macrophages but not for WT LVS-infected macrophages. Increased cleavage of PARP and caspases-3 and -9 was observed at 12 and 36 h postinfection for $\Delta tolC$ -infected cells, whereas BMDM infected with the WT LVS showed strong cleavage of caspase-3, caspase-9, and PARP only after 36 h of infection (Fig. 2). In contrast, apoptotic cleavage of caspase-8 was not apparent at any time point during infection with either the WT or $\Delta tolC$ LVS (Fig. 2). As a positive control, caspase-8 cleavage was detected in cells treated with TNF- α (Fig. 2). These results demonstrate that *F. tularensis* delays induction of the intrinsic apoptotic host cell response in a TolC-dependent manner.

FIG 2 *F. tularensis* delays activation of the intrinsic apoptotic pathway in a TolC-dependent manner. BMDM were infected with the WT or $\Delta tolC$ LVS as described for Fig. 1A. At the times indicated postinfection, cell lysates were collected and examined for the presence of the indicated proteins via SDS-PAGE and immunoblotting. Analysis of BMDM treated with TNF- α served as a positive apoptosis control. Sizes of full length (FL) and cleaved (C) proteins are as follows. Caspase-8, 57 kDa (FL) and 43 kDa (C); caspase-9, 49 kDa (FL) and 39 and 37 kDa (C); caspase-3, 19 and 17 kDa (C); PARP, 116 kDa (FL) and 89 kDa (C); α -tubulin, 52 kDa (FL). Results are representative of at least 2 independent experiments per time point.

The hypercytotoxicity of the LVS $\Delta tolC$ mutant is not due to compromised bacterial integrity. Several *Francisella* genes in addition to *tolC* have been identified that, when mutated, result in a hypercytotoxic phenotype (29, 33, 50, 51). Investigation of these mutants revealed that the structural integrity of the bacteria is compromised, rendering the mutants more susceptible to lysis upon uptake by macrophages. In activated macrophages, DNA released by lysed intracellular *F. tularensis* is detected by AIM2, which induces inflammasome assembly and leads to pyroptotic cell death characterized by activation of caspase-1 and secretion of interleukin 1 β (IL-1 β) and IL-18 (33, 34). We demonstrated previously that the hypercytotoxicity of the LVS $\Delta tolC$ mutant toward BMDM is caspase-1 independent (27), suggesting that detection of DNA from lysed bacteria is not the basis for the phenotype. To determine if the activation state of the macrophage impacts hypercytotoxicity of the LVS $\Delta tolC$ mutant, we compared infection of untreated BMDM (our standard infection condition) with LPS-stimulated BMDM. Infection with the $\Delta tolC$ mutant resulted in increased toxicity compared to infection with the WT LVS regardless of the activation state of the macrophages (see Fig. S1A in the supplemental material). Growth of *F. tularensis* in brain heart infusion (BHI) medium compared to that in Mueller-Hinton broth (MHB) results in bacteria more closely resembling organisms isolated from infected macrophages (52). These different growth conditions have been shown to affect structural integrity of *F. tularensis* and cytotoxicity toward host cells (53). We found that the LVS $\Delta tolC$ mutant was hypercytotoxic to BMDM compared to the WT strain regardless of bacterial growth medium used (see Fig. S1B).

We next conducted a series of experiments to compare directly the structural integrity of the $\Delta tolC$ mutant with the WT LVS. *Francisella* mutants that are structurally compromised exhibit increased sensitivity to reactive oxygen and nitrogen species (ROS and RNS, respectively) and are susceptible to the bactericidal action of complement (50, 54, 55). We observed no differences between the WT and $\Delta tolC$ LVS in sensitivity to ROS- or RNS-inducing compounds (H_2O_2 , tert-butyl hydroperoxide, and

peroxynitrite) (see Fig. S2A to C in the supplemental material). We also detected no difference between the WT and $\Delta tolC$ LVS in resistance to the membrane-active antimicrobial peptide polymyxin B (see Fig. S2D), indicating that the $\Delta tolC$ mutant does not have gross alterations in its envelope. In support of this, the protein profiles of outer membrane fractions isolated from the WT and $\Delta tolC$ LVS were indistinguishable, as determined by SDS-PAGE (data not shown). In addition, the WT and mutant bacteria exhibited similar resistance to mouse serum (see Fig. S2E), indicating no change in complement sensitivity and suggesting that the exopolysaccharide capsule of the $\Delta tolC$ mutant is not altered (54). To test further the integrity of the bacteria, we assessed permeability to Sytox green, a DNA binding stain normally excluded by the bacterial envelope, and examined the ultrastructure of the bacteria by electron microscopy. These studies revealed no differences between the WT and $\Delta tolC$ LVS (data not shown). Taken together, these results demonstrate that the LVS $\Delta tolC$ mutant is not structurally compromised and that the hypercytotoxicity of the $\Delta tolC$ mutant is not due to increased bacterial lysis.

***F. tularensis* actively delays death of infected macrophages.** If the hypercytotoxicity of the LVS $\Delta tolC$ mutant is not due to compromised bacterial integrity, this suggests that *F. tularensis* may actively inhibit host cell death pathways in a TolC-dependent manner. Treatment of host cells with staurosporine (STS) activates caspase-3 and induces apoptotic cell death (56). To test if *F. tularensis* is capable of interfering with cell death caused by proapoptotic stimuli, we examined STS-treated or untreated BMDM left uninfected or infected with the WT or $\Delta tolC$ LVS. Infection with WT bacteria blocked STS-induced cell death early during infection (6 and 21 h postinfection), whereas infection with the $\Delta tolC$ mutant did not (Fig. 3A). At a later time point (45 h postinfection), suppression of STS-induced cell death in WT LVS-infected cells was no longer apparent (Fig. 3A), consistent with *F. tularensis* initially delaying, but ultimately inducing, host cell death. Thus, *F. tularensis* is able to inhibit cell death induced by proapoptotic stimuli in a TolC-dependent manner. This result is not compatible with the hypercytotoxicity of the $\Delta tolC$ mutant being due to a passive structural defect.

If the TolC-dependent activity of *F. tularensis* in delaying host cell death is an active process due to factors secreted via TolC, then coinfection of WT with $\Delta tolC$ mutant bacteria should rescue the premature cell death induced by the mutant bacteria. To test this, we infected BMDM with either the WT or $\Delta tolC$ LVS alone (MOI = 50) or performed experiments in which the $\Delta tolC$ mutant (MOI = 50) was added together with increasing amounts of the WT LVS (MOI = 10 to 50). The single-strain control infections showed that the $\Delta tolC$ mutant was hypercytotoxic to the macrophages compared to the WT LVS (Fig. 3B), as seen previously. However, coinfection of BMDM with the $\Delta tolC$ mutant and the WT strain led to a dose-dependent decrease in cytotoxicity, reaching a level indistinguishable from infection with the WT LVS alone (Fig. 3B). These results support a model in which *F. tularensis* secretes effectors via TolC that function to interfere with activation of host cell death pathways during infection of BMDM.

***F. tularensis* delays apoptosis in mice in a TolC-dependent manner.** To determine if *F. tularensis* interferes with host cell death pathways during infection *in vivo*, we inoculated mice intranasally with the WT or $\Delta tolC$ LVS and examined bacterial burdens and caspase-3 activation in spleens at early times postinfection (days 2, 3, and 4). As observed previously (27), organ burden

FIG 3 *F. tularensis* actively delays macrophage cell death in a TolC-dependent manner. (A) BMDM were infected with the WT or $\Delta tolC$ LVS at an MOI of 50. At 3 h postinfection, the BMDM were left untreated or treated with staurosporine (125 ng/ml) to trigger apoptotic cell death. Cytotoxicity was determined by measuring LDH release at the indicated times postinfection. (B) BMDM were infected with the WT or $\Delta tolC$ LVS alone at an MOI of 50 or coinfecting with the WT and $\Delta tolC$ LVS at the indicated MOI. Cytotoxicity was quantified by measuring LDH release at 24 h postinfection. Bars represent means $\pm$ SEM from three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, calculated by one-way ANOVA and Tukey's posttest for the indicated comparisons in panel A or for comparison to infection with WT LVS alone in panel B.

analysis showed that CFU levels in spleens from mice infected with the $\Delta tolC$ mutant were lower by ~ 1 to 2 logs at each time point compared to mice infected with the WT LVS (Fig. 4A). Despite this decreased bacterial burden, examination by immunohistochemistry using an anticleaved caspase-3 antibody revealed that overall levels of caspase-3 cleavage in $\Delta tolC$ LVS-infected spleens were similar to levels in WT LVS-infected spleens at each time point (Fig. 4B). All spleens examined from PBS-inoculated control mice were negative for cleaved caspase-3 at all time points (data not shown). These results indicate that the $\Delta tolC$ mutant is hypercytotoxic compared to the WT LVS during infection of host tissues. The immunohistochemical analysis revealed that cleaved caspase-3 was localized to two distinct subcellular compartments: the cytoplasm, indicative of early stages of apoptosis; or condensed, fragmented nuclei, indicative of late stages of apoptosis (Fig. 4C) (49, 57, 58). Notably, at each time point, the proportion of cleaved caspase-3-positive cells undergoing late-stage apoptosis was significantly higher for mice infected with the $\Delta tolC$ mutant than for mice infected with the WT LVS (Fig. 4D). This difference was particularly stark at day 3 postinfection, where only 8% of cleaved caspase-3-positive splenocytes were late apoptotic for the WT LVS, but 80% of cleaved caspase-3-positive splenocytes were late apoptotic for the $\Delta tolC$ mutant (Fig. 4D). Thus, *F. tularensis* suppresses and delays caspase-3-mediated host cell death *in vivo* by a mechanism dependent on TolC.

Mice vaccinated with the $\Delta tolC$ mutant clear challenge doses more efficiently than mice vaccinated with the WT LVS. The defect of the LVS $\Delta tolC$ mutant in delaying apoptosis during infection *in vivo* should result in premature exposure of the bacteria to the extracellular environment due to loss of the intracellular replication niche. We hypothesized that this premature exposure

of the $\Delta tolC$ mutant could lead to altered innate immune responses and more efficient adaptive immune responses compared to infection with the WT LVS. In addition, the attenuation in virulence and lower organ burden levels of the $\Delta tolC$ mutant could make it a safer vaccine strain than the WT LVS. To test this, we immunized mice via the intranasal route with a sublethal dose (10^3 CFU) of the WT or $\Delta tolC$ LVS. Six weeks later, the mice were challenged intranasally with a lethal dose (10^8 CFU) of the WT LVS and monitored for survival over 21 days. All mock-vaccinated control mice died 5 to 15 days postchallenge, whereas the majority of mice vaccinated with WT LVS (73%) or the $\Delta tolC$ mutant (82%) survived the lethal challenge (Fig. 5). Therefore, vaccination with the LVS $\Delta tolC$ mutant protects mice from lethal challenge at least as well as the WT LVS, despite the fact that it colonizes host organs to lower levels (Fig. 4A) (27).

In parallel experiments, we vaccinated mice intranasally with 10^3 CFU of the WT or $\Delta tolC$ LVS or mock vaccinated with PBS as a control and challenged with 10^8 CFU of WT LVS as described above. We then quantified bacterial burdens in the lungs, livers, and spleens on days 3, 5, and 9 postchallenge. Consistent with the protection afforded by immunization with either strain, organ burdens for mock-vaccinated mice were significantly higher than those in the WT and $\Delta tolC$ LVS-vaccinated mice, and all mock-vaccinated mice died by day 9 postchallenge (see Fig. S3 in the supplemental material). Notably, a difference in organ burden patterns between mice vaccinated with the WT or $\Delta tolC$ LVS emerged from these studies. On day 3 postchallenge, WT LVS-vaccinated mice had significantly lower bacterial burdens than $\Delta tolC$ LVS-vaccinated mice in all organs analyzed (Fig. 6). In contrast, on day 5 postchallenge, similar organ burdens were obtained for both the WT- and $\Delta tolC$ -vaccinated mice. This change was due

FIG 4 *F. tularensis* delays induction of apoptosis during infection of mice in a TolC-dependent manner. C3H/HeN mice were intranasally infected with a sublethal dose (5×10^3 CFU) of the LVS or $\Delta tolC$ mutant. (A) Bacterial burdens in the spleen on days 2, 3, and 4 postinfection were determined by plating for CFU. (B) Spleens harvested on days 2, 3, and 4 postinfection were sectioned and labeled using an anticleaved caspase-3 antibody. Cleaved caspase-3-positive cells were quantified based on two sections per spleen and 10 fields per section. (C) Representative immunohistochemistry analysis of infected spleens. Arrows indicate intact cells expressing cleaved caspase-3 in the cytoplasm. Arrowheads indicate fragmented apoptotic nuclei containing cleaved caspase-3. Scale bar = 10 μ m. (D) Caspase-3-positive cells from panel B were characterized as early or late apoptotic based on cleaved caspase-3 localization and nuclear fragmentation. The percentage of total caspase-3-positive cells undergoing late-stage apoptosis was calculated for each time point. Bars represent means $\pm$ SEM from 2 to 3 independent experiments (total $n = 4$ to 6 mice per strain per time point). ***, $P < 0.001$; calculated by Student's *t* test.

to an increase in bacterial burdens from days 3 to 5 for mice vaccinated with the WT LVS, whereas levels remained similar or decreased in mice vaccinated with the $\Delta tolC$ mutant (Fig. 6). Moreover, greater numbers of $\Delta tolC$ LVS-vaccinated mice had undetectable challenge doses in their organs on day 5 compared to day 3, indicating clearance of the bacteria by the host. This increased clearance was not observed for WT LVS-vaccinated mice (Fig. 6). By day 9 postchallenge, organ burdens for all mice decreased compared to those at day 5. However, bacterial levels in the liver were significantly lower for mice vaccinated with the $\Delta tolC$ mutant than for mice vaccinated with the WT LVS, revers-

ing the pattern seen on day 3 (Fig. 6). In addition, all mice vaccinated with the $\Delta tolC$ mutant had completely cleared challenge doses from the liver and spleen by day 9, whereas bacteria were still detected in organs from mice vaccinated with the WT LVS (Fig. 6). These data demonstrate that the kinetics of challenge dose clearance are different between the WT and $\Delta tolC$ LVS-vaccinated mice, with mice immunized with the $\Delta tolC$ mutant able to clear challenge doses more efficiently. Taken together, these findings support our hypothesis that primary infection with the $\Delta tolC$ mutant leads to altered and more effective adaptive immune responses.

DISCUSSION

F. tularensis is a highly virulent human pathogen. A key feature of *F. tularensis* virulence is its ability during early stages of infection to evade and subvert host innate immune responses, including induction of programmed cell death pathways. The ability of *F. tularensis* to dampen host responses during infection likely provides time for the bacteria to replicate and spread within the host, contributing to the morbidity and mortality associated with tularemia. However, the molecular mechanisms underlying the immunomodulatory capacity of *F. tularensis* are not well understood. TolC is an outer membrane channel protein required for secretion by the type I secretion pathway. Previously, we identified TolC as critical for the virulence of the *F. tularensis* LVS. TolC has also been shown to contribute to the virulence of the type A human-pathogenic Schu S4 strain (59). We show here that TolC is

FIG 5 Mice vaccinated with the LVS $\Delta tolC$ mutant are protected from lethal LVS challenge. Mice were intranasally vaccinated with PBS (mock) or with 1×10^3 CFU of the WT or $\Delta tolC$ LVS. All mice were intranasally challenged with 1×10^8 CFU of the WT LVS 6 weeks postvaccination. Mice were monitored for survival for 21 days following challenge. Data represent three independent experiments (total $n = 9$ to 12 mice per vaccinating strain).

FIG 6 Mice vaccinated with the LVS $\Delta tolC$ mutant clear challenge doses faster than mice vaccinated with the WT LVS. Mice were intranasally vaccinated with PBS (mock) or with 1×10^3 CFU of the WT or $\Delta tolC$ LVS. All mice were intranasally challenged with 1×10^8 CFU of the WT LVS 6 weeks postvaccination and then sacrificed at days 3, 5, or 9 postchallenge. Bacterial burdens in the lungs, liver, and spleen were determined by plating for CFU and are expressed as CFU/g tissue. The limit of detection was 10 CFU. Results represent three independent experiments (total $n = 10$ mice per vaccinating strain per time point). *, $P < 0.05$; ns, not significant; calculated by the Mann-Whitney test for nonparametric data.

necessary for the LVS to delay induction of the intrinsic apoptotic pathway during infection of macrophages and that loss of TolC function results in premature loss of the intracellular replicative niche. We also demonstrate that the LVS delays induction of apoptosis during infection of mice in a TolC-dependent manner. Despite the virulence attenuation and replication defect of the LVS $\Delta tolC$ mutant, immunization of mice with this strain provides protection against lethal challenge with WT LVS. Moreover,

mice immunized with the $\Delta tolC$ mutant clear challenge bacteria more effectively than mice immunized with the WT LVS. Taken together, our results demonstrate that TolC is an important *F. tularensis* virulence determinant that may modulate host innate and adaptive immune responses during pathogenesis.

Although *Francisella* has a detectable extracellular phase in the host, the bacteria are thought to replicate primarily within the intracellular niche during infection (10, 60). *F. tularensis* mutants that are unable to survive within host cells, such as FPI mutants that are defective for phagosomal escape, are severely attenuated for virulence (17–19). The LVS $\Delta tolC$ mutant has a distinct phenotype, in that it replicates intracellularly, but reaches numbers consistently lower than those of the WT strain. We found that the LVS $\Delta tolC$ mutant replicates similarly to the WT LVS in macrophages during the first ~ 1 to 4 h postinfection. Given that *F. tularensis* escapes from the phagosome within ~ 1 to 4 h after uptake by macrophages (14–16), this observation indicates that TolC is not required for phagosomal escape or initiation of replication within the cytoplasm. After this initial period, the LVS $\Delta tolC$ mutant exhibits a replication defect compared to the WT strain. This timing parallels the kinetics of macrophage cell death induced by the $\Delta tolC$ mutant. Our results support a model in which *F. tularensis* delays induction of host cell death by a TolC-dependent mechanism. This delay in cell death allows *F. tularensis* to preserve its intracellular replicative niche and maximize bacterial growth. The LVS $\Delta tolC$ mutant also exhibits decreased replication compared to that of the WT strain during infection of host organs (Fig. 4A) (27), demonstrating that TolC function is required to preserve the bacterial replicative niche *in vivo*.

We previously showed that the hypercytotoxicity of the LVS $\Delta tolC$ mutant in BMDM was associated with increased activation of caspase-3 and apoptosis and independent of caspase-1 activity (27). We show here that TolC is required for the LVS to delay activation of the intrinsic pathway of apoptosis, as evidenced by earlier cleavage of caspase-3, caspase-9, and PARP during infection of macrophages with the $\Delta tolC$ mutant compared to WT bacteria and the absence of caspase-8 cleavage (Fig. 2). Our results are in contrast to analyses of several other *Francisella* mutants that exhibit hypercytotoxicity toward host cells (29, 33, 50, 51, 61). The hypercytotoxicity of these other mutants was correlated with compromised structural integrity of the bacteria leading to release of bacterial DNA, activation of caspase-1, and pyroptotic rather than apoptotic cell death. We directly confirmed through a number of tests that the structural integrity of the LVS $\Delta tolC$ mutant is not compromised compared to that of the WT LVS. Moreover, we present two separate lines of evidence that the TolC-dependent delay in apoptosis during *F. tularensis* infection is an active process. We show that the LVS suppresses STS-induced activation of apoptosis in a TolC-dependent manner and that coinfection of WT bacteria with the $\Delta tolC$ mutant reduces cytotoxicity back to levels observed upon infection with the WT alone. In additional coinfection experiments, we found that WT bacteria can suppress cytotoxicity in macrophages preinfected with the $\Delta tolC$ mutant, demonstrating that the WT bacteria do not have to be taken up simultaneously with the mutant (C. R. Doyle and D. G. Thanassi, unpublished data). Together, these results are incompatible with the hypercytotoxicity of the LVS $\Delta tolC$ mutant being due to a structural or some other passive defect. Instead, they support a model wherein *F. tularensis* secretes effector proteins via TolC that actively interfere with the innate cell death responses of macro-

phages. The identity of the TolC-secreted effector protein(s) remains to be determined, as does the conservation of TolC function in fully virulent *F. tularensis* strains.

Inhibition of host cell apoptosis appears to be a general strategy employed by intracellular pathogens to combat innate immune responses and facilitate infection. For example, *Ehrlichia*, *Neisseria*, *Salmonella*, and *Chlamydia* inhibit cytochrome *c* release from mitochondria (62–65), *Bartonella*, *Rickettsia*, and enteropathogenic *E. coli* disrupt signaling pathways leading to the induction of apoptosis (66–68), and *Shigella* and *Legionella* modulate the activity of proapoptotic caspases (69, 70). Although a role for multi-drug efflux in modulation of host inflammatory responses has been described in *Listeria monocytogenes* (71), none of the anti-apoptotic activities characterized to date for intracellular pathogens has been attributed directly to TolC or the type I secretion system. Thus, *F. tularensis* likely employs unique mechanisms to inhibit innate host immune responses.

Using the mouse model of tularemia, we found that *F. tularensis* delays induction of caspase-3-mediated apoptosis in a TolC-dependent manner in infected host tissues. The inability of the Δ tolC mutant to delay apoptosis *in vivo* could result in premature loss of the intracellular replicative niche and greater exposure of the Δ tolC mutant to host immune surveillance mechanisms in the extracellular environment. We previously observed that the LVS Δ tolC mutant elicits increased proinflammatory responses from human macrophages compared to those of the WT strain (27). Taking these possibilities together, the greater exposure of the Δ tolC mutant bacteria to the extracellular environment and increased proinflammatory responses may in turn affect the development of adaptive immune responses. Consistent with this hypothesis, we found that mice immunized with the Δ tolC mutant cleared challenge doses of the WT LVS more efficiently than mice immunized with the WT strain (Fig. 6). Future studies are needed to determine the basis for the differences in initial colonization levels and kinetics of challenge dose clearance between the WT and Δ tolC LVS-vaccinated mice. Nevertheless, our results demonstrate a role for TolC during primary *F. tularensis* infection that impacts the adaptive immune response.

There is currently no licensed tularemia vaccine in the United States. The LVS offers incomplete protection against challenge with fully virulent *F. tularensis*, and the basis for the attenuation of the LVS is not fully understood (5, 72, 73). Therefore, there is need for an improved tularemia vaccine. FPI mutants and other *Francisella* strains that are unable to escape the phagosome or replicate intracellularly do not provide significant protection when used as vaccines, presumably because their growth *in vivo* is so compromised that they do not effectively trigger adaptive immune responses (74, 75). The problem of overattenuation is also seen during vaccination attempts with *F. tularensis* O-antigen mutants (76, 77). In this regard, the LVS Δ tolC mutant may represent an improved live vaccine strain, as it (i) contains a defined genetic lesion, (ii) is attenuated for virulence, (iii) replicates and disseminates within the host, but to lower levels than WT bacteria, and (iv) appears to trigger more effective immune responses compared to those of the WT LVS. On a broader level, the construction of mutant strains that cause premature induction of host cell death during infection may represent a general strategy to generate effective vaccines against intracellular pathogens.

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