MECHANISMS OF *FRANCISELLA TULARENSIS* VIRULENCE AS REVEALED BY RIPA AND ICLR

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ABSTRACT

BRITTANY LYNNE MORTENSEN: Mechanisms of *Francisella tularensis* virulence as revealed by RipA and IclR
(Under the direction of Thomas H. Kawula)

F. tularensis is a Gram-negative coccobacillus that is the etiologic agent of the zoonotic disease tularemia. With a low infectious dose via the inhalational route and the ability to cause a potentially severe disease in humans, *F. tularensis* is a very successful pathogen. While it is known that *F. tularensis* depends on intracellular replication and immune suppression of the host, little is known about the specific mechanisms of virulence. Using a screen to identify genes required for intracellular replication, we identified a locus FTL_1914 which was subsequently named *ripA.* A mutant containing a deletion of *ripA* (LVSΔ*ripA*) escaped the phagosome; however, it failed to replicate intracellularly in the cytoplasm of macrophages and epithelial cells and was attenuated in a mouse model of pulmonary tularemia. Later studies showed that RipA is a conserved cytoplasmic membrane protein with similarity to hypothetical proteins of unknown function in a few randomly-distributed bacterial strains. Therefore, the function of RipA is not known. Investigation into protein-protein interactions involving RipA identified a putative RipA-interacting protein termed IclR (FTL_1364). In studies to determine the biological relevance of the RipA-IclR interaction, we analyzed the role of IclR to the virulence of *F. tularensis*. Deletion of *iclR* in *F. tularensis* LVS and Schu S4 suggested that unlike the non-pathogenic *F. novicida,* IclR was not required for virulence of human-virulent strains of *F. tularensis* and therefore, likely not required for RipA function. Further studies into RipA function using LVSΔ*ripA* led to a

greater understanding of innate immune signaling pathways that are being suppressed by *F. tularensis*. Finally, biochemical determination of the unique topology of RipA and identification of functional domains revealed new insights into a potentially new family in proteins conserved throughout Prokaryotes. Together, not only do these studies provide general characterization of two *F. tularensis* proteins, they help elucidate mechanisms of virulence utilized by the highly pathogenic *F. tularensis*.

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LIST OF ABBREVIATIONS

AIM2	Absent in melanoma 2
ASC	Apoptotic speck-containing protein with a CARD
BALF	Broncheolar lavage fluid
BHI	Brain heart infusion
BM(D)M	Bone marrow-derived macrophages
CARD	Caspase recruitment domain
CDM	Chamberlain's defined medium
CFU	Colony forming units
DC	Dendritic cell
DSP	Dithiobis (succinimidyl propionate)
EEA1	Early endosomal antigen 1
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
FCV	*Francisella*-containing vacuole
FPI	*Francisella* Pathogenicity Island
FRET	Fluorescence resonance energy transfer
i.d.	Intradermal
IFN	Interferon
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL	Interleukin
i.n.	Intranasal

i.p.	Intraperitoneal
JNK	c-Jun NH2-terminal kinases
LAMP	Lysosomal-associated membrane protein
LB	Lysogeny broth
LD_{50}	Median lethal dose
LD_{100}	Lethal dose
LPS	Lipopolysaccharide
LVS	Live vaccine strain
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MOI	Multiplicity of infection
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nucleotide binding domain-leucine rich repeats containing
NBD	Nucleotide Binding domain
ORF	Open reading frame
P/MAMPs	Pathogen/microbe- associated molecular patterns
PGE_2	Prostaglandin E2
PhoA	Alkaline phosphatase
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PMN	Polymorphonuclear

ppGpp	Guanosine-tetraphosphate
PTEN	Phosphatase and tensin homolog
PYCARD	Pyrin-CARD
RIG-I	Retinoic acid-inducible gene 2
RNAP	RNA polymerase
SAM	Significance analysis for microarrays
s.c.	Subcutaneous
SOE	Splice overlap extension
SQ	Starting quantity
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TMS1	Target of methylation-induced silencing
TNF-α	Tumor necrosis factor alpha
T6SS	Type VI secretion system
U112	Utah 112

CHAPTER 1

Introduction

FRANCISELLA CLASSIFICATION AND EPIDEMIOLOGY

Francisella tularensis is a Gram-negative, coccobacillus belonging to the family *Francisellaceae* of the γ-Proteobacteria. *Francisellaceae* are found throughout the environment and commonly isolated from marine and fresh water (30, 37, 135); however, the environmental reservoir is not known. Additionally, *Francisellaceae* have been isolated from a wide-range of animal species. The most common mammalian hosts are lagomorphs such as rabbits, but *Francisellaceae* have been isolated from over 500 other species including rodents (188), birds (211), cats (238), dogs (125, 126), sheep and oxen (13), fish (214), and even crayfish (8). *F. tularensis* is also frequently recovered from arthropod vectors, mainly ticks (188), flies (155), and mosquitoes (86). Certain species within *Francisellaceae* have been reported to infect amoeba, including *Acanthamoeba castellani* (1, 85, 164, 277) and *Hartmannella vermiformis* (250) suggesting that amoeba may serve as an environmental reservoir; however, *F. tularensis* has not yet been isolated from amoeba in nature.

F. tularensis infection in many of the animal hosts, including humans, results in the development of the zoonotic disease tularemia. In fact, *F. tularensis* was first identified as *Bacterium tularense*, the organism causing a disease in ground squirrels in Tulare County, California in 1911 (196, 197). Soon after, a febrile disease in humans called "deer-fly fever" was reported in Utah (219). Dr. Edward Francis is attributed with the identification and naming of *Bacterium tularense* as the agent causing "deer-fly fever" in the Utah patients (105, 106), and in 1947 the organism was renamed to *Francisella tularensis* in recognition of his contributions (215, 221). *F. noatunensis* (203, 204), *F. philomiragia* (214), and *F. piscicida* (216) species are now known as common fish pathogens, and *F. philomiragia* has caused disease in humans in a few isolated cases with immune-compromised patients or

near-drowning victims (107, 262, 285). The diverse host range and geographic distribution of *Franciselleaceae* reflects the success of the organism to adapt to many environments, including humans. Based on the ability to infect and cause disease in so many hosts, there is a repertoire of animal models, including non-human primates (287), mice (27), rabbits (27), guinea pigs (27), rats (144, 233, 289), zebrafish (279), *A. castellani* (1), *H. vermiformis* (250), and *Drosophila melanogaster* (280), that have been used for studying the pathogenesis in *F. tularensis* infections.

There are three main subspecies of *F. tularensis*, the species that causes tularemia in humans: *tularensis, holarctica,* and *mediasiastica*. *F. tularensis* subspecies *tularensis* has only been reported in North America and is considered the most highly infectious subspecies for humans (269). Subspecies *tularensis* is also known as Type A and the primary strain used in research is the Schu S4 strain, which requires biosafety level 3 conditions to handle in the laboratory. *F. tularensis* subspecies *holarctica* is found throughout the northern hemisphere, and while *holarctica* still infects humans, this subspecies has a higher infectious dose (<1000 organisms) and usually causes a less severe form of tularemia (269). Nonetheless, subspecies *holarctica* is a source of significant endemic disease in European countries (269). Subspecies *holarctica* was also used to generate the live vaccine strain (LVS) which is one of the frequently used strains in laboratories due to the ability to use this strain in under biosafety level 2 conditions and retained virulence for mice (269). *F. tularensis* subspecies *mediasiatica* has been associated with several reports of tularemia in Asia (152); however, not much is known about this subspecies. Finally, *F. novicida* is a commonly-studied species of *Francisella* due to the high genetic similarity to *tularensis* species and the ability to cause disease in mouse models of tularemia. Generally, *F. novicida* is considered non-pathogenic

for humans; however, it has been reported to cause disease, predominantly in immune-compromised patients (29, 32, 58, 94, 135, 165, 286). The Utah 112 (U112) strain, which was isolated from a water sample in Utah in 1955 (161), is a frequently-used laboratory strain for *F. novicida*. Within the field, some consider *F. novicida* as a subspecies of *F. tularensis*; however, based on divergence realized by whole genome single-nucleotide polymorphism analysis as well as differences in virulence and in at least 11 metabolic processes, *novicida* is considered a separate species by others (143, 147, 152, 162, 163). Nevertheless, due to journal policies and the current classification debate in the field (45, 147), chapters 2-4 refer to *F. novicida* as *F. tularensis* subspecies *novicida*.

In humans, tularemia is manifested in several different forms depending on the route of transmission of *F. tularensis*. General symptoms can include fever, headache, chills, joint pain, muscle aches, cough, and diarrhea, which begin to appear usually 3-5 days post infection (252, 253). Regardless of the route of inoculation, inflammation and swelling of the lymph nodes is also typical (269). The most common transmission route is contact with *F. tularensis* through the skin either through bites from arthropod vectors or handling contaminated animals, tissues or indirectly through items such as tools or bedding (269). There have been multiple cases of tularemia resulting from scratches or bites from house pets, commonly cats (205, 255). Dermal contact results in the ulceroglandular form of tularemia and the characteristic formation of a lesion at the site of infection (269). Transmission via the eye is rare but results in the oculoglandular form of tularemia characterized by conjunctivitis and occasionally other complications (269). Ingestion of *F. tularensis* in contaminated food or water results in oropharyngeal tularemia characterized by pharyngitis and ulcerative-exudative stomatitis (269). Typhoidal tularemia is the term given

when the source of infection is not known (269). The most severe form of tularemia is pneumonic tularemia that can be a result of complications from another form of tularemia or from inhalation of aerosolized bacteria. Often aerosols are generated by disruption of materials such as hay, soil, or grass contaminated with *F. tularensis* and therefore farmers and lawncare workers are often infected in this way (269). A famous set of cases in the United States occurred in Martha's Vineyard where multiple lawn care workers were diagnosed with pneumonic tularemia (95, 273). There have also been at least two cases of pneumonic tularemia resulting from transmission by dogs (241, 263). Historically, lab workers were infected via inhalation, although these incidences have decreased due to increased biosafety in handling the organism. Surprisingly, there is no evidence that *F. tularensis* is transmitted human to human (269).

F. tularensis subspecies *tularensis* has a very low infectious dose via the inhalational route, with as few as 10 organisms sufficient to cause disease (252, 253), and pneumonic tularemia has a mortality rate as high as 60% if left untreated (80). Infection can be controlled with prompt antibiotic treatment, and there is partial protection by previous vaccination using LVS (80). Due to the lack of knowledge on why LVS is attenuated, only being partially protective as a vaccine, and the inability to vaccinate immune-compromised individuals with a live strain, LVS is not licensed in the United States. The preferred antibiotics for treating tularemia patients are streptomycin or gentamicin, though alternative antibiotics can be used such as doxycycline, chloramphenicol, or ciprofloxacin (80). During the Cold War, several countries, including the former Soviet Union and the United States, stockpiled antibiotic-resistant strains of *F. tularensis* (5, 217, 254). This fact combined with the ease of aerosolization, the low infectious dose via the inhalational route, and the high

mortality rate after pulmonary infection, *F. tularensis* subspecies *tularensis* is designated as a Select Agent by the Centers for Disease Control (80).

PATHOGENESIS AND INTRACELLULAR LIFECYCLE

In a pulmonary infection, once in the lung, *F. tularensis* travels to the alveolus where it infects a number of cell types including macrophages, dendritic cells (DCs), neutrophils, and Type II alveolar epithelial cells (21, 36, 129, 130). The distribution of infected cells changes over the course of infection and among subspecies (130). *F. tularensis* has also been reported to infect erythrocytes (140), fibroblasts (108), kidney epithelial cells (138), and hepatocytes (68), cell types that could presumably be infected in systemic disease. Through the use of a uracil biosynthesis mutant (Δ*pyrF*), it has been suggested that intracellular replication in non-macrophage cells is sufficient to cause infection (139). There is also evidence that *F. tularensis* undergoes an extracellular phase during an infection (21, 99, 292). Overall, the ability to infect and manipulate so many cell types reflects the success of *F. tularensis* as a pathogen.

Central to *F. tularensis* virulence is its ability to replicate to high numbers within the cytoplasm of host cells. The ability of the bacteria to reach the cytoplasm is dependent on both the opsonization of the bacteria and whether the bacteria can escape the phagosome. First, in serum-opsonizing conditions, *F. tularensis* is taken up by looping phagocytosis through interaction with complement receptor C3 and actin microfilaments (60). Other receptors that may be involved in macrophage uptake under opsonizing conditions are Fcγ receptors, surfactant protein A, class A scavenger receptors, and nucleolin (17, 20, 115, 223, 258). Under non-opsonizing conditions, bacterial phagocytosis is mediated by the mannose

receptor with additional contributions by class A scavenger receptors and nucleolin (20, 115, 223, 258). After phagocytosis, *F. tularensis* escapes the phagosome as early as 20 minutes post invasion, and by approximately 1 hour, begins replicating within the cytoplasm. The kinetics of phagosomal escape depends on opsonization status of the bacteria. when compared to non-opsonizing conditions, serum opsonization of bacteria delays phagosomal escape and thereby impairs intracellular growth (115).

After uptake of bacteria and prior to phagosomal escape, the phagosome undergoes a maturation process in which the phagosome acquires early endosomal markers such as EEA1 and late endosomal markers such as CD63, LAMP-1, and LAMP-2 (59, 119). Studies using lysosomotropic agents revealed that these late-stage phagosomes are not acidified, even by 3 hours post infection (59). On the other hand, additional studies demonstrated that there is an initial, transient acidification as well as acquisition of the vacuolar ATPase that drops by 1 hour post infection, and escape is delayed if acidification is blocked (57, 102, 247). In contrast, another study suggests that phagosomal acidification is not required for phagosomal escape or intracellular replication (61). Therefore, the requirement for phagosomal acidification for phagosomal escape by the bacteria remains under debate. Once in the cytoplasm, *F. tularensis* can replicate up to several logs by 24 hours post infection (10). At this point, some bacteria are located in a membranous vacuole shown to be autophagic vacuoles, as is discussed below, and it is not yet clear how bacteria exit host cells to spread and infect other host cells.

The mechanisms by which other cell types take up *F. tularensis* and the lifecycle within these other cell types are not as well understood. Epithelial cells take up both viable and nonviable *F. tularensis* bacteria with similar kinetics, suggesting that there is a bacterial

surface ligand required for entry, but the host cell receptors involved are not known (75). Entry into epithelial cells also requires actin polymerization, microtubule rearrangement, and associated upstream signaling by phosphatidylinositol 3-kinase (PI3K) and tyrosine kinase pathways (75). Once inside the epithelial cell, *F. tularensis* escapes the phagosome to replicate within the cytoplasm following the same endocytic pathway and with similar kinetics as in the macrophage (75). DCs are thought to engulf *F. tularensis* via a mechanism that is dependent on serum opsonization of bacteria and the DC integrins CR3 and CR4 (28). Like DCs, neutrophils also take up *F. tularensis* in a serum-dependent manner (177, 227). After entry, *F. tularensis* bacteria escape the phagosome to persist in the cytoplasm, though no reports demonstrate any intracellular replication in neutrophils (194). In fact, *F. tularensis* has been shown to actively recruit neutrophils to the lung at least in part through the activity of matrix metalloproteinase-9, suggesting that neutrophils may provide an additional *in vivo* niche for *F. tularensis* (186). Finally, both complement-dependent and independent mechanisms are involved in uptake into erythrocytes, but nothing else is known about the lifecycle of *F. tularensis* in this cell type (140). We still have much to learn about the life cycle of *F. tularensis* within non-macrophage cell types.

As noted above, after approximately 24 hours post infection, *F. tularensis* can be observed within multi-membranous vacuoles termed *Francisella*-containing vacuoles or FCVs (54). The study initially characterizing FCVs showed that these vacuoles are juxtanuclear and fused with lysosomes, yet there are intact Schu S4 bacteria within. Furthermore, FCVs acquired the protein LC3, accumulated monodansylcadaverine, and formation was inhibited by 3-methyladenine, together suggesting that the FCV represents an autophagosome. Subsequent studies have expanded on the autophagy concept by showing

that FCV formation also occurs with LVS and that other autophagy-associated molecules such as cathepsin D, phosphatase and tensin homolog (PTEN), and p53 may have altered expression in infected cells (141). Finally, microarray studies at 24 hours post infection demonstrated that infection with Schu S4 or U112 results in decreased expression of other autophagic response proteins and the autophagy-related PI3K signaling pathway (46, 76). This suggests that *Francisella* may be suppressing and thereby delaying the autophagic response to infection; however, it is not known what bacterial effectors may be involved.

Related to this, some host cell death is induced by *F. tularensis*, even with bacteria-mediated suppression of the process. Several reports have shown that *F. tularensis* induces caspase-3 activation and cell death in macrophages and *in vivo* using *F. novicida,* LVS, and Type A strains KU49 and KU54 (33, 159, 230, 251, 288). To build on this idea, in two studies using *F. novicida*, it was demonstrated that caspase-3 activation and cell death are downregulated at early time points as a result of activation of Ras signaling pathways and that although there is eventually upregulation of caspase-3, apoptosis is delayed further, potentially as a result of anti-apoptotic signaling (6, 251). Therefore, although apoptotic pathways are eventually activated, the delayed kinetics suggest the possibility that the bacteria are downregulating these apoptotic pathways, yet the specifics of caspase-3 and apoptosis signaling pathways during *F. tularensis* infections are not completely clear.

HOST IMMUNE RESPONSE TO *FRANCISELLA* INFECTION

Innate immune response

Another pathogenic strategy of *F. tularensis* is to suppress the host pro-inflammatory immune response. This is one important difference between *novicida* and the more virulent

holarctica and *tularensis* subspecies. Whereas *F. novicida* stimulates the innate immune system, *F. tularensis* subspecies *holarctica* and *tularensis* does so to a much lesser extent and in a delayed manner due to active suppression on the part of these organisms (9, 35, 36, 46, 53, 176, 270, 271). In fact, it has also been reported that both LVS and Schu S4 upregulate expression of the immunosuppressive cytokine TGF-β (35, 36). The pathways involved in response to *F. tularensis,* and which *F. tularensis* is suppresses, have only been partially elucidated. In terms of extracellular recognition, unlike many other Gram-negative bacteria, *F. tularensis* lipopolysaccharide (LPS) does not stimulate the host cell sensor protein toll-like receptor (TLR) 4 (2, 62, 128, 270). This low stimulatory effect is likely due in part to changes in lipid A, specifically the lack of a 4'-phosphate, which is removed by LpxF, and hypoacylation of the fatty acid chains (222, 278, 281, 282). There has been a fair bit of controversy on the exact structure of lipid A between the strains of *Francisella* (25, 222, 261, 278, 282). Despite the inability of *F. tularensis* LPS to activate the host response, *F. tularensis* does stimulate TLR2, which recognizes lipoproteins, and whether this recognition is specific to TLR1/TLR2 or TLR6/TLR2 heterodimers is not clear, but *F. tularensis* may possibly stimulate both heterodimers with different ligands (2, 150, 168, 187, 274). Following stimulation of TLR2, several downstream pathways are required for immune activation. Overall, signaling occurs through MyD88 with a partial contribution of the MyD88 adaptor molecule TIRAP (2, 63, 65, 66, 199). This leads to downstream activation at least p38 and ERK1/2 MAPK, which presumably leads to activation of NF-κB and AP-1 transcription factors for several pro-inflammatory cytokines, most notably pro-IL-1β (142, 199, 270). LVS-infected cells display a TLR2- and MyD88-independent activation of PI3K that corresponded to a downregulation of p38 and ERK1/2, suggesting that PI3K and MAPK

signaling pathways may be involved in the suppression of the immune response observed for *F. tularensis* (199) . The specific downstream signaling pathways involved in suppression of pro-IL-1β transcription need further elucidation.

Processing of pro-IL-1β to mature IL-1β for secretion requires a two-step process. First, there must be transcription and synthesis of pro-IL-1β as described above. A second signal is required for processing into mature IL-1β in which a cytoplasmic sensor molecule recognizes a pathogen and then recruits additional protein components to form a complex called the inflammasome. The most well-characterized families of cytoplasmic sensors are the nucleotide binding domain leucine rich repeats containing proteins (NLRs) (145); however, there are reports of inflammasomes containing the cytoplasmic sensors absent in melanoma 2 (AIM2) or retinoic acid-inducible gene 2 (RIG-I) (44, 96, 97, 137, 225, 239). The respective sensor molecule will recruit procaspase-1, and then depending on the sensor molecule involved, also recruit the adaptor protein PYCARD/ASC (Pyrin-CARD/apoptotic speck-containing protein with a CARD) (71, 193). The formation of the complete inflammasome complex results in the auto-catalytic cleavage of pro-caspase-1 into caspase-1, which can then process pro-IL-1β into mature IL-1β for secretion.

In terms of *Francisella,* ASC and caspase-1 are involved in detection of cytoplasmic *Francisella* bacteria (64, 113, 191). A recent study demonstrates that TLR2 and MyD88 are also required for inflammasome activation and assembly, but the mechanism is not clear (148). Once in the cytoplasm, *F. tularensis* stimulates the inflammasome via an unknown cytoplasmic receptor, although we know that *F. tularensis* does not stimulate the well-characterized NLRP3 or NLRC4 (190, 191). There is some evidence that pyrin, a non-NLR cytoplasmic sensor, is required for inflammasome activation in response to *F. novicida* (114).

Several groups have published that a non-NLR sensor AIM2 recognizes *F. tularensis* DNA, which leads to inflammasome activation (97, 149, 232, 276); however, it is likely that this is not the only mechanism in play, and whether AIM2 is important for recognition by human cells is not clear. Type I interferon (IFN-α/β) is secreted in response to *F. tularensis* in a TLR-independent, interferon regulatory factor (IRF) 3-dependent manner and is necessary for caspase-1 processing, macrophage cell death, and IL-1β secretion (133). While Type I interferon secretion does not require the AIM2 inflammasome activation, Type I interferon and IRF-3 are required for activation of the AIM2 inflammasome in response to *F. tularensis* infection (97, 149). In contrast to these studies, another report argues that IFN-β is not required for inflammasome activation in DC's or control of replication in this cell type but rather is important for the production of IL-12p40 (24). There is still a lot to learn about the inflammasome pathways activated in response to *F. tularensis* infection, and if and how *F. tularensis* is suppressing this arm of the pro-inflammatory pathway is not completely clear.

Many immunological studies with *F. tularensis* have been done *in vivo* or in macrophages, but several studies have addressed the specific roles of other cell types to the host innate immune response to *F. tularensis* infection. Interestingly, mast cells are recruited to the lymph nodes and lungs during pulmonary infection with *F. tularensis* LVS, and inhibit *F. tularensis* uptake and growth in macrophages as well as provide protection in a mouse model (153). The role of mast cells to control *F. tularensis* infection requires the cytokine IL-4 and involves increased ATP production and phagosomal acidification of infected cells (153, 240). In endothelial cells, *F. tularensis* does not induce secretion of significant levels of pro-inflammatory chemokines and cytokines, specifically CCL2, MCP-1 and IL-8 by this cell type, and although neutrophil recruitment was involved, the neutrophils displayed

blunted responsiveness (98, 210). The above data demonstrating downregulation of cytokines and chemokines makes sense in light of a study showing that both LVS and Schu S4 actively suppress the pro-inflammatory response of endothelial cells by acting through the anti-inflammatory endothelial protein C receptor (EPCR) (41). Type II epithelial cells exposed to *F. tularensis* secrete several cytokines and chemokines including IL-8, MCP-1, and GRO-α, and this secretion is NF-κB-dependent and promotes recruitment of neutrophils (116). Overall, these studies support the idea that *F. tularensis* also modulates the pro-inflammatory response by altering chemokine release, leukocyte adhesion molecule expression, and subsequent recruitment of neutrophils, albeit less successfully.

Adaptive immune response

The adaptive immune response to *F. tularensis* is largely a T-cell-mediated response and several T-cell populations are essential for resolution of infection (70, 72, 73, 89, 90, 237). Interferon gamma (IFN-γ) was first shown to be required for an effective response to *F. tularensis* infection (103), with contribution of TNF-α that is thought to activate natural killer cells to secrete IFN-γ (166, 180). B-cells also play a minor role in protection against *F. tularensis,* and this B-cell response is independent of antibodies (77, 87). Not surprisingly, the immune response is dependent on the route of infection. Specifically, while IFN-γ is important in controlling infection following an intradermal inoculation, after intranasal inoculation during the subsequent infection in the lung, IFN-γ is not as important. Although T-cells are still involved, the composition and kinetics of the T-cell response in the lung is different than that of an intradermal infection (66, 67, 290). Instead, a role for Th17 cells has been shown following intranasal inoculation (290). Finally, there have been several *F.*

tularensis T-cell epitopes identified, with the most prominent epitope being a peptide from the outer membrane protein Tul4 (104, 120, 264, 265).

In addition to suppressing the innate immune response, *Francisella* also modulates the adaptive immune response to promote infection. As mentioned above, *F. tularensis* has been shown to suppress IFN-γ secretion (218). Another mechanism of suppression is through the induction of infected macrophages to secrete prostaglandin E2 (PGE_2), which skews the T-cell response to an anti-inflammatory, Th2-like response by increasing IL-5 production (291). PGE_2 has also been shown to be important *in vivo* following intranasal inoculation where it delays production of IFN-γ-positive T-cells and promotes IL-17 secretion and Th17 cell production (74, 192, 290). Blocking IL-12 and producing IL-23, both of which result in an increase of Th17 cells, are also important in pulmonary infections of *F. tularensis* (47, 82, 88).

FRANCISELLA RESPONSE TO THE HOST

Despite the fact that *F. tularensis* is a successful pathogen, there is still a lot to learn about its virulence mechanisms, in part, because many of the classic virulence factors present in other bacterial pathogens have not been identified in *F. tularensis* (163). Furthermore, only recently have the genetic systems for large mutagenesis screens and generation of mutant libraries of *F. tularensis* been possible (23, 43, 112, 151, 168, 175, 184). In order to identify *F. tularensis* virulence factors, several genome-wide screens have been completed focusing either on intracellular replication, host cell cytotoxicity, phagosome biogenesis, or *in vivo* (3, 4, 15, 156, 158, 183, 212, 228, 259, 267, 272, 284). From these screens, as well as *in silico* analyses, *F. tularensis* has been shown to have a capsule, Type IV pili, several proteases, iron

acquisition systems, and the *Francisella* Pathogenicity Island (FPI), all of which are discussed below. Other *Francisella* virulence factors include *mviN* (276), citrulline ureidase (182), a potassium uptake protein TrkH (7), *minD* (11), *valAB* (198), an alanine racemase (123), a purine biosynthetic enzyme with homology to glutamine phosphoribosylpyrophosphate amidotransferases (123), *purMCD* (220), *purF* (229), an AcrB RND efflux pump (31), OppB oligopeptide transporter (40), and FTL_0200 encoding a putative AAA+ ATPase of the MoxR subfamily (81). Finally, numerous hypothetical proteins were identified as virulence factors in the screens mentioned above and remain to be characterized.

Francisella pathogenicity island

The FPI is an approximately 30 kb region consisting of 17 genes and located between inverted repeat sequences in the *F. tularensis* genome (213). Furthermore, the FPI is present in all *F. novicida* and *F. tularensis* strains, but while there is only one copy in *F. novicida*, the FPI is duplicated in *F. tularensis* (213). There are other differences in this region between strains. One major difference is found in the 3' end of the FPI where *holarctica* strains are missing *pdpD* and *anmK*, and *F. novicida* encodes an additional 48 amino acids in PdpD (181, 213). The *anmK* gene has stop codon(s) inserted, differing on location and number between subspecies and clades, and the specific role for *anmK* in virulence is not clear (181). Before discovery of the FPI, one its component genes, *iglC*, was identified as encoding a 23 kD protein that was upregulated during intracellular growth (121) and soon after, both *iglC* and *iglABCD*, one of the putative operons within the FPI, was shown to be required for intracellular growth (123, 157). Other individual genes within the FPI have been shown to be

required for virulence including *pdpD* (181, 213), *pdpA* (213, 256, 257), *iglA* and *iglB* (78), *iglD* (248), *pmcA/anmK* (181), FTT_1347 (22), *iglI* (22, 38), and *iglG* (38). Interestingly, PdpE is not required for virulence (38). Of the FPI proteins, at least IglC (34, 170, 249), PdpA (256), IglD (34), IglG (38) and IglI (38), are important for phagosomal biogenesis and/or escape. Additionally, IglA and IglB have been shown to interact (78), but otherwise, the function of the FPI proteins are unknown.

There is some evidence that the FPI may encode a secretion system that is distantly related to the Type VI secretion system (T6SS) found in several other bacterial species (22, 78). One study in *F. novicida* shows that the VgrG homolog (FTT_1347), is secreted into macrophages and in culture supernatants as observed for VgrG in other T6SS; however, unlike other T6SS, secretion of VgrG is independent of the known FPI T6SS homologs (22). Also unlike other characterized T6SS, the Hcp-homolog PdpE is not secreted in *F. novicida* (22). Curiously, using deletion mutants of either the FPI, *vgrG*, or the *icmF* homolog *pdpB* in *F. novicida*, it was shown that IglI is secreted and that this secretion is dependent on the FPI, VgrG, and PdpB (22). Overall, the studies on the FPI suggest that the proteins are part of a secretion system, potentially a novel secretion system similar but distinct from the T6SS.

Regulation of virulence factors

Regulation of virulence factors in *F. tularensis* has been largely focused on the FPI genes. MglA is one of the key regulators of FPI gene transcription as well as at least 100 other genes, including ones encoding secreted proteins PepO and BglX and genes involved in oxidative and general stress response (40, 124). MglA is also required for phagosomal escape, intracellular replication, and virulence *in vivo* (23, 34, 164, 249) and for replication in

mosquito and *D. melanogaster* cells (235, 246). In order to function as a transcriptional regulator, MglA binds to the RNA polymerase (RNAP) to direct transcription of the subset of genes mentioned above (52). This interaction with RNAP is dependent on MglA first binding to a protein called SspA and the MglA-SspA heterodimer then binding to RNAP; thus, the SspA regulon overlaps with that of MglA (52). Another regulator, MigR, regulates the FPI and other genes in the MglA/SspA regulon (39). FevR (also called PigR) has also been shown to regulate both FPI genes and other genes, and this regulation involves the small alarmone guanosine-tetraphosphate (ppGpp) which promotes the interaction between FevR and MglA to coordinate regulation of the same set of genes (42, 51).

Another distinctive aspect of *F. tularensis* is that the genome does not appear to encode any classic paired two-component regulatory systems, but does contain orphaned members (163). The orphan response regulator PmrA has been shown regulate the FPI as well as numerous other virulence genes and is required for both intracellular replication and *in vivo* pathogenesis (207, 244). More recently it was shown that PmrA is phosphorylated by a kinase KdpD and may interact with MglA and SspA (26). QseC is another orphan two-component system member, which its partner, QseB, was only recently identified, and both genes were required for biofilm formation, typical of QseBC in other bacteria (84).

Another important *F. tularensis* regulator is Hfq, which traditionally acts as a post-transcriptional regulator that facilitates the interaction between small non-coding RNAs in and RNA transcripts, which in turn decreases or enhances translation of the RNA transcripts. Hfq is not only valuable as a global regulator for stress tolerance, but also for its contribution to the regulation of virulence genes, including some genes found in the FPI (50, 200). Additionally, recent *in silico* and experimental analyses suggest that *F. tularensis* has several

non-coding small RNAs that are involved in gene regulation, although interactions with Hfq were not determined (226). In addition to virulence regulators, *F. tularensis* encodes the major sigma-70 factor (σ^{70}), as well as a σ^{32} factor RpoH that regulates numerous genes required for heat-shock response (122).

Regulation of both gene and protein expression occurs in response to numerous environmental stimuli including inside the host macrophage (121, 283), different culture medias (43, 132, 293), heat shock (91, 122), osmotic and membrane stress (200), starvation and oxidative stress (91, 124, 167), mammalian body temperature (91, 138), iron limitation (79, 167), and glucose (138). A surprising result from a recent study demonstrated that *F. tularensis* alters gene expression in response to spermine and that this gene regulation is largely mediated through adjacent insertion sequence elements (48).

Iron acquisition

As mentioned, *F. tularensis* gene expression changes in response to iron levels, which is not unexpected considering the iron is an essential metal that bacteria require for normal metabolism and is actively sequestered from pathogens by the host. The role of iron in *F. tularensis* infection was first observed in the context of iron acquisition upon phagosomal acidification in *F. tularensis*-infected cells (102). A common mechanism of iron uptake by bacteria is the use of siderophores, secreted iron-binding molecules. In *F. tularensis*, the *fslABCDEF* operon, is required for production of a siderophore similar to the polycarboxylate siderophore rhizoferrin and is required for iron acquisition and for growth in iron-limiting conditions (79, 154, 268). A highly conserved transcriptional regulator required during iron-limiting conditions is the ferric uptake regulator Fur, and *F. tularensis* encodes a

Fur protein that regulates genes involved in iron acquisition (79, 92, 93, 131). Later, *fslE* was also shown to be required for siderophore utilization, growth in iron-limiting conditions, and was under control of the Fur promoter as part of the *fsl* operon in Schu S4 (231). Interestingly, the *F. tularensis* genome does not appear to have *tonB, exbB*, or *exbD*, three genes encoding proteins that are typically required for siderophore uptake (163). Another protein recently described as being involved in both siderophore-dependent and -independent iron uptake by Schu S4 is FupA (FTT_0918) (171). In LVS, the FupA homolog (FTL_0439), which is a fusion protein of two adjacent genes in Schu S4, FupA/B, is required for siderophore-mediated iron uptake, and unlike Schu S4, FslE in LVS is considered a secondary player in siderophore-mediated iron uptake (260). FupA is required for virulence for both LVS and Schu S4 (171, 260, 275). These studies display some differences in iron uptake between *Francisella* strains, and both these differences as well as specific mechanisms of iron uptake need to be explained.

Protection from oxidative stress

Oxidative stress can also induce the expression of iron uptake genes through activation of Fur as well as the regulator OxyR (294). In fact, *F. tularensis* subspecies *tularensis* displays decreased susceptibility to hydrogen peroxide killing that is associated with decreased intracellular iron content (172). The role of reactive oxygen and nitrogen species in controlling intracellular bacterial infections in general is well understood. *F. tularensis* was first shown to be killed by nitric oxide in IFN-γ-activated macrophages and soon after, host nitric oxide production was shown to be correlated with increased resistance to *F. tularensis* infection *in vivo* in a mouse model (103, 134). Using both peritoneal exudate

cells and mice deficient for production of reactive nitrogen and oxygen species, another group demonstrated more definitively that there are distinctive roles of reactive nitrogen and oxygen species to controlling both *F. tularensis* replication in host cells and an infection *in vivo* (170, 174) Macrophages infected with *F. tularensis* have also been shown to upregulate proteins involved in the oxidative stress response of the macrophage (9, 283). Despite this host response, *F. tularensis* replicates within macrophages, as discussed above. In neutrophils, *F. tularensis* survives intracellularly, at least in part due to blocking the oxidative response through prevention of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase formation and gp91/p22^{phox} or p47/p67^{phox} acquisition on the phagosome, and this blockage leads to a failure to control infection *in vivo* as well (83, 178, 179, 194, 227).

F. tularensis encodes for several proteins involved in the response to oxidative stress common to other bacteria, though not many have been well-characterized. The superoxide dismutase SodB is required for virulence and for resistance to redox cycling agents paraquat and hydrogen peroxide (16). KatG, the catalase present in *F. tularensis,* is also required for resistance to the oxidative burst as well as for inhibition of pro-inflammatory signaling pathways, likely through PTEN, which is activated in oxidative conditions (173, 201). Finally, an attenuated strain of *F. tularensis* expressing a highly active form of AcpA was purified and shown to specifically inhibit the superoxide formation in neutrophils (236). AcpA in *F. novicida* was also shown to function as a phosphatase required for phagosomal escape (206). Later it was demonstrated that all four acid phosphatases, AcpABC and Hap, in *F. novicida* are required for the inactivation of the NADPH oxidase, resistance to phagocyte oxidative burst, and virulence *in vivo* (208, 209). Notably, in *F. tularensis* subspecies

tularensis and *holarctica,* AcpA is not required for inhibition of the oxidative burst, and AcpABC proteins are not required for Schu S4 virulence *in vitro* or *in vivo* (56, 195). Together these studies highlight a key difference between the human-virulent *F. tularensis* species and *F. novicida.*

The capsule

A polysaccharide capsule is one common virulence factor found on many but not all pathogenic bacteria, likely including *Francisella.* The presence and type of capsule expressed by *F. tularensis* is a subject of debate. *F. tularensis* was originally shown to have a capsule-like substance surrounding the bacterium over thirty years ago (55, 60, 136, 245). Mutants lacking this capsule structure demonstrate increased sensitivity to serum but reduced sensitivity to killing by neutrophils and are also avirulent in mouse models of tularemia (19, 55, 245, 266). Furthermore, the composition and expression of the capsule can change depending on the growth conditions (55, 293). The *F. tularensis capBCA* locus has partial homology to the *cap* locus in *B. anthracis* that encodes for proteins needed to synthesize the poly-D-glutamic acid capsule (163, 185). Interestingly, while *F. tularensis capBCA* is required for intracellular growth and in mouse models of tularemia for both Schu S4 and LVS (69, 146, 202, 267), *F. tularensis* capsule has not been reported to consist of poly-D-glutamic acid (202, 234). Early studies suggest that the capsule consists of carbohydrates, including mannose, rhamnose, and two other unidentified sugars, amino acids, and fatty acids (136). A more recent study found that the *F. tularensis* capsule is made up of O-antigen capsular polysaccharide and its production is dependent on the *wbt* O-antigen glycosyltransferases gene cluster (12). Three additional genes, FTT1236-1238 are also

required for O-antigen and/or O-antigen capsule formation (169). Besides the O-antigen capsular polysaccharide, higher molecular weight carbohydrate complex has also been identified in isolated capsule and consists of glucose, galactose, mannose, as well as Proteinase K-resistant protein, and this complex synthesis is dependent on the glycosyltransferase genes FTT_1422 and FTT_1423 (19). So although it is largely accepted that *Francisella* has a capsule, the composition, biosynthesis, and regulation of the capsule are not well defined.

Type IV Pili

The use of type IV pili is a common way bacteria adhereto host cells and/or use for motility, yet the presence, composition, and function of type IV pili in *Francisella* are also subjects of debate. Type IV pili-like structures were first observed on LVS using microscopy (117) and later observed on *F. novicida* (117, 295) and Schu S4 (14). Pili formation in LVS and *F. novicida* is dependent on both the assembly ATPase PilB as well as the disassembly ATPase PilT (49, 295). Surprisingly, in *F. novicida* pili formation is not dependent on PilC or PilQ, which are proteins typically required for pilus assembly in other bacteria, though it has yet to be determined whether this is the case for other *Francisella* strains (295).

In terms of pilus composition, PilA is typically the pilin subunit that makes up the pilus in Gram-negative bacteria; however, *Francisella* encodes six different pilin genes, *pilA/pilE1, pilE/pilE2, pilV/pilE3, pilE4, pilE5, and pilE6*. *F. tularensis* subspecies *holarctica* is the most divergent in the putative pilin genes. In several *holarctica* strains including LVS, *pilA* is absent from the genome, there are non-sense mutations in *pilE/pilE2* and *pilV/pilE3,* and the *pilE4* sequence longer in length (163, 243). In *F. tularensis*

subspecies *tularensis* and *holarctica* and *F. novicida* strains that do encode for PilA, the only difference is found in the 3' end of *pilA* in *F. novicida* (295). The role of these individual proteins in pilus composition has yet to be determined, and there are differences between subspecies. There is some evidence that the major pilin for *F. novicida* is PilE/PilE2 (295). Using heterologous expression of Schu S4 and *F. novicida pilA* in *Neisseria gonnorhea,* another group suggested that *pilA* is sufficient for pilus formation (242). Another recent study showed that in both LVS and Schu S4, *pilE4* was required for pilus formation (14). Interestingly, *pilE4* was not required for virulence; however, *pilE5* and *pilE6* were not required for pilus formation but were required for virulence in LVS only, again highlighting subspecies and species differences (14).

The role of the *pil* genes and pili formation to virulence is also different between strains. In terms of Schu S4, only recently was it demonstrated that *pilA, pilE5, pilE6, pilC*/*pilG* and *pilQ* are required for virulence *in vivo*, while *pilT* and *pilE4* are not (14, 101). As explained above, of these, only *pilE4* has been shown to be involved in formation of pili. For LVS, *pilE5* and *pilE6* were shown to be important for virulence *in vivo*, yet in two other *holarctica* strains, *pilA* was required for virulence *in vivo* (100). Finally, in *F. novicida,* the roles of *pilA/pilE1* and *pilE4* in virulence *in vivo* are variable between studies (127, 295). These discrepancies in a role for pili formation and the *pil* genes to virulence as well as among strains of *Francisella* only further complicate elucidation of the function of type IV pili. Some have ascribed virulence differences to a role for pili in Type II secretion, as discussed below.

Protein secretion

To date, all bacteria have (or are predicted to have/encode) mechanisms for secretion of effector molecules and proteins into the environment in order to support survival and replication, and *Francisella* is no exception. Although no toxins or other well-characterized secreted effectors have been identified in *Francisella*, several secreted proteases have been identified including ClpB heat shock protease (123), FTT1209c metallopeptidase family M13 protein (40), PepO zinc protease (127), BglX beta-glucosidase (127), and hemolysins (160). Unlike many other pathogens, *Francisella* does not encode any classic Type III, Type IV, or Type V secretion system; however, there are Type I and Type II secretion systems as well as the cytoplasmic membrane Sec translocon (163, 189). For Type I secretion, TolC is the prototypical outer membrane efflux pump that interacts with inner membrane ATPases for secretion of toxic products. *F. tularensis* encodes a functional TolC protein involved with multidrug resistance and is required for intracellular replication and virulence *in vivo* (118, 224). *Francisella* also encodes a putative secretion system in the FPI, as described above.

The Type II secretion system in *Francisella* is thought to be synonymous with Type IV pili encoded by the *pil* genes. The key difference is the use of GspC, GspL, and GspM as well as several periplasmic proteins in Type II secretion; however, *Francisella* does not encode GspC, GspL, and GspM, so it's possible that the Type IV pili system is sufficient. Evidence of Type II secretion being functional in *Francisella* was determined by the observation that secretion of seven *F. novicida* proteins was dependent on *pilA/pilE1, pilB/pilF, pilC/pilG, and pilQ,* although these genes were not required for intracellular replication, they were important *in vivo* (127, 128, 295). Furthermore, *pilE4,* which is required for pilus formation in *F. novicida,* is not required for secretion (295). In LVS, *pilE5*

and *pilE6* were not required for pilus formation but were required for virulence and the authors suggest a role for these proteins in secretion (14). Clearly, much remains to be elucidated in regards to mechanism and secreted effectors of Type II secretion as well as its relation to type IV pili and virulence.

DISCOVERY AND INITIAL CHARACTERIZATION OF RIPA

Using a LVS transposon mutant library screen for intracellular replication in epithelial cells, our lab identified the locus FTL_1914. Deletion of this locus via allelic exchange resulted in an LVS mutant (LVSΔ*ripA*) that is deficient for intracellular replication in both epithelial cells and macrophages and therefore was named *ripA*, required for intracellular proliferation, factor A (110). Interestingly, LVSΔ*ripA* escapes the phagosome and re-enters the autophagosome-like vacuoles with the same kinetics as wild-type (110). Both *ripA* gene expression and RipA protein expression are upregulated at higher pH 7.5 and *ripA* expression is also upregulated upon host cell infection, especially between 1-6 hours post invasion when bacteria have begun to enter the cytoplasm and replicate (111). Furthermore, deletion of the FPI transcriptional regulators *mglA* and *sspA* in LVS results in increased *ripA* expression, suggesting that these regulators inhibit *ripA* transcription. LVSΔ*ripA* is also attenuated in a mouse model of pulmonary tularemia (110). A Δ*ripA* mutant in the highly virulent Schu S4 strain is also attenuated in a pulmonary mouse model and defective for intracellular replication (our unpublished results). Altogether these data suggest that *ripA* is required for virulence of *F. tularensis.*

RipA is a cytoplasmic membrane protein of 179 amino acids and approximately 17 kD (110). While BLASTp analysis of the RipA protein sequence did not identify any

functional motifs or homologs with known functions, it revealed that there are RipA-like proteins found in select strains of a few randomly-distributed bacterial species (110). None of these RipA-like proteins have any known function. Interestingly, in a recent study RipA was identified as a glycosylated protein in a screen using lectin affinity chromatography (18). However, despite the importance of *ripA* to *F. tularensis* pathogenesis, the function of this protein is unknown. To address the question of RipA function, our lab began a search for RipA-interacting proteins. Since RipA was predicted to have two large cytoplasmic domains, we hypothesized that RipA likely interacts with cytoplasmic proteins. The search for RipA-interacting proteins was initiated by means of immunoprecipitation using HA-tagged RipA and LVS lysates (109). These studies revealed a list of putative RipA-interacting proteins, one of which was confirmed using reciprocal pull-down experiments (109). This RipA-interacting protein was named IclR due to its homology to the IclR family of transcriptional regulators (109). The *F. novicida* U112 IclR homolog had previously been described as being required for *F. novicida* virulence (284). Due to the role of IclR for the virulence of *F. novicida* and our results demonstrating an interaction with RipA, we considered that its role in virulence may be at least in part through its interactions with RipA.

Therefore, as described in chapter 2, we investigated the biological relevance of the IclR-RipA interactions through characterization of *F. tularensis* strains lacking *iclR.* In chapter 3, through the use of the *ripA* deletion mutant, we uncover a role for RipA in suppression of the immune response and elucidate pathways suppressed by wild-type *F. tularensis*. Finally, chapter 4 details work that characterizes RipA at the molecular and biochemical level in order to determine topology and to identify functional domains.

Together these studies help to elucidate RipA's role in virulence and function, as well as reveal insights into the overall pathogenesis of *F. tularensis.*

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CHAPTER 2

Effects of the putative transcriptional regulator IclR on *Francisella tularensis* pathogenesis.

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ABSTRACT

Francisella tularensis is a highly virulent Gram-negative bacterium and is the etiological agent of the disease tularemia. IclR, a presumed transcriptional regulator, is required for full virulence of the animal pathogen, *F. tularensis* subspecies *novicida* U112 (53). In this study, we investigated the contribution of IclR to the intracellular growth, virulence and gene regulation of human pathogenic *F. tularensis* subspecies. Deletion of *iclR* from the Live Vaccine and SchuS4 strains of *F. tularensis* subspecies *holarctica* and *tularensis*, respectively, did not affect their ability to replicate within macrophages or epithelial cells. In contrast to *F. tularensis* subspecies *novicida iclR* mutants, LVS and SchuS4 Δ*iclR* strains were equally virulent as their wild-type parental strains in intranasal inoculation mouse models of tularemia. Furthermore, wild-type LVS and LVSΔ*iclR* were equally cytotoxic and induced equivalent levels of IL-1β expression by infected bone marrow-derived macrophages. Microarray analysis revealed that the relative expression of a limited number of genes differed significantly between LVS wild-type and *iclR* strains. Interestingly, many of the identified genes were disrupted in LVS and SchuS4 but not in their corresponding *novicida* U112 homologs. Thus, in spite of the impact of *iclR* deletion on gene expression, and in contrast to the effects of *iclR* deletion on *F. tularensis* subsp. *novicida* virulence, IclR does not contribute significantly to the virulence or pathogenesis of *F. tularensis*

INTRODUCTION

Francisella tularensis is a Gram-negative bacterium and the etiological agent of tularemia or "rabbit fever". While zoonotic hosts include small mammals such as rabbits and voles, *F. tularensis* is also found in ticks, mosquitoes, and flies, and can replicate within amoebae as well (29). Human infection with *F. tularensis* can occur by several routes including bites by arthropod vectors (4, 5, 34), contact with contaminated tissues, ingestion of contaminated food or water (28, 43), or inhalation of aerosolized bacteria (18, 48). *F. tularensis* is considered a Select Agent by the Centers for Disease Control due to its low infectious dose (as few as 10 organisms) via the pulmonary route and its potential as a biological threat agent (15, 46).

There are two *F. tularensis* subspecies most commonly associated with disease in humans: *F. tularensis* subspecies *tularensis* (Type A) and *F. tularensis* subsp. *holarctica* (Type B). The Live Vaccine Strain (LVS) of subsp. *holarctica* is a useful model for studying the virulent *F. tularensis* subspecies, because it causes disease in mice, is attenuated in humans (19), and shares genomic and proteomic similarity with *F. tularensis* subsp. *holarctica* and *tularensis* (51). *F. tularensis* subsp. *novicida*, which does not cause disease in healthy humans, has significant similarity with subsp. *holarctica* and *tularensis* and is also used as model organism for studying *F. tularensis* pathogenesis. Although there are reports of subsp. *novicida* causing disease, these cases are commonly associated with immunocompromised individuals (2, 9, 24, 32). However, subsp. *novicida* does cause a severe disease in *in vivo* mouse models (40).

Francisella is known to predominately infect and replicate within macrophages but also infects and replicates within neutrophils (37), dendritic cells (3) and Type II alveolar

epithelial cells (23). After phagocytosis, *F. tularensis* escapes the phagosome and replicates within the cytoplasm of host cells (1, 10). Numerous *in vitro* and *in vivo* screens have identified virulence factors required for this intracellular life cycle (13, 14, 27, 30, 35, 41, 47, 49, 53); however, many of the identified virulence factors have little or no similarity to known proteins of other bacteria and their functions remain, for the most part, unknown.

Weiss et al. recently identified a locus (FTN_0720) in *F. tularensis* subsp. *novicida* U112 that is important for virulence in mice as determined by an *in vivo* competition assay between a FTN_0720 deletion mutant and wild-type U112 (53). FTN_0720 encodes a protein with homology to the IclR family of transcriptional regulators. IclR family members activate and repress genes in a wide range of bacteria including genes involved in sporulation, metabolism, drug-efflux pumps and organic solvent tolerance, and phytopathogenicity (39). Given the close genetic relationship among the *F. tularensis* subspecies, the phenotype of the subsp. *novicida iclR* deletion strain suggests that IclR may be involved in the pathogenicity of the *holarctica* and *tularensis* subspecies. We investigated the contribution of IclR homologs in the pathogenicity of subsp. *holarctica* and *tularensis* by evaluating the role of IclR in gene expression, host cell interactions and virulence of *F. tularensis* subsp. *holarctica* LVS (FTL_1364) and subsp. *tularensis* SchuS4 (FTT_0748) strains.

MATERIALS AND METHODS

Bacterial strains.

F. tularensis subsp. *holarctica* LVS was obtained from the CDC, Atlanta, GA. *F. tularensis* subsp. *tularensis* SchuS4 was obtained from BEI Resources. *F. tularensis* subsp. *novicida* U112 was obtained from the American Type Culture 88 Collection (ATCC). An

iclR transposon mutant was one of two mutants from the transposon mutant library (21) and was received as a gift from Colin Manoil. All strains were maintained on chocolate agar supplemented with 1% IsoVitaleX (Becton-Dickson), brain heart infusion (BHI) broth supplemented with 1% IsoVitaleX or Chamberlain's defined medium (CDM) (6). *Escherichia coli* TOP10 (Invitrogen) were used for cloning purposes. *E. coli* was propagated in Luria broth supplemented with hygromycin at 200 μg/ml or kanamycin at 20 μg/ml as necessary for antibiotic selection. All cultures were grown at 37°C.

Cell Culture.

J774A.1 (ATCC TIB-67) cells are a macrophage-like cell line derived from mouse sarcoma reticulum cells and were cultured in Dulbecco's minimal essential medium with 4.5 g/L glucose, 10% fetal bovine serum, and 2 mM L-glutamine. TC-1 (ATCC CRL-2785) cells are a tumor cell line derived from mouse primary lung epithelial cells and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10 mM HEPES and 0.1 mM nonessential amino acids. Bone marrow-derived macrophages were generated by flushing bone marrow cells from C57BL/6 mouse femurs and recovered cells were incubated for 6 days on 15 cm^2 non-tissue culture-treated dishes in L929 cell-conditioned DMEM. Nonadherent cells were removed by washing with phosphate-buffered saline (PBS) and bone marrow-derived macrophages were recovered from the dish using 1 mM EDTA in PBS.

Molecular techniques and allelic exchange.

For both LVS and SchuS4 the *iclR* deletion was generated by splice overlap extension (SOE) PCR using primers designed to amplify the 5' and 3' regions of the *iclR* locus, which were then annealed to their complementary, homologous genomic DNA tags (20, 25). The subsequent deletion left only the first six amino acids and the stop codon of *iclR*. Each construct was cloned into the pCR-Blunt II TOPO vector (Invitrogen), verified by DNA sequence analysis, and subsequently cloned into pMP590 (*sacB* Kan^r) using BamHI and NotI restriction sites (20, 33). For allelic exchange, plasmids were electroporated into LVS or SchuS4 and integrants were selected on chocolate agar containing kanamycin (10 μg/ml). Kan^r strains were grown overnight and plated on 10% sucrose for counterselection (loss of plasmid) (20, 33). Both the LVS and SchuS4 *iclR* deletion strains were confirmed for loss of *iclR* by PCR. For complementation of the *iclR* deletion in LVS, *iclR* and its predicted promoter were PCR-amplified and subcloned into the pCR-Blunt II TOPO vector (Invitrogen). After a MluI/EcoRV restriction digest, the construct was ligated into the pMP633 low-copy *Francisella* shuttle vector (20) and electroporated into LVSΔ*iclR*. Complementation was determined by detection of *iclR* in the complementation strain via PCR as well as demonstration of increased *iclR* transcript levels via microarray analysis (data not shown).

Gentamicin protection assays.

Gentamicin protection assays were performed as described (20, 23). Briefly, J774A.1 murine macrophages, TC-1 murine lung epithelial cells, or bone marrow-derived macrophages were infected with LVS or SchuS4 at an MOI 100. Cells were incubated with

the bacterial inoculum for 2 hr (J774A.1 and bone marrow-derived macrophages) or 4 hr (TC-1) and then incubated with media containing 25 μg/ml gentamicin for an additional 2 hr to kill extracellular bacteria. At time points of 4 hr (or 6 hr for TC-1) and 24 hr, medium was removed, cells were washed with PBS and then scraped from the dish, and the bacteria serially diluted and plated to determine the number of viable bacteria.

Mouse infections.

6- to 8-week-old C57BL/6 mice were anesthetized intraperitoneally (i.p) with avertin and then inoculated intranasally (i.n.) with bacteria suspended in 50 μl PBS by application to the nares of each mouse or inoculated intradermally (i.d.) by injection into the tail using the same volume. Concentrations of LVS and U112 were determined by klett and concentrations of SchuS4 by spectrophotometer (OD600), and inocula were serially diluted and plated on chocolate agar to confirm the CFU administered. At the designated time points, mice were euthanized and the lungs, liver and spleen of each mouse were removed and homogenized. Serial dilutions of the homogenates were plated on chocolate agar to enumerate the bacterial organ burdens. Statistical significance between strains in each organ and at each time point was determined by the Mann-Whitney nonparametric test using GraphPad Prism v.5 software. All animal experiments were performed according to the animal care and use guidelines as established by IACUC-approved protocols.

IL-1β ELISAs and cytotoxicity assays.

Bone marrow-derived macrophages were seeded in 12-well dishes at $1x10^6$ cells per well, and infected with bacteria at an MOI 500 in a final volume of 1 ml medium per well

and incubated at 37°C. After 24 hr, the supernatants from each well were collected, centrifuged to pellet cellular debris, and stored at -20°C. The IL-1β ELISA was performed using the BD OptEIA mouse IL-1β ELISA kit (BD Biosciences) according to the manufacturer's protocol. The OD_{450} was read using a TECAN Infinite M200 and analyzed using Magellan v6 software. Cytotoxicity assays were performed using the ToxiLight® BioAssay kit (Lonza) following the manufacturer's protocol for cytokine detection from supernatants, and the luminescence was read using a TECAN Infinite M200 and analyzed using Magellan v6 software. Statistical significance between each strain was determined by the student's t-test using GraphPad Prism v.5 software.

Microarrays.

RNA was obtained using the RiboPure-Bacteria kit (Ambion) according to the manufacturer's protocol. Briefly, bacteria were grown to early mid-log phase in CDM and pelleted. Cells were disrupted by suspension in Trizol and vortexing with 0.1 mm glass beads. Purified RNA was recovered by chloroform extraction followed by treatment with DNase I to remove DNA. Microarray analysis was performed following the guidelines provided by the Venter Institute for Genomic Research (SOP#M007, M008). Briefly, aminoallyl labeled cDNA was generated from 2 μg total RNA using SuperScript III reverse transcriptase (Invitrogen), random hexamers, and dNTPs containing aa-UTP. After removal of unincorporated aa-dUTP and free amines, labeled cDNA was coupled to Cy3 or Cy5 mono-reactive dye (GE Healthcare). The *Francisella* microarray slides (Pathogen Functional Genomics Resource Center; PFGRC) contained 2331 70mer oligonucleotides in quadruplicates of the *F. tularensis* SchuS4 genome and several LVS genes as well as

quadruplicates of 70mer oligonucleotides for 500 *Arabidopsis thaliana* genes as controls. Slides were prehybridized in 5x SSC, 10% SDS and 1% BSA, washed and then hybridized with cDNA probes at 42°C. After post-hybridization washes, the slides were scanned using the GenePix 4000B scanner and GenePix Pro v6.0 software. The microarray data were normalized using the TIGR MIDAS v2.22 and analyzed using the TIGR Multiexperiment Viewer v 4.2.1 (MeV) as part of the TM4 Suite software (45). In MeV, pooled, normalized Cy5/Cy3 intensities from wild-type LVS control arrays were compared to pooled, normalized Cy5/Cy3 intensities from LVSΔ*iclR* arrays. This list was filtered by statistical significance using Significance Analysis for Microarrays (SAM) provided on MeV after 179 an 80% cut-off filter and using a false discovery rate of 5%.

Quantitative RT-PCR.

Quantitative RT-PCR was performed in a 96-well format using the SensiMix™ SYBR & Fluorescein One-Step kit (Bioline) following the manufacturer's protocol. Briefly, 50 ng of RNA isolated from wild-type or *iclR* mutant strains was mixed with SensiMix™ SYBR & Fluorescein, RNase inhibitors, and designated primers in a 20 µl volume. A genomic DNA ladder and a no reverse transcriptase control were analyzed using the SensiMix™ SYBR & Fluorescein kit following the manufacturer's protocol with primers to *gyrA*. Thermocycling and detection was performed using the iCycler Thermal Cycler (Bio-Rad). All starting quantity (SQ) values were normalized to the mean SQ value for *gyrA*.

Antibiotic sensitivity assays.

F. tularensis LVS was grown to mid-log phase in BHI broth supplemented with 1% IsoVitaleX, the bacterial suspension was spread onto chocolate agar plates, and antibiotic-containing filter paper discs were placed in the center of each plate. The rafampin (5μg), tetracycline (30μg), and colistin (10μg) were purchased pre-loaded from Becton Dickinson. The ampicillin and polymixin B discs were self-prepared by adding a 10 μl or 20 μl volume of antibiotic per disc at 10 μg ampicillin or 20 μg polymixin B. Bacteria were grown for 36 hr and the diameter of the zone of inhibition was measured.

Microarray data accession numbers.

The raw and normalized microarray 199 data is available on the GEO database under the following accession numbers: GSM574374, GSM574375, GSM574376, GSM574377, GSM574379, GSM574380, and GSE23454.

RESULTS

Comparison of *iclR* alleles among *F. tularensis* subspecies and construction of *iclR* deletion mutants.

The locus FTL_1364 is annotated as a hypothetical protein in NCBI; however, some of its homologs in other *Francisella* species are annotated as proteins belonging to the IclR family of transcriptional regulators. A search for conserved domains found within FTL_1364 resulted in several related hits including a helix-turn-helix (HTH) domain conserved among IclR family members. Additionally, *Francisella* IclR has a C-terminal domain with high similarity to the IclR family profile Pfam01614. A recent publication describes a highly

specific IclR family member profile that lies outside the HTH domain and covers less than 100 amino acids in the central region towards the C-terminal end (31). These authors classify current Pfam01614 members as belonging to the IclR family based on the new profile. Furthermore, BLASTp analysis of *F. tularensis* LVS or SchuS4 IclR reveals high similarity to IclR family proteins found across many bacterial species. *F. tularensis* IclR proteins share considerable amino acid identity (30-40%) and amino acid similarity (60%) with non-*Francisella* IclR family proteins. Overall, the bioinformatic analysis strongly suggests that *Francisella* FTL_1364 and its homologous loci in other *Francisella* species encode a protein belonging to the IclR family of transcriptional regulators.

Using NCBI and the *Francisella* genome browser (www.francisella.org) for annotations and synteny analysis, we found that the *iclR* locus has shared characteristics among *F. tularensis* subsp. *novicida* U112, *F. tularensis* subsp. *holarctica* LVS, and *F. tularensis* subsp. *tularensis* SchuS4 strains (FTN_0720, FTL_1364 and FTT_0748, respectively), (Figure 1A). On one side of *iclR* in each strain is a gene encoding a predicted protein with similarity to an esterase lipase (FTL_1363, FTN_0721, and FTT_0749). On the other side of *iclR* is a gene encoding a predicted protein with similarity to the multidrug efflux protein EmrA (FTL_1365-66, FTN_0718, and FTT_0747). One difference is that EmrA is divided into two ORFs in LVS. There are other differences in the length and coding sequences of this genetic region, including an additional open reading frame in U112 that encodes a predicted protein of unknown function FTN_0719. Nevertheless, in each strain, *iclR* is located in a similar region of the genome.

Additionally, *iclR* itself is highly conserved among the three *F. tularensis* strains U112, LVS and SchuS4. SchuS4 *iclR* has three nucleotide differences compared to *iclR* from

LVS that translate into two amino acid differences, S22G and H78Y, between LVS and SchuS4 IclR. U112 *iclR* has 95 nucleotide differences compared to LVS *iclR* and 94 nucleotide changes compared to SchuS4 *iclR*. Although this results in a three nucleotide truncation of U112 *iclR*, there is 80% amino acid identity between U112 IclR and SchuS4 and LVS IclR proteins (Figure 1B). While these similarities suggest that IclR is conserved among the U112, LVS, and SchuS4, there are a sufficient number of differences to account for possible functional deviations between these strains as well. Due to genetic similarity and the contribution of IclR to the virulence for *F. tularensis* subsp. *novicida*, we investigated the potential contribution of IclR to the virulence of *F. tularensis* subspecies *holarctica* and *tularensis*. To do this, we made a clean deletion of the *iclR* gene in the *F. tularensis* subspecies *holarctica* LVS (LVSΔ*iclR*) and *tularensis* SchuS4 (SchuS4Δ*iclR*) using SOE PCR and allelic exchange in LVS (FTL_1364). We also generated an *iclR* complementation strain by expression of *iclR* on a low-copy shuttle vector.

LVS and SchuS4 *iclR* deletion mutants are competent for intracellular replication.

One method to assess the contribution of IclR to *F. tularensis* virulence is to determine what role IclR plays in intracellular replication. We used gentamicin protection assays in the J774A.1 murine macrophage-like cell line and the TC-1 murine lung epithelial cell-like cell line to assess intracellular replication by *iclR* deletion mutant strains. Both LVSΔ*iclR* and wild-type LVS replicated approximately two logs by 24 hr in both J774A.1 and TC-1 cells (Figure 2A-B). We also performed these assays in bone marrow-derived macrophages, and both wild-type LVS and LVSΔ*iclR* replicated intracellularly in these cells (Figure 2C). Similarly, the intracellular replication of SchuS4Δ*iclR* was similar to wild-type

SchuS4 in J774A.1 cells (Figure 2D). These results demonstrate that IclR is not required for intracellular replication of LVS or SchuS4 in these cell types.

LVSΔ*iclR* is not attenuated following intranasal or intradermal inoculation of mice.

Properties other than intracellular replication contribute to *F. tularensis* pathogenesis. We therefore determined whether IclR was required for LVS virulence *in vivo*. To test this, we used a mouse model of pulmonary tularemia in which we inoculated C57BL/6 mice i.n. with a lethal dose ($1x10^5$ CFU) of LVS or LVSΔ*iclR*. At 1, 3, 7 and 8 days post inoculation the lungs, liver and spleen were harvested to enumerate the bacterial organ burdens (Figure 3A). These initial experiments revealed that there were no differences in the organ burdens at 1 or 3 days post inoculation. At day 7, there appeared to be slight differences in the organ burdens in the liver and spleen, and by day 8 the organ burdens in the liver and spleen had not increased. These initial experiments suggested that LVSΔ*iclR* may demonstrate enhanced clearance in the mouse. This would correlate with previously published data demonstrating a decrease in competitive index in the spleen at 48 hr for the subsp. *novicida* U112 *iclR* deletion mutant compared to wild-type *novicida* U112 (53).

To further investigate the possibility of a more subtle phenotype of enhanced clearance, we used a low dose ($1x10^3$ CFU) i.n. inoculation of groups of six wild-type C57BL/6 mice with LVS or LVSΔ*iclR*. At days 1, 3, 7 and 10 post inoculation, we again harvested the lungs, liver and spleen to calculate the bacterial organ burdens. There were no significant differences between the bacterial organ burdens of LVSΔ*iclR* or wild-type LVS at any time point (Figure 3B). This suggests that LVSΔ*iclR* is not attenuated in a mouse model of pulmonary tularemia.

Since the experiments with subsp. *novicida* U112 *iclR* deletion mutant were performed using subcutaneous (s.c.) and i.p. inoculation, we investigated whether a role for *iclR* in pathogenesis may be route-specific. Groups of 6 to 7 wild-type C57BL/6 mice were infected i.d. with $3x10^5$ CFU of LVS or LVSΔ*iclR*. The i.d. route has a comparable LD_{50} dose and is similar in nature to the s.c. route (17). At 1, 3, and 7 days post inoculation, we again harvested the lungs, liver and spleen and determined bacterial organ burdens. At each time point and in each organ, there was no significant difference in the bacterial burdens comparing LVS and LVSΔ*iclR* (Figure 3C). These data indicate that in LVS, *iclR* is not required for pathogenesis in the mouse via the i.n. or i.d. route.

SchuS4Δ*iclR* is not attenuated following intranasal inoculation of mice.

Although *iclR* does not appear to be required for LVS pathogenesis, it is possible that *iclR* plays a role in SchuS4 pathogenesis. We inoculated groups of four wild-type C57BL/6 mice i.n. with a lethal dose (100 CFU) of wild-type SchuS4 or SchuS4Δ*iclR*. At 1 and 3 days post inoculation, the lungs, liver and spleen were harvested to enumerate the bacterial organ burdens of infected mice (Figure 4). At both time points and in each organ, there were no differences in bacterial burden between wild-type SchuS4 and SchuS4Δ*iclR*. These data suggest that IclR does not play a role in the in vivo virulence of SchuS4 when assessed by the mouse model of pulmonary tularemia.

A subsp. *novicida* U112 *iclR* transposon mutant is attenuated following intranasal inoculation of mice.

As noted above, an *iclR* deletion mutant in subsp. *novicida* U112 displays decreased competitive index in the spleen following s.c. and i.p inoculation of mice (53). Therefore, we wanted to determine whether *iclR* is required for subsp. *novicida* U112 pathogenesis in a pulmonary mouse model. We inoculated groups of six wild-type C57BL/6 mice i.n. with a dose of approximately 10 CFU of wild-type U112 or a U112 *iclR* transposon mutant. At 1 and 5 days post inoculation, the lungs, liver and spleen were harvested and the bacterial organ burdens were enumerated. Each organ had reduced burdens of the *iclR* transposon mutant compared to wild-type U112, and at day 5 these differences were statistically significant in the liver and spleen (Figure 5). These data suggest that *iclR* is required for U112 pathogenesis via the i.n. route and correlates with the previously published data using the s.c. and i.p. routes.

Deletion of *iclR* does not affect IL-1β expression or cytotoxicity of infected cells.

To determine if there is an altered cellular response to LVSΔ*iclR* compared to wild-type LVS, we measured the production of pro-inflammatory cytokines by infected cells. Bone marrow-derived macrophages were infected at an MOI 500 with LVS or LVSΔ*iclR* and the supernatants were analyzed for IL-1β at 24 hr post infection (Figure 6A). The levels IL-1β measured in the supernatants of LVSΔ*iclR*-infected cells was similar to that of cells infected with wild-type LVS, and no differences between strains were statistically significant.

F. tularensis is also reported to induce cytotoxicity of infected macrophages. To determine whether there was a change in cytotoxicity induced by LVSΔ*iclR*, we infected murine bone marrow-derived macrophages with LVS or LVSΔ*iclR* at an MOI 500 and performed cytotoxicity assays on supernatants collected at 24 hr post infection. As shown in Figure 6B, LVSΔ*iclR* induces cytotoxicity in infected cells to a level similar to that of wild-type LVS, and no differences between strains were statistically significant.

The effects of IclR on gene expression.

Due to its homology to transcriptional regulators, we used microarray analysis to determine what genes in LVS were affected by IclR by comparing gene expression between the LVSΔ*iclR* mutant and wild-type LVS. We grew LVS and LVSΔ*iclR* to mid-log phase to harvest RNA for reverse transcription and amino-allyl labeling of cDNA, and the labeled cDNA was hybridized to microarray slides. The slides are printed for every annotated ORF for SchuS4, plus LVS alleles that are either not present or are variant in SchuS4, but they are not tailored to *F. tularensis* subsp. *novicida*. Three separate microarrays from independent RNA samples were pooled and statistically significant gene expression differences between LVSΔ*iclR* and wild-type LVS were determined by SAM (Table 1). Genes exhibiting significant changes in expression are listed by the provided locus annotations, LVS or SchuS4, as printed on the slides.

Using the above criteria, we identified 13 downregulated and 4 upregulated genes in LVSΔ*iclR*. The list of genes identified comprises diverse functional groups suggesting that IclR does not impact expression of one specific functional group of proteins. There were several IclR-affected genes annotated as encoding hypothetical proteins. To get a better idea

of what types of proteins these genes may be encoding and possibly obtain insight on IclR function, we performed BLASTp analyses. Many of the proteins were only conserved in *Francisella* with no similarity to proteins or conserved domains in other bacteria. However, there were several with similarity to known proteins in other bacteria and these are described in Table 1. Although most of the genes were represented exclusively by the SchuS4 allele, there were two cases where the SchuS4 and LVS homologs were both printed on the microarray slide and also appeared on the gene list as having significant expression changes in the absence of IclR. FTT_0741c and its FTL_1373 homolog were both upregulated in LVSΔ*iclR*, and both FTL_0388 and its homolog FTT_0885 were downregulated in LVSΔ*iclR*. Overall, although further studies need to be performed to demonstrate a function of IclR, both bioinformatic and microarray data suggest that *Francisella* IclR could function as a transcriptional regulator.

Comparison of IclR-regulated genes between LVS, SchuS4 and U112.

One explanation for the phenotypic differences observed for *iclR* mutants among the *F. tularensis* U112, LVS and SchuS4 strains could be due to differences in the genes affected by IclR among the strains. To address this we performed a more detailed examination of the genes on our microarray list. First, we performed synteny analysis using the genome synteny tool at www.francisella.org to determine whether each gene was annotated in SchuS4, LVS and U112. We observed that there were a few genes that were not annotated or not present in all three strains, as shown in Table 1. Secondly, we generated alignments and protein translations of the genes using Vector NTI software based on the NCBI annotation or the putative loci of non-annotated genes from the synteny analysis, if they were found. For

example, sequence alignments revealed that in LVS there is an unannotated ORF between FTL_1120 and FTL_1121 bearing homology to FTT_1082. Nearly half of the genes were similarly annotated and encoded one intact open reading frame (ORF) in SchuS4, LVS and U112. However, a significant percentage of genes displayed considerable sequence differences between strains as described in Table 1.

Of these genes, many were not intact in the virulent strains LVS and/or SchuS4, whereas the homologous genes in U112 were intact. For example, FTL_1506 and FTL_1507 are pseudogenes because they encode two ORFs while their SchuS4 (FTT_0723c) and U112 (FTN_0634) encode only one ORF. One special case is FTT0715, which along with its LVS homolog FTL_1521, has two large deletions, 131bp (119bp in LVS) and 197bp, when compared to the U112 homolog FTN_0627. The significance of these deletions cannot be inferred, and though these genes are not pseudogenes, the fact that these large deletions are present only in SchuS4 and LVS is noteworthy. This also highlights the fact that many of the intact genes on the microarray list have greater overall sequence differences between U112 and LVS or SchuS4 when compared to that of the differences between LVS and SchuS4.

We next wanted to determine whether the set of genes that were changed in expression in LVSΔ*iclR* were also changed in the absence of IclR in U112. First, we performed quantitative RT-PCR on six genes that were differentially-regulated in the microarray for LVS versus LVSΔ*iclR* (Figure 7A) and normalized to the housekeeping gene *gyrA*. We also included *iclR*. As expected, we detected a dramatic decrease in *iclR* transcript in LVSΔ*iclR* and negligible change in *gyrA*. Of the six genes analyzed, four repeated the trend seen in the microarray analysis. For the two genes that did not, the primers appeared to amplify with similar efficiencies to other primers (data not shown). Overall, the qRT-PCR

data supports the fact that the genes identified in our microarray are changed in expression in the absence of IclR using a different method. We then tested the same set of gene homologs on RNA isolated from wild-type U112 and the U112 *iclR* transposon mutant (Figure 7B). Quantitative RT-PCR first verified that *iclR* transcripts were substantially lower in the transposon mutant. Overall, the six selected genes appear to be changed similarly to their LVS homologs, suggesting a similar set of genes affected by IclR in U112. These analyses do not account for any additional genes affected by U112 IclR that were not affected by LVS IclR as detected by microarray. Furthermore, these analyses alone are not sufficient to extrapolate any correlations in terms of IclR function or which of the IclR-affected genes are likewise impacted at the protein level or functional.

The effects of IclR on antibiotic resistance.

Other IclR family proteins are known to be involved in the regulation of multi-drug efflux pumps (39). In all three *F. tularensis* subspecies, *iclR* is located near ORFs encoding hypothetical proteins that have homology to the EmrA multidrug efflux pump. In LVS, the two ORFs encoding proteins with EmrA homology that are found upstream of *iclR* were not changed in expression as determined by our microarray analysis. Nevertheless, the microarray data for LVSΔ*iclR* showed increased expression of a gene encoding a protein with homology to organic solvent tolerance proteins, suggesting that IclR may be involved in repression of some genes involved in drug efflux. Organic solvent tolerance is often associated with multi-drug efflux pumps, most notably in *Escherichia coli* and *Pseudomonas putida* (42). Furthermore, our BLASTp analyses of hypothetical genes that appear in the LVSΔ*iclR* microarray gene list also reveal proteins with homology to other transporter

proteins. To determine whether *iclR* is involved with drug efflux, we performed disc diffusion assays using a panel of antibiotics. Antibiotics selected for analysis were chosen as representatives from several classes of antibiotics targeting cell wall synthesis, protein synthesis, nucleic acid synthesis, and cell membrane integrity. There was no difference in antibiotic sensitivity between wild-type LVS and LVSΔ*iclR* using this method (Figure 8).

DISCUSSION

Herein we investigated the contribution of the putative transcriptional regulator IclR to *F. tularensis* pathogenicity. In this study, we found that the LVSΔ*iclR* was not attenuated for intracellular replication in J774A.1 macrophage-like cells, TC-1 epithelial cells, or bone marrow-derived macrophages. Similarly, SchuS4Δ*iclR* was not attenuated for replication in J774A.1 cells. These data are consistent with published data by Weiss et al. for the *novicida* U112 *iclR* deletion mutant strain in bone-marrow derived macrophages (53).

When compared to wild-type LVS, LVSΔ*iclR* did not impact IL-1β induction or cytotoxicity of infected cells, which is different from that of the published *novicida* studies (53). It is important to note that the methods used for these analyses were different between the two studies. The levels of IL-1β that we reported in this study are near but not below the limit of detection for the ELISA. The fact that the levels of IL-1β induced are low is consistent with other studies evidencing that LVS suppresses the inflammatory response (26, 50). Furthermore, Weiss et al. used pre-stimulated bone marrow-derived macrophages, whereas we used naïve bone marrow-derived macrophages. Macrophages pre-treated with LPS or heat-killed *F. tularensis* subp. *novicida* as well as thioglycolate-elicited macrophages produce higher levels of IL-1β in response to infection (11, 12, 36, 52). Another possibility is

that there are strain-specific differences in the role of IclR, as evidenced by the results of the *in vivo* studies discussed below.

Unlike the *novicida iclR* deletion mutant, neither LVSΔ*iclR* nor SchuS4Δ*iclR* were attenuated in mice following i.n. inoculation. There were differences in the experimental design between our studies and the *novicida* study. We initially inoculated mice i.n. and monitored lung, liver, and spleen over several days post infection. Weiss et al. used s.c. and i.p. inoculations in competition assays examining the spleen at 2 days post infection. It is possible that inoculation route may have an impact on the importance of IclR on establishing infection. To address the possibility that the phenotype is route-specific, we performed a reciprocal analysis by evaluating the virulence of LVS and U112 *iclR* mutants in i.d. and i.n. infection models, respectively. The results confirmed that the *Francisella* virulence-specific properties of IclR are restricted to subspecies *novicida*.

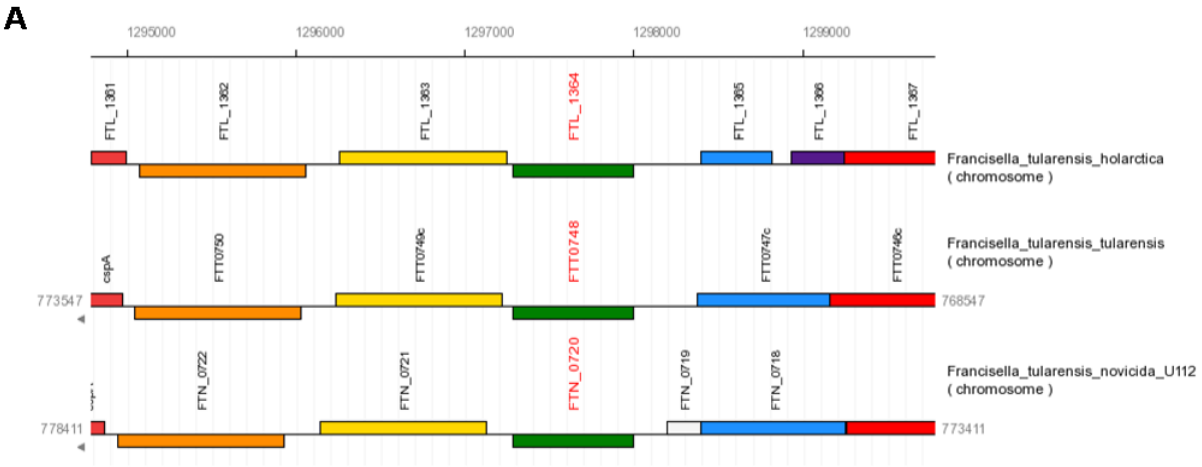
It is not clear why IclR is required for virulence in U112 but not LVS and SchuS4. Based on our microarray analysis, the subspecies-specific sequence differences among IclR-affected genes could contribute to the functional differences we observe for IclR between subspecies. Many of the genes are intact in U112, but in LVS and/or SchuS4, the homologous genes are pseudogenes or displayed significant sequence variation (e.g. two large deletions in FTT0715/FTL_1521). The virulent subspecies of *F. tularensis* are noted for their genome decay as characterized by smaller genomes as well as increased numbers of pseudogenes, transposases and gene rearrangements (51). Genome-wide analyses of *Francisella* strains support this idea and many of the genes changed in LVSΔ*iclR* that we identified to be pseudogenes correlate with those found in other studies (7, 44). It is possible that IclR in subspecies *novicida* exerts its effects on genes that are intact whereas in LVS and

SchuS4, IclR affects genes that are similar those in *novicida* but because of disruptions or changes to the ORFs, many of these genes are transcribed but do not encode functional proteins. We must also consider that there are two genes in the list that are absent in U112 that are present in LVS and SchuS4, and the absence of a gene affected by IclR in *novicida* could also contribute to the different phenotypes. Overall, analysis of the genes identified in our microarray suggest that the majority of genes affected by IclR have differences in sequence between the three subspecies and that this variation could contribute to the phenotypic disparities observed.

Taken together, our data suggest that IclR contributes to the virulence of U112 but not to that of LVS or SchuS4, highlighting the fact that there are significant differences among these strains. Another example of differences among strains is seen in the conserved acid phosphatases AcpA, AcpB, and AcpC. These proteins were shown to be required for the virulence of subsp. *novicida*, but not for the virulence of SchuS4 (8, 38). Even though IclR may not play a major role in SchuS4 or LVS virulence, there are other potential roles that IclR could be have as a functional transcriptional regulator. Quite a few of the microarray-identified genes encode hypothetical proteins, but there are others that encode proteins with known functions or are homologous to proteins with known functions. Investigation into these proteins may provide an additional understanding of the function of IclR in *F. tularensis*. For example, in *Pseudomonas putida*, the IclR family proteins TtgT and TtgV regulate operons encoding genes that form efflux pumps for organic solvent extrusion (16, 22). Although our antibiotic sensitivity assays showed no role for IclR in drug efflux by LVS, we cannot rule out the involvement of IclR in the regulation of a system specific for organic solvent efflux or the role of IclR in drug efflux in other *F. tularensis* subspecies.

Finally, direct comparison of the complete transcriptional profiles of subspecies *novicida*, *tularensis* and *holarctica iclR* deletion strains might reveal some clues to the properties that are responsible for the phenotypic differences. Unfortunately, the currently available microarrays do not contain targets for genes found exclusively in subspecies *novicida*.

FIGURES



B

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                  1                                                  50
FTN_0720    (1)   MSEDKRIQVISRAVNVLKSIGSKPDGMSLGEIAKQIYLPRSTVQRIVAAL
FTL_1364    (1)   MSEDKRIQVISRAVNVLKSIGSKPDGMSLGEIAKQIYLPRSTVQRIVAAL
FTT_0748    (1)   MSEDKRIQVISRAVNVLKSIGGKPDGMSLGEIAKQIYLPRSTVQRIVAAL
                  51                                                 100
FTN_0720   (51)   EEEGFIRSEGAGKILLGPGIFKLISSCYVDIITLTQDSLSKLSEKVQETV
FTL_1364   (51)   EEEGFIKSEGAGKILLGPGIFKLISSSHADIISLTQDSLSKLSAKIQETV
FTT_0748   (51)   EEEGFIKSEGAGKILLGPGIFKLISSSYADIISLTQDSLSKLSAKIQETV
                  101                                                150
FTN_0720  (101)   SLTQSYDKNLLVLHRFVVNRELQIIPRVGALVPLHKTAAGRALLSLHTDD
FTL_1364  (101)   VITQPYDSDLLIVHRFIINRELQVLPRVGALIPIYNSSSGRAILALHTDD
FTT_0748  (101)   VITQPYDSDLLIVHRFIINRELQVLPRVGALIPIYNSSSGRAILALHTDD
                  151                                                200
FTN_0720  (151)   KIIKILGEQITDDKELFEKIAKIRVKGIDIDYSQTIKGIVTIGVAIKSFL
FTL_1364  (151)   KIIEILGEQIPDDKELFEKIAKIRADGIEFDYGKVIKGIVSIGVAINTFL
FTT_0748  (151)   KIIEILGEQIPDDKELFEKIAKIRADGIEFDYGKVIKGIVSIGVAINTFL
                  201                                240
FTN_0720  (201)   GDFGVSILAPQHRFDQNKDLYVDELLKVKSKIIDEIGV--
FTL_1364  (201)   GTFGISILTPQHRFDKYKEFYVNELLKTKSKIIDEIGIK-
FTT_0748  (201)   GTFGISILTPQHRFDKYKEFYVNELLKTKSKIIDEIGIK-
```

Figure 1. Comparison of *iclR* in three Francisella strains. (A) Synteny diagram of the genomic organization of the *iclR* locus in *F. tularensis* subspecies *novicida* U112 (FTN_0720), *F. tularensis* subspecies *holarctica* LVS (FTL_1364), and *F. tularensis* subspecies *tularensis* SchuS4 (FTT_0748). (B) Amino acid sequence alignment of *F. tularensis* subspecies *novicida* U112, *F. tularensis* subspecies *holarctica* LVS, and *F. tularensis* subspecies *tularensis* SchuS4 IclR. Alignment was created using VectorNTI software and *iclR* sequences uploaded from NCBI annotated genomes of each strain and translated using VectorNTI. Red letters highlight residues conserved between all three strains. Blue letters highlight the residues conserved between two strains.

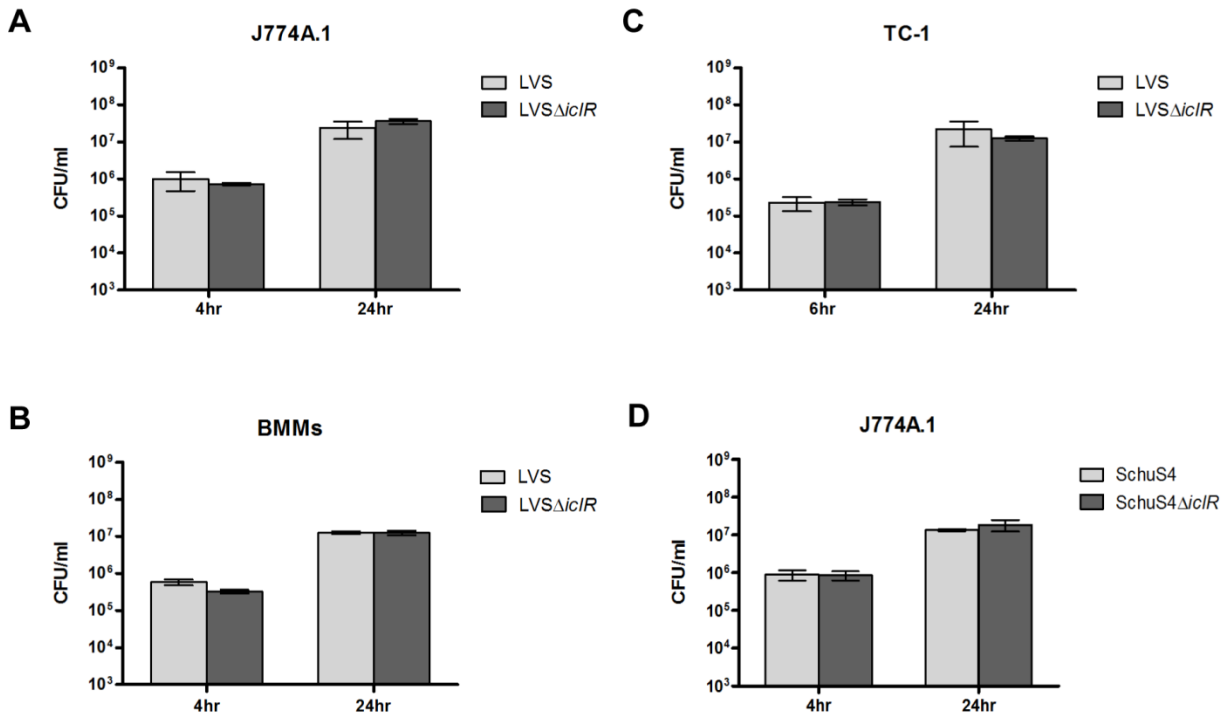

Figure 2. Intracellular replication of LVSΔ*iclR* and SchuS4Δ*iclR* in murine macrophages or lung epithelial cells. Gentamicin protection assays were performed by infecting (A) J774A.1 murine macrophages, (B) TC-1 murine lung epithelial cells, and (C) bone marrow-derived macrophages with wild-type LVS or LVSΔ*iclR* at an MOI 100. (D) Gentamicin protection assay was performed using J774A.1 cells infected with wild-type SchuS4 or SchuS4Δ*iclR*. Bars represent the standard deviation of three replicate wells and each graph is representative of two separate experiments.

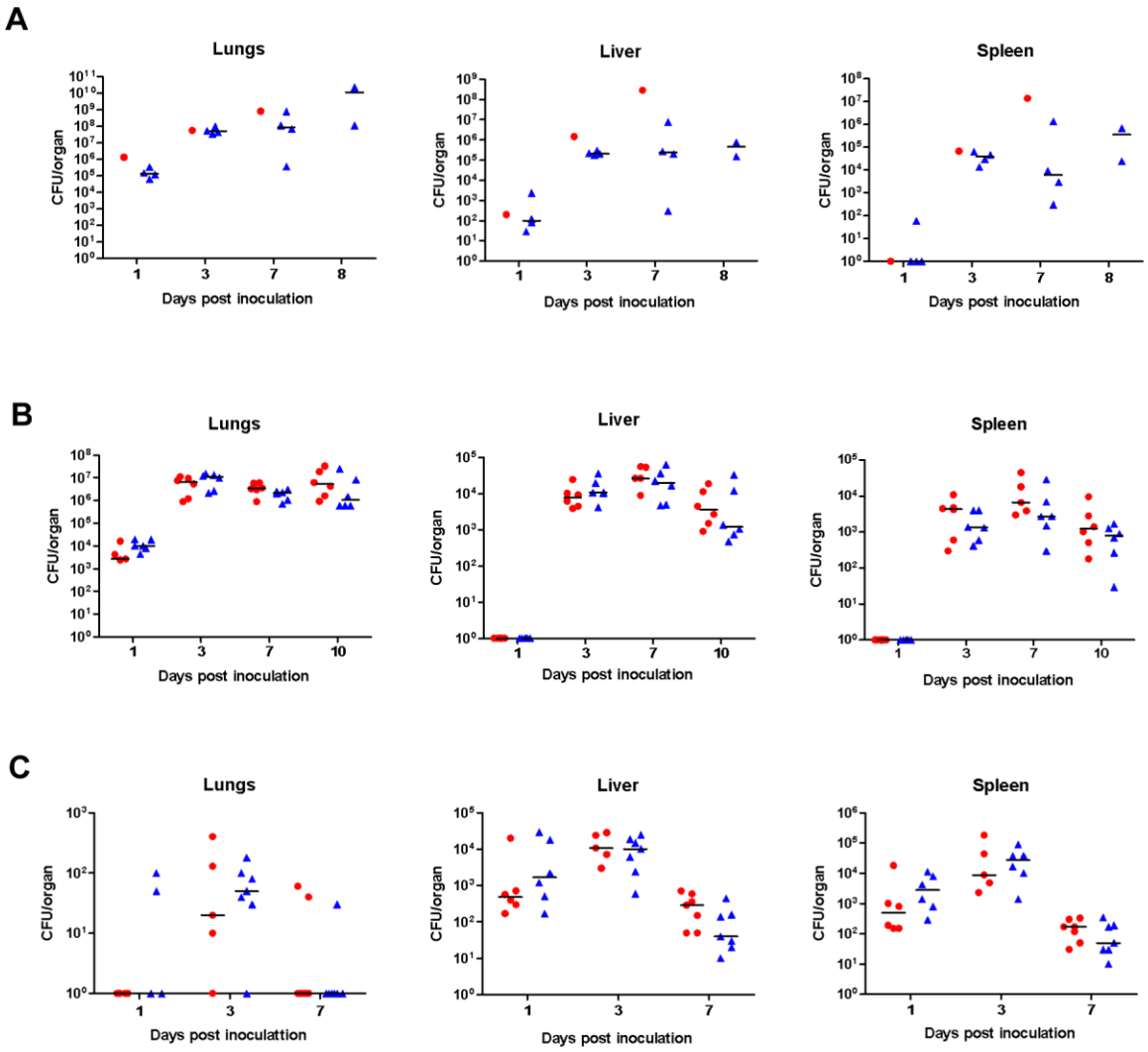

Figure 3. Recovery of LVSΔ*iclR* mutant in mice following i.n. or i.d. inoculation. C57BL/6 mice were inoculated with either wild-type LVS (circles) or LVSΔ*iclR* (triangles) i.n. at (A) a lethal dose of ~1x10^5 CFU or (B) a low dose of ~1x10^3 CFU. (C) C57BL/6 mice were inoculated with either wild-type LVS (circles) or LVSΔ*iclR* (triangles) i.d. at a dose of ~3x10^5 CFU. Each symbol represents data from a single mouse. There were no significant differences in recovery of mutant versus wild-type organisms from any organ at any time point as determined by the Mann-Whitney nonparametric test in the low dose (B) and i.d. (C) experiments.

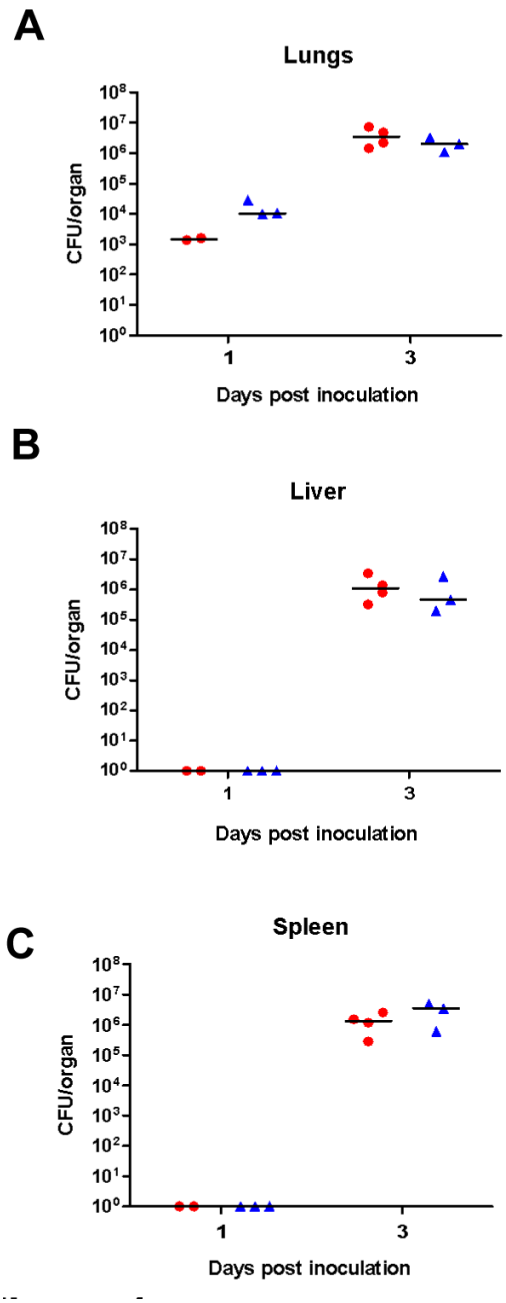

Figure 4. Recovery of SchuS4Δ*iclR* mutant in mice following i.n. inoculation. C57BL/6 mice were inoculated with either wild-type SchuS4 (circles) or SchuS4Δ*iclR* (triangles) i.n. at a dose of ~100 CFU. No differences in recovery of mutant versus wild-type organisms from any organ at any time point were significant using the Mann-Whitney nonparametric test.

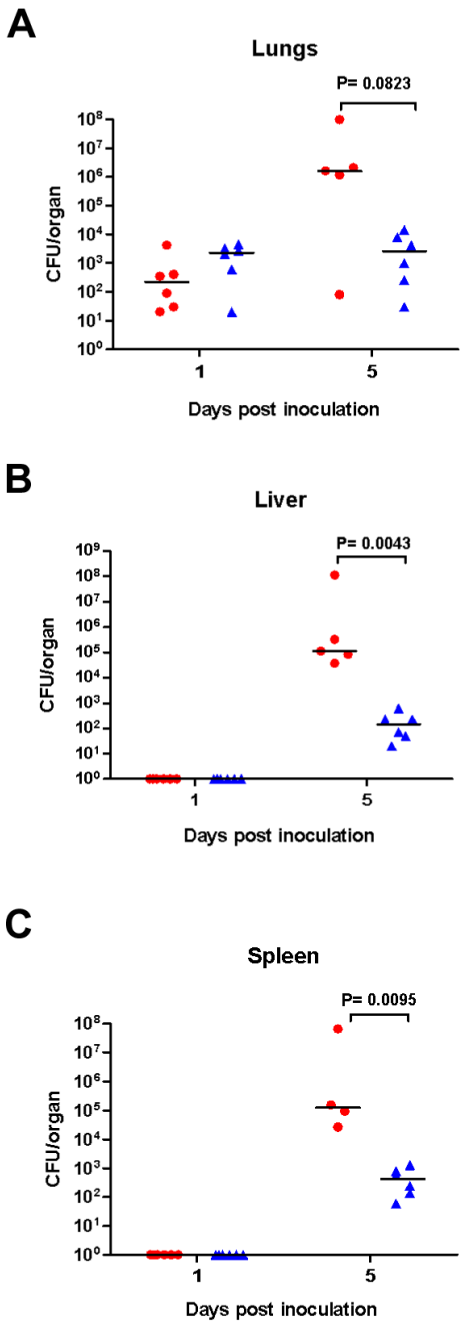

Figure 5. Recovery of U112 *iclR* transposon mutant in mice following i.n. inoculation. C57BL/6 mice were inoculated with either wild-type U112 (circles) or U112 *iclR* mutant (triangles) i.n. at a dose of ~10 CFU. Differences in recovery of mutant versus wild-type organisms at day 5 for the liver and spleen were significant using the Mann-Whitney nonparametric test.

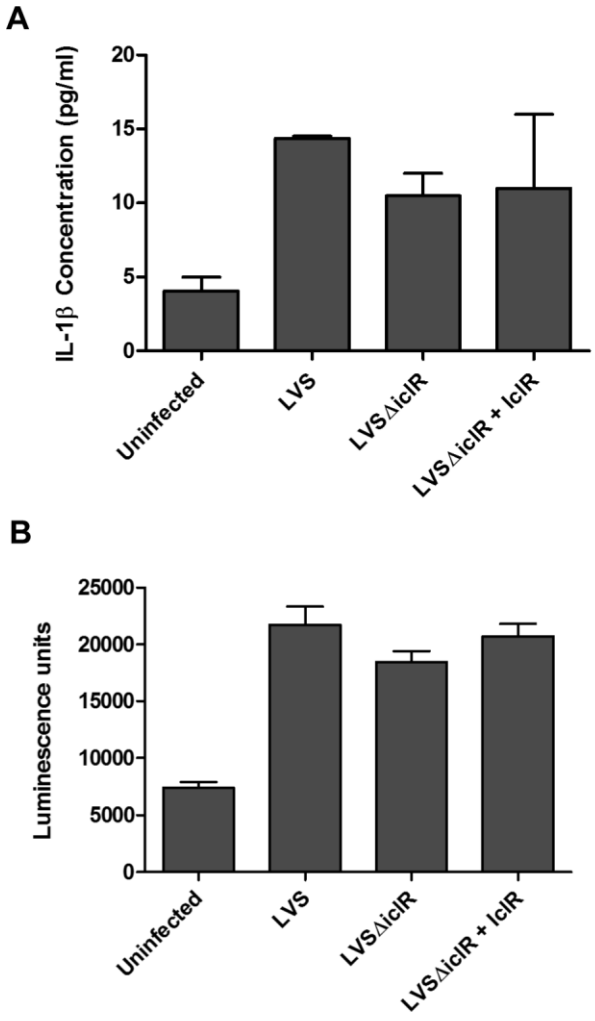

Figure 6. IL-1β release and cytotoxicity in murine bone marrow-derived macrophages infection with LVSΔ*iclR*. Infections were carried out at an MOI 500 for wild-type LVS, LVSΔ*iclR*, and LVSΔ*iclR* + IclR (complementation). (A) IL-1β was quantified via ELISA and (B) cytotoxicity was quantified via ToxiLight bioassay (Lonza), both at 24 hr post infection. Graphs are representative of at least three separate experiments, with duplicate or triplicate wells for each strain per experiment. No differences were significant by any strain comparison using the student's t-test.

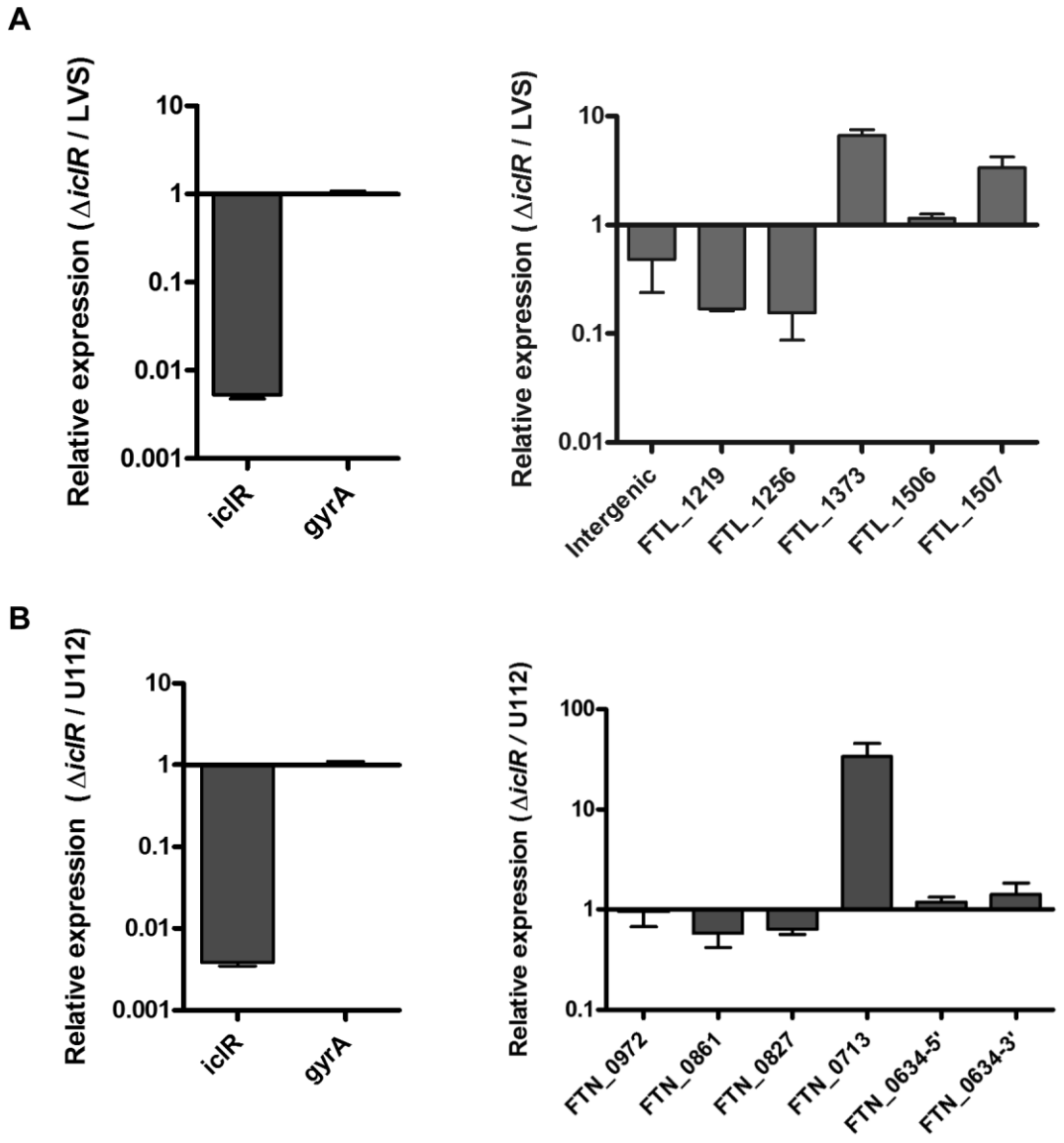

Figure 7. Transcript levels of genes found significantly changed in microarray analysis comparing LVS and LVSΔ*iclR*. RNA was isolated from (A) wild-type *F. tularensis* subsp. *holarctica* LVS and LVSΔ*iclR* or (B) wild-type *F. tularensis* subspecies *novicida* U112 and a U112 *iclR* transposon mutant and used in qRT-PCR analysis for several genes that were significantly changed in the microarray. Data is presented as relative expression of log change in wild-type over the respective *iclR* mutant after normalization to *gyrA*. Graph is representative of two or three experiments.

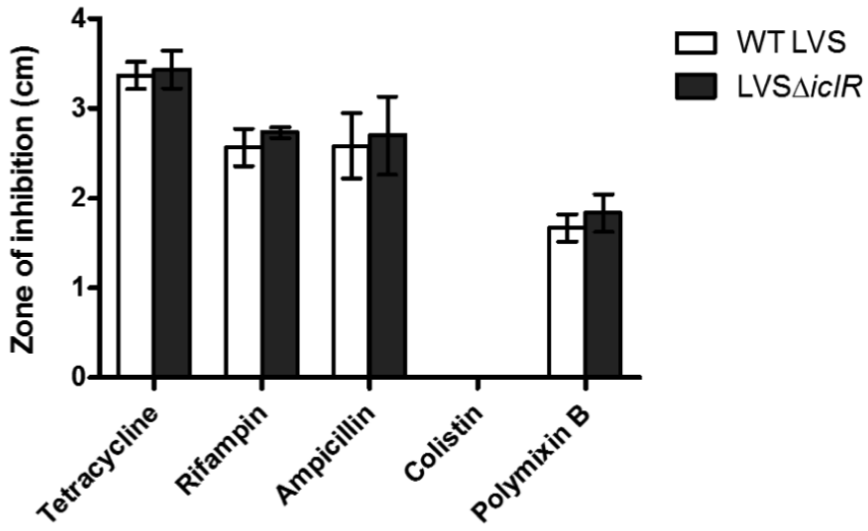

Figure 8. Antibiotic sensitivity of LVSΔ*iclR*. Wild-type LVS and LVSΔ*iclR* were grown to mid-log phase, bacteria spread on chocolate agar, and an antibiotic-containing paper disc was added to the center. Bacteria were grown for 36 hr and the diameter of the zone of inhibition was measured. Experiment was performed in triplicate and the averages and standard deviations were calculated.

TABLE 1. Microarray gene expression in LVSΔ*iclR*

Gene regulation and locus	Fold change	Description or annotation (BLASTp)[a]	Result of gene comparison between strains[b]		
			SchuS4	LVS	U112
Downregulated					
FTT0748	34.57	*iclR*	Intact	Intact	Intact
FTT0980	5.02	Hypothetical protein (aminotransferase class II)	Intact	Intact	Intact
FTT0987	2.84	Hypothetical protein (membrane protein of unknown function)	Pseudogene	Pseudogene	Intact
FTL_1506	2.82	Short-chain dehydrogenase (reductase family protein)	Intact	Pseudogene	Intact
FTT1082	2.64	T1082 protein	Intact	Intact	Intact
FTL_0388/FTT0885	2.63/2.18	Cation transporter (cobalt zinc cadmium cation transporter)	Pseudogene	Intact	Intact
FTL_1256	2.62	Pseudogene (carbon-nitrogen hydrolase family protein)	Pseudogene	Pseudogene	Intact
FTL_1507	2.57	3-Oxoacyl-[acyl-carrier protein] reductase	Intact	Pseudogene	Intact
FTT1081	2.53	Hypothetical protein (hemolysin-type binding protein)	Intact	Intact	Absent
FTT1507	2.37	Hypothetical protein (thymosin beta-4 family protein)	Intact	Absent	Intact
FTL_1122	2.19	Hypothetical membrane protein	Intact	Pseudogene	Absent
FTT0715	2.19	Chitinase family 18 protein	Two large deletions	Two large deletions	Intact
FTT0389	2.16	Acetyltransferase	Intact[c]	Intact[c]	Intact[c]
FTT0203	1.97	Bifunctional purine biosynthesis protein (*purH*)	Intact	Intact	Intact
Upregulated					
FTL_1373/FTT0741	4.28/6.40	Organic solvent tolerance protein	Pseudogene	Pseudogene	Intact
FTT1555	1.93	RNase III (*rnc*)	Intact	Intact	Intact
FTT1554	1.88	tRNA pseudouridine synthetase B (*truB*)	Intact	Intact	Intact
FTT0554	1.59	Hypothetical protein	Intact	Intact	Intact

[a] Annotation based on the microarray; BLASTp results (shown in parentheses), when included, provided additional information.
[b] Following alignments, genes were designated as intact, absent, or a pseudogene (introduced stop codon and two shorter predicted ORFs); genes those without such designations are discussed in the text.
[c] Possible alternative start site (see text for details).

Table 2.1

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CHAPTER 3

Deletion of *ripA* alleviates suppression of the inflammasome and MAP kinase by *Francisella tularensis*

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ABSTRACT

Francisella tularensis is a facultative intracellular pathogen and potential biothreat agent. Evasion of the immune response contributes to the extraordinary virulence of this organism although the mechanism is unclear. While wild type strains induced low levels of cytokines, an *F. tularensis ripA* deletion mutant (LVSΔ*ripA*) provoked significant release of IL-1β, IL-18 and TNF-α by resting macrophages. IL-1β and IL-18 secretion was dependent on inflammasome components PYCARD/ASC and Caspase-1, while the TLR/IL-1R signaling molecule, MyD88, was required for inflammatory cytokine synthesis. Complementation of LVSΔ*ripA* with a plasmid encoding *ripA* restored immune evasion. Similar findings were observed in a human monocytic line. The presence of *ripA* nearly eliminated activation of MAP kinases including ERK1/2, JNK and p38 while pharmacologic inhibitors of these three MAPKs reduced cytokine induction by LVSΔ*ripA*. Animals infected with LVSΔ*ripA* mounted a stronger IL-1β and TNF-α response than mice infected with wild type LVS. This analysis revealed novel immune evasive mechanisms of *F. tularensis*.

INTRODUCTION

Francisella tularensis is a Gram-negative, facultative intracellular pathogen that is the causative agent of the zoonotic disease tularemia. The organism can be transmitted to a host through insect bites, handling of infected carcasses, and inhalation of aerosolized bacteria (1-3). *F. tularensis* is a potential agent of biological warfare classified as a Select Agent by the Center for Disease Control (CDC) due to its highly infectious nature; roughly 50% mortality is caused by an infectious dose of as few as 10 bacteria via inhalation (4). During the Cold War, both the former Soviet Union and the United States of America weaponized and stockpiled *F. tularensis* in their biological weapons programs (5). The World Health Organization (WHO) has estimated that a 50 kilogram release of *F. tularensis* over a 5 million metropolis would cause 250,000 incapacitating casualties and 19,000 deaths (WHO 1970). The CDC also estimated that a *F. tularensis* attack would cost 5.4 billion to the society for every 100,000 infected individuals (6). Four subspecies of *F. tularensis* exist, including *tularensis, holarctica, mediasiatica,* and *novicida* (7). The most virulent subspecies for humans is the subsp. *tularensis* which is found primarily in North America. Subsp. *holarctica* is less virulent and was used to generate a live vaccine strain (LVS). LVS is attenuated in humans but remains highly virulent for mice making LVS a useful model to study *F. tularensis* pathogenesis.

Since exposure to the respiratory tract results in the most aggressive form of tularemia, several laboratories have developed mouse models of pulmonary tularemia to study this mode of infection (8-10). Using an intranasal delivery model, the median lethal dose (LD_{50}) of LVS is 10^3 CFU (9). By comparison, the LD_{100} for the highly virulent *F. tularensis* subsp. *tularensis* strain SchuS4 is less than 20 CFU (11). Upon inhalation, the

bacteria are restricted to the lung for approximately 36 to 48 hours where the organism persists and multiplies (12). During this time, little host immune response occurs as evidenced by the lack of pro-inflammatory cytokines IL-1β, TNF-α, IL-6 and IFN-γ (13). 2-4 days post infection, inflammatory cells infiltrate the lung and pro-inflammatory cytokines can be detected *in vivo*. By this time, bacteria have overwhelmed the lung and disseminated to other organs such as the spleen and liver. Mice eventually succumb to infection 5-7 days post inoculation. How *F. tularensis* circumvents the host immune response is not well understood.

F. tularensis is found *in vivo* within alveolar macrophages, dendritic cells (DC) and lung epithelial cells (9). Following entry into host cells, *F. tularensis* escapes the phagosome and enters the cytosol where it replicates. This process is apparently unhindered by innate immune defenses that typically detect and facilitate a response to eliminate foreign microbes. IFN-γ, TNF-α, and IL-1β are critical mediators of an effective defense against *Francisella* infection (14, 15). SchuS4 suppresses pro-inflammatory cytokine induction in mouse lung resident DCs in early stages of the disease (13). Similarly, LVS dampens intracellular signaling and TNF-α in human monocytes and mouse macrophages (16). Administration of anti-IFN-γ, and anti-TNF-α antibodies greatly reduces the LD_{50} of LVS in an intradermal mouse model of mouse tularemia (17). IL-1β also is critical in innate defense against *F. tularensis* subsp. *novicida* (14). Disrupting the signaling pathway that leads to IL-1β processing greatly enhances host susceptibility to subsp. *novicida* infection.

IL-1β, one of the more potent pro-inflammatory cytokines, is tightly regulated by a two-step signaling process. The first step requires the transcriptional activation of pro-IL-1β followed by the translation of the pro-protein. This is achieved by engagement of the toll-

like receptors (TLR) and subsequent activation of MyD88-dependent signaling pathways, leading to NF-κB and MAP kinase induction. This leads to translocation of the NF-κB subunit p65 from the cytosol to the nucleus and activation of the MAP kinase phosphorylation cascade to cause the transcription of pro-IL-1β message. A second and separate signal is required after pro-IL-1β protein is produced, and this occurs in the cytosol, leading to the assembly of an inflammasome complex. There are several types of inflammasomes, including those which contain an NLR (nucleotide binding domain-leucine rich repeats containing) component, and those which contain a non-NLR component, such as AIM2 (absent in melanoma 2) or RIG-I (retinoic acid-inducible gene 2) (18-22). Procaspase-1 is a requisite member of the complex, while the adaptor PYCARD/ASC/TMS1 (Pyrin-CARD, apoptotic speck-containing protein with a CARD, or target of methylation-induced silencing) is found in most inflammasome complexes (23, 24). Upon inflammasome formation, procaspase-1 undergoes auto-catalytic cleavage into an active, mature form which subsequently cleaves pro-IL-1β and pro-IL-18 into their active forms.

NLR family members share a conserved structure, with most of the members bearing an N-terminal Pyrin or CARD (caspase recruitment domain) domain followed by an NBD domain; and a C-terminal LRR domain that is homologous to those found in TLRs (25). A current working hypothesis is that, upon induction, the NLR recruits PYCARD via a Pyrin-Pyrin homotypic interaction. PYCARD possesses its own CARD domain which recruits Caspase-1 into the inflammasome complex for IL-1β processing. Several NLRs that contribute to pathogen induced IL-1β release in macrophages have been identified (26). The absence of NLRs or their adaptors renders hosts more susceptible to a variety of pathogens. The NLRP3 inflammasome senses a wide variety of intracellular pathogens including Gram-

positive and Gram-negative bacteria, RNA viruses, DNA viruses, yeast, microbial toxins and a host of damage-associated molecular patterns such as silica, asbestos and alum (27-29). The NLRC4/Ipaf inflammasome responds to bacterial virulence factors from the type III and IV secretion systems from bacteria such as *Salmonella typhi, Burkholderia pseudomallei, Escherichia coli* (30-32) and *Pseudomonas aeruginosa* (33).

Unlike most Gram-negative bacterial pathogens, *F. tularensis* subspecies *tularensis* and *holarctica* do not provoke a substantial initial inflammatory response *in vitro* or *in vivo* (13, 33, 34). *F. tularensis* LPS does not stimulate significant signaling through TLR4 (35), but this property alone does not account for the muted host response to infection. The goal of this study was to identify mechanisms by which *F. tularensis* actively suppresses the host innate immune response. To achieve this goal, we used an attenuated *F. tularensis* mutant that elicited a robust inflammatory response to reveal signaling pathways that are normally suppressed by wild type organisms and that dampen IL-1β, IL-18, and TNF-α responses to infection.

MATERIALS AND METHODS

Cell lines and Reagents

Bone marrow derived macrophages were harvested from 6-8 week old mice and cultured for 7 days in 30% M-CSF conditioned media. THP-1 cells (ATCC) were cultured as described (36). Anti-IL-1β antibody was obtained from R&D Systems; anti-IκBα, phospho-p65, phospho-ERK1/2, phospho-JNK, phospho-p38 from Cell Signaling; and anti-GAPDH from Santa Cruz Biotechnology. Detailed methods for preparation of retroviral shuttle

vectors, transduction and sorting to generate THP-1 cell lines stably expressing shRNA have been described (36).

Western blotting and ELISA

Western blots were performed as described (37, 38). Quantification of western blots by densitometry was performed using Adobe Photoshop. For ELISAs, mouse and human cell supernatants were collected 24 hours post-infection and assayed with BD OptEIA Mouse IL-1β, IL-18, and TNF-α ELISA Sets (BD Biosciences).

Cytotoxicity assays

Cytotoxicity assays were performed using the ToxiLight® BioAssay kit (Lonza) following the manufacturer's protocol for detection from supernatants. The luminescence was read using a TECAN Infinite M200 and analyzed using Magellan v6 software.

Experimental Animals

All studies were conducted in accordance with the National Institutes of Health Guidelines for the care and use of Laboratory Animals and the Institutional Animal Care and Use Committee guidelines of University of North Carolina, Chapel Hill. The generation of mice lacking functional *Nlrp3*, *Nlrc4*, *Pycard*, *Caspase-1*, and *MyD88* has been previously described (38). C57BL/6 mice were purchased from Jackson Laboratories (Maine).

Bacteria Preparation

F. tularensis LVS was obtained from the CDC, Atlanta, GA. *F. tularensis* subsp. *novicida* U112 was obtained from ATCC. The LVSΔ*ripA* strain has been described (12). All *Francisella* strains were maintained on chocolate agar supplemented with 1% IsoVitaleX (BD Biosciences) and grown in Chamberlain's defined media (39). Other bacteria were grown in lysogeny broth (LB) medium or brain-heart infusion (BHI) medium. LVS and LVS*ΔripA* were killed by incubation for 5 minutes at room temperature in 1 ml of 2% paraformaldehyde in PBS, washed three times with PBS, and suspended in DMEM, 10% PBS. All cultures were grown at 37°C.

***Francisella* infection of mouse primary macrophages and human monocytic cell lines**

LVS and LVSΔ*ripA* were grown overnight in Chamberlain's defined media prior to infection, and U112 was grown overnight on chocolate agar prior to infection. Concentrations of bacteria were determined by klett reading, and cells were exposed to the designated *Francisella* strain at the indicated MOI. Infected cells were incubated at 37^{o}C and supernatants were harvested at select time points for cytokine analysis. For pharmacological assessments, cells were treated with Y-VAD-fmk (10 μM), U0126, SP-600125, and SB-202190 (0.5 – 25 μM) as described (36).

***In vivo F. tularensis* infection**

F. tularensis LVS from an overnight culture in Chamberlain's media was centrifuged, and the pellet was suspended in PBS. These suspensions were enumerated by klett reading and diluted in Dubecco's phosphate buffered saline (DPBS) for the inoculums. Wild-type 8-

to 10-week-old female C57BL/6 mice were anesthetized with avertin, as determined by insensitivity to a toe pinch, and were then infected intranasally with bacterial inoculums in a 50 µl volume in DPBS. Mice were monitored for recovery from anesthesia.

Statistical Analysis

Statistical significance in Figures 1A-H, 2A-C, 3A-D, 5B-D was determined by two-way analysis of variance (ANOVA) followed by a Tukey post-hoc test using Graph Pad Software (La Jolla, CA). Statistical significance in Figure 6 was determined by two-tailed Mann-Whitney U tests using Graph Pad Software (La Jolla, CA). The p values < 0.05 were considered statistically significant. Unless otherwise specified, data are presented as the mean ± standard deviation (s.d.).

RESULTS

F. tularensis LVSΔ*ripA* fails to suppress the release of pro-inflammatory cytokines IL-1β, IL-18 and TNF-α by mouse primary macrophages.

In response to foreign microbes, macrophages secrete pro-inflammatory cytokines including IL-1β, IL-18 and TNF-α which activate neutrophils, fibroblasts and endothelial cells to mount an anti-microbial response. *F. tularensis* can actively down-regulate host immune cytokine production *in vivo* and *in vitro* (7, 15). To assess the ability of *F. tularensis* to induce pro-inflammatory cytokines, we first compared the induction profile for the cytokine IL-1β in response to various bacterial pathogens in macrophages. We selected IL-1β due to its central role in initiating a variety of host immune defense cascades (40). When

compared to *Salmonella typhi*, *Klebsiella pneumoniae* and *Shigella flexneri*, mouse primary macrophages exposed to *F. tularensis* LVS released significantly less IL-1β (Figure 1A) suggesting that *F. tularensis* LVS represses this particular inflammatory response. One possible source of this difference is the LPS. LPS from *S. typhi*, *K. pneumoniae*, and *S. flexneri* may be more immune-stimulatory than *F. tularensis* LPS as described by another report (33).

RipA (required for intracellular proliferation, factor A; FTL_1914) is a cytoplasmic membrane protein that is conserved among *Francisella* species and is required for adaptation of the bacteria to the host cell cytoplasm. Mutants lacking *ripA* (Δ*ripA* strains) enter macrophages and escape from the phagosome at the same frequency and kinetics as wild type *F. tularensis* but fail to replicate intracellularly in the host cell (12). In that study, we demonstrated that LVSΔ*ripA* was unable to replicate intracellularly in host epithelial cells or macrophages. We considered that Δ*ripA* strains might also be affected in their ability to suppress host cell immune responses. To test this possibility, we monitored IL-1β release by macrophages infected with wild type or LVSΔ*ripA* strains. Over a range of MOI, LVSΔ*ripA* induced roughly 5-10 fold higher levels of IL-1β, than wild type- infected macrophages (Figure 1B). Suppression of IL-1β was restored to LVSΔ*ripA* by *in trans* complementation with a *ripA* containing construct, p*ripA* (Figure 1B), demonstrating a direct but inverse cause-and-effect relationship between *F. tularensis* RipA expression and IL-1β response.

We next asked whether the effect of RipA on cytokine expression was widespread or limited to IL-1β. IL-18 production was measured since the inflammasome is also involved in processing and release of this cytokine. Increased levels of IL-18 similar to that of IL-1β were released by the LVSΔ*ripA* stimulated macrophages, and complementation reduced

induction nearly to wild type levels (Figure 1C). Since *F. tularensis*-induced TNF-α is not regulated by inflammasome components (14) but is regulated by MyD88 (35), we sought to test whether these findings hold true for LVSΔ*ripA*-induced TNF-α release. LVSΔ*ripA* induced 2-3 fold more TNF-α than wild type LVS (Figure 1D). The magnitude of the difference between LVS and LVSΔ*ripA* induced TNF-α release by macrophages was less than that of IL-1β. This suggests that *ripA* affects *F. tularensis* suppression of both inflammasome and non-inflammasome cytokines, but that the impact on inflammasome cytokines is more significant. Neither paraformaldehyde (PFA)-fixed wild type LVS or LVSΔ*ripA* mutant strains provoked significant levels of IL-1β (Figure 1E) demonstrating that the observed impact of RipA on cytokine expression was not due simply to lack of intracellular replication by the deletion mutant strain.

One possible mechanism for the different levels of IL-1β induced by LVS versus LVSΔ*ripA* is that the former might cause more cell death, thus interfering with IL-1β production and processing. To assess cell viability differences between LVS and LVSΔ*ripA*, we measured cytotoxicity of primary mouse macrophages after infection with LVS, LVSΔ*ripA*, or the LVS *ripA* complementation strain. As shown in Figure 1F, infection with LVSΔ*ripA* resulted in increased cytotoxicity when compared to cells infected with wild type LVS or the *ripA* complementation strain. These data together suggest that LVSΔ*ripA* is both hyper-inflammatory and hyper-cytotoxic compared to wild type LVS. These results are consistent with the frequent association of inflammasome activation and cell death (41) as shown here for LVSΔ*ripA*, but inconsistent with the possibility that LVS causes more cell death which interferes with IL-1β production and release.

To further address the potential contribution of MOI to the observed phenotypes, primary mouse macrophages were exposed to wild type LVS at MOI 10, 50, or 500 and IL-1β secretion was assayed by ELISA at 24 hours post infection. As shown in Figure 1G, IL-1β production increased with MOI; however, the levels of IL-1β produced at the lower MOI of 10 was near the limit of detection. Therefore, for the subsequent LVS infections in primary mouse macrophages, MOI 50 or 500 was used.

In contrast to the relatively low levels of IL-1β that we observe for wild type LVS, several studies have shown that infection with subsp. *novicida* result in high levels of IL-1β secretion and cell death, albeit the Mariathasan et al. study used LPS-pre-treated, thioglycolate-elicited peritoneal macrophages (14, 42). To determine if the differences in IL-1β secretion are strain-specific, we infected resting BMDM with *F. tularensis* subsp. *novicida* U112 at MOI 10, 100, or 500 and assayed for IL-1β secretion at 24 hours post infection (Figure 1H). Similar to the results of Henry et al. (42) for subsp. *novicida*-induced IL-1β secretion in bone marrow-derived macrophages, our data shows that subsp. *novicida* strain U112 induced higher levels of IL-1β secretion by primary mouse macrophages (Figure 1H).

LVSΔ*ripA*-induced IL-1β is PYCARD, Caspase-1 and MyD88 dependent.

In the regulation of IL-1β release by macrophages, bacterial-derived pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) first activate the TLR signaling pathway, thereby promoting the synthesis of pro-IL-1β transcript and later protein. A second signal initiates assembly of the inflammasome to process pro-IL-1β into IL-1β for release. To determine the level at which IL-1β release is regulated upon infection, we tested whether

disruption of either TLR or NLR/inflammasome signaling pathways can abrogate LVSΔ*ripA* induced IL-1β release. MyD88 was chosen for TLR signaling due to its central role in TLR/IL-1R pathways. To disrupt inflammasome formation, mice deficient in either *Pycard* or *Caspase-1* were used since these two gene products are critical in subsp. *novicida*-induced IL-1β release by mouse macrophages and the ulceroglandular form of tularemia (14). Furthermore, two commonly interacting NLRs, NLRP3 and NLRC4 were tested because they are critical in the detection and response to several pathogens (26). In the absence of *Pycard*, *Caspase-1* and *MyD88*, macrophages did not release IL-1β upon LVS or LVSΔ*ripA* exposure (Figure 2A). However, absence of *Nlpr3* or *Nlrc4* did not impair the LVSΔ*ripA*-induced release of IL-1β suggesting that these NLRs are not involved in the sensing of *F. tularensis*.

Similar to IL-1β release, PYCARD, Caspase-1, and MyD88 but not NLRP3 or NLRC4 were required for LVSΔ*ripA* induced IL-18 release by macrophages, further confirming effects on an additional inflammasome cytokine (Figure 2B). As expected, MyD88 but not PYCARD, Caspase-1, NLRP3 or NLRC4 is required for LVSΔ*ripA*-induced release of the non-inflammasome cytokine TNF-α by primary mouse macrophages (Figure 2C). Taken together, LVSΔ*ripA* induced IL-1β production is mediated by PYCARD, Caspase-1 and MyD88 which suggests that *F. tularensis* LVS may target the PYCARD/Caspase-1 axis as well as other TLR-dependent signaling pathways to suppress IL-1β release by macrophages.

***F. tularensis* LVSΔ*ripA* fails to suppress pro-inflammatory cytokine response by human monocytic THP-1 cells.**

To determine if the observed effects are also applicable to human cells, we measured cytokine expression by the human monocytic cell line, THP-1, in response to wild type LVS and LVSΔ*ripA*. THP-1 cells exposed to LVSΔ*ripA* released 10-fold more IL-1β (Figure 3A) and 2-3 fold more TNF-α (Figure 3B) than cells exposed to wild type LVS, which is consistent with the mouse primary macrophage response. Also consistent, the difference in amount of TNF-α induced between LVS and LVSΔ*ripA* is of smaller magnitude than that of IL-1β.

We next investigated whether PYCARD/ASC was required for LVSΔ*ripA* induced IL-1β release by THP-1 cells using cells stably expressing either a control scramble PYCARD retroviral ShRNA vector (Sh-Ctrl) that does not confer knock down, a specific PYCARD retroviral ShRNA vector (Sh-PYCARD) to knock down PYCARD, or a specific NLRP3 retroviral ShRNA vector (Sh-NLRP3) to knock down NLRP3 (36). In our previous studies, all pathogens investigated induced IL-1β in a PYCARD dependent manner in a system where PYCARD expression was reduced by shRNA (36). Cells were infected with LVS and LVSΔ*ripA*, and IL-1β was monitored. 24 hours post infection, Sh-PYCARD-bearing THP-1 cells treated with either LVS or LVSΔ*ripA* did not release detectable IL-1β (Figure 3C). In contrast, induction of IL-1β release was similar in sh-NLRP3-bearing cells and Sh-Ctrl-bearing cells. This is consistent with our studies using gene-deficient mouse primary macrophages. To determine whether caspase-1 is required for LVSΔ*ripA* induced IL-1β release by THP-1 cells, THP-1 cells were pretreated with a specific Caspase-1 inhibitor (10 μM Y-VAD-fmk). *Porphyromonas gingivalis*, which induces a Caspase-1-dependent IL-

1β release in THP-1 cells (37), served as control. Y-VAD blocked IL-1β release in response to infection with *P. gingivalis,* and also with LVS and LVSΔ*ripA* (Figure 3D). Based on this data, we concluded that PYCARD and Caspase-1 are required in LVSΔ*ripA*-induced IL-1β release by human monocytic THP-1 cells.

F. tularensis LVSΔ*ripA* fails to suppress the processing and synthesis of IL-1β.

To elucidate the mechanisms by which *F. tularensis* suppresses host macrophage release of pro-inflammatory cytokines, we first sought to determine kinetics of intracellular IL-1β synthesis and processing using western blot analysis to detect pro-IL-1β (33KD) and processed IL-1β (17KD) present in LVSΔ*ripA*-infected mouse macrophages over a time course following inoculation. Pro-IL-1β levels increased starting at 30 minutes post LVSΔ*ripA* exposure (Figure 4A), a time corresponding with the kinetics of phagosome escape by both wild type *F. tularensis* and LVSΔ*ripA* (12). IL-1β processing occurred 30-45 minutes post infection, and processed IL-1β accumulated over several hours (Figure 4A). By 24 hours post infection, the intracellular level of processed IL-1β decreased, likely due to release of IL-1β into the extracellular space and death of macrophages. To determine whether the response to LVS and LVSΔ*ripA* differs in the synthesis and processing of IL-1β by macrophages, we repeated a time course of infection. By 60 minutes, LVS induced the synthesis of pro-IL-1β, and no processed IL-1β was evident. Pro-IL-1β increased significantly 120 minutes post-inoculation with LVS; however, only a small fraction of the pro-protein was processed to mature IL-1β at this point as shown by western blotting for pro-IL-1β and processed IL-1β (Figure 4B) and quantification of processed IL-1β by densitometry (Figure 4C). In contrast, pro-IL-1β was present in LVSΔ*ripA*-infected

macrophages by 60 minutes post infection, with the majority processed to mature IL-1β. Pro-IL-1β continued to increase by 120 minutes post-inoculation, with half of the pro-protein processed to mature IL-1β.

Next, we sought to determine if *F. tularensis* LVS also disrupted of the synthesis of pro- IL-1β. To accomplish this objective, we exposed *Pycard*$^{-/-}$ mouse primary macrophages to LVS and LVSΔ*ripA* and monitored the synthesis of pro-IL-1β. Since the absence of PYCARD abolishes the processing of pro-IL1β into mature IL-1β, we can directly compare the macrophage synthesis of pro-IL-1β. In the absence of IL-1β processing, LVSΔ*ripA*-infected macrophages induced significantly more pro-IL-1β than LVS as shown by western blot (Figure 4D) and quantified by densitometry in Figure 4E. Only pro-IL-1β is observed in LVS and LVSΔ*ripA*-infected *Pycard*$^{-/-}$ BMDM, because the absence of PYCARD prevents processing of IL-1β. Thus, *F. tularensis* LVS disrupts both the synthesis and processing of pro-IL-1β by macrophages. Collectively, these data suggest that the removal of RipA affected signaling pathways required for both the synthesis and processing of pro-IL-1β.

The effect of RipA on both pro- IL-1β and TNF-α synthesis led us to test the effect of RipA on pathways that can affect the production of both cytokines. Since NF-κB is a master transcriptional regulator that controls a wide range of host immune responses, including cytokine production, we sought to determine if *F. tularensis* LVS interfered with NF-κB signaling in macrophages. Degradation of IκBα and phosphorylation of p65 were monitored because both events are involved in NF-κB activation. Degradation of IκBα allows phosphorylation and translocation of p65 into nucleus which subsequently binds to promoters of pro-inflammatory genes. LVS and LVSΔ*ripA* each induced degradation of IκBα 30-60 minutes post infection, and phosphorylation of p-65 15-30 minutes post infection (Figure

4F). LVSΔ*ripA* appeared to induce slightly faster degradation of IκBα and phosphorylation of p65; however, these small differences alone are unlikely to account for the significant differences between LVSΔ*ripA* and LVS in the induction of TNF-α and pro-IL-1β.

***F. tularensis* LVSΔ*ripA* fails to dampen the activation of MAP kinase pathways**.

Another common group of signaling pathways that contributes to cytokine activation by bacteria and bacterial components are the MAP kinase pathways. *F. tularensis* LVS initially activates MAP kinase signaling pathways but subsequently down-regulates their activity (43). To determine whether the difference between the cytokine activation by LVS and LVSΔ*ripA* might be explained by differences in the induction of MAP kinases, we profiled the phosphorylation of ERK1/2, JNK and p38 in macrophages following infection. LVS induced modest levels of ERK1/2 and p38 phosphorylation which peaked at 30 and 60 minutes, but induced very little JNK activation (Figure 5A). In contrast, LVSΔ*ripA* induced dramatic levels of ERK1/2 phosphorylation at 15-30 minutes, and of JNK and p38 at 30-60 minutes post inoculation. These data suggest that LVS dampened the induction of MAP kinase signaling pathways by a mechanism that is missing or ineffective in the Δ*ripA* mutant strain.

To determine whether differences in MAP kinase activation might explain the increased inflammatory nature of LVSΔ*ripA*, we abrogated MAP kinase activity in Δ*ripA* infected cells with pharmacological inhibitors of ERK, JNK and p38 signaling pathways. Primary mouse macrophages were treated with U0126, SP-600125 or SB-202190 to prevent the phosphorylation of ERK1/2, JNK and p38, respectively. These cells were subsequently exposed to either LVS or LVSΔ*ripA*, and IL-1β release was measured 24 hours post

infection. Both U0126 and SP-600125 reduced the release of IL-1β by macrophages by 1.5-2 fold following infection with LVSΔ*ripA* over a range of doses (Figure 5B). Thus, ERK and JNK pathways each partially contribute to IL-1β activation in Δ*ripA*-infected cells. The addition of U0126 or SP-600125 also reduced the release of TNF-α by macrophages upon exposure to LVS and LVSΔ*ripA* (Figure 5C). Finally, the combination of ERK, JNK and p38 inhibitors blocked LVSΔ*ripA*-induced IL-1β release by macrophages more effectively than each inhibitor alone (Figure 5D). Taken together, these data demonstrate that wild type *F. tularensis* LVS interferes with the activation of multiple MAP kinase signaling pathways that are important for release of cytokines following infection.

***F. tularensis* LVSΔ*ripA* fails to suppress pro-inflammatory cytokine responses *in vivo*.**

Our previous study showed that LVSΔ*ripA* is attenuated in mice as evidenced by a reduction in organ burden at days 1, 3 and 7 post intranasal infection when compared to LVS (12). In this study we confirm and expand this finding by monitoring mice morbidity, mortality and cytokine responses post infection. Mice were intranasally inoculated with LVS, LVSΔ*ripA* and mock/PBS, and weight loss was monitored among the three experimental groups. When using a LD_{100} dose of LVS (10^5), LVS-inoculated animals exhibited severe weight loss, a measure of the severity of respiratory tularemia, and eventually succumbed to the disease (Figure 6A). In contrast, LVSΔ*ripA*-inoculated animals only showed a modest weight loss when compared to mock/PBS treated animals. We also measured the bacterial burdens in the lungs of mice during the early stages of the disease (6 and 24 hours post infection). Similar lung burdens were observed 6 hours post infection when comparing LVS and LVSΔ*ripA*, indicating that the initial bacterial loads of LVS and LVSΔ*ripA* are

indistinguishable (Figure 6B). By 24 hours post inoculation, LVS lung burdens rose to 10^6 CFU/organ. In contrast, the lung burden of LVSΔ*ripA* inoculated animals did not increase beyond that of the 6 hour time point. In our previous report, the *ripA* complementation strain of LVSΔ*ripA* inoculated animals exhibited similar lung, spleen, and liver burdens when compared to LVS inoculated animals (12).

The *in vitro* analyses shown earlier indicate that LVSΔ*ripA* failed to suppress the release of IL-1β, IL-18 and TNF-α by primary mouse macrophages and human THP-1 cells. To determine if the absence of *ripA* similarly affected cytokine responses *in vivo*, we measured IL-1β and TNF-α in the lungs of infected mice. After testing multiple doses of LVSΔ*ripA* to determine the amount required to detect IL-1β and TNF-α in the lung (Figure 6C), we found that mice inoculated with 10^{10} CFU of LVSΔ*ripA* expressed on average 5-10 fold more IL-1β than animals inoculated with the same number of wild type LVS (Figure 6D). Interestingly, at that dose of bacteria, IL-1β levels in the BALF of animals infected with wild type LVS was indistinguishable from mock/PBS-treated mice. We next examined levels of TNF-α in mouse lungs of mock/PBS, LVS and LVSΔ*ripA*-inoculated animals. LVSΔ*ripA* induced more TNF-α than LVS, but the difference was not as significant as that of IL-1β (Figure 6E). These results are consistent with the *in vitro* results obtained with mouse and human cells (Figures 1D, 3B) and support the effects of *ripA* deletion on both inflammasome and non-inflammasome mediated cytokines. Taken together, these data suggest that the expression of *ripA* in LVS suppresses macrophage pro-inflammatory cytokine production during an infection both *in vitro* and *in vivo.*

DISCUSSION

The innate immune response is indispensible to eradicate the invasion of microbial pathogens, and monocytes/macrophages are major arsenals in combating infectious diseases. Proper release of cytokines and chemokines by these cells is critical in the migration of polymorphonuclear (PMN) cells and other effector cells to the site of infection. To establish a successful infection, a pathogen must either evade immune surveillance or modulate host anti-microbial response. Over time, pathogens have developed sophisticated strategies to manipulate host immune responses to their advantage. In this study, we demonstrated that *F. tularensis* actively suppresses the release of pro-inflammatory cytokines, and that the *F. tularensis* gene *ripA* is critical in this process. In response to *F. tularensis* LVSΔ*ripA*, macrophages are able to mount an effective initial response by releasing higher amounts of IL-1β, IL-18 and TNF-α when compared to LVS. This observation is also seen *in vivo*, which correlates with the reduced morbidity caused by LVSΔ*ripA*. These data suggest that *ripA* contributes to effective suppression of host immunity, and hence *F. tularensis* survival. Of equal importance is the role of IFN-γ and IFN-β during host response to *F. tularensis*, and it would be interesting to test whether the IFN-β and IFN-γ responses are altered in LVSΔ*ripA*-treated cells and animals during an infection.

An emerging theme in the literature is that a variety of pathogens can suppress IL-1β release by macrophages. *Pseudomonas aeruginosa* induces the release of IL-1β by macrophages in a NLRC4 dependent manner (32). A subset of *P. aeruginosa* expresses the effector molecule ExoU, and this molecule inhibits Caspase-1 activation thereby preventing the release IL-1β and IL-18 by macrophages. *Mycobacterium tuberculosis* (*Mtb*) circumvents both innate and adaptive immune responses. Similar to our observations with *F. tularensis*,

the *Mtb* gene product *zmp1* prevents the activation of the inflammasome to inhibit IL-1β processing (44). Among viruses, Myxoma virus (MYXV) inhibits release of pro-inflammatory cytokines IL-1β, IL-18, TNF-α, IL-6 and MCP-1. MYXV encodes a pyrin containing protein m103 which has been shown to bind to PYCARD and thereby disrupt the activation of inflammasome (45). The m103 protein also binds NF-κB which suppresses the degradation of IκBα and phosphorylation of IKK. Cowpox virus, via gene *crmA*, directly inhibits Caspase-1 activity, and suppresses IL-1β response to infection (46). Our study with *F. tularensis* LVS and LVSΔ*ripA* together with these and other reports exemplifies the multiple strategies employed by pathogens to disrupt host immune activation of the production of IL-1β and other cytokines by innate immune cells.

To elucidate the pathways by which LVS suppresses the IL-1β release by macrophages, we have identified PYCARD, Caspase-1 and MyD88 mediated signaling that the LVS strain can disrupt. Mariathasan and coworkers have shown that PYCARD and Caspase-1 mediated host IL-1β production is key in combating subsp. *novicida* infection (14). In their study, mice lacking *Pycard* or *Caspase-1* were far more susceptible to challenge by *F. tularensis* subsp. *novicida*. Moreover, mice intraperitoneally injected with IL-18 and IL-1β neutralization antibody prior to subsp. *novicida* challenge exhibited higher organ burdens than those treated with isotype control, further suggesting the importance of IL-1β and IL-18 in tularemia. Recently, AIM2 has been identified as candidate proteins required in *F. tularensis* induced inflammasome formation and subsequent IL-1β release (19, 47, 48). Thus far, TLR2, MyD88 and type I Interferon β have been implicated in *F. tularensis* induced host cytokine response, including IL-1β by macrophages (14, 35, 49-51). Our data

suggest that *F. tularensis* LVSΔ*ripA* induced IL-1β is PYCARD, Caspase-1 and MyD88 dependent and that LVS disrupts these signaling pathways.

In macrophages and epithelial cells, PAMPs or MAMPs activate NF-κB and MAP kinase pathways to initiate the pro-inflammatory responses. There are three major families of MAP kinase including the extracellular signal-regulated protein kinases (ERK), the c-Jun NH2-terminal kinases (JNK) and the p38 kinases (52). Many pathogens have developed strategies to disrupt the MAP kinase pathway to subvert the immune response. Arbibe and coworkers demonstrated that *Shigella flexneri* virulence factor *OspF* inactivates ERK1/2 and p38 by preventing their phosphorylation (53). *YopJ*, a type III effector protein of *Yersinia* species has been shown to acetylate the serine and threonine residues on MAP kinase kinase (MAPKK) thereby interrupting the downstream MAP kinase phosphorylation (54). Telepnev and coworkers have demonstrated that LVS may interfere with MAP kinase signaling pathways and that *iglC* is critical in this process (43). Our data suggest that LVS may be able to interfere with both the synthesis and processing of IL-1β at multiple points and that *ripA* is critical in these processes. The degradation of IκBα and phosphorylation of p65 is not likely the major mechanism by which *ripA* mediates immune suppression. However, LVSΔ*ripA* induced a significant increase in phosphorylation of these three MAP kinases when compared to wild-type LVS. Moreover, our studies showed that the LVSΔ*ripA* induced IL-1β and TNF-α release can be partially reduced by pre-treating cells with inhibitors of ERK, JNK and p38 alone, while the use of all three inhibitors resulted in an additive reduction of cytokine production that approaches the lower level caused by LVS infection. These results further validate that *ripA* contributes to the MAP kinase suppressive nature of *F. tularensis*. LVS

synergistically down-regulates the synthesis of pro-IL1β, and prevents the processing of IL-1β, thereby resulting in strong suppression of IL-1β release by macrophages.

A specific function for RipA protein has not yet been identified. Our experiments using PFA-killed LVS and LVSΔ*ripA* demonstrated that RipA-mediated suppression of the induction of IL-1β by LVS-infected host cells is an active process. In other words, the induction is not solely the result of gross morphological change in the bacterial cell or a cell surface marker that is present in LVSΔ*ripA* regardless of the bacterial viability. Preliminary data from our lab also indicates that the outer membrane profile and LPS profile of LVSΔ*ripA* is unchanged. Furthermore, our previous study demonstrated that RipA is localized to the cytoplasmic membrane of *F. tularensis* (12). This finding suggests that RipA does not mediate immune response suppression by directly interfering with or binding to innate immune receptors or sensors. More likely RipA is interacting with another protein or proteins that interfere with the pro-inflammatory signaling pathways.

Others have also found that infection of macrophages with LVS blocks TLR-induced signaling (43); however, there are seemingly conflicting reports that *F. tularensis* subsp. *novicida* or LVS is a potent inducer of pro-inflammatory cytokines (14, 33, 55, 56). These apparent contradictions may be due to variations in the experimental designs, such as the differences in MOI, the activation state of the cell population used, or the different subspecies used. Ulland et al. reported nanogram levels of IL-1β secretion from LPS-pre-stimulated BMDM that were subsequently infected with LVS (56), whereas we infected naïve BMDM. We also have found that following pre-stimulation of BMDM cells with *E. coli* LPS, LVS induced high levels of IL-1β (data not shown). Thus, the activation state of the cell likely affects the cytokine levels produced in response to *F. tularensis*. Consistent

with this possibility, Cole et al (55) found substantial LVS-induced IL-1β response by thioglycolate-elicited peritoneal macrophages. We believe that the use of non-stimulated macrophages, as in our study, might better reflect the host response during a primary infection.

An additional difference between our study and the latter is that our study primarily used LVS at MOIs of 50 to 500, while the paper by Cole and colleagues used MOI of 5-10 (55). We found that infection of resting macrophages with a low MOI of LVS induced little IL-1β, and the increased MOI is therefore unlikely to explain the difference in cytokine induction levels. Furthermore, the fact that LVSΔ*ripA* is both hyper-inflammatory and hyper-cytotoxic compared to wild type LVS, suggests that differences in cell death induction in LVS cannot explain the differences in IL-1β production and release (Fig. 1F). Finally, it is possible that these differences are due to infection by different subspecies. *F. tularensis* subsp. *novicida*, a less virulent subspecies, caused substantial IL-1β and IL-18 production and caspase-1-dependent cell death at the low MOI (14). Likewise, we found that a similar range of doses of subsp. *novicida* U112 caused a significant amount of IL-1β release in resting BMDM (Fig. 1H) whereas LVS did not (Fig. 1G). This suggests that the high levels of IL-1β in other studies relative to ours might also be explained by the use of different *F. tularensis* subspecies. While we acknowledge that LVS may induce various levels of cytokines depending on the methods of preparing primary mouse macrophages, it is clear that the deletion of *ripA* alleviated the immuno-suppressive nature of LVS with respect to IL-1β and TNF-α.

In conclusion, we elucidated a mechanism by which *F. tularensis* actively evades host immune response by an analysis of the attenuated LVSΔ*ripA* strain. These results indicate

that the *ripA* gene product functions by targeting two prominent innate immune circuits: the circumvention of the host inflammasome response and the dampening of the activation of MAP kinase signaling pathways. During the review of this paper, there was a report of another immune evasion gene in LVS that when removed, resulted in elevated IL-1β and cell death accompanied by reduced *in vivo* bacterial replication (56). Thus, the phenomenon described herein might be a common pathway for immune evasion by *F. tularensis*.

FIGURES

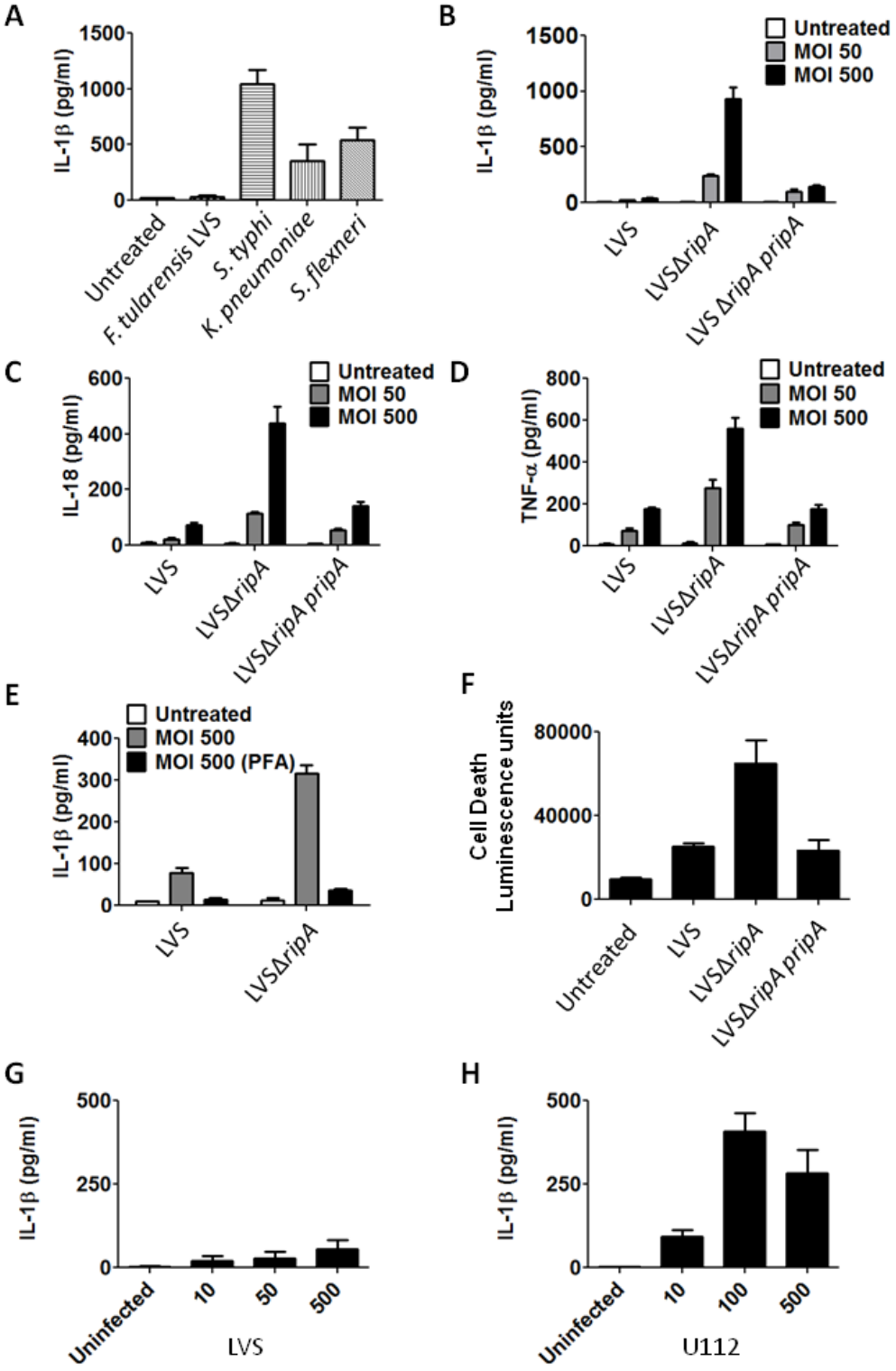
A
IL-1β (pg/ml)
1500
1000
500
0
Untreated
F. tularensis LVS
S. typhi
K. pneumoniae
S. flexneri
B
IL-1β (pg/ml)
Untreated
MOI 50
MOI 500
LVS
LVSΔripA
LVS ΔripA pripA
C
IL-18 (pg/ml)
600
400
200
0
Untreated
MOI 50
MOI 500
LVS
LVSΔripA
LVSΔripA pripA
D
TNF-α (pg/ml)
800
600
400
200
0
Untreated
MOI 50
MOI 500
LVS
LVSΔripA
LVSΔripA pripA
E
IL-1β (pg/ml)
400
300
200
100
0
Untreated
MOI 500
MOI 500 (PFA)
LVS
LVSΔripA
F
Cell Death
Luminescence units
80000
40000
0
Untreated
LVS
LVSΔripA
LVSΔripA pripA
G
IL-1β (pg/ml)
500
250
0
Uninfected
10
50
500
LVS
H
IL-1β (pg/ml)
500
250
0
Uninfected
10
100
500
U112

Figure 1. *F. tularensis* LVSΔ*ripA* fails to suppress pro-inflammatory cytokine release by primary mouse macrophages. (A) IL-1β secretion levels in mouse macrophages following exposure to *F. tularensis* LVS, *Salmonella typhi, Klebsiella pneumoniae,* or *Shigella flexneri* at MOI 50 for 24 hours as determined by ELISA. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$ (B) IL-1β secretion levels in mouse macrophages exposed to LVS, LVSΔ*ripA*, or LVSΔ*ripA* complementation strain at MOI 50 and 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$ (C) IL-18 secretion levels in mouse macrophages exposed to LVS, LVSΔ*ripA*, or LVSΔ*ripA* complementation strain at MOI 50 and 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$ (D) TNF-α secretion levels in mouse macrophages exposed to LVS, LVSΔ*ripA*, or LVSΔ*ripA* complementation strain at MOI 50 and 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$ (E) IL-1β secretion levels in mouse macrophages exposed to live and PFA-treated LVS and LVSΔ*ripA* at MOI 50 and 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$ (F) Cytotoxicity of mouse macrophages exposed to LVS, LVSΔ*ripA*, or LVSΔ*ripA* complementation strain at MOI 500 for 24 hours as measured by luminescence. Data represent mean± s.d. for at least three independent experiments performed in duplicate or triplicate. A representative experiment is shown. *, $p < 0.05$ (G) IL-1β secretion levels in mouse macrophages exposed to LVS at MOIs 10, 50, 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. (H) IL-1β secretion levels in mouse macrophages exposed to U112 at MOIs 10, 100, 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown.

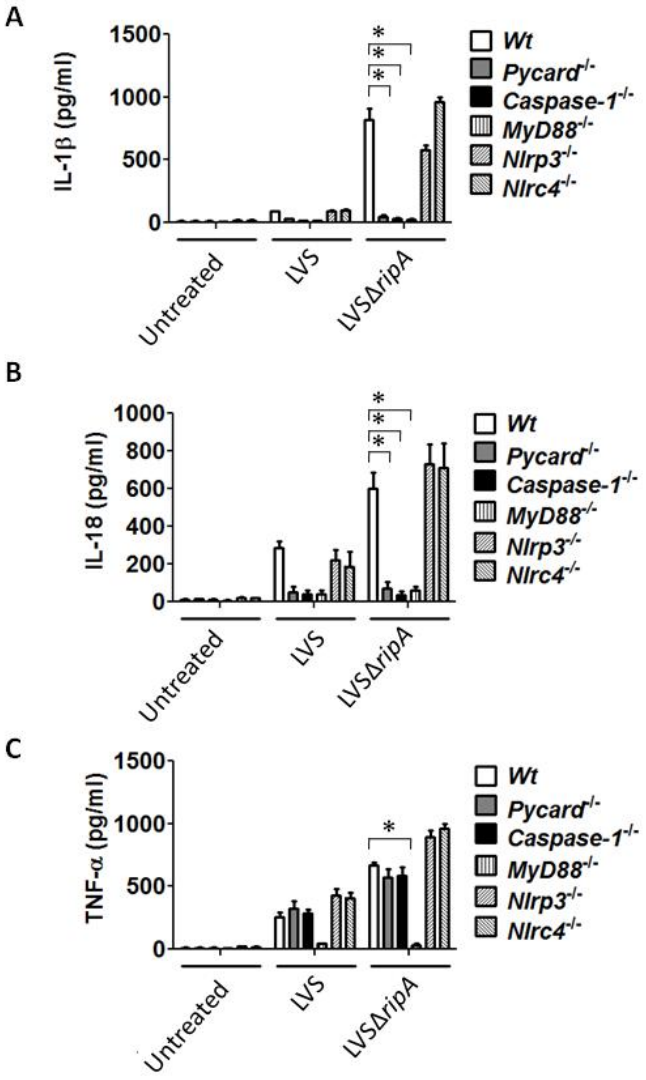

Figure 2. LVSΔ*ripA* induced pro-inflammatory cytokines are regulated by inflammasome components and MyD88. (A) IL-1β secretion levels in mouse macrophages derived from *Wt, Pycard*$^{-/-}$, *Caspase-1*$^{-/-}$, *MyD88*$^{-/-}$, *Nlrp3*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice exposed to LVS and LVSΔ*ripA* at MOI 500 for 24 hours as assessed by ELISA of cell supernatants. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$ (B) IL-18 secretion levels in macrophages derived from *Wt, Pycard*$^{-/-}$, *Caspase-1*$^{-/-}$, *MyD88*$^{-/-}$, *Nlrp3*$^{-/-}$, and *Nlrc4*$^{-/-}$ mice exposed to LVS and LVSΔ*ripA* at MOI 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$ (C) TNF-α secretion levels in mouse macrophages derived from *Wt, Pycard*$^{-/-}$, *Caspase-1*$^{-/-}$, *MyD88*$^{-/-}$, *Nlrp3*$^{-/-}$, *Nlrc4*$^{-/-}$ exposed to LVS and LVSΔ*ripA* at MOI 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$

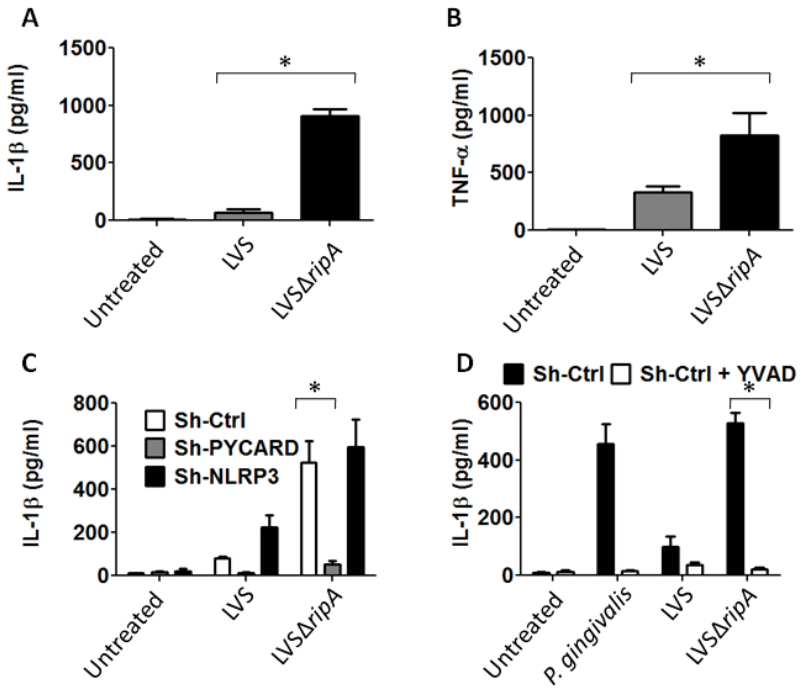

Figure 3. LVS *ripA* is necessary for suppression of pro-inflammatory cytokine release by human THP-1 cells. (A) IL-1β secretion levels in THP-1 cells exposed to LVS and LVSΔ*ripA* at MOI 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$ (B) TNF-α secretion levels in THP-1 cells exposed to LVS and LVSΔ*ripA* at MOI 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$ (C) IL-1β secretion levels in THP-1 cells expressing stable Sh-Ctrl, Sh-PYCARD and Sh-NLRP3 exposed to LVS and LVSΔ*ripA* at MOI 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$ (D) IL-1β secretion levels in mock pre-treated THP-1 cells and cells pre-treated with Y-VAD and exposed to LVS and LVSΔ*ripA* at MOI 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$

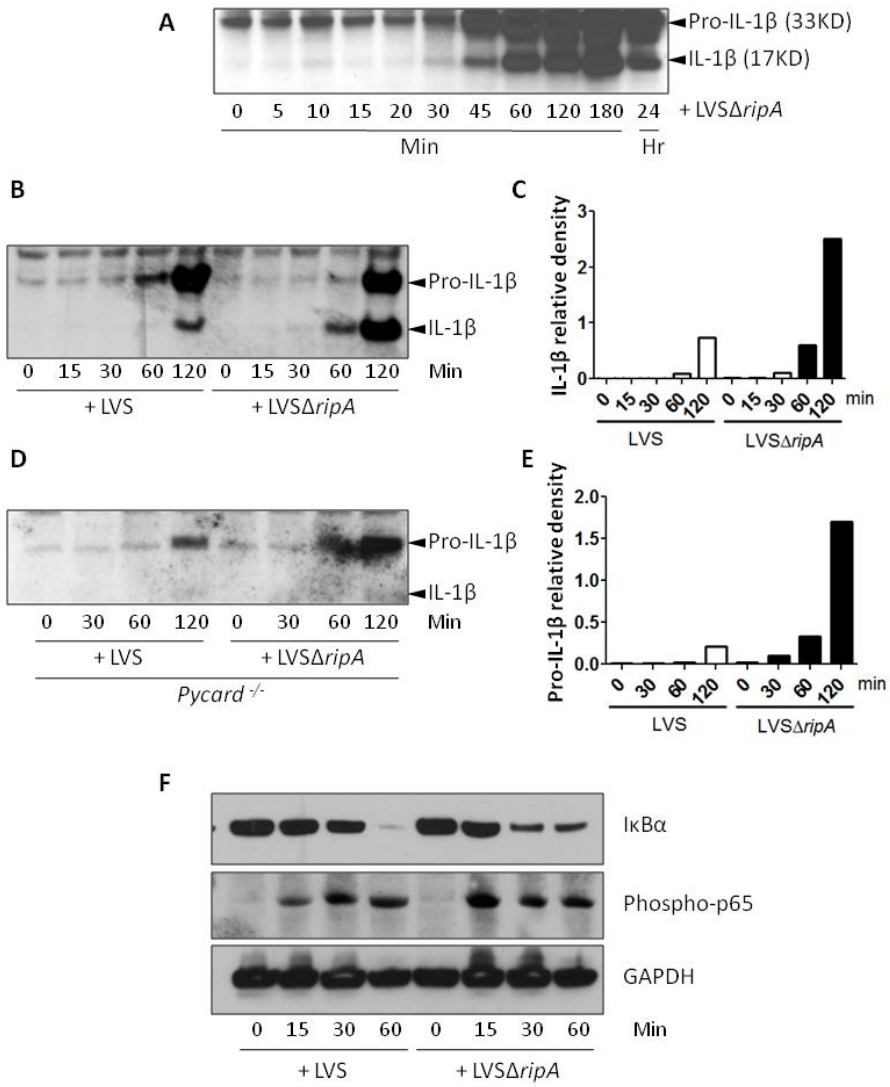

Figure 4. *F. tularensis* LVS suppresses IL-1β response in macrophages by interfering with IL-1β synthesis and processing. (A) Western blot analysis of IL-1β expression in mouse macrophages exposed to LVSΔ*ripA* at MOI 500 for 0, 5, 15, 20, 30, 45, 60, 120, 180 minutes and 24 hours. At least three independent experiments were performed. A representative western blot is shown. (B) Western blot analysis of IL-1β expression in mouse macrophages exposed to LVS and LVSΔ*ripA* at MOI 500 for 0, 15, 30, 60 and 120 minutes. At least three independent experiments were performed. A representative western blot is shown. (C) Densitometric quantification of the bands for processed IL-1β (17KD) is shown in B. (D) Western blot analysis of IL-1β expression in macrophages derived from *Pycard*$^{-/-}$ mice following exposure to LVS and LVSΔ*ripA* at MOI 500 for 0, 30, 60 and 120 minutes. At least three independent experiments were performed. A representative western blot is shown. **(**E) Densitometric quantification of the bands for pro-IL-1β (33KD) shown in D. F. Western blot analysis of IκBα and phospho-p65 in mouse macrophages exposed to LVS and LVSΔ*ripA* at MOI 500 for 0, 15, 30 and 60 minutes. At least three independent experiments were performed. A representative western blot is shown.

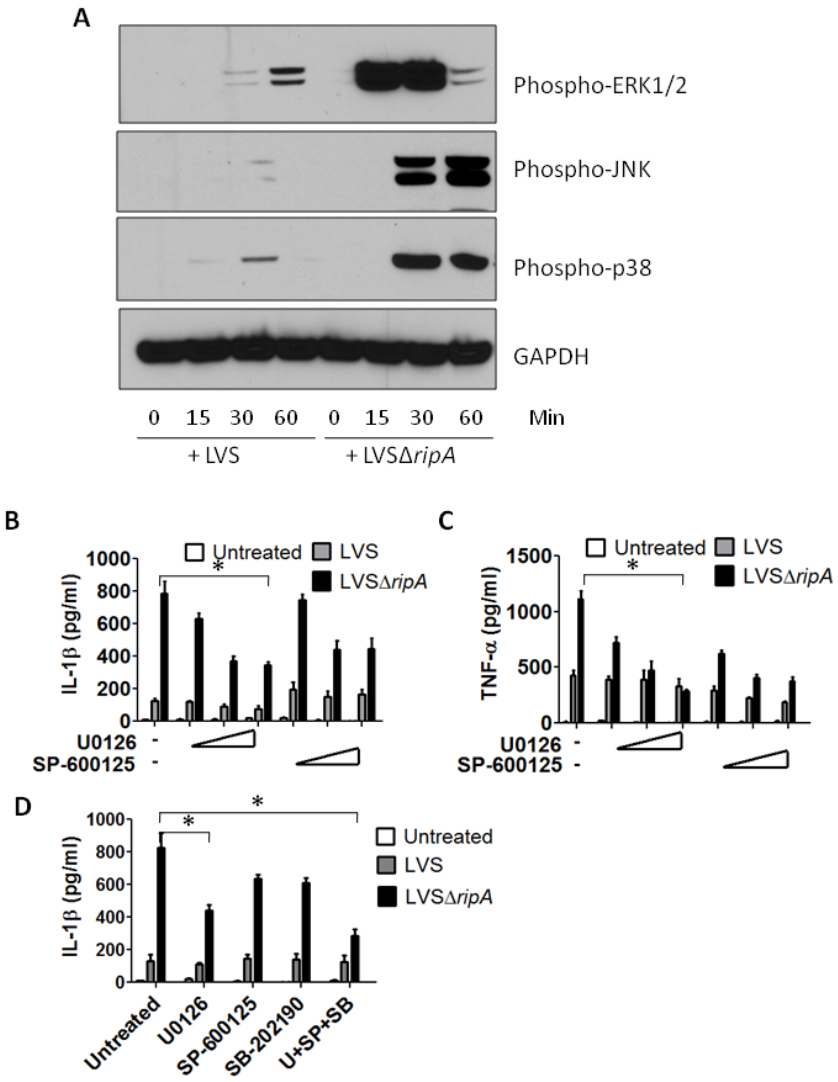

Figure 5. *F. tularensis* LVS suppresses pro-inflammatory cytokine release by disrupting MAP kinase signaling pathways. (A) Western blot analysis of phospho-ERK1/2, phospho-JNK, phospho-p38 and GAPDH in mouse macrophages exposed to LVS and LVSΔ*ripA* at MOI 500 for 0, 15, 30 and 60 minutes. At least three independent experiments were performed. A representative western blot is shown. (B) IL-1β secretion levels in mouse macrophages pre-treated with ERK inhibitor U0126 and JNK inhibitor SP-600125 and exposed to LVS and LVSΔ*ripA* at MOI 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$**.** (C) TNF-α secretion levels in mouse macrophages pre-treated with U0126 or SP-600125 and exposed to LVS or LVSΔ*ripA* at MOI for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$ (D) IL-1β secretion levels in mouse macrophages pre-treated with, U0126, SP-600125, p38 inhibitor SB-202190 or a combination of U0126, SP-600125 and SB-202190 and exposed to LVS or LVSΔ*ripA* at MOI 500 for 24 hours. Data represent mean ± s.d. for at least three independent experiments performed in triplicate. A representative experiment is shown. *, $p < 0.05$

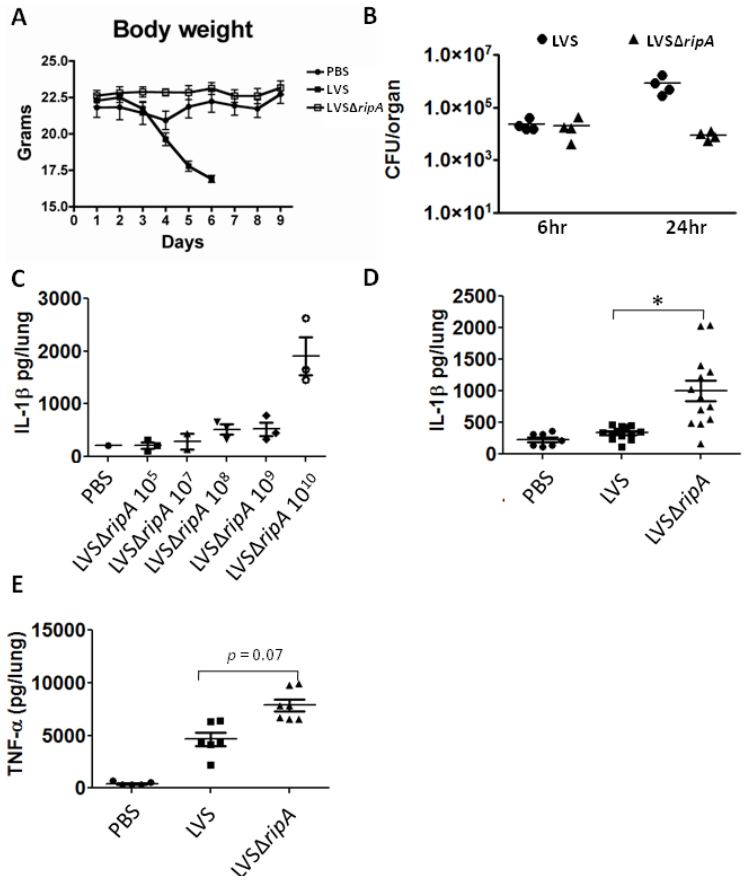

Figure 6. LVSΔ*ripA* fails to suppress pro-inflammatory cytokine responses in a mouse respiratory tularemia model. (A) Body weights of mice intranasally (i.n.) exposed to mock, LVS and LVSΔ*ripA* at dose of 10^5 for 1-10 days. At least two independent experiments with 7 animals per experimental condition (PBS, LVS, and LVSΔ*ripA*) were performed. A representative experiment is shown. (B) Lung organ burdens of mice i.n. exposed to LVS and LVSΔ*ripA* at a dose of 10^5 for 6 and 24 hours. At least three in, p ndent experiments with more than 4 animals per group were performed. Each symbol represents one animal. A representative experiment is shown. (C) IL-1β levels in mouse bronchoalveolar lavage fluid (BALF) following i.n. exposure to LVSΔ*ripA* at increasing doses for 24 hours. Data represent mean ± s.d. for at least two independent experiments with more than 3 animals per group. Each symbol represents one animal. A representative experiment is shown. (D) IL-1β levels in mouse BALF following mock treatment or i.n. exposure to LVS or LVSΔ*ripA* (10^{10} bacteria per animal for both LVS and LVSΔ*ripA*) for 24 hours. Data represent mean ± s.d. for at three independent experiments with more than 6 animals per group. Each symbol represents one animal. All data are shown. *, $p < 0.05$ (E) TNF-α levels in mouse BALF following mock treatment or i.n. exposure LVS or LVSΔ*ripA* (10^{10} bacteria per animal for both LVS and LVSΔ*ripA*) for 24 hours. Data represent mean ± s.d. for at least three two independent experiments with more than 6 animals per group. Each symbol represents one animal. All data are shown.

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CHAPTER 4
Francisella tularensis RipA protein topology and identification of functional domains.

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ABSTRACT

Francisella tularensis is a Gram-negative coccobacillus and is the etiological agent of the disease tularemia. Expression of the cytoplasmic membrane protein RipA is required for *Francisella* replication within macrophages and other cell types; however, the function of this protein remains unknown. RipA is conserved among all sequenced *Francisella* species, and RipA-like proteins are present in a number of individual strains of a wide variety of species scattered throughout the prokaryotic kingdom. Crosslinking studies revealed that RipA forms homoligomers. Using a panel of RipA-GFP and RipA-PhoA fusion constructs, we determined that RipA has a unique topology within the cytoplasmic membrane with the N- and C- termini in the cytoplasm and periplasm, respectively. RipA has two significant cytoplasmic domains, one comprised roughly of amino acids 1 – 50, and the second flanked by the second and third transmembrane domains and comprising amino acids 104 – 152. RipA functional domains were identified by measuring the effects of deletion mutations, amino acid substitution mutations and spontaneously arising intragenic suppressor mutations on intracellular replication, induction of IL-1β secretion by infected macrophages, and oligomer formation. Result from these experiments demonstrated that each of the cytoplasmic domains and specific amino acids within these domains were required for RipA function.

INTRODUCTION

Francisella tularensis is a Gram-negative coccobacillus that is the etiological agent of the zoonotic disease tularemia. *F. tularensis* infects a wide range of hosts, which includes humans, but predominately infects small mammals such as rabbits, voles, and squirrels (24). *F. tularensis* has also been isolated from arthropod vectors such as ticks (29, 34), mosquitoes (28, 43), and deerflies (24), which are also a source of transmission to humans (5, 6). Additional modes of transmission to humans include contact with infected animals (30, 31, 36), ingestion of contaminated food or water (23, 25), and inhalation of aerosolized bacteria (37, 42). There are four main subspecies of *F. tularensis* that differ in their virulence for humans*: novicida, mediasiatica, holarctica,* and *tularensis.* Subspecies *novicida* is not generally considered pathogenic for humans; however, there have been a few cases reported (1, 9, 20, 26). Subspecies *mediasiatica* is also associated with human disease in Asia, though less is understood about its pathogenesis (24). Subspecies *holarctica* and *tularensis* are most commonly associated with disease in humans with the latter being associated with the lowest infectious dose and most severe disease. For subspecies *tularensis*, transmission to humans via the inhalational route can occur at an infectious dose of as few as ten organisms (35). The ease of aerosolization and low infectious dose resulted in the designation of *F. tularensis* subspecies *tularensis* a select agent by the Centers for Disease Control, especially considering the historical development of *F. tularensis* bioweapons by several countries during the Cold War (13).

Within the lung, *F. tularensis* travels to the alveoli where it infects a wide range of cell types including alveolar macrophages, neutrophils, dendritic cells, monocytes, and alveolar Type II epithelial cells (2, 18, 19). *F. tularensis* is taken up by host cells via looping

phagocytosis, and after internalization, the bacteria escape the phagosome to replicate to high numbers in the cytoplasm (10, 11). In addition to the ability to replicate intracellularly, *F. tularensis* is able to suppress the pro-inflammatory immune response (2, 3, 8, 22, 40, 41). Several *F. tularensis* proteins have been reported as virulence factors that are required for this intracellular lifecycle; however, many of the identified virulence factors have little or no similarity to known proteins of other bacteria and their functions remain, for the most part, unknown.

Previously we identified a locus called *ripA* that is required for *F. tularensis* virulence. More specifically, deletion of this locus in the live vaccine strain (LVS) resulted in a mutant (LVSΔ*ripA*) that escapes the phagosome but is defective for intracellular replication (17) and fails to suppress the pro-inflammatory immune response (22). Not surprisingly, LVSΔ*ripA* is also attenuated in a pulmonary mouse model of tularemia (17). Deletion of *ripA* in the highly virulent *F. tularensis* Schu S4 strain results in a mutant that is defective for intracellular replication and attenuated in a mouse model as well, suggesting that RipA is required for virulence in the highly pathogenic *F. tularensis* subspecies *tularensis* (our unpublished results).

RipA is a cytoplasmic membrane protein that is conserved among *Francisella* species (17). Interestingly, bioinformatic analyses by our lab show that there is sporadic representation of RipA-like proteins among Prokaryotes, where RipA-like proteins are found in only specific strains of a given bacterial species. Similar to RipA, these other proteins are annotated as hypothetical proteins with no known functions or conserved domains; thus, the function of RipA remains unknown. RipA is a small protein (17 kD) with three transmembrane domains and has a single cysteine found within the second membrane-

spanning domain, which are unusual properties for a membrane protein, and examples of similar proteins in the literature are sparse. The unusual biochemical properties, combined with RipA's random distribution but obvious conservation within Prokaryotes, led us to study RipA for its potential for providing insight into what may be a novel class of proteins and/or for revealing a novel virulence mechanism employed by *F. tularensis*.

In this report, we address the question of RipA function by analyzing RipA at the biochemical and molecular level. Our results confirm a predicted topology model of RipA in the cytoplasmic membrane and show that RipA forms homoligomers. Using the topology model and alignments to other RipA-like proteins, amino acid substitutions were generated at conserved amino acids and the two individual cytoplasmic domains were deleted within RipA. These amino acid substitution and domain deletion mutants were analyzed for their ability to replicate intracellularly, suppress the immune response, and to form RipA oligomers. We also identified and analyzed an intragenic suppressor mutant. Using the suppressor mutant as well as the substitution and deletion mutants of RipA, we showed that both cytoplasmic domains and at least four amino acids are required for RipA function.

MATERIALS AND METHODS

Bacterial strains.

Francisella tularensis subsp. *holarctica* live vaccine strain (LVS) was obtained from the CDC, Atlanta, GA. All *Francisella* strains were maintained on chocolate agar supplemented with 1% IsoVitaleX (Becton-Dickson) and when applicable, 10 μg ml^{-1} kanamycin (Kan10) for selection. For growth of bacteria for infection or monitoring *in vitro* growth, strains were propagated in Chamberlain's defined medium (7). *Escherichia coli*

TOP10 (Invitrogen) or DH10b (Invitrogen) were used for cloning purposes. *E. coli* CC118 or BL21 (DE3)pLysS were used for expression of topology fusion reporter proteins. *E. coli* was propagated in Luria broth supplemented with ampicillin at 100 μg ml^{-1} (Amp100) or kanamycin at 50 μg ml^{-1} (Kan50) as necessary for antibiotic selection. All cultures were grown at 37°C with aeration.

Cell culture.

TC-1 (ATCC CRL-2785) cells are a tumor cell line derived from mouse primary lung epithelial cells and were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM $_L$-glutamine, 1.5 g L^{-1} sodium bicarbonate, 10 mM HEPES and 0.1 mM nonessential amino acids. J774A.1 (ATCC TIB-67) cells are a macrophage-like cell line derived from mouse sarcoma reticulum cells and were cultured in Dulbecco's minimal essential medium with 4.5 L^{-1} glucose, 10% fetal bovine serum, and 2 mM $_L$-glutamine. Bone marrow-derived macrophages were generated by flushing C57BL/6 mouse femurs and recovered cells were incubated for 6 days on 15 cm^2 non-tissue culture-treated dishes in L929 cell-conditioned DMEM. Nonadherent cells were removed by washing with phosphate-buffered saline (PBS) and bone marrow-derived macrophages were recovered from the dish using 10 mM EDTA in PBS. For experiments, BMMs were maintained in DMEM plus 10% FBS.

Molecular techniques and mutagenesis.

To generate constructs for RipA fusion protein expression, using the Phusion polymerase (New England Biosciences) we PCR-amplified *ripA* from LVS genomic DNA using primers that introduced XhoI and BamHI restriction sites directly before the start

codon and in place of the stop codon of *ripA*, respectively. For cloning of truncated *ripA* constructs, the reverse primer introduced a BamHI restriction site at the respective locations in *ripA* sequence but used the same forward primer. These constructs were first cloned into the pCR-Blunt II TOPO vector (Invitrogen) and subsequently subcloned using BamHI and XhoI restriction digest and ligation into pWaldo-TEV-GFPe and pHA-4 (33) to generate the fusion protein expression constructs. For expression of fusion proteins, the plasmids were transformed into *E. coli* CC118 (pHA-4) or *E. coli* BL21 (DE3)pLysS (pGFPe) by electroporation with selection on ampicillin at 100 μg ml^{-1} (pHA-4) or kanamycin at 50 μg ml^{-1} (pGFPe).

The cytoplasmic loop deletions in *ripA* were generated by gene synthesis of *ripA* containing the designated nucleotide deletions (corresponding to amino acids 4-47 or 105-151) and linker sequence insertion while maintaining the integrity of the flanking regions (Blue Heron). Each construct was PCR-amplified from the synthesis vector and cloned into the pCR-Blunt II TOPO vector (Invitrogen), verified by DNA sequence analysis, and subsequently subcloned into pMP590 (*sacB* Kan^r) using the BamHI and NotI restriction sites (27). For allelic exchange, plasmids were electroporated into LVS and integrants were selected on chocolate agar containing kanamycin (10 μg ml^{-1}). Kan^r strains were grown overnight and plated on 10% sucrose for counterselection (loss of plasmid) (17). Deletions were confirmed by PCR analysis of genomic DNA using primers external to deleted regions. The strategy for generation of the *ripA* deletion in LVS and for complementation of the *ripA* deletion has been described (17).

Single amino acid changes in RipA were generated by introducing 1-2 bp changes in the respective codon of the *ripA* DNA sequence using QuikChange II Site-directed

Mutagenesis kit (Stratagene) following the manufacturer's protocol. The plasmid used for mutagenesis was the multi-copy *Francisella* shuttle vector pKKMCS that expressed *ripA* under its native promoter and containing both 5' and 3' flanking regions. After DNA sequence confirmation of the mutations within *ripA*, the plasmids were each transformed into the LVSΔ*ripA* strain by electroporation and selection on chocolate agar Kan10.

To generate HA-tagged *ripA*, a fusion construct was made by splice overlap extension (21) with primers that introduced the HA tag in-frame to the C-terminus of the coding sequence of *ripA* and including a glycine linker sequence between the end of the coding sequence and the HA tag. This construct was sequenced before use and then cloned into pMP590 suicide vector and used for allelic exchange to generate a chromosomally-expressed HA-tagged protein. Clones were screened using one primer specific to the tag and the second specific to sequence on the chromosome. Additionally, this same construct was cloned into pKKMCS to generate a plasmid-expressed HA-tagged protein. Integrity of the sequence was confirmed by DNA sequence analysis.

GFP fluorescence and alkaline phosphatase activity assays.

Procedures for protein expression, GFP fluorescence and PhoA activity were adapted from published protocols (12, 15, 33). For expression of the GFP fusion proteins, *E. coli* BL21(DE3)pLysS containing each construct was grown in 1ml LB Kan50 overnight at 37°C and then diluted 1:50 into 500 µl LB Kan50 per well of a 48-well plate and grown at 37°C, 250 rpm to OD_{600}= 0.3-0.4. Expression was then induced by addition of 0.4 mM IPTG and growth to a final OD_{600}=0.6-0.8. Cell pellets were resuspended in 200 µl of buffer containing 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 15 mM EDTA and incubated for 30 minutes

at room temperature. Samples were then transferred to a black 96-well plate (Nunclone) and analyzed for GFP fluorescence using an excitation filter of 485 nm, emission filter of 512 nm, and a cutoff of 495 nm with the TECAN Infinite M200 and analyzed using Magellan v6 software. For calculated GFP fluorescence, after subtraction of background cells-only fluorescence, GFP emission was normalized against the OD_{600} of the culture.

For expression of the PhoA fusion proteins, *E. coli* CC118 with each construct was grown in 1 ml LB Amp100 overnight at 37°C and then diluted 1:50 into 500 µl LB Amp100 per well of a 48-well plate and grown at 37°C, 250 rpm to OD_{600}=0.13-0.18. Arabinose was added to a final concentration of 0.2% and bacteria were grown to OD_{600} 0.3-0.6. To prevent spontaneous PhoA activation, 1 mM iodoacetamide was added 10 minutes prior to harvesting and to all subsequent buffers. Samples were washed once in cold 10 mM Tris-HCl, pH 8.0 containing 10 mM $MgSO_4$ and resuspended in 1 ml cold 1 M Tris-HCl, pH 8.0. Then, 100 µl of each sample was added to 900 µl 1M Tris-HCl, pH 8.0 containing 1 mM $ZnCl_2$ and an OD_{600} measurement was taken on the remaining sample. We next added 50 µl 0.1% SDS and 50 µl chloroform, vortexed for 15 seconds, and incubated at 37°C for 5 minutes to permeabilize cells, then placed on ice for 5 minutes to cool. Finally, 100 µl of 0.4% *p*-nitrophenyl phosphate (in 1 M Tris-HCl, pH 8.0) was added and incubated at 37°C. The tubes were monitored until each turned pale yellow, and then 120 µl of 1:5 0.5 M EDTA, pH 8.0 containing 1 M KH_2PO_4 was added to stop the reaction. For each sample, the OD_{550} and OD_{420} were determined and to calculate the activity, the following equation was used: Units Activity= ((OD_{420} - (1.75 x OD_{550}))*1000)/ (time (min) x OD_{600} x volume cells (ml)).

Protein crosslinking.

Overnight cultures of LVS were grown in CDM pH 6.3 and used either for crosslinking or re-seeded to grow to mid-log (Klett 150). Each culture was aliquoted into 250-500 µl samples, washed once in 1X PBS, and then resuspended in the same buffer. Crosslinker was added to samples at a final concentration of 0.5% for formaldehyde or 0.5 mM for dithiobis (succinimidyl propionate) (DSP). Samples were incubated at room temperature for 30 minutes. For DSP-treated samples, 100 mM Tris-HCl (pH 7.4) was added to quench the reaction. All formaldehyde-treated samples were then washed once in ice-cold 1X PBS and resuspended in 200 µl SDS-PAGE loading buffer plus β-mercaptoethanol (βME) and heated at 60°C for 10 minutes (to maintain crosslinking) or at 100°C for at least 20 minutes (to cleave crosslinking). All DSP-treated samples were resuspended in SDS-PAGE buffer without βME (to maintain crosslinking) or with βME (to cleave crosslinking) and heated at 100˚C for 10 minutes. All samples were analyzed by SDS-PAGE and Western blotting as described below.

Membrane fractionations.

LVS was grown overnight in 15 ml cultures in CDM pH 6.3 at 37°C, pelleted, and washed once in lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.5). Cells were lysed in Lysis Matrix B tubes (MP Biomedicals) and beating in a Mini-Beadbeater (Biospec Products) for 45 seconds twice at 4°C. Cell lysates were collected after the beads settled and clarified by centrifugation at 12,000 x g for 3 minutes. Crude membrane fractions were separated from cytoplasmic fractions by ultracentrifugation at 100,000 x g for 90 minutes, with the cytoplasmic fraction being in the supernatant. The remaining crude membrane fraction pellet

was solubilized in lysis buffer containing a final concentration of 0.5% sarkosyl and incubated at room temperature with shaking for 30 minutes. Insoluble outer membrane components were isolated from the cytoplasmic membrane fractions (supernatant) by centrifugation at 100,000 x g for 60 minutes (17). Protein concentrations for each fraction were determined using a standard BCA Assay (Thermo Scientific).

SDS-PAGE and Western blotting.

Either 4-20% or 12% Pierce Precise™ protein gels (Thermo Scientific) were loaded with the designated samples at equal concentrations as determined by BCA Assay and run using BupH Tris-HEPES-SDS running buffer at 120 V. To determine molecular weights, either Benchmark™ Pre-stained Protein Ladder (Invitrogen) or PageRuler™ Plus Prestained Ladder (Fermentas) was used. For Western blotting, gels were transferred to nitrocellulose membranes at 400 mA for 45 minutes and then blocked overnight in 1% BSA in PBST. All antibodies were incubated at room temperature with rocking for 1 hr with PBST washes between incubations. Primary antibodies used were rabbit anti-RipAaa1-19 (described below) or mouse anti-HA monoclonal (Sigma), and secondary antibodies used were goat anti-rabbit IgG IRDye 680 or goat anti-mouse IgG IRDye 800CW. Protein was detected by near infrared fluorescence at 700 nm or 800 nm using the Odyssey Infrared Imaging System (LI-COR Biosciences).

Generation of affinity purified antibodies against RipA peptides.

Two RipA peptide sequences corresponding to amino acids 1-19 and amino acids 112-128 were synthesized by YenZyme Antibodies, LLC for the production of each peptide

and then rabbit antiserum and affinity purification of antibodies against each peptide. Pre-immune sera were also collected from the rabbits and used during initial tests of the antibodies for reactivity and specificity in Western blots of LVS lysates.

Gentamicin protection assays.

TC-1 epithelial cells or J774A.1 macrophages were inoculated with LVS at MOI 100 after determining bacterial concentrations via Klett. All LVS strains were grown overnight in CDM pH 6.3 prior to inoculation. The cells were incubated with the bacterial inoculum for 2 hr (J774A.1) or 4 hr (TC-1), and then incubated with media containing 25 μg ml^{-1} gentamicin for an additional 2 hr to kill the extracellular bacteria. At 4 hr (J774A.1) or 6 hr (TC-1) and 24 hr post infection, medium was removed, cells were washed with PBS and then scraped from the dish, and the bacteria serially diluted and plated to determine the number of viable bacteria.

IL-1β ELISAs.

Bone marrow-derived macrophages were prepared as described above and seeded in 12-well dishes at $1x10^6$ cells per well the same day as infection. One to two hours after plating, cells were then inoculated with bacteria at an MOI 500 and incubated at 37°C. After 24 hr, the supernatants from each well were collected and centrifuged to pellet cellular debris. The IL-1β ELISA was performed using the BD OptEIA mouse IL-1β ELISA kit (BD Biosciences) according to the manufacturer's protocol. The OD_{450} was read using a TECAN Infinite M200 and analyzed using Magellan v6 software.

Statistical analysis

For intracellular replication assay data, the differences for total CFU values at the 24 hr time point for each strain was analyzed for significance using the unpaired, two-tailed student's t-test. For IL-1β ELISA data, significance was determined using the repeat measures one-way ANOVA with a Dunnett multiple comparison post-test. Other details and the p-values are included in the figure legends. All statistical values were calculated using GraphPad Prism v.5 software (GraphPad).

RESULTS

Mapping RipA topology of RipA using carboxyl-terminal fusion reporter proteins.

RipA is a 179 amino acid cytoplasmic membrane protein estimated to be approximately 17 kD (17). Using the annotated amino acid sequence for RipA and the TMHMM software (38), the topology of RipA was predicted. The data output was used in TMRPres2D (39) to generate the image depicted in Figure 1A. This model predicted that RipA has three membrane-spanning domains (amino acids 51-71, 83-103, and 152-172) with the amino terminus (N-terminus) in the cytoplasm and the carboxyl terminus (C-terminus) in the periplasm. Also predicted are two large cytoplasmic domains (amino acids 1-50 and 104-151) and two smaller periplasmic domains within RipA (amino acids 72-82 and 173-179). It is rare for a protein to have only three transmembrane domains with this orientation, and very few such proteins have been thoroughly characterized.

Considering the value of validating such a unique topology for RipA, we made use of a fusion reporter protein system that has been used to map *Escherichia coli* cytoplasmic membrane proteins (12, 15, 33). In this system, either GFP or alkaline phosphatase (PhoA)

was fused at the C-terminal end of recombinant proteins or peptides, expressed in *E. coli,* and assayed for GFP fluorescence and PhoA activity. GFP is only fluorescent in the cytoplasm and PhoA is only active in the oxidizing environment of the periplasm (14). Thus, by fusing GFP and PhoA to a protein, one can determine the cytoplasmic or periplasmic location for the C-terminus of the protein. GFP or PhoA was fused at the C-terminus of RipA and at four locations predicted to be near transmembrane junctions of RipA thereby generating fusion proteins expressing amino acids 1-47, 1-80, 1-106, 1-148, and 1-179 (full length RipA). C-terminal fusion proteins of DcuB and GlpT, two *E. coli* proteins that have been identified as having C-termini with periplasmic and cytoplasmic locations, respectively, were included as controls (12). The GFP fusion proteins were expressed in BL21 (DE3)pLysS *E. coli* and PhoA fusion proteins were expressed in *phoA*$^{-}$ CC118 *E. coli.* GFP fluorescence or PhoA activity was measured for each construct, and graphed as a ratio of PhoA to GFP (Figure 1B). As expected, the control DcuB fusion protein had high PhoA to GFP ratio designating periplasmic location, and the control GlpT fusion protein had a low PhoA to GFP ratio designating a cytoplasmic location. In terms of RipA, fusion proteins for amino acids 1-47, 1-106, and 1-148 had a low PhoA to GFP ratio (i.e. high GFP fluorescence) corresponding to a cytoplasmic location for RipA at amino acids 47, 106, and 148. Constructs for amino acids 1-80 and 1-179 had a higher PhoA to GFP ratio (i.e. high PhoA activity) corresponding to a periplasmic location at amino acids 80 and 179. Thus, the fusion protein reporter system supports that the predicted RipA topology is correct.

RipA forms homoligomers as revealed by *in vivo* crosslinking in LVS.

As a small, integral cytoplasmic membrane protein, it is possible that RipA interacts with itself and/or other proteins. Additionally, under non-reducing conditions, higher molecular weight (MW) bands have been observed on Western blots probed with anti-RipA aa1-19 antibody. For example (Figure 2A), under non-reducing conditions (i.e. no addition of β-mercaptoethanol (βME)), there are at least three higher MW bands, whereas there is only one higher MW band in reducing conditions. This result hints that RipA may be interacting with itself as a homoligomer. In order to capture potential RipA oligomerization *in vivo*, two different bifunctional, reversible chemical crosslinkers, formaldehyde and DSP, were used to treat LVS grown in chemically-defined media. Formaldehyde was added to cells at concentrations ranging from 0.1-1% with the optimal percentage being 0.5%, which was used for subsequent experiments. Cleavage of formaldehyde-mediated protein interactions is accomplished by boiling samples. DSP was added to cells at concentrations ranging from 0.5-1 mM with the optimal concentration being 0.5 mM, which was used for subsequent experiments. DSP-mediated interactions are reversed by the addition of βME. Crosslinking with either formaldehyde or DSP revealed that RipA formed 2-4 higher molecular weight bands of 40 kD, 55 kD, 70 kD, and sometimes 100 kD that disappear upon cleavage of the crosslinker (Figure 2B). These higher molecular weight bands likely represent a range of oligomeric states for RipA.

To determine whether the higher MW complexes correspond to RipA interacting with itself as opposed to other proteins, crosslinking experiments were performed on two complementary strains: HA-tagged *ripA* expressed on a plasmid in wild-type LVS and chromosomally-expressed HA-tagged *ripA* with wild-type *ripA* on a plasmid. For each strain,

both native and HA-tagged RipA were expressed by the *ripA* promoter on the same multi-copy plasmid backbone. Each of these strains as well as wild-type LVS were crosslinked with formaldehyde and analyzed via Western blot using antibodies against both RipA aa1-19 and against the HA tag. Secondary antibodies conjugated to different wavelength infrared dyes allowed for differential labeling of native and HA-tagged RipA (RipA-HA), and the HA tag allowed for differentiation by size. Due to the lower resolution of the higher MW bands, we focused on the ~40 kD band that corresponded to a potential RipA dimer. As expected, in place of a single band, there were three bands differing by only a few kD in the two strains containing HA-tagged and wild-type *ripA.* The lowest MW band corresponds to RipA/RipA dimers, the middle MW band to RipA/RipA-HA dimers, and the highest MW band to RipA-HA/RipA-HA dimers (Figure 2C). These data suggest that RipA interacts with itself and forms a homodimer and possibly higher number homoligomers. Work to identify whether RipA also interacts with other proteins is underway.

The cytoplasmic domains of RipA are important for RipA function.

Having determined the topology of RipA in the cytoplasmic membrane, we set out to identify which domains of RipA were required for function. Since protein-protein interactions often occur in the cytoplasm and because there are two large domains within RipA in the cytoplasm, the two cytoplasmic domains were targeted for analysis as putative functional domains using intracellular replication, induction of IL-1β secretion by infected macrophages, and RipA oligomer formation as functional read-outs. Independent deletions of each domain corresponding to amino acids 4-47 and 105-151 were constructed by replacement with a 15 amino acid linker sequence rich in glycines and serines and also two

prolines for flexibility. This resulted in two LVS mutants designated LVS *ripA*Δaa4-47 and LVS *ripA*Δaa105-151. These mutants were analyzed for intracellular replication in J774A.1 murine macrophages (Figure 3A) and TC-1 epithelial cells (Figure 3B). The results demonstrate that LVS *ripA*Δaa4-47 and LVS *ripA*Δaa105-151 are both defective for intracellular replication in each cell type, similar to LVSΔ*ripA*. Each mutant was also tested for the ability to induce IL-1β secretion as measured by ELISA on supernatants from infected bone marrow-derived macrophages (BMMs) (Figure 3C). Similar to what is seen for LVSΔ*ripA*, LVS *ripA*Δaa4-47 and LVS *ripA*Δaa105-151 both induced increased levels of IL-1β by infected BMMs. To validate that each protein was expressed in the proper location, cytoplasmic membrane fractions of each mutant were analyzed via Western blot using antibodies against RipA aa1-19 or RipA aa112-128 (Figure 3D). Deletion of amino acids 4-47 and 105-151 resulted in RipA proteins that are expressed in the correct location, though at a slightly lower level. The apparent reduction in membrane-localized protein could contribute to or even account for the observed phenotypes for this mutant. Finally, to determine the role of each domain to the ability of RipA to oligomerize, we performed *in vivo* crosslinking with formaldehyde or DSP as described above, excluding the first deletion mutant, whose protein expression was too low to detect any crosslinking (Figure 3E). Unlike LVS, which again displayed several higher molecular weight complexes, no RipA oligomer formation was observed for LVS *ripA*Δaa105-151, suggesting that the second cytoplasmic domain is required for binding. Together, these data suggest that both cytoplasmic domains, corresponding to amino acids 4-47 and 105-151 are required for RipA function, and that at least the second cytoplasmic domain is required for RipA oligomerization.

At least four amino acids within RipA are required for intracellular replication and suppression of the pro-inflammatory response.

Analysis of the results from a BLASTp search using the protein sequence of RipA revealed a relatively short list of RipA-like proteins that have E-values below zero. Moreover, these proteins are found only in individual strains of a given species, which seem to be randomly distributed across Prokaryotes (Table S1). Thirteen amino acids are identical or highly conserved among the 15 RipA-like proteins with E-values less than 10^{-3}. As an additional approach to identifying regions required for RipA function, mutants expressing alanine substitutions were made at each of these 13 conserved amino acids found among the RipA-like proteins discussed above, as well as the single cysteine, totaling 14 amino acid substitution mutants. Site-directed mutagenesis was performed on a plasmid containing *ripA* and the mutated plasmid was transformed into LVSΔ*ripA*. Each mutant was screened for expression of RipA in the cytoplasmic membrane using the anti-RipAaa1-19 antibody via Western blot on lysates of cytoplasmic membrane fractions (Figure 4A). To determine whether any mutant could trans-complement LVSΔ*ripA,* each strain was analyzed for the ability to replicate intracellularly in J774A.1 macrophages (Figure 4B) or TC-1 epithelial cells (Figure 4C) and for induction of IL-1β secretion by infected BMMs (Figure 4D). The mutants S46A, R48A, R49A, N53A, F55A, N60A, C97A, and E134A *trans*-complemented LVSΔ*ripA* in all assays, therefore suggesting that none of these residues are required for RipA function. Of the remaining mutants, W100A and P154A had variable or intermediate phenotypes; however, mutants Y35A, K114A, E122A and E150A did not *trans*-complement LVSΔ*ripA* in any of the assays. Thus, at least these four respective amino acids, Y35, K114,

E122 and E150, are required for RipA function and corroborates with the data for the domain deletion mutants that both cytoplasmic domains are required for RipA function.

Conservative and charge reversal amino acid changes confirm a role for K114, E122, and E150 in RipA function.

Due to their location in the second cytoplasmic domain and to further investigate the importance of K114, E122 and E150 to RipA function, both conservative and charge reversal substitutions were made at each amino acid. The substitution mutations were again generated using site-directed mutagenesis and the mutants assessed for intracellular replication (Figure 5A, B) and induction of IL-1β secretion (Figure 5C). For conservative changes, E122D and E150D, each mutant *trans*-complemented the LVSΔ*ripA* phenotype, whereas the respective charge reversal substitution mutants, E122R and E150R, did not. These data not only confirm a role of E122 and E150 in RipA function, they suggest that the charge at these sites is also important. For K114R, the conservative change mutant, an intermediate phenotype was observed, or partial *trans*-complementation. For K114E, the charge reversal mutant, there was no *trans*-complementation observed. Based on these data, we conclude that K114 is important for RipA function, but cannot determine whether the charge is responsible for its function. To verify that each mutant was expressed in the cytoplasmic membrane, protein expression was determined via membrane fractionations and Western blot using anti-RipAaa1-19 as described above (Figure 5D). Lastly, crosslinking experiments were performed with K114A, E122A, and E150A to determine whether any of these amino acids were required for RipA oligomerization (Figure 5E, 6D). Interestingly, each mutant was still able to form RipA oligomers, suggesting either that these amino acids are not involved in

oligomerization or that there are multiple amino acids mediating the binding such that changing one amino acid is not sufficient to eliminate the interaction.

Identification of an intragenic suppressor mutant supports a role for the first cytoplasmic domain in RipA function.

During the course of experiments with the alanine point mutants, the plasmids from LVS were repeatedly sequenced to verify the integrity of the *ripA* sequence and the desired mutations within RipA. In this process for K114A, E122A and E150A, we frequently observed mutations that resulted in a frameshift, loss of the alanine substitution mutation (rare), or single amino acid changes within *ripA.* Due to the relatively high frequency of mutations found within RipA-E150A, of the single amino acid changes identified, one was selected for further characterization, E150A with an additional V39A change, which was designated E150A/V39A. Assays for intracellular replication (Figures 6A, B) and also for IL-1β secretion (Figure 6C) revealed that this mutant *trans*-complemented LVSΔ*ripA*, suggesting that this new mutation was an intragenic suppressor. To confirm that the phenotype was the result of the intragenic mutation, and not the result of an extragenic mutation, the plasmid containing RipA E150A/V39A was isolated and re-transformed into LVSΔ*ripA* three independent times. Each of these new E150A/V39A transformants was assayed for intracellular replication and for IL-1β secretion and displayed the same phenotype as the original suppressor mutant, confirming that the V39A substitution was responsible for *trans*-complementation. After formaldehyde crosslinking, the E150A/V39A mutant also displayed the same higher MW bands as wild-type and E150A, suggesting that this *trans*-complementation was not a result of altered oligomeric complex formation (Figure

6D). Of note, a mutant containing only the V39A substitution in LVSΔ*ripA* deletion background did not display a defect in intracellular replication (data not shown). Overall, when considering the intragenic suppressor data, one can infer that the two cytoplasmic domains may be interacting with each other or alternatively with another protein or molecule.

DISCUSSION

F. tularensis is a highly successful pathogen; however, many of the known virulence factors still have no identified function. RipA is a virulence factor that was previously shown to be required *in vivo* in a pulmonary mouse model of tularemia, likely in part due to its inability to suppress the pro-inflammatory response like wild-type *F. tularensis* LVS or Schu S4 (17, 22, unpublished observations). Furthermore, RipA is required for *F. tularensis* intracellular replication in both macrophages and epithelial cells (17). However, LVSΔ*ripA* escapes the phagosome with similar kinetics to wild-type *F. tularensis* (17). This phenotype suggests that a lack of RipA results in a strain unable to adapt to the environment of the host cell cytoplasm. Not surprisingly, LVSΔ*ripA* grows poorly *in vitro* at higher pH and both *ripA* transcript and RipA protein expression are upregulated at higher pH and between 1 and 6 hours post infection of host cells, all correlating with a role for RipA in adapting to the cytoplasm (16).

This study addresses the question of RipA function at the biochemical and molecular level. Preceding studies demonstrated that RipA localizes to the cytoplasmic membrane of *F. tularensis* (17). Here, the topology of RipA within the cytoplasmic membrane was determined in terms of orientation and subcellular location of functional domains. RipA has three transmembrane domains, two large cytoplasmic domains and two smaller periplasmic

domains with the N-terminus in the cytoplasm and the C-terminus in the periplasm. Additionally, RipA was shown to form homoligomers in the membrane using *in vivo* crosslinking with either formaldehyde or DSP, and even in the absence of protein crosslinkers, the protein can exist in higher order forms. The first of several higher MW bands appears to correspond to at least a dimer size (~40 kD), suggesting that RipA forms a homodimer. RipA homodimerization was confirmed by the experiments using strains expressing both RipA and RipA-HA where in place of the single 40 kD band there were 3 bands corresponding to RipA-RipA dimers, RipA/RipA-HA dimers and RipA-HA/RipA-HA dimers. Due to the lack of resolution for the higher bands in these experiments and since these bands are not exactly trimer, tetramer, etc. in size, we cannot definitively say whether or not these bands are solely RipA or RipA crosslinked to another protein; however, based on the characteristic banding pattern, we suspect that these forms represent homoligomers as well. We are in the process of determining whether or not RipA interacts with any other *F. tularensis* proteins. Further insight into RipA topology, structure, and ultimately function, could be gained through biochemical experiments such as size exclusion chromatography and solving the crystal structure.

Francisella RipA proteins and some of the other RipA-like proteins have only a single cysteine found in the second transmembrane domain, which is uncommon. The highly reactive thiol group of a cysteine is known to be involved in several biological functions, most prominently the formation of disulfide bonds with other cysteine residues. These disulfide bonds can occur within the same protein to maintain tertiary structure or with a cysteine from another protein to mediate intermolecular complex formation, making unpaired cysteines quite rare. Due to the high reactivity of the thiol group, cysteine is not usually

found in isolation, and proteins with a single cysteine form oligomers or bind metals or other molecules via this amino acid. The fact that the RipA C97A mutant still formed oligomers (data not shown), was not attenuated for intracellular growth, and did not induce IL-1β secretion by infected macrophages suggests that this is not the case for RipA. Thus, the role of this single cysteine in RipA function remains unknown. One explanation is that there is no specific role for C97 in RipA function, especially considering that the cysteine is not completely conserved in all RipA-like proteins and that in some RipA-like proteins there is more than one cysteine present. Alternatively, the cysteine has a function unique to only *Francisella* RipA or a subset of RipA-like proteins. An interesting possibility is that the cysteine is involved in conformational changes of the RipA protein or binds to a small molecule as opposed to directly binding to protein partners.

Employing the topology model of RipA, we targeted specific domains for assessment of their role in RipA function. Using domain deletion mutants we found that each cytoplasmic domain was required for RipA function in terms of intracellular replication in macrophages and epithelial cells and suppression of the pro-inflammatory immune response and that at least the second cytoplasmic domain was required for RipA oligomer formation. This is further supported by analysis of the Y35, K114, E122, and E150 substitution mutants, each of which is within the cytoplasmic domains, and showed that these amino acids were also required for intracellular replication in both macrophages and epithelial cells as well as for suppression of the pro-inflammatory immune response. Unlike the RipA Δaa105-151 cytoplasmic domain deletion mutant, the single amino acid changes were not sufficient to prevent RipA oligomerization, suggesting that the interactions are mediated by multiple amino acids or that the individual amino acids are involved in some other aspect of RipA

function. In terms of the first cytoplasmic domain, the inability to determine the involvement of aa4-47 to RipA oligomer formation was due to RipAΔaa4-47 decreased cytoplasmic membrane expression. Nevertheless, there is a possibility that the first cytoplasmic domain is involved in formation of RipA oligomers and even that the two cytoplasmic domains interact. The intragenic suppressor mutation V39A in the E150A mutant restores the ability of the E150A mutant to replicate intracellularly and suppress the IL-1β response. Considering that V39 and E150 are in the first and second cytoplasmic domain, respectively, suggests that the two cytoplasmic domains interact with each other or alternatively with another shared substrate. Overall, our data show that both cytoplasmic domains, and even certain amino acids within each domain are important for RipA function.

The fact that there are other RipA-like proteins in a wide range of mainly less-studied species, including pathogenic, non-pathogenic, Gram-positive, Gram-negative, Mycobacteria, soil-dwelling, marine-dwelling, freshwater-dwelling and even one Archaea member is intriguing. Stranger still is that there have only been RipA proteins identified in select strains of a given species with the exception being *Francisella*, in which RipA is conserved among all sequenced species and strains. This could be due in part to a lack of genome sequences for a given species, or RipA could truly be a rare protein, or one for which there are functional homologs that share little protein sequence homology. So far RipA-like proteins have been identified in only four strains of pathogenic bacterial species: *Mycobacterium avium*, which can cause disease in birds as well as children, elderly and the immune-compromised, *Streptomyces scabiei*, which causes disease in plants, and *Aeromonas caviae*, which is a cause of gastrointestinal disease in humans, and *Actinomyces odontolyticus*, which is an opportunistic pathogen causing invasive disease in elderly or

immune-compromised patients with advanced dental cavities. Whether or not these RipA-like proteins play a role in pathogenesis is not known. Since we know that RipA is an important virulence factor for *F. tularensis,* it is possible that the function of RipA will represent a conserved virulence mechanism among pathogenic bacteria. Alternatively, and equally exciting would be that RipA functions as a novel virulence mechanism unique to *Francisella.* Being that the bacteria in which other RipA-like proteins are found are so varied in classification, it will be interesting to determine if there are differences in function of RipA among the pathogenic and non-pathogenic strains. The fact that a virulence gene homolog is found in environmental bacteria is not unfounded. A recent *in silico* analysis of marine bacteria genomes for virulence factors revealed that there was an abundance of well-characterized virulence genes, including pathogenicity islands and genes encoding secretion systems and toxins, represented among the analyzed strains (32). Genes encoding virulence factors are also widely distributed among soil bacteria as well (4). Perhaps RipA belongs to a novel family of proteins that will be revealed as more strains are sequenced and deposited into NCBI resulting in more RipA-like proteins being identified. Overall, determining the function of RipA is relevant not only for understanding the virulence of *F. tularensis,* but also perhaps for understanding the function of a potential class of proteins distributed throughout the Prokaryotes.

FIGURES

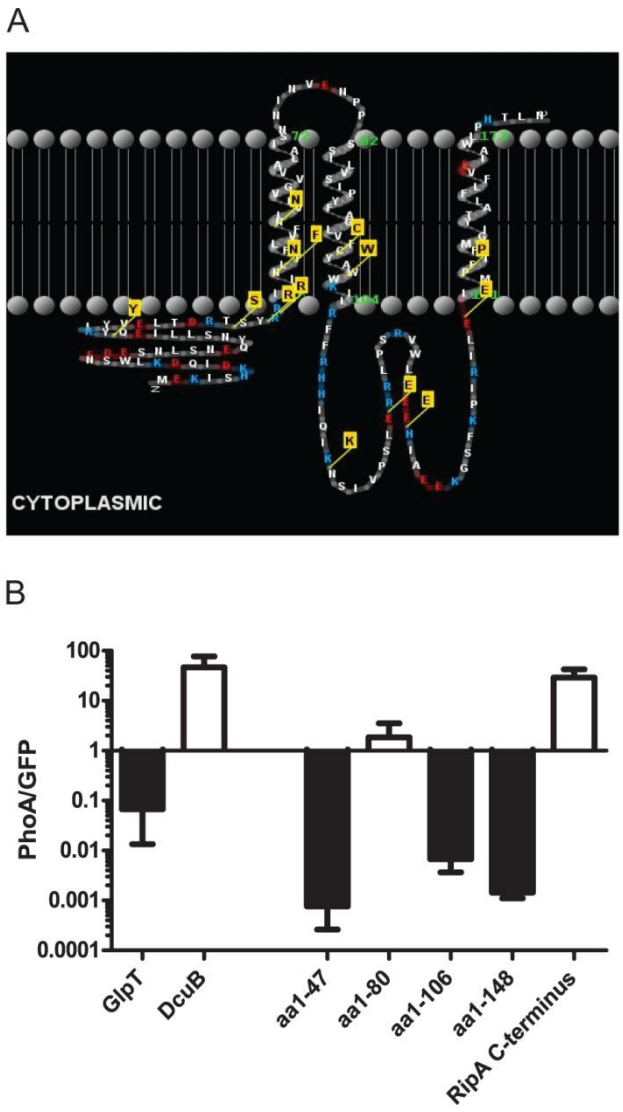

Figure 1. Mapping of RipA within the cytoplasmic membrane using C-terminal fusion reporter proteins in *Escherichia coli*. (A) The predicted topology model for RipA was determined using TMHMM and the image made using TMRPres2D. The yellow tags label the 13 amino acids that are conserved among RipA-like proteins in other bacterial strains as well as the single cysteine found within *Francisella* RipA. The colors designate electrostatic potential with red highlighting negatively-charged amino acids and blue highlighting positively-charged amino acids. (B) RipA C-terminal fusion proteins were expressed in *E. coli* alongside GlpT and DcuB C-terminal fusion proteins as GFP and PhoA positive controls, respectively. Each strain was assayed for PhoA activity or GFP fluorescence as appropriate. Data are represented as PhoA to GFP ratio consolidated from at least three experiments for each construct performed in triplicate. Error bars represent the standard deviation of the PhoA to GFP ratio values composite from all experiments.

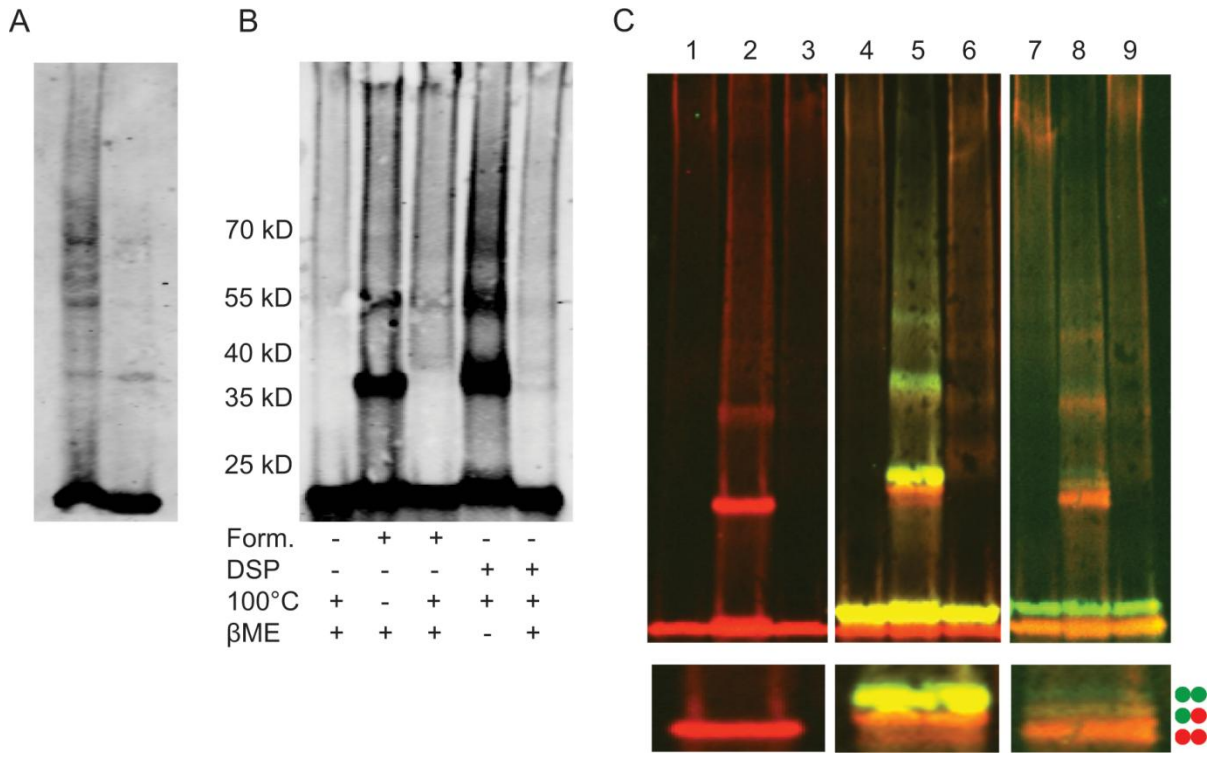

Figure 2. *In vivo* protein crosslinking of RipA. (A) Western blot of LVS lysates probed with anti-RipAaa1-19 after SDS-PAGE under non-reducing conditions (no βME) in Lane 1 and under reducing conditions in Lane 2. (B) LVS treatment either with 0.5 mM DSP or 0.5% formaldehyde (Form.) and analyzed by Western blot using anti-RipAaa1-19. Incubation of samples at 100°C (formaldehyde) or addition of βME (DSP) cleaved selected samples as designated. (C) LVS (Lanes 1-3), LVS with plasmid-expressed RipA-HA (Lanes 4-6), and LVS with chromosomally-expressed RipA-HA plus plasmid-expressed wild-type RipA (Lanes 7-9) were grown overnight and incubated with 0.5% formaldehyde and analyzed by Western blot probed with anti-RipAaa1-19 (red) and anti-HA (green). Lane 1, 4, 7- untreated; Lanes 2, 6, 8- 0.5% formaldehyde; Lanes 3, 7, 9- 0.5% formaldehyde plus 100°C. The images below each panel of the Western blot are cropped and enlarged from the Western blot to focus on the bands of interest. Although from the same blot, the panel containing lanes 7-9 was exposed at a lower intensity in the red channel in order to distinguish the fainter upper band (green channel) and this results in the upper band appearing more green than the expected yellow as seen in lanes 4-6. All data shown are representative of at least three experiments.

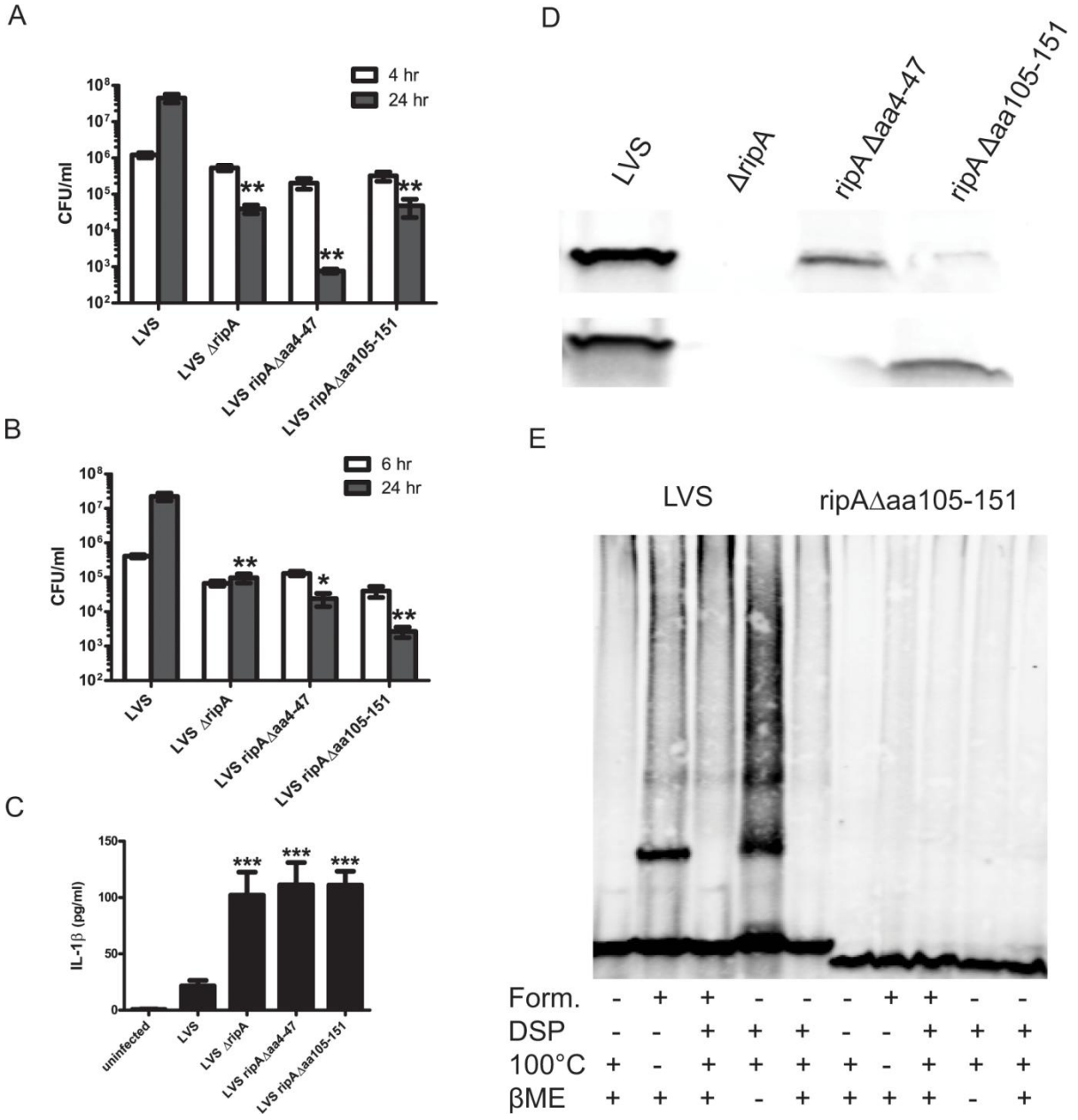
A
4 hr
24 hr
CFU/ml
LVS
LVS ΔripA
LVS ripAΔaa4-47
LVS ripAΔaa105-151
B
6 hr
24 hr
CFU/ml
LVS
LVS ΔripA
LVS ripAΔaa4-47
LVS ripAΔaa105-151
C
IL-1β (pg/ml)
uninfected
LVS
LVS ΔripA
LVS ripAΔaa4-47
LVS ripAΔaa105-151
D
LVS
ΔripA
ripAΔaa4-47
ripAΔaa105-151
E
LVS
ripAΔaa105-151
Form. - + + - - - + + - -
DSP - - + + + - - + + +
100°C + - + + + + - + + +
βME + + + - + + + + - +

Figure 3. Characterization of two RipA cytoplasmic domain deletion mutants. (A) Intracellular replication of wild-type LVS, LVSΔ*ripA*, LVS *ripA*Δaa4-47, and LVS *ripA*Δaa105-151 was assessed within J774A.1 macrophages (A) and within TC-1 epithelial cells (B) as determined by gentamicin protection assay. Each graph is representative of one of three experiments each performed in triplicate, and the error bars signify standard deviation between triplicates. (C) Secretion of IL-1β by BMMs at 24 hr as measured by ELISA on supernatants of cells left untreated or incubated with LVS, LVSΔ*ripA*, LVS *ripA*Δaa4-47, or LVS *ripA*Δaa105-151. The graph represents the results from one of at least three experiments performed in triplicate with error bars representing the standard deviation between replicates. (D) Protein expression of LVS, LVSΔ*ripA*, LVS *ripA*Δaa4-47, and LVS *ripA*Δaa105-151 in LVS cytoplasmic membrane fractions was determined by Western blot probed with anti-RipAaa1-19. (E) *In vivo* crosslinking on wild-type LVS (lanes 1-5) and LVS *ripA*Δaa105-151 (lanes 5-10) was performed by treatment with 0.5% formaldehyde or 0.5 mM DSP. For some samples, cleavage was achieved by addition of βME (DSP) or incubation at 100°C (formaldehyde) as designated. Crosslinking was followed by Western blot analysis probed with anti-RipAaa1-19. The Western blots shown are representative of at least three experiments. Statistical significance was determined by comparing the values for each respective mutant to the values for LVSΔ*ripA*. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

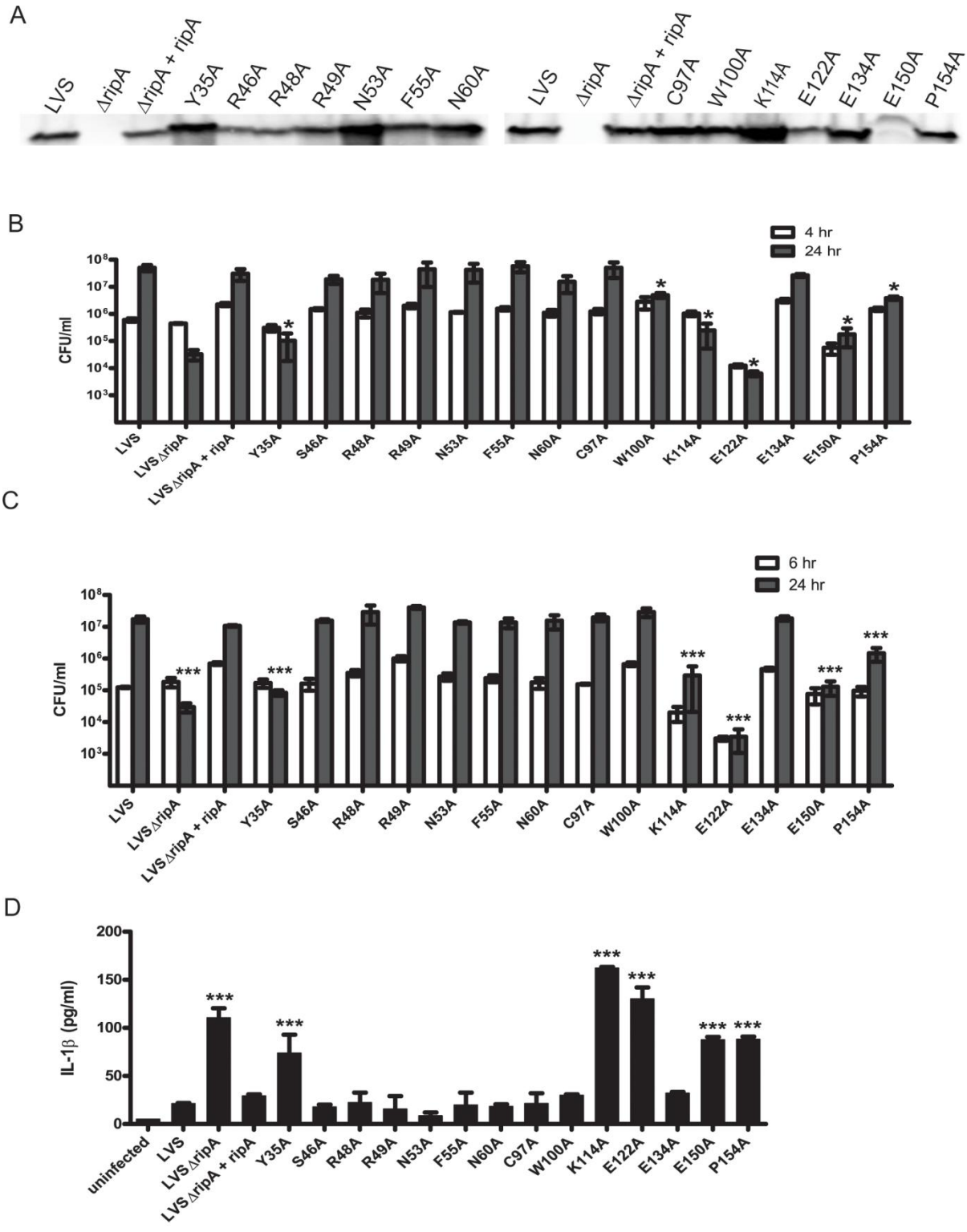
A
LVS
ΔripA
ΔripA + ripA
Y35A
R46A
R48A
R49A
N53A
F55A
N60A
LVS
ΔripA
ΔripA + ripA
C97A
W100A
K114A
E122A
E134A
E150A
P154A
B
4 hr
24 hr
CFU/ml
LVS
LVSΔripA
LVSΔripA + ripA
Y35A
S46A
R48A
R49A
N53A
F55A
N60A
C97A
W100A
K114A
E122A
E134A
E150A
P154A
C
6 hr
24 hr
CFU/ml
LVS
LVSΔripA
LVSΔripA + ripA
Y35A
S46A
R48A
R49A
N53A
F55A
N60A
C97A
W100A
K114A
E122A
E134A
E150A
P154A
D
IL-1β (pg/ml)
uninfected
LVS
LVSΔripA
LVSΔripA + ripA
Y35A
S46A
R48A
R49A
N53A
F55A
N60A
C97A
W100A
K114A
E122A
E134A
E150A
P154A

Figure 4. Characterization of RipA alanine substitution mutants RipA for intracellular replication and suppression of the pro-inflammatory response. (A) The 14 RipA alanine substitution mutants were expressed in LVSΔ*ripA* and analyzed for protein expression in the cytoplasmic membrane fractions of LVS by Western blot probed with anti-RipAaa1-19. Each mutant was assessed for intracellular replication in J774A.1 macrophages (B) and TC-1 epithelial cells (C) as measured by gentamicin protection assay. The graphs represent a consolidation of data for each mutant from a representative of at least two experiments performed in triplicate with the error bars signify standard deviation between triplicates. (D) Each mutant was analyzed for induction of IL-1β secretion by infected BMMs at 24 hr as measured by ELISA. The graph represents a consolidation of data for each mutant from a representative of at least two experiments performed in duplicate or triplicate, and the error bars signify standard deviation between replicates. Statistical significance was determined by comparing the values for each respective mutant to the values for LVSΔ*ripA* + *ripA* since this strain has the backbone plasmid on which the mutants were made. *, $p < 0.05$; ***, $p < 0.001$.

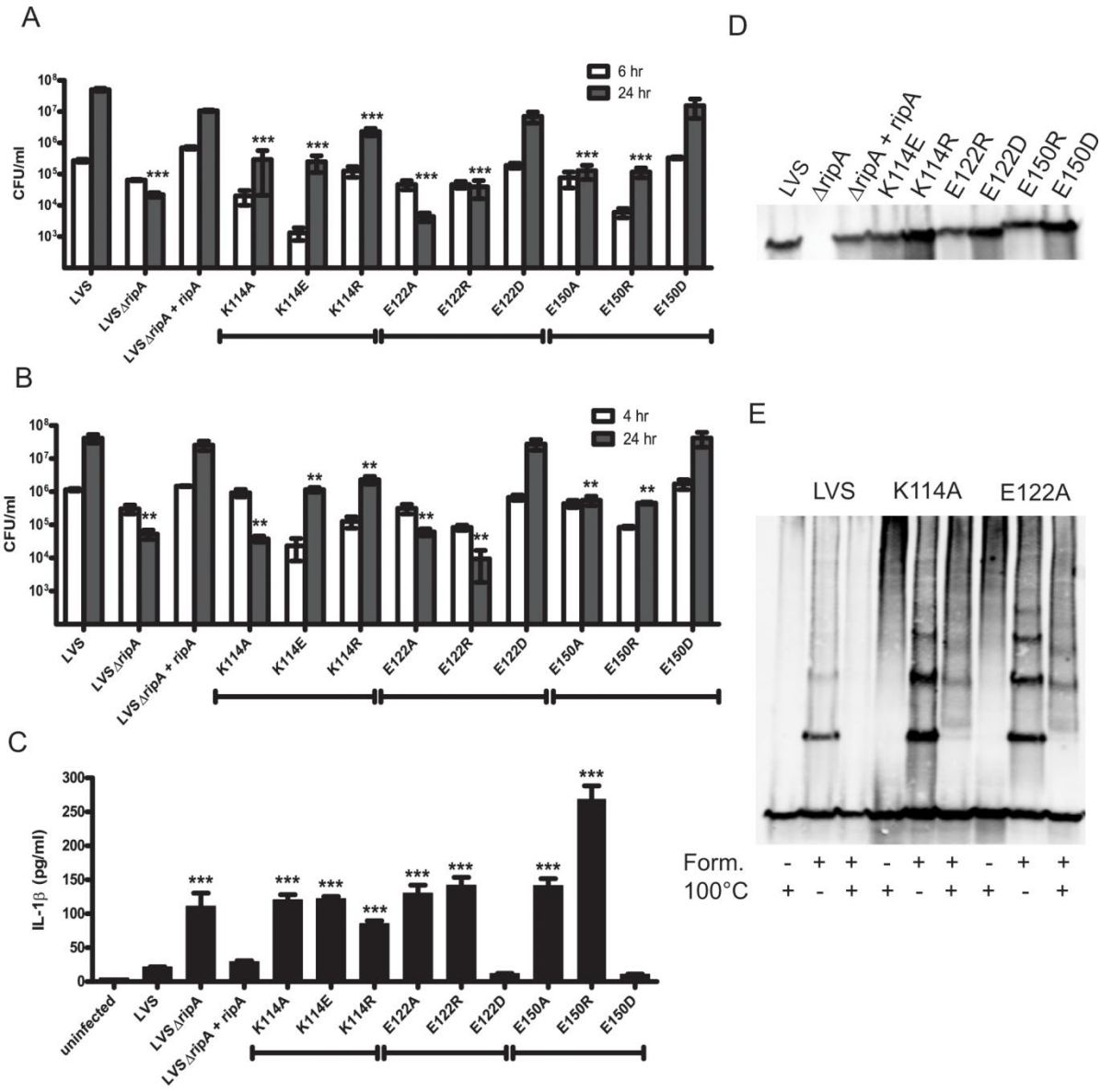
A
6 hr
24 hr
CFU/ml
LVS
LVSΔripA
LVSΔripA + ripA
K114A
K114E
K114R
E122A
E122R
E122D
E150A
E150R
E150D
B
4 hr
24 hr
C
IL-1β (pg/ml)
uninfected
D
E
Form.
100°C

Figure 5. Further analysis of amino acids K114, E122 and E150 to RipA function. The charge reversal and conservative amino acid substitution mutants for K114, E122, and E150 were assessed for intracellular replication within J774A.1 macrophages (A) and within TC-1 cells (B) using gentamicin protection assays. The graphs represent a consolidation of data for each mutant from a representative of at least two experiments performed in triplicate, and the error bars signify standard deviation between triplicates. (C) Each mutant was analyzed for induction of IL-1β secretion by BMMs at 24 hr as measured by ELISA on supernatants of infected cells. The graph represents a consolidation of data for each mutant from a representative of at least two experiments performed in duplicate or triplicate, and the error bars signify standard deviation between replicates. (D) Each mutant was assessed for protein expression in the cytoplasmic membrane fraction of LVS by Western blot analysis probed with anti-RipAaa1-19. (E) Wild-type LVS and the K114A and E122A mutants were crosslinked with 0.5% formaldehyde and analyzed by Western blot probed with anti-RipAaa1-19. Statistical significance was determined by comparing values for each respective mutant to the values for LVSΔ*ripA* + *ripA*. **, $p < 0.01$; ***, $p < 0.001$.

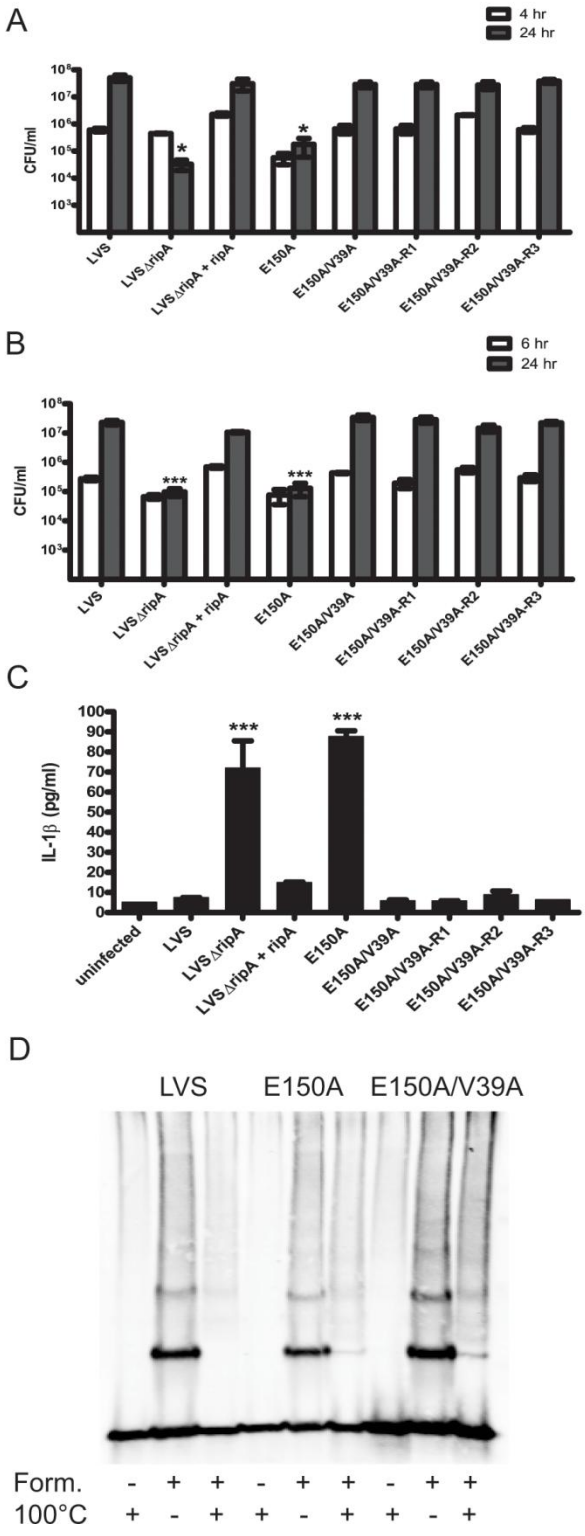
A
4 hr
24 hr
CFU/ml
LVS
LVSΔripA
LVSΔripA + ripA
E150A
E150A/V39A
E150A/V39A-R1
E150A/V39A-R2
E150A/V39A-R3
B
6 hr
24 hr
CFU/ml
C
IL-1β (pg/ml)
uninfected
D
LVS
E150A
E150A/V39A
Form.
100°C

Figure 6. Identification and characterization of an intragenic suppressor mutation in RipA E150A. A spontaneous V39A suppressor mutation appeared in the E150A mutant (E150A/V39A), and this plasmid was used to re-transform three LVSΔ*ripA* strains and generate E150A/V39A-R1, R2, R3. Wild-type LVS, LVSΔ*ripA*, LVSΔ*ripA* + *ripA*, the E150A mutant, E150A/V39A, and E150A/V39AR1-3 were assessed for intracellular replication within J774A.1 macrophages (A) and within TC-1 epithelial cells (B) as determined by gentamicin protection assay. The graphs represent a consolidation of data for each mutant from a representative of at least two experiments performed in triplicate with the error bars signify standard deviation between triplicates. (C) Secretion of IL-1β by macrophages at 24 hr as measured by ELISA on supernatants of infected cells. The graph shows a representative of at least two experiments performed in duplicate or triplicate, and the error bars signify standard deviation between replicates. (D) Wild-type LVS, the E150A mutant, and the E150A/V39A mutant were crosslinked using 0.5% formaldehyde and analyzed by Western blot probed with anti-RipAaa1-19. The Western blot shown is representative of at least two experiments. Statistical significance was determined by comparing the values for each respective mutant to the values for LVSΔ*ripA* + *ripA*. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Bacterial Strain	Accession #	E-value	Description of strain/species
Acidovorax delafieldii 2AN Alicycliphilus denitrificans K601	ZP_04761593.1 YP_004388625	$2x10^{-14}$	Gram-negative, β-Proteobacteria, aerobic, water-dwelling Gram-negative, β-Proteobacteria, aerobic, de-nitrifying, breaks down cholates, soil
Thermomonospora curvata DSM43183	YP_003299058.1	$4x10^{-14}$	Gram-positive, Actinobacteria, aerobic, cellulolytic thermophile, isolated from straw compost
Hylemonella gracilis ATCC 19624	ZP_08407204.1	$1x10^{-13}$	Gram-negative, β-Proteobacteria, aerobic, spirillum-shaped, isolated from pond water, stagnant or still freshwater
Streptomyces lividans TK24	ZP_05525530.1	$2x10^{-13}$	Gram-positive, Actinobacteria, aerobic, producer of small molecule natural products
Streptomyces cattleya NRRL 8057	CCB74526.1	$4x10^{-13}$	Gram-positive, Actinobacteria, aerobic, fluorinase producer
Aeromonas caviae Ae398	ZP_08522295.1	$4x10^{-13}$	Gram-negative, γ-Proteobacteria, facultative anaerobe, cause of gastrointestinal diseases
Streptomyces coelicolor A3(2)	NP_627504.1	$2x10^{-12}$	Gram-positive, Actinobacteria, aerobic, soil-dwelling, lifecycle involving mycelial growth and spore formation
Beggiatoa sp. PS	ZP_02000104.1	$4x10^{-12}$	Gram-negative, γ-Proteobacteria, aerobic, filamentous, marine environment, chemosynthesizer, isolated originally from the German coast of the Baltic Sea, one of the largest Prokaryotes
Nocardioidaceae bacterium Broad-1	ZP_08194929.1	$2x10^{-11}$	Gram-positive, Actinobacteria, contaminant during the assembly of *Coccidioides* genomes
Moritella sp. PE36	ZP_01896445.1	$3x10^{-11}$	Gram-negative, γ-Proteobacteria, aerobic, deep-sea piezophile heterotroph, adapted to high pressure, cryophilic
Streptomyces scabiei 87.22	YP_003490655.1	$4x10^{-10}$	Gram-positive, Actinobacteria, aerobic, mesophilic, motile, soil-dwelling, causes scab diseases of potatoes and other root crops
Mycobacterium avium subsp. avium ATCC 25291	ZP_05215248.1	$3x10^{-8}$	Mycobacteria/Gram-positive, Actinobacteria, aerobic, mesophilic, isolated from the

			liver of a hen and causes disease in birds as well as children, elderly and immune-compromised
Desulfobacterium autotrophicum HRM2	YP_002602733.1	$4x10^{-8}$	Gram-negative, δ-Proteobacteria, anaerobic, mesophilic, found in marine sediments, sulfate-reducing, isolated from Mediterranean Sea
Actinomyces odontolyticus F0309	ZP_06609016.1	$1x10^{-6}$	Gram-positive, Actinobacteria, aerobic, mesophilic, non-motile oral bacterium isolated from dental caries, can cause systemic disease in elderly and immune-compromised humans with advanced dental cavities
Archaeoglobus profundus DSM 5631	YP_003401486.1	$3x10^{-6}$	Archaea, Euryarchaeota, anaerobic, hyperthermophilic, motile, isolated from a deep sea hydrothermal vent,
Actinomyces sp. oral taxon 178 str. F0338	ZP_08026401.1	$1x10^{-5}$	Gram-positive, Actinobacteria, oral bacterium

Table 4.1 List of bacterial strains (other than those belonging to the *Francisella* genus) that have a RipA-like protein, as identified by BLASTp to *Francisella* RipA at the time of manuscript submission. Descriptive information was obtained from NCBI.

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CHAPTER 5

Summary and Future Directions

SUMMARY AND FUTURE DIRECTIONS

F. tularensis has a low infectious dose and causes the potentially fatal disease tularemia, but despite the knowledge of *F. tularensis* being such a successful pathogen, not much is known about its mechanisms of virulence. Part of the struggle to understanding *F. tularensis* virulence is the seeming lack of typical virulence factors employed by other pathogenic bacteria such as toxins or the Type III, IV, or V secretion systems. In fact, approximately 30% of the *F. tularensis* genome encodes hypothetical proteins, many of which have little or no homology to other characterized proteins (16). Another factor contributing to the relative lack of knowledge on *F. tularensis* virulence mechanisms is that only recently have the necessary genetic tools become available. Within the last decade, as these means have been utilized, numerous screens have been performed and through which, a significant number of proteins that contribute to *F. tularensis* pathogenesis were revealed (1-3, 14, 15, 18, 21, 24-26, 29, 31). Nonetheless, many of the studies remain descriptive in nature, and therefore, the functions of the majority of these virulence factors remain unknown.

In a search for proteins that were required for intracellular replication within the host lung epithelial cell, our lab identified the locus FTL_1914, which we called *ripA* (required for intracellular proliferation factor A). Deletion of *ripA* in LVS resulted in a mutant (LVSΔ*ripA*) that was defective for intracellular replication in both epithelial cells and macrophages (12). Interestingly, unlike many of the previously described mutants, LVSΔ*ripA* escapes the phagosome with the same kinetics as wild-type LVS, and therefore, the decreased replication is not the result of an inability to escape the phagosome (12). Furthermore, at later time points post-infection, like wild-type LVS, LVSΔ*ripA* is reported to

re-enter the FCV (12). Deletion of *ripA* in the highly virulent Schu S4 strain (Schu S4Δ*ripA*), results in a mutant that is defective for intracellular replication as well (our unpublished results, Dr. Sharon Taft-Benz). In addition, both *ripA* gene expression and RipA protein expression are upregulated at a higher pH and within macrophages during the first several hours of infection, and LVSΔ*ripA* grows poorly in broth media cultures at a high pH (13). All of this data suggest LVSΔ*ripA* is unable to adapt to the host cytoplasmic environment, although the specific reasons are not clear.

Further evidence to a role for RipA to *F. tularensis* virulence was shown through the studies described in chapter 3, in which we demonstrated the inability of LVSΔ*ripA* and Schu S4Δ*ripA* to suppress the pro-inflammatory immune response. Using PFA-killed LVS and LVSΔ*ripA*, we found that the suppression of the immune response is an active process, i.e. the reason for the increased LVSΔ*ripA*-mediated immune induction cannot be only due to gross morphological changes in the bacterial cell surface or to a cell surface protein that is present in LVSΔ*ripA* regardless of bacterial viability. Moreover, mixed infections with LVS and LVSΔ*ripA*, led to a loss in IL-1β induction, suggesting that the presence of wild-type bacteria can suppress the immune response induced by LVSΔ*ripA* (our unpublished results). Active suppression of the immune response by wild-type *F. tularensis* is further supported by other studies (5-7, 9, 17, 27, 28).

Since RipA is an inner membrane protein, it is probable that the effects on the immune response are indirect and that RipA does not directly interfere with or bind to host immune receptors. RipA could either be interacting with other proteins that interfere with the pro-inflammatory signaling pathways or alternatively, the loss of RipA be altering the bacterial membrane in such a way to expose other immune-interacting proteins or molecules,

in a manner that requires viable bacteria. Additional studies in our lab also indicate that the outer membrane, LPS, and lipid A profiles of LVSΔ*ripA* are unchanged and that LVSΔ*ripA* LPS does not stimulate IL-1β or TNF-α secretion by infected macrophages (our unpublished results, Cheryl N. Miller). Therefore, further studies are needed to determine if a more subtle or specific change is the source of the enhanced induction of the pro-inflammatory response in the absence of *ripA*. Significantly, these studies helped to provide further insight into the pathways that are being suppressed by wild-type *F. tularensis*, most notably the role for the MAP kinases ERK1/2, JNK, and p38. Future studies will include further dissection of the signaling pathways targeted by *F. tularensis* and activated by LVSΔ*ripA* with a priority on the identification of cytoplasmic sensors upstream of inflammasome activation. Identification of the protein that is responsible for sensing LVSΔ*ripA*, as well as other specific downstream pathways and proteins, could be useful in determining RipA function. In general, understanding the mechanisms by which *F. tularensis* modulates the host response to infection is principal in prophylactic and vaccine development not only in accounting for host responses but also for identification of bacterial drug targets.

For a small inner membrane protein of 17 kD, RipA has an unusual topology. More specifically, with a large cytoplasmic N-terminus, two transmembrane alpha-helices linked by a short periplasmic domain, a second large cytoplasmic domain, a third transmembrane domain, and a short periplasmic C-terminus. What's more, RipA contains only a single cysteine residue found within the second transmembrane domain. Cysteines contain a highly reactive thiol group that is usually involved in forming intramolecular disulfide bonds and occasionally binding to metals or other molecules. This reactivity makes unpaired cysteines quite rare; however, such cases often involve the cysteine binding to a cysteine from another

protein to mediate an intermolecular complex formation. With this in mind, we tested RipA for the ability to form oligomers using crosslinking agents, formaldehyde and DSP, and through this we learned that RipA forms homodimers. The characteristic banding pattern observed when crosslinking RipA suggests that RipA may be forming trimers, tetramers, and pentamers as well. Surprisingly, the cysteine in RipA was not required for oligomer formation nor for *F. tularensis* intracellular replication or suppression of IL-1β, so the role of RipA's single cysteine remains unknown.

Nevertheless, further experiments are required to clarify whether these higher MW complexes represent homo-oligomers and/or RipA interacting with another protein(s). Mass spectrophotometry on crosslinked RipA complexes as well as size exclusion chromatography on RipA-containing inner membrane fractions are currently being performed in pursuit of further defining the composition of RipA complexes. Localization studies using electron microscopy or immunofluorescence would also be useful in determining the distribution of RipA within the inner membrane of the bacterium. Future experiments also include further characterization of the role of various amino acids and domains to RipA function. We have already established that the two cytoplasmic domains are required for virulence and RipA oligomer formation. We would like to address the possibility that these two domains interact, for example, by FRET, yeast two-hybrid, or immunoprecipitation. By targeting additional amino acids or groups of amino acids by mutagenesis we can hope to narrow down regions of RipA that are mediating oligomerization and potentially binding to other proteins. Finally, as discussed in chapter 1, RipA was identified in a screen looking for glycosylated proteins, so we also plan to determine whether or not RipA is glycosylated at a predicted glycosylation site within the N-terminal cytoplasmic domain (4). Overall, additional biochemical

experiments will be beneficial to understanding RipA topology, oligomer formation, and eventually, RipA function.

Since RipA has two large cytoplasmic domains, RipA likely interacts with cytoplasmic proteins. The search for RipA-interacting proteins was initiated by means of immunoprecipitation using HA-tagged RipA and LVS lysates (11). These studies revealed a list of putative RipA-interacting proteins, one of which was confirmed using reciprocal pull-down experiments. This RipA-interacting protein (FTL_1364) had significant homology to proteins within the IclR family of transcriptional regulators, and therefore, we named this protein IclR. The homologous locus in *F. novicida* (FTN_0720) had previously been described as being required *in vivo* in the mouse after subcutaneous or intraperitoneal inoculation, for suppression of the pro-inflammatory response and cytotoxicity (31). Interestingly, the *novicida iclR* mutant was still able to replicate intracellularly in macrophages (31). Despite this, the other data in the study suggested that IclR contributes to virulence, and that its role may be at least in part through its interactions with RipA.

Therefore, we deleted the gene encoding IclR in the more highly pathogenic LVS (FTL_1364) and in Schu S4 (FTT_0748), and analyzed these mutants for intracellular replication, ability to induce IL-1β secretion and cytotoxicity in infected macrophages, and *in vivo* in the mouse following intranasal or intradermal inoculation. Surprisingly, all of the *iclR* deletion mutants behaved like wild-type strains in all assays; however, our examination of a *F. novicida iclR* transposon mutant confirmed the previously published results that *iclR* is required for *F. novicida* virulence *in vivo.* Microarray studies and subsequent synteny analysis of the results show that loss of IclR results in expression changes in genes that are disrupted in LVS and Schu S4, but not in *F. novicida* U112. These data suggest that in spite

of the changes in gene expression in all of the *iclR* mutants, the loss or disruption of affected genes in LVS and Schu S4 may at least partially account for why *iclR* is not required for virulence in these virulent strains. The precedence for this conclusion is also supported by the fact that the *tularensis* and *holarctica* species are noted for their genome decay. For example, both *tularensis* and *holarctica* genomes have a few hundred pseudogenes, while the *F. novicida* U112 genome has only fourteen (16, 30). The *tularensis* and *holarctica* genomes are also distinguished from the U112 genome in the increased presence of transposase sequences and gene rearrangements (16, 23, 30). Therefore, it's possible that IclR affects a similar set of genes shared among the species of *Francisella,* but due to the genome decay in the virulent strains, many of the transcripts are unable to be translated into functional proteins. Moreover, as discussed in chapters 1 and 2, the acid phosphatases AcpABC are required for the virulence of U112, but not for Schu S4 (10, 19, 20). Overall, combined with the different pathogenicity of each species for humans, these studies highlight differences between the *Francisella* species.

Even though IclR does not appear to be required for virulence of *F. tularensis*, the fact that expression of limited numbers of transcripts were significantly changed between LVS wild-type and Δ*iclR* strains suggest that IclR may in fact be a transcriptional regulator. Future studies with IclR include characterizing IclR as a transcriptional regulator by means of DNA binding assays, mutagenesis, and more detailed transcriptional studies in *novicida*. We would like to investigate further the microarray-identified genes to help us gain further insight into the function of IclR in *Francisella.* It is possible that IclR still plays an important role for *Francisella* survival in the environment or other hosts. Finally, we plan to further investigate the IclR-RipA interaction and whether or not this interaction is biologically

relevant. To do this, we would need to first validate the *in vitro* interaction with other assays, e.g. microscopy, FRET, or the crosslinking with mass spectrophotometry studies mentioned above, as a true interaction could provide insight into RipA function. Preliminary studies suggest that there is no overlap in gene expression changes identified by microarray for LVSΔ*iclR* compared to LVSΔ*ripA*, which goes against the hypothesis that the IclR-RipA interaction is related to the putative transcriptional activity of IclR.

The original immunoprecipitation studies with RipA identified multiple putative RipA-interactors, so there are more proteins that we can investigate that may help elucidate RipA function. One promising contender is the protein LpxA, which we have since confirmed the interaction with RipA using reciprocal pull-downs with tagged proteins (our unpublished results, Cheryl N. Miller). Spontaneous extragenic suppressor mutations of LVSΔ*ripA* were also identified within *lpxA* and *glmU,* both genes involved in lipid A biosynthesis, which complemented the LVSΔ*ripA* intracellular growth defect (our unpublished results, Cheryl N. Miller). These data suggest that RipA may be involved in LPS biosynthesis and/or membrane biogenesis. Studies looking at membrane permeability suggest that there are only mild differences between LVS and LVSΔ*ripA* susceptibility to antibiotics and other compounds, and more detailed studies exploring this phenotype are underway (our unpublished results, James R. Fuller, Cheryl N. Miller and myself). Changes to the membrane also would correspond with the LVSΔ*ripA* inability to suppress the immune response as such changes could uncover epitopes that could be recognized by the host, although other mechanisms could account for the lack of immune suppression by LVSΔ*ripA*.

The *ripA* gene is conserved among the species of *Francisella,* suggesting that the RipA protein functions the same among *Francisella.* To substantiate this observation, we

have also found that plasmid-expressed *F. novicida ripA* can functionally complement LVSΔ*ripA* for intracellular replication in both macrophages and epithelial cells as well as for suppression of the IL-1β secretion by infected cells (our unpublished results). While we have not investigated the role of *ripA* to virulence in *F. novicida,* we expect that based on the above data and gene conservation, that *ripA* is also required for the virulence of *F. novicida* as well as other pathogenic species of *Francisella.* Alignments of the genomic regions that immediately surround *ripA* show that there are some sequence differences in the 5' and 3' regions among the three strains, most notably in U112. Therefore, it's possible that the differences in this region could result in differences in the role of RipA to the virulence of *F. novicida,* and future studies could include investigation into these differences.

Along those lines, we have observed that there are other RipA-like proteins in strains of several Prokaryotic species. The fact that there are RipA-like proteins in such a randomly-distributed group of Prokaryotes, including mainly non-pathogens, is intriguing. As discussed in chapter 4, to date there are only four pathogenic bacteria known have a RipA-like protein, and whether or not these RipA-like proteins play a role in pathogenesis is not known. There is the possibility that RipA is a conserved virulence protein, but on the other hand, RipA may function as a novel virulence factor unique to *Francisella*. Future studies could include investigating whether the loss of the RipA-like protein in these organisms result in decreased virulence, and if so, whether *F. tularensis* RipA can complement the observed attenuated phenotype. It will also be worthwhile to establish if there are differences in RipA function among the pathogenic and non-pathogenic organisms, as many non-pathogenic bacteria encode virulence factors in their genomes (8, 22). It is exciting to speculate that RipA belongs to a novel family of proteins. To that end, future experiments could include

complementation studies in which RipA-like proteins from other Prokaryotes are expressed in *F. tularensis ripA* deletion mutants to see if they complement the deletion mutant phenotypes. Though the current list is small, as more strains are sequenced and deposited into public databases, more RipA-like proteins may be identified that will contribute to our determining RipA function. In the end, determining the function of RipA will not only help understand the function of perhaps a widely-distributed, larger class of proteins, but also bring a better understanding of the virulence of the highly-infectious and successful pathogen *F. tularensis.*

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