

Adherence and uptake of *Francisella* into host cells

G Brett Moreau¹ and Barbara J Mann^{1,2,*}

¹Department of Microbiology, Immunology and Cancer Biology; University of Virginia; Charlottesville, VA USA;

²Department of Medicine; Division of Infectious Diseases and International Health; University of Virginia; Charlottesville, VA USA

Keywords: attachment, adherence, internalization, *Francisella tularensis*, LVS, *novicida*

Abbreviations: LVS, live vaccine strain; EF-Tu, elongation factor-Tu; MR, mannose receptor; LPS, lipopolysaccharide; CR1, complement receptor 1; CR3, complement receptor 3; CR4, complement receptor 4; FcγR, Fc receptor; SRA, macrophage scavenger receptor class A; SP-A, surfactant protein-A; O-Ag, O antigen; HMDM, human monocyte-derived macrophage; GPI, glycosylphosphatidylinositol; GFP, green fluorescent protein; PI3K, phosphoinositol-3-kinase

Francisella tularensis is a highly virulent bacterial pathogen that is easily aerosolized and has a low infectious dose. As an intracellular pathogen, entry of *Francisella* into host cells is critical for its survival and virulence. However, the initial steps of attachment and internalization of *Francisella* into host cells are not well characterized, and little is known about bacterial factors that promote these processes. This review highlights our current understanding of *Francisella* attachment and internalization into host cells. In particular, we emphasize the host cell types *Francisella* has been shown to interact with, as well as specific receptors and signaling processes involved in the internalization process. This review will shed light on gaps in our current understanding and future areas of investigation.

Francisella tularensis is a highly infectious gram-negative bacteria and the causative agent of the potentially life-threatening disease, tularemia. *F. tularensis* can be acquired through a number of infectious routes, including inhalation, ingestion, percutaneous, or direct infection of the eye.¹ *F. tularensis* consists of several subspecies that vary in virulence potential. *Francisella tularensis* ssp. *tularensis*, which is also referred to as Type A, is found mostly in North America and causes the most severe form of the disease. Schu S4 is the most commonly studied Type A strain, and due to its high virulence must be handled under biosafety level 3 conditions. *F. tularensis* ssp. *tularensis* has also been classified as a Tier 1 select agent by the United States Centers for Disease Control, signifying that it poses a severe health threat if deliberately misused. *Francisella tularensis* ssp. *holarctica*, which is also referred to as Type B, is found mostly in northern Europe and causes a milder form of the disease. The live vaccine strain (LVS) is an attenuated type B strain that is useful for experimental models because it can be manipulated under biosafety level 2 conditions and, although attenuated in humans, is still capable of causing disease in mice.² *Francisella novicida*, which primarily

causes disease in immunocompromised individuals, is also used as a model for virulent *Francisella* species because it is virulent in mice, and shares a high degree of DNA and protein sequence similarity with the virulent species.²

F. tularensis is primarily an intracellular pathogen. Once intracellular *Francisella* escapes from the phagosome it replicates in the cytosol until the host cell lyses, allowing the released bacteria to infect other cells.¹ Although evidence for an extracellular phase of *Francisella* has emerged,³ the intracellular phase is thought to be dominant during infection. Indeed, mutants that are unable to survive and replicate intracellularly are typically attenuated for virulence.⁴⁻⁶

The processes of attachment and internalization into host cells are key steps necessary for *Francisella* to reach its intracellular niche and cause disease. It has been demonstrated both in vitro and in vivo that *Francisella* infects a wide variety of cell types. This review will discuss potential mechanisms by which *Francisella* attaches to host cells, as well as specific receptor interactions and processes that mediate internalization of the bacteria. A better understanding of these initial interactions could provide novel targets for therapies that could reduce virulence and thus disease.

Host Cells Supporting *Francisella* Infection

F. tularensis is a zoonotic bacterium that can infect a wide variety of species, ranging from arthropod vectors to many species of mammals.^{1,2} *F. tularensis* can also replicate in vitro within a variety of cell types, including phagocytic cells such as macrophages, neutrophils,⁷ dendritic cells,⁸ the murine macrophage-like cell lines, J774A.1⁹⁻¹¹ and RAW264.7 cells,¹²⁻¹⁴ and the human monocytic cell line THP-1.^{10,15} *Francisella* uptake and replication also occurs in non-phagocytic cells such as murine¹⁶ and human lung epithelial cell lines,^{17,18} hepatocyte cell lines,^{5,19} and fibroblasts.²⁰ Although *F. tularensis* replicates within all of these different cell lines, there are some differences in the ability of different cell types to interact with and support *F. tularensis* growth. For example, *F. novicida* and LVS associate with and are taken up by human monocyte-derived macrophages (HMDMs) in greater numbers than by human monocytes or J774A.1 cells.^{21,22} Lindemann et al.

*Correspondence to: Barbara J Mann; Email: bjm2r@virginia.edu
Submitted: 04/30/2013; Revised: 07/02/2013; Accepted: 07/03/2013
<http://dx.doi.org/10.4161/viru.25629>

found that the degree of attachment of LVS to several different epithelial cell lines (HEP-2, human bronchial epithelial [HBE], and A549 cells) was similar.²³ However, Lo et al. systematically examined uptake and growth of *F. novicida* in nine different epithelial cell lines using colony counts and immunofluorescent techniques, and found that while all tested cell lines supported replication, there were differences in the degrees of internalization, rates of replication, and bacterial loads.²⁴ Replication within host non-phagocytic cells may be sufficient for virulence, as a mutant in *Francisella* Schu S4 that was unable to replicate within macrophages maintained the ability to replicate within epithelial cells in vitro, and retained virulence in a mouse respiratory model.²⁰ *Francisella* LVS has also been observed within purified human erythrocytes,²⁵ which could represent a mechanism of dissemination within the host and possibly transmission to other hosts via arthropod vector.

Francisella infection of host cells has also been investigated in vivo. Hall et al. examined infected cell types in the lungs of mice over the course of a respiratory infection with Schu S4, LVS, and *F. novicida* by flow cytometry.²⁶ Twenty-four hours post-infection, the primary cell type infected by all three bacteria was alveolar macrophages, though the percentage of these cells was different for the various strains; over 70% of infected cells isolated from Schu S4 and LVS infected lungs were alveolar macrophages, whereas only 51% of the cells in *F. novicida* infected lungs were of this cell type. CD11b⁺ macrophages, dendritic cells, and Type II alveolar epithelial cells were also infected during this time period. Interestingly, nearly one fourth of all cells infected by *F. novicida* at 24 h post-infection were neutrophils, whereas only 0.4% of LVS infected cells were neutrophils, and no Schu S4-infected neutrophils were detected at this time point. However, by three days post-infection roughly 50% of the infected cells in both LVS and Schu S4 infected lungs were neutrophils, and in *F. novicida*-infected mice this percentage had risen to 80%. These differences in infected cell populations and their timing may be due to differences in the inflammatory responses elicited by *F. novicida* compared with LVS or Schu S4.²⁶

Arthropods including ticks and mosquitoes are natural vectors for *F. tularensis*. Thus, it is not too surprising that *F. tularensis* replicates well within *Drosophila*-derived cell lines.^{27,28} *F. tularensis* also infects and replicates within whole *Drosophila*, which could potentially provide a model to study aspects of the vector stage of *Francisella*. *Drosophila* flies have been used to screen for virulence factors,²⁹ and investigate interactions with the insect innate immune system.³⁰ Non-mammalian hosts, such as *Acanthamoeba castellanii*,^{31,32} and *Hartmannella vermiformis*,³³ also support *F. tularensis* replication, suggesting that protozoans could serve as a reservoir, or perhaps a mode of waterborne transmission.

Attachment to Host Cells

As the first step in the process of bacterial uptake, attachment to the surface of host cells can influence the internalization process itself, as well as downstream signaling events, through specific adhesin–receptor interactions. Although several host cell

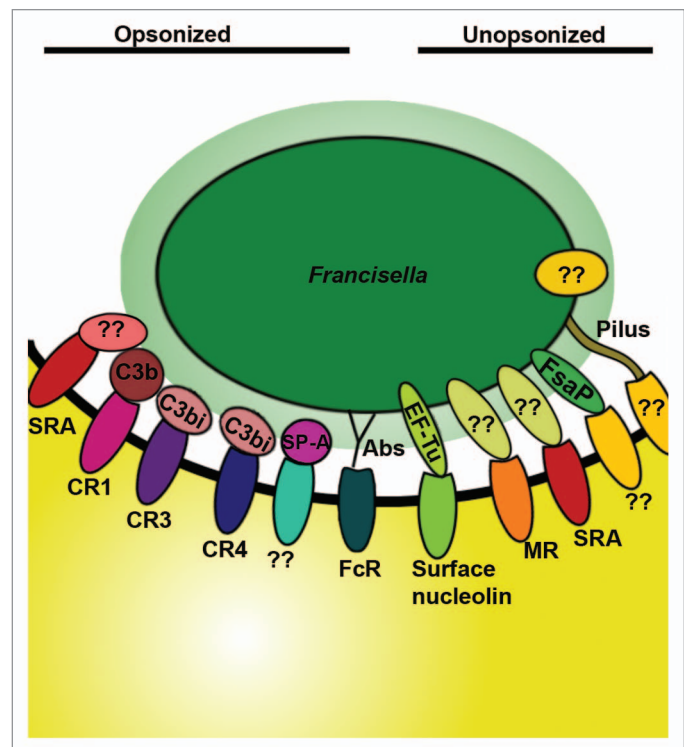


Figure 1. Overview of initial interactions between *Francisella tularensis* and host cells. *Francisella* attaches to the host cell surface via the bacterial pilus, FsaP, bacterial elongation factor-Tu (EF-Tu), uncharacterized adhesins (??), or opsonins. Host cell ligands for the pilus and FsaP have not been characterized (??). Once adhered, bacteria engage in specific adhesin–host receptor interactions to mediate internalization. In the absence of opsonins, *Francisella* engages the mannose receptor (MR), macrophage scavenger receptor (SRA), and surface nucleolin or uncharacterized receptors (??) to initiate uptake. In the presence of opsonins, *Francisella* internalization is redirected to the Fc γ R for antibody opsonized (Abs), or complement receptors (CR) and scavenger receptor (SRA) for serum-opsonized with C3b or C3bi. Opsonins bound by SRA have not been elucidated. Lung surfactant protein A (SP-A) can also serve as an opsonin to enhance internalization of *Francisella*.

receptors have been identified, the bacterial factors that contribute to *Francisella* attachment are not well characterized (Fig. 1). One potential adhesin is a Type IV pilus, which contributes to host cell attachment and virulence of a number of different bacterial species.^{34,35} Schu S4, LVS, and *F. novicida* all encode a Type IV pilus, though there are some differences.³⁶ LVS has deletions or frameshift mutations in 2 out of 6 genes encoding pilin-like proteins, and in *pilT*, which in other bacteria encodes the retraction motor.³⁷ Pili-like structures have been observed on *F. novicida*, LVS, and Schu S4,^{37–39} but the functional role of these structures in attachment and virulence appears to differ among the various subspecies. LVS deletion mutants in *pilF* and *pilT*, which encode orthologs of two proteins required for Type IV pilus fiber assembly and disassembly, exhibited significantly decreased attachment to both macrophage and epithelial cell lines.⁴⁰ However, deletion of genes encoding pilin-like components *pilE4*, *pilE5*, or *pilE6* in Schu S4 had no effect on attachment to J774A.1 macrophage-like cells or virulence, and in an LVS background actually

increased attachment.³⁹ Forslund et al. also determined that *pilA*, another gene encoding a pilin-related protein in a virulent Type B strain, was required for full virulence, but not attachment to HeLa cells.⁴¹ In *F. novicida* *pilF* is required for a Type II secretion system.³⁸

Francisella outer membrane protein FsaP (FTL_1658) has also been shown to contribute to host cell attachment.¹⁷ Melillo et al. identified FsaP as a prominent outer membrane protein by surface biotinylation of LVS. Expression of FsaP in *E. coli* resulted in an 8-fold enhancement of attachment to A549 lung epithelial cells. While expressed in all three major *Francisella* spp., FsaP does not localize to the outer membrane in *F. novicida*. This may be due to an amino acid variation at the signal peptide cleavage site that may prevent cleavage of the signal peptide and proper protein localization. However, there is also some discrepancy with the localization of FsaP; Zarrella et al. detected FsaP in the inner membrane during fractionation of LVS.⁴² FsaP is upregulated in a Type A strain isolated from infected mice,⁴³ but its significance or contribution to virulence has not yet been elucidated. FsaP has some similarity to FimV from *Pseudomonas aeruginosa*, which is involved in assembly of Type IV secretin,⁴⁴ and it also contains a conserved LysM domain, which in other proteins has been shown to bind peptidoglycan.⁴⁵

Surface-expressed *Francisella* elongation factor-Tu (EF-Tu) has been identified as mediating attachment to THP-1 cells.¹⁵ EF-Tu was found to interact with host cell nucleolin by pull-down assays. Both anti-EF-Tu antibodies and pseudopeptide HB-19, which binds irreversibly to nucleolin, blocked LVS attachment to THP-1 cells. Although EF-Tu is generally thought to be a cytoplasmic protein, there is a growing list of bacteria that utilize surface-expressed EF-Tu to bind host cells or factors.⁴⁶⁻⁴⁸

Internalization into Host Cells

Francisella has a low infectious dose of 25 colony forming units or less.¹ This low infectious dose suggests that *Francisella* can efficiently enter and replicate within host cells. However, *Francisella* internalization into host cells in vitro is fairly inefficient. Opsonization with complete serum or complement enhances internalization into host cells at least 10-fold.^{8,49,50} Schu S4, LVS, and *F. novicida* are resistant to complement-mediated killing through several mechanisms.⁵¹ LVS has been shown to bind Factor H, a host regulator that downregulates activation of the complement alternative pathway.⁵² Additionally, Schu S4, LVS, and *F. novicida* can all cleave C3b into the inactive form C3bi much more efficiently than a complement-sensitive LVS phase variant, implicating this cleavage in protection from complement-mediated killing.⁵¹ Deposition of C3bi on a bacterial surface does not lead to the recruitment of the membrane attack complex and cell lysis, but retains the ability to opsonize and enhance phagocytosis of the bacteria.

Host receptors mediating internalization

Several host cell receptors have been implicated in the internalization of *Francisella* (Fig. 1). The predominant receptor mediating uptake depends on whether *Francisella* is opsonized or unopsonized. In the absence of opsonins, *Francisella* uptake

by macrophages appears to be primarily mediated by the mannose receptor (MR), as bone marrow-derived macrophages from MR knockout mice internalize significantly less unopsonized Schu S4 than macrophages from wild-type mice.⁵⁰ Additionally, blocking MR with specific antibodies or soluble mannan significantly inhibits internalization of both *F. novicida* and LVS by monocytes.^{21,22} However, deletion of the mannose receptor does not completely eliminate *Francisella* internalization,^{21,22,50} implicating other host molecules in the internalization of unopsonized *Francisella*.

Uptake of opsonized *Francisella* can be mediated by different cell surface receptors depending on the opsonin. Uptake of antibody-opsonized *Francisella* occurs almost exclusively through the Fc receptor (FcγR), because uptake of Schu S4 exposed to opsonizing antibodies is completely ablated in macrophages from FcγR knockout mice.⁵⁰ Uptake of antibody-opsonized *F. novicida* also uses the FcγR, as fewer opsonized bacteria are internalized by monocyte-derived macrophages that are depleted of Fc receptors compared with control macrophages.²² Uptake of opsonized Schu S4 through the FcγR is associated with superoxide production, delayed escape from the phagosome, and decreased cytosolic growth compared with unopsonized bacteria,⁵³ suggesting that this route of entry would be associated with bacterial destruction in the presence of a humoral response.

Several host receptors have demonstrated roles in the internalization of serum-opsonized *Francisella*. The macrophage scavenger receptor class A (SRA) has been shown to contribute to the uptake of opsonized *Francisella*.^{49,50} This is somewhat unusual because in other gram-negative and some gram-positive bacteria it has been shown that bacteria directly bind to SRA through interactions with LPS or lipoteichoic acid, and that this binding is not influenced by the presence of serum.^{54,55} Treatment of J774A.1 cells with SRA agonists or blocking antibodies significantly decreases the uptake of serum-opsonized LVS, and macrophages isolated from SRA knockout mice have reduced ability to internalize LVS or Schu S4 compared with wild-type.^{49,50} In addition, expression of SRA in HEK293T cells significantly enhances the attachment of LVS, though not internalization, suggesting that additional receptors or signals are required for uptake. *Francisella* is also opsonized in the presence of SP-A, a surfactant found abundantly in the lungs. Pre-opsonization of *F. novicida* with SP-A results in increased association with monocyte-derived macrophages.²²

Multiple complement receptors have been shown to have roles in the uptake of serum-opsonized *F. tularensis*. Macrophage knockouts for the complement receptor 3 (CR3) internalize significantly less serum-opsonized Schu S4 than wild-type macrophages.^{21,50,51} Complement receptor 4 (CR4) plays a role in the uptake of serum-opsonized LVS by immature dendritic cells⁸ and monocyte-derived macrophages,⁷ whereas complement receptor 1 (CR1) and CR3 are critical for uptake of opsonized LVS and Schu S4 by neutrophils.⁷ Entry into host cells via the complement receptor could represent a mechanism for delaying immune responses and enhancing *Francisella* virulence. Typically, ligation of CR3 on monocytes does not result in production of reactive oxygen species or stimulation of inflammatory responses.^{56,57}

Additionally, uptake of Schu S4 by CR3 seems to dampen signaling through Toll-like receptor 2, leading to a less robust proinflammatory response.^{58,59} Geier and Celli found that when opsonized Schu S4 entered bone marrow-derived macrophages there was delayed maturation and escape from phagosomes, as well as delayed cytosolic replication compared with entry of unopsonized bacteria.⁵³ These results suggest that opsonization may represent a restrictive route of entry for Schu S4. However, more controlled replication could potentially be beneficial in delaying proinflammatory responses; Dai et al. showed that entry of Schu S4 into human monocyte-derived macrophages via CR3 limits proinflammatory cytokine responses.⁵⁹

Mechanism of phagocytosis

Uptake of *Francisella* is dependent on actin polymerization and microtubules in both phagocytic and nonphagocytic cells.^{23,60,61} However, *Francisella* can use different mechanisms of uptake depending on conditions and cell type. Clemens et al. used electron microscopy to observe phagocytosis of a Type A clinical isolate, RCI, and LVS by human monocyte-derived macrophages (HMDMs) and THP-1 human monocytic cells.⁶² This study found that a majority of bacteria appeared to be taken up by spacious asymmetrical pseudopod loops. This was the primary mechanism used for the uptake of both live and killed bacteria, suggesting that it is dependent on a pre-formed factor on the bacterial cell surface. Interestingly, they found that treatment of bacteria with 1% periodic acid to degrade surface carbohydrates resulted in bacterial uptake via conventional phagocytosis, implicating surface components such as LPS, or bacterial capsule in this asymmetrical looping phagocytosis. In support of this hypothesis, they found that, in the presence of serum that had been depleted of C7 to avoid issues with increased serum sensitivity, LVS O-antigen (O-Ag) mutants were taken up by aberrant tight pseudopod loop structures that had multiple, “onion-skin”-like layers.⁶³ These aberrant loops were only observed when O-Ag mutant bacteria were serum-opsonized, as both unopsonized wild-type and O-Ag mutant LVS were taken up by similar appearing spacious asymmetrical loop structures.

In their system Clemens et al. rarely (around 5%) observed LVS internalization via a ruffling mechanism resembling macropinocytosis by HMDMs and THP-1 cells.⁶² Macropinocytosis is the non-specific uptake of extracellular fluid due to the interaction and fusion of plasma membrane extensions.⁶⁴ This process is not constitutive, but rather transiently turned on by specific activating ligands.⁶⁵ Using microarray analysis, Bradburne et al. observed that genes regulating macropinocytosis were highly upregulated during the initial period of infection of A549 human lung epithelial cells, and have proposed that LVS uses macropinocytosis as a mechanism for uptake into epithelial cells.¹⁸ In support of this mechanism they showed that uptake of LVS was inhibited by amiloride, an inhibitor of macropinocytosis, and that infection with LVS allowed A549 cells to internalize FITC-dextran, which is known to be taken up by macropinocytosis.⁶⁶ This uptake was not seen when FITC-dextran was added to A549 cells alone, indicating that LVS infection facilitated uptake of FITC-dextran by macropinocytotic pathways. Further research is necessary to determine if specific bacterial ligands trigger this process.

Lai et al. identified several hypercytotoxic mutants in *F. novicida* that were able to kill host cells more quickly than wild-type bacteria, but were less virulent in mice.¹¹ These mutants also exhibited enhanced uptake; they were taken up by macrophages at higher rates than wild-type bacteria, even when the cells were pretreated with the actin polymerization inhibitor cytochalasin D.¹¹ Some of these mutants lacked O-Ag, and also lacked or had defects in the LPS core. However, other mutants that did not have any LPS-related defect exhibited a similar phenotype. These authors speculate that these mutants all have surface alterations that change the mechanism of uptake, or expose a surface molecule that enhances or signals uptake. Enhanced uptake of LPS mutants in other gram-negative bacteria has been reported, so this could represent a common mechanism shared with other bacteria.¹¹

Role of lipid rafts in *F. tularensis* uptake

Plasma membrane microdomains, such as lipid rafts, are targeted by a number of intracellular pathogens as a mechanism for invasion.⁶⁷ Lipid rafts provide a highly concentrated region of receptors and signaling molecules that can interact with pathogens, granting them entrance into host cells.⁶⁸ Using fluorescent microscopy, it has been shown that GFP-expressing LVS colocalized with filipin III, a fluorescent agent that binds cholesterol.¹² Depletion of lipid rafts from the plasma membrane of J774A.1 cells also decreased LVS uptake, implicating lipid rafts as hot spots of *Francisella* internalization. This internalization was associated with glycosylphosphatidylinositol (GPI)-anchored proteins located in the lipid rafts, as removal of GPI results in significantly decreased intracellular LVS.

The role of lipid rafts in internalization of *Francisella* draws attention to the role of caveolin, a host protein usually associated with pits in cholesterol-rich microdomains of the host cell plasma membrane.⁶⁹ Immunofluorescent imaging shows colocalization between caveolin and GFP-expressing LVS, and disruption of caveolin with cholera toxin prevents LVS entry into macrophages.¹² Interestingly, Law et al. failed to see involvement of caveolin in uptake of *F. novicida* in hepatocytes,¹⁹ but they did see involvement of clathrin-coated pits in this process. Differences in clathrin and caveolin involvement in uptake may be due to differences in cell types analyzed and *Francisella* subspecies.

Cell signaling pathways that influence *Francisella* uptake and attachment

Francisella interaction with host cells activates signaling pathways that promote uptake of the bacteria, and limit the production of proinflammatory cytokines. The specifics of this interaction are dependent on the subspecies or species of *Francisella*, growth conditions,^{42,70} cell types (including mouse or human),⁵⁹ and whether the bacteria have been opsonized.^{53,59}

Using HMDMs Dai et al. found that C3 opsonization of Schu S4, and uptake via CR3 was critical for Schu S4-mediated suppression of proinflammatory cytokine responses.⁵⁹ HMDMs incubated with Schu S4 opsonized with C3-depleted serum or unopsonized bacteria produced relatively high levels of proinflammatory cytokines compared with C3 opsonized bacteria. The immune suppression induced by opsonized Schu S4 and CR3 ligation in these cells was characterized by early inhibition

of ERK1/2, p38 MAPK, and NFκB activation, and activation of Lyn kinase, Akt, and MKP-1. Lyn kinase was shown to have a critical role, because siRNA knockdown of Lyn kinase decreased Schu S4 uptake via CR3, and despite this decrease, these cells produced higher levels of proinflammatory cytokines. Lyn kinase has been shown to have a role in the phagocytosis of *Pseudomonas aeruginosa* and subsequent inflammatory cytokine suppression,^{59,71} as well as in the negative regulation of TLR2 signaling.⁷² TLR2 is the major pattern recognition receptor recognized by Schu S4,⁷³⁻⁷⁶ as unlike many other LPS molecules, *Francisella* LPS is not recognized by TLR4. Dai et al. also found evidence that cross-talk between CR3 and TLR2 controls both uptake and immune suppression. SiRNA knockdown of TLR2 resulted in a 40% decrease in attachment and uptake of serum opsonized Schu S4; proinflammatory cytokines were also decreased, supporting the role of TLR2 in mediating proinflammatory responses, and cross-talk between CR3. Thus, their data suggests that CR3 mediated uptake of Schu S4 serves to dampen TLR2 mediated proinflammatory responses.

In an LVS model of infection of mouse bone marrow-derived macrophages Medina et al. identified a role for phosphatidylinositol 3-Kinase (PI3K)/Akt activation in limiting the TLR2-mediated proinflammatory responses.⁷⁷ Consistent with Dai et al., they identified a link between ERK1/2 and p38 MAPK activation and proinflammatory cytokine expression in LVS infected mouse bone marrow-derived macrophages; inhibition of PI3K activity resulted in activation of ERK1/2 and p38 MAPK and increased TNF-α and IL-6 expression. However, while activation of p38 MAPK and cytokine expression was TLR2-dependent, ERK1/2 activation was MyD88-dependent, but TLR2-independent, suggesting that there are additional pathways that contribute to immune suppression. Dai et al. were unable to recapitulate their results in Schu S4 infected mouse bone marrow-derived macrophages. Instead they found that ERK1/2 activation was enhanced in response to interaction with C3 opsonized Schu S4 when compared with unopsonized Schu S4. Medina et al. only examined PI3K activity in host cells infected with unopsonized LVS, so it is not known if they would have observed a similar activation with opsonized LVS. However, together these results suggest that there are differences in the signaling responses of mouse and human cells to *Francisella* infections.

In contrast to Schu S4 and LVS, Parsa et al. found that activation of the ERK1/2 and Syk signaling pathway was key to the uptake of *F. novicida*, as ERK overexpression in RAW 264.7 cells enhanced bacterial internalization.⁶¹ Additionally, inhibitors of ERK1/2 as well as an upstream signaling activator, Syk, led to decreased bacterial internalization.⁶¹ While uptake of *F. novicida* was significantly reduced in cells with inhibited ERK1/2, this reduction was not to the level seen in cytochalasin D treated cells, implicating additional signaling pathways in this process.

These investigators used unopsonized bacteria with both human and mouse-derived macrophage cell lines, but found that these two cell types responded similarly to *F. novicida* infection. Dai et al. also observed that *F. novicida* stimulated higher levels proinflammatory cytokines in HMDMs but was not influenced by serum opsonization.^{12,59,60} The inability of *F. novicida* to suppress initial proinflammatory responses likely contributes to its attenuation in humans, but *F. novicida* is still fully virulent in mice despite a seemingly limited ability to dampen proinflammatory responses. The bacterial factors and host mechanisms that account for the increased sensitivity of mice is unclear and still need to be defined.

Conclusion

Francisella has developed efficient means of attaching and internalizing into a wide variety of host cells. As access to the intracellular environment is a crucial niche for *Francisella* within the host, a better understanding of initial interactions with host cells should reveal key components required for full virulence, as well as downstream consequences of the bacterial–host interaction. Targeting steps in these processes may also identify novel targets for therapies to treat infection.

The various subspecies of *F. tularensis* vary considerably in their virulence potential, and yet, with the exception of the Type IV pili, few differences in the mechanisms of attachment and internalization into host cells have been delineated. Like many bacteria, *F. tularensis* has multiple adhesins and uses a variety of receptors to gain access to the intracellular space. However, because of the incomplete knowledge regarding specific adhesins and the consequences of specific adhesin–receptor interactions, subspecies differences that contribute to virulence differences have yet to be identified or completely understood. However, the expanding knowledge of adhesin–receptor interactions allows for greater understanding of how these interactions impact virulence. For example, uptake via specific receptors can influence the induction of proinflammatory cytokines. Serum-opsonized Schu S4 induces low levels of proinflammatory cytokine production in human monocyte-derived macrophages compared with unopsonized Schu S4 or *F. novicida*.⁵⁹ Furthermore, siRNA knockdown of CR3 results in higher proinflammatory productions, indicating a dependence on the C3-CR3 mediated uptake in suppressing proinflammatory responses. Understanding the specific bacterial factors that engage host cells and influence the internalization process is critical for fully understanding how *Francisella* is able to suppress and evade the immune response.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

42. Zarella TM, Singh A, Bitsaktis C, Rahman T, Sahay B, Feustel PJ, et al. Host-adaptation of *Francisella tularensis* alters the bacterium's surface-carbohydrates to hinder effectors of innate and adaptive immunity. *PLoS One* 2011; 6:e22335; PMID:21799828; <http://dx.doi.org/10.1371/journal.pone.0022335>
43. Twine SM, Mykytczuk NC, Petit MD, Shen H, Sjöstedt A, Wayne Conlan J, et al. In vivo proteomic analysis of the intracellular bacterial pathogen, *Francisella tularensis*, isolated from mouse spleen. *Biochem Biophys Res Commun* 2006; 345:1621-33; PMID:16730660; <http://dx.doi.org/10.1016/j.bbrc.2006.05.070>
44. Wehbi H, Portillo E, Harvey H, Shimkoff AE, Scheurwater EM, Howell PL, et al. The peptidoglycan-binding protein FimV promotes assembly of the *Pseudomonas aeruginosa* type IV pilus secretin. *J Bacteriol* 2011; 193:540-50; PMID:21097635; <http://dx.doi.org/10.1128/JB.01048-10>
45. Buist G, Steen A, Kok J, Kuipers OP, LysM, a widely distributed protein motif for binding to (peptidoglycan). *Mol Microbiol* 2008; 68:838-47; PMID:18430080; <http://dx.doi.org/10.1111/j.1365-2958.2008.06211.x>
46. Granato D, Bergonzelli GE, Pridmore RD, Marvin L, Rouvet M, Corthésy-Theulaz IE. Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins. *Infect Immun* 2004; 72:2160-9; PMID:15039339; <http://dx.doi.org/10.1128/IAI.72.4.2160-2169.2004>
47. Kunert A, Losse J, Gruszyn C, Hühn M, Kaendler K, Mikkat S, et al. Immune evasion of the human pathogen *Pseudomonas aeruginosa*: elongation factor Tuf is a factor H and plasminogen binding protein. *J Immunol* 2007; 179:2979-88; PMID:17709513
48. Balasubramanian S, Kannan TR, Baseman JB. The surface-exposed carboxyl region of *Mycoplasma pneumoniae* elongation factor Tu interacts with fibronectin. *Infect Immun* 2008; 76:3116-23; PMID:18411296; <http://dx.doi.org/10.1128/IAI.00173-08>
49. Pierini LM. Uptake of serum-opsonized *Francisella tularensis* by macrophages can be mediated by class A scavenger receptors. *Cell Microbiol* 2006; 8:1361-70; PMID:16882038; <http://dx.doi.org/10.1111/j.1462-5822.2006.00719.x>
50. Geier H, Celli J. Phagocytic receptors dictate phagosomal escape and intracellular proliferation of *Francisella tularensis*. *Infect Immun* 2011; 79:2204-14; PMID:21422184; <http://dx.doi.org/10.1128/IAI.01382-10>
51. Clay CD, Soni S, Gunn JS, Schlesinger LS. Evasion of complement-mediated lysis and complement C3 deposition are regulated by *Francisella tularensis* lipopolysaccharide O antigen. *J Immunol* 2008; 181:5568-78; PMID:18832715
52. Ben Nasr A, Klimpel GR. Subversion of complement activation at the bacterial surface promotes serum resistance and opsonophagocytosis of *Francisella tularensis*. *J Leukoc Biol* 2008; 84:77-85; PMID:18430786; <http://dx.doi.org/10.1189/jlb.0807526>
53. Geier H, Celli J. Phagocytic receptors dictate phagosomal escape and intracellular proliferation of *Francisella tularensis*. *Infect Immun* 2011; 79:2204-14; PMID:21422184; <http://dx.doi.org/10.1128/IAI.01382-10>
54. Peiser L, Gough PJ, Kodama T, Gordon S. Macrophage class A scavenger receptor-mediated phagocytosis of *Escherichia coli*: role of cell heterogeneity, microbial strain, and culture conditions in vitro. *Infect Immun* 2000; 68:1953-63; PMID:10722588; <http://dx.doi.org/10.1128/IAI.68.4.1953-1963.2000>
55. Dunne DW, Resnick D, Greenberg J, Krieger M, Joiner KA. The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. *Proc Natl Acad Sci U S A* 1994; 91:1863-7; PMID:8127896; <http://dx.doi.org/10.1073/pnas.91.5.1863>
56. Wright SD, Silverstein SC. Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes. *J Exp Med* 1983; 158:2016-23; PMID:6227677; <http://dx.doi.org/10.1084/jem.158.6.2016>
57. Underhill DM, Ozinsky A. Phagocytosis of microbes: complexity in action. *Annu Rev Immunol* 2002; 20:825-52; PMID:11861619; <http://dx.doi.org/10.1146/annurev.immunol.20.103001.114744>
58. Leander R, Dai S, Schlesinger LS, Friedman A. A mathematical model of CR3/TLR2 crosstalk in the context of *Francisella tularensis* infection. *PLoS Comput Biol* 2012; 8:e1002757; PMID:23133361; <http://dx.doi.org/10.1371/journal.pcbi.1002757>
59. Dai S, Rajaram MV, Curry HM, Leander R, Schlesinger LS. Fine tuning inflammation at the front door: macrophage complement receptor 3-mediates phagocytosis and immune suppression for *Francisella tularensis*. *PLoS Pathog* 2013; 9:e1003114; PMID:23359218; <http://dx.doi.org/10.1371/journal.ppat.1003114>
60. Clemens DL, Horwitz MA. Uptake and intracellular fate of *Francisella tularensis* in human macrophages. *Ann N Y Acad Sci* 2007; 1105:160-86; PMID:17435118; <http://dx.doi.org/10.1196/annals.1409.001>
61. Parsa KV, Butchar JP, Rajaram MV, Cremer TJ, Tridandapani S. The tyrosine kinase Syk promotes phagocytosis of *Francisella* through the activation of Erk. *Mol Immunol* 2008; 45:3012-21; PMID:18295889; <http://dx.doi.org/10.1016/j.molimm.2008.01.011>
62. Clemens DL, Lee BY, Horwitz MA. *Francisella tularensis* enters macrophages via a novel process involving pseudopod loops. *Infect Immun* 2005; 73:5892-902; PMID:16113308; <http://dx.doi.org/10.1128/IAI.73.9.5892-5902.2005>
63. Clemens DL, Lee BY, Horwitz MA. O-antigen-deficient *Francisella tularensis* Live Vaccine Strain mutants are ingested via an aberrant form of looping phagocytosis and show altered kinetics of intracellular trafficking in human macrophages. *Infect Immun* 2012; 80:952-67; PMID:22202123; <http://dx.doi.org/10.1128/IAI.05221-11>
64. Bohdanowicz M, Grinstein S. Role of phospholipids in endocytosis, phagocytosis, and macropinocytosis. *Physiol Rev* 2013; 93:69-106; PMID:23303906; <http://dx.doi.org/10.1152/physrev.00002.2012>
65. Mercer J, Helenius A. Gulping rather than sipping: macropinocytosis as a way of virus entry. *Curr Opin Microbiol* 2012; 15:490-9; PMID:22749376; <http://dx.doi.org/10.1016/j.mib.2012.05.016>
66. West MA, Antoniou AN, Prescott AR, Azuma T, Kwiatkowski DJ, Watts C. Membrane ruffling, macropinocytosis and antigen presentation in the absence of gelsolin in murine dendritic cells. *Eur J Immunol* 1999; 29:3450-5; PMID:10556799; [http://dx.doi.org/10.1002/\(SICI\)1521-4141\(199911\)29:11<3450::AID-IMMU3450>3.0.CO;2-A](http://dx.doi.org/10.1002/(SICI)1521-4141(199911)29:11<3450::AID-IMMU3450>3.0.CO;2-A)
67. Lafont F, van der Goot FG. Bacterial invasion via lipid rafts. *Cell Microbiol* 2005; 7:613-20; PMID:15839890; <http://dx.doi.org/10.1111/j.1462-5822.2005.00515.x>
68. Vieira FS, Corrêa G, Einicker-Lamas M, Coutinho-Silva R. Host-cell lipid rafts: a safe door for micro-organisms? *Biol Cell* 2010; 102:391-407; PMID:20377525; <http://dx.doi.org/10.1042/BC20090138>
69. Parton RG, Richards AA. Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. *Traffic* 2003; 4:724-38; PMID:14617356; <http://dx.doi.org/10.1034/j.1600-0854.2003.00128.x>
70. Singh A, Rahman T, Malik M, Hickey AJ, Leifer CA, Hazlett KR, et al. Discordant results obtained with *Francisella tularensis* during in vitro and in vivo immunological studies are attributable to compromised bacterial structural integrity. *PLoS One* 2013; 8:e58513; PMID:23554897; <http://dx.doi.org/10.1371/journal.pone.0058513>
71. Kannan S, Audet A, Knittel J, Mullegama S, Gao GF, Wu M. Src kinase Lyn is crucial for *Pseudomonas aeruginosa* internalization into lung cells. *Eur J Immunol* 2006; 36:1739-52; PMID:16791881; <http://dx.doi.org/10.1002/eji.2006035973>
72. Keck S, Freudenberg M, Huber M. Activation of murine macrophages via TLR2 and TLR4 is negatively regulated by a Lyn/PI3K module and promoted by SHIP1. *J Immunol* 2010; 184:5809-18; PMID:20385881; <http://dx.doi.org/10.4049/jimmunol.0901423>
73. Cole LE, Elkins KL, Michalek SM, Qureshi N, Eaton LJ, Rallabhandi P, et al. Immunologic consequences of *Francisella tularensis* live vaccine strain infection: role of the innate immune response in infection and immunity. *J Immunol* 2006; 176:6888-99; PMID:16709849
74. Cole LE, Shirey KA, Barry E, Santiago A, Rallabhandi P, Elkins KL, et al. Toll-like receptor 2-mediated signaling requirements for *Francisella tularensis* live vaccine strain infection of murine macrophages. *Infect Immun* 2007; 75:4127-37; PMID:17517865; <http://dx.doi.org/10.1128/IAI.01868-06>
75. Hajjar AM, Harvey MD, Shaffer SA, Goodlett DR, Sjöstedt A, Edebro H, et al. Lack of in vitro and in vivo recognition of *Francisella tularensis* subspecies lipopolysaccharide by Toll-like receptors. *Infect Immun* 2006; 74:6730-8; PMID:16982824; <http://dx.doi.org/10.1128/IAI.00934-06>
76. Li H, Nookala S, Bina XR, Bina JE, Re F. Innate immune response to *Francisella tularensis* is mediated by TLR2 and caspase-1 activation. *J Leukoc Biol* 2006; 80:766-73; PMID:16895974; <http://dx.doi.org/10.1189/jlb.0406294>
77. Medina EA, Morris IR, Berton MT. Phosphatidylinositol 3-kinase activation attenuates the TLR2-mediated macrophage proinflammatory cytokine response to *Francisella tularensis* live vaccine strain. *J Immunol* 2010; 185:7562-72; PMID:21098227; <http://dx.doi.org/10.4049/jimmunol.0903790>