Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org

Mechanisms of *Francisella tularensis* Intracellular Pathogenesis

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Francisella tularensis is a zoonotic intracellular pathogen and the causative agent of the debilitating febrile illness tularemia. Although natural infections by *F. tularensis* are sporadic and generally localized, the low infectious dose, with the ability to be transmitted to humans via multiple routes and the potential to cause life-threatening infections, has led to concerns that this bacterium could be used as an agent of bioterror and released intentionally into the environment. Recent studies of *F. tularensis* and other closely related *Francisella* species have greatly increased our understanding of mechanisms used by this organism to infect and cause disease within the host. Here, we review the intracellular life cycle of *Francisella* and highlight key genetic determinants and/or pathways that contribute to the survival and proliferation of this bacterium within host cells.

Francisella are nonmotile, encapsulated, Gram-negative coccobacilli and are facultative intracellular pathogens of humans and many animals. The genus consists of three recognized species: *Francisella tularensis*, *Francisella novicida*, and *Francisella philomiragia*. *F. tularensis* is highly infectious and causes a potentially debilitating febrile illness known as tularemia. *F. novicida* and *F. philomiragia* are rarely pathogenic in man and usually only in individuals who are severely immunocompromised. Although *F. novicida* has a limited ability to cause disease in humans, this organism continues to serve as an important surrogate model to study aspects of *F. tularensis* pathogenesis and host response to infection owing to its reduced biosafety requirements, the conserved nature of its genome relative to pathogenic *F. tularensis* derivatives, its apparently similar intracellular life cycle, and its ability to cause a tularemia-like disease in in vivo model systems of infection.

F. tularensis is transmitted from infected animals to humans by multiple routes and can cause disease of varying severities depending on the portal of entry, infectious dose, and subspecies (biovar) of the infecting strain. Person-to-person transmission of *F. tularensis* has not yet been reported. *F. tularensis* subspecies *tularensis* is the most infectious biovar ($ID_{50} < 10$ cfu) and is responsible for most cases of tularemia in North America (Saslaw et al. 1961a). This subspecies causes the most severe disease symptoms

Editors: Pascale Cossart and Stanley Maloy
Additional Perspectives on Bacterial Pathogenesis available at www.perspectivesinmedicine.org

 doi: 10.1101/cshperspect.a010314
Cite this article as *Cold Spring Harb Perspect Med* 2013;3:a010314

and has mortality rates approaching 60% if untreated (Saslaw et al. 1961a,b; Dienst 1963). Type A strain Schu S4 is the most commonly studied isolate from this subspecies. *F. tularensis* subspecies *holarctica* has an infectious dose $<10^3$ cfu and is the primary cause of tularemia in Europe and other regions in the Northern Hemisphere. Infections by this subspecies are generally associated with milder disease symptoms and are rarely fatal. The live vaccine strain (LVS) is an attenuated isolate derived from this subspecies and was developed in the former Soviet Union. However, it is not licensed for use in the United States. The remaining biovar, *F. tularensis* subspecies *mediastica*, only occasionally causes disease in humans.

Infection by *F. tularensis* occurs primarily after inadvertent exposure to infected wildlife species, most frequently rodents, hares, and rabbits. Transmission to humans occurs via direct contact, through arthropod or insect vectors, by ingestion of contaminated material(s), or by inhalation of aerosolized organisms. Regardless of the entry route, *F. tularensis* can disseminate from the initial infection site to the lungs where it can cause respiratory tularemia, the most severe form of the disease. The low infectious dose, with the ability to be transmitted to humans via multiple routes, and potential to cause life-threatening illness has resulted in the designation of *F. tularensis* by the United States Centers for Infectious Disease Control and Prevention as a Category A Select Agent with potential to be weaponized and/or intentionally released into the environment. These characteristics have resulted in a renewed interest in the study of *Francisella*, including characterization of the *F. tularensis* life cycle, and identification of bacterial and/or host determinants important for aspects of its pathogenesis.

OVERVIEW OF THE *Francisella* LIFE CYCLE

Although *Francisella* shows an extracellular phase during bacteriemia in mice (Forestal et al. 2007), survival and replication within host cells is thought to be a key aspect of its life cycle. This is exemplified by the ability of various strains of *F. tularensis* subsp. *tularensis* and *holarctica* and of *F. novicida* to enter, survive, and proliferate within a variety of host-cell types, including macrophages, dendritic cells, polymorphonuclear neutrophils, hepatocytes, endothelial, and type II alveolar lung epithelial cells (Oyston et al. 2004; McCaffrey and Allen 2006; Hall et al. 2007, 2008). Because intracellular proliferation is essential to *Francisella* virulence, much research has focused on understanding and characterizing specific steps in the intracellular cycle of this bacterium. It has become clear that *Francisella* survival and proliferation strategies rely on physical escape from its original phagosome and replication in the host-cell cytosol (Fig. 1), making this bacterium a typical cytosol-dwelling pathogen.

Francisella ENTRY INTO MAMMALIAN CELLS

Although entry into nonphagocytic cells remains to be further defined, phagocytosis of *Francisella* by macrophages has been extensively studied and involves the engagement of different phagocytic receptors depending on the bacterium's opsonization conditions. The mannose receptor (MR) plays a significant role in nonopsonic uptake of *F. novicida* and *F. tularensis* strains by either human monocyte-derived macrophages (MDMs), murine bone marrow-derived macrophages (BMMs), or J774A.1 macrophage-like cells (Balagopal et al. 2006; Schulert and Allen 2006; Geier and Celli 2011). Additional, yet-to-be-identified receptors are also likely engaged by nonopsonized *Francisella*. Serum opsonization, which markedly enhances *Francisella* uptake (Clemens et al. 2004, 2005; Balagopal et al. 2006; Schulert and Allen 2006; Geier and Celli 2011), mostly redirects the bacterium to the complement receptor CR3 in human and murine macrophages, human neutrophils, and dendritic cells (Balagopal et al. 2006; Ben Nasr et al. 2006; Schulert and Allen 2006; Barker et al. 2009a). The scavenger receptor A (SR-A) (Pierini 2006; Geier and Celli 2011), Fcγ receptors (Balagopal et al. 2006), nucleolin (Barel et al. 2008), and the lung surfactant protein A (SP-A) (Balagopal et al. 2006) have also been implicated to various degrees in uptake of serum-opsonized *Francisella* by murine or human macrophages.

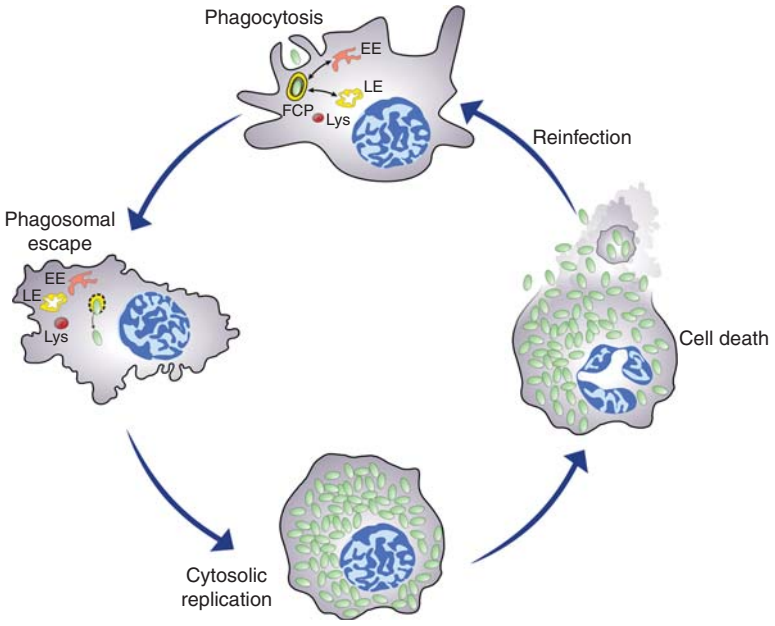

Figure 1. Model of the *Francisella* intracellular cycle depicting stages that are common to murine and human phagocytes. Upon phagocytosis, bacteria reside in an early phagosome (FCP) that interacts with early (EE) and late (LE) endocytic compartments but not lysosomes (Lys). Bacteria rapidly disrupt the FCP membrane and reach the cytosol where they undergo extensive replication, a process followed by cell death, bacterial release, and subsequent infection.

Conversely, Fcγ receptors are the main phagocytic receptors engaged during uptake of antibody-opsonized *Francisella*, which is also enhanced compared with nonopsonic conditions (Balagopal et al. 2006; Geier and Celli 2011). Opsonophagocytosis of microbes is generally associated with enhanced uptake, as is the case for *Francisella*. This suggests that this pathogen exposes a limited number of ligands to nonopsonic phagocytic receptors, possibly providing the bacterium with means to limit uptake by bactericidal phagocytes. Additionally, such bacterial ligands may engage receptors that trigger less bactericidal pathways, thereby ensuring better intracellular survival. Consistent with this hypothesis, intracellular proliferation of nonopsonic SchuS4 in murine BMMs is superior to that of opsonized bacteria (Geier and Celli 2011).

THE *Francisella*-CONTAINING PHAGOSOME

Following uptake, *Francisella* resides within a phagosome, the *Francisella*-containing phagosome (FCP), an initial vacuolar compartment along the endocytic degradative pathway that is normally subjected to progressive maturation into a bactericidal phagolysosome. Newly formed FCPs sequentially acquire markers of early endosomes and late endosomes, such as EEA-1, CD63, LAMP-1, LAMP-2, and Rab7 (Clemens et al. 2004, 2009; Santic et al. 2005a, 2008; Checroun et al. 2006; Chong et al. 2008; Wehrly et al. 2009), indicative of a normal maturation process. Yet, such interactions do not proceed toward fusion with lysosomes, as FCPs do not seem to accumulate lysosomal luminal hydrolases, such as cathepsin D, or lysosomal tracers (Anthony et al. 1991; Clemens et al. 2004; Santic et al. 2005b; Bonquist et al. 2008) and bacteria physically disrupt the phagosomal membrane and escape into the host-cell cytosol (see below). Another important feature of phagosomal maturation is the progressive acidification of the phagosomal lumen, via the recruitment of the vacuolar ATPase (v-ATPase), which is both a requirement and a consequence

of phagosomal maturation (Huynh and Grinstein 2007). Whether the FCP becomes acidified or not before disruption remains contentious. Some studies have shown that FCPs containing either LVS, Schu S4, or a *F. tularensis* clinical isolate resist acidification and acquire limited amounts of v-ATPase (Clemens et al. 2004; Bonquist et al. 2008; Cremer et al. 2009), whereas others report that FCPs containing either *F. novicida* strain U112 or SchuS4 become acidified and acquire the v-ATPase proton pump before phagosomal disruption (Chong et al. 2008; Santic et al. 2008). Additional controversy resides in the fact that studies that found that FCPs become acidified show a role for acidification in optimal phagosomal escape (Chong et al. 2008; Santic et al. 2008), whereas a study in which FCPs were found to resist acidification did not observe any effect of acidification inhibitors (Clemens et al. 2009). It is noteworthy that acidified FCPs are observed using nonopsonic infection conditions, whereas nonacidified FCPs are generated using serum-opsonized *Francisella*, suggesting that the mode of uptake may affect acidification of the FCP and account for discrepancy in the literature. Although FCP intraluminal pH remains to be measured under different opsonization conditions, these studies nonetheless suggest that physicochemical cues within the FCP contribute to *Francisella* intracellular fate. This reinforces the importance of the FCP in the *Francisella* intracellular cycle, in agreement with the fact that it constitutes the site of intracellular induction of virulence genes encoded within the *Francisella* pathogenicity island (FPI), a genetic loci that has been predicted to encode a type VI secretion system (Nano et al. 2004; Chong et al. 2008; Wehrly et al. 2009).

REACTIVE OXYGEN SPECIES AND INHIBITION OF NADPH OXIDASE ACTIVATION

A major bactericidal function of phagosomes is production of reactive oxygen species (ROS) during the oxidative burst, a process mediated by recruitment and assembly of a multiprotein complex called the NADPH oxidase onto the phagosomal membrane. This enzyme mediates the conversion of molecular oxygen to superoxide (O_2^-) anions, an ROS that can be subsequently converted to other reactive species including hydrogen peroxide (H_2O_2), hypochlorous acid (bleach), and peroxynitrite ($ONOO^-$) within certain cell types or under certain conditions (Zahrt and Deretic 2002). The NADPH oxidase complex consists of two integral membrane proteins ($gp91^{phox}$ and $p22^{phox}$) that together form flavocytochrome b_{558}, and various soluble components that are translocated to the phagosomal membrane on activation. Flavocytochrome b_{558} contains the redox center of NADPH oxidase and is the docking site for cytosolic subunits of the enzyme complex including $p47^{phox}$, $p40^{phox}$, and $p67^{phox}$. Translocation of these determinants to flavocytochrome b_{558} requires phosphorylation of $p47^{phox}$ and $p40^{phox}$; activation of NADPH oxidase also requires association with a small G protein such as Rac1 or Rac2 (Babior 2004).

To ensure their survival, many bacterial pathogens have evolved strategies to resist and/or interfere with ROS generation. For *Francisella*, these survival strategies have been best elucidated in human polymorphonuclear neutrophils (PMNs) (McCaffrey and Allen 2006; Allen and McCaffrey 2007; Buchan et al. 2009; Schulert et al. 2009; McCaffrey et al. 2010; Mohapatra et al. 2010), a cell type normally encountered by the bacterium in vivo and a potent producer of NADPH oxidase-generated O_2^- (Allen and McCaffrey 2007). In these cells, *F. tularensis* and *F. novicida* have been shown to actively disrupt ROS generation by altering at least two different steps in NADPH oxidase activation: (1) by inhibiting assembly of flavocytochrome b_{558} in the phagosomal membrane (McCaffrey et al. 2010), and/or (2) by inhibiting the phosphorylation of $p47^{phox}$ and $p40^{phox}$ (McCaffrey et al. 2010; Mohapatra et al. 2010). Inhibition of NADPH oxidase assembly by *F. tularensis* is dependent on opsonization, and is observed with strains from both *F. tularensis* subspecies *tularensis* and *holarctica*, including the LVS (McCaffrey et al. 2010; Geier and Celli 2011). Interestingly, *F. tularensis* also possesses the unique ability to inhibit NADPH oxidase

activity postcomplex assembly, an event that limits subsequent PMN activation by other stimuli and diminishes ROS production following phagocytosis of antibody-opsonized *F. tularensis* (McCaffrey et al. 2010). Regardless of mechanism(s), inhibition of NADPH oxidase activity enhances survival of both *F. tularensis* and *F. novicida* in this cell type (McCaffrey and Allen 2006; McCaffrey et al. 2010; Mohapatra et al. 2010; Geier and Celli 2011). Similarly, monocytes or MDMs that have been infected with *F. tularensis* or *F. novicida* also fail to trigger a robust and/or productive oxidative burst (Schulert et al. 2009; Mohapatra et al. 2010; Geier and Celli 2011). However, in contrast to PMNs, the inability of monocytes and MDMs to produce ROS following infection is likely owing to *Francisella* engagement of and/or internalization by receptors not coupled to NADPH oxidase activation (Schulert et al. 2009). Consistent with this observation, intracellular growth of *F. tularensis* or *F. novicida* is similar following ex vivo infection of monocytes and/or MDMs from wild-type mice compared with $p47^{phox-/-}$ mice (Lindgren et al. 2005; Mohapatra et al. 2010). Furthermore, IgG-opsonization of *F. tularensis* results in NADPH oxidase-dependent ROS generation in wild-type murine BMMs, an affect that is not observed in macrophages lacking $gp91^{phox}$ (Geier and Celli 2011).

Although the ability of *Francisella* to inhibit NADPH oxidase activity in neutrophils in vitro has been well established, the importance of neutrophils and/or NADPH oxidase activity in this and other cell types in vivo remains less clear. Antibody-mediated depletion of neutrophils from mice, or targeted recruitment of neutrophils to the lungs using intranasally delivered recombinant MIP-2, a neutrophil-chemotactic chemokine, does not affect bacterial burden or time-to-death kinetics in mice that have been infected with *F. tularensis* compared with untreated control animals (KuoLee et al. 2011). Similarly, $gp91^{phox-/-}$ mice show only slight differences in time-to-death and overall bacterial burden in target organs of infection compared with wild-type animals following respiratory infection with *F. tularensis* (KuoLee et al. 2011). Thus, although *Francisella* may possess specific strategies to inhibit, counteract, or bypass the production of ROS by neutrophils and other phagocytes, the importance of the NADPH oxidase in controlling infection by pathogenic biovars of *Francisella* appears limited in murine tularemia.

Several bacterial determinants have been implicated in the resistance of *Francisella* to ROS. For example, *Francisella* encodes a number of putative acid phosphatases, enzymes that hydrolyze monoesters at acidic pH, that may inhibit the respiratory burst. Purified AcpA, the major contributor of acid phosphatase activity in *Francisella*, is able to inhibit the oxidative burst in porcine neutrophils that have been activated with exogenous stimulants (Reilly et al. 1996). Recent evidence suggests that AcpA is secreted by *F. tularensis* and *F. novicida* strains in vitro (Dai et al. 2011), and is detectable in the cytosol of macrophages infected with *F. novicida* shortly after infection (Dai et al. 2011). These findings indicate that AcpA would be in a physical location (near the phagosomal membrane or in the cytosol) to interact with membrane-bound or soluble components of the NADPH oxidase (Dai et al. 2011). Consistent with these observations, an *F. novicida* mutant lacking AcpA is partially compromised in its ability to suppress the oxidative burst following infection of human neutrophils or MDMs (Mohapatra et al. 2010), a phenotype that becomes more pronounced as additional predicted acid phosphatases (*acpB*, *acpC*, and *hap*) are deleted from the bacterium (Mohapatra et al. 2010). Furthermore, loss of AcpA and/or other acid phosphatases reduces the ability of *F. novicida* to survive following infection of these and other cell types in vitro (Mohapatra et al. 2007, 2008, 2010), and in vivo following intraperitoneal or intranasal infection of mice (Mohapatra et al. 2007, 2008). The importance of acid phosphatases in resistance of *F. novicida* to ROS can also be seen following treatment of neutrophils and MDMs with reagents that block assembly and/or function of NADPH oxidase, or in macrophages from $p47^{phox-/-}$ mice, where the ability of *F. novicida* Δ*acpA* and/or Δ*acpABCH* mutants to survive intracellularly is

improved (Mohapatra et al. 2010). Additionally, ascorbate treatment of murine or human macrophages infected with the LVS blocks acid phosphatase production and inhibits intracellular growth of the bacterium, a phenotype that is dependent on production of AcpA (McRae et al. 2009). Thus, AcpA and other predicted acid phosphatases appear to play an important role in NADPH oxidase inhibition and resistance to ROS following infection by *F. novicida*.

It is, however, important to note that AcpA and/or the other acid phosphatases appear to play little if any role in the inhibition of NADPH oxidase activity or resistance to ROS in phagocytes that have been infected with human-virulent strains of *F. tularensis*. An *acpA* mutant generated in Schu S4 does not trigger a respiratory burst and shows similar growth characteristics to the wild-type parent following infection of human PMNs, even though this bacterium lacks detectable acid phosphatase activity (McCaffrey et al. 2010). Similarly, deletion of *acpA*, *acpB*, or *acpC* alone or sequentially does not alter growth and/or trafficking characteristics of Schu S4 in vitro in murine BMMs or human MDMs, or in vivo following intranasal infection of mice (Child et al. 2010). Thus, acid phosphatases may play a different role in *F. novicida* compared with *F. tularensis*, or strains of *Francisella* that are pathogens of humans may possess/use additional determinants/strategies for ROS resistance that are not conserved or functional in *F. novicida*.

Apart from acid phosphatases, other determinants have also been implicated in *Francisella* resistance to ROS. A number of determinants involved in pyrimidine biosynthesis (*carA*, *carB*, and *pyrB*) mediate resistance to ROS in *F. tularensis* LVS, although it is unclear whether these genes act indirectly by simply enhancing the fitness of the bacterium (Schulert et al. 2009). FevR, a transcription factor that regulates expression of ~100 genes including those from the FPI (Brotcke and Monack 2008; Meibom et al. 2009), is also required for the inhibition of NADPH oxidase activation and subsequent ROS production in PMNs by both *F. tularensis* LVS (Buchan et al. 2009) and *F. tularensis* subspecies *tularensis* (McCaffrey et al. 2010). *FTN1133* (*FTL0803*) encodes a hypothetical gene product that is required for resistance of *F. novicida* and *F. tularensis* LVS to organic hydroperoxides (Llewellyn et al. 2011). Mutants lacking this enzyme are attenuated for survival in macrophages in vitro and show reduced burdens in mice following intradermal infection, a phenotype that is partially or completely ablated in cells or animals lacking $gp91^{phox}$ (Llewellyn et al. 2011). *F. tularensis* also encodes at least two superoxide dismutases that mediate the detoxification of superoxide to H_2O_2 and O_2. *sodB*, encoding an FeSOD, and *sodC*, encoding CuZn SOD, mediate resistance to paraquat, H_2O_2, and/or pyrogallol in *F. tularensis* LVS (Bakshi et al. 2006; Melillo et al. 2009), and are required for virulence of *F. tularensis* LVS in macrophages and mice (Bakshi et al. 2006). The attenuation in virulence observed with these strains in vitro and in vivo is attributable to NADPH oxidase, as addition of NADPH oxidase inhibitors or utilization of cells or mice unable to make this enzyme complex fail to control infection (Melillo et al. 2009). Finally, *F. tularensis* LVS mutants lacking KatG, a catalase, show increased sensitivity to H_2O_2 in vitro and are attenuated for virulence in mice in vivo (Lindgren et al. 2007). Interestingly, a similar phenotype is not observed in strains from subspecies *tularensis* (Lindgren et al. 2007). Thus, *Francisella* likely uses multiple strategies and a variety of determinants to mediate resistance to ROS.

Francisella PHAGOSOMAL ESCAPE

Several laboratories have described phagosomal escape by various strains of *F. tularensis* or *F. novicida* in either human or murine macrophages, and other cell types (Golovliov et al. 2003; Clemens et al. 2004; Santic et al. 2005a, 2008; Checroun et al. 2006; Chong et al. 2008), clearly indicating that this process is conserved and is a hallmark of the *Francisella* intracellular life cycle. The study of phagosomal escape kinetics have nonetheless brought controversy to the field, as early studies have reported phagosomal disruption and bacterial access to the cytosol that range anywhere from 1 h to 8 h postinfection (Golovliov et al. 2003; Clemens et al.

2004; Santic et al. 2005a; Checroun et al. 2006; McCaffrey and Allen 2006). Such large variations in kinetics are likely attributed to technical differences in the assays and criteria used to assess phagosomal disruption, and also to variations in the models studied between laboratories where different species and strains of *Francisella*, and different macrophage models were examined. In support of the latter, more recent side-by-side comparisons of phagosomal escape kinetics of *F. tularensis* and *F. novicida* strains have highlighted differences in this process (Chong et al. 2008). For example, more rapid phagosomal escape is observed under nonopsonic conditions (Golovliov et al. 2003; Lindgren et al. 2004; Checroun et al. 2006; Chong et al. 2008; Santic et al. 2008; Barker et al. 2009a; Wehrly et al. 2009; Child et al. 2010; Edwards et al. 2010), whereas slower escape kinetics occur when opsonic conditions are used (Clemens et al. 2004; McCaffrey and Allen 2006; Schulert et al. 2009). Recent systematic comparisons of phagosomal escape kinetics by nonopsonic and opsonized SchuS4 in murine macrophages have clarified this issue further by demonstrating that opsonization of *Francisella* with either complement or antibodies targets bacteria to phagocytic pathways that restrict the extent and timing of phagosomal escape (Geier and Celli 2011). Although the basis for restricted phagosomal escape on serum opsonization is unclear, that occurring on antibody opsonization and bacterial targeting to Fcγ receptors depends on the rapid and transient NADPH oxidase-dependent oxidative burst (Geier and Celli 2011) that the bacteria do not seem to be able to prevent, further confirming that conditions encountered by phagocytosed *Francisella* within the FCP can influence its intracellular fate.

Francisella phagosomal escape is requisite to intracellular proliferation, as exemplified by the inability of numerous phagosomal escape-deficient mutants to grow within macrophages (Lindgren et al. 2004; Santic et al. 2005b; Bonquist et al. 2008; Barker et al. 2009b; Wehrly et al. 2009; Broms et al. 2012). Given the important role of phagosomal escape in the *Francisella* intracellular cycle, much effort has been made to identify bacterial factors that contribute to this process. Unlike *Listeria monocytogenes* or *Shigella flexneri*, *Francisella* does not encode lipid hydrolases or phospholipases classically involved in phagosomal membrane disruption (Larsson et al. 2005). However, *F. tularensis* does appear to express a type VI-like secretion system (encoded by the FPI) (Nano et al. 2004), and numerous genes that are expressed from this genetic locus or that regulate expression from this locus are important for this phagosomal egress (reviewed in Chong et al. 2008). These include *iglC* and *iglD* (Lindgren et al. 2004; Santic et al. 2005b; Bonquist et al. 2008; Chong et al. 2008), *pdpA* (Schmerk et al. 2009), *iglI* and *iglJ* (Barker et al. 2009b; McCaffrey et al. 2010), *vgrG* (Barker et al. 2009b; Broms et al. 2012), *dotU* (Broms et al. 2012), *mglA* (Baron and Nano 1998; Santic et al. 2005b; Bonquist et al. 2008), *fevR* (Brotcke and Monack 2008; Buchan et al. 2009; Wehrly et al. 2009), and *migR* (Buchan et al. 2009). However, other determinants not linked to the FPI have also been implicated in phagosomal escape by *Francisella* including the acid phosphatases of *F. novicida* (AcpA, AcpB, AcpC, and Hap) (Mohapatra et al. 2008), pyrimidine biosynthetic genes (*carA*, *carB*, and *pyrB*) (Schulert et al. 2009), as well as several genes of unknown function including *FTT1103* (Qin and Mann 2006; Qin et al. 2009) and *FTT1676* (Wehrly et al. 2009). Thus, it remains unclear whether *Francisella* use a single mechanism for escape from the phagosome; further delineation of this process, including the genes required, remains a top priority in the field given its importance for subsequent stages of *Francisella* survival.

Francisella REPLICATION IN THE CYTOSOL

Besides phagosomal escape, the ability of *Francisella* to proliferate within the host-cell cytosol is another key aspect of its intracellular life cycle, as exemplified by the avirulence in mice of the large number of identified replication-deficient mutants (Brotcke et al. 2006; Tempel et al. 2006; Su et al. 2007; Weiss et al. 2007; Alkhuder et al. 2009; Wehrly et al. 2009). Although the cytosol may be viewed as permissive for bacterial

proliferation, compared with a vacuole along the endocytic degradative pathway, only cytosol-adapted pathogens can readily proliferate within this host-cell compartment (O'Riordan and Portnoy 2002), through the expression of dedicated factors and interactions with host components that either favor or antagonize bacterial growth. Because phagosomal escape is a prerequisite to cytosolic replication, identifying bacterial factors that are specifically required for growth in the cytosol (and not for phagosomal escape) via mutagenesis and in vitro and/or in vivo screens has been difficult. In general, identification of specific *Francisella* cytosolic replication-defective mutants has required use of more specialized secondary readouts including those based on electron and/or immunofluorescence microscopy. To date, only purine biosynthetic genes (*purMCD*) (Pechous et al. 2006, 2008), a γ-glutamyl transpeptidase (*ggt*) (Alkhuder et al. 2009), and several genes of unknown function including *FTT0369c/dipA* (Wehrly et al. 2009; Chong et al. 2012), *FTT0989* (Brotcke et al. 2006), and *ripA* (Fuller et al. 2008), have been identified as being specifically required for cytosolic replication by *Francisella*. However, it is likely that there remain a large number of additional gene products that also contribute to this process (reviewed in Chong et al. 2008).

Although *Francisella* have evolved mechanisms to adapt to a cytosolic lifestyle, some host factors have been identified that either contribute or interfere with cytosolic proliferation. Recently, Akimana et al. have identified through a genome-wide RNAi screen in *Drosophila melanogaster* $S2R^+$ cells the type III PI4-kinase α subunit PI4KC and the ubiquitin-specific peptidase USP22 as required for cytosolic replication of *F. novicida* (Akimana et al. 2010). Whether the same host proteins are required for replication of virulent *F. tularensis* and what their function in bacterial replication is remains to be established. Nonetheless, these findings argue that this bacterium modulates specific host pathways associated with lipid signaling and ubiquitin systems to foster replication.

Not surprisingly, innate immune mechanisms have been shown to negatively control intracellular proliferation of *Francisella*. The cytokine interferon (IFN)-γ is essential to control primary infections (Leiby et al. 1992; Elkins et al. 1996), and has long been known to restrict intracellular growth of various *Francisella* strains in different host-cell models (Anthony et al. 1992; Fortier et al. 1992; Polsinelli et al. 1994), although the effector mechanisms are unclear. IFN-γ control of either LVS or Schu S4 intracellular growth in murine peritoneal exudate cells (PECs) seems to depend on generation of nitric oxide by the inducible nitric oxide synthase (iNOS) (Lindgren et al. 2005, 2007), but not in murine alveolar macrophages (Polsinelli et al. 1994), nor in either human blood-derived or murine bone marrow-derived primary macrophages (Edwards et al. 2010). Nonetheless, independent studies have established that cytosolic replication rather than phagosomal escape of *F. tularensis* is the target of IFN-γ, as this cytokine does not affect phagosomal escape of either LVS or SchuS4 in either J774A.1 cells and human or murine primary macrophages (Bonquist et al. 2008; Edwards et al. 2010), but restricts cytosolic proliferation (Edwards et al. 2010). These findings are, however, inconsistent with those of Santic et al. (2005a), who reported that IFN-γ activation of human blood-derived macrophages prevented phagosomal escape of *F. novicida*. Although variations in the experimental designs of these studies may account for such contrasting conclusions, and further work is required to reconcile these results and clarify the effector mechanisms induced by IFN-γ, these findings clearly illustrate that phagocytes express specific mechanisms that control cytosolic proliferation of *Francisella*. In addition to the effect of IFN-γ on *Francisella* intracellular growth, alternative activation of macrophages by mast cells via interleukin-4 (IL-4) controls intramacrophage growth of LVS (Ketavarapu et al. 2008). Although increased ATP production, prolonged FCP acidification, and up-regulation of the MR have been invoked in this control (Rodriguez et al. 2010), whether IL-4 affects *Francisella* phagosomal escape and/or cytosolic replication remains to be examined in more depth.

Francisella AND INNATE IMMUNE RECOGNITION

Francisella phagocytosis by macrophages and intracellular trafficking through vacuolar and cytosolic compartments likely subjects the bacterium to innate immune recognition by various pattern recognition receptors (PRRs) either located on the cell surface or endosomes, such as the Toll-like receptor (TLRs), or in the cytosolic compartment such as Nod-like receptors (NLRs). TLRs or NLRs typically recognize pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) to trigger proinflammatory responses, among which the activation of the inflammasome, a cytosolic molecular complex, orchestrates caspase-1 activation, processing and release of the proinflammatory cytokines IL-1β and IL-18, and cell death via pyroptosis.

Because of the unique structure of its lipid A moiety (Hajjar et al. 2006), *Francisella* LPS shows very low endotoxicity and the bacterium does not induce TLR4-mediated signaling. Instead, LVS significantly stimulates TLR2-mediated signaling resulting in proinflammatory cytokine production (Katz et al. 2006; Cole et al. 2007), although it is also capable of subsequently suppressing TLR-mediated signaling and secretion of proinflammatory cytokines induced by other agonists (Telepnev et al. 2003). This ability of *Francisella* to suppress or dampen proinflammatory responses has been extended to the virulent subspecies *tularensis* in human dendritic cells, where infection impairs their activation and function (Bosio and Dow 2005; Chase et al. 2009), further supporting the notion that *Francisella* interferes with immune detection and responses.

INFLAMMASOME ACTIVATION

Given its cytosolic location, *Francisella* has become a model pathogen to study the function of inflammasomes, which has also expanded our understanding of how this bacterium may interfere with immune recognition. Release of *F. novicida* in the macrophage cytosol is sensed in a type I IFN-dependent manner (Henry et al. 2007), and activates the absent in melanoma 2 (AIM2)-containing inflammasome (Fernandes-Alnemri et al. 2010; Rathinam et al. 2010; Jones et al. 2011), a type I IFN-inducible complex whose activation induces secretion of the proinflammatory cytokines IL1β and pro-IL18 and pyroptosis (Mariathasan et al. 2005). AIM2 inflammasome activation is potentiated by TLR2 signaling (Jones et al. 2010) and is required in vivo in mice to control bacterial burden by eliminating infected macrophages that constitute the *Francisella* proliferation niche (Mariathasan et al. 2005; Fernandes-Alnemri et al. 2010; Jones et al. 2011). Consistent with AIM2 recognizing cytosolic double-stranded DNA (Rathinam et al. 2010), *F. novicida* activation of the AIM2 inflammasome is associated with bacterial release of DNA through lysis in the cytosol (Jones et al. 2011). These studies have focused on murine systems and the nonhuman pathogenic *F. novicida* species, so an important question that remains unanswered is whether the conclusions drawn from these studies also apply to humans and virulent strains. For example, recent evidence indicates that infection of human cells with *F. novicida*, LVS, and the highly virulent *F. tularensis* Schu S4 strains activate the NLRP3 inflammasome in addition to the AIM2 inflammasome (Atianand et al. 2011), suggesting some functional differences in the innate immune complexes involved in *Francisella* recognition between mice and humans. Moreover, several studies using *F. tularensis* subspecies suggest that infections of BMMs with the LVS strain (Huang et al. 2010; Ulland et al. 2010), or of human primary macrophages with virulent Schu S4 (Lindemann et al. 2010) do induce little cytotoxicity compared with *F. novicida*, raising the question of relevance of cytotoxicity in infections with virulent *tularensis* strains.

Given the high adaptation of *Francisella* to the cytosol and the potential of proinflammatory pathways to restrict its proliferation, this bacterium has likely evolved mechanisms to counteract inflammasome activation to preserve its replication niche. In support of this concept, many laboratories have identified hypercytotoxic mutants of *F. novicida* (Brotcke et al. 2006; Hager et al. 2006; Weiss et al. 2007; Lai

et al. 2010), LVS (Huang et al. 2010; Ulland et al. 2010), and Schu S4 (Lindemann et al. 2010), which typically induced increased inflammasome-dependent cell death and secretion of proinflammatory cytokines in either mouse or human macrophages. Intriguingly, the hypercytotoxic phenotype results from the interruption of genes encoding a variety of proteins, such as the oligopeptide permease OppB (Brotcke et al. 2006), the metallopeptidase PepO (Brotcke et al. 2006; Hager et al. 2006), the inner membrane protein RipA (Huang et al. 2010), the IclR family transcriptional factor FTT0784 and an unknown protein FTT0584 (Weiss et al. 2007), and several proteins involved in biosynthesis of type IV pili, the LPS and the O-antigen polysaccharidic capsule (Huang et al. 2010; Ulland et al. 2010), bringing some confusion as to whether *Francisella* specifically suppress inflammasome activation. The hypothesis of *Francisella* evasion of inflammasome activation was recently challenged by Peng et al., who showed that many previously identified hypercytotoxic mutants of *F. novicida* and LVS are deficient in membrane-associated proteins and lyse more extensively in the cytosol than wild-type bacteria, consequently triggering increased inflammasome activation, secretion of proinflammatory cytokines, and pyroptosis (Peng et al. 2011). Although this study does not completely rule out the existence of *Francisella* inflammasome suppression factors, it elegantly argues that most genes identified as potential suppressors of inflammasome activation do not act specifically and that the hypercytotoxic phenotype is in many cases an indirect consequence of bacterial cell wall fragilization and enhanced release of PAMPs. Because this study has been performed in *F. novicida* and LVS, it will also be interesting to verify that these conclusions also apply to virulent strains.

XENOPHAGIC CAPTURE

Another intracellular innate immune defense mechanism is xenophagy, a selective process of capture of intracellular microorganisms within a double-membrane-bound vacuole, the autophagosome, for delivery to and degradation into the lysosomal compartment (Levine et al. 2011). *Francisella* cytosolic location makes this pathogen an ideal substrate for selective autophagic recognition and capture, a process that shares many molecular machineries with nonselective, canonical autophagy. Yet, surprisingly, this bacterium is able to replicate within murine and human macrophages over long periods of time ($>$18 h) without evidence of a xenophagic response, suggesting it is either capable of avoiding recognition or inhibiting autophagic processes. A belated autophagic response has nonetheless been observed in murine BMMs that encloses a fraction of cytosolic *Francisella* into large vacuoles with autophagic features (Checroun et al. 2006), but the role of these vacuoles and their relevance is unclear, as they do not form in human macrophages (Akimana et al. 2010; Edwards et al. 2010) and may not constitute a bona fide stage of the bacterium's intracellular cycle. Current evidence that *Francisella* may interfere with the autophagy pathway stems from the down-regulation of several autophagy-related genes, such as *beclin1*, *ATG5*, *ATG12*, *ATG16L*, *ATG7*, and *ATG4a* in either Schu S4- or *F. novicida*-infected human monocytes (Butchar et al. 2008; Cremer et al. 2009), which suggests that *Francisella* may suppress an autophagic response at the gene expression level. Yet, it remains to be shown whether down-regulation of some autophagy gene during infection is sufficient to block this constitutive pathway rapidly enough to prevent bacteria that have reached the cytosol from being targeted. Interestingly, a Δ*dipA* (FTT0369c) deletion mutant of SchuS4, which is deficient in cytosolic replication, is eventually captured and cleared by autophagy following a loss of viability in the cytosol (Chong et al. 2012), suggesting that viable *Francisella* may possess mechanisms that prevent their recognition by the autophagic machinery. Additionally, whether *Francisella* infection negatively modulates nonselective autophagy also needs to be examined to further understand *Francisella* potential interference with this degradative pathway.

CONCLUDING REMARKS

The ability of *Francisella* to survive and replicate within phagocytes and other host cells follow-

ing infection is a key aspect of its life cycle and represents an essential step required by this bacterium to cause disease within the host. Alterations in mechanisms that mediate this ability, including the engagement of appropriate receptors, resistance to ROS, escape from the phagosome, replication within the cytosol, and/or avoidance of innate immune recognition or other defense mechanisms, can lead to altered survival characteristics and potential attenuation of *F. tularensis* in virulence in vitro and/or in vivo. Continued study of the genetic determinants contributing to these mechanisms will be essential for the development of therapeutics or vaccines that are able to specifically inhibit or prevent disease caused by this bacterium.

ACKNOWLEDGMENTS

The authors apologize to those investigators in the field whose excellent work we were not able to reference. We are grateful to Anita Mora at the Rocky Mountain Laboratory for her assistance with graphic art. T.C.Z. acknowledges support from the National Institutes of Health, National Institute of Allergy and Infectious Diseases (NIAID) (1R21 AI097597-01A1), and the NIAID's Regional Center of Excellence for Biodefense and Emerging Infectious Diseases Research Program. Membership within and support from the Region V Great Lakes Regional Center of Excellence (National Institutes of Health Award 2-U54-AI-057153) is also acknowledged. J.C. acknowledges support from the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases.

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