Francisella tularensis interactions with the lung

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ABSTRACT

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***Francisella tularensis* interactions with the lung**
(Under the direction of Thomas H. Kawula)

Francisella tularensis, a gram-negative bacterium, is the etiological agent of the human disease, tularemia. Inhalation of as few as 10 bacteria can result in a rapidly progressing, disseminating, and often lethal infection. The biology and pathogenesis of *Francisella* within the lung following inhalation is not well understood. *In vitro*, the bacterium replicates within macrophages, but the cellular targets and intracellular niche within an animal host are unknown.

We determined the suite of cells infected by *Francisella* during inhalation-acquired tularemia and assessed how pulmonary innate immune cell populations are impacted during infection. Initially, alveolar macrophages were the predominant host cell for replicating *Francisella*. Later, monocytes and neutrophils were recruited to the lung and neutrophils, normally potent microbial killers, became a major host cell for bacterial replication. *Francisella* was also found within interstitial macrophages, monocytes, dendritic cells, and alveolar type II epithelial (ATII) cells.

Given the relative abundance of infected macrophages and monocytes during pulmonary disease, we assessed the importance of chemokine receptor, CX3CR1, which facilitates cell recruitment from the blood into infected tissues. Responding to *Francisella* infection, CX3CR1-deficient mice demonstrated increased monocyte and neutrophil

recruitment into the lung over wild-type mice, though the infected cell profile and bacterial burden in lungs, liver, or spleen throughout the disease course were similar, indicating that this receptor did not play a critical role during pulmonary tularemia.

Of all the identified infected cell types in the lung, only ATII cells are stationary structural cells. ATII cells possess many properties important to respiratory function including the production of pulmonary surfactant which maintains lung surface tension and contributes to protection against infectious agents. *Francisella* invaded ATII cells via host-cell dependent processes, and the bacterium replicated equally well within ATII cells as within macrophages. We isolated an insertion mutation disrupting the *Francisella* gene, *ripA*, that ablated the bacterium's ability to replicate within ATII cells but not macrophages. This strain was significantly attenuated in a mouse model of tularemia suggesting that infection of ATII cells is an important component of Francisella virulence and pathogenesis.

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µg	microgram
µm	micrometer
ATCC	American Type Culture Collection
ATII	alveolar type II epithelial cells
BEI	Biodefense and Emerging Infections
bp	base pair
BSA	bovine serum albumin
CDC	Centers for Disease Control and Prevention
CFU	colony forming unit
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
E.	*Escherichia*
F.	*Francisella*
FPI	*Francisella* pathogenicity island
g	gram
GFP	green fluorescent protein
IACUC	Institutional Animal Care and Use Committee
kb	kilobase
L	liter
LAMP	lysosome-associated membrane glycoproteins

LPS	lipopolysaccharide
LVS	Live Vaccine Strain
mL	milliliter
MOI	multiplicity of infection
^{o}C	degree Celsius
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PE	phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
SP	surfactant protein
T4P	type 4 pili
TEM	transmission electron microscopy
TLR	toll-like receptor
U	unit
Δ	deletion
::	insertion

CHAPTER 1

Introduction and Background

Discovery of *Francisella tularensis*

During the spring of 1911 in Tulare County, California, a plague-like disease afflicting hundreds of ground squirrels was documented by Dr. George McCoy (65). Within a year, McCoy and his associate isolated and identified the causative agent as a gram-negative bacterium which they named *Bacterium tularense* (66). Edward Francis, a prominent scientist of the day, named the disease caused by *Bacterium tularense*, "tularemia" and demonstrated that this disease of small mammals could be transmitted to man through various routes including handling of infected carcasses and biting insects (34). Dr. Francis determined that similar diseases observed in Europe and Japan were, in fact, tularemia and were caused by the same bacterial pathogen. As methods of bacterial taxonomy became more sophisticated, it was evident that the agent of tularemia did not fit into previously described bacterial phylogenetic groups (88). Thus, in 1947 the bacterial genus *Francisella* was named in honor of Dr Francis, and the causative agent of tularemia was renamed, *Francisella tularensis*.

Francisella in humans

Soon after isolation and identification it became apparent that *Francisella* infections were not limited to squirrels, but affected a wide range of mammals. McCoy and Chapin in their initial characterization of *Bacterium tularense* demonstrated that the organism caused

severe disease in guinea pigs, rabbits, rats, mice, ground squirrels, gophers, and monkeys (67). Infections in monkeys led the researchers to believe that the organism could also cause disease in humans. Soon afterwards, infections of meat-packers and animal hunters were reported with the causative agent identified as *B. tularense* (98). As scientific interest in studying this newfound pathogen grew, so did the incidence of laboratory-acquired infections. *Francisella* infections in the laboratory have been common since the very beginning of tularemia research. In the initial papers describing *Bacterium tularense,* McCoy reported that his serum and the serum from a fellow lab worker agglutinated the bacteria (66), indicating prior exposure during the processing of hundreds of diseased rodents. There are reports of McCoy taking extended leaves of absence from his research due to acute illness which included high fever (36). By 1959, there were 55 reported laboratory-acquired infections although other accounts estimate this number at as much as 200 (47).

Seven clinical types of human tularemia were recognized by 1947 including, ulceroglandular (80% of recorded cases), oculoglandural, glandular with no evident primary lesion, typhoidal (potentially via gastrointestinal route), meningeal, oropharyngeal, and pulmonary (35, 87). For some time, there was debate among clinicians regarding whether the lungs were a primary site of infection or were infected secondarily following skin exposure. Later, human trials demonstrated that aerosolized *Francisella* could cause illness (85). More recently, several tularemia outbreaks were reported in Martha's Vineyard and the majority resulted from inhalation of *F. tularensis* (63). A CDC task force identified lawn mowing and brush cutting to be significant risk factors for disease acquisition in this region (63).

Worldwide Incidence of *Francisella* infections

Incidence of human tularemia is widespread among the northern hemisphere, though the degree of incidence varies greatly from region to region. Almost 60% of the 778 reported cases of tularemia in the United States between 2000 and 2005 occurred in 5 states: Missouri, Arkansas, Oklahoma, Massachusetts, and South Dakota (49). Tularemia is likely to be highly underreported due to general symptoms, such as fever, which often results in antibiotic treatment which could allow recovery from infection in the absence of identification. Reported incidence of tularemia in European countries is higher than in the United States with 2,697 reports of tularemia in Finland and 1,828 in Sweden from 2000-2005 (49). Severity of disease varies widely among *Francisella* subspecies, and various subspecies are localized within different geographic regions.

Taxonomy

In 1959 it was first proposed that *Francisella tularensis* from North America and Europe divided into two groups which were indistinguishable serologically, but displayed extreme differences with regard to virulence in small mammals and humans (75). Today, *Francisella tularensis* is divided into four subspecies based on biochemical determinants and virulence in rabbits; *tularensis, holarctica, novicida,* and *mediasiatica*. Found predominantly in North America (49), subspecies, *tularensis* is the most virulent in humans, causing incapacitating disease with the potential of a lethal infection from a small infectious dose (85). Subspecies *holarctica*, found throughout the Northern Hemisphere, also causes a severe disease in humans, but rarely does this infection result in lethality (49). Subspecies *novicida* does not cause disease in immuno-competent humans, while *mediasiatica* displays similar virulence in rabbits as *holarctica* (49, 78). Incidence of *novicida* and *mediasiatica*

are isolated and rare. Subspecies *tularensis, holarctica,* and *novicida* all cause lethal infections in mice which has resulted in the mouse being the most common animal model for the study of tularemia.

Natural reservoirs

The environmental reservoir allowing *F. tularensis* to persist in nature is unclear. Tularemia is a zoonosis, as the bacterium establishes infection within a wide range of mammals, however all of these species develop acute infections making it unlikely that any mammal species is a true environmental reservoir of *Francisella*. Infections of water-dwelling animals such as beavers and lemmings (8) have been observed indicating a potential water reservoir. *F. tularensis* has been detected in water samples taken from sources near human tularemia outbreaks (8, 50), though it is unclear if bacteria are free-living or associated with a unicellular host. Evidence for the latter is that *Francisella* has the ability to survive and replicate within amoebae species, though once infected, these cells are eventually killed by *Francisella in vitro* (2, 9). Insect vectors could provide an environmental reservoir for *Francisella.* The organism has been isolated from tick populations (44, 45, 63) and early work on *Francisella* identified disease acquired from biting flies (33). In Martha's Vineyard, as many as 5% of dog ticks in certain areas contained *F. tularensis*, though the most common primary form of disease is pneumonic for unknown reasons (63). A likely conclusion from these varied isolation sources is that *Francisella* persists in many different environmental niches among different geographical locations.

***Francisella* as a biological weapon**

In 2001, letters containing anthrax spores were received by two members of the United States Senate as well as multiple major U.S. news outlets. These events resulted in 22

confirmed cases of anthrax including 5 deaths (6). The estimated cost of dealing with this event was greater than US $1 billion (52).

In response to this act of bioterrorism, the U.S. government greatly increased the amount of funding for organisms that pose a threat for purposeful release. Those given highest priority were defined as "Category A Diseases/Agents"(1). Organisms in this group pose a risk to national security due to their ease of dissemination or transmission, high mortality rate, and potential for causing panic and social disruption. Members of this category are *Bacillus anthracis* (anthrax), toxin produced by *Clostridium botulinum, Yersinia pestis* (plague), *variola major* (smallpox), viral hemorrhagic fevers (ebola, lassa), and *Francisella tularensis* (tularemia).

Characteristics of *Francisella tularensis* have made it attractive for development as a biological weapon. The organism is extremely infectious, with a dose of less than 10 bacteria necessary to cause disease in humans (85). The organism causes the most acute and severe disease when inhaled (26), and *F. tularensis* is easily aerosolized (18, 19). In the environment, *Francisella* can survive for weeks to months in water (31), and potentially as long in dead animal carcasses, or grasses and hay which would result in prolonged and repeated exposure long after the initial release event. Since *Francisella* is a natural host to a wide variety of animal species, bacterial populations could persist in numerous and varied animal reservoirs which would lead to continued exposure for quite some time.

In the 1930s and 40s, the Japanese government considered the feasibility of using *Francisella* as a biological weapon as a part of their active germ-warfare unit (43). Biological weapons programs in the U.S. led to the development and stockpiling of weaponized *Francisella tularensis* during the 1940s through the 1960s (14). In 1970, an

executive order terminated U.S. biological weapons programs and all stocks of these weapons were destroyed by 1973 (14). Parallel programs existed in the former Soviet Union until the 1990s which allegedly led to the production of *Francisella* strains resistant to antibiotics and vaccines (3). There are claims that a massive tularemia outbreak that affected more than 10,000 German and Soviet soldiers during World War II was the result of an intentional Soviet release (3), though this assertion is challenged and the real result of such an outbreak could be due to increased proximity to infected rodents during times of war (20).

In 1969, the World Health Organization developed a committee to assess the medical, social, and financial impacts of biological attacks. The committee estimated that an aerosol dispersal of 50 kg of live *Francisella tularensis* over a metropolitan area would result in 250,000 incapacitated individuals including 19,000 deaths (102). The appearance of acute, non-specific, respiratory illness would appear in the exposed population 3-5 days following release. In the early stages, it would be difficult for public health authorities to diagnose a tularemia outbreak due to the initial similarities with more common community-acquired outbreaks such as influenza (discussed in 23). Illness would persist for weeks to months with multiple relapses being probable. The CDC estimates that the financial burden of dealing with a tularemia outbreak would be approximately $5.4 billion per 100,000 exposed people (48).

Pathogenic factors of *Francisella tularensis*

Despite the potential risks of a purposeful *Francisella* release, very little is understood about how this highly infectious organism causes disease. At the outset of this work, in 2002, the number of publications examining pathogenesis of *F. tularensis* was limited. Much of the following information concerning *Francisella* pathogenesis has come

to light concurrently with the studies presented here. In Chapter 8, I will attempt to frame this body of work within the current (April 2008) literature and discuss how these data filled a knowledge gap within the field, as well as will influence future studies.

Replication within macrophages

The most commonly studied *Francisella* phenotype related to pathogenesis is the ability to replicate within macrophages. Intra-macrophage replication has been observed within cell lines and primary cells from mice, humans, and other mammalian hosts (5, 39). *Francisella* entry into macrophages may occur via a novel mechanism involving spacious pseudopod loops (15). Immediately following phagocytosis, *Francisella* is contained within a membrane-enclosed compartment (39) that contains the early endosomal marker, EEA-1 (13). This vacuole becomes associated with lysosome-associated membrane glycoproteins (LAMPs), but acidification does not take place (13, 16). Instead, by an unknown mechanism, the phagosomal membrane degrades within 1-4 hours and *Francisella* replicates within the host cell cytoplasm (13, 16, 39). There is some evidence that intracellular *Francisella* re-enters a LAMP-positive compartment by 24 hours following phagocytosis (13) prior to death of the host cell.

The ability of *F. tularensis* to cause disease may be directly tied to the organism's ability to escape the endocytic pathway and avoid degradation by the lysosome. *Francisella* mutants that are unable to replicate within macrophages are attenuated for virulence in animal hosts (69, 72, 84, 94). Three of these intracellular replication-deficient mutants, Δ*iglC*, Δ*mglA*, Δ*acpA* are unable to escape to the cytoplasm of the host cell (59, 69, 84). In contrast, in Chapter 7 a mutant is discussed that escapes the phagosome but is unable to replicate within the cytoplasm.

Dissemination in an animal host

Independent of inoculation route, *Francisella* disseminates from the initial site of infection to distal tissues. Within a day following infection, bacteria are found in the draining lymph nodes in close proximity of the infection site, followed by bacterial presence in the lungs, liver, and spleen (32). Once bacteria arrive in these tissues, they begin replicating logarithmically. Due to the extreme bacterial burden in various tissues, multiple organ failure is a probable cause of death in fatal cases of tularemia.

Pathogenicity island

The sequencing of *Francisella* genomes, as well as genetic studies within *F. tularensis novicida*, have facilitated the discovery of a *Francisella* pathogenicity island (FPI). The FPI has been classified as such due to it's deviation in G+C content from the rest of the chromosome as well as the fact it contains a cluster of genes required for full virulence (71). The FPI is made up of 17-19 genes and is duplicated within *holarctica* and *tularensis* strains. Because of this, much of the preliminary work on the FPI has been performed in the *novicida* strain U112, which has only one copy, making genetic manipulations of the FPI more straightforward. Transposon mutagenesis studies identified adjacent *Francisella* genes within the FPI that were necessary for replication within macrophages, *iglB* and *iglC* (40). These genes are predicted to be part of a four gene operon, *iglABCD* that resides within a region, along with *pdpD*, that has a G+C content of 31% compared to 33.2% G+C for the rest of the genome. The central region of the FPI, containing *pdpC, pdpB, pdpA*, and several predicted small genes ranging in sizes from 125-554 bp, has a G+C content of 26.6% which is extremely low among bacteria. Also within the FPI is a 5 kb region that is 51% G+C predicted to encode rRNA (72). All four of the *igl* genes as well as *pdpA, pdpB,* and *pdpD*

are required for intracellular replication within macrophages and for full virulence within an animal host (21, 72, 83, 92). The FPI genes are clearly critical for pathogenesis of *Francisella*, however their function remains a mystery. Sequence homology of several FPI ORFs has led to the proposal that these genes function as a type VI secretion system (71), though further work is needed to substantiate this claim.

LPS

A major component of the gram-negative outer membrane is lipopolysaccharide (LPS). Taking advantage of this, many organisms produce receptors and pattern-recognition molecules that detect the presence of LPS which results in stimulation of an immune response aimed at clearing the bacterial invader. LPS recognition occurs primarily through toll-like receptor 4 (TLR4) which is present on macrophages and dendritic cells among other cell types (68). Activation of the TLR4 complex leads to translocation of NFκB to the nucleus where transcription of genes important in the inflammatory response are initiated (24).

Francisella immune evasion appears to partly be due to a lack of host-cell recognition. In the presence of increasing amounts of *Francisella* LPS, human and mouse monocytic cells produce very little, if any, inflammatory cytokines such as TNF-α, IL-6, and IL- 8 (17, 25, 42). In contrast, *Salmonella* and *E. coli* LPS induce high levels of these cytokines (25, 42). The lack of host immune stimulation is most likely because *Francisella* LPS does not interact with TLR4 (17, 41).

Pilus

Contact with and adhesion to host cells is an important consideration for a bacterial pathogen. One method for facilitating these interactions are type 4 pili (T4P), which are

mainly homopolymeric structures that extend from the bacteria and can mediate attachment to a host cell (79). The *F. tularensis holarctica* Live Vaccine Strain (LVS), and the highly virulent *F. tularensis tularensis* Schu S4 strain displayed long thin fibers on their surface that were reminiscent of type 4 pili (T4P) produced by other gram-negative pathogens (38, 62, 79). Analysis of *Francisella* genome sequences revealed the presence of gene clusters with homology to T4P genes found in *Neisseria* spp. and *Pseudomonas aeruginosa* (38). The Schu S4 genome contained a *pilAEV* locus predicted to encode three full length genes. In *holarctica* isolates, *pilE* and *pilV* are truncated due to nonsense mutations that introduce a stop codon. In a subset of *holarctica* isolates, including LVS, the majority of *pilA* is deleted due to homologous recombination of two 120bp direct repeats flanking the gene (30). *Novicida* ssp. are similar to *tularensis* ssp. with the exception of differences in the C-terminal portion of *pilA*. In other gram-negative pathogens, PilA is the major pilin subunit. Functional studies demonstrate the *pilA* of *Francisella* is expressed and PilA protein is exported to the cell surface where it forms multimeric complexes (30). Schu S4 and *F. tularensis novicida* U112 *pilA* cloned into a heterologous *Neisseria* T4P expression system resulted in expression of a functional pilus (29). A *pilA* deletion strain retained the ability to replicate in macrophages, though to a lesser degree than wildtype, but was attenuated for dissemination to the spleen following sub-cutaneous infection of mice (30). Though LVS lacks full length *pilA,* TEM studies have identified T4P-like structures on the bacterial surface, whose expression has been linked to *pilB* (29). These results indicate that *Francisella* produces T4P, though there may be strain-specific differences in pilus expression based on genetic differences between strains. Further studies are needed to determine the

major pilin subunits between subspecies; particularly in strains that lack full length *pilA*, such as LVS.

Capsule

Evading the host immune system is an important criteria for a successful pathogen. One strategy employed by several bacterial human pathogens is the presence of a polysaccharide capsule which surrounds the bacterial outer membrane and prevents host recognition of bacterial components. Often, the presence of a capsule is required for full virulence of an organism, as is the case with *Staphylococcus aureus* and *Streptococcus pneumoniae* (76, 80). The capsule often confers the ability of bacteria to avoid phagocytosis, however intracellular replication of *Francisella* may be an important component of the bacterium's patho-biology within an animal host.

Francisella strains lacking an electron dense zone, proposed to be a capsule, have been isolated (82, 89). These strains are more sensitive to serum killing and are attenuated in mice (82). However, these strains also contain significant differences in their outer membrane profile which casts some doubt that the nature of attenuation is entirely capsule related. Also, the makeup of the electron dense zone is unclear. Confusing the issue, others have observed an electron translucent zone surrounding *Francisella* within host cells which has also been proposed to be capsule (39). Mutagenesis studies have identified attenuated mutants with disruptions in *Francisella* genes resembling a capsule biosynthesis gene in *Bacillus anthracis* (90). These studies provide some evidence that the production and presence of capsule may be an important component of *Francisella* pathogenesis, though further studies are needed to better understand the contribution.

Inhalation tularemia

From a biological weapon perspective, the most serious tularemia transmission route is via inhalation (77). An inhalation exposure is predicted to incapacitate the largest number of civilians and carry the largest financial burden to an affected community (102). To understand how *Francisella* instigates a lethal infection following inhalation, it is important to understand the host mechanisms in place to deal with inhaled foreign particles. This will enable deciphering of how *Francisella* subverts these pulmonary immune functions in order to establish disease.

Pulmonary innate immunity

The lung is the largest epithelial surface in the body, and is important for efficient gas exchange from the air to the blood stream. Respiration moves 15,000 liters of air over 150 m^2 of epithelial surface area (64). A byproduct of this interaction is constant exposure of external particles and potentially harmful pathogens to the lung during respiration. To protect the host, a complex defense system has evolved that combines physical barriers, pathogen-ingesting cells, microbicidal molecules, and molecular signals that enable communication among all of the components. These systems must work in concert to control potential infections without interfering with respiration, a primary function necessary for the life of the organism.

Physical barrier

The lung's first line of defense against foreign would-be pathogens are physical barriers that block entry into the lung environment. The walls of the upper airway, the nose, trachea, and upper bronchi are covered with mucous and ciliated cells (60). Inhaled particles become trapped in the mucous layer, and the cilia lining the airway beat in unison away from

the lung, sending foreign matter back from whence it came, up the airway where it can be coughed out or swallowed.

Alveolar macrophages

The alveolus is a sterile environment, and alveolar macrophages are key scavengers and sentinels that play a critical role in maintaining alveolar sterility. These cells are efficient at phagocytosing organisms that invade the alveolar space (37). Following phagocytosis, alveolar macrophages often single-handedly eliminate intruding microorganisms through production of microbicidal molecules such as reactive oxygen radicals and nitric oxides (37). If inhaled pathogenic microorganisms cannot be quickly and efficiently contained and eliminated, alveolar macrophages become potent producers of cytokines, such as TNF-α and IL-6 which recruit neutrophils and monocytes from the blood stream into the lung to aid in clearing the infection (70). In this way, the alveolar macrophage is central to initiating an innate immune response within the lung in response to respiratory pathogens.

Dendritic cells

Another important phagocytic cell for maintaining a healthy respiratory system within a mammalian host is the dendritic cell (DC). DCs are present throughout the airway, within the trachea, bronchi, and alveolus (86). Under normal conditions, DCs within the alveolar space are immature, at which time they actively sample the environment and process antigens. Upon detection of antigenic patterns found on pathogenic microorganisms or as a result of tissue destruction, DCs enter a maturation state. During maturation, DCs cease uptake and antigen processing functions and begin presenting the stimulating antigen on the cell surface where it can be recognized by T-cells (96). The activated DCs become highly

attracted to chemokines produced by T-cells which are found in large numbers within lymph nodes. This leads to DC migration from the initial infection site to the draining lymph node where the antigen is presented to lymphocytes, leading to proliferation of antigen-specific T-cells which can travel to the site of infection and aid in controlling the disease (96). In this way, the DC acts as a bridge joining the innate and adaptive immune systems together.

Monocytes

Monocytes are cells that circulate in the bloodstream and can be recruited to infected tissues by cytokine signals. Monocytes are precursors to macrophages (95) and dendritic cells (81) and upon entry into tissue, differentiate into mature phagocytes that can aid in the clearance of invading pathogens (10). During infection, endothelial cells respond to inflammatory signals produced by macrophages and DCs which leads to upregulation of chemokines on their cell surface (7). Through interaction with cognate molecules on the surface of recruited cells, such as monocytes and neutrophils, extravasation of these cells into infected tissues is facilitated (46).

Neutrophils

Perhaps the cell with the most potent antimicrobial potential is the neutrophil. These cells are highly phagocytic and have several pathways available to eliminate invading microorganisms (73). Neutrophils are crucial to the protection of the host from microbial infections. People with inherited disorders affecting neutrophil number or function suffer from frequent and severe microbial diseases (74).

One such pathway is the oxygen-dependent "respiratory burst", which utilizes nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to convert O_2 into highly toxic superoxide anions (93). Reactive oxygen species (ROS) are toxic, not only to

pathogens, but also to host cells. Therefore regulation of their production is tightly controlled. When a neutrophil is in an inactivated state, NADPH subunits are segregated into different cellular compartments which prevents the enzyme from being active. Two subunits, gp91^{phox} and p22^{phox} are targeted to the membranes of phagosomes whereas the remaining NADPH subunits, p47^{phox}, p67^{phox}, and rac2, are distributed within the cytoplasm of resting neutrophils (73). Within 30 seconds after particle binding on the neutrophil cell surface, oxidase subunits localize to the invaginating membrane that will become the phagosome (4, 22). Assembled and active NADPH oxidase is limited to the particle-containing phagosome which is critical in effectively killing potentially pathogenic organisms while simultaneously protecting host cells and tissues from oxidative damage (22, 73).

Neutrophils also possess non-oxidative means to kill microorganisms. Anaerobic killing in neutrophils can occur via phagosome maturation and fusion with a lysosome, similar to macrophages. However, this process occurs more rapidly in neutrophils and is augmented through the fusion of the phagosome with granules containing a suite of cationic anti-microbial peptides (27, 51). Activated neutrophils also possess the ability to expel the contents of the anti-microbial containing vesicles into the extracellular environment as a means of controlling an infection, however this often occurs at the expense of damage to host tissues (27). Neutrophils are often critical to controlling bacterial infections with a significant intra-macrophage growth component (61). Proteins associated with virulence of intracellular pathogens *Shigella*, *Salmonella*, and *Yersinia* are degraded by neutrophil elastase (97).

ATII cells

The primary function of the lung is to facilitate gas exchange from the air into the bloodstream. To mediate efficient gas transfer, the lung epithelial cell surface is quite large. Mucociliary clearance is an important host defense mechanism utilized in the airways, but it requires an air-liquid interface. Surface tension at this interface creates collapsing forces that would make respiration difficult (100). The system that has evolved to circumvent this problem is the production of pulmonary surfactant, which creates a lipid-rich monolayer separating liquid and gas at the epithelial cell surface, thus reducing the surface tension and allowing respiration to occur easily. A lack of sufficient surfactant leads to respiratory failure (99).

The primary producer of pulmonary surfactant is the alveolar type II (ATII) epithelial cell. Surfactant is made up of 90% phospholipids and 10% protein, which includes two hydrophilic proteins, surfactant protein A (SP-A) and surfactant protein D (SP-D), and two hydrophobic proteins, surfactant protein B (SP-B) and surfactant protein C (SP-C) (28, 64). SP-A and SP-D have homology to proteins labeled "collectins" found in serum. These proteins have the ability to recognize common patterns found on the surface of microorganisms which facilitates opsonization and phagocytosis by macrophages (64). SP-A and SP-D bind and aggregate bacterial, viral, and fungal pathogens, enhance phagocytosis of these organisms, and activate macrophages. Both SP-A and SP-D bind to the surface of macrophages, enhancing phagocytosis (64), and SP-A deficient mice display delayed clearance of multiple pathogens while not impacting normal respiration, underscoring the importance of pulmonary surfactant in host defense in the lung (53-58).

The hydrophobic surfactant proteins, SP-B and SP-C do not have identified roles in host defense, but are necessary for maintaining proper surface tension at the liquid-air interface within the lung (100). Both proteins are produced primarily by ATII cells and are stored in lamellar bodies before being secreted into the alveolus. Once secreted, interactions with phospholipids occur which leads to the formation of stable monolayers and bilayers, thus reducing surface tension in the lung.

The ATII cell has been referred to as the "stem cell of the alveolar epithelium" (28). In response to alveolar injury, ATII cells proliferate and become new ATII cells or differentiate into ATI cells. In this way, ATII cells are critical for repair of damaged alveolar epithelium. In addition, ATII cells aid in the innate immune response to invading pathogens by producing pro-inflammatory cytokines as well as responding to signals produced by macrophages and DCs (28). This leads to increased epithelial layer permeability which facilitates the extravasation of neutrophils and monocytes from the blood stream into the alveolus to aid in battling an infection.

Pulmonary immune response to *Francisella*

Within one hour following inhalation of *Francisella*, bacteria are found within macrophages and dendritic cells from bronchoalveolar lavage fluid (12). Normally, upon detection of bacteria, these cells activate and produce pro-inflammatory cytokines which leads to the recruitment of monocytes and neutrophils from the blood to aid in controlling the infection. Upon infection with *Francisella*, pulmonary macrophages and dendritic cells do not produce pro-inflammatory cytokines, TNF-α and IL-6 (11, 12). This lack of activation appears to be a result of active suppression of their production. *Francisella*-infected macrophages and dendritic cells fail to react to potent TLR4 agonists, such as *E. coli* LPS,

resulting in no production of TNF-α (91). This suppression may be a result of TGF-β production, which is activated during *Francisella* infection (11). *Francisella* infection also suppresses T-cell activation by macrophages via production of prostaglandin-E2 (101). In this way, *Francisella* pathogenesis in the lung is most likely achieved through a balance of avoiding immune recognition and actively subverting the host response.

Key questions

Understanding pathogenic mechanisms utilized during pulmonary tularemia is important to deciphering more effective vaccines or therapeutics to combat the disease. Also, understanding *Francisella*'s manipulation of the host immune response can provide more specific insight into how lung pathogens interact with and exploit the host immune response to cause disease. *Francisella* replicates within macrophages *in vitro* and the bacterium can avoid phagosomal killing and suppresses pro-inflammatory signaling, clearly aiding the organism's survival. However the suite of cells infected by *Francisella* following inhalation is unknown. Also, it is unclear how infection by *Francisella* affects cell populations in the lung as disease progresses. These are fundamental pieces of information that must be in place to most efficiently make directed hypotheses concerning how *Francisella* causes disease when inhaled. A major goal of this work was to fill in these gaps and determine which cells become infected by *Francisella* in the lung and how those populations are affected over time.

Answers to these questions enable more specific inquiries into how *Francisella* survives and thrives within unique cell types as well as the contribution of each cell type to disease progression and outcome. Chapter 5 is an in depth analysis of the entry mechanism and intracellular lifestyle of *Francisella* within a specific infected cell type that was

identified; the ATII cell. This work follows a top-down approach, beginning from the understanding that inhaling *F. tularensis* causes a rapidly progressing lethal disease. Knowing that *Francisella* survives in host cells *in vitro*, this led us to determine which cells were infected within the lung following inhalation. Subsequently, we could ask more specific questions about how the bacterium gains entry into those cells and what is the intracellular biology of the organism. To discover the bacterial factors important, or even necessary, for aspects of *Francisella's* lifestyle within the host, we needed the ability to make mutants in specific genes and decipher how disruption of those genes changed specific components of the bacterium's pathogenesis. Lastly, understanding how *Francisella* causes disease in a host gives us a baseline to study how host factors are important during inhalation tularemia through the use of mice deficient for specific immune components. In this body of work, we move from gross pathology progressively towards the molecular details of disease.

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CHAPTER 2

Francisella tularensis replicates within alveolar type II epithelial cells *in vitro* and *in vivo* following inhalation

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ABSTRACT

Francisella tularensis replicates in macrophages and dendritic cells, but interactions with other cell types have not been well described. *F. tularensis* LVS invaded and replicated within alveolar epithelial cell lines. Following intranasal inoculation of C57BL/6 mice, *Francisella* localized to the alveolus and replicated within alveolar type II epithelial cells.

INTRODUCTION

Francisella tularensis, the etiological agent of tularemia, is a zoonotic pathogen with a broad host range spanning from protists to humans. The organism is transmitted to humans through handling of contaminated material, insect bites, or inhalation (1, 10). It has been determined that contact with 25 or fewer bacteria is sufficient to cause disease (16, 17). Following contact, the bacteria disseminate to liver, spleen and lungs regardless of initial transmission route (7, 10).

The low infectious dose, myriad of transmission routes, broad host range, and severity of disease caused by *F. tularensis* led a number of nations to develop and stockpile

this organism as a biological weapon (9), yet little is known about the basis of its pathogenesis or virulence. It has been reported that macrophages and potentially dendritic cells serve as the primary host cells for *Francisella* growth in humans and other animals (6, 18). This conclusion is supported by the observation that *F. tularensis* and other closely related *Francisella* species survive and replicate in human and rodent macrophages (2, 5), and that many of the identified attenuating mutations impair intra-macrophage growth (3, 11, 12, 14, 15).

Clearly intra-macrophage survival is an important *Francisella* virulence property. However, apart from one report of hepatocyte association in infected liver (8), the potential contribution of other host cell interactions to *F. tularensis* virulence and pathogenesis remains untested. Given the severity of inhalation-acquired tularemia, we sought to address this possibility by using *in vitro* and *in vivo* models to examine whether *Francisella* invades and replicates within epithelial cells of the lung.

RESULTS

Francisella tularensis LVS invades and replicates within lung epithelial cells *in vitro.*

To determine if *Francisella* could invade and replicate within lung epithelial cells, *in vitro* invasion and replication assays were performed using a human alveolar type II (ATII) epithelial-derived cell line A549 (ATCC), a mouse ATII-derived cell line MLE-12 (ATCC), and a mouse lung epithelial-derived cell line Tc-1 (ATCC). For comparison, the mouse macrophage-like cell line, J774A.1 (ATCC) was also used. Intracellular bacteria were enumerated using a gentamicin protection assay. The cell lines were grown to confluency in 24-well plates and inoculated with *F. tularensis* LVS at a multiplicity of infection (MOI) of

100:1. Four hours following inoculation, the cells were washed with PBS and media containing gentamicin (25 μg/mL) was added to kill extracellular bacteria.

Intracellular organisms were recovered from all cell lines 6 and 24 hours post-inoculation (Figure 1A). The number of intracellular organisms recovered 6 hours post-inoculation as well as the percentage of infected cells varied among the lung epithelial cell lines with the fewest intracellular *Francisella* recovered from the human ATII cell line, A549. The percentage of infected epithelial cells ranged from 0.2% infected A549 cells infected to 17% infected Tc-1 cells (Figure 1A). The number of intracellular bacteria recovered from each lung epithelial cell line at 6 hours was less than the recovery from J774A.1 cells as was the percentage of infected cells, indicating that entry into epithelial cells may be a less efficient process than uptake by macrophages.

The number of intracellular bacteria increased by an average of 100 fold during the following 18 hours in each cell line representing an intracellular doubling time of roughly 3 hours (Figure 1A). Intracellular localization and proliferation was confirmed using fluorescence microscopy and digital image deconvolution (Figure 1B-E). From 6 to 24 hours post-inoculation, the relative increase of intracellular bacteria within lung epithelial cells was similar to that observed within J774A.1 cells, indicating that *F. tularensis* LVS replicated to the same degree within lung epithelial cells as in macrophages.

There are many reports of *Francisella* replicating within macrophages *in vitro* and here we report *Francisella's* ability to replicate within cultured lung epithelial cells. Whereas some work has been done to examine *Francisella* localization in the liver (8), little is known about the localization of inhaled *Francisella* in the lung. Bosio *et al* recently reported that *Francisella* was associated primarily with dendritic cells isolated from

bronchioalveolar lavage fluid of mice one hour following inhalation (6). However, the localization of bacteria in lung tissue throughout the course of infection has not been addressed.

Following inhalation, *Francisella* localizes to the alveolus.

To determine the localization of *Francisella* in the airway following inhalation, anesthetized 6-8 week old female C57BL/6 mice were inoculated intranasally with 10^5 CFU of *F. tularensis* LVS expressing GFP suspended in 50 µl of PBS (All mouse work was performed under IACUC-approved protocol.). Immunofluorescence analysis was performed on formalin fixed and paraffin embedded tissue sections obtained from nasal turbinates, trachea, and lungs harvested 1, 3 and 7 days post-inoculation. No bacteria were observed in turbinates or trachea. In the lung, few bacteria associated with the apical surface of bronchial epithelial cells while the majority localized with alveolar cells (Figure 2). Fluorescence imaging of sequential vertical planes revealed that most infected alveolar cells contained multiple bacteria throughout the cytoplasm, indicative of intracellular replication (data not shown). Bacterial replication within ciliated or non-ciliated (Clara) bronchial epithelial cells was not observed. The absolute number of bacteria (Figure 2D) and the number of infected alveolar cells increased by day 3 (Figure 2B). By day 7, *F. tularensis* within the lung was widespread but remained exclusively in the alveolus (Figure 2C). Throughout the course of infection, the amount of extracellular space observed in the network of alveolar cells decreased leading to the alveolar cells appearing more condensed (Figure 2).

Inhaled *Francisella* co-localizes to and replicates within ATII cells *in vivo*.

Given that *Francisella* invaded and replicated within ATII-derived cell lines *in vitro*, we probed infected lung tissue sections with antibodies to non-secreted surfactant protein

precursors proSP-B and proSB-C (Chemicon) to determine if *Francisella* localized to and replicated within ATII cells *in vivo* following inhalation. proSP-B is produced by ATII cells and non-ciliated bronchiolar Clara cells (13) whereas proSP-C is produced by ATII cells exclusively (4). Three days following inoculation, the majority of alveolar cells associated with *F. tularensis* also bound antibodies to non-secreted surfactant protein precursors, proSP-B and proSB-C (Figure 3A-D). Some cells associated with bacteria were not positive for proSP-B or proSP-C indicating that *Francisella* interacts with other cell types in the lung following inhalation.

In addition to the co-localization with proSP proteins, microscopy of sequential vertical planes confirmed that bacteria were inside infected ATII cells (data not shown). Rabbit antibody specific for β-tubulin (Abcam) did not co-localize with *F. tularensis* (Fig 3E) indicating that the co-localization of *Francisella* with rabbit proSP antibodies was not due to bacteria non-specifically binding rabbit antibodies, nor did the proSP antibodies cross-react with cultured *Francisella* (data not shown). These collective data demonstrate that *F. tularensis* replicates within ATII cells following inhalation, and the proportion of ATII cells harboring bacteria increases during the course of pneumonic tularemia.

Bacteria were also observed in probed sections that did not co-localize with proSP-B and proSP-C (Figure 3) indicating that ATII cells are not the only alveolar cell type harboring replicating *Francisella*. Macrophages and dendritic cells have been reported as potential host cells for replicating *Francisella* (2, 5, 6). Due to difficulties in staining for surface macrophage and dendritic cell markers in embedded lung sections, we utilized dispase digestion of infected mouse lungs to stain for surface markers and also to assess our results from the embedded tissue staining.

Three days following inoculation with LVS expressing GFP, lungs were infused and incubated with the neutral protease, dispase (BD Biosciences). Digested tissue was washed in PBS and subsequently filtered through 40 μm and 20 μm mesh. Staining with fluorescently-labeled antibody specific for the surface markers F4/80 (eBioscience) and CD11c (eBioscience) was performed prior to fixation with 4% paraformaldehyde. Staining for the intracellular markers proSP-B and proSP-C occurred following fixation and required the use of Cytoperm (BD Biosciences) to permeabilize the cells. *F. tularensis* LVS was observed within cells expressing F4/80, CD11c, proSP-B, and proSP-C (Figure 4) indicating that following inhalation, bacteria survive and replicate within macrophages, dendritic cells, and ATII cells.

Here we report that in addition to replicating within macrophages and dendritic cells, *F. tularensis* also invades and replicates within alveolar type II epithelial cells, indicating that interaction with these cells following inhalation may be an important component of pneumonic tularemia. Future work will analyze the proportion of various cell types that are infected in the lung as well as attempt to identify the contribution of ATII cell invasion and replication to the disease progression of pneumonic tularemia.

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ATTRIBUTIONS

I performed all experiments presented in this chapter with the following exceptions. Robin Craven assisted with mouse infections, lung collection, organ burden experiments, and acquired J774 fluorescence microscopy images. This work was published in the journal Infection and Immunity, Volume 75, pages 1034-1039. Permission has been granted to reprint this material.

FIGURES

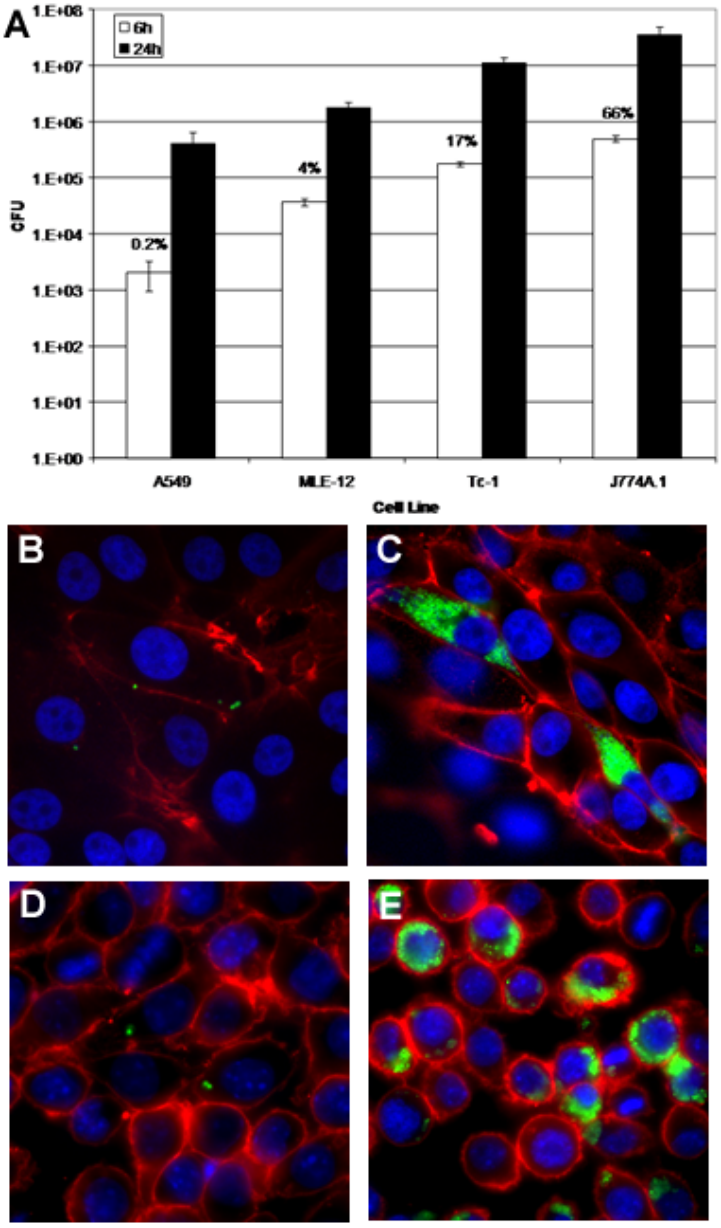

Figure 2.1. *F. tularensis* LVS invades and replicates within ATII cell lines *in vitro*. (A) Intracellular bacteria recovered from A549, MLE-12, Tc-1, and J774A.1 cells 6 and 24 hours post inoculation. Percentages above bars represent percentage of infected cells. (B) Fluorescence imaging of A549s inoculated with GFP-expressing LVS 6 and (C) 24 hours following inoculation. Cell borders were visualized by rhodamine-phalloidin (Molecular Probes) staining (red) and nuclei visualized with DAPI (blue). (D) Fluorescence imaging of J774A.1 cells inoculated with GFP-expressing LVS (green) 6 and (E) 24 hours post-inoculation. Cell borders were visualized using biotinylated lectin from *Lens culinaris* and streptavidin-conjugated Alexa Fluor 647 (Molecular Probes) (red) and nuclei were stained with DAPI (blue). Intracellular replication experiments were carried out in triplicate with error bars representing standard deviations of the means.

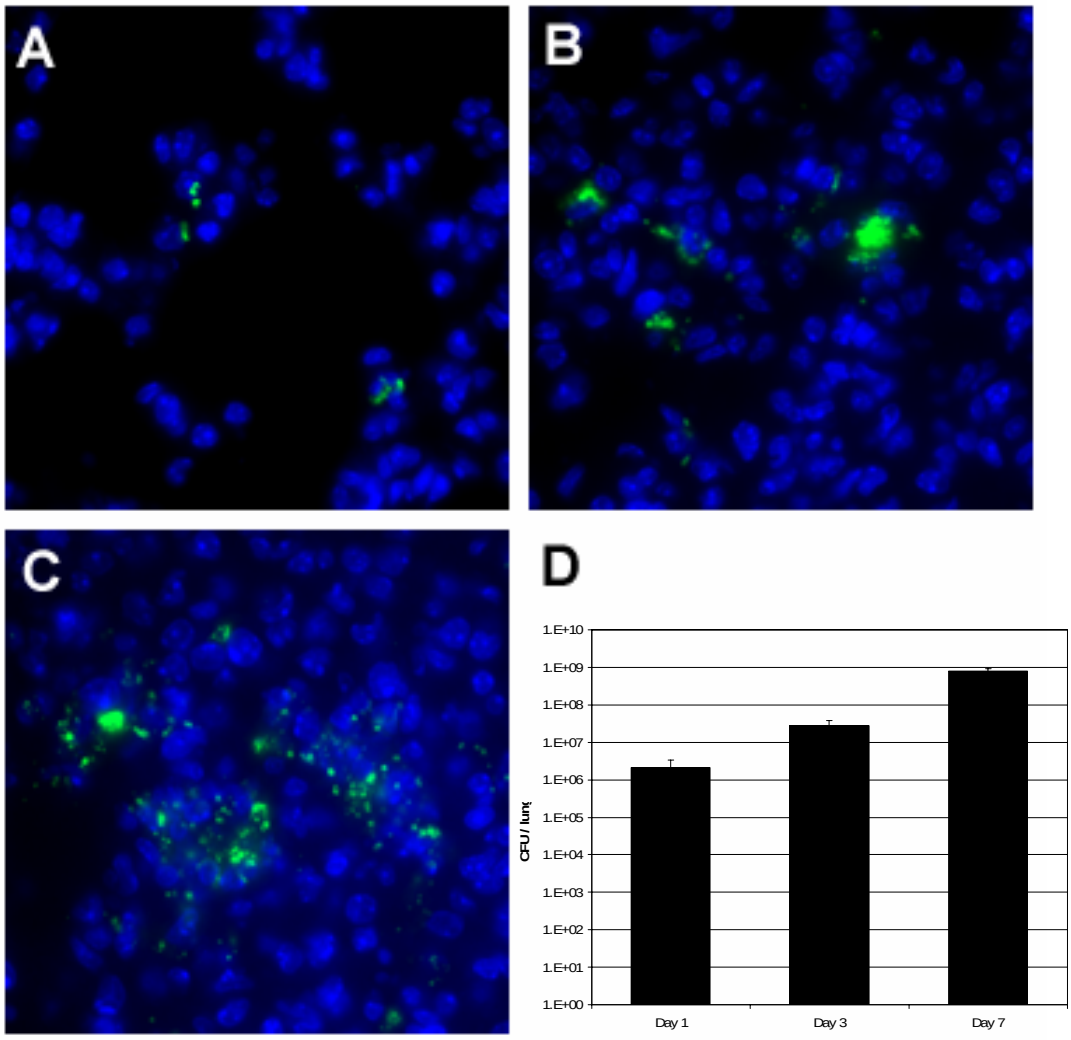

Figure 2.2. Following inhalation *F. tularensis* localizes to the alveolus. Mice were intranasally inoculated with 10^5 CFU of *F. tularensis* LVS expressing GFP. One, three, and seven days post-inoculation, lungs were harvested and prepared for immunofluorescence analysis. Bacterial localization was determined by probing lung sections with a fluorescently-labeled antibody to GFP (green). Nuclei were stained with DAPI (blue) to visualize lung cells. Representative images of the alveolus of infected mice (A) 1, (B) 3, and (C) 7 days post-inoculation. (D) Bacterial recovery from lungs 1, 3, and 7 days following intranasal inoculation with 10^5 CFU LVS. Each bar represents mean recovery from 3 mice with error bars representing standard deviations of the means.

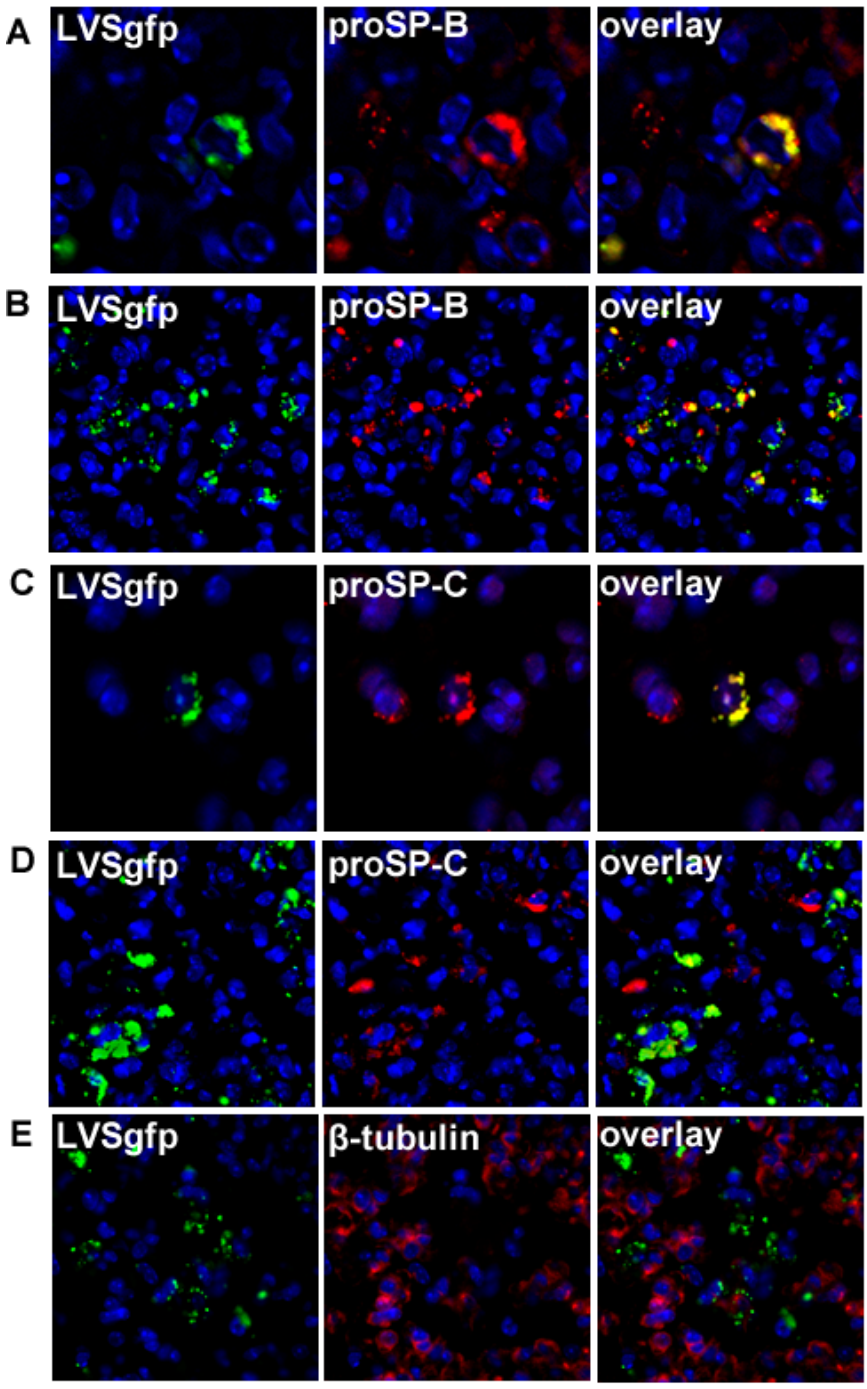

Figure 2.3. Following inhalation, *F. tularensis* LVS expressing GFP co-localized with proSP-B and proSP-C; proteins produced by ATII epithelial cells. Bacterial localization was determined with a fluorescently-labeled antibody to GFP (green). Nuclei were stained with DAPI (blue). Sections were probed with fluorescently-labeled antibody to (A-B) proSP-B (red), (C-D) proSP-C (red) to identify ATII cells and (E) β-tubulin (red). Representative images are from lung sections 3 days post-inoculation.

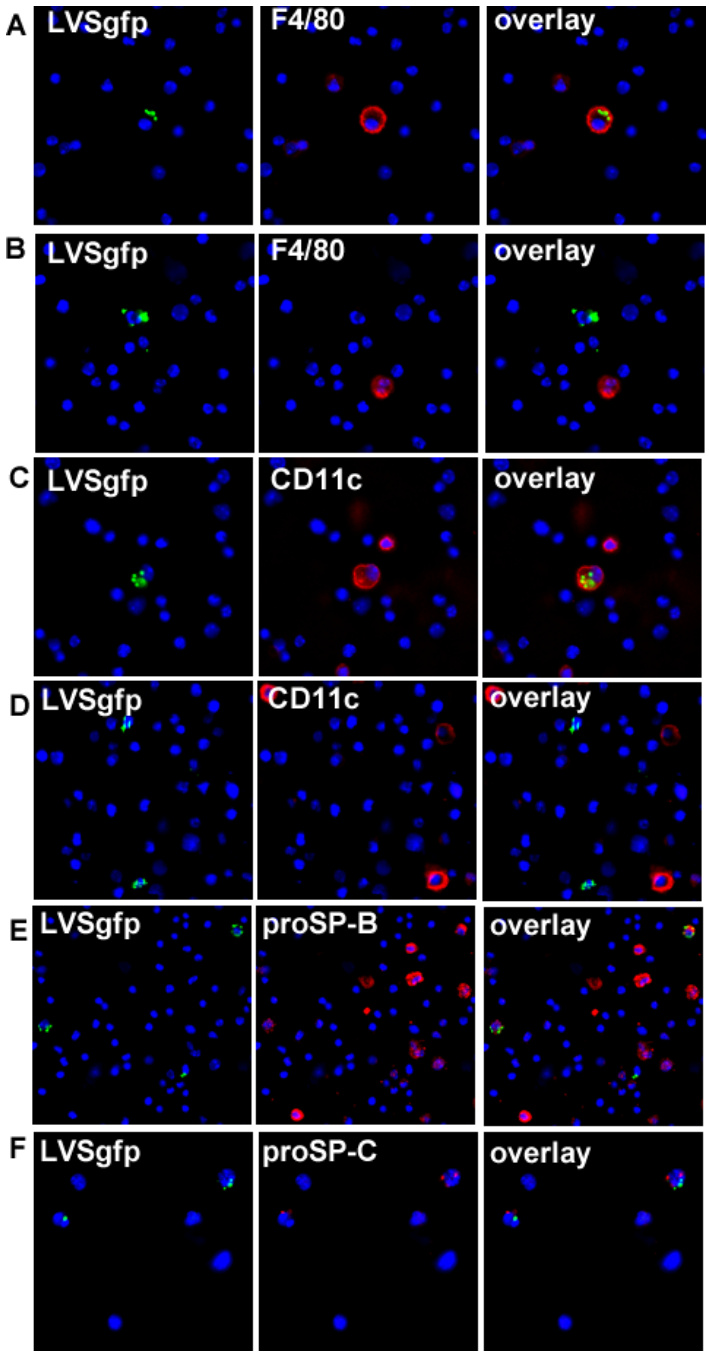

Figure 2.4. *F. tularensis* LVS expressing GFP co-localized with cells expressing the macrophage marker F4/80, the dendritic cell marker CD11c, and the ATII cell markers proSP-B and proSP-C. Nuclei were stained with DAPI (blue). Mouse lung cells were probed with fluorescently-labeled antibody to (A-B) F4/80 (red), (C-D) CD11c (red), and (E) proSP-B (red). Representative images are from lung cells 3 days post-inoculation.

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CHAPTER 3

Infected host cell repertoire and cellular response following inhalation of *Francisella tularensis* Schu S4, LVS, or subspecies *novicida* U112

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ABSTRACT

Background: *Francisella tularensis* causes systemic disease in humans and other mammals with high morbidity and mortality associated with inhalation-acquired infection. *F. tularensis* is a facultative intracellular pathogen, but the scope and significance of cell-types infected during disease is unknown. **Methods:** Using flow cytometry, we identified and quantified infected cell-types and assessed the impact of infection on cell populations following inhalation of *F. tularensis* U112, LVS, and Schu S4. **Results:** Initially alveolar macrophages comprised over 75% of Schu S4- and LVS-infected cells whereas approximately 51% and 27% of U112-infected cells were alveolar macrophages and neutrophils, respectively. After three days roughly half the Schu S4- and LVS- and nearly 80% of U112-infected cells were neutrophils. All strains infected interstitial macrophages, dendritic cells, monocytes and ATII cells throughout infection. Macrophage, monocyte, and dendritic cell populations were reduced during U112, but not Schu S4, infection. **Conclusions:** These results demonstrate directly for the first time that *F. tularensis* is a

promiscuous intracellular pathogen that invades and replicates within cell-types ranging from migratory immune cells to structural tissue cells. However, the proportion of cell-types infected, and the cellular immune response evoked by the human pathogenic strain Schu S4 differs from that of the human avirulent U112.

INTRODUCTION

Since 2001, there has been increased interest in understanding pathogens with virulence characteristics making them dangerous for purposeful release. One such organism is the gram-negative bacterium, *Francisella tularensis,* the etiological agent of tularemia. Virulent strains of *Francisella* can cause incapacitating or lethal disease in humans, mice, and other mammals (24). Tularemia can be acquired via insect-bites (13), handling infected animal carcasses (30) , contaminated water (11), or inhalation (15). Inhalation exposure results in the most acute, rapidly progressing, manifestation of disease (6). When inhaled, as few as 10 organisms can cause a debilitating, and potentially fatal, infection in humans (22).

Given the seriousness of inhalation-acquired tularemia, surprisingly little is known about *Francisella* biology in the host lung. Within one hour after inhalation, *Francisella* is found in airway macrophages and dendritic cells (DCs) (3, 4). However, infection of these cells does not trigger production of TNF-α or IL-6 (3, 4). Instead, *Francisella* infection induces immuno-suppressive mediators such as TGF-β (3) and prostaglandin E2 (31) through yet unknown mechanisms. *F. tularensis* also infects other cell-types important for host-defense against lung infections, such as monocytes (23), neutrophils (16), and alveolar type II (ATII) epithelial cells (10).

Growth within host cells is recognized as an important aspect of *Francisella* pathogenesis, however the range and scope of cells infected throughout disease have not been determined. In order to understand mechanisms pivotal to the virulence of this organism we identified and quantified the repertoire of infected lung cells and characterized the cellular immune response following inhalation of three different *F. tularensis* subspecies.

RESULTS

Identifying *Francisella*-Infected Lung Cells

U112, Live Vaccine Strain (LVS), and Schu S4 are commonly studied closely related *F. tularensis* strains that exhibit differences in host range, virulence, and host response (Table 1). Single cell suspensions prepared from whole lung digests of mice inoculated intranasally with *Francisella* strains expressing green fluorescent protein (GFP) were analyzed by flow cytometry and fluorescence microscopy to identify the host-cell niche occupied by these organisms. GFP+ lung cells, indicating association with *Francisella*, were readily detectable by flow cytometry (Figure 1A). Microscopic examination of cells sorted from the high GFP+ population presented significant punctate GFP+ fluorescence, indicative of highly infected cells (Figure 1B), whereas low GFP+ cells had as few as 1 GFP+ bacterium indicating that cells containing a single bacterium could be detected by flow cytometry. No GFP+ bacteria were observed in the negative population. Projection images compiled from multiple planes throughout the Z-axis of membrane-stained cells revealed that bacteria were intracellular (data not shown).

To directly compare *Francisella* strains, we inoculated mice with 10^2 - 10^3 colony forming units (CFU) for each strain. Actual dose ranges were 513-550 CFU for U112, 1100-

1260 for LVS, and 337-410 for Schu S4. For each strain there were more infected cells 24 hours post-inoculation than the initial inocula, indicating that bacteria had replicated within this time frame (Figure 1C). It is unlikely that *Francisella* could have replicated intracellularly, killed host cells, and infected additional cells within 24 hours, so this result suggests that a significant degree of extracellular replication occurred concurrently with host cell infection. From day 1 to day 3 post-inhalation, there was a significant increase in the number of infected lung cells for each strain (Figure 1C). On day 3 there was a similar number of infected cells between mice infected with U112 and Schu S4. Fewer GFP+ cells were observed in LVS-infected mice at day 3 *($p<.05$)*, potentially because LVS infection was better controlled by the innate immune system.

Composition of the *Francisella* host cell niche

To assess the suite of cells infected by *Francisella* following inhalation, we identified GFP+ cells with cell-type-specific staining patterns (Figure 2, Table 2). Twenty-four hours post-inoculation the predominant infected cell-type in mice infected with U112 (51.6%), LVS (76.9%), and Schu S4 (78.9%) were alveolar macrophages (Figure 3). Other cell types infected at day 1 were interstitial macrophages (11.0% U112, 5.4% LVS, 6.2% Schu S4), $CD11b^{low/mid}$ DCs (1.2% U112, 4.3% LVS, 2.0% Schu S4), and ATII cells (2.4% U112, 2.4% LVS, 6.0% Schu S4). Only 0.2% of LVS-associated cells were neutrophils and no Schu S4-associated neutrophils were observed on day 1. In contrast, 27.3% of U112-infected cells at day 1 were neutrophils.

By day 3, neutrophils were the predominant infected cell-type for each strain (79.3% U112, 56.9% LVS, 56.2% Schu S4), although more prevalent in U112-infected mice (Figure 3). The percentage of infected cells that were alveolar macrophages decreased to 2.3% for

U112, 18.0% for LVS, and 14.3% for Schu S4. A higher fraction of Schu S4-infected cells (8.8%) at day 3 were monocytes than LVS (1.6%) or U112 (1.4%).

Number of infected cells

Examining the proportion of *Francisella*- infected cell-types in the lung gave us insight into the *Francisella* host-cell suite over time, but another important consideration for understanding inhalation tularemia is how the abundance of infected cells changes in the lung during the disease course. Therefore, we determined the number of each cell-type infected at day 1 and 3 following inhalation. There was a significant increase in the number of infected interstitial macrophages, CD11b^{high} DCs, monocytes, neutrophils, and ATII cells in mice infected with each strain between day 1 and day 3 (Figure 4B,D,E,F,G). In contrast, the number of infected alveolar macrophages and CD11b$^{low/mid}$ DCs increased from day 1 to day 3 in mice infected with LVS or Schu S4, whereas the number of these cells infected with U112 was unchanged (Figure 4A,C). Most strikingly, on day 1 following inoculation, there were 2,670±1,287 U112-infected neutrophils compared to 10±16 for LVS and none detected for Schu S4. (Figure 4F). These data demonstrate that increasing numbers of alveolar macrophages and DCs continue to become infected from day 1 to day 3 following inhalation of LVS or Schu S4 but not during infection with U112. Schu S4-infected lungs had more infected monocytes than U112- or LVS- infected lungs indicating a potential propensity by this strain to infect these cells.

Effects on cell populations during disease progression

In addition to analyzing the number and proportion of infected cell-types, we determined how *Francisella* infection impacted total cell populations in the lung as disease progressed. Strain U112 imparted a 2.4-fold decrease in the number of alveolar macrophages

from day 1 to day 3 (Figure 5A). LVS-infected mice displayed a more modest decrease in these cells from day 1 to day 3, but this difference was statistically significant *($p<.05$)*. Alveolar macrophage numbers in Schu S4- infected mice did not change significantly from day 1 to day 3. We also observed more alveolar macrophages in lungs of mice infected with each strain at day 1 than from PBS control mice, but only in LVS-infected mice was this difference significant *($p<.05$)*.

Similar to alveolar macrophages, DC populations significantly decreased from day 1 to day 3 in U112-infected mice (Figure 5C,D). $CD11b^{low/mid}$ DCs decreased 2.3-fold and $CD11b^{high}$ DCs decreased 1.8-fold from day 1 to day 3 in U112-infected mice. A modest increase in $CD11b^{high}$ DCs was observed for LVS-infected mice from day 1 to day 3, however the number of $CD11b^{low/mid}$ DCs remained the same. There was no significant change in the number of DCs in Schu S4-infected mice from day 1 to day 3. No significant difference was observed between the number of DCs from infected mice at day 1 and PBS control mice.

There was no significant difference in the number of monocytes on day 1 following infection with each strain compared to PBS control mice (Figure 5E). The number of monocytes increased significantly in LVS- and Schu S4-infected mice from day 1 to day 3, whereas U112-infected mice had fewer monocytes in the lung over time.

Lungs from LVS- and Schu S4-infected mice had more interstitial macrophages at day 3 than day 1, while U112-infected mice displayed no significant change in interstitial macrophage numbers (Figure 5B). Considering that alveolar macrophage, DC, and monocyte populations decreased in response to U112 infection by day 3, it is plausible that there is a balance between macrophage recruitment to the lung and macrophage killing which

would result in no net change from day 1 to day 3, though more experimental work is needed to determine if this is the case. There was no difference between the number of interstitial macrophages in infected lungs and lungs from PBS control mice at day 1.

In response to *Francisella* infection, there was a significant increase in the number of neutrophils in the lungs of mice infected by each strain from day 1 to day 3 (Figure 5F) indicating that infection resulted in neutrophil recruitment, but not until after at least 24 hours following exposure. One day post-inhalation there was no increase in the number of neutrophils in the lungs of infected mice compared to PBS-inoculated control mice. We consistently observed fewer neutrophils at day 1 in Schu S4-infected mice than PBS control mice *($p<.05$)*, suggesting that Schu S4 may initially suppress neutrophil recruitment more efficiently than U112 or LVS.

Unlike other cell types analyzed, ATII cells are stationary structural cells. As expected, there was no significant change in the number of ATII cells in response to *Francisella* infection by U112, LVS, or Schu S4 from day 1 to day 3 (Figure 5G), but these studies do not address any effects that infection may have on ATII cell physiology or function.

Taken together, these data demonstrate that the innate immune response to LVS and Schu S4 in the lung is similar despite differences in the lethal dose of each strain. Upon inhalation, *Francisella* infects a variety of host cells, though alveolar macrophages are the predominant infected cell early during infection while neutrophils are recruited and become the main host cell in the later stages of pulmonary tularemia. U112 differed from both LVS and Schu S4 in that it was taken up by neutrophils more rapidly following inhalation, and this apparently occurs without significant recruitment of new neutrophils to the lung. U112

infection also impacted macrophage, DC, and monocyte populations differently during the disease course than LVS or Schu S4.

DISCUSSION

A goal of this study was to determine which cells in the lung become infected by *F. tularensis* following inhalation, and identify how these populations are altered during pulmonary tularemia. At the same time, we sought to compare three commonly studied *Francisella* strains with regard to cell infection and impact on population dynamics in the lung. Understanding the similarities and differences among strains will enable more accurate interpretations of other studies using these strains, as well as facilitate more specific inquiries into *Francisella* disease mechanisms.

To directly compare lung infections of *Francisella* strains, we initiated infection with similar numbers of each strain. Our dose range allowed detection of infected cell-types from whole lung digests 24 hours post-inoculation. Infected cells were not consistently distinguished from background at this time point from mice inoculated with less than 100 bacteria. However, mice similarly infected with 10 CFU of Schu S4 displayed a similar suite of infected cells and similar recruitment of monocytes and neutrophils at day 3 as mice inoculated with 300-400 CFU (data not shown) indicating that *Francisella* biology in the lung at a higher dose was not appreciably different than that of a very low dose.

During the early stages of infection, LVS- and Schu S4-mediated disease appeared similar while U112 displayed fundamental differences. The most common infected cell-type 24 hours after inhalation of each strain was alveolar macrophages, although interstitial macrophages, CD11b$^{low/mid}$ DCs, and ATII cells were also infected. In contrast, U112 was

found in neutrophils at 24 hours but LVS and Schu S4 were not. While there was no apparent influx of neutrophils overall at this stage of infection, there were almost 1000-fold more neutrophils associated with U112 than with LVS or Schu S4, indicating that neutrophils responded to and phagocytosed U112 to a significantly greater extent than LVS or Schu S4.

Also, U112-infected mice lost alveolar macrophages, DCs, and monocytes during the course of disease, whereas depletion of these cell-types was not observed in LVS- or Schu S4-infected mice. Potentially, phagocytes that are infected in the early stages of disease begin to undergo apoptosis or necrosis by day 3. More studies are needed to determine if U112 infection results in more rapid killing of infected cells than infection by LVS or Schu S4.

Other studies have noted differences in U112 when compared to LVS or Schu S4. For example, subspecies *novicida* exhibits different immuno-stimulatory properties in a mammalian host than subspecies *holarctica* or *tularensis*. U112 LPS stimulates macrophages to produce pro-inflammatory cytokines such as TNF-α, which are important in signaling the recruitment of neutrophils and monocytes, whereas LPS of LVS and Schu S4 does not (21). We therefore might have expected that U112 would provoke a more rapid or robust neutrophil influx than LVS or Schu S4, but this was not the case. Though significantly more U112 organisms were observed in neutrophils early during infection than LVS or Schu S4, there was no difference in the abundance of neutrophils in U112-infected lungs than the other strains or PBS control mice. A different study reported that inhalation of U112 did not lead to neutrophil recruitment in the lung by 24 hours (9), which is consistent with our observations. In addition to neutrophil recruitment, we observed an increase in the number of monocytes in LVS- and Schu S4-infected mouse lungs, however the number of

these cells in U112-infected mice decreased. Different studies are needed to determine whether monocyte recruitment is being blocked by U112 or whether these cells are being killed at a more rapid rate than they are recruited.

Francisella's high capacity to cause disease in mammals is not entirely explained by lack of host-recognition. *Francisella*-infected macrophages are unable to respond to pro-inflammatory stimuli, such as *E. coli* LPS (25), indicating an active suppression of this response. Though the complete mechanisms for inflammatory cytokine suppression is unclear, there is evidence that LVS and Schu S4 stimulate production of the immuno-suppressive cytokine, TGF-β within infected pulmonary macrophages and DCs (3, 4). TGF-β leads to up-regulation of Fc-receptor on macrophages which increases the phagocytic capability of these cells (29), while at the same time limits production of IFNγ and other pro-inflammatory molecules (12). Normally, increased phagocytosis by macrophages would expedite clearance of bacterial infection. However, given *Francisella*'s ability to survive and replicate within these cells, this may exacerbate replication of the organism within the host. While production of TGF-β and other immuno-suppressive mediators may delay influx of inflammatory cells, this study demonstrates that eventual neutrophil and monocyte recruitment did occur in response to *Francisella* infection in the lung, however these cells contribute to disease progression by becoming host cells for *Francisella* replication.

Normally, neutrophils are key to controlling bacterial infections with a significant intra-macrophage growth component (14). Despite the evolution of neutrophils as efficient microbial killers, the data presented here demonstrate that interactions with neutrophils are a key component of *Francisella* pathogenesis within the lung. Recent *in vitro* studies demonstrated that LVS is rapidly taken up by human neutrophils, but the respiratory burst is

prevented due to disruption in NADPH oxidase assembly within the phagosome. This process is apparently due to an undefined active bacterial process, as *Francisella* infection also prevented neutrophils from responding to potent heterologous stimuli (1, 16).

Another observation from this study was that infected ATII cells, which are non-migratory, were as abundant as infected DCs or interstitial macrophages during the early stages of disease. *Francisella* interactions with ATII cells could play a unique role in pulmonary tularemia. Cell-cell junctions of the epithelium and endothelium are affected by infection-induced cytokines and chemokines which facilitate chemotaxis of neutrophils and monocytes from the blood stream to infected tissue. The alveolar epithelium is closely associated with blood vessels to provide efficient oxygen exchange, and in addition to facilitating inflammatory cell recruitment into the lung, increasing numbers of *Francisella* within ATII cells during disease progression could provide a method of bacterial entry into the blood stream. In this way, ATII cells could provide a "gateway" to the blood stream where bacteria could then disseminate to distal organs such as liver and spleen. Further studies are ongoing in our lab to decipher the unique contribution of ATII cells during inhalation-acquired tularemia.

EXPERIMENTAL PROCEDURES

Bacterial Culture

Francisella strains were maintained on chocolate agar supplemented with Isovitalex (BD Biosciences). U112 was a gift from Colin Manoil, LVS was obtained from the CDC in Atlanta GA, and Schu S4 from BEI Resources. GFP strains contained a modified pKK214gfp plasmid (a kind gift from Mats Forsman).

Mouse infections

Female 7-10 week-old C57BL/6 mice were inoculated with *Francisella* strains diluted in sterile PBS and enumerated by Klett reading or OD 600. Dose was verified by plating inoculum on chocolate agar. Mice were anesthetized with avertin until unresponsive to toe pinch, and 50 µL of bacterial suspension was dispensed onto nares of the mouse. Previous results suggest this is an effective procedure for establishing a pulmonary *Francisella* infection (10). All animal experiments were conducted in accordance with animal care and use guidelines, and animal protocols were approved by the IACUC at UNC-Chapel Hill.

Lung cell isolation

Mice were anesthetized with avertin+heparin (1000 U/mL) and profused with 4-7 mL of PBS+heparin (200 U/mL). Tracheas were cannulated using a 16-gauge blunt-tipped needle, and lungs were inflated with approximately 1 mL of dispase (BD Biosciences). The trachea was tied off with surgical sutures and lungs were removed and incubated in 3.0 mL dispase at room temperature for 45 minutes. Tracheas were removed, and lungs were transferred to a Petri plate along with 7 mL of PBS+DNaseI (250 µg/mL) and tissue was teased apart using forceps. Cells were gently swirled for 1-2 minutes, and the suspension was filtered through 40 µm mesh. Filtered suspensions were pelleted by centrifugation at 300 x g for 5 minutes at 4^{o}C and resuspended in 1 mL red blood cell lysis solution for 2 minutes at room temperature before adding 9 mL PBS to neutralize osmolarity. Cells were pelleted and resuspended in PBS and enumerated.

Cell sorting and fluorescence microscopy

Cell sorting was performed using a MoFlo (Dako) cell sorter, and collected cells were dispensed onto poly-L-lysine coated glass coverslips and allowed to adhere for 1 hour at 4^{o}C. Cells were stained with DAPI mounting media and observed using a Zeiss Axioplan 2 epifluorescence microscope and analyzed using SlideBook digital deconvolution software (Intelligent Imaging Innovation).

Staining of lung cells for flow cytometry

Cells were kept on ice and all incubations were done at 4^{o}C. Lung cells were incubated in 24.G2 culture supernatant for 20 minutes to block Fc receptors. 10^6 cells were stained with the following fluorescently-labeled antibodies to cell surface components, F4/80 PE (clone BM8 - eBioscience), GR-1 PerCP (clone RB6-8C5 - BD Biosciences), CD11b PE-Cy7 (clone M1/70 - eBioscience), and CD11c Alexa 647 (clone N418 - eBioscience) in flow buffer (1% BSA and .09% sodium azide in PBS) for 30 minutes. Cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 30 minutes. Cells were washed and resuspended in PBS and stored at 4^{o}C until analysis by flow cytometry.

Intracellular staining for ATII cells

The 3C9 antibody (Abcam), which is specific for LBM180 expressed in ATII cells (32), was labeled using a Alexa 647 Zenon mouse IgG2a antibody labeling kit (Invitrogen) according to manufacturers instructions. Lung cells were incubated in clone 24.G2 culture supernatant for 20 minutes to block Fc receptors, fixed, and permeabilized with Cytofix/Cytoperm buffer (BD Biosciences) for 30 minutes and were washed with Cytoperm buffer (BD Biosciences). Permeabilized cells were incubated with labeled 3C9 antibody in

Cytoperm buffer for 30 minutes and washed with Cytoperm buffer before resuspending in PBS and stored at 4^{o}C until analysis by flow cytometry.

Flow cytometry of lung cells and data analysis

Cells were analyzed using a CyAn™ ADP LX 9 Color flow cytometer (Dako). Data was analyzed using Summit v4.3 (Dako). Compensation was performed using lung cells stained with each labeled antibody individually, and gates were drawn based on n-1 controls. Data bars represents the mean of 3-6 mice with error bars representing standard deviation of the mean. Significance was determined using an unpaired two-tailed T-test assuming unequal variance. P-values of $<.05$ were characterized as significant.

Identifying Lung Cell Types

Lung homogenates were treated with cell-type-specific fluorescently-labeled antibodies for the identification of cell populations (Figure 2, Table 1). The cell type we have defined as alveolar macrophages have phenotypic characteristics of this cell-type (28). ATII cells were defined as highly expressing LBM180. No LBM180 expressing cells were observed in splenocytes (data not shown) and >96% of LBM180 staining cells were GFP+ in an EGFP/SP-C transgenic mouse (data not shown) where GFP expression is driven by the SP-C promoter which is only active in ATII cells (20).

ACKNOWLEDGEMENTS

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ATTRIBUTIONS

I performed all experiments and data analysis presented in this chapter with the following exceptions. Bronwyn Gunn processed and collected data from a subset of U112- and LVS-infected mice under my direction. Robin Craven assisted with lung cell isolation procedures. This chapter has been submitted for publication.

TABLES AND FIGURES

Table 3.1: Comparison of *Francisella tularensis* strains

	U112	LVS	Schu S4
Subspecies	*novicida*	*holarctica*	*tularensis*
Genome (19):			
Size	1,910,031 bp	1,895,998 bp	1,892,819 bp
G+C content	32.47%	32.15%	32.26%
Predicted ORFs	1731	1380	1445
Pseudogenes	14	303	254
Sequence similarity of common genes	97.8% to LVS 98.1% to Schu S4	97.8% to U112 99.2% to Schu S4	98.1% to U112 99.2% to LVS
Intracellular replication:			
Macrophages[a] (2, 27)	+	+	+
ATII cells[a] (10)	+	+	+
Virulence in mice (inhalation) (8, 17)	High	Moderate	High
Virulence in humans[b] (inhalation) (22, 26)	None reported	Low	High
Dissemination to distal organs post-inhalation[a] (5, 8, 18)	+	+	+
Lethal inhalation dose in mice[a] (7, 8)	10-100	500-5000	< 10

a - unpublished observations from our lab for all strains

b - for immuno-competent adults

Table 3.2: Identification of specific cell-types from lung homogenates.

Cell Type	Properties
Alveolar Macrophage	F4/80^{high}, CD11b^{low}, CD11c^{high}, GR-1^{low}, FS^{high},SS^{high}
Interstitial Macrophage	F4/80^{high}, CD11b^{high}, CD11c^{var}, GR-1^{low}, FS^{var},SS^{var}
CD11b$^{low/mid}$ DC	F4/80^{low}, CD11b$^{low/mid}$, CD11c^{high}, GR-1^{low}, FS^{mid},SS^{mid}
CD11b^{high} DC	F4/80^{low}, CD11b^{high}, CD11c^{high}, GR-1^{low}, FS$^{low/mid}$,SS$^{low/mid}$
Monocyte	F4/80^{low}, CD11b^{mid}, CD11c$^{low/mid}$, GR-1$^{low/mid}$, FS^{low},SS^{low}
Neutrophil	F4/80^{low}, CD11b^{high}, CD11c^{low}, GR-1^{high}, FS^{var},SS^{var}
ATII Epithelial	LBP18^{high}, FS^{mid}, SS^{high} , *(low for other markers)*

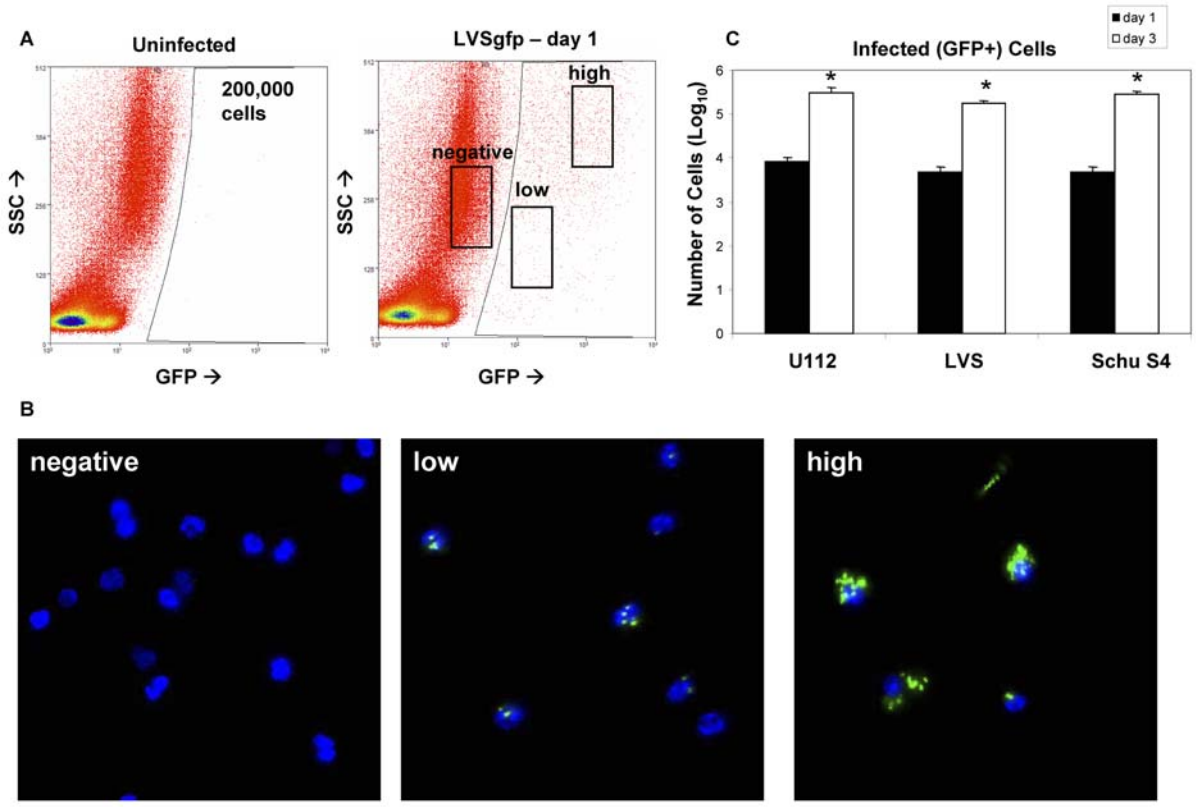

Figure 3.1. Identifying *Francisella*-infected lung cells.
Lung cells infected with GFP+ *Francisella* are readily detectable by flow cytometry. Mice were intranasally inoculated with GFP+ *Francisella* strains and on days 1 and 3 post-inoculation, lungs were harvested and digested to produce a single cell suspension which could be further analyzed. (A) GFP+ lung cells were detected on day 1 following intranasal inoculation with LVS*gfp*. (B) GFP^{high}, GFP^{low}, and $GFP^{negative}$ populations were collected and plated on coverslips for analysis by fluorescence microscopy. (C) The absolute number of GFP+ lung cells from mice infected with U112, LVS, or Schu S4 were determined on day 1 and day 3 post-inoculation. Error bars represent standard deviation of the mean (*n=3-6 mice*). Statistical significance of differences between day 1 and day 3 was determined by unpaired two-tailed T-test assuming unequal variance (* $p<.05$).

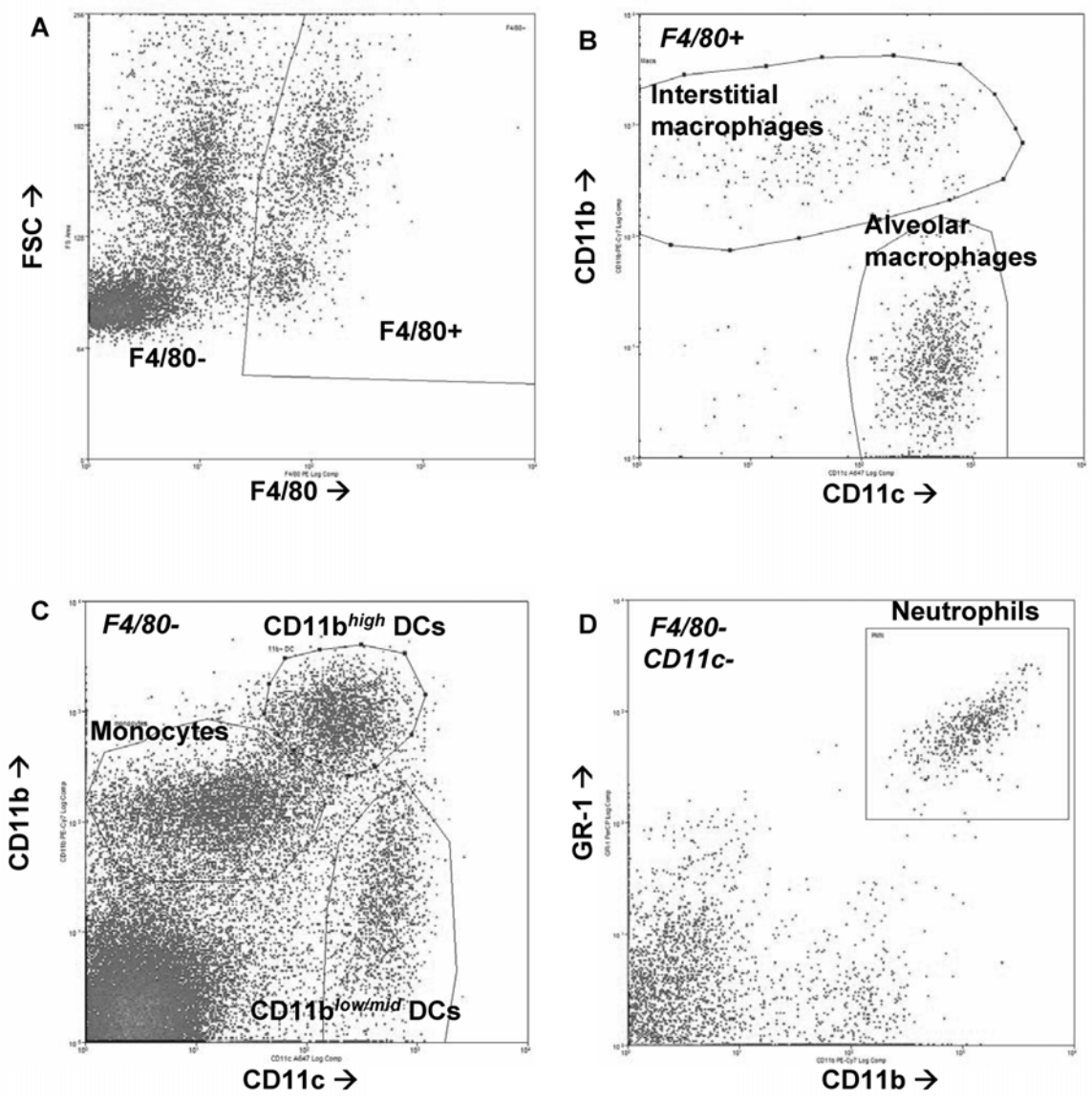

Figure 3.2. Identifying cell types in the lung by flow cytometry.
Cell types were identified based on differential expression of F4/80, CD11b, CD11c, and GR-1. Mouse lungs were digested with dispase and cells were stained with fluorescently-labeled cell-type-specific antibodies. (A) F4/80+ cells (B) that were $CD11b^{low}$ were classified as alveolar macrophages while $F4/80^{high}CD11b^{high}$ cells were classified as interstitial macrophages. (C) $F4/80^{low}CD11c^{high}$ cells were classified as DCs and subdivided into $CD11b^{low/mid}$ DCs and $CD11b^{high}$ DCs. $F4/80^{low}CD11c^{low}$ $CD11b^{mid}$ cells were classified as monocytes. (D) $F4/80^{low}CD11c^{low}$ $CD11b^{high}GR\text{-}1^{high}$ cells were classified as neutrophils.

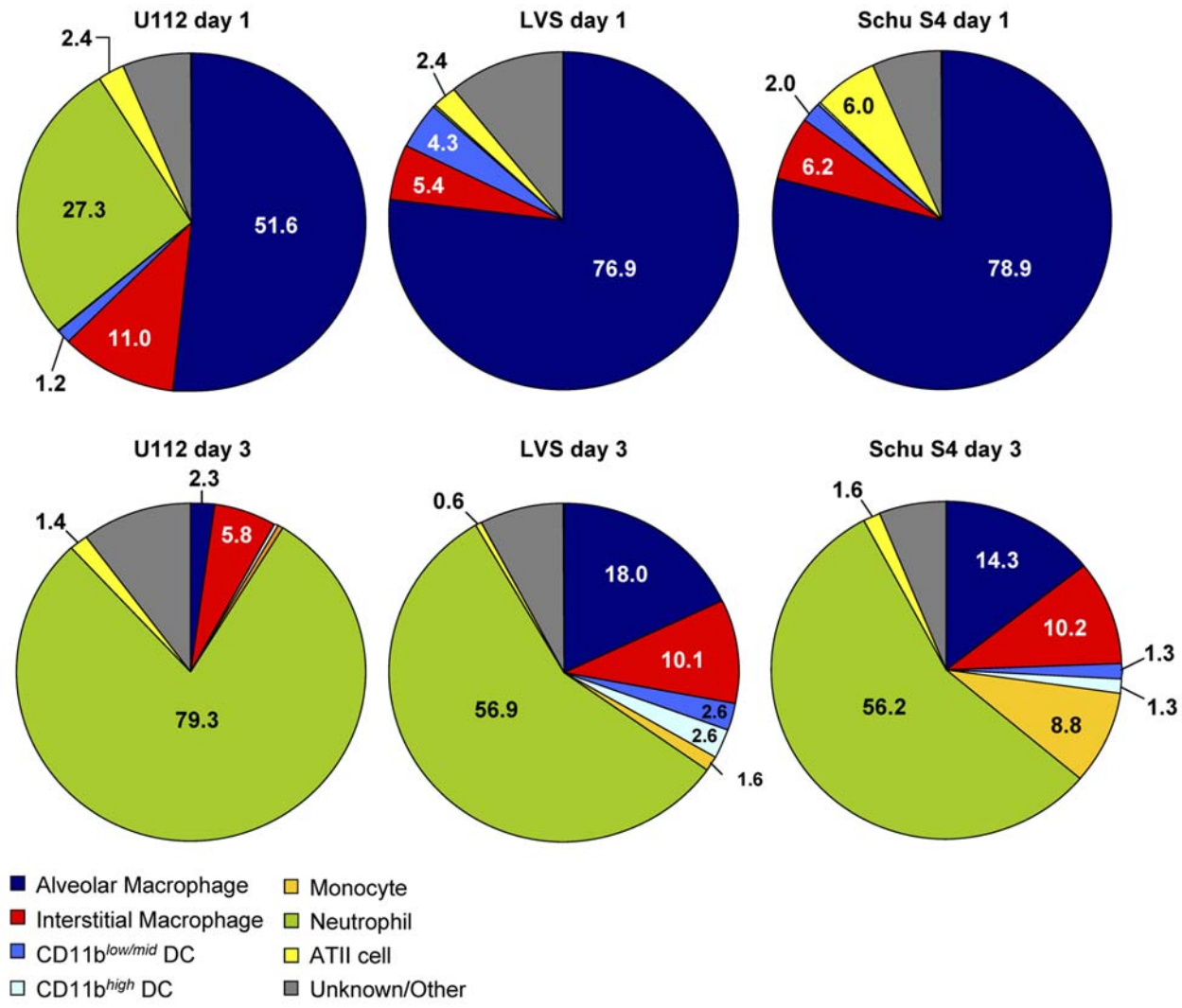

Figure 3.3. Lung cell types infected by *Francisella* following inhalation.
Proportion of lung cell-types infected with *Francisella* strains U112, LVS, or Schu S4 on day 1 and day 3 post-inoculation.

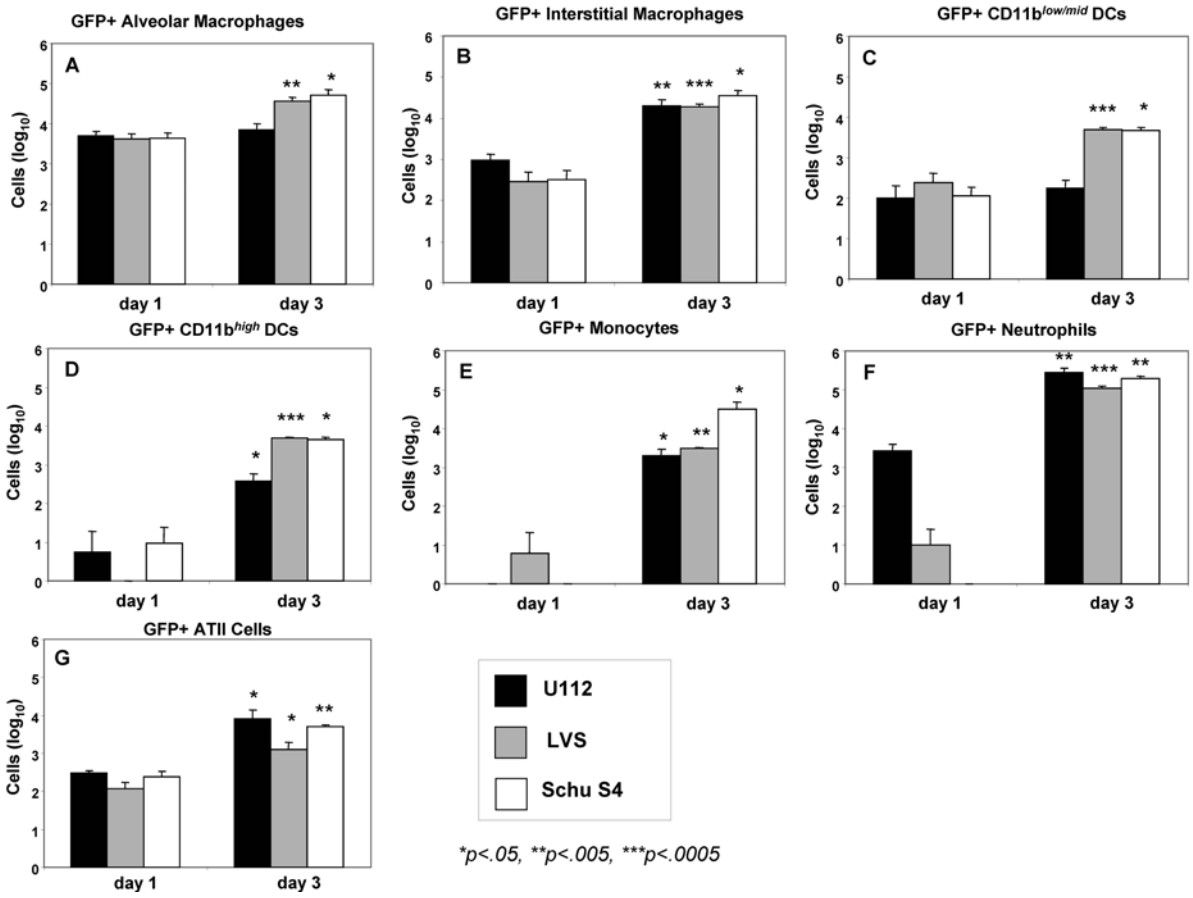

Figure 3.4. Number of *Francisella*-infected cells following inhalation.
Absolute number of infected (A) alveolar macrophages, (B) interstitial macrophages, (C) CD11b$^{low/mid}$ DCs, (D) CD11b^{high} DCs, (E) monocytes, (F) neutrophils, and (G) ATII cells from mouse lungs on day1 and day 3 following intranasal inoculation with U112, LVS, or Schu S4. Error bars represent standard deviation of the mean *(n=3-6 mice)*. Statistical significance of differences between day 1 and day 3 was determined by unpaired two-tailed T-test assuming unequal variance (* $p<.05$, ** $p<.005$, *** $p<.0005$).

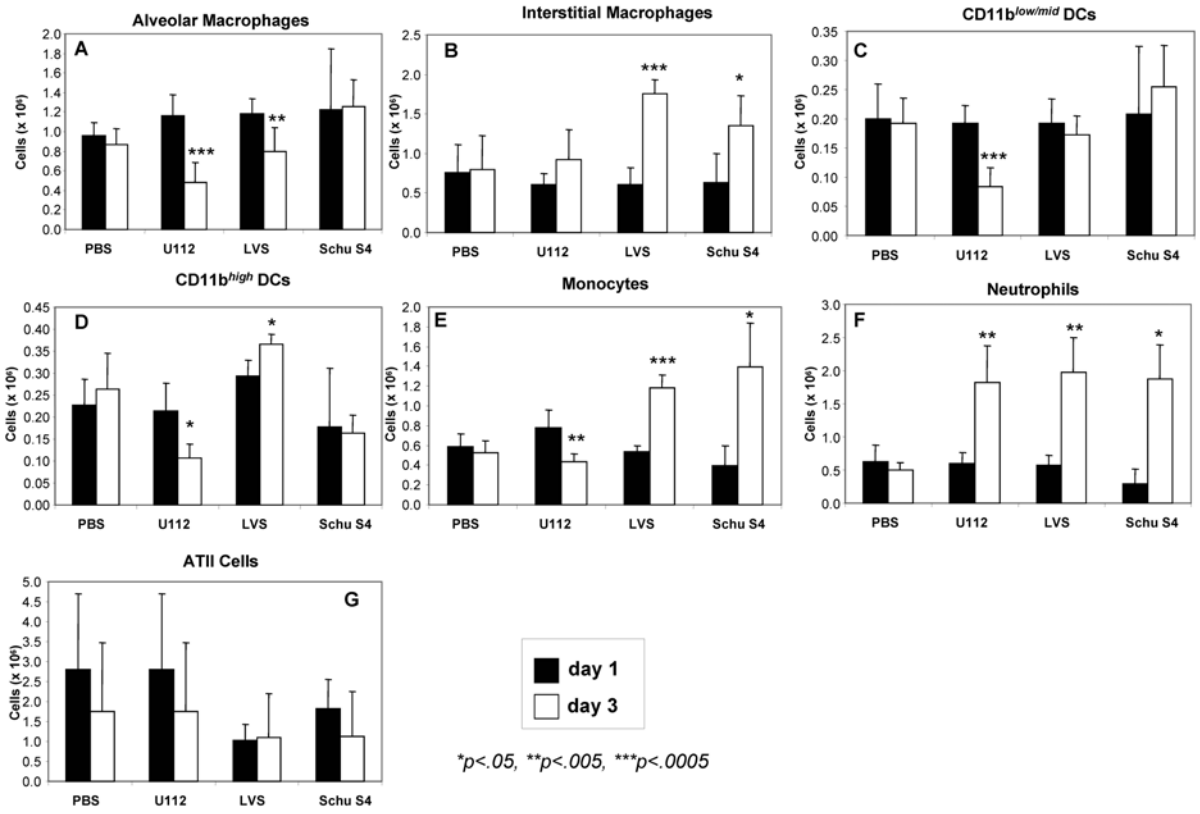

Figure 3.5. Effects of *Francisella* infection on cell number within lung populations. Absolute number of (A) alveolar macrophages, (B) interstitial macrophages, (C) CD11b$^{low/mid}$ DCs, (D) CD11b^{high} DCs, (E) monocytes, (F) neutrophils, and (G) ATII cells from mouse lungs on day1 and day 3 following intranasal inoculation with U112, LVS, or Schu S4. Error bars represent standard deviation of the mean *(n=3-6 mice)*. Statistical significance of differences between day 1 and day 3 was determined by unpaired two-tailed T-test assuming unequal variance (* *p<.05*, ** *p<.005*, *** *p<.0005*).

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CHAPTER 4

Chemokine receptor CX3CR1 deficiency leads to increased cell recruitment in the lung following inhalation of *Francisella tularensis* but does not impact disease outcome

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ABSTRACT

When inhaled, the gram negative bacterium, *Francisella tularensis*, causes a rapidly progressing infection in humans, mice, and other mammals that is often lethal. During the middle to late stages of pulmonary disease, monocytes and neutrophils are recruited to the lung and become infected with *Francisella*. The fractalkine receptor, CX3CR1, is highly expressed on the surface of monocytes and contributes to the docking and extravasation of these cells through the endothelium and into infected tissues. In CX3CR1-deficient mice, pulmonary *F. tularensis* infection progressed and was controlled similarly to wildtype mice. Surprisingly, the absence of CX3CR1 led to increased monocyte and neutrophil influx into the lung. Despite this, there was no difference in the abundance of these cells that were infected. Therefore, we conclude that CX3CR1 plays a role in modulating inflammatory cell entry into the lung following infection with *Francisella*, however it does not impact disease outcome.

INTRODUCTION

Respiration moves 15,000 liters of air over the extensive epithelial surface of the human airway each day (8). As a result, the lung environment is prone to constant exposure to foreign particles and potentially harmful pathogens. Proper respiratory function is essential for life of the organism and maintaining sterility within this system is critical. The host accomplishes this through a combination of physical barriers, pathogen-ingesting cells, microbicidal molecules, and molecular signals that facilitate communication among the various components. Often the initial response to an invading pathogen within the lung is recognition and phagocytosis by macrophages and dendritic cells (DCs). If these cells are unable to efficiently dispose of the pathogenic microbes, cytokine signals lead to the recruitment of monocytes and neutrophils from the bloodstream to infected tissues. Monocytes are the precursors to mature macrophages (2, 10, 11) and DCs (9), and during an immune response they replenish apoptotic or necrotic cells as well as provide increased numbers of phagocytic cells to aid in controlling infection.

The chemokine receptor CX3CR1 and it's ligand, fractalkine (CX3CL1) are important components that facilitate cell migration from the bloodstream into tissues. During infection, endothelial cells respond to inflammatory signals such as TNF-α, IL-1,IFN-γ, and LPS by activating expression and secretion of fractalkine, a transmembrane protein (1). Through the interaction of fractalkine with CX3CR1 on the surface of certain circulating cell populations, particularly monocytes and T-cells, capture and migration into tissues is facilitated (1, 7).

An influx of monocytes and neutrophils is often a critical component of the innate immune response to pulmonary bacterial pathogens. Following inhalation of *Francisella*

tularensis, there is an initial delay in recruitment of these cells followed by infiltration later during disease progression (6). The number of infected monocytes and neutrophils increases logarithmically during disease, indicating that these cell types provide a host cell niche for replicating *Francisella.* Clearly, cell recruitment to the lung is an important component of both the host response and *Francisella*'s patho-biology within the lung.

In this study, we examine the role of chemokine receptor CX3CR1 during pulmonary bacterial infections. Specifically, we will examine disease outcomes following inhalation of *F. tularensis* in mice deficient for CX3CR1. Also, since cell recruitment is an important component of pulmonary tularemia, we will assess the contribution of CX3CR1 to monocyte and neutrophil migration to the lung during the disease course.

RESULTS

Inhalation tularemia in CX3CR1-deficient mice

To assess the importance of CX3CR1 during pneumonic tularemia, CX3CR1$^{-/-}$ mice were inoculated intranasally with 1000 CFU of *F. tularensis* Live Vaccine Strain (LVS). At this dose, C57BL/6 mice develop disease, but recover and clear the infection (data not shown) (12). Three and seven days post-inoculation, lungs, liver, and spleen were harvested from infected mice and processed to determine bacterial organ burdens in wildtype C57BL/6 versus CX3CR1$^{-/-}$ mice. On day 3, logarithmic bacterial growth was evident in the lungs of both wildtype and CX3CR1$^{-/-}$ mice (Figure 1) and similar numbers of bacteria were recovered from each strain. Dissemination from the lung to liver and spleen, a hallmark of tularemia, also had occurred by day 3 to a similar extent for wildtype and CX3CR1$^{-/-}$ mice.

For both mouse strains, by day 7, bacterial burdens in the lung were less than day 3, indicating that the immune system was controlling the infection (Figure 1). During a lethal infection, logarithmic bacterial growth continues in the lungs between days 3 and 7 (5). Similarly, bacterial burdens in the liver and spleen did not increase significantly from day 3 to day 7 in wildtype and CX3CR1$^{-/-}$ mice. Bacterial burdens in all organs were similar between mouse strains at day 7. These data indicate that the absence of CX3CR1 did not alter disease progression of pulmonary tularemia, and mice deficient for CX3CR1 controlled *Francisella* infection similarly to wildtype mice.

Cellular response to *Francisella* in the lungs of CX3CR1-deficient mice

Given that CX3CR1-fractalkine interactions play a role in monocyte recruitment to the lungs, we sought to determine whether cell recruitment to the lung in response to *Francisella* infection was altered in CX3CR1$^{-/-}$ mice. Specific cell types were identified as described previously in Chapter 3 (6). *Francisella* infection instigates an increase in the number of monocytes and neutrophils by three days post-inhalation and neutrophils become the predominant infected cell type (6). No increase in monocyte number was observed on day 1 following inhalation for wildtype or CX3CR1$^{-/-}$ mice (Figure 2A). On day 3, monocyte recruitment to the lung was evident for both wildtype and CX3CR1$^{-/-}$ mice, however the magnitude of recruitment differed between mouse strains. There was a 2.2-fold increase in monocyte number between days 1 and 3 post-inhalation in wildtype mice and a 4.9-fold increase in CX3CR1$^{-/-}$ mice. At both time points there were more monocytes in mock inoculated CX3CR1$^{-/-}$ mice than in wildtype mice *($p<.05$)* suggesting that CX3CR1$^{-/-}$ mice had more resident monocytes within the lung than wildtype mice.

There was no increase in neutrophil number in the lung at day 1 for infected wildtype mice compared to PBS-inoculated mice (Figure 2B). However, infected CX3CR1$^{-/-}$ mice displayed significantly more neutrophils at day 1 than PBS-inoculated mice or than LVS-infected wildtype mice *($p<.05$)*. From day 1 to 3, the number of neutrophils increased by 3.5-fold in wildtype mice and 2.2-fold for CX3CR1$^{-/-}$ mice. There were more neutrophils in the lungs of CX3CR1-deficient mice on day 3 than in wildtype mice *($p<.05$)*. These data demonstrate that in CX3CR1-deficient mice, there was earlier neutrophil influx in response to inhaled *Francisella*, and there was increased neutrophil influx in CX3CR1$^{-/-}$ mice compared to wildtype mice.

Once monocytes enter into tissue, they have the ability to differentiate into macrophages (2) or DCs (9). Therefore, we examined the number of these cell types within infected wildtype or CX3CR1$^{-/-}$ mice. In response to *Francisella* infection, CX3CR1$^{-/-}$ mice did not have increased numbers of interstitial macrophage from day 1 to 3 whereas wildtype mice did (Figure 2C). Alveolar macrophage numbers also stayed constant within CX3CR1-/- mice compared to wildtype mice that exhibited a decrease in this population during disease (Figure 2D). With regard to DCs, CX3CR1-/- mice had increased numbers of these cells in the lung compared to wildtype mice, however there was no change in DC number in response to infection (Figure 2E).

Francisella-infected cells following inhalation by CX3CR1$^{-/-}$ mice

Since CX3CR1$^{-/-}$ mice had higher numbers of monocytes and neutrophils recruited to the lung in response to *Francisella* infection, we assessed whether this led to greater numbers of these cells becoming infected during the disease course. To do this, we infected C57BL/6 and CX3CR1$^{-/-}$ mice with LVS expressing GFP which enabled the identification of infected

lung cells. On day 1 following inhalation, there were <10 detected GFP+ neutrophils detected in the lungs of either mouse strain (Figure 3B). By day 3, there were approximately 100,000 infected neutrophils in the lungs of each strain indicating that neutrophils became infected to the same extent in CX3CR1-deficient mice as in wildtype mice. Also, despite there being increased neutrophil numbers in the lungs of $CX3CR1^{-/-}$ mice early during infection, this did not lead to increased numbers of infected neutrophils at this time. Similarly, there was no significant difference in the number of infected monocytes in the lungs of $CX3CR1^{-/-}$ mice when compared to wildtype mice despite there being increased monocyte recruitment during infection in a CX3CR1-deficient background (Figure 3A).

Absence of CX3CR1 had no impact on the number of *Francisella*-infected alveolar macrophages (Figure 3D). However, CX3CR1-/- mice had significantly fewer infected interstitial macrophages on day 3 (Figure 3C). This may be related to the observation that there was no increase in this population in the lungs of infected CX3CR1-/- mice during disease. There were initially fewer infected DCs in CX3CR1-deficient mice than wildtype mice, however by day 3 similar numbers of infected DCs were found in the lungs of each mouse strain (Figure 3E).

These data, taken together, indicate that the absence of CX3CR1 impacted the recruitment of monocytes and neutrophils into the lung, and this alteration affected the population dynamics of macrophages and DCs during infection of bacterial pathogens. However, these differences only subtly, if at all, affected disease outcomes following inhalation of *F. tularensis*.

DISCUSSION

An important component of the innate immune response is the recruitment and extravasation of circulating inflammatory cells into infected tissues. Here we demonstrated that the absence of the fractalkine receptor, CX3CR1, did not have an appreciable impact on disease outcome during pulmonary infections with *F. tularensis*. There were some notable differences in the innate immune response to *Francisella* in CX3CR1-deficient mice that were observed in this study. The absence of CX3CR1 resulted in elevated numbers of monocytes and neutrophils present in the lungs of infected animals during the later stages of pulmonary tularemia. This was somewhat surprising since the known function of CX3CR1 is to facilitate binding of monocytes, and other CX3CL1-expressing cells, to endothelial cells which proceeds migration into infected tissues. Therefore we might have expected to observe fewer monocytes recruited to the lung during pulmonary tularemia in CX3CR1-deficient mice. Also, we observed earlier influx of neutrophils into the lungs of CX3CR1-deficient mice than infected wildtype mice and subsequently more neutrophils in the later stages of disease progression in $CX3CR1^{-/-}$ mice than wildtype. CX3CR1 is not highly expressed on neutrophils, nor does fractalkine stimulate migration of these cells (1, 7), therefore it is unclear why the absence of CX3CR1 would impact the kinetics or magnitude of the neutrophil response.

One explanation for increased inflammatory-cell recruitment within CX3CR1-deficient mice is that mice lacking chemokine receptors might have much higher serum or tissue levels of corresponding chemokines that may act similarly on analogous receptors. $CX3CR1^{-/-}$ mice have 300-fold higher levels of fractalkine circulating in the blood than wildtype mice (3), which may provide some evidence for this hypothesis. Elevated

fractalkine levels could lead to increased interactions with other chemokine receptors important for cell recruitment and extravasation such as CCR2 and CCR5.

After receiving an intranasal dose of 1000 CFUs of *Francisella* LVS, C57BL/6 mice will recover and eventually clear the bacterial infection. During a fatal infection, the bacterial burden in lungs, liver, and spleen continues to increase exponentially from day 3 to day 7, whereas in recovering animals, the number of *Francisella* decreases during this time (4, 12). In mice lacking CX3CR1, the *Francisella* organ burden declined from day 3 to day 7 indicating that these mice could recover from tularemia similarly wildtype. Despite differences in the abundance of various cell populations between CX3CR1 expressing and deficient strains, the makeup of *Francisella*-infected cells was not affected. These data indicate that CX3CR1 plays a role in regulating inflammatory cell recruitment to the lung, however it is not critical in the disease outcome of *F. tularensis.* These results also indicate that CX3CR1-deficiency leads to a modest, yet significant, increase in the number of monocytes and DCs in the lung in the absence of infection. Given that these mice were not more susceptible to respiratory bacterial pathogens, it seems likely that these subtle differences do not impact immunity to respiratory infections.

EXPERIMENTAL PROCEDURES

Bacterial Culture

F. tularensis Live Vaccine Strain was obtained from the Centers for Disease Control and Prevention in Atlanta GA. LVS was maintained on chocolate agar supplemented with Isovitalex (BD Biosciences). GFP strains contained a modified pKK214gfp plasmid (a kind gift from Mats Forsman).

Mouse infections

C57BL/6 or CX3CR1$^{-/-}$ mice were inoculated with *Francisella* diluted in sterile PBS and enumerated by Klett reading or OD600. Inoculation dose was verified by plating and counting inoculum on chocolate agar. Mice were anesthetized with avertin until unresponsive to a toe pinch, and 50 μL of bacterial suspension was dispensed onto anterior nares of the mouse. Previous results suggest this is an effective procedure for establishing a pulmonary *Francisella* infection (5). All animal experiments were conducted in accordance with animal care and use guidelines, and animal protocols were approved by the IACUC at UNC Chapel Hill.

Lung cell isolation

Mice were anesthetized with avertin + heparin (1000 U/mL) and profused with 4-7 mL of PBS + heparin (200 U/mL) until lungs were fully blanched. The trachea was cannulated using a 16 gauge blunt-tipped needle, and lungs were inflated with approximately 1 mL of the neutral protease, dispase (BD Biosciences). The trachea was tied off with surgical sutures and lungs were removed and incubated in 3.0 mL dispase at room temperature for 45 minutes. Tracheas were removed, and lungs were transferred to a Petri plate along with 7 mL of PBS + DNaseI (250 μg/mL) and tissue was teased apart using forceps. Cells were gently swirled in the Petri plate for 1-2 minutes, and the suspension was filtered through 40 μm mesh. Filtered suspensions were pelleted by centrifugation at 300 x g for 5 minutes at 4^{o}C and resuspended in 1 mL red blood cell lysis solution for 2 minutes at room temperature before adding 9 mL PBS to neutralize osmolarity. Cells were pelleted and resuspended in PBS and enumerated.

Staining of lung cells for flow cytometry

Cells were kept on ice and all incubations were done at 4^{o}C. Lung cells were incubated in 24.G2 culture supernatant for 20 minutes to block Fc receptors. 10^6 cells were stained with the following fluorescently-labeled antibodies to cell surface components, F4/80 PE (clone BM8 - eBioscience), GR-1 PerCP (clone RB6-8C5 - BD Biosciences), CD11b PE-Cy7 (clone M1/70 - eBioscience), and CD11c Alexa 647 (clone N418 - eBioscience) in flow buffer (1% BSA and .09% sodium azide in PBS) for 30 minutes. Cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 30 minutes. Cells were washed and resuspended in PBS and stored at 4^{o}C until analysis by flow cytometry.

Flow cytometry of lung cells and data analysis

Cells were analyzed using a CyAn™ ADP LX 9 Color flow cytometer (Dako). Data was analyzed using Summit v4.3 (Dako). Compensation was performed using lung cells stained with each labeled antibody individually, and gates were drawn based on n-1 controls. Each data bar represents the mean of 6 mice for inoculations of wildtype C57BL/6 mice, 4 mice for LVS-infected CX3CR1$^{-/-}$ mice, and 2 mice for PBS-inoculated CX3CR1$^{-/-}$ mice with error bars representing the standard deviation of the mean. Significance was determined using an unpaired two-tailed T-test. P-values of $<.05$ were characterized as significant.

Identifying Lung Cell Types

Cell types were identified based on differential staining with fluorescently-labeled antibodies to F4/80, CD11b, CD11c, and GR-1 as described (6) (Chapter 3). Monocytes were defined as F4/80^{low}, CD11b^{mid}, CD11c^{low}, GR-1$^{low/mid}$ with low forward and side scatter. Neutrophils were defined as F4/80^{low}, CD11b^{high}, CD11c^{low}, GR-1^{high} with low forward scatter and heterogeneous side scatter properties. F4/80+ cells that were CD11b^{low} were

classified as alveolar macrophages while F4/80^{high}CD11b^{high} cells were classified as interstitial macrophages. F4/80^{low}CD11c^{high} cells were classified as DCs and subdivided into CD11b$^{low/mid}$ DCs and CD11b^{high} DCs. F4/80^{low}CD11c^{low} CD11b^{mid} cells were classified as monocytes. F4/80^{low}CD11c^{low} CD11b^{high}GR-1^{high} cells were classified as neutrophils. F4/80^{high}CD11b^{high} cells were classified as interstitial macrophages.

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ATTRIBUTIONS

I performed all of the experiments and data analysis described in this chapter with the following exceptions. Bronwyn Gunn processed and collected data for a subset of the CX3CR1$^{-/-}$ mice. Robin Craven assisted with lung cell isolation and organ burden experiments.

FIGURES

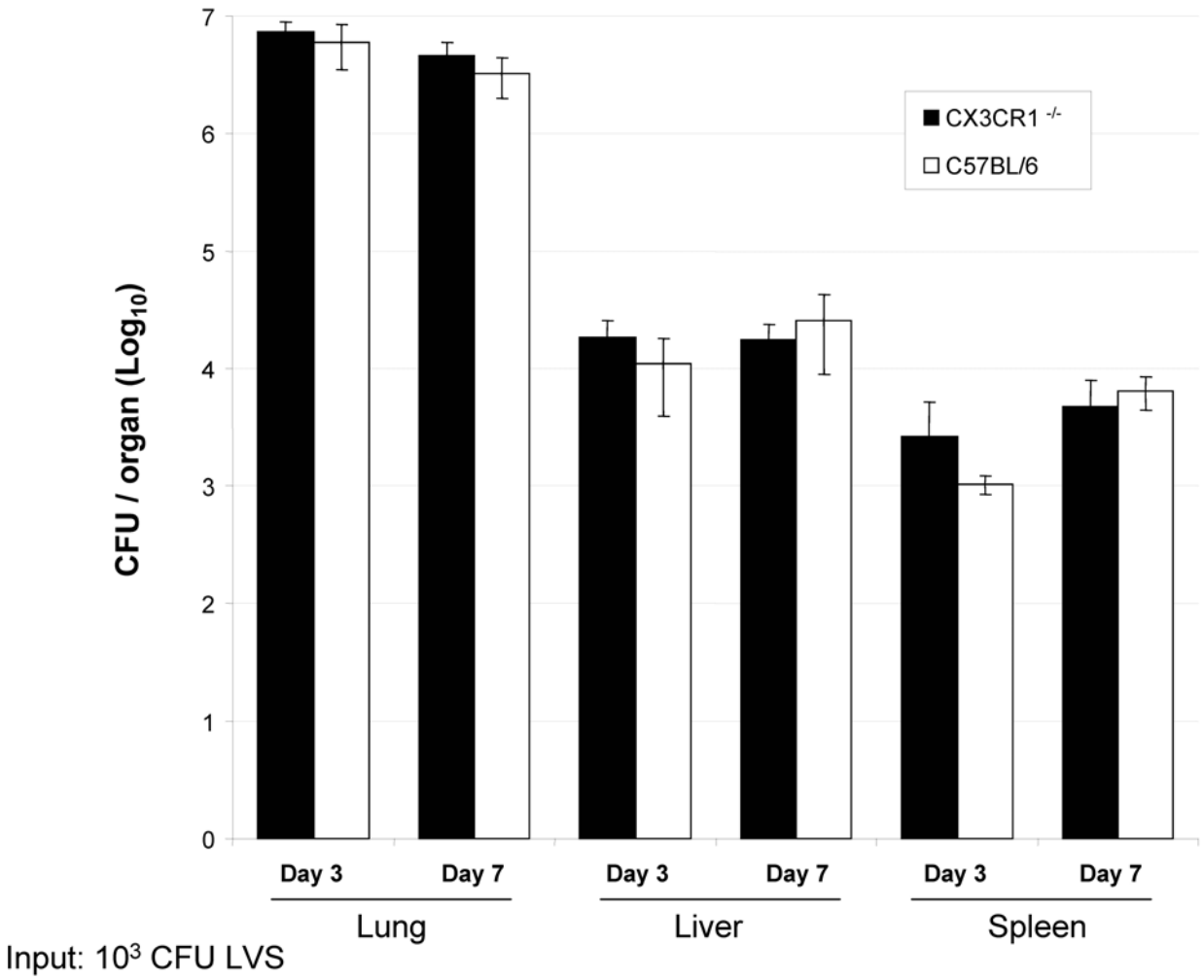

Figure 4.1. *Francisella* recovery from CX3CR1-deficient mice following intranasal inoculation. Mice were intranasally inoculated with 10^3 CFU of LVS. Lungs, liver, and spleen were collected and processed to determine bacterial burden at days 3 and 7. Error bars represent standard deviation of the mean (n=4 mice).

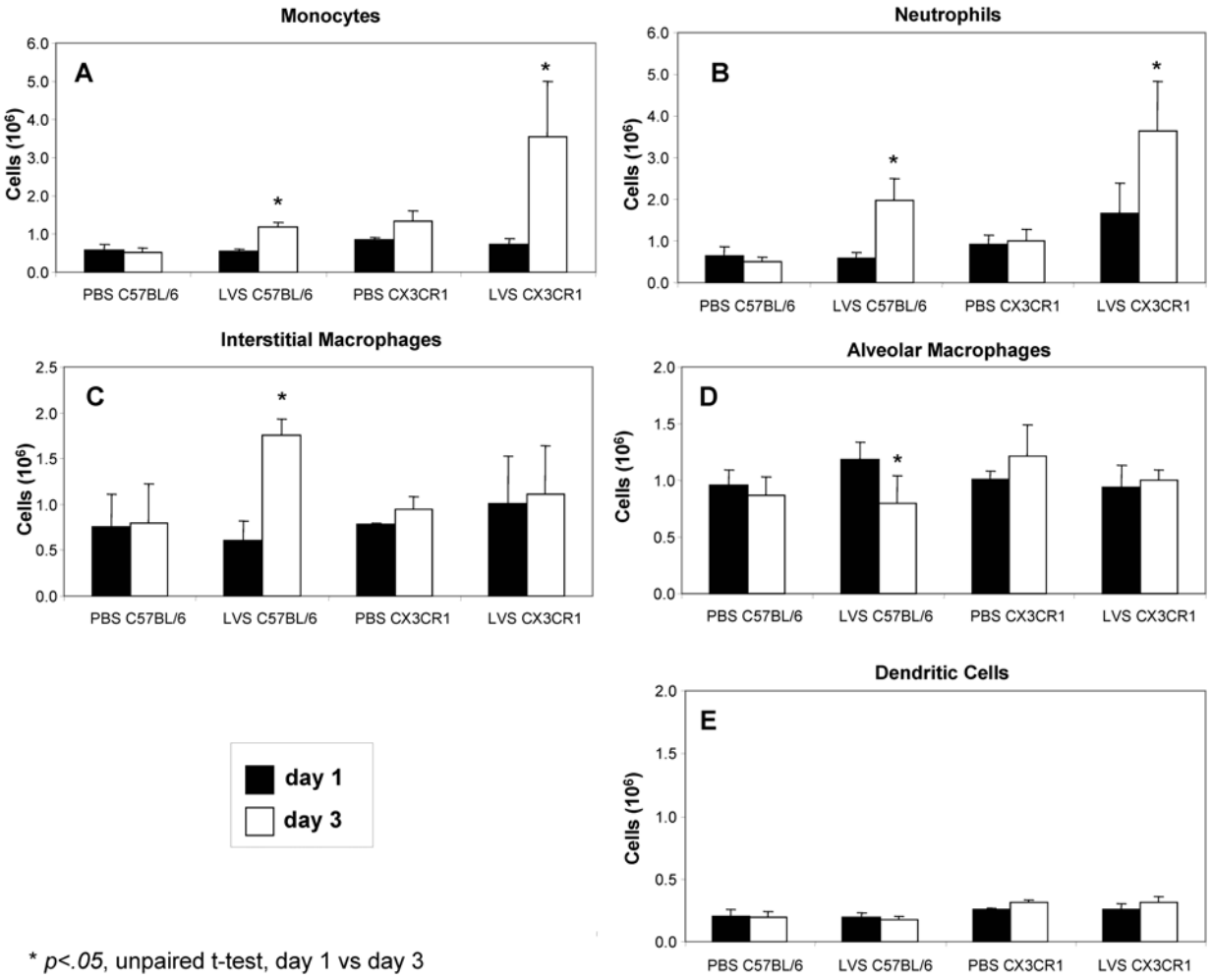

Figure 4.2. Impact of *Francisella* infection on pulmonary cell populations from CX3CR1-deficient mice. Absolute number of (A) monocytes, (B) neutrophils, (C) interstitial macrophages, (D) alveolar macrophages, and (E) DCs from mouse lungs on day1 and day 3 following intranasal inoculation with LVS. Error bars represent standard deviation of the mean *(n=3-6 mice)*. Statistical significance of differences between day 1 and day 3 was determined by unpaired two-tailed T-test (* $p<.05$).

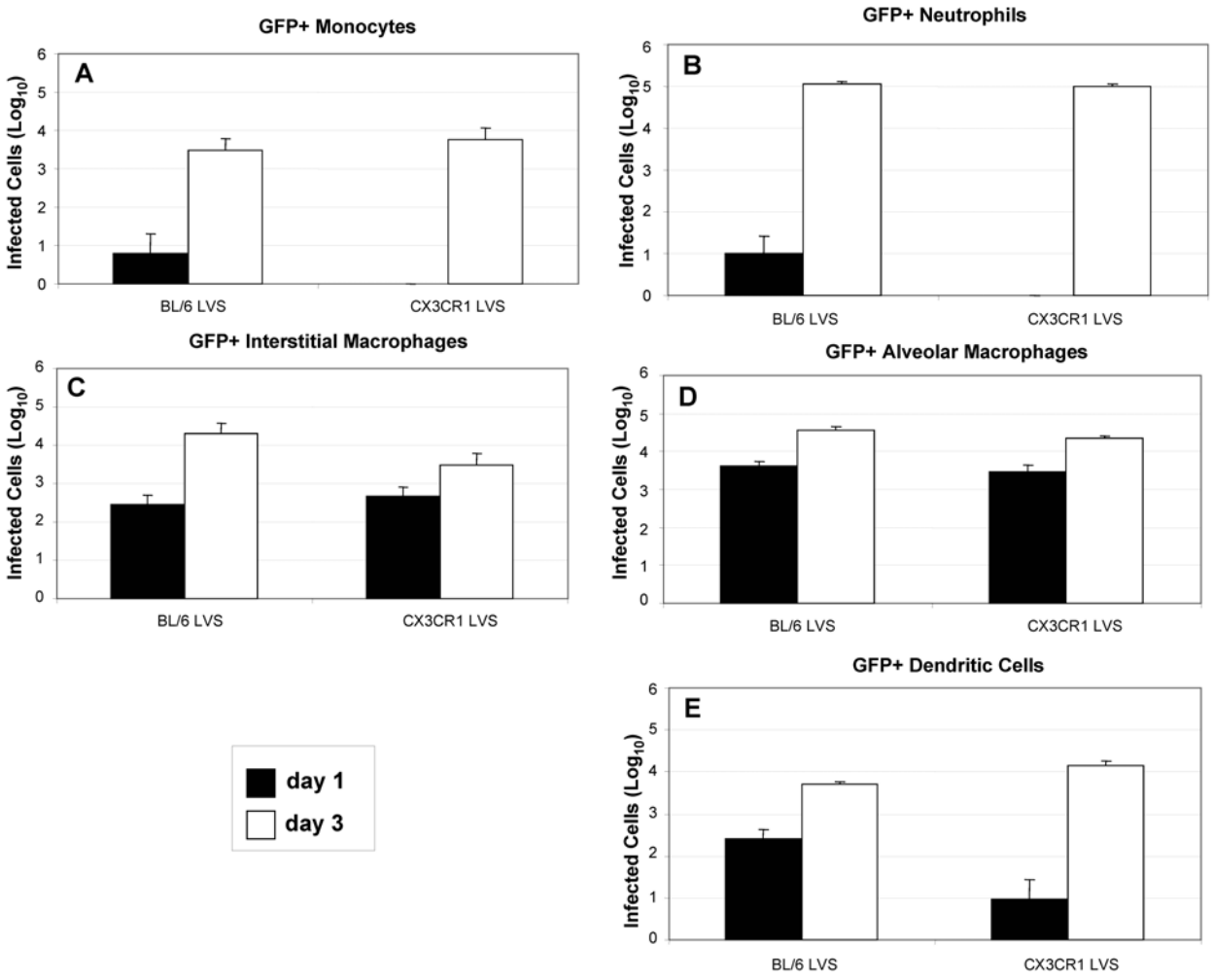

Figure 4.3. Abundance of *Francisella*-infected cells in CX3CR1-deficient mice during pulmonary infection. Absolute number of infected (GFP+) (A) monocytes, (B) neutrophils, (C) interstitial macrophages, (D) alveolar macrophages, and (E) DCs from mouse lungs on day1 and day 3 following intranasal inoculation with LVSgfp. Error bars represent standard deviation of the mean *(n=3-6 mice)*.

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CHAPTER 5

Francisella tularensis invasion of lung epithelial cells

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ABSTRACT

Francisella tularensis, a gram-negative facultative intracellular bacterial pathogen, causes disseminating infections in humans and other mammalian hosts. Macrophages and other monocytes have long been considered the primary site of *F. tularensis* replication in infected animals. However, recently it was reported that *F. tularensis* also invades and replicates within alveolar epithelial cells following inhalation in a mouse model of tularemia. TC-1 cells, a mouse lung epithelial cell line, were used to study the process of *F. tularensis* invasion and intracellular trafficking within nonphagocytic cells. Live and paraformaldehyde fixed *F. tularensis* live vaccine strain (LVS) associated with, and were internalized by, TC-1 cells at a similar frequency and with indistinguishable differences in kinetics. Inhibitors of microfilament and microtubule activity resulted in significantly decreased *F. tularensis* invasion, as did inhibitors of PI3 kinase and tyrosine kinase activity. Collectively these results suggest that *F. tularensis* epithelial cell invasion is mediated by a preformed ligand on the bacterial surface and driven entirely by host cell processes. Once internalized, *F.*

tularensis containing endosomes associated with EEA1 followed by LAMP-1 with peak co-association frequencies occurring at 30 and 120 minutes post-inoculation, respectively. By 2 hours post-inoculation 70.0% (±5.5%) of intracellular bacteria were accessible to antibody delivered to the cytoplasm indicating vacuolar breakdown and escape into the cytoplasm.

INTRODUCTION

Francisella tularensis, the causative agent of the disease tularemia, infects a wide range of animal hosts. Humans can be infected by a variety of routes, including physical contact with infected animals, insect bites, ingestion of contaminated food or water, and inhalation of organisms (13, 52). Disease severity is affected by both the route of inoculation and bacterial subtype (13, 48, 52). *F. tularensis* strains are subdivided into two groups, A and B; the more severe form of tularemia in humans is caused by type A strains (13, 48, 52). The type A strain *F. tularensis* subspecies *tularensis* is found almost exclusively in North America, whereas the type B strain *F. tularensis* subspecies *holarctica* is found throughout Europe, as well as in North America (35). There is a 5% to 15% mortality rate associated with untreated human tularemia caused by type A strains, however that rate reaches 30% to 60% for untreated pneumonic and typhoid forms of the disease (11). The live vaccine strain (LVS) is an attenuated type B strain that causes a tularemia-like disease in mice, and is used as a model organism to study *F. tularensis* pathogenesis. There has been heightened interest in the study of this organism in recent years due to its history of weaponization and potential for use as an agent of biological warfare. *F. tularensis* is a category A select agent on the CDC's bioterrorism agent list, which includes organisms with the potential to cause high numbers of casualties if disseminated in an aerosol form (26).

Much of the *Francisella* pathogenesis research has focused on survival and replication of *F. tularensis* in macrophages and dendritic cells, and many of the genes identified to date that are required for full virulence contribute to survival or replication in the macrophage (1, 6, 17, 20, 27, 30, 32, 37, 42). We recently demonstrated that *F. tularensis* also invades and replicates in alveolar type II (ATII) epithelial cells of infected mice (21). ATII cells have a number of biological functions, including the production, secretion and recycling of surfactant, proliferation to produce additional type II cells as well as transdifferentiation into type I cells, maintenance of alveolar fluid balance, and production of antimicrobial and anti-inflammatory substances(29). Due to the close proximity between the blood and alveolar epithelial cells required for gas exchange and fluid balance, these cells are ideally located to provide a portal through which bacteria could disseminate to distal organs.

F. tularensis attaches to and invades nonphagocytic cells, including the ATII cell lines A549 and MLE 12 and the lung epithelial cell line TC-1 (21, 28). Melillo *et al.* also reported that *E. coli* expressing the *F. tularensis* surface protein FsaP bind to A549 cells (31), identifying a surface protein that may play a role in ATII cell association.

A primary function of monocytes is to engulf bacterial cells and other foreign particles (24, 44). However, invasion of epithelial and other nonphagocytic host tissue cells requires bacterially mediated exploitation of host cell functions to gain entry (10, 15, 16). Alveolar epithelial cells provide a site where *F. tularensis* can replicate in the infected host (21), and as such it is important to understand how bacterial interaction with these cells may differ from interaction with macrophages. Herein we describe our efforts to understand how *F. tularensis* invades lung epithelial cells.

MATERIALS AND METHODS

Bacterial strains

Francisella tularensis LVS was obtained from the Centers for Disease Control and Prevention, Atlanta, Ga. LVSgfp was constructed using the pKK214GFP plasmid (a gift of Mats Forsman). *F. tularensis* LVS and LVSgfp were propagated on chocolate agar supplemented with 1% IsoVitaleX (Becton-Dickinson). *Listeria monocytogenes* EGD1/2a, *Salmonella enterica* serovar *Typhimurium*, *Yersinia pseudotuberculosis*, and *Campylobacter jejuni* strain 81-176 were gifts from Paul Orndorff, Craig Altier, Ralph Isberg, and Deborah Threadgill, respectively. All bacterial strains were grown on LB agar, with the exception of *C. jejuni*, which was grown on Mueller-Hinton agar with 5% CO_2, and *F. tularensis* LVS, which was grown on chocolate agar as described above. All bacterial strains were grown at 37°C. *Salmonella enterica* was grown overnight in LB broth under static conditions for invasion assays.

Cell culture

TC-1 (ATCC CRL-2785) is a tumor cell line derived from primary lung epithelial cells of C57BL/6 mice. The cells were immortalized with HPV-16 E6 and E7 and transformed with the c-H-*ras* oncogene. These cells were grown in RPMI 1640 supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.1 mM non-essential amino acids and 10% FBS. Cell cultures were maintained at 37°C and 5% CO_2.

Attachment, Invasion and Vacuolar Escape Assay

To evaluate the percentage of TC-1 cells with associated bacteria synchronized infections were carried out by adding *F. tularensis* LVS expressing GFP (LVSgfp) at a

multiplicity of infection (MOI) of 100 to TC-1 cells chilled to 4°C. Bacterial suspensions were made in tissue culture media from bacteria grown as described above. Bacterial concentration of suspensions was determined using a Klett meter. Dilutions of suspensions were plated to verify MOI. Plates were centrifuged at 300 x g for 5 minutes at 4°C, and then rapidly warmed by placing in a 37°C water bath for 2 minutes before transferring to a 37°C, 5% CO_2 incubator. For experiments using killed LVS, organisms were treated with 4% paraformaldehyde (PFA) for 10 minutes. There was no growth from a PFA treated bacterial aliquot that was plated on chocolate agar, demonstrating that PFA treatment killed all organisms. At 10 minutes post-inoculation samples were washed with PBS to remove unattached bacteria and pre-warmed media was added to monolayers. Cells were collected by trypsinization at 10, 20, 30 and 60 minutes post-inoculation and samples were processed at 4°C. Cells to be analyzed for bacteria association were fixed in 4% PFA for 10 minutes, and analyzed by flow cytometry.

Parallel wells were analyzed for intracellular bacteria by collecting samples as described above. Extracellular bacteria were labeled using anti-*F. tularensis* LPS antibody (USBiological) conjugated to Pacific Blue (Molecular Probes) 1:1000 for 30 minutes at 4°C. Cells were then lysed with water and centrifuged at 300 x g to removed eukaryotic cell debris. Supernatant containing bacteria was centrifuged at 16,000 x g, fixed with 4% PFA, and analyzed by flow cytometry to differentiate intracellular (GFP only) from extracellular (GFP and Pacific Blue) bacteria.

To evaluate bacterial escape into the cytoplasm, synchronized inoculations of TC-1 cells and staining of extracellular bacteria was done as described above. Cells were collected by trypsinization at 10, 20, 30, 60 and 120 minutes post-inoculation. TC-1 cells were treated

with 50 μg/ml digitonin in KHM buffer (110 mM potassium acetate/20 mM Hepes/2 mM $MgCl_2$, pH 7.3) for 1 minute, washed in KHM buffer, and incubated with anti-*F. tularensis* LPS antibody conjugated to Alexa Fluor 647 (Molecular Probes) 1:1000 for 30 minutes at 4°C. Cells were then washed in KHM buffer, lysed with water and centrifuged at 300 x g to removed cellular debris. Supernatants were centrifuged at 16,000 x g and bacterial pellets were fixed in 4% PFA and analyzed by flow cytometry to differentiate extracellular (GFP, Pacific Blue and Alexa Fluor 647), cytoplasmic (GFP and Alexa Fluor 647) and vacuolar (GFP only) bacteria.

Flow cytometry of whole cells and bacteria was performed using a CyAn ADP flow cytometer (Dako Cytomation). Data was analyzed using Summit Software (Dako), with gating for single events for whole cell samples and GFP positive single events for bacterial samples.

Inhibitor Assays

Inhibitor assays were carried out with cytochalasin D, colchicine, wortmannin and genistein. Cytochalasin D, an actin polymerization inhibitor, was used at 0.5 and 1.0 μM concentrations. Colchicine, a microtubule polymerization inhibitor, was used at 0.1 and 0.25 μM concentrations. Wortmannin, an inhibitor of PI3 kinase activity, was used at 100 and 200 nM concentrations. Genistein, an inhibitor of tyrosine kinase activity, was used at 50 and 100 μM concentrations. Monolayers were preincubated for one hour with escalating concentrations of inhibitor, and then bacteria suspended in media containing inhibitor were added at an MOI of 100 for LVS, or 25 to 50 for other bacteria. Samples without inhibitor included bacteria suspended in tissue culture media alone, or media containing the carrier in which the inhibitor was reconstituted where appropriate. After four hours, cells were washed

with PBS to remove inhibitor. Extracellular bacteria were killed by adding media containing 25 μg/ml of gentamicin and incubated 2 hours before monolayers were washed with PBS and scraped from the plate using sterile applicator sticks. Serial dilutions were plated on chocolate agar to quantify colony forming units (CFU) of intracellular bacteria. Inhibitors did not affect LVS viability as demonstrated by incubating bacteria in tissue culture media containing inhibitor for four hours, followed by plating onto chocolate agar to quantify CFU. Inhibitors did not affect eukaryotic cell viability at the concentrations used as determined by Trypan Blue exclusion. The effect of inhibitors on bacterial attachment to cells was determined as described above for invasion, except that gentamicin was not added, allowing bacteria attached to cells but not internalized to be included in the CFU recovered. Inhibitors had no effect on LVS attachment to TC-1 cells. Results are expressed as the percentage of LVS or control organism that survived gentamicin treatment, relative to the sample without inhibitor inoculated with the same organism. Invasion without inhibitor is defined as 100% invasion. Data presented are the results of assays done in triplicate, and are representative of multiple repetitions of each experiment. Data were analyzed for statistical significance by paired two-tailed *t* test, and considered significantly different from the untreated control when $P<0.01$.

Fluorescence microscopy

Cells were grown on poly-L-lysine coated coverslips in 24-well cell culture plates to ~90% confluency. LVSgfp was added at an MOI of 100 to pre-chilled TC-1 plates, and centrifuged at 300 x g for three minutes to synchronize infection as described above. At 10, 20, 30, 60, 120 and 180 minutes post-inoculation monolayers were washed with PBS to remove unattached bacteria and fixed using 4% PFA. Samples were blocked with PBS/Fc

block/5% donkey serum, incubated with anti-*F. tularensis* LPS diluted 1:1000 for 30 minutes at 4°C followed by donkey anti-mouse AMCA (7-amino-4-methylcoumarin-3-acetic acid, Jackson ImmuoResearch) diluted 1:100, 30 minutes, 4°C to label extracellular bacteria. For early endosome antigen 1 (EEA1) or lysosome associated membrane protein 1 (LAMP-1) staining samples were blocked using PBS/0.1% saponin/5% serum of host species of secondary antibody followed by goat polyclonal anti-EEA1 (N-19; Santa Cruz Biotechnologies) diluted 1:200 in blocking solution, or rat monoclonal anti-mouse LAMP-1 (1D4B**,** developed by J. T. August, obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242) diluted 1:200 in blocking solution. Secondary antibodies used were donkey anti-goat Cy5 (Jackson ImmunoResearch) or goat anti-rat Alexa Fluor 647 (Molecular Probes), diluted 1:500 in PBS/0.1% saponin. Samples were examined using a Zeiss Axioplan 2 epifluorescence microscope and SlideBook digital deconvolution software (Intelligent Imaging Innovations). 100 intracellular bacteria were counted for each time point and condition. Images of sequential vertical planes were acquired to determine the location of bacteria within EEA1 or LAMP-1 containing vacuoles. Data presented are the results of three independent experiments.

Electron microscopy

Cell monolayers grown on polystyrene plates were rinsed with PBS or serum-free medium and fixed in 3% glutaraldehyde/0.15M sodium phosphate, pH 7.4. Following three rinses with sodium phosphate buffer, the monolayers were post-fixed for 1 hour in 1% osmium tetroxide/1.25% potassium ferrocyanide/0.15M sodium phosphate buffer, rinsed in deionized water, dehydrated using increasing concentrations of ethanol (30%, 50%, 75%,

100%, 100%, 10 minutes each) and embedded in Polybed 812 epoxy resin (Polysciences, Inc.,Warrington, PA). The embedded samples were sectioned parallel and perpendicular to the substrate at 70nm using a diamond knife. Ultrathin sections were collected on 200 mesh copper grids and stained with 4% aqueous uranyl acetate for 15 minutes, followed by Reynolds' lead citrate for 7 minutes. Sections were observed using a LEO EM910 transmission electron microscope at 80kV (LEO Electron Microscopy, Thornwood, NY) and photographed using a Gatan Bioscan Digital Camera (Gatan, Inc., Pleasanton, CA).

RESULTS

Kinetics of F. tularensis epithelial cell attachment and invasion

Francisella tularensis LVS invades and replicates within TC-1, MLE 12 and A549 lung epithelial cell lines (21). While the intracellular replication rates within these lines are indistinguishable, TC-1 cells support the highest initial invasion frequency (21). In addition, TC-1 cells are a mouse cell line, and LVS infection causes a tularemia-like disease in mice similar to that seen with virulent strains in humans. We therefore used TC-1 cells to examine the initial stages of *F. tularensis* invasion.

To determine kinetics of lung cell attachment and invasion by *F. tularensis* we synchronized infection of TC-1 cells at an MOI of 100 with LVSgfp. At 10, 20, 30 and 60 minutes post-inoculation, cells were washed to remove nonadherent bacteria and collected to quantify TC-1 cells associated with GFP expressing bacteria by flow cytometry (Fig 1A). At these same time points, in parallel samples, extracellular bacteria were stained using a Pacific Blue labeled antibody to *F. tularensis* LPS and TC-1 cells were lysed to recover cell-associated bacteria. GFP positive bacteria recovered from cells were analyzed by flow

cytometry to determine intracellular (GFP alone) versus extracellular (GFP and Pacific Blue) localization (Fig 1B). At 10 minutes 1.7% (±0.4%) of TC-1 cells had associated bacteria, of which 1.4% (±0.1%) of TC-1 cell-associated bacteria were intracellular**.** At 20 minutes post-inoculation 1.1% (±0.2%) of TC-1 cells had cell-associated bacteria, of which 7.0% (±0.6%) were internal. At 30 minutes post-inoculation 1.0% (±0.2%) of TC-1 cells had cell-associated bacteria, of which 21.3% (±6.3%) were internal. At 60 minutes post-inoculation 1.4% (±0.1%) of TC-1 cells had cell-associated bacteria, of which 23.6% (±5.8%) were intracellular.

To determine if LVS entry into lung epithelial cells required viable bacteria, we repeated the above experiments using killed LVSgfp. At 10 minutes 0.9% (±0.2%) of TC-1 cells had dead bacteria associated with them, of which 1.2% (±0.4%) of TC-1 cell-associated bacteria were intracellular**.** At 20 minutes post-inoculation 1.1% (±0.7%) of TC-1cells had cell-associated dead bacteria, of which 5.7% (±0.6%) were internal. At 30 minutes post-inoculation 0.6% (±0.2%) of TC-1 cells had cell-associated dead bacteria, of which 17.3% (±3.5%) were internal. At 60 minutes post-inoculation 0.4% (±0.1%) of cells had dead bacteria associated with them, of which 25.7% (±1.6%) were intracellular. These results indicated that both live and dead LVS attached to and invaded lung epithelial cells. Further, invasion frequency and kinetics were not significantly different between live and dead bacteria ($P<0.01$), except cell association at 60 minutes (Figure 1). The ability of nonviable *F. tularensis* to invade lung epithelial cells suggested that a preformed ligand on the bacterial surface may interact with the host cell.

Effects of cytoskeleton and signaling pathway inhibitors on F. tularensis invasion of epithelial cells.

Inhibitors of eukaryotic cell function were used to determine the contribution of host cell signaling and cytoskeleton rearrangement to LVS invasion of lung epithelial cells. Bacterial entry into host cells generally requires rearrangement of cytoskeletal structures; either microfilaments alone, as is the case with *Salmonella, Shigella, Listeria* and *Yersinia* spp. (12), or both microfilaments and microtubules, as is the case with *Neisseria gonorrhoeae* and *Campylobacter jejuni* (4, 43).

To determine the contribution of actin polymerization to LVS invasion we incubated TC-1 lung epithelial cells with cytochalasin D, an actin polymerization inhibitor that disrupts microfilaments, and measured bacterial internalization by gentamicin protection assay. Treatment of TC-1 cells with 0.5 and 1.0 μM cytochalasin D decreased LVS invasion more than 99% (±0.1%) (Figure 2A), whereas *Salmonella enterica* invasion, which is known to be actin dependent (36), decreased by 84.6% (±2.3%) and 88.1% (±2.5%) at the same inhibitor concentrations, respectively. TC-1 cell invasion by *Campylobacter jejuni* strain 81-176, a strain which does not require actin for invasion of intestinal epithelial cells (34), was not decreased by actin inhibition.

Microtubules are responsible for the cytoplasmic organization of eukaryotic cells, control of organelle transport, and are a primary component of cilia and flagella (55). Microtubule polymerization has been shown to contribute to the epithelial cell invasion of *Neisseria gonorrhoeae* and *Campylobacter jejuni* (4, 43). To determine the contribution of microtubules to LVS invasion we treated lung epithelial cells with colchicine, which binds tubulin and inhibits microtubule polymerization, and determined invasion by gentamicin

protection assay. Treatment of TC-1 cells with colchicine decreased LVS invasion by 55.2% (±18.3%) and 94.2% (±2.4%) at 0.1 and 0.25 μM concentrations, respectively (Figure 2B). TC-1 cell invasion by *Campylobacter jejuni* strain 81-176, a strain for which invasion is blocked by microtubule depolymerization in intestinal epithelial cells (34), was decreased significantly at the higher colchicine concentration. *Salmonella enterica* invasion, which is not considered microtubule dependent (4), did not demonstrate a statistically significant decrease in invasion at these concentrations.

Signaling pathways are frequently manipulated by bacteria to cause the cytoskeletal rearrangement necessary to gain entry into nonphagocytic cells (10, 15, 16). PI3 kinase and tyrosine kinase signaling are exploited by other pathogens for invasion (25, 45). These pathways were examined for their contribution to *Francisella tularensis* entry into lung epithelial cells.

PI3 kinases phosphorylate inositol phospholipids, forming lipid products that are in turn involved in cellular functions such as cell growth, actin rearrangement and vesicular trafficking (51). To determine the importance of PI3 kinase signaling to *F. tularensis* invasion, lung epithelial cells were treated with wortmannin, an inhibitor of PI3 kinase activity, and invasion was assessed by gentamicin protection assay. Wortmannin decreased LVS invasion of TC-1 cells by 69.4% (±4.6%) and 84.1% (±1.8%) when cells were exposed to 100 and 200 nM concentrations, respectively (Figure 3A). Invasion by *Listeria monocytogenes*, which is PI3 kinase dependent (25), was significantly decreased. *Salmonella enterica* invasion, which is not PI3 kinase dependent (49), was not significantly decreased at these concentrations in TC-1 cells.

Cells monitor and respond to their external environment via receptors that lead to intracellular signaling events (53). Activation of receptor tyrosine kinases can lead to receptor internalization, as well as initiation of a cascade of downstream signaling events (53). Some organisms, such as *Yersinia pseudotuberculosis*, exploit signaling through host cell receptors to gain entry into cells, and this entry can be blocked by tyrosine kinase inhibitors (45). To determine the contribution of tyrosine kinase signaling to LVS invasion, the inhibitor genistein was added to lung epithelial cells and the number of intracellular organisms measured by gentamicin protection assay. Genistein decreased LVS invasion of TC-1 cells by 68.1% (±1.1%) and 77.1% (±1.1%) at 50 and 100 μM concentrations, respectively (Figure 3B). Invasion by *Yersinia pseudotuberculosis* was significantly reduced at these concentrations, while *Salmonella enterica* invasion, which is tyrosine kinase independent (45), was not significantly decreased in TC-1 cells.

Collectively, these results indicate that actin and microtubule rearrangement both contribute to the entry of *F. tularensis* into lung epithelial cells. PI3 kinase and tyrosine kinase, proteins which regulate cytoskeletal rearrangement, both impact *F. tularensis* invasion of lung epithelial cells.

LVS traffics along the endocytic pathway in lung epithelial cells

To characterize the trafficking of *F. tularensis* LVS along the endocytic pathway in lung epithelial cells, we synchronized the addition of LVSgfp to TC-1 cells and evaluated these cells microscopically for the presence of bacteria inside vacuoles containing the early endosomal marker EEA1 or the late endosomal/lysosomal marker LAMP-1 (Figure 4A and 4B). LVS association with EEA1 containing phagosomes increased from 1.4% (±2.4%) at 10 minutes to 40.4% (±5.3%) at 30 minutes, before decreasing (Fig 4C). LVS association with

LAMP-1 containing vacuoles peaked two hours post-inoculation at 49.3% (±3.5%) (Figure 4C). These results indicate that LVS traffics along the endocytic pathway in lung epithelial cells, associating first with early endosomes before progressing to late endosomes/lysosomes.

LVS escapes the phagosome and replicates in the cytoplasm of lung epithelial cells

Once inside host cells invasive bacteria either replicate within the endosome or escape the vacuole and replicate in the cytoplasm. To determine the intracellular location of bacteria, TC-1 cells were inoculated with LVS at an MOI of 100 and examined at one and 24 hours by transmission electron microscopy. At 1 hour post-inoculation LVS were TC-1 cells in a membrane-bound vacuole (Figure 5A). The membrane was easily visualized, and in some cases was beginning to degrade (Figure 5A). By 24 hours all observed intracellular bacteria were no longer contained within a visible membrane, and appeared to be free in the cytoplasm (Figure 5B), though they were surrounded by an electron lucent zone that has been noted by other researchers (8, 19).

To determine the kinetics of LVS escape from the endocytic pathway into the cytoplasm of lung epithelial cells, we differentially labeled extracellular, cytoplasmic and vacuolar LVSgfp and analyzed recovered bacteria by flow cytometry (modified from Checroun *et al*.).We first labeled bacteria that were extracellular and attached to TC-1 cells using Pacific Blue conjugated anti-*F. tularensis* LPS. We then permeabilized the cytoplasic membrane of TC-1 cells using digitonin, allowing Alexa Fluor 647 conjugated anti-*F. tularensis* LPS to access cytoplasmic bacteria, but not those enclosed in intact vacuoles, which are digitonin impermeable. TC-1 cells were lysed and cell-associated bacteria recovered and analyzed by flow cytometry, gating on GFP to differentiate bacteria from residual TC-1 debris. The resulting three populations of bacteria were analyzed: extracellular

(Pacific Blue, Alexa Fluor 647 and GFP), cytoplasmic (Alexa Fluor 647 and GFP), and vacuolar (GFP only) (Figure 6A and 6B). At 10 minutes post-inoculation 19.3% (±8.1%) of intracellular bacteria were accessible to antibody delivered to the cytoplasm, and therefore considered cytoplasmic (Figure 6C). This number increased to 70.0% (±5.5%) at 2 hours post-inoculation, indicating that the majority of intracellular bacteria were no longer contained within intact endocytic vacuoles. It should be noted that once the vacuolar membrane begins to degrade bacteria are accessible to antibody. As a result, bacteria that were associated with LAMP-1 staining vacuoles at 2 hours post-inoculation (Figure 4C) would be identified as cytoplasmic by this assay if the vacuolar membrane was no longer intact. When TC-1 cells were treated with saponin, which permeabilizes both vacuolar and cytoplasmic membranes, greater than 99% of bacteria were accessible to antibody labeling (data not shown), demonstrating that a population of bacteria were protected from staining when only the cytoplasmic membrane was permeabilized. Microscopic examination of saponin treated TC-1 cells revealed that anti-*F. tularensis* antibody was able to access bacteria in EEA1 containing vacuoles, while antibody was excluded from these vacuoles in digitonin treated cells (data not shown). When TC-1 cells were inoculated with PFA killed bacteria >90% of organisms were vacuolar at 60 minutes post-inoculation, indicating that killed bacteria did not escape into the cytoplasm (data not shown). Thus, bacterial viability was required for escape from the epithelial cell endosome.

DISCUSSION

The ability of many facultative intracellular bacterial pathogens to cause disease is dependent upon their ability to invade and replicate within various host cells (14, 22, 41). *F.*

tularensis survival and replication within macrophages and dendritic cells has been well described (1, 5, 6, 8, 33). We previously demonstrated that *F. tularensis* LVS also localizes to and replicates within alveolar type II epithelial cells following inhalation of organisms in a mouse model of infection (21), prompting us to investigate lung epithelial cell invasion by this organism.

Alveolar type II cells account for about 12% of the total cells, and 2% of the surface area, of the alveolar region in mice (50). Approximately 98% of the surface area, and 10% of the total cell number, is comprised of type I cells, which provide structure and are the site of gas exchange (50). Type II cells have a number of biological functions, including the production, secretion and recycling of surfactant, proliferation to produce additional type II cells as well as transdifferentiation into type I cells, maintenance of alveolar fluid balance, and production of antimicrobial and anti-inflammatory substances (29). Due to the close proximity between the blood and alveolar epithelial cells required for gas exchange and fluid balance, these cells are ideally located to provide a portal through which bacteria could disseminate to distal organs.

To characterize the interactions of *F. tularensis* with lung epithelial cells we investigated the initial interactions of LVS with the lung epithelial cell line TC-1. We determined that bacteria associated with and were internalized by lung epithelial cells within 10 minutes of inoculation, and that viable bacteria were not necessary for gaining entry into these cells. We previously found that the frequency with which *F. tularensis* invaded lung epithelial cell lines was low (21). The low invasion frequency may be due to the inherent inability of a transformed cell line grown in a non-polarized fashion to recapitulate the complex environment within the host lung. These cells may also intermittently express a

relevant receptor or other process that facilitates *F. tularensis* invasion *in vivo*. A system using primary cells grown in a polarized manner may more realistically reproduce the environment in the lung (18). Given the quantity of cells required for studies, cell culture lines provide a reasonable place to begin the investigation of the interaction of bacteria with epithelial cells, and have been described as a model for such interactions (28).

Pathogens have developed numerous means of exploiting host cell functions to gain entry into nonphagocytic cells (10, 15). These invasion strategies are typically classified as either a zipper mechanism, which is utilized by pathogens such as *Yersinia pseudotuberculosis* and *Listeria monocytogenes* or a trigger mechanism, which is utilized by organisms such as *Salmonella typhimurium* and *Shigella flexneri*. The zipper mechanism is characterized by bacterial surface proteins binding to host cell receptors leading to internalization. The trigger mechanism is characterized by the injection of bacterial effector proteins into the host cell via a type III secretion system resulting in bacterial engulfment via induced macropinocytosis. Entry by either of these mechanisms requires the manipulation of host cell cytoskeletal components and signaling pathways. A number of host cell receptors play a role in *F. tularensis* entry into macrophages, including Fcγ, CR3, MR and SRA receptors (3, 38, 47). However, very little is known about how this organism is able to access cells that are not considered to be professional phagocytes.

To better understand the process of *F. tularensis* entry into lung epithelial cells we investigated cytoskeletal components and signaling pathways that are involved in epithelial invasion by other bacterial pathogens for their contribution to internalization of LVS by a lung epithelial cell line. Lindemann *et al*. previously demonstrated that actin and microtubules are necessary for *F. tularensis* invasion the human epithelial cell line HEp-2

(28). We determined that both actin and microtubules contribute to *F. tularensis* invasion of lung epithelial cells, as do PI3 kinases and tyrosine kinases, both of which can control signaling events leading to cytoskeletal rearrangement. We showed that *F. tularensis* internalization by lung epithelial cells is dependent upon a variety of host cell mechanisms, and that interruption of any of these mechanisms interferes with bacterial invasion.

We were unable to identify the characteristic membrane ruffling that is seen with *Salmonella* invasion either by phalloidin staining or Field Emission Scanning Electron Microscopy (FESEM) under conditions where we were able to clearly identify membrane ruffling with *Salmonella* (data not shown). While this points toward *F.tularensis* not causing massive actin reorganization at the site of entry it is possible that membrane ruffling occurs but was not detected by us. *Listeria monocytogenes,* another organism that is able to invade epithelial cells, also requires PI3 kinase and tyrosine kinase function for invasion, in a cell type dependent manner (39, 40). This organism has two invasion proteins, InlA and InlB, which interact with different host receptors, leading to different signaling events. It is possible that *F. tularensis* also uses multiple receptors for entry into lung epithelial cells, and that interruption of various signaling pathways could decrease uptake via various receptors. Toll-like receptors (TLRs) may play a role in modulation of the immune response to *F. tularensis* (9, 23, 54), and functional TLRs are present on ATII cells (2). However, TLRs have not been implicated among macrophage receptors for *F. tularensis* internalization identified to date, which include Fcγ, CR3, MR and SRA receptors (3, 38, 47). Melillo *et al.* have reported that *E. coli* expressing the *F. tularensis* surface protein FsaP were able to bind A549 cells (31). We have also demonstrated that killed *F. tularensis* was taken up by lung epithelial cells. This information, taken together with the absence of genes predicted to

encode a type III secretion system, point toward the presence of a preformed ligand receptor interaction as with the zipper mechanism of uptake, rather than injection into the host cell of effector proteins as with organisms that gain entry via the trigger mechanism. Further studies are needed to identify receptors necessary for uptake of *F. tularensis* by nonphagocytic cells.

To investigate *F. tularensis* trafficking along the endocytic pathway in lung epithelial cells we examined association of LVS with EEA1 and LAMP-1 containing vacuoles, as well as escape into the cytoplasm. *F. tularensis* traffics along the endocytic pathway in macrophages before escaping to the cytoplasm where replication occurs. The timing of this escape seems to be dependent upon the *Francisella* species and host cell type tested. *F. tularensis* subsp.*novicida* begins to disrupt the phagosomal membrane of quiescent human macrophages at 4 hours and is free in the cytoplasm by 12 hours post-infection (46). LVS and clinical isolates of *F. tularensis* associate with EEA1 then LAMP-1 containing vacuoles in mouse bone marrow derived macrophages, human macrophages, and mouse and human macrophage-like cell lines before degrading the phagosomal membrane and beginning to escape into the cytoplasm between one and two hours post-inoculation (7, 8, 19). We determined that LVS was initially associated with EEA1 containing vacuoles, then LAMP-1 containing vacuoles, before being found free in the cytoplasm of lung epithelial cells, in a manner and kinetic consistent with that seen in macrophages.

F. tularensis LVS is found in ATII cells in the lungs of C57BL/6 mice 1, 3 and 7 days post-intranasal inoculation, replicates in these cells as disease progresses, and by day 7 organisms are widespread in the alveolar epithelium (21). These observations demonstrate that that ATII cell invasion and replication is a part of the *F. tularensis* disease process. Understanding how this bacterium gains access to these cells, and just as importantly, how

they replicate and establish a stronghold in the lung epithelium, is necessary to understanding the progression of respiratory tularemia, as well as potentially providing insight into methods that may be used to block bacterial uptake or replication, and thus prevent disease.

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ATTRIBUTIONS

I developed the inhibitor assay protocol and did preliminary experiments examining the effects of cytochalasin D, colchicine, wortmannin, and genistein on invasion of ATII-like cell lines by LVS. I infected TC-1 cells and prepared samples for TEM experiments presented in this chapter. This work was previously published in the journal Infection and Immunity. Permission has been granted to reprint this material.

FIGURES

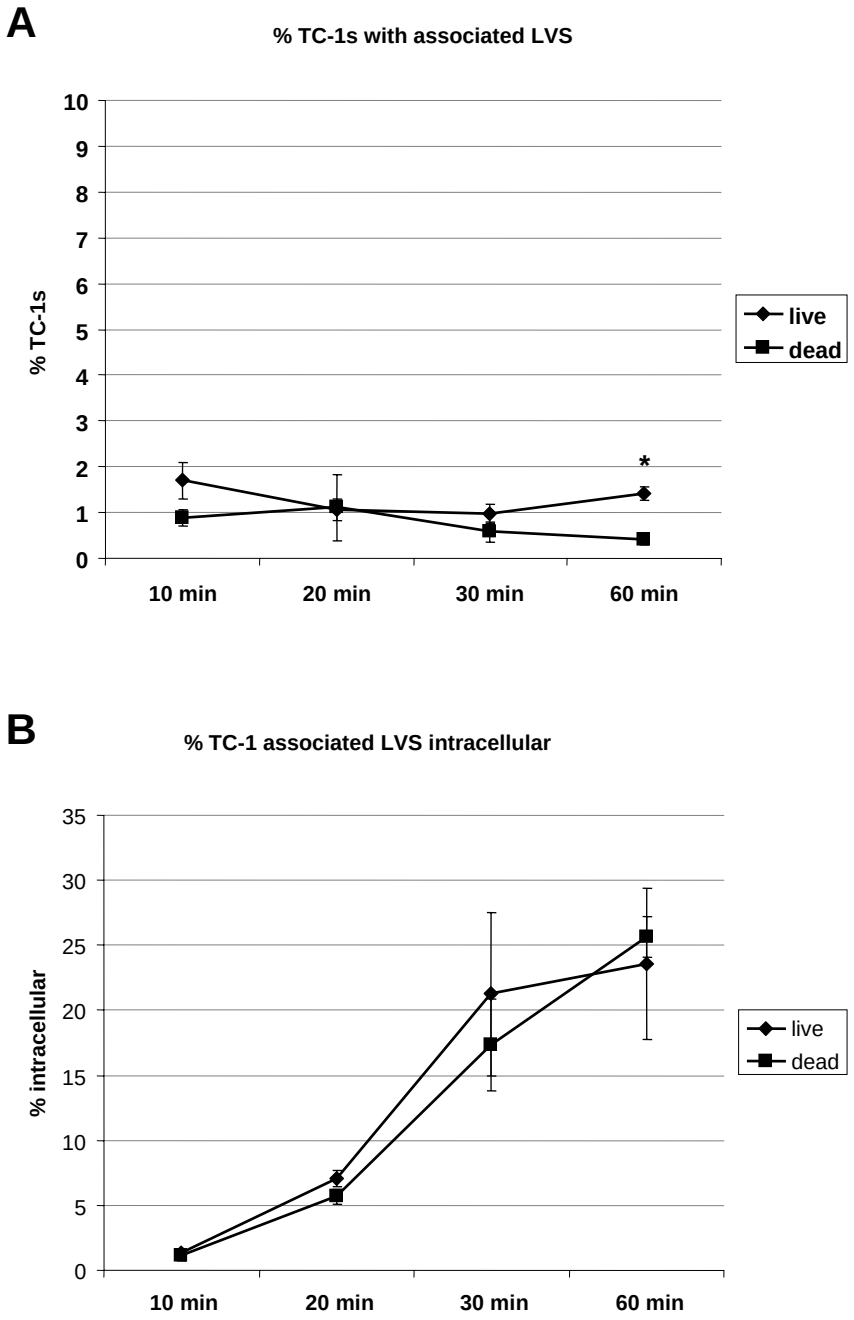

Figure 5.1. *F. tularensis* LVS association with and internalization by TC-1 lung epithelial cells. (A) Percent of TC-1 cells with associated live (♦) or PFA-fixed (■) LVS at designated times post-inoculation. * Data are significantly different from untreated control, $P<0.01$ by paired two-tailed *t* test. (B) Percent of TC-1 cell-associated live (♦) or PFA-fixed (■) LVS that are intracellular at designated times post-inoculation.

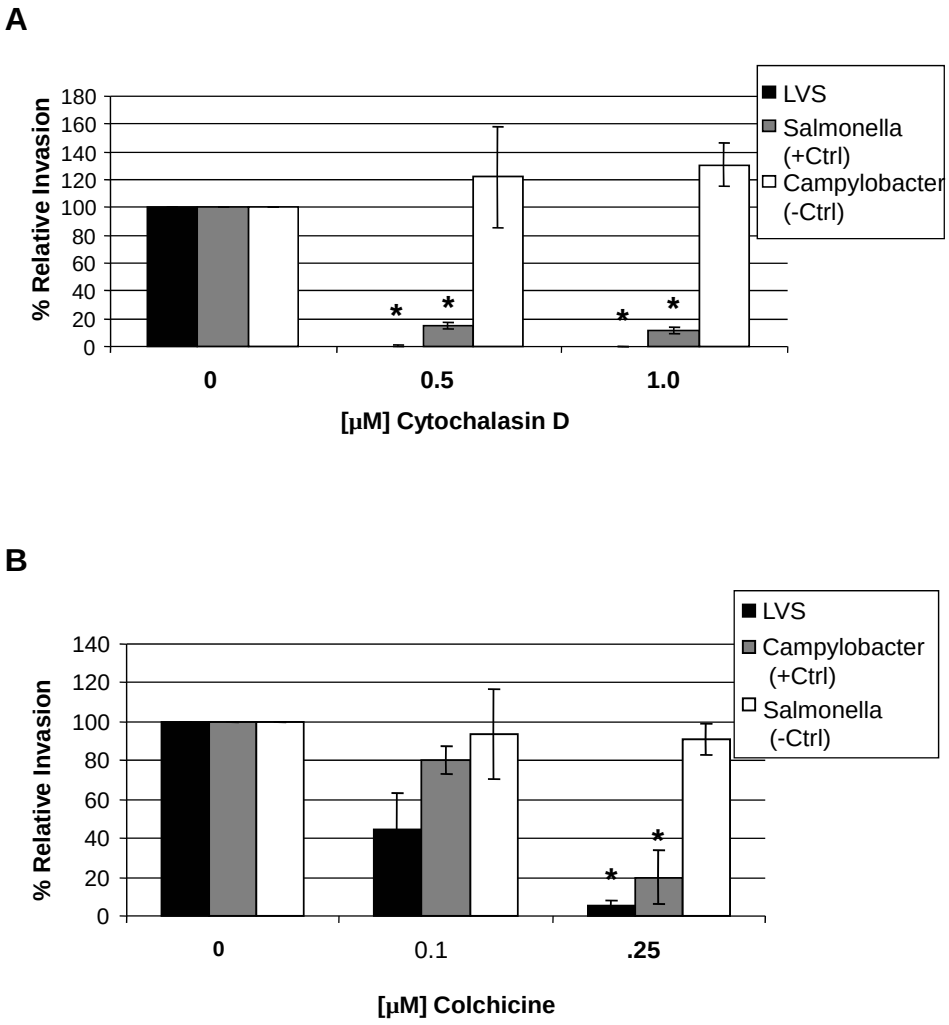

Figure 5.2. The effect of actin and microtubule polymerization on *F. tularensis* LVS invasion of lung epithelial cells. TC-1 cells were treated with designated concentrations of (A) cytochalasin D or (B) colchicine. Results are expressed as the percentage of LVS, *Salmonella* or *Campylobacter* that survived gentamicin treatment, relative to the sample without inhibitor inoculated with the same organism (defined as 100% invasion). * Data are significantly different from untreated control, $P<0.01$ by paired two-tailed *t* test.

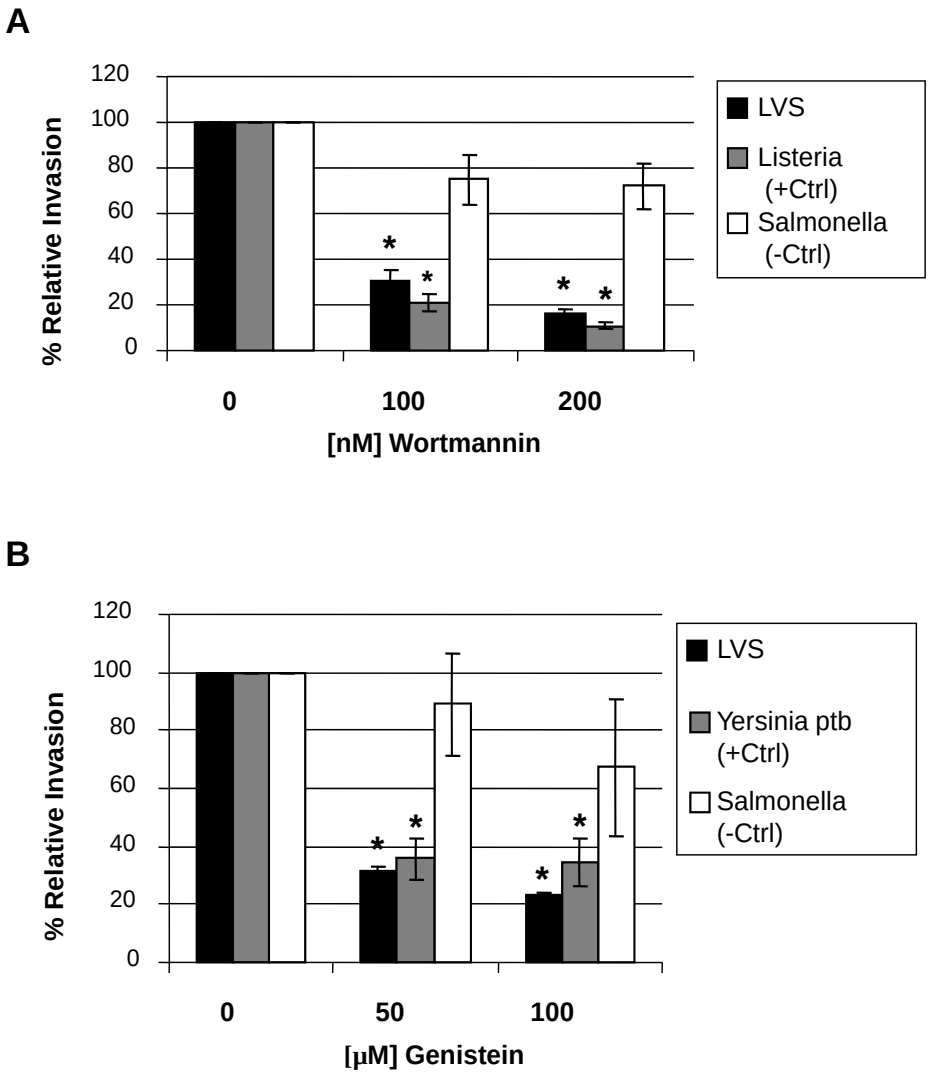

Figure 5.3. The effect of PI3 kinase and tyrosine kinase activity on LVS invasion of lung epithelial cells. (A) Wortmannin or (B) genistein were added to TC-1 cells at the indicated concentrations. Results are expressed as the percentage of LVS or control organism that survived gentamicin treatment, relative to the sample without inhibitor inoculated with the same organism(defined as 100% invasion). * Data are significantly different from untreated control, $P<0.01$ by paired two-tailed *t* test.

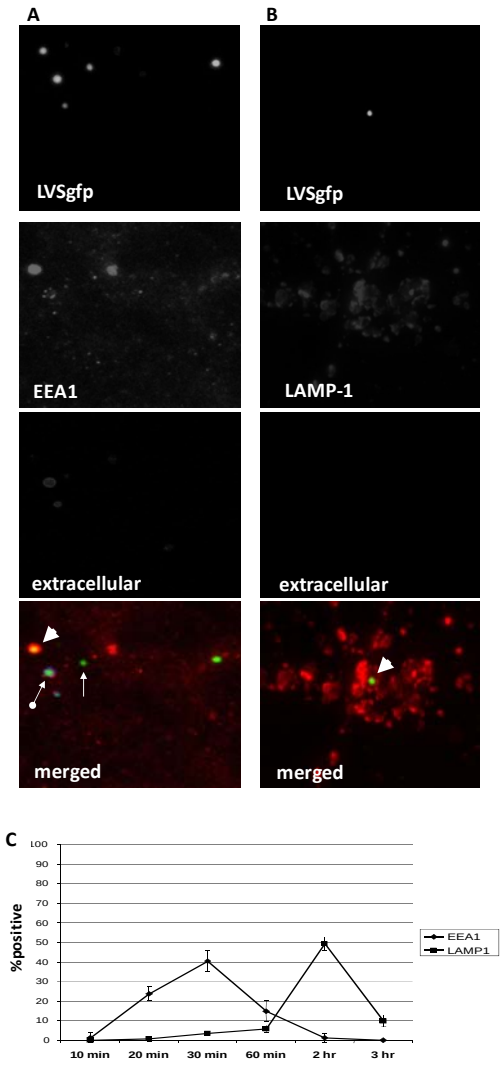

Figure 5.4. Representative fluorescence microscopy images demonstrating LVSgfp localization within EEA1 or LAMP-1containing endosomes in TC-1 cells. Images depict (A) TC-1 cells stained for EEA1 30 minutes post-inoculation with LVSgfp or (B) 2 hours post-inoculation stained for LAMP-1. Single color images: LVSgfp images depict bacteria alone, EEA1 and LAMP-1 images depict staining for EEA1 or LAMP-1 only, and extracellular images depict staining of extracellular bacteria (no extracellular bacteria were present in B). The merged color images depict LVSgfp (green), vacuoles labeled with anti-EEA1 antibody (N19)(red) or anti-LAMP-1 (1D4B) (red), or extracellular LVS labeled with anti-*F. tularensis* LPS antibody conjugated to Pacific Blue (blue). Extracellular LVSgfp (arrow with round end), intracellular but not EEA1 associated LVSgfp (small arrow), and LVS associated with EEA1 containing vacuoles (large arrowhead). (C)Trafficking of LVSgfp in TC-1 cells. 100 intracellular bacteria were counted for each condition and scored for association with EEA1 (♦) or LAMP-1 (▪) containing vacuoles. Three replicates were examined for each time point and condition.

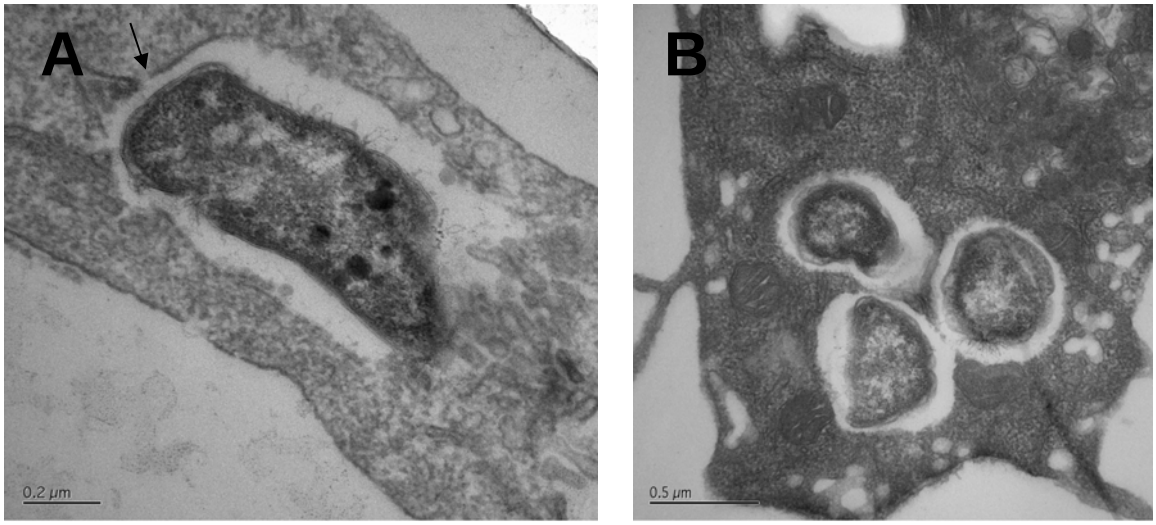

Figure 5.5. Transmission electron micrographs of TC-1 infected with *F. tularensis* LVS. (A) One hour post-inoculation TC-1 cells showing LVS in a membrane bound vacuole that in some cases appeared to be degrading (arrow). (B) 24 hours post-inoculation LVS were free in the cytoplasm.

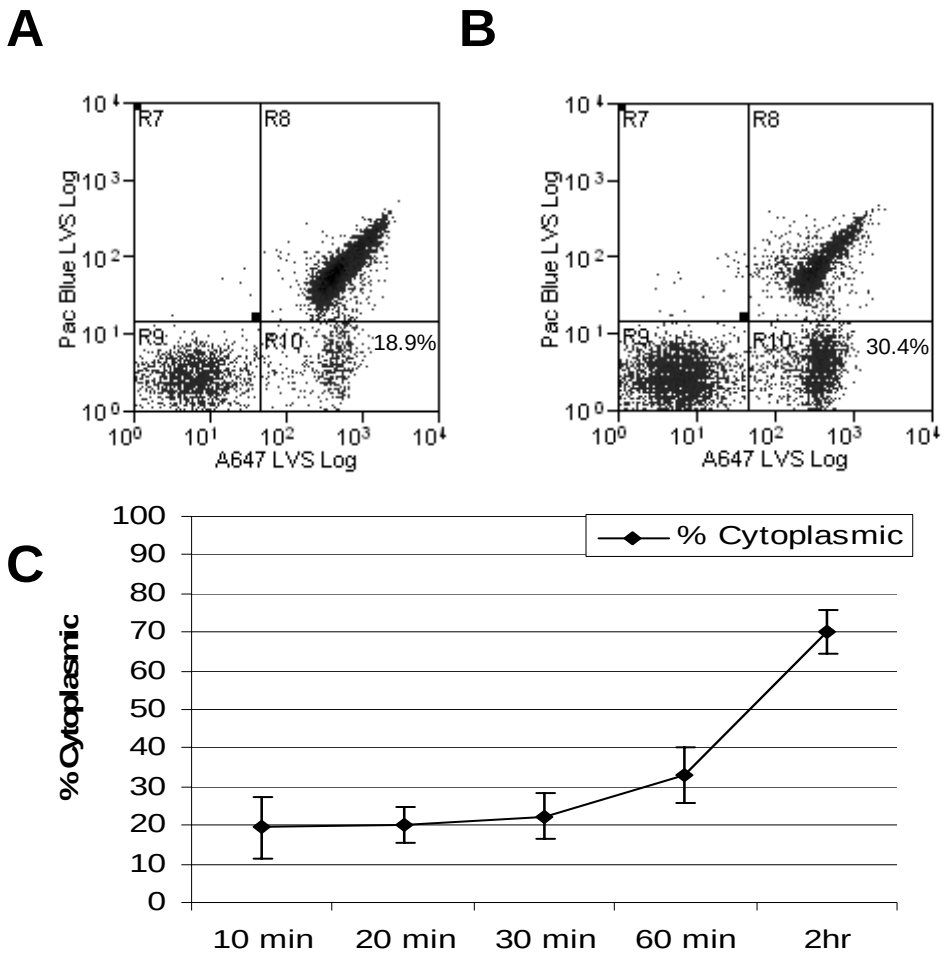

Figure 5.6. LVSgfp was analyzed by flow cytometry for escape from vacuoles into the cytoplasm of TC-1 cells. Extracellular bacteria were labeled with anti-*F. tularensis* LPS antibody conjugated to Pacific Blue (region R8). Cytoplasmic bacteria were identified by labeling with anti-*F. tularensis* LPS conjugated to Alexa Fluor 647 after digitonin permeabilization of the cytoplasmic membrane (region R10). Vacuolar bacteria were inaccessible to antibody, and therefore GFP positive only (region R9). Representative flow cytometry data of bacteria recovered (A) 10 minutes or (B) 60 minutes post-inoculation. The value shown in region R10 represents the percent of intracellular bacteria that are cytoplasmic. (C) Percent of intracellular bacteria present in the cytoplasm at designated times post-inoculation

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CHAPTER 6

Use of transposon-transposase complexes to create stable insertion mutant strains of *Francisella tularensis* LVS

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ABSTRACT

Francisella tularensis is a highly virulent zoonotic bacterial pathogen capable of infecting numerous different mammalian species, including humans. Elucidation of the pathogenic mechanisms of *F. tularensis* has been hampered by a lack of tools to genetically manipulate this organism. Herein we describe the use of transposome complexes to create insertion mutations in the chromosome of the *F. tularensis* live vaccine strain (LVS). A Tn*5*-derived transposon encoding kanamycin resistance and lacking a transposase gene was complexed with transposase enzyme and transformed directly into *F. tularensis* LVS by electroporation. An insertion frequency of $2.6 \times 10^{-8} \pm 0.87 \times 10^{-8}$ per cell was consistently achieved using this method. There are 178 described Tn*5* consensus target sites distributed throughout the *F. tularensis* genome. Twenty-two of 26 transposon insertions analyzed were within known or predicted open reading frames, but none of these insertions was associated with the Tn*5* target site. Analysis of the insertions of sequentially passed strains indicated that the transposons were maintained stably at the initial insertion site after more than 270

generations. Therefore, transformation by electroporation of Tn*5*-based transposon-transposase complexes provided an efficient mechanism for generating random, stable chromosomal insertion mutations in *F. tularensis*.

INTRODUCTION

Francisella tularensis is a gram-negative bacterial pathogen and is the etiologic agent of tularemia. The manifestations of tularemia depend on the initial route of inoculation, but all modes of contact can result in sepsis and disseminated disease, with organisms found in the liver, spleen, lymph nodes, kidney, and lungs (6, 20, 24, 25). Skin contact results in ulcer formation at the site of inoculation, where the organisms multiply and spread to the draining lymph nodes. Inhalation of *F. tularensis* leads to bronchial hemorrhaging, mediastinal lymphadenopathy (27), and pneumonia without a corresponding productive cough. Ingestion of the organisms can result in oropharyngeal tularemia, where patients typically develop exudative ulcerative pharyngitis and pharyngeal lymphadenopathy (1, 4).

F. tularensis strains are divided into two groups, A and B, which are distinguished by acid production from glycerol and by citrulline ureidase activity. The two groups of organisms exhibit similar pathogenesis; however, group A strains are considered to be highly virulent for humans and other animals, whereas group B strains typically cause milder disease (28). A live attenuated vaccine strain (LVS) derived from a group B *F. tularensis* strain has been developed and used to vaccinate laboratory workers (7). This vaccine provides significant protection against the highly virulent strains initiated by skin contact (21) and inhalation (13, 22). However, the basis for attenuation of this strain in humans is not known, and it has not been licensed for general public use in the United States.

F. tularensis can survive within macrophages, a feature that is thought to be important in the pathogenesis of this organism (5, 12, 17, 26). *F. tularensis* subsp. *novicida*, a related organism historically referred to as *Francisella novicida*, also survives within macrophages, but it is an animal pathogen that does not infect humans. Two different genetic loci, termed *mglAB* for macrophage growth locus (2) and *iglABCD* for intracellular growth locus (11), have been identified in *F. tularensis* subsp. *novicida* that contribute to its survival in macrophages. The function of the *igl* gene products in promoting this survival has not been determined. MglA of *F. tularensis* subsp. *novicida* has recently been shown to function as a positive regulator for at least seven different genes (16) that are normally induced in macrophages, including *iglC*. An *F. tularensis* subsp. *novicida* mutant lacking *mglA* is unable to survive in cultured macrophages and is attenuated in mice (16). Homologues of these genes are present in both group A and B *F. tularensis* strains, and they presumably serve similar functions in these organisms.

Apart from the described macrophage survival phenotype and the identification of a limited number of genetic loci that contribute to intracellular survival (11), very little is known about the molecular mechanisms that support *F. tularensis* pathogenesis and virulence. The lack of tools for the genetic manipulation of *F. tularensis* has made it difficult to identify and dissect the bacterial products and processes that contribute to this organism's extraordinary virulence and pathogenesis. Mechanisms of transformation and allelic exchange have only recently been described (3, 8, 15), and most of these procedures have been developed in *F. tularensis* subsp. *novicida*. Lauriano et al. (15) recently described the use of allelic exchange to generate targeted insertion mutations in *F. tularensis* LVS. They also reported that Tn*10*- and Tn*1721*-based transposon insertions were unstable in *F.*

tularensis and that bacterial gene-encoded transposase-complementing activity may function to promote the movement of these transposons.Herein we describe a procedure that uses a Tn*5* derivative to create insertion mutations in *F. tularensis* LVS which, unlike Tn*10* and Tn*1721*, were stably maintained at the initial insertion site.

MATERIALS AND METHODS

F. tularensis LVS was obtained from the Centers for Disease Control and Prevention, Atlanta, Ga., and were propagated at 37°C on chocolate medium supplemented with 1% IsoVitalex (BBL). Multiple colonies from an overnight culture of LVS grown on chocolate agar were picked and swabbed onto fresh plates to achieve confluent growth, and they were then incubated for 16 h. Cells from one plate of freshly confluent LVS were suspended in 6 ml of wash buffer, consisting of 0.5 M sucrose and 10% glycerol, and then were centrifuged at 16,000 x *g* for 3 min. The pellet was suspended in wash buffer and centrifuged again for a total of four washes. The pellet resulting from the final centrifugation was suspended in wash buffer to a total volume of 100 µl.

A procedure initially described by Goryshin et al. (9) to create mutations in *Salmonella*, *Proteus*, and *Escherichia* species was used to produce similar transposon insertion mutations in *F. tularensis* LVS. One microliter of EZ::TN <kan-2> transposome complex (Epicentre) containing 0.1 pmol of transposon and 1 U of transposase was added to 100 µl of the washed cell suspension, consisting of 10^9 to 10^{10} cells in 0.5 M sucrose and 10% glycerol. The contents were then mixed and transferred to a 0.1-cm-gap electroporation cuvette.

The EZ::TN <kan-2> transposome contains a derivative of Tn*5* that lacks a transposase gene and has the transposase enzyme bound to the inverted repeat ends of the transposon. The transposase is stably associated with the transposon but is inactive in the absence of Mg^{2+}. Magnesium ions present inside the bacterium activate the transposase following transformation, facilitating transposition into the *Francisella* chromosome. Thus, transposition is dependent simply upon activation of the enzyme and not the expression of a foreign transposase gene.

The transposome was introduced into *F. tularensis* LVS by electroporation using a Bio-Rad Gene Pulsar set at 2.5 kV, 25 μF, and 200 Ω. Immediately following electroporation the cells were suspended in 1 ml of brain heart infusion broth (BBL) supplemented with 50 μg of hemin/ml, incubated for 1 h at 37°C, and then plated on chocolate agar containing 10 μg of kanamycin/ml.

RESULTS

We consistently achieved an insertion frequency of $2.6 \times 10^{-8} \pm 0.87 \times 10^{-8}$ (Table 1), as determined by the number of antibiotic-resistant colonies divided by the total number of potential recipient organisms. The insertion frequency was not appreciably affected by the number of bacteria, nor was it improved by increasing the concentration of transposome complexes. Recovering the organisms with cold or prewarmed media following electroporation as described for other organisms (23) also did not alter the frequency with which we isolated antibiotic-resistant organisms.

Ideally a transposon will insert randomly throughout a genome in order to be a useful tool for creating insertion mutation libraries. The transposon insertion sites were mapped by directly sequencing the transposon-chromosome junctions by using oligonucleotide primers that hybridize within the transposon. Primers Kan-2 FP-1 (ACCTACAACAAAGCTCTCATCAACC) and JF119 (GGATCAGATCACGCATCTTC) hybridize 70 and 150 bp, respectively, from the end of the transposon adjacent to the 3' end of the Kan resistance gene, and primer KAN RP-1 (GCAATGTAACATCAGAGATTTTGAG) hybridizes 43 bp from the other end of the transposon. Individual kanamycin-resistant colonies were picked and restreaked on selective media. Chromosomal DNA was prepared from strains by using the MasterPure DNA purification kit according to the manufacturer's instructions (Epicentre).

The precise transposon-chromosome junction sites were determined in a total of 26 kanamycin-resistant colonies from each of three different transformations. One microgram of chromosomal DNA was mixed with 100 pM primer, and DNA sequence was generated by the University of North Carolina Genome Analysis Center. The transposon insertion site was determined by aligning the sequence to the *F. tularensis* LVS strain genome database produced by the Biology and Biotechnology Research Program Sequencing Group at Lawrence Livermore National Laboratory (http://bbrp.llnl.gov/bbrp/html/microbe.html).

Tn*5*, the transposon upon which EZ::TN is based, has a reported insertion bias for the sequence A-GNTYWRANC-T (where W is A or T, R is A or G, Y is C or T, and N is any base) (10). The *F. tularensis* 1.8-Mbp LVS genome has 178 of these insertion bias sequences, but none of the 26 insertions that we analyzed occurred within one of these sites. Of the 26

junction sites sequenced, 21 were within potential open reading frames and 1 was in the transcription termination sequence of an open reading frame, which is consistent with the observation that, apart from the Tn*5* insertion bias site, this transposon has a propensity for inserting into actively transcribed DNA and regions of high superhelical density (18, 19). One insertion, B5, was in a gene encoding the RNA polymerase ß subunit; this is an essential gene. The insertion is located 12 bases from the 3' end of the gene. *F. tularensis* LVS does not possess a second copy of this gene; thus, it is likely that the B5 mutant still produces a functional RNA polymerase ß subunit. Ironically, one of the insertions (strain A10; Table 2 and Fig. 1) was within a gene reported to encode a *Francisella* transposase (14). The insertions did not appear to cluster within a specific chromosomal segment (Fig. 1).

Insertions in potential reading frames were examined further by translating the sequence and blasting the protein database to tentatively identify the products of interrupted genes. The majority, but not all, of these mutations occurred in genes that encoded proteins with significant homology to proteins with known functions or conserved hypothetical proteins (Table 2).

Lauriano et al. reported that insertion mutations created by derivatives of the Tn*10* and Tn*1721* transposons in the closely related organism *F. tularensis* subsp. *novicida* were unstable and subject to movement to other chromosomal locations, possibly through the activity of a host-encoded transposase (15). We picked five transposon insertion mutation strains representing insertions within and outside of potential reading frames, including the strain with the insertion in the reported *Francisella* transposase gene. These strains were passed daily for 10 days on kanamycin-containing media, and chromosomal DNA was

prepared from cells on passages 0, 5, and 10. Southern blots were performed by digesting 5 µg of chromosomal DNA with EcoRI, which does not cleave the transposon. Digested DNA segments were separated by agarose gel electrophoresis, transferred to nylon, and probed with digoxigenin-labeled Tn*5* probe. The Tn*5* probe hybridized to identically sized fragments in each of the DNA samples prepared from different passages of the same insertion strain (Fig. 2). Given that a single colony is composed of at least 10^8 organisms, a single passage equates to at least 27 generations of growth. Thus, after 10 passages the analyzed insertions were stable for a minimum of 270 generations.

Electroporation of a Tn*5*-derived transposon-transposase enzyme complex into *F. tularensis* LVS resulted in the creation of random insertion mutant strains at a frequency that is sufficient to generate mutant libraries of this organism. These insertions are genetically stable and are apparently unaffected by the activities of any potential chromosomally encoded transposases. Thus, this scheme will facilitate the use of genetic approaches to study the mechanisms of *F. tularensis* physiology and pathogenesis.

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ATTRIBUTIONS

James Fuller and I each performed replicates of the transposon mutagenesis reactions used to calculate insertion frequencies. I isolated and characterized the 27 mutants described in the chapter. Julie Clark performed Southern blot analysis for 5 of the mutants under the direction of Robin Craven. This work was published in the journal Applied and Environmental Microbiology, Volume 70, pages 6901-6904. Permission has been granted to reprint this material.

TABLES AND FIGURES

Table 6.1. **Transposon insertion frequencies**

Input (CFU)	No. of mutants	Mutants/CFU
2.8×10^{10}	344	1.0×10^{-8}
4.9×10^{9}	62	1.2×10^{-8}
1.1×10^{9}	50	4.5×10^{-8}
2.6×10^{9}	207	7.0×10^{-8}
5.7×10^{10}	1,097	1.9×10^{-8}
2.3×10^{10}	550	1.0×10^{-8}
1.4×10^{10}	210	1.5×10^{-8}

Table 6.2. Identified transposon insertion sites

Strain designation	Predicted insertion
A1	Extragenic
A2	Deacyclase
A3	Guanosine polyphosphate pyrophosphohydrolases
A4	Hydrolase of HD superfamily
A5	Metal-dependent hydrolase
A6	Extragenic
A8	Hypothetical protein gi34496297
A9	Extragenic
A10	*Francisella* transposase (14)
B1	ATPase
B2	Extragenic
B4	Eflux pump protein
B5	RNA polymerase ß subunit
B6	Transcription terminator
B7	Predicted membrane protein of unknown function, pfam03956
B8	Phosphate acetyltransferase
B9	Adenylosuccinate synthase
B10	UsoAp of *Emericella nidulans*
C1	Pyruvate phosphate dikinase
C2	Methyltransferase
C3	Isocitrate dehydrogenase
C4	Chloride channel protein EriC
C6	Anthranilate synthase
C7	Tryptophan synthase alpha chain
C8	Multidrug resistance protein
C10	Integral membrane protein

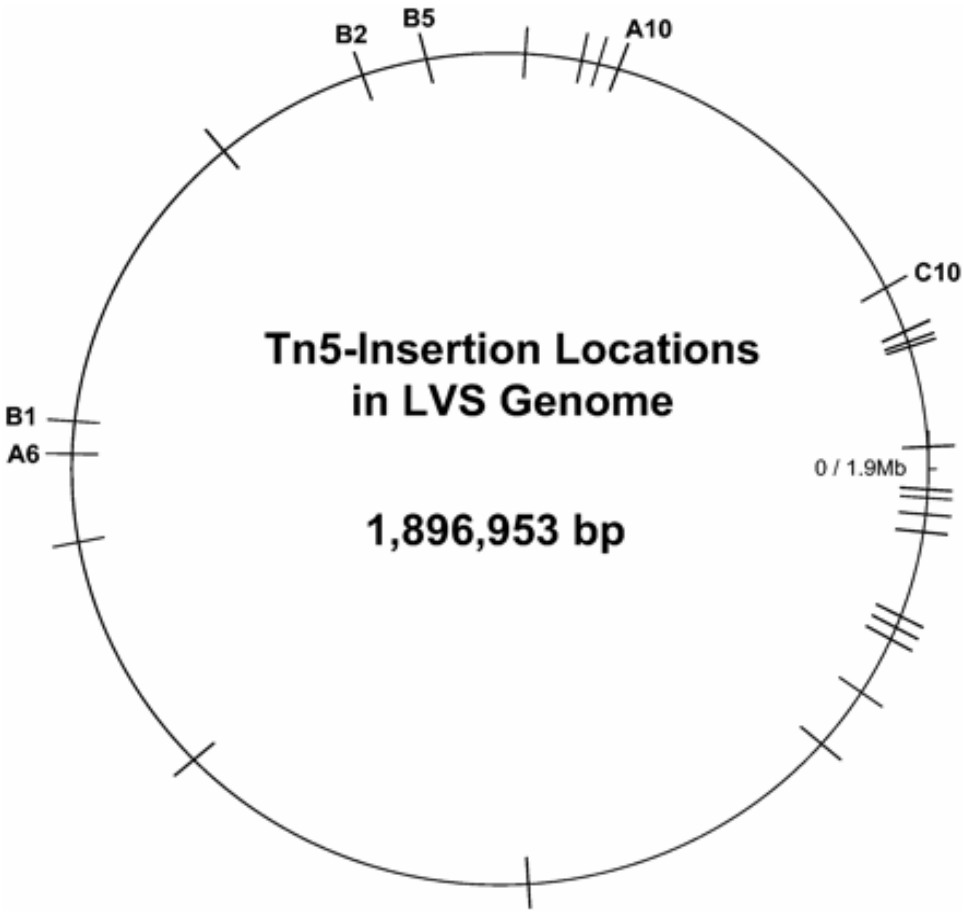

Figure 6.1. Graphic representation of the chromosomal positions of the 27 identified insertion sites. The insertions assessed for stability and depicted in Fig. 2 are labeled.

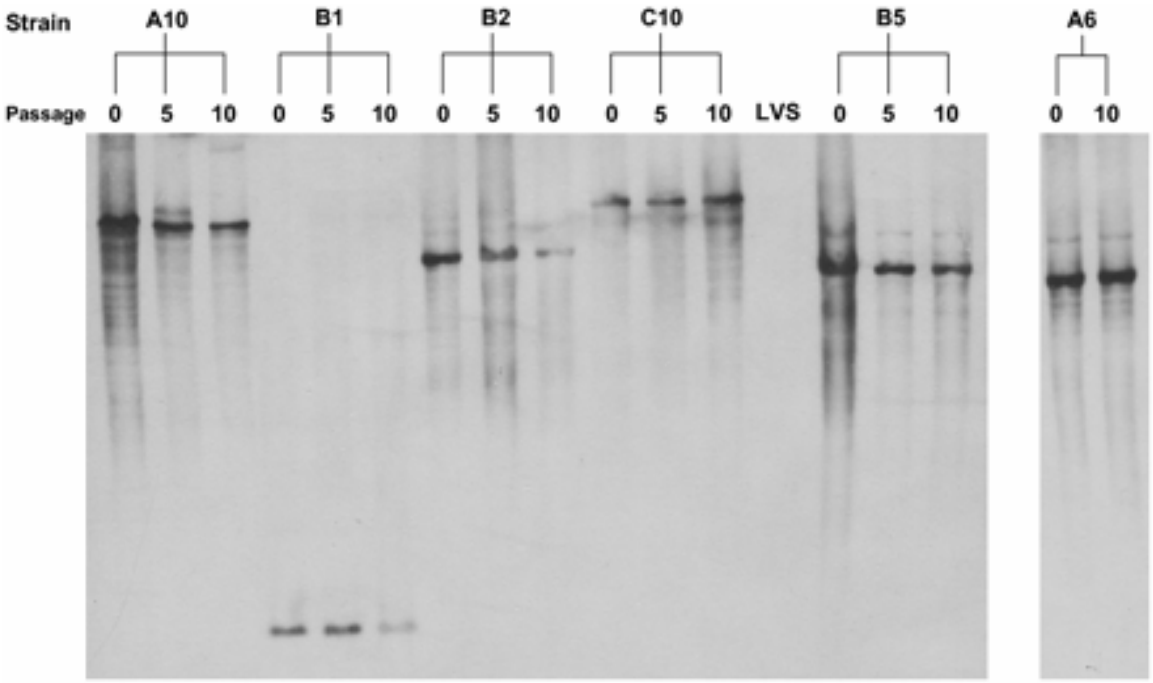

Figure 6.2. Southern blot of five insertion mutants (A6, A10, B1, B2, B5, and C10) **and wild-type *F. tularensis* LVS probed with labeled Tn*5*.** Chromosomal DNA was prepared from mutants after 0, 5, and 10 passages, digested with EcoRI, and probed. All insertions analyzed in this manner retained the transposon at the initial insertion site.

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CHAPTER 7

RipA, a cytoplasmic membrane protein unique to *Francisella* species is required for intracellular survival

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ABSTRACT

Francisella tularensis is a highly virulent facultative intracellular pathogen that is the etiologic agent of the zoonotic disease tularemia. An F. tularensis LVS strain with a transposon insertion into FTL1914 invaded, but failed to replicate in tested epithelial cell lines. This strain did, however, replicate within macrophages, albeit to a lesser extent than the parental strain. A deletion strain lacking FTL1914 was constructed. This strain did not replicate in either epithelial or macrophage–like cells, and intracellular replication was restored by the wild type allele in trans. Based on the deletion mutant phenotype FTL1914 was termed ripA (Required for Intracellular Persistence, Factor A). Following uptake by J774.A1 cells, F. tularensis ΔripA escaped from the phagosome at the same rate and frequency as wild type F. tularensis LVS. In a mouse model of pulmonary tularemia, *F. tularensis* ΔripA was significantly impaired in its ability to persist in the lung and in its capacity to disseminate and colonize the liver and spleen. Molecular characterization of ripA revealed that it is expressed by an independent promoter as a single transcript. The RipA

protein is expressed during growth in laboratory media, and localizes to the cytoplasmic membrane. RipA is a novel factor that is necessary for F. tularensis intracellular survival and virulence.

INTRODUCTION

Francisella tularensis is a highly virulent zoonotic pathogen that is the etiologic agent of the disease tularemia. *Francisella* has been isolated from over 250 animal species including mice, rabbits, and squirrels (5). Transmission to humans occurs through infected arthropod bites (47), physical contact with infected animal tissues (10), contaminated water (9, 58), or inhalation of aerosolized organisms (20). Transmission by inhalation results in the most aggressive form of tularemia with as few as 10 colony forming units (CFU) leading to a disease (64) that can rapidly progress (72) resulting in mortality rates as high as 60% if left untreated (18). Individual cases of tularemia occur throughout the Northern hemisphere (41) with clusters of outbreaks occurring in Scandinavia (73), Europe (31, 39), and the American Midwest (66). Reported cases of tularemia peaked in the United States during the 1930's and 1940's, but have since declined with only 1368 cases between 1990 and 2000 (11). Because of it's high virulence and relative ease of propagation, research into the weaponization of *F. tularensis* was conducted by Japan during World War II, and by the United States and Soviet Union during the Cold War era (18). The Department of Health and Human Services (HHS) and the United States Department of Agriculture (USDA) recently categorized *F. tularensis* as a Select agent based on the potential risk to public health resulting in a revitalized interest in *F. tularensis*.

F. tularensis is subdivided into Type A and Type B. Type A is characterized by high virulence and geographic localization to North America; although recently Type A was

isolated in Europe as well (32). Type B is distributed throughout the Northern hemisphere and has a lower associated mortality than Type A. Type B *F. tularensis* was used to create an attenuated live vaccine strain (LVS) for use in the Soviet Union (69); however, this vaccine is not licensed for use in the United States. *F. tularensis* LVS is attenuated in humans, but pathogenic in mouse and cell culture models making it an excellent model for the study of *Francisella* pathogenesis (22).

F. tularensis is a facultative intracellular pathogen. The ability to replicate in various cell types such as alveolar macrophages (3, 53, 56, 68), dendritic cells (8), and lung alveolar Type II epithelial cells (23, 34) are all considered to be important in the pathogenesis of *Francisella*. Virulence factors such as *mglA* (4), *iglA* (17), *iglC* (44, 45, 63), *tolC* (25), *clpB* (48), Type IV pili (21, 24, 33), and a recently identified 58 kDa protein (70) have all been characterized using macrophage and mouse models of infection. Yet the actual function of many of these proteins remains to be determined. We screened a transposon insertion library to identify additional genes that contribute to the intracellular growth of *F. tularensis*. Herein we describe one such gene that is required for intracellular growth and virulence in a mouse model of tularemia.

MATERIALS AND METHODS

Bacterial strains and Cell culture

Francisella tularensis Live Vaccine Strain (LVS) was obtained from the Centers for Disease Control and Prevention, Atlanta, Ga. *F. tularensis* LVS was propagated on chocolate agar (25g/l BHI, 50µg/ml hemoglobin, 15g/l agarose) supplemented with 1% IsoVitaleX (Becton-Dickson), complete BHI broth (35g/l BHI, 50µg/ml hemin, 1% IsoVitalex), or

Chamberlains Defined Media (12). All bacterial strains cultured on chocolate agar were grown at 37°C. Broth cultures were incubated in a shaking water bath at 37 ºC.

TC-1 (ATCC CRL-2785) is a tumor cell line derived from primary lung epithelial cells of C57BL/6 mice. J774A.1 (ATCC TIB-67) is a reticulum cell sarcoma mouse macrophage-like cell line. Cell lines were cultured in the ATCC recommended media at 37 ºC and 5% CO_2 atmosphere.

Plasmids and Molecular Techniques

Cloning of *F. tularensis* LVS DNA was conducted by PCR amplification of genomic DNA using Pfu turbo DNA polymerase (Stratagene) and cloned into pCR Blunt-II TOPO vector (Invitrogen) using the manufacturer's protocols. Plasmids for complementation experiments were created by ligating cloned regions of the *F. tularensis* LVS genome into pKK MCS, a pKK214 GFP (1) derivative where a *gfp* tet^R fragment was removed and replaced with a fragment containing a multiple cloning site (MCS) and a kan^R allele. The kanamycin resistance gene was a *F. tularensis* codon optimized version of *aphA1* synthesized by Blue Heron Biotechnology that is expressed from a modified *F. tularensis* *groEL* promoter (19).

Gentamicin Protection Assay

To determine the rate of intracellular invasion and replication, *F. tularensis* LVS strains were cultured to mid-exponential phase in Chamberlains Defined Media and then added to J774 or Tc-1 monolayers at a multiplicity of infection (MOI) of 100 in 200µl prewarmed tissue culture media. Gentamicin protection assays were then conducted as described previously (34). Assays were done in triplicate and statistical significance was

determined using unpaired t tests with unequal variance on the log transforms of recovered CFU to compare mutant strains to the wild type.

Mutagenesis and Allelic Exchange.

A *ripA* deletion construct was made by Splice Overlap Extension (SOE) PCR (40) using primers designed to delete the *ripA* locus and maintain 1kb flanking regions. All *ripA* sequence between the start to the stop codon was deleted. The construct was sequenced to confirm the deletion was in frame and for the integrity of the flanking DNA sequence. A *BamHI-NotI* fragment containing the deleted allele was ligated into pMP590 $kan^{R}sacB$ for allelic exchange (46). Kanamycin (10µg/ml) was used to select for plasmid integration and 10% sucrose to counterselect for resolution as described by LoVullo et el. (46). Based on sucrose resistance and kanamycin sensitivity strains were screened for the loss of the wild type allele and retention of the deletion allele by PCR of genomic DNA.

Invasion and Vacuolar Escape Assay.

Intracellular bacteria accessible to cytoplasmically delivered antibodies were enumerated by the methods described previously (16). *F. tularensis* LVS Δ*ripA* accessibility to the cytoplasm was compared to wild type at 20 minutes, 60 minutes, and 180 minutes post invasion in J774 cells in triplicate assays per time point. Bacteria accessible to the cytoplasm are described as vacuolar and bacteria inaccessible to the cytoplasm as cytoplasmic.

Mouse Model of Pulmonary Infection.

Francisella strains were prepared for intranasal inoculation by culturing in Chamberlains Defined Media to mid-exponential phase prior to suspension in PBS. Anesthetized 6- to 8-week-old female C57BL/6 mice were inoculated intranasally with 10^5 CFU of *F. tularensis* suspended in 50 µl of PBS. Inoculations were conducted in groups of four mice for each time point. Organ burdens were evaluated by homogenization and serial dilution before plating on Chocolate agar to determine recovered CFU per organ as described previously (Hall). To compare mutant strain to wild type organ burdens statistical analysis was conducted on log transforms of organ burdens using independent unpaired t-test.

Reverse Transcriptase PCR

Total RNA was isolated from mid-exponential phase *F. tularensis* LVS in Chamberlains Defined Media using a mirVana RNA isolation kit (Ambion). Contaminating DNA was removed using RQ1 DNase (Promega) in a 1 hour digestion at 37 °C and first strand cDNA generated using SuperScript III Reverse transcriptase (Invitrogen) as per the manufacturer's protocols with 250ng of Random Primers. First strand cDNA was buffer exchanged using a Qiagen QIAquick PCR purification kit. cDNA was then quantified using a ND-1000 (Nanodrop) spectrophotometer. PCR analysis for expression of *ripA*, *FTL1913*, and *FTL1912* was conducted using 20ng cDNA per 50µl PCR reaction amplified for 25 cycles. As a control for DNA contamination a Reverse transcriptase (RT) reaction was conducted without the RT enzyme. Analysis of expression was conducted by agarose gel electrophoresis and ethidium bromide staining.

Agarose Formaldehyde Electrophoresis and Northern Analysis

Total RNA was harvested from mid-exponential phase *F. tularensis* LVS grown in Chamberlains Defined Media using RNAeasy columns (Qiagen). Total RNA was prepared for analysis by concentration using ethanol/sodium acetate precipitation followed by quantitation with a ND-1000 spectrophotometer (Nanodrop). RNA was separated using agarose-formaldehyde (2% agarose, 2.2M Formaldehyde) electrophoresis followed by capillary transfer to nitrocellulose as in Rueger et al. (61). Additional lanes of the membrane were cut off and stained with methylene blue to assess rRNA bands for degradation and equality of loading. Digoxigenin labeled RNA probes were generated using a Northern Starter Kit (Roche) and the manufacturer's protocols. Hybridization, washing, and detection were done the manufacturer's (Roche) protocols.

Membrane Fractionation

F. tularensis LVS was cultured to mid-exponential phase in Chamberlains Defined Media (12), pelleted by centrifugation, and resuspended in lysis buffer (10mM Tris pH 7.5, 150mM NaCl). Lysozyme was added to 0.1mg/ml and the bacterial suspension incubated on ice for 30 minutes. A 1:1 cell suspension with 0.1mm silica beads was beaten for 15 minutes in a Disruptor Genie (Scientific Industries). Cell lysates were collected after the beads settled and the beads were washed 3 times with lysis buffer. The cell lysate was clarified by centrifugation at 12,000 x g for 4 minutes. The crude membrane fraction was collected by ultracentrifugation at 100,000 x g for 90 minutes, and the supernatants were saved as the cytosolic fraction. The crude membrane fraction was washed one time by re-suspension in lysis buffer and ultracentrifugation. The cytoplasmic membrane fraction was solubilized

with lysis buffer and 10% sarkosyl (Sigma) added to a 0.2% final concentration. The outer membrane fraction was then pelleted by ultracentrifugation at 100,000 x g for 60 minutes (17, 52).

Recombinant RipA and anti-RipA Antiserum Production

The *F. tularensis* LVS *ripA* gene was cloned and sequenced to confirm the integrity of the DNA sequence. The *ripA* allele was ligated into a pQE30 (Qiagen) vector to construct an N terminal 6x His tag fusion. The *ripA::6His* allele was induced using 0.1mM IPTG for 4 hours and harvested by centrifugation. The cell pellet was lysed by lysozyme treatment and sonication, before clarification by centrifugation. The crude membrane fraction was pelleted by ultracentrifugation at 115,000 x g for 60 minutes. RipA::6His was purified from the crude membrane fraction using a Qiagen native purification protocol with buffers containing 1% Triton X-100 and 10mM β mercaptoethanol. The purified RipA::6His was analyzed for purity by SDS-PAGE and Coomassie staining. The purified protein was methanol chloroform precipitated (71) and resuspended in PBS. Purified RipA::6His was sent to Proteintech Group, Inc for the production of Rabbit antiserum by the standard Proteintech long protocol. Preimmune bleeds were collected from each rabbit used in the protocol. Antiserum was screened for reactivity and specificity by Western blotting of the purified protein.

SDS-PAGE and Western Analysis

SDS-PAGE was conducted by the methods of Laemmli et al (43) utilizing a denaturing discontinuous 12.5% acrylamide gel. Total protein loaded in each sample was equivalent as determined by a BCA assay (Pierce). Proteins were transferred to

nitrocellulose and blocked in 1% BSA blocking buffer (0.05% Tween 20, PBS) overnight. Antibody incubations were conducted in blocking buffer. Membranes were washed in 0.05% Tween 20 PBS. Primary incubations and secondary antibody incubations were for 60 minutes. Rabbit anti-RipA serum was used at 1:1000, Chicken anti-AtpB IgY at 1:5000 (AgriSera), Mouse anti-GFP IgG (Abcam) at 1:1000, Goat anti-rabbit IgG-HRP at 1:10,000 (Sigma), and Goat anti-Chicken IgG HRP at 1:10,000 (Sigma). Development was conducted using Pierce SuperSignal Pico substrate as the manufacturer's protocols and exposed to autoradiography film.

RESULTS

Identification of RipA as a Virulence Factor

To identify genes involved in *Francisella* virulence, we created and screened a transposon mutant library of *F. tularensis* Live Vaccine Strain (LVS). Library construction was done as described previously (42). Gentamicin protection assays were conducted in the TC-1 lung epithelial cell line to identify strains defective for intracellular growth (Figure 1). One mutant strain was identified that invaded epithelial cells at the same frequency as *F. tularensis* LVS, but did not replicate after invasion. At 6 hours post inoculation there was no significant difference between the numbers of intracellular mutant or wild type CFU recovered from TC-1 cells; however, by 24 hours post inoculation significantly fewer mutant organisms, $1.9x10^5$ versus $1.1x10^7$ CFU ($P < 0.001$) were recovered from the LVS and Tn*5* mutant infected cells, respectively.

Identical assays were performed using the J774A.1 mouse macrophage-like cell line to compare the intracellular growth of wild type LVS and the Tn*5* mutant strain in epithelial and macrophage – like cell lines (Figure 1). As in the TC-1 epithelial cell line, the mutant

and wild type strains entered J774 cells at the same frequency. However, in contrast to TC-1 cells, the Tn*5* mutant replicated within J774 cells, but to a lesser extent than wild type LVS. At 24 hours post inoculation, $8.2x10^7$ versus $8.7x10^6$ CFU ($P < 0.01$) were recovered from LVS and Tn*5* mutant infected cells, respectively. The notable difference when TC-1 and J774A.1 cell lines were compared was the amount of intracellular replication of the Tn5 mutant strain. No significant replication was observed in TC-1 cells, while moderate replication was seen in J774 cells. These findings were also observed in other lung epithelial cell lines (MLE-12, A549) and in bone marrow derived mouse macrophages (data not shown).

Sequence analysis of the DNA flanking the Tn5 insertion showed that the interrupted allele was *FTL1914* (Figure 2). This allele was termed *ripA* (required for intracellular persistence, factor A). The *ripA* locus is predicted to encode a 178 amino acid membrane protein conserved in all sequenced *Francisella* strains. There is only one copy of the locus in each sequenced genome. pBLAST (26) was used to identify similar non-*F. tularensis* proteins ($E < 1$). These were hypothetical membrane proteins of unknown function in *Streptomyces coelicolor A3(2)* ($E = 1x10^{-10}$), *Beggiatoa sp. PS* ($E = 2x10^{-10}$), *Moritella sp. PE36* ($E = 1x10^{-9}$), *Sulfitobacter sp. NAS-14.1* ($E = 0.006$), *Clostridium perfringens C strain JGS1495* ($E = 0.044$), a predicted membrane protein linked to a retron element in *Escherichia coli* ($E = 0.36$), and a nitric oxide reductase (NorW) in *Aeromonas salmonicida* ($E = 0.60$). NorW plays a role in nitric oxide detoxification through interactions with NorR and NorV; however, there are no NorR and NorV homologs encoded in the *Francisella* genome and the amino acid similarity is not with the functional oxidoreductase domain of

NorW. All the other homologous proteins revealed by the pBLAST analysis were of unknown function.

ripA is Required for Intracellular Survival

A *F. tularensis* LVS *ripA* deletion strain, LVS Δ*ripA,* lacking all but the start and stop codons, was created via allelic exchange (29, 46) using a deletion construct generated by splice overlap extension (SOE) PCR (Figure 2). Allelic exchange was achieved using the pMP590 vector as described by LuVullo et al. (46). The doubling time of the resulting mutant strain in Chamberlains Defined Media was approximately 150 minutes. This was not significantly different than the growth rate of the wild type LVS seeded in parallel cultures. The maximum density of the mutant strain was equivalent to wild type after 24 hours of growth in Chamberlains Defined Media (data not shown).

To assess the effect of deleting *ripA* on intracellular growth, gentamicin protection assays were conducted using the J774A.1 mouse macrophage-like and TC-1 lung epithelial cell lines as described previously (34). Invasion frequencies of the deletion mutant were not significantly different from wild type (P values>0.4) in all cases (Figure 3). However, the CFU recovered had decreased by 24 hours in both cell types indicating that this mutant had an intracellular survival defect. Mean recovery for LVS and LVS Δ*ripA* was 1.23×10^7 versus 2.60×10^2 CFU ($P<0.001$), respectively, at 24 hours in TC-1 lung epithelial cells (Figure 3A). Mean recovery for LVS and LVS Δ*ripA* was 1.04×10^8 versus 1.07×10^5 CFU ($P<0.001$), respectively, at 24 hours in J774 cells (Figure 3B). Intracellular replication of LVS Δ*ripA* was restored by *trans* complementation with *ripA* (Figure 3). The intracellular

growth defect of LVS □*ripA* was not due to increased sensitivity to gentamicin (data not shown).

Intracellular Trafficking of *F. tularensis* Δ*ripA*

Following uptake by host cells, *F. tularensis* escapes the phagosome and replicates within the cytoplasm (13, 27). Most mutant strains studied to date that fail to replicate intracellularly do not escape the phagosome (45, 63) with the notable exception of *F. novicida* Δ*iglD* (62) and *clpB* (48). LVS □*ripA* intracellular trafficking in J774A.1 cells was compared to LVS using a modification of methods described by Checroun et al. (13). J774A.1 chilled monolayers were inoculated with GFP-expressing mutant and wild type strains at an MOI of 100 and invasion synchronized by rapid warming At 20, 60 and 180 minutes post inoculation the number of cytoplasmic versus vacuolar bacteria was determined by antibody accessibility following differential membrane permeablization with digitonin. At each time point the percentage of antibody accessible mutant and wild type bacteria were statistically identical (Figure 4). Thus, the LVS Δ*ripA* strain escaped the phagosome at the same rate as wild type LVS. Further, the intracellular growth defect of this mutant strain is not because of a failure to reach the cytoplasm.

RipA is Required for Virulence in a Mouse Model of Infection

A mouse model of pulmonary tularemia was used to assess the role of *ripA* in *F. tularensis* pathogenesis. Anesthetized C57BL/6 mice were intranasally inoculated with 10^5 CFU. Organ burdens were measured by organ homogenization, serial dilution, and plating to determine viable CFU. Lung burdens were determined two hours post inoculation to monitor

the delivery of each strain to the lung. Lung, liver, and spleen organ burdens were determined 1, 3, 7, and 14 days post inoculation (Figure 5).

The CFU recovered from the lungs at 2 hours post inoculation were 2.48 x 10^4, 1.18 x 10^4, and 1.12 x 10^4 for LVS, LVS Δ*ripA,* and LVS Δ*ripA* pJRF146, respectively (Figure 5A). An independent two tailed t-test with unequal variance was used to compare the data at 2 hours post inoculation (log transforms of the data used to compare later time points). The differences in lung burdens at 2 hours were not statistically different, ($P > 0.2$) thus the strains were delivered to the lung at equivalent frequencies. However, by day 1, the organ burdens of LVS Δ*ripA* infected animals were significantly lower than those of wild type and complemented mutant strains ($P<0.001$). The number of LVS Δ*ripA* organisms in the lung decreased in the first 24 hours, during the same time period, the number of both wild type and complemented strains increased by at least one order of magnitude. LVS Δ*ripA* was not detected in the liver or spleen before day seven. Recovery of this strain was particularly low with only 1 mouse (day 7) out of 16 mice having a detectable spleen burden (> 30 CFU). By day 7 post inoculation, significant pathology was noted in mice infected with either LVS or LVS Δ*ripA* pJRF146 *ripA* (enlarged spleens 4 out of 4, liver lesions 1 out of 4.) Mice infected with wild type LVS were euthanized at day 7 due to significant mouse morbidity, reflecting the lack of day 14 data for the wild type organ burdens. Organ burdens were reduced by day 14 in mice infected with LVS Δ*ripA* or LVS Δ*ripA* pJRF146 *ripA* relative to day 7 ($P<0.01$); however, one mouse infected with the complemented mutant strain died on day 8. These data indicate that deletion of *ripA* results in significant attenuation of LVS.

The *ripA* gene is independently transcribed

Analysis of the *ripA* locus using FGENESB (www.softberry.com) suggested that *ripA* may be co-transcribed with *FTL1913*. A combination of reverse transcriptase (RT) PCR and Northern analysis was used to address this possibility. RT-PCR utilizing primer sets in *ripA*, *FTL1913*, and *FTL1912* were used to map transcripts (Figure 6A). *FTL1912* was included because there is a predicted Rho independent terminator between *FTL1912* and *FTL1913*. The presence of bridging transcripts was assessed by RT-PCR using primers in adjacent reading frames (Figure 6A).

FTL1912, *FTL1913* and *ripA* were all expressed under the conditions tested. There was a lack of any detectible product from reaction utilizing primers bridging *FTL1912* and *FTL1913* suggesting that the predicted Rho independent terminator prevented transcription bridging the reading frames (Figure 6B). A faint amplification product was present in reactions using *FTL1913 – ripA* bridging primers. However, the band intensity was significantly lower than that of gene specific products (Figure 6B) suggesting that transcriptional termination occurred 3' of *FTL1913* and that *ripA* was predominantly contained in an independent transcript.

Northern analysis of total RNA harvested from mid exponential phase LVS and LVS *ripA::tn5* was used to measure the size of the *ripA* containing transcript. *In vitro* transcription was used to incorporate digoxigenin into a 500 nucleotide RNA probe specific to the 3' end of *ripA*. A 600 nucleotide *ripA* RNA was present in the wild type strain based on a digoxigenin labeled RNA ladder (Figure 6C), which is consistent with the 537

nucleotide *ripA* gene being transcribed independently. No *ripA* message was detected in The LVS *ripA::tn5* RNA samples.

Characterization of RipA

Subcellular fractions of LVS, LVS *ΔripA*, LVS *ΔripA* pJRF146 *ripA*, and LVS *ripA::tn5* were analyzed by Western blotting using anti-RipA antiserum (Figure 7A). RipA migrated at a relative molecular weight of 17 kDa. RipA protein was not detected in LVS Δ*ripA* or LVS *ripA::tn5*. Complementation with the native *ripA* allele in a multicopy plamid led to overexpression relative to the wild type strain.

When the translated amino acid sequence was analyzed with TopPred (14), RipA was predicted to be localized to the cytoplasmic membrane with three transmembrane domains and the amino terminus in the cytoplasm. Analysis of LVS pKK214gfp cytosolic, cytoplasmic membrane, and outer membrane enriched fractions (17, 52) by Western blot revealed that RipA localized to the cytoplasmic membrane fraction (Figure 7B). RipA was differentiated from proteins that reacted non-specifically with the RipA anti-serum by probing corresponding samples with preimmune serum. Antibodies to AtpB (Agrisera) and Gfp (Abcam) were used to determine subcellular fraction purity. GFP, a soluble cytosolic protein was detected with a Mouse anti-GFP monoclonal antibody. No GFP was detected in either membrane fraction. Low levels of AtpB and RipA were detected in the outer membrane fraction, indicating that the outer membrane fraction had low level contamination with cytoplasmic membrane proteins. However, the relative band intensities demonstrate that the majority of RipA was present in the cytoplasmic membrane fraction of *F. tularensis*.

DISCUSSION

The highly virulent nature of *F. tularensis* was recognized even before it was isolated and identified in the 1920's as the etiologic agent of a plague like disease in rodents. It is evident that the ability to survive and replicate within diverse host cell types is fundamental to *F. tularensis* pathogenesis. While the processes of host cell entry, phagosome escape, and intracellular replication have been described, the mechanisms used by which *F. tularensis* to achieve these have not. The recent creation and improvement of tools and procedures for the genetic manipulation of *F. tularensis* has led to the identification of a number of genes that are required for intracellular growth and/or virulence in animal models of tularemia.

Various *F. tularensis* mutants including *mglA* (4), *iglA* (17, 30), *iglC* (28, 44, 45) , *wbtA* (15, 65), *purF* (57), *purMCD* (55), *pmrA* (50), *clpB* (48), *acpA* (49), and *tolC* (25) exhibit decreased or absent intracellular replication in macrophages and are attenuated in mouse models of tularemia. Phagosomal escape has been studied in five of these mutants. *F. tularensis clpB* and *iglD* mutants escaped to the cytoplasm (48, 62), while *mglA*, *iglC*, and *acpA* mutants failed to escape the phagosome (45, 49, 63). Little is known about *F. tularensis* mechanisms responsible for phagosome escape or the mechanisms responsible for intracellular survival either inside or outside the phagosome.

Screening of a transposon insertion mutant library resulted in the identification of a gene that is required for *F. tularensis* persistence within host cells. RipA has homology to predicted proteins in other unrelated bacteria, but none of these homologies provide much insight into the function of RipA. We studied the expression and cellular localization of RipA in an effort to characterize RipA. The intracellular trafficking and replication of LVS

Δ*ripA* was studied as well as its ability to initiate and sustain infection in a mouse model of pulmonary tularemia as an indicator of the importance of *ripA* in *Francisella* pathogenesis.

Intracellular pathogens must overcome the host innate immune response to successfully colonize the intracellular niche. The primary host defense is centered on the antimicrobial properties of the phagosome. Most successful intracellular pathogens either escape the phagosome or divert phagosome maturation to their own ends. *Francisella* quickly escapes the phagosome into the cytoplasmic environment where it replicates (13, 27, 45). Recovery of LVS Δ*ripA* within epithelial cells or macrophages decreased dramatically after initially invading at the same rate as wild type LVS. When we evaluated the intracellular trafficking of LVS Δ*ripA,* it escaped from the phagosome at the same rate and frequency as wild type LVS. Thus, the intracellular survival defect was not due to failure to escape from the phagosome and subsequent killing in the phagolysosome.

The survival defect of LVS Δ*ripA* is likely due to host innate immunity. Innate immunity in the host cytoplasm can be characterized into compartmentalization by autophagy, direct microbiocidal activity, and nutritional deprivation. Nutritional deprivation in the cytoplasm is generally considered to have a bacteriostatic effect (2). This is exemplified by *F. tularensis* Δ*purF*, a purine auxotroph. *F. tularenis* Δ*purF* fails to replicate in the cytoplasm and persists for an extended period before declining gradually (57). This is not the phenotype demonstrated by *F. tularensis* Δ*ripA*. It declines dramatically between 6 hours and 24 hours in the intracellular niche. Though not conclusive, this rapid loss of viability is consistent with the possibility that *F. tularensis* Δ*ripA* is being degraded intracellularly.

This leaves either sensitivity to direct antimicrobial factors in the cytoplasm or autophagy to explain the intracellular killing of *F. tularensis* Δ*ripA*. Factors such as ubiquicidin (37) and histones have been purified from both the macrophage cytoplasm and compartmentalized vesicles and have been shown to have a direct bacteriocidal effect (36, 38); however, the significance of these factors in pathogenesis is unknown. Autophagy is considered to be an important means by which host cells clear pathogens from the host cytoplasm (51, 59). Most cytoplasmic pathogens studied to date either evade or hijack autophagic mechanisms. For example, *Shigella flexneri* and *Listeria monocytogenes* evade autophagy in a process requiring *de novo* protein synthesis (54, 59). While *Legionella pneumophila*, *Salmonella enterica*, and *Coxiella burnetti* exploit the autophagic machinery (6, 7, 35, 60, 67). *Francisella* is different in that it escapes to the cytoplasm, and that evasion of autophagy, after escape from the vacuole, is not dependent on *de novo* protein synthesis (13). We hypothesize that autophagy of intracellular *F. tularensis* Δ*ripA* is the most likely mechanism for the decrease in intracellular viability and that the decreased intracellular viability is responsible for the severe attenuation in the mouse model of infection.

In an effort to connect RipA with the observed phenotypes of the LVS Δ*ripA* mutant, we studied the predicted amino acid sequence to hypothesize a function for RipA. RipA has homology to a group of hypothetical membrane proteins of unknown function in unrelated bacterial species. Homology is limited to conserved domains of RipA suggesting conservation of function; however, significant divergence is displayed in other regions of RipA. We propose to investigate the function of RipA through protein-protein interactions with the aim of inferring its function based on interacting proteins. From these interactions a

function for RipA may be inferred leading to a viable hypothesis on the role of RipA in *F. tularensis* pathogenesis.

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ATTRIBUTIONS

I isolated the *ripA*::Tn*5* mutant and characterized the intracellular growth phenotype in macrophages and ATII cells as well as the attenuated growth characteristics of this mutant in mice. These observations provided the basis for in depth analysis of this locus. Additionally, I assisted with mouse inoculations and organ burden studies performed in this chapter.

FIGURES

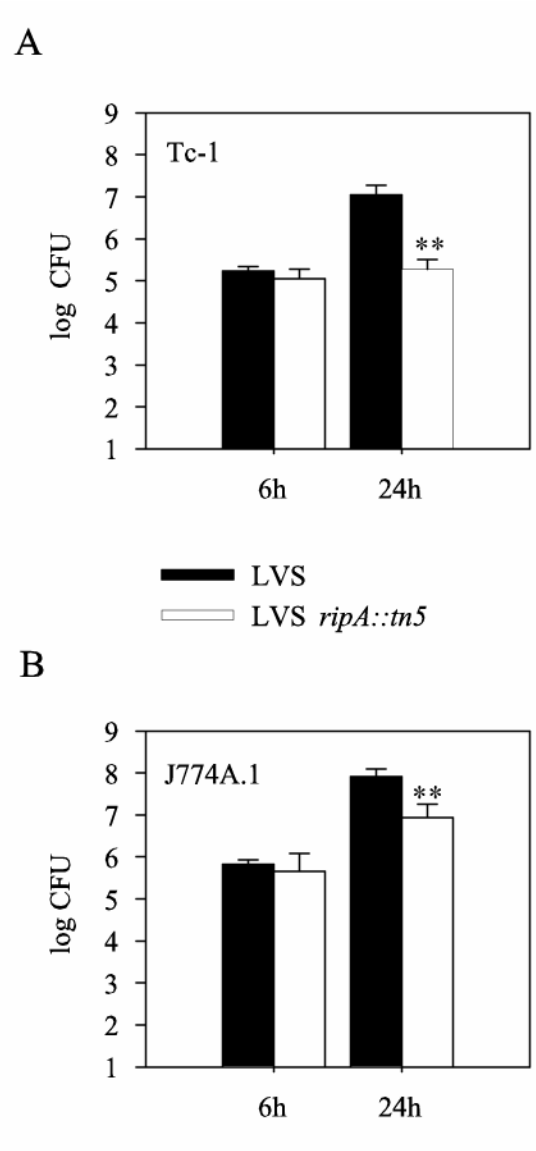

Figure 7.1. Gentamicin protection assays of LVS and LVS *ripA::tn5* were conducted in TC-1 (A) lung epithelial cells or J774A.1 (B) mouse macrophage-like cells at an MOI of 100. Mean CFU (N=3) at 6 hours and 24 hours post inoculation with error bars ± one standard deviation. Student's t-tests were conducted comparing LVS to LVS *ripA::tn5* at each time point ($P<0.01$ demarcated by **).

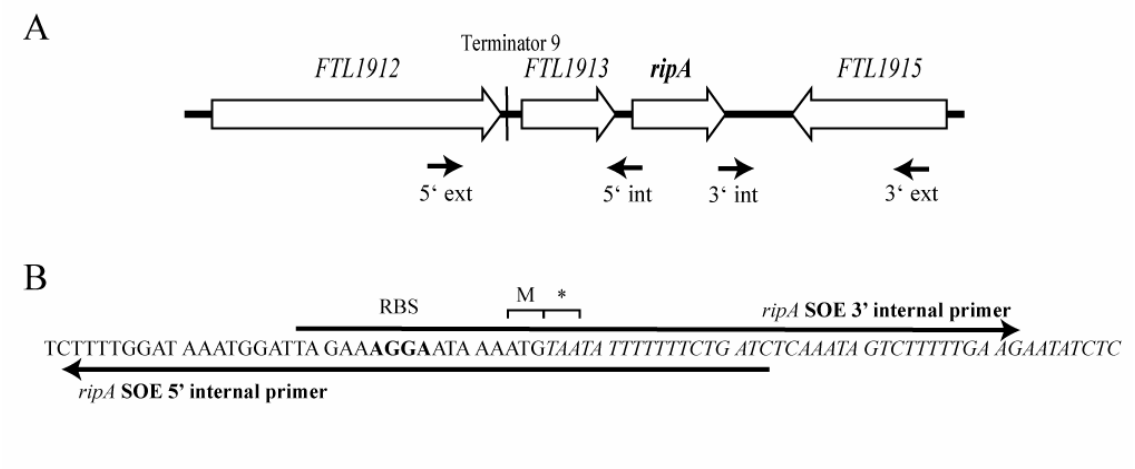

Figure 7.2. (A) Graphical representation of the genomic organization of the LVS *ripA* locus. Loci in close proximity to *ripA* (*FTL 1914*) on the chromosome are *FTL 1912* (30S ribosomal protein S1), *FTL 1913* (Sua5/YciO/YrdC family protein), and *FTL 1915* (Acetyltransferase). Primers utilized for SOE PCR are marked by arrows. (B) DNA sequence of the *ripA* deletion marked with the forward and reverse overlapping internal primers used in the SOE PCR. The predicted ribosome binding site (RBS) and the remaining *ripA* codons (M and *) are marked.

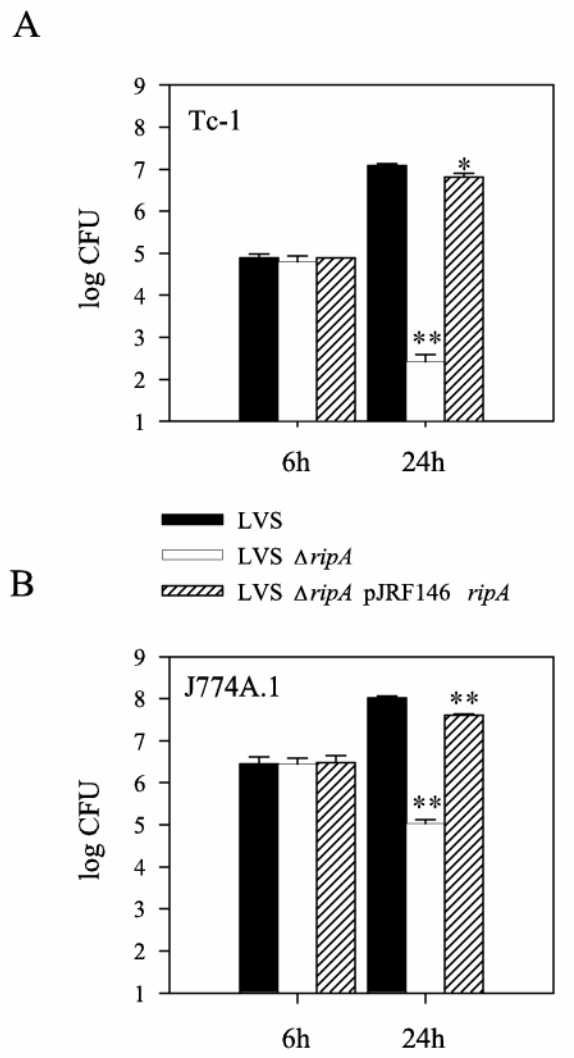

Figure 7.3. Gentamicin protection assays were conducted in TC-1 (A) lung epithelial cells or J774A.1 (B) mouse macrophage-like cells at an MOI of 100. Mean CFU (N=3) at 6 hours and 24 hours post inoculation with error bars ± one standard deviation. Student's t-tests were conducted comparing strains to wild type LVS at each time point ($P<0.05$ emarcated by * and $P<0.01$ demarcated by **).

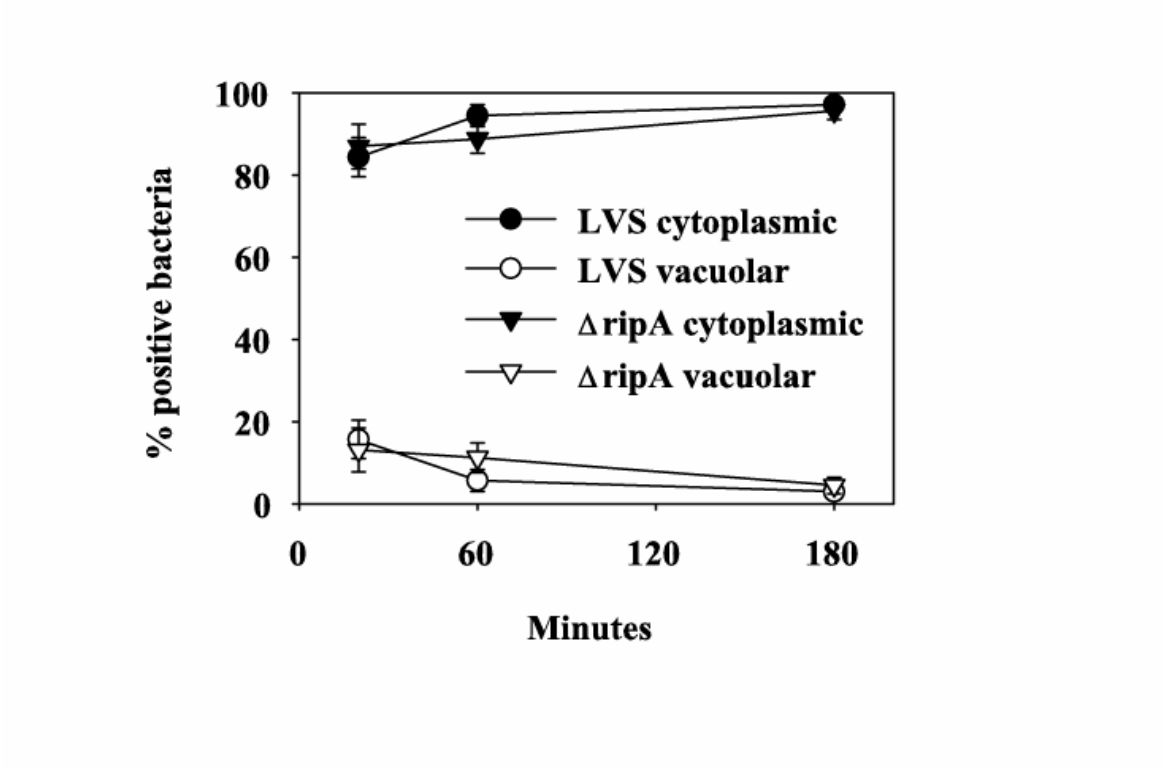

Figure 7.4. Mean percentage (N=3) of intracellular bacteria in the cytoplasm or vacuole at 20 minutes, 1 hour, and 3 hours post invasion of J774A.1 mouse macrophage-like cells with error bars representing one standard deviation. Bacteria in the cytoplasm determined by accessibility to cytoplasmically delivered antibodies. Vacuolar bacteria defined as bacteria protected from cytoplasmically delivered antibodies.

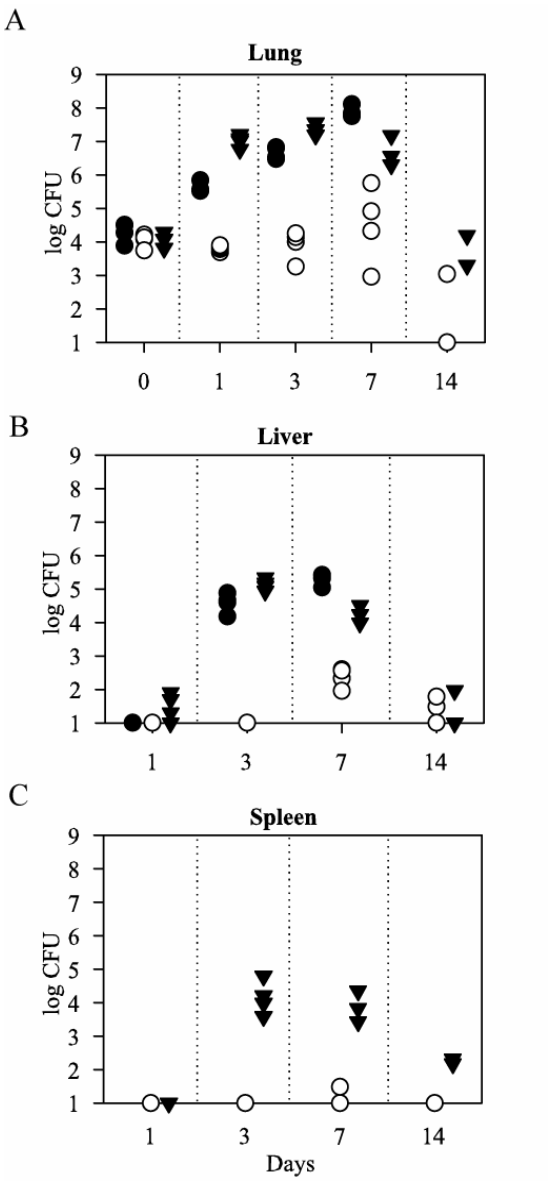

Figure 7.5. Bacterial organ burdens in infected mice (N=4) after intranasal inoculation quantitated as CFU and graphed as individual data points. Organ burdens in mice inoculated with LVS vector only (●), LVS Δ*ripA* vector only (○), and LVS Δ*ripA* pJRF146 *ripA* (▼) strains. Lung (A), liver (B), and spleen (C) burdens were determined at 2 hours (lung only), 1 day, 3 days, 7 days, and 14 days (LVS Δ*ripA* vector only and LVS Δ*ripA* pJRF146 *ripA*) post inoculation. Burdens that were below the limit of detection (~30 CFU) are plotted on the X axis.

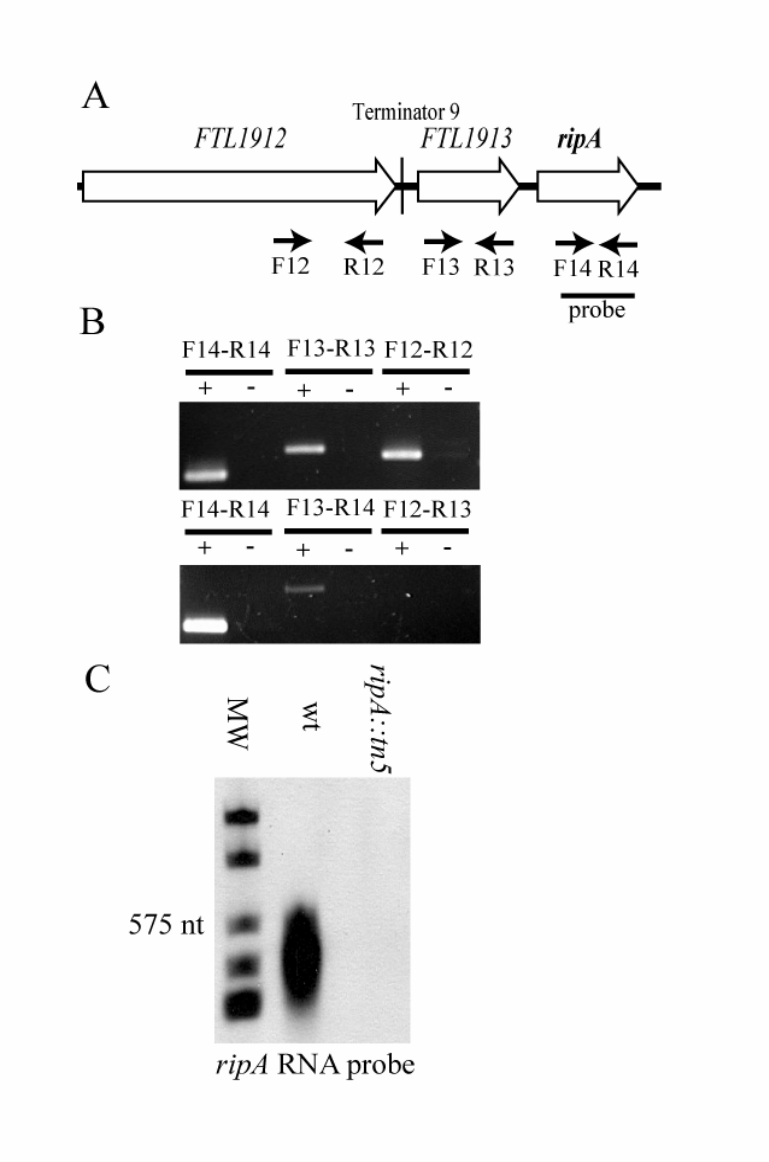

Figure 7.6. (A) Graphical representation of the *ripA* region. Primers utilized for RT-PCR marked are marked with arrows while the region complementary to the RNA probe used in the Northern analysis is demarcated by a solid line. (B) RT-PCR analysis of the expression of genes *FTL1912* (F14-R14), *FTL1913* (F13-R13), and *ripA* (F14-R14) shown in the upper image. Analysis for transcripts bridging *FTL1912* to *FTL1913* (F12-R13) and *FTL1913* to *ripA* (F13-R14) shown in lower image. PCR of cDNA demarcated by a + and reverse transcriptase negative reactions to assess DNA contamination marked as -. (C) Northern analysis to determine the transcript size of *ripA* containing RNA. Roche digoxigenin labeled RNA ladder is present in the left most lane followed by total RNA from LVS (wt) and LVS *ripA::tn5*. This analysis used a *ripA* complementary digoxigenin labeled RNA probe demonstrating the presence of monocistonic *ripA* transcript in LVS and the absence of the transcript in LVS *ripA::tn5*.

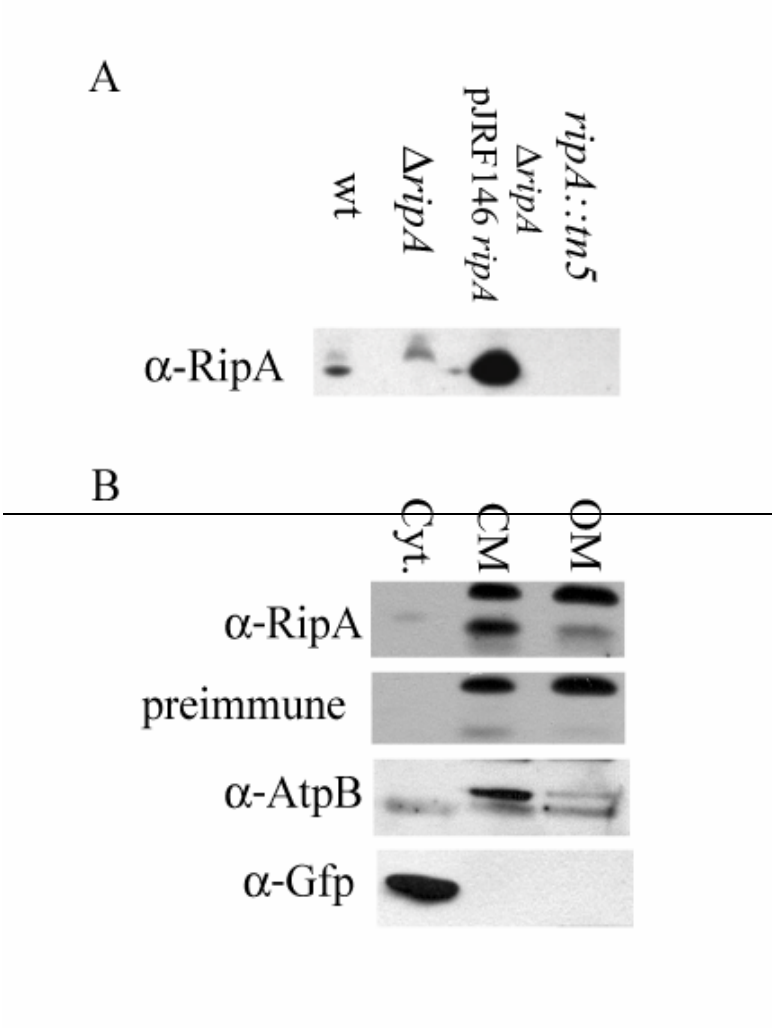

Figure 7.7. (A) Western blot analysis of cytoplasmic membrane enriched fractions of LVS, LVS Δ*ripA*, LVS Δ*ripA* pJRF146 *ripA*, and LVS *ripA::tn5*. Rabbit anti-RipA (1:1000) antiserum was used as the primary antibody. (B) Subcellular localization of RipA. Western analysis of cytosolic (Cyt.), cytoplasmic membrane (CM), and outer membrane (OM) enriched fractions of LVS pKK214 gfp. Non specific bands were determined with preimmune serum from the mouse used to generate the mouse anti-RipA serum. AtpB was used as a marker for the cytoplasmic membrane and GFP for the cytoplasm

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CHAPTER 8

Discussion and Conclusions

The ability of *Francisella tularensis* to replicate intracellularly within macrophages has been documented for quite some time (2). Several *Francisella* virulence factors have been related to *in vitro* intra-macrophage replication (5, 7-9). At the outset of these studies, very little was understood about the cellular targets for *Francisella* replication during disease *in vivo*. A goal of this research was to gain a deeper understanding of *Francisella*'s life within an animal host to enable more specific inquiries into how the bacterium causes disease. Specifically, we were interested in how, and to what extent, the bacterium interacts with cells of the lung following inhalation since this route of infection is associated with the most acute and rapidly progressing form of disease (3).

An important observation from Chapter 3 was that disease progression differs between closely related and commonly used *Francisella* strains. It is known that these different strains have vastly different disease outcomes in humans, though they all can initiate lethal infections in mice. Our studies determined that though disease outcomes were similar, mice infected with a subspecies *novicida* strain displayed fundamental differences in the impact on the lung environment when compared to subspecies *holarctica* or *tularensis*, both of which are pathogenic in humans. Subspecies *novicida* was taken up by neutrophils as early as 24 hours following inhalation, whereas *holarctica* and *tularensis* strains were not observed in neutrophils until day 3. Also, *novicida*-infected mice had decreased numbers of

macrophages, dendritic cells, and monocytes during disease progression that was not seen in *holarctica*- or *tularensis*- infected mice. Further work is needed to elucidate the molecular basis for these differences, however, these results illuminate the caution needed when drawing conclusions based on studies performed using different *Francisella* strains.

The data presented in Chapters 2 and 3 describe which cells become infected in the lung following inhalation of *F. tularensis* and how these cell populations change during the course of pulmonary tularemia. These are important considerations when attempting to understand how the bacteria initiates disease and avoids the immune system and likewise to understand how the host reacts to the bacterium and attempts to control infection. We have determined that in a mammalian host, *Francisella* is a promiscuous intracellular pathogen. Certain cell types, specifically alveolar macrophages and neutrophils, are predominant host cells for *Francisella* during the early and later stages of disease, respectively. However, bacteria also infect and replicate within interstitial macrophages, dendritic cells, monocytes, and alveolar type II epithelial cells. Understanding the makeup and timing of cells recruited to the lung during infection as well as which cells become infected by *Francisella* enables the formation of specific inquiries into the contribution and importance of each observation to the pathogenesis and progression of pulmonary tularemia.

The nature of these inquiries is two-pronged; an analysis of how the bacterium initiates infection and avoids the host response as well as how the host responds in an attempt to control infection. From the pathogen side, we can assess which bacterial factors are necessary for specific components of disease by generating genetic mutations and characterizing the resultant phenotypic outcome. In Chapter 6 we present a strategy for creating random transposon-insertion mutants that can be screened for deficiencies in

specific disease processes. An example of this is presented in Chapter 7 where a transposon insertion within the *Francisella* gene, *ripA*, ablated the ability of the bacterium to replicate within ATII cells and modestly reduced replication within macrophages. Further characterization was then accomplished for *ripA* via deletion analysis, complementation, and expression analysis as described in Chapter 7. This provides just one example of how genetic tools for making mutants can be applied to determine factors important in specific components of disease. The ability to isolate and characterize attenuated mutants paired with our detailed analysis of *Francisella* disease progression in the lung provides many avenues of inquiry into disease processes important during pulmonary tularemia.

Research presented in Chapter 3 demonstrates that association with neutrophils is a key component of *Francisella*'s lifestyle within the lung of an animal host. What remains unknown is how *Francisella* survives and replicates within neutrophils when a hallmark of this cell type is it's bactericidal nature. Understanding how *Francisella* subverts or avoids neutrophil killing could provide key insight into why *Francisella* infection is so severe and could provide targets for therapeutics against the bacterium. We demonstrate that neutrophils become the predominant host cell for *Francisella* during the later stages of pulmonary tularemia. Therefore, thwarting *Francisella*'s ability to avoid neutrophil-mediated killing could prevent disease progression. Other studies have shown that *Francisella* has the ability to block NADPH-oxidase assembly and function in human neutrophils (1, 6) which is likely a major factor in survival within these cells.

Using genetic tools and mutant screens to determine bacterial factors necessary for subverting oxidative-killing would be a major contribution to understanding *Francisella* pathogenesis. *Francisella* disruption of oxidative burst was studied in human neutrophils

using strains that can causes disease within humans. Many tularemia studies in mice make use of the *F. tularensis* subspecies *novicida* U112 strain that is highly virulent in mice, but avirulent in healthy humans. One possible explanation for this difference could be a strain-specific ability to survive in human neutrophils versus murine neutrophils. Strains virulent in humans, such as wildtype *tularensis* and *holarctica* strains could have the ability to survive and replicate within human neutrophils whereas strains avirulent in man, such as *novicida*, could be unable to survive in human neutrophils. Future studies will be needed to address this possibility.

To further understand the importance of neutrophils during infection, future studies will examine the impact of neutrophil depletion on disease progression and host response. Specifically, how the infected lung cell repertoire and cell recruitment is impacted in a lung environment depleted of neutrophils. Using analysis techniques described in Chapter 3, we can assess the degree and duration of neutrophil depletion following treatments that ablates these cells. Past studies determined that the absence of neutrophils led to more severe disease outcome following intradermal and intravenous challenge with LVS when compared to wildtype mice (10). Analysis of neutrophil populations after treatment with neutralizing antibody was missing from these studies as well as the impact of neutrophil depletion on inhalation-acquired tularemia. Analyzing the disease outcome, infected suite of cells, and cellular response in neutrophil depleted mice compared to untreated mice would provide significant insight into the importance of these cells during pulmonary tularemia.

Developing techniques to analyze *Francisella* disease progression in the lungs also gives us the ability to assess the importance of host factors in controlling infection. In Chapter 4, we examined the role of the chemokine receptor, CX3CR1, in cell recruitment to

the lung following bacterial inhalation as well as it's importance in controlling infection. Using flow cytometry-based lung cell analysis described in Chapter 3, we determined that the absence of CXC3R1 led to increased migration of monocytes and neutrophils into the infected lung, however this did not greatly impact which cells became infected nor did it impact the ability of the host to control infection. Therefore, we were able to conclude that CX3CR1 is not critical for the host response to *Francisella*, nor is it critical for the bacterium to cause disease. However, a deficiency in CX3CR1 does have implications in cell recruitment from the blood to the infected lung, as would be expected. The fact that recruitment was enhanced was a surprise since CX3CR1 is expressed on the cell surface of monocytes and through interaction with fractalkine (CX3CL1) expressed on the cell surface of endothelial cells, this interaction facilitates extravasation of circulating cells into infected tissues.

Another important consideration is the unique contribution of each cell type to disease progression during pulmonary tularemia. We identified several infected cell types that were likely to ingest an invading bacterial pathogen such as macrophages, dendritic cells, and neutrophils. However, we also observed replicating *Francisella* within alveolar type II epithelial (ATII) cells. These are stationary structural cells that are unable to chemotax to the site of infection. While these cells are not classified as professional phagocytes, they have high levels of metabolic activity, as they are the primary producers and exporters of lung surfactant (4). In Chapter 5, we demonstrated that entry into these cells did not require active bacterial processes, as killed bacteria were internalized into ATII cells just as efficiently as live bacteria. ATII cells constantly are exporting and recycling pulmonary surfactant, and this could provide a method of bacterial entry into these cells.

Once inside, *Francisella* replicated within these cells just as well as within other cell types, such as macrophages, known to harbor these organisms.

Interestingly, the *ripA* transposon mutant, described in Chapter 7, displayed cell type-specific intracellular replication. This strain was unable to replicate within ATII cells but retained the ability to replicate within macrophages. The *ripA* transposon mutant highlights the fact that there are differences between *Francisella* replication within epithelial cells and within macrophages. Unpublished results from our lab demonstrated that the *ripA* transposon mutant was defective for colonization of distal organs following inhalation, though it is unclear if this is a defect in dissemination from the lung or an inability to replicate within the liver or spleen. The strain persists in the lung for longer than a week, potentially due to it's ability, though diminished slightly from wildtype, to replicate within macrophages. We have begun studies in our lab to examine *Francisella* gene expression differences within macrophages versus epithelial cells using microarray analysis. By identifying genes that are uniquely expressed within a specific cell type, we can use genetic tools described here to generate mutants and subsequently analyze phenotypes *in vitro* and *in vivo*.

These studies have advanced our understanding of how *Francisella* causes disease in the lung following inhalation and have enabled us to develop a model of *Francisella* patho-biology in the lung during disease progression. Soon after inhaling *Francisella* into the lung, alveolar macrophages efficiently take up the bacteria. Bacteria are also phagocytosed by DCs and interstitial macrophages present in the lung. These cells are constantly patrolling the lung environment for invading pathogens, however, if *Francisella* comes into contact

with an ATII cell, it can be actively taken up by these cells, potentially during the uptake process used to recycle pulmonary surfactant.

Regardless the cell type, *Francisella* escapes from the phagosome and replicates within the cytoplasm. This leads to production of anti-inflammatory cytokines which, along with surface properties that enable the bacterium to avoid detection, prevent production of pro-inflammatory cytokines such as TNF-α and IL-6. This delays recruitment of circulating inflammatory cells such as monocytes and neutrophils. Early infected cells eventually become apoptotic or necrotic, and this may lead to production of molecular signals that influence neutrophils and monocytes to migrate into the infected lung. This migration is not dependent on the chemokine receptor CX3CR1. Neutrophils efficiently take up *Francisella*, however the bacteria are not killed but these cells become reservoirs for bacterial intracellular replication. Additional macrophages, DCs, and ATII cells become infected as the bacterial burden in the lung increases. Bacteria disseminate from the lung to distal tissues, such as liver and spleen through unknown mechanisms.

One explanation is that ATII cells, which are in close proximity to blood vessels due to the necessity of gas exchange with the blood, may provide a gateway to the bloodstream. Alternatively, infected DCs could carry *Francisella* to the lymph nodes and bacteria could travel via the lymphatic system to other locations in the body. In these ways, cells that are crucial for host defense against invading pathogens become facilitators for the disease progression of this successful intracellular pathogen.

Gaining a better understanding of *Francisella* pathogenesis can facilitate discovery of effective vaccines and treatments that would be beneficial to public health in the event of a large scale tularemia outbreak. More broadly, with the advent of antibiotic resistant bacterial

strains and the emergence of previously unknown pathogens, uncovering mechanisms utilized by pathogenic microbes will aid in the discovery of novel targets for antimicrobials that certainly will be required to combat infectious disease in the future.

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APPENDIX A

Supplemental Methods and Materials for Chapter 2

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Bacterial Culture

Francisella LVS was maintained on chocolate agar supplemented with Isovitalex (BD Biosciences). LVS was obtained from the CDC in Atlanta GA. GFP strain contained a modified pKK214gfp plasmid (a kind gift from Mats Forsman).

Cell culture and gentamicin protection assays

A549, MLE-12, and J774 cells were cultured as described by ATCC. Human macrophages were derived from peripheral blood by isolating adherent peripheral blood mononuclear cells from anti-coagulated venous blood. Adherent cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and L-glutamine for two weeks to enrich for macrophages. All *in vitro* gentamicin protection assays were performed with approximately 10^6 cells per well in a 24-well tissue culture dish. Bacteria were added at a multiplicity of infection of 100:1 in a volume of 200 µL per well. Supernatants were aspirated and replaced with 1 mL of media containing gentamicin (25 µg/mL) to kill extracellular bacteria 2 hours post-inoculation (J774, human macrophages) or 4 hours post inoculation (A549, MLE-12). Six and 24 hours post-inoculation cells were rinsed with PBS and scraped from the wells with sterile wooden applicator sticks, vortexed,

diluted, and plated on chocolate agar for bacterial recovery. All gentamicin protection experiments were performed in triplicate and standard deviations of the means were calculated and are represented by error bars. Fluorescence microscopy was performed on *in vitro* cell cultures by growing cells on sterile glass cover slips. Gentamicin was added to cells as described above. At the indicated time points, cover slips were washed and fixed with 4% paraformaldehyde prior to antibody staining. Following staining, cover slips were washed and mounted to slides with nail polish.

Mouse infections

Female 7-10 week-old C57BL/6 mice were inoculated with *Francisella* strains diluted in sterile PBS and enumerated by Klett reading or OD 600. Dose was verified by plating inoculum on chocolate agar. Mice were anesthetized with avertin until unresponsive to toe pinch, and 50 µL of bacterial suspension was dispensed onto nares of the mouse. All animal experiments were conducted in accordance with animal care and use guidelines, and animal protocols were approved by the IACUC at UNC-Chapel Hill.

Immunofluorescence staining of tissues

One, 3, and 7 days following intranasal-inoculation of female 6-8 week old C57BL/6 mice, infected tissues were aseptically removed and fixed in 10% neutral buffered formalin prior to paraffin embedding. 5 micron sections of nasal turbinates, trachea, and lungs were mounted to slides. Prior to staining, slides were de-paraffinized with xylene and rehydrated through a series of washes with decreasing concentrations of ethanol followed by a 30 minute incubation in 95^{o}C Target Retrieval Solution (DakoCytomation) pH=6.1. Slides were blocked with 3% bovine serum albumin (BSA) in PBS++ (1mM $CaCl_2$, 1mM $MgCl_2$, pH=7.4) for 2 hours and primary antibody incubations were performed in 1% BSA in PBS++

overnight at room temperature. Slides were washed 3X with PBS++ followed by incubation with secondary antibodies in PBS++ for 1 hour. Slides were washed 3X with PBS++ and mounting media + DAPI (Vector) was added. Probed sections were sealed with cover slips using nail polish. All fluorescence microscopy was performed using a Zeiss Axioplan 2 microscope.

Lung cell isolation

Mice were anesthetized with avertin+heparin (1000 U/mL) and profused with 4-7 mL of PBS+heparin (200 U/mL). Tracheas were cannulated using a 16-gauge blunt-tipped needle, and lungs were inflated with approximately 1 mL of dispase (BD Biosciences). The trachea was tied off with surgical sutures and lungs were removed and incubated in 3.0 mL dispase at room temperature for 45 minutes. Tracheas were removed, and lungs were transferred to a Petri plate along with 7 mL of PBS+DNaseI (250 µg/mL) and tissue was teased apart using forceps. Cells were gently swirled for 1-2 minutes, and the suspension was filtered through 40 µm mesh. Filtered suspensions were pelleted by centrifugation at 300 x g for 5 minutes at 4^{o}C and resuspended in 1 mL red blood cell lysis solution for 2 minutes at room temperature before adding 9 mL PBS to neutralize osmolarity. Cells were pelleted and resuspended in PBS and enumerated.