The Interaction of *Francisella tularensis* with Lung Epithelial Cells

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ABSTRACT

Robin R. Craven: The Interaction of *Francisella tularensis* with Lung Epithelial Cells
(Under the direction of Thomas H. Kawula)

Francisella tularensis, a gram-negative facultative intracellular bacterial pathogen, is

the causative agent of the disease tularemia in humans and other mammalian hosts. While

tularemia can be acquired by a number of routes, the respiratory route results in the most

severe disease. As few as 10 organisms can lead to pneumonic tularemia, which can have a

fatality rate as high as 30-60% if untreated.

Macrophages have long been considered the primary site of *F. tularensis* replication

in infected animals, and many of the genes identified as necessary for virulence are related to

survival and replication in the macrophage. However, we demonstrate that *F. tularensis* also

invades and replicates within type II alveolar epithelial (ATII) cells in a mouse model of

respiratory tularemia. We used TC-1 cells, a mouse lung epithelial cell line, to study *F.

tularensis* invasion and intracellular trafficking within nonphagocytic cells. Live and killed

F. tularensis live vaccine strain (LVS) associated with, and was internalized by, TC-1 cells

with a similar frequency and kinetics. Inhibitors of microfilament and microtubule activity

resulted in significantly decreased *F. tularensis* invasion, as did inhibitors of PI3 kinase and

tyrosine kinase activity. Once internalized, *F. tularensis* containing endosomes associated

with EEA1 then LAMP-1, followed by bacterial escape into the cytoplasm. Collectively

these results suggest that *F. tularensis* epithelial cell invasion is mediated by a preformed

ligand on the bacterial surface and driven by host cell processes, and that internalized *F.

tularensis trafficks along the endocytic pathway before escape into the epithelial cell

cytoplasm where replication takes place.

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AMCA   7-amino-4-methylcoumarin-3-acetic acid

ATI   type I alveolar epithelial cells

ATII   type II alveolar epithelial cells

CDC   Centers for Disease Control and Prevention

cDNA   complementary DNA

CFU   colony forming units

EEA1   early endosome antigen 1

FPI   *Francisella* pathogenicity island

GFP   green fluorescent protein

IAHP   IcmF-associated homologous proteins

LAMP-1   lysosome associated membrane protein 1

LAMP-2   lysosome associated membrane protein 2

LVS   Live Vaccine Strain

mg   milligram

ml   milliliter

mM   millimolar

MOI   multiplicity of infection

nM	nanomolar
OM	outer membrane
PCR	polymerase chain reaction
PFA	paraformaldehyde
SP-A	surfactant protein A
SP-B	surfactant protein B
SP-C	surfactant protein C
SP-D	surfactant protein D
T1SS	type I secretion system
T2SS	type II secretion system
T3SS	type III secretion system
T4P	type IV pili
T4SS	type IV secretion system
T5SS	type V secretion system
T6SS	type VI secretion system
μg	microgram
μl	microliter

μm   micrometer

μM   micromolar

WHO   World Health Organization

CHAPTER 1

Introduction

History

The initial descriptions of the disease tularemia and the bacterium that causes it emerged almost simultaneously from several American labs during 1911-1914 (102). The U. S. Public Health Service established a lab in California in response to plague cases occurring after the San Francisco earthquake of 1906. Thousands of rodents from the region were cultured for the presence of the plague-causing bacteria, then known as *Bacillus pestis*. Observant researchers recognized that some ground squirrels tested had an infection that was indistinguishable from plague, except that they were unable to culture *Bacillus pestis* from these animals. In 1911 George McCoy published these findings in an article entitled "A Plague-Like Disease of Rodents" (71). McCoy reported that the disease could be transmitted to a variety of healthy animals, and could be experimentally transmitted by fleas. While researchers were unable to grow the causative agent, they did observe small bacilli in some samples. McCoy went on to point out in this paper that the number of species susceptible to this disease lead to suspicion that man might be susceptible as well. In 1912 McCoy and C.W. Chapin announced that they had cultured the organism causing the plague-like disease, and given it the name *Bacterium tularense* (72).

During the same time period that McCoy and Chapin were studying rodents in California, a physician in Utah noticed a sickness in his patients that was associated with biting flies (102). Dr. R. A. Pearse presented a paper titled "Insect Bites" to the Sixteenth Annual Meeting of the Utah State Medical Association in 1910 (78). The paper, describing an ulcer at the site of a fly bite, followed by swollen and suppurated lymph nodes in the region of the bite, fever and chills was published in March, 1911, one month before McCoy's description of the disease in squirrels. Pearse described cases in 6 patients, with severity ranging from mild to deadly (78).

In 1914 Wheery and Lamb described a case of a 21 year old meat cutter in Cincinnati, Ohio, who was treated for an ulcerative conjunctivitis (115). Though initial cultures of the lesions were negative, a guinea pig injected with material from the ulcer died six days later of a disease resembling the plague-like disease of rodents described by McCoy. Wheery and Lamb were able to grow *Bacterium tularense* using the egg-yolk culture medium described by McCoy and Chapin, hence identifying the first confirmed human case of the disease that would come to be known as tularemia (102).

Numerous incidents of a disease resembling tularemia were described in the Soviet Union in the 1920s (82), most frequently associated with contact with water rats. This disease could be propagated in animals following injection of the lymph node contents of infected people, and the causative agent of the animal infections could be cultured using egg yolk medium. However, definitive identification of the Russian illness as tularemia would not occur until 1929-1930, when the serum of G.I. Zarkhi, a researcher who had contracted the illness, was sent to McCoy for testing. The serum agglutinated the American *B. tularense* culture, and bacteria cultured from infected Russian lab animals had the same characteristics

as the American strain, confirming the presence of tularemia in Europe (82). Once tularemia had been identified and described, many illnesses in North America, Europe and Japan that had previously been attributed to other organisms, or to unknown causes, were classified as probable cases of tularemia in retrospect (82, 103).

By 1937 6,149 cases of human tularemia had been documented in the U.S. (55), a number that peaked with 2,291 cases of tularemia reported in 1939 alone (16). However, cases were rare in New England, which was attributed to the absence of cottontail rabbits in that region (55). While early reports of tularemia implicated rodents as having an important role in the transmission of tularemia to man, Jellison and Parker reported that approximately 90% of human cases were the result of contact with infected lagomorphs, particularly cottontail rabbits, and the remaining 10% of cases included transmission by arthropod vectors, rodents, sheep and other animals (56). In the 1930s massive numbers of rabbits were imported to New England from Missouri, Kansas and Oklahoma, areas of endemic tularemia, for hunting purposes (55). Since that time tularemia has been seen in New England. Tularemia is now endemic on Martha's Vineyard, Massachusetts, the site of the only known outbreaks of primary pneumonic tularemia in the U.S. (70).

Routes of exposure and types of disease

I know of no other infection of animals communicable to man that can be acquired from sources so numerous and so diverse. In short, one can but feel that the status of tularemia, both as a disease in nature and of man, is one of potentiality.—R. R. Parker (76)

Tularemia is caused by the small gram-negative coccobacillus, *Francisella tularensis*. About 200 cases of tularemia occur each year in the United States (CDC). The disease is endemic throughout much of Europe, where large outbreaks involving hundreds of cases occur periodically (110). Humans contract tularemia via a number of routes. The most typical naturally occurring form of the disease is ulceroglandular tularemia, which results from skin or mucous membrane contact with *F. tularensis* (2). Exposure may occur by direct contact with an infected animal (31, 118), or through the bite of an arthropod vector (2, 62, 67). An ulcer develops, and if untreated the draining lymph nodes may enlarge and suppurate (109). Oculoglandular and oropharyngeal tularemia result from bacterial contact with the eyes, or ingestion of contaminated food or water, respectively (9, 10, 51, 91). Primary pneumonic tularemia results from inhalation of *F. tularensis*, while dissemination from initial sites of infection to the lung can result in secondary pneumonic tularemia (30, 70). Patients with pneumonic tularemia experience symptoms that may include sporadic fever, fatigue, chills, malaise, myalgia, headache, and nonproductive cough (32). Chest X-rays are usually normal in the early stage of infection, then may begin to show infiltrate and pleural effusion as the disease progresses (70). Patients with typhoidal tularemia present with generalized symptoms, similar to those of pneumonic tularemia, but without an identifiable route of infection (30). The non-specific nature of symptoms, which can overlap with those caused by illnesses such as Rocky Mountain Spotted Fever and Lyme Disease, complicates the diagnosis of tularemia (70). The overall mortality rate due to tularemia in the United States is less than 2%. Untreated, that rate rises to 5-15% for infections with virulent strains, and 30-60% for pneumonic and typhoid forms of tularemia (30).

Biodefense

Francisella tularensis is an ideal candidate for development as a biological weapon due to its very low infectious dose and ability to cause disease following inhalation, and as such was designated a Category A select agent by the CDC (93). Japan's infamous research unit 731 studied the effects of *Francisella* and other agents on human subjects in the years leading up to and including World War II (52). *Francisella* was a part of the biological weapons programs of both the United States and the former USSR during the cold war (5, 22, 30, 101) Ken Alibek, a former Soviet bioweapons researcher, suggests that *F. tularensis* was used offensively against the Germans during WWII (5). However, evidence suggests that the large outbreaks of tularemia during WWII were likely naturally occurring and related to ideal conditions for the disease that existed during wartime (39). Alibek also claims that the Soviets continued to develop *Francisella* as a biological weapon into the 1990s (5).

A World Health Organization (WHO) model developed in 1969 predicted that release into the air of 50 kg of virulent *F. tularensis* over an area inhabited by 5 million people would result in 250,000 illnesses and 19,000 deaths (1). Using this model the CDC extrapolated the economic impact of such an attack, and predicted it to be $5.4 billion for every 100,000 people exposed (58). The Biological and Toxin Weapons Convention, which prohibits development of biological weapons, has been in effect since 1975 and signed by 144 countries (88). However, recent world events including the September 11, 2001 terrorist attacks, and the intentional dispersion of *Bacillus anthracis* spores through the U.S. mail that same year (4), have lead to renewed interest in bioterrorism preparedness research.

Strains and Distribution

There are four subspecies of *Francisella tularensis*. *F. tularensis* subsp. *tularensis* (Type A) and *F. tularensis* subsp. *holarctica* (Type B) are implicated in human disease. The two other subspecies, *mediasiatica*, and *novicida*, are considered non-virulent in immune competent people.

F. tularensis subsp. *tularensis* (Type A), found primarily in North America, is highly virulent in humans and animals. The highest incidence of tularemia in the United States is seen in Arkansas and Missouri, in the eastern parts of Oklahoma and Kansas, in southern South Dakota and Montana, and on the island of Martha's Vineyard in Massachusetts (3). Inhalation of as few as 10 organisms of a virulent type A strain can cause disease (97). Two subpopulations of Type A *F. tularensis* have been defined by pulsed-field gel electrophoresis that differ in distribution, transmission and disease outcome (106). These subpopulations are designated Type A-east for those isolates coming from states transecting or east of the 100th meridian, and Type A-west for those from states west of the 100th meridian (106). Infection is most frequently associated with ticks (type A-east) or biting flies (type A-west), or exposure to infected cats or lagomorphs (106). There is a marked difference in fatality rates between the type A subpopulations, with type A-east being 14% and type A-west being 0% (106). The less virulent *F. tularensis* subsp. *holarctica* (Type B) is found throughout Europe, Asia and North America. The geographic distribution of type B cases in the United States tends to fall along major waterways and areas with high rainfall (106), and is associated with tick bites and exposure to rodents or cats (106). Tularemia caused by type B strains is widely distributed throughout Europe and Asia, with highest incidences occurring in the Nordic region, and the former USSR (108). European tularemia transmission has been

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associated with rodents, lagomorphs, ticks, horse flies and mosquitoes, as well as with

contaminated water supplies (86, 103). Analysis of all clinical isolates of *F. tularensis*

submitted to the Centers for Disease Control and Prevention (CDC) between 1964 and 2004

indicated that of American tularemia cases 66% were Type A and 34% were type B isolates

(106).

The live vaccine strain (LVS) is an attenuated type B strain that was created by

repeated passage of a virulent strain. It is not approved for general use in the United States,

as the basis of its attenuation is unknown (89). However, LVS does cause a tularemia-like

disease in mice, and is used as a model for research purposes (8). *Francisella tularensis*

supsp. *novicida* (*F. novicida*), while not virulent in immune competent humans also causes a

tularemia-like disease in mice, and is also used in tularemia research.

Genomic Analysis and Genetic Tools

In order to discover the means by which pathogenic bacteria cause disease it is useful

to have tools available to genetically manipulate the bacteria. While spontaneous or

chemically induced mutants may yield phenotypes of interest, it is very difficult to identify

the gene(s) interrupted. Therefore, it is ideal to be able to introduce random mutations, with

associated markers, into the genome. Early mutant libraries were constructed in *F. novicida*

using strategies that involved manipulation and reintroduction of chromosomal DNA

fragments. Cassette mutagenesis allowed introduction of antibiotic resistance markers into

restriction sites of chromosomal DNA fragments, which were then transformed into *F.*

novicida (7). Antibiotic resistant transformants, which contained a genomic copy of the

mutated gene resulting from homologous recombination, were used to screen for a phenotype of interest, such as the inability to replicate in macrophages. Shuttle mutagenesis allowed more complete genome coverage when introducing mutations by using transposon insertions to randomly insert into genes rather than antibiotic resistance markers ligated into restriction sites (37). Transposon mutagenesis was carried out in *E. coli* on 5-10 kb fragments of *F. novicida* genomic DNA ligated into pUC18. Mutagenized plasmids were then transformed into *F. novicida*, where they were forced to integrate into the chromosome due to inability of the plasmid to replicate in *Francisella*, and transformants screened for the phenotype of interest (27, 48). It was subsequently reported that the Tn10 and Tn1721-based transposon insertions created by these shuttle mutagenesis strategies are not stable in *Francisella* (64). These early strategies were ineffective in human pathogenic strains of *Francisella*.

Of the predicted genes in *Francisella* subspecies *novicida*, *holarctica* and *tularensis*, 86.4% are common to all three (90). When comparing the nucleotide sequences of those common genes, *novicida* and LVS are 97.8% identical; *novicida* and Schu S4 are 98.1% identical, and LVS and Schu S4 are 99.2% identical (90). In spite of the genetic similarity between these species, it is obvious based on variability in infectious dose, disease severity, and host range that there are important differences between these strains. As the genetic determinants of *Francisella* virulence have begun to emerge, inter-species differences in some of those loci have been observed.   Examples of virulence related genes/operons that differ between subspecies include the pathogenicity island (*pmcA* and *pdpD*) and T4P/T2SS related genes (*pilAEV* and pilT), both of which are described in the following sections. For this reason, it is crucial that genetic tools be available for use in *Francisella* subspecies other than *novicida*. Chapter 5 describes our development of a strategy to create stable *F.*

tularensis LVS transposon insertion mutants (59). This method has now been used successfully in *F. tularensis* Schu S4 as well (83).

In addition to the creation of random mutant libraries, methods have been developed for the disruption of targeted genes by allelic exchange. This has been achieved using a one step strategy involving the introduction of PCR products consisting of upstream and downstream regions of homology to the gene of interest flanking an antibiotic resistance cassette (64). This method has only been used successfully in *F. novicida*, and the efficiency of allelic exchange is low (37). Suicide vectors have been used in type A (66, 113) and B (45, 66, 79) strains, as well as *F. novicida* (15, 17), to generate either marked or unmarked gene deletions. Merodiploids created by single crossover events resolve efficiently in *F. novicida* (84), while *sacB* counter selection for second crossover events can be used in subspecies *holarctica* and *tularensis* (45, 66). Rodriguez et al. recently described the development of group II introns for use in subspecies *tularensis*, *holarctica*, and *novicida* (87). Group II introns are mobile genetic elements composed of excised intron RNA and intron encoded reverse transcriptase (57). This catalytic RNA inserts into a target DNA sequence by a process known as retrohoming, and by designing introns that recognize sequence from genes of interest targeted mutations can be created (57). This strategy has the advantage of allowing simultaneous and efficient targeting of duplicate genes, such as the pathogenicity island genes in the human pathogenic *Francisella* strains (87).

Genes related to pathogenesis

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Due to easier genetic manipulation, and the lack of tools available for use in the other strains of *Francisella*, much of the early *Francisella* pathogenesis work was done in *F. novicida. Francisella tularensis* is a facultative intracellular pathogen, and the genes identified for virulence are largely associated with the ability to replicate in macrophages. A spontaneous mutation of *F. novicida* that decreases the organism's ability to replicate in macrophages was mapped to an operon that was termed *mglAB*(14). MglA expression peaks during exponential phase growth, and is induced during growth in macrophages and amoebae (13, 65). MglA has homology to the *E. coli* transcriptional regulatory protein SspA, and regulates the expression of genes potentially involved in pathogenesis (17, 65). PmrA also regulates a number of *F. tularensis* genes, and a *F. novicida pmrA* mutant is attenuated in macrophages and mice (12).

The *Francisella* pathogenicity island (FPI) is a 30 kb section of genomic DNA, surrounded by transposable elements, and containing a lower G+C content than the rest of the genome (75). Expression of FPI genes is regulated by both MglA and PmrA. The *F. novicida* pathogenicity island contains 17 ORFs, including *iglABCD* and *pdpABC*, which are predicted to form two convergently transcribed operons (12). Type A and B strains of *Francisella* contain nearly identical, but duplicate, copies of the FPI (75). The notable differences between the subspecies lie upstream of *iglA*, where *F. novicida* contains *pmcA* and *pdpD* genes. The type A strain Schu4 contains 3 small ORFs in place of *pmcA*, and *pdpD* contains a 150bp deletion, while in the type B strain LVS *pmcA* and the majority of *pdpD* are missing (74).

Mutants of many FPI genes have been evaluated for their effect on pathogenesis, which is associated with the inability of these mutants to replicate in macrophages. *F.*

novicida iglA and *iglC* mutants are defective for growth in macrophages (28, 46, 96). IglC was first identified as a 23 kd LVS protein that was up-regulated during growth in macrophages or after exposure to hydrogen peroxide, and the LVS *iglC* mutant is unable to replicate in macrophages and is avirulent in mice (44, 45). A *F. tularensis* Schu S4 *iglC* mutant is avirulent in mice as well (113). *F. novicida pdpA* and *pdpB* mutants are defective for replication in macrophages and are attenuated in mice (75, 111). The mechanisms by which these gene products cause disease largely remain to be elucidated.

Genes outside the FPI that are required for survival and replication in the macrophage have been identified as well. A gene necessary for survival and replication in macrophages and epithelial cells, as well as virulence in mice, *ripA*, will be described in detail in Chapter 4. ClpB is a heatshock protein which is responsible for disaggregating and reactivating proteins in *E. coli* (119), and is involved in stress resistance in a number of bacteria (19, 104). *F. novicida* mutant screens have identified *clpB* as necessary for replication in macrophages (46, 111). A LVS *clpB* mutant exhibits increased sensitivity to stess (i.e., heat, low pH, ethanol, oxidative stress), and decreased replication in macrophages compared to wildtype LVS and is severely attenuated in mice (73, 107). A mutant of another heat shock protein gene, *htpG*, which encodes an hsp90 chaperone homologue, was deficient for virulence in mice and replication in macrophages (114). A LVS mutant in a gene involved in LPS O-antigen biosynthesis, *wbtA*, is serum sensitive, unable to replicate in macrophages, and attenuated in mice (85, 100). Interestingly, O-antigen mutants in *F. novicida* were serum sensitive and attenuated in mice, but were able to replicate normally in macrophages (112). Additionally, mice immunized with LPS from a type A strain were protected against infection with a type B strain, and mice immunized with *F. novicida* LPS are protected

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against *F. novicida* infection, but neither group are protected against heterologous challenge (112). Other *F. novicida* genes required for growth in macrophages and disease in mice include *dsbB*, a gene whose product is used for proper folding of periplasmic proteins in *E. coli*, as well as genes which contribute to purine and pyrimidine biosynthesis (46, 111).

Genes required for virulence that are not required for replication in the macrophage have been identified as well. Two mutants identified in a F. *novicida in vivo* negative selection screen, FTT0748 and FTT0584, replicate normally in macrophages, but are severely attenuated in mice. These mutants induce rapid cell death in macrophages in an ASC and Caspase-1 dependent manner, indicating that these gene products may be responsible for modulating the host immune response to intracellular *Francisella* (114). *F. tularensis tolC*, is predicted to encode a protein whose homologues in other organisms form components of a multi-drug efflux pump or type I secretion system(29, 63). An LVS mutant of *tolC* is able to replicate in macrophages, but is severely attenuated in mice inoculated intradermally (42).

In contrast to genes that contribute to the virulence of *Francisella*, *pepO* may have the opposite effect. PepO is a zinc metalloprotease that is transcriptionally regulated by MglA in *F. novicida* (50). Interestingly, *pepO* mutants are hyper-virulent in mice, possibly due to the absence of a potent vasoconstrictor that is formed by the PepO mediated cleavage of pro-endothelin (50), and which could limit bacterial dissemination. Only *F. novicida* contains a version of this gene predicted to encode both a secretion signal and an intact protease domain, which could potentially contribute to this organism's status as non-pathogenic in humans.

In vivo screens have identified a number of additional genes that are necessary for *F. novicida* and LVS virulence in mice (107, 114). The majority of these virulence determinants remain to be characterized, but should provide a wealth of information as to the mechanisms by which *Francisella* causes disease.

Protein Secretion in *F. tularensis*

All bacteria need to secrete proteins for a variety of reasons, such as acquisition of nutrients and production of outer membrane structures. Bacteria use the general secretory pathway (Sec) to translocate proteins with the appropriate signal sequence across the cytoplasmic membrane. Gram-negative pathogens frequently make use of secreted toxins or virulence factors, and have evolved a variety specialized systems to translocate these proteins across the outer membrane, and in some cases directly into the host cell (reviewed in (40).

The *F. tularensis* genome does not contain homologues to type III secretion systems (T3SS) and type IV secretions systems (T4SS), which are utilized by many pathogens for secretion of virulence factors. T3SS are responsible for the ability of *Salmonella* and *Shigella* spp. to invade nonphagocytic cells (26). *F. tularensis* does contain two genes, *tolC* and *ftlC*, whose products are homologous to *E. coli* TolC, which could encode a protein that functions as part of a T1SS, or participates in efflux of toxins. LVS mutants *tolC* and *ftlC* demonstrate increased susceptibility to drugs and detergents, and the *tolC* mutant is attenuated in mice (42).

There is considerable overlap among genes used for Type IV pili (T4P) formation and those that play a role in type II secretion. The type A Schu S4 genome contains homologues

to all genes necessary for T4P formation (35). The *F. novicida* genome contains homologues

to all genes as well, though the C-terminal half of the *pilA* gene is significantly altered, as is

the intergenic region of the *pilAEV* gene cluster (35). Some *F. holarctica* (type B) strains

lack *pilA*, and all appear to lack functional copies of the downstream genes *pilE*, *pilV*, and

pilT due to nonsense mutations (36, 80). LVS is lacking functional copies of both the *pilAEV*

genes and *pilT* (36). Protein secretion is abrogated in transposon insertion mutants of *F.*

novicida pilB, pilC and pilQ, which are respectively homologues of an ATPase, an inner

membrane protein, and a secretin involved in T4P secretion. The *pilA* gene is also necessary

for *in vitro* protein secretion in *F. novicida* (50). A *pilA* negative type B strain is able to

replicate normally in macrophages, but is attenuated in mice when given subcutaneously

(36). Similarly, a *F. novicida pilC* mutant is able to replicate normally in macrophages (50).

Interestingly, in contrast to the attenuated type B mutant, the *F. novicida* pilC mutant is

hyper-virulent when given to mice intradermally (50). Type A and B strains contain

mutations in the *pepO* gene that result in a deletion of the secretion signal, while type B

strains also contain a premature stop codon that eliminates the protease domain from PepO

(50). It is hypothesized that this increase in virulence is due to failure to secrete the PepO

protease that is predicted to activate a vasoconstrictor, thereby limiting dissemination.

Hager et al. suggest that *F. novicida* protein secretion takes place via the T4P system,

rather than the T2SS, since there are no *Francisella* homologues to *gspC*, *gspM* and *gspL*,

genes necessary to meet the definition of type II secretion (50, 77). This idea is not

unprecedented, as *Vibrio cholerae* secretes a virulence factor, TcpA, via a T4P system (60).

Whether these genes are also used to produce type IV pili seems to be strain dependent and

still open to investigation. Pili have not been identified on Type A strains or *F. novicida*,

though the expression of *pilA* from both induced pili formation in a *Neisseria* system (35). T4P-like structures have been identified microscopically on LVS, however this strain does not contain a functional *pilA* gene, so the exact nature of these structures remains to be elucidated (41).

There is speculation that the *F. tularensis* pathogenicity island may encode a type VI secretion system. *IglA* and *iglB* homologues are present in a number of plant and animal pathogens, where they invariably appear together and their order is conserved (74). *IglA* and *iglB* homologues cluster into a group of genes that include members with IcmF motifs (74). IcmF-associated homologous proteins (IAHPs) are associated with T6SSs in some organisms. Two other PI genes, *pdpB* and *pigF*, also show homology with IAHPs (74). However, it remains to be determined whether PI genes encode a T6SS system in *Francisella*.

Respiratory Tularemia and Lung Biology

Although the primary type of naturally occurring tularemia is ulceroglandular (2), the low infectious dose of as few as 10 organisms by the respiratory route (97), along with the mortality rate of 30-60% for untreated pneumonic infections with a type A strain (30), have lead to our lab's focus on respiratory tularemia. The potential for aerosol dissemination of this organism necessitates research to understand the mechanisms of respiratory tularemia.

A person inhales about 10,000 liters of air each day (117). This air, which passes through the airways (trachea, bronchi, bronchioles) to the alveoli, may contain particulate matter, bacteria and viruses. Ciliated epithelial cells coated with mucus line the airways, and

make up the "mucociliary escalator", which moves particles captured in mucus up and out of

the airway by the action of the cilia (105, 117). The airways terminate at the alveoli, the site

of gas exchange with the blood. Alveolar macrophages patrol the alveolar space, where they

phagocytose bacteria and modulate the host immune response (68). The alveoli are made up

of type I and type II alveolar epithelial cells. Type I cells (ATI) are responsible for gas

exchange, and make up about 95% of the surface area of the alveolar epithelium (69). Type II

cells (ATII) are the progenitors of ATI cells, make up 5% of the surface area of the alveolar

epithelium, and are responsible for the production and secretion of pulmonary surfactant

(69). Surfactant is composed primarily of phospholipids, along with the surfactant proteins

SP-A, SP-B, SP-C and SP-D (117). Surfactant phospholipids reduce the surface tension at the

lung's air-liquid interface, allowing respiration. SP-B and SP-C are small hydrophobic

proteins that help to stabilize the surfactant film (69, 117). SP-A and SP-D are collectins

(collagen-containing C-type lectins) capable of binding a number of microorganisms leading

to several possible outcomes, including bacterial aggregation, lysis, and opsonization for

phagocytosis, and modulation of the immune response (61, 117). Both macrophages and

ATII cells express surfactant protein receptors (61). ATII cells employ these receptors for the

re-uptake of surfactant, which is constantly being recycled in the lung (116).

Most pathogens that are able to cause disease in the lung do so by virtue of their

ability to survive in macrophages. However, there is some evidence of pathogens that also

invade alveolar epithelial cells. *Mycobacterium tuberculosis*, considered to primarily infect

macrophages, is capable of invading and replicating in the alveolar epithelial cell line A549

(38). While *in vivo* uptake and replication has not been demonstrated, bacterial DNA is found

in ATII cells of human lung tissue (38). *Burkholderia cepacia* invades ATII cells both in

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vitro and in vivo (18, 21). Chapter 1 and 2 of this work describe the ability of *F. tularensis* to invade and replicate in ATII cells both *in vivo* and *in vitro*.

A primary function of macrophages is to engulf bacterial cells and other foreign particles (54, 92). *F. tularensis* invasion of and trafficking within macrophages has been characterized, and is described below. However, invasion of epithelial and other host tissue cells that lack professional phagocytic receptors requires bacterially mediated exploitation of host cell cytoskeleton and signaling to gain entry (26, 33, 34). Pathogens that invade nonphagocytic cells, such as *Yersinia pseudotuberculosis*, *Listeria monocytogenes*, and *Salmonella* spp. have developed strategies to gain access to these cells, and to survive intracellularly (26). Chapter 3 describes our lab's efforts to characterize *F. tularensis* entry into lung epithelial cells and trafficking inside these cells.

F. tularensis **uptake by the macrophage**

Much of the research on *F. tularensis* pathogenesis to date has focused on the interaction of *Francisella* with the macrophage, as many of the genes that have been identified as being required for virulence are related to surviving in these cells. *Francisella* is taken up by macrophages by a unique process that has been termed looping phagocytosis (24). LVS and type A clinical isolates of *F. tularensis* have been identified being taken up by human macrophages by this process, which involves the formation of a single pseudopod loop which encircles the bacteria before fusing with the plasma membrane to form a large vacuole. This vacuole shrinks once the bacterium has been internalized by the macrophage. This uptake mechanism differs from the well characterized zipper and trigger mechanisms, as

well as from the coiling phagocytosis previously described for *Legionella pneumophila* (53).

Both live and dead *F. tularensis* is taken up by looping phagocytosis, and uptake is dependent upon actin polymerization and PI3 kinase signaling (23). However, oxidation of bacterial carbohydrates results in uptake that microscopically resembles conventional receptor-mediated (zipper) phagocytosis, suggesting that bacterial LPS or capsular material may mediate looping phagocytosis (23).

Invasion frequency of macrophages is far lower in the absence of fresh serum, and C3 deficient serum does not promote uptake, indicating the involvement of the macrophage complement receptor (CR3) in uptake of LVS and type A strains by human macrophages (23, 98). Opsonization with fresh serum leads to human macrophage uptake of *F. novicida* via complement receptors and Fcγ receptors (11, 98). Competitive inhibition of the macrophage mannose receptor (MR) decreases uptake of both LVS and *F. novicida* by human macrophages the absence of serum opsonization, indicating a MR role in phagocotosis (11, 98). *F. novicida* phagocytosis by human macrophages is also increased when bacteria are pretreated with surfactant protein A (SP-A) (11). SP-A is a collectin produced in the lung, and will be described in greater detail in the lung biology section. Phagocytosis of LVS by the mouse macrophage-like cell line J774, is mediated by class I and II A scavenger receptors (SRA) and CR3 (81). Of interest, Fcγ receptors do not appear to have an effect on *Francisella* phagocytosis in J774 cells (81), and this cell line does not express mannose receptors (98). In conclusion, the suite of receptors responsible for the uptake of the various species of *Francisella* by macrophages include CR3, SRA, MR, Fcγ and SP-A receptors.

F. tularensis **lifestyle in the macrophage**

Once particles are taken up by phagosomes they are set on a path toward eventual destruction by lysosomal enzymes (47, 99). Nascent phagosomes interact with components of the endocytic pathway resulting in phagosome maturation as evidenced by acquisition of membrane markers. Markers are acquired in a sequential manner demarcating progression from phagosome to early endosome, late endosome, to lysosome. During this maturation process the contents of the vacuole become increasingly acidic and hydrolytic, leading to the digestion of the contents.

Bacterial pathogens have evolved strategies to survive the hostile environment of the phagosome. Some, such as *Ehrlichia chaffeensis* and *Mycobacterium* spp. are able to survive in an endocytic vacuole by halting maturation of the vacuole and fusion with the lysosome (49). Others, such as *Legionella pneumophila* and *Brucella abortus* , modify the vacuole to create a unique intracellular niche separate from the endocytic pathway (49). Still others, such as *Listeria monocytogenes* and *Shigella flexneri*, survive by escaping the endocytic vacuole to replicate in the cytoplasm (49).

Francisella taken up by rodent or human macrophages is contained within a membrane bound vacuole which rapidly acquires early endosome markers (EEA1), then eventually acquires late endosome markers (LAMP1 and LAMP2), before escaping into the cytoplasm to replicate (6, 20, 25, 43, 95). The *Francisella* containing vacuole does not acquire lysosomal markers, such as cathepsin D (95). This same general path is followed by *F. novicida*, type A and type B strains of *Francisella*. The timing of escape from the vacuole into the cytoplasm has been reported as occurring between 1 and 6 hours post-infection (20,

19

25, 43, 95). This wide range can largely be attributed to how researchers choose to define escape from the vacuole. Those that use a flow cytometry based assay in which bacteria are defined as cytoplasmic if they are accessible to cytoplasmically delivered antibody report 95% escape by 60 minutes (20), while those that define escape as less than 50% of the circumference of a particular intracellular bacteria surrounded by membrane report escape times of 3 to 6 hours (25).

There is some disagreement as to whether the vacuole becomes acidified before escape into the cytoplasm. Clemens et al. indicate that a type A strain resides in a vacuole that does not become significantly acidified in human macrophages (25). However, Santic et al. recently reported that *F. novicida* containing vacuoles in human macrophages become acidified via transient acquisition of the proton vATPase pump 30-60 minutes post-infection, and that this acidification is vital to bacterial escape into the cytoplasm (94). *F. novicida* mutants of the pathogenicity island gene *iglC*, or its regulator *mglA*, are unable to prevent lysosomal fusion, and are unable to escape the phagosome (96). Phagosomes containing the *iglC* mutant retain the proton vATPase pump, and go on to lysosomal killing (94, 96). It is possible that these conflicting results regarding vacuole acidification could be attributed to the different strains being tested, or more likely, to the timing of the experiments. Those experiments demonstrating that there is no acidification of the vacuole were carried out 3 hours post-infection (25), a time at which the vacuolar membrane is degraded and the vacuolar contents are likely in equilibrium with the cytoplasm; those showing acidification were done 30-60 minutes post-infection, a time at which the membrane is more likely intact (94).

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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.

Text

   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 the liver, spleen, and lungs regardless of the 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 growth of *Francisella* in humans and other animals (6, 18). This conclusion is supported by the observations 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 intramacrophage growth (3, 11, 12, 14, 15).

Clearly, intramacrophage survival is an important virulence property of *Francisella*. 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 tularemia acquired by inhalation, 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.

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

To determine whether *Francisella* could invade and replicate within lung epithelial cells, in vitro invasion and replication assays were performed using the human alveolar type

II (ATII) epithelial cell-derived cell line A549 (ATCC), the mouse ATII cell-derived cell line MLE-12 (ATCC), and the mouse lung epithelial cell-derived cell line Tc-1 (ATCC). For comparison, the mouse macrophage-like cell line J774A.1 (ATCC) was also used. Intracellular bacteria were enumerated by a gentamicin protection assay. The cell lines were grown to confluence in 24-well plates and inoculated with *F. tularensis* LVS at a multiplicity of infection of 100:1. Four hours after inoculation, the cells were washed with phosphate-buffered saline (PBS), and medium containing gentamicin (25 μg/ml) was added to kill extracellular bacteria.

Intracellular organisms were recovered from all cell lines 6 and 24 h postinoculation (Figure 1A). The number of intracellular organisms recovered 6 h postinoculation and the percentage of infected cells varied among the lung epithelial cell lines, with the fewest intracellular *Francisella* organisms recovered from the human ATII cell line, A549. The percentage of infected epithelial cells ranged from 0.2% of A549 cells to 17% of Tc-1 cells (Figure 2.1A). The number of intracellular bacteria recovered from each lung epithelial cell line at 6 h was smaller than the number recovered 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 h in each cell line, representing an intracellular doubling time of roughly 3 h (Figure 2.1A). Intracellular localization and proliferation were confirmed using fluorescence microscopy and digital image deconvolution (Figure 2.1B to E). From 6 to 24 h postinoculation, 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 its ability to replicate within cultured lung epithelial cells. Whereas some work has been done to examine localization of *Francisella* in the liver (8), little is known about the localization of inhaled *Francisella* in the lung. Bosio and Dow recently reported that *Francisella* was associated primarily with dendritic cells isolated from bronchioalveolar lavage fluid of mice 1 hour after 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- to 8-week-old female C57BL/6 mice were inoculated intranasally with 10^5 CFU of *F. tularensis* LVS expressing green fluorescent protein (GFP) suspended in 50 μl of PBS. (All mouse work was performed according to 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 postinoculation. 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 colocalized with alveolar cells (Figure 2.2). Fluorescence imaging of sequential vertical planes revealed that most infected alveolar cells contained multiple bacteria throughout the cytoplasm,

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indicative of intracellular replication (data not shown). Bacterial replication within ciliated or

nonciliated (Clara) bronchial epithelial cells was not observed. The absolute number of

bacteria (Figure 2.2D) and the number of infected alveolar cells increased by day 3 (Figure

2.2B). By day 7, *F. tularensis* within the lung was widespread but remained exclusively in the

alveolus (Figure 2.2C). Throughout the course of infection, the amount of extracellular space

observed in the network of alveolar cells decreased, causing the alveolar cells to appear more

condensed (Figure 2.2).

Inhaled *Francisella* colocalizes 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 the nonsecreted surfactant protein

precursors proSP-B and proSB-C (Chemicon) to determine whether *Francisella* localized to

and replicated within ATII cells in vivo following inhalation. proSP-B is produced by ATII

cells and nonciliated 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 the nonsecreted surfactant protein precursors

proSP-B and proSB-C (Figure 2.3A to 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 colocalization with proSP proteins, microscopy of sequential

vertical planes confirmed that bacteria were inside infected ATII cells (data not shown).

Rabbit antibody specific for ß-tubulin (Abcam) did not colocalize with *F. tularensis* (Figure

2.3E), indicating that the colocalization of *Francisella* with rabbit proSP antibodies was not due to bacteria nonspecifically binding rabbit antibodies, nor did the proSP antibodies cross-react with cultured *Francisella* organisms (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 colocalize with proSP-B and proSP-C (Figure 2.3), 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* organisms (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 after 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- and 20- μm-pore-size 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 2.4),

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* 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.

Acknowledgements

Attributions

I performed the fluorescence microscopy on J774 cells and assisted with the mouse experiments described in this paper. This work has been previously published in the journal Infection and Immunity, Volume 75, pages 1034-1039. Permission has been granted to reprint this material.
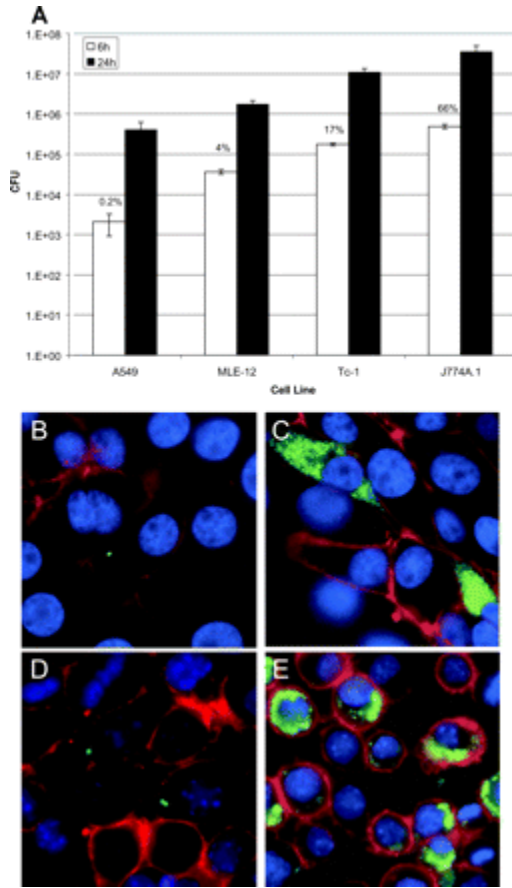
Figure 2.1. *F. tularensis* LVS invades and replicates within ATII cell lines in vitro. (A) Intracellular bacteria recovered from A549, MLE-12, Tc-1, and J774A.1 cells 6 and 24 h postinoculation. The percentages above the bars represent percentages of infected cells. (B and C) Fluorescence imaging of A549 cells inoculated with GFP-expressing LVS 6 h (B) and 24 h (C) following inoculation. Cell borders were visualized by rhodamine-phalloidin (Molecular Probes) staining (red), and nuclei were visualized with 4',6'-diamidino-2-phenylindole (DAPI; blue). (D and E) Fluorescence imaging of J774A.1 cells inoculated with GFP-expressing LVS (green) 6 h (D) and 24 h (E) postinoculation. 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; error bars represent standard deviations of the means.
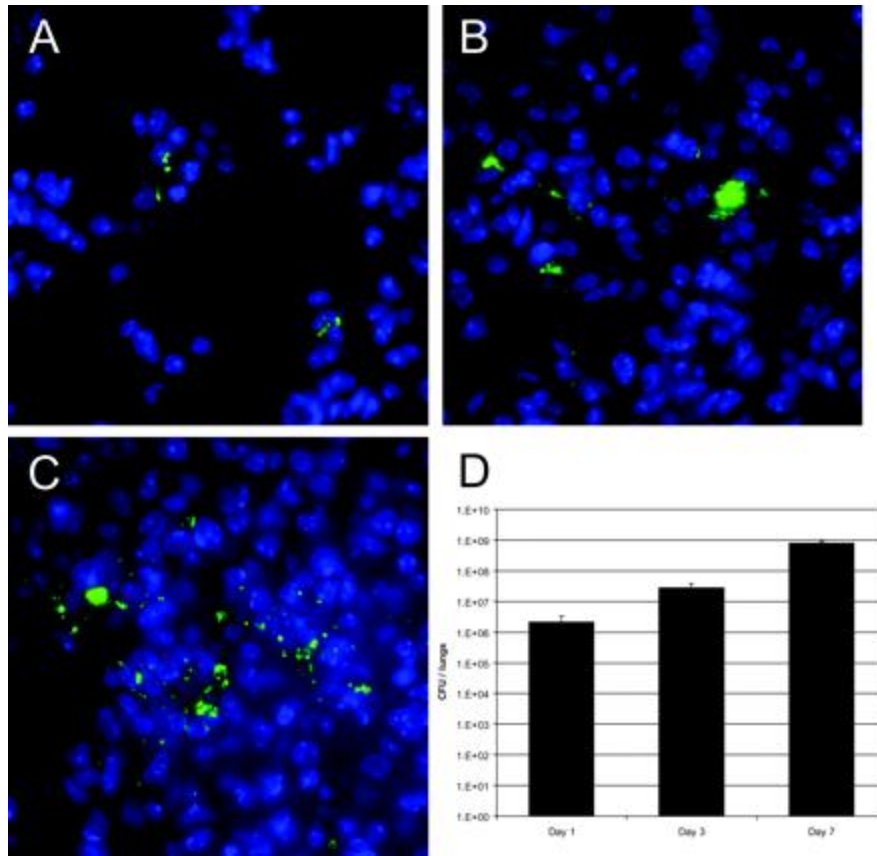
Figure 2.2. *F. tularensis* localizes to the alveolus following inhalation. Mice were intranasally inoculated with 10^5 CFU of *F. tularensis* LVS expressing GFP. One, three, and seven days postinoculation, lungs were harvested and prepared for immunofluorescence analysis. (A to C) Bacterial localization 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 alveoli of infected mice 1 (A), 3 (B), and 7 (C) days postinoculation. (D) Bacterial recovery from lungs 1, 3, and 7 days following intranasal inoculation with 10^5 CFU LVS. Each bar represents mean recovery from three mice; error bars represent standard deviations of the means.
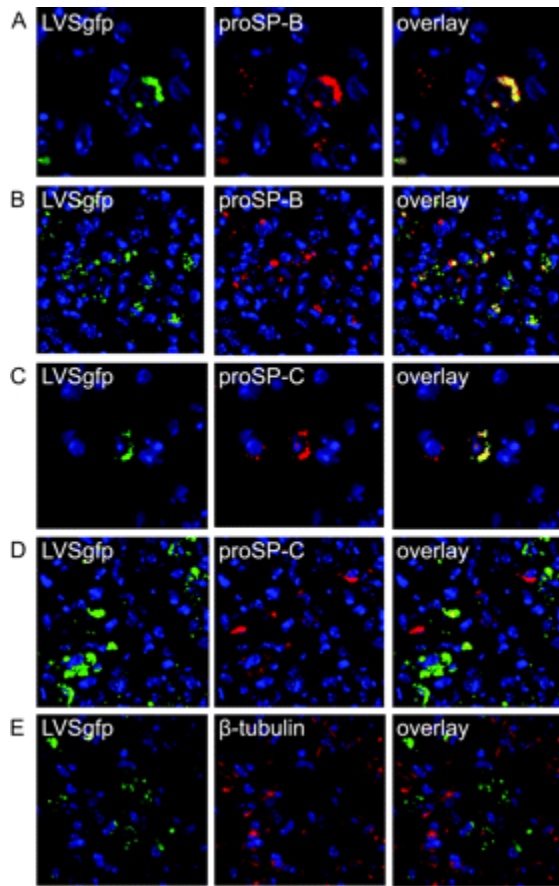
Figure 2.3. Following inhalation, *F. tularensis* LVS expressing GFP colocalized with proSP-B and proSP-C, proteins produced by ATII epithelial cells. Bacterial localization was determined with a fluorescently labeled antibody to GFP (green). Nuclei were stained with DAPI (blue). Sections were probed with fluorescently labeled antibody to proSP-B (red) (A and B), proSP-C (red) (C and D) to identify ATII cells and ß-tubulin (red) (E). Representative images are from lung sections 3 days postinoculation.
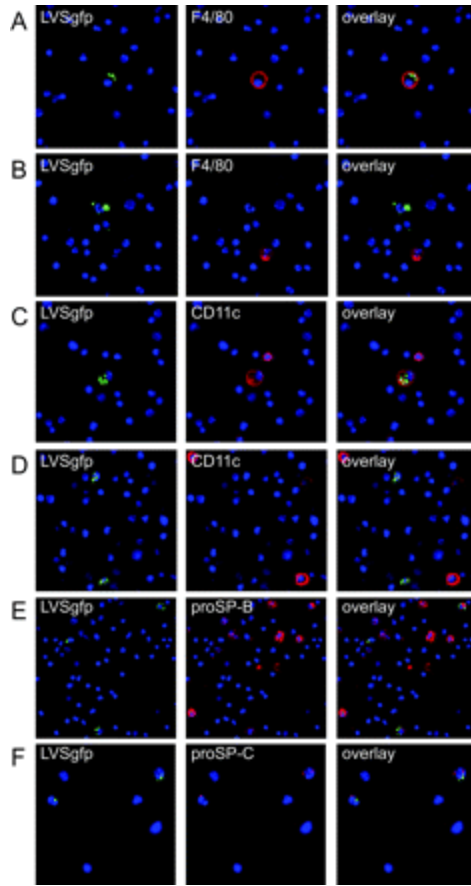
Figure 2.4. *F. tularensis* LVS expressing GFP colocalized with cells expressing the macrophage marker F4/80, the dendritic cell marker CD11c, and the ATII cell markers proSP-B and proSP-C. Nuclei were stained with DAPI (blue). Mouse lung cells were probed with fluorescently labeled antibody to F4/80 (red) (A and B), CD11c (red) (C and D), proSP-B (red) (E), and proSP-C (red) (F). Representative images are from lung cells 3 days postinoculation.

Supplemental Methods for Chapter 2

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°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.

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.

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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 (14), handling infected animal carcasses (30), contaminated water (12) 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 3.1A). Microscopic examination of cells sorted from the high GFP+ population presented significant punctate GFP+ fluorescence, indicative of highly infected cells (Figure 3.1B), 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 3.1C). 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 3.1C). 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 3.2, Table 3.2). 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 3.3). 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 3.4B,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 3.4F). 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 3.5A). 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 3.5C,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 3.5E). 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 3.5B). 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 3.5F) 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 3.5G), 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 (13). 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 (11). 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 OD600. 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°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°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°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°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°C until analysis by flow cytometry.

Flow cytometry of lung cells and data analysis

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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 represent 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).

Attributions

I assisted with the mouse lung cell isolation experiments presented in this chapter.

	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 3.1. Comparison of *Francisella tularensis* strains

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)*

Table 3.2. Identification of specific cell-types from lung homogenates**.**
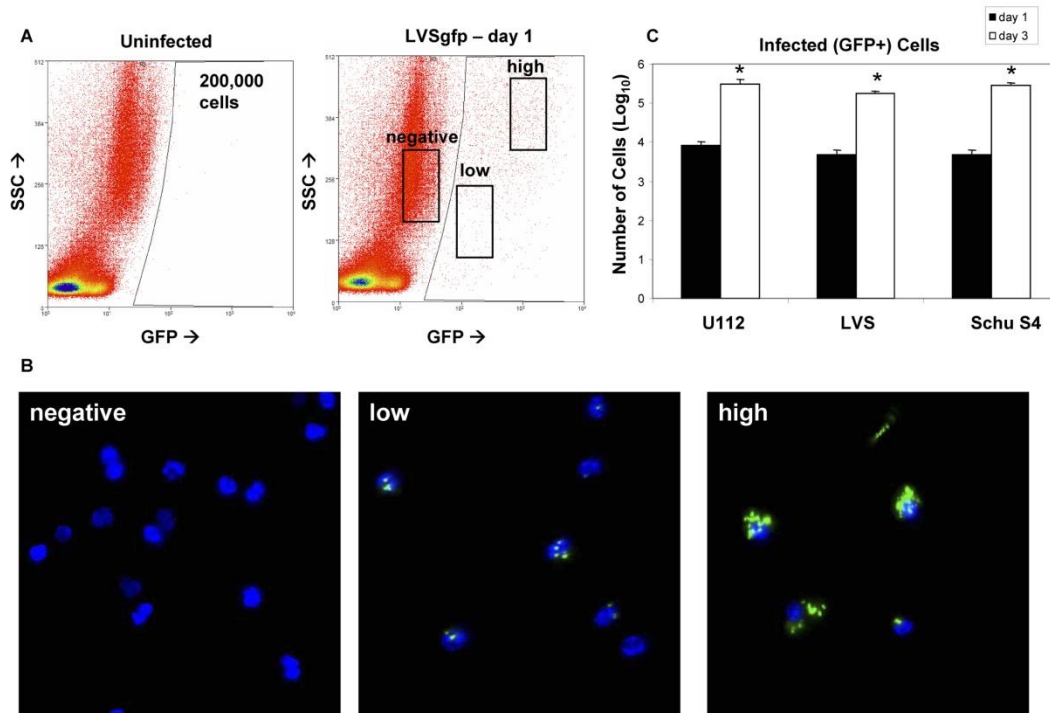
Figure 3.1. Identifying *Francisella*-infected lung cells. Lung cells infected with GFP+ *Francisella* are readily detectable by flow cytometry. Mice were intranasally inoculated with GFP+ *Francisella* strains and on days 1 and 3 post-inoculation, lungs were harvested and digested to produce a single 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 was 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*).
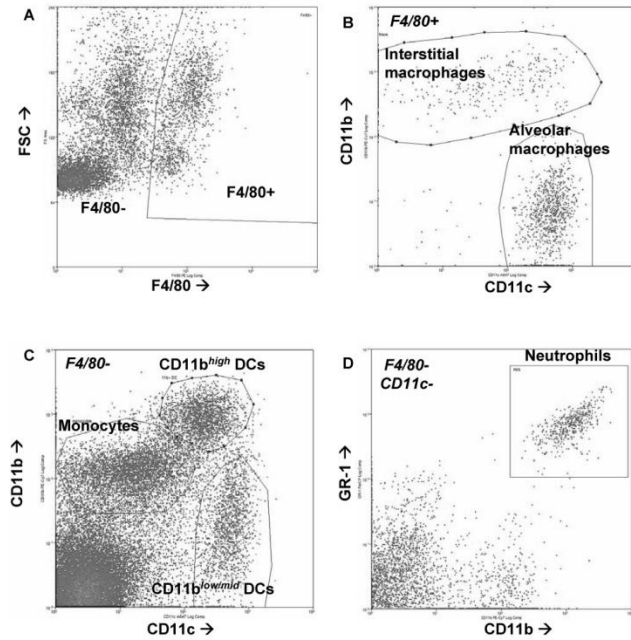
Figure 3.2. Identifying cell types in the lung by flow cytometry. Cell types were identified based on differential expression of F4/80, CD11b, CD11c, and GR-1. Mouse lungs were digested with dispase and cells were stained with fluorescently-labeled cell-type-specific antibodies. (A) F4/80+ cells (B) 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-1^{high} cells were classified as neutrophils.
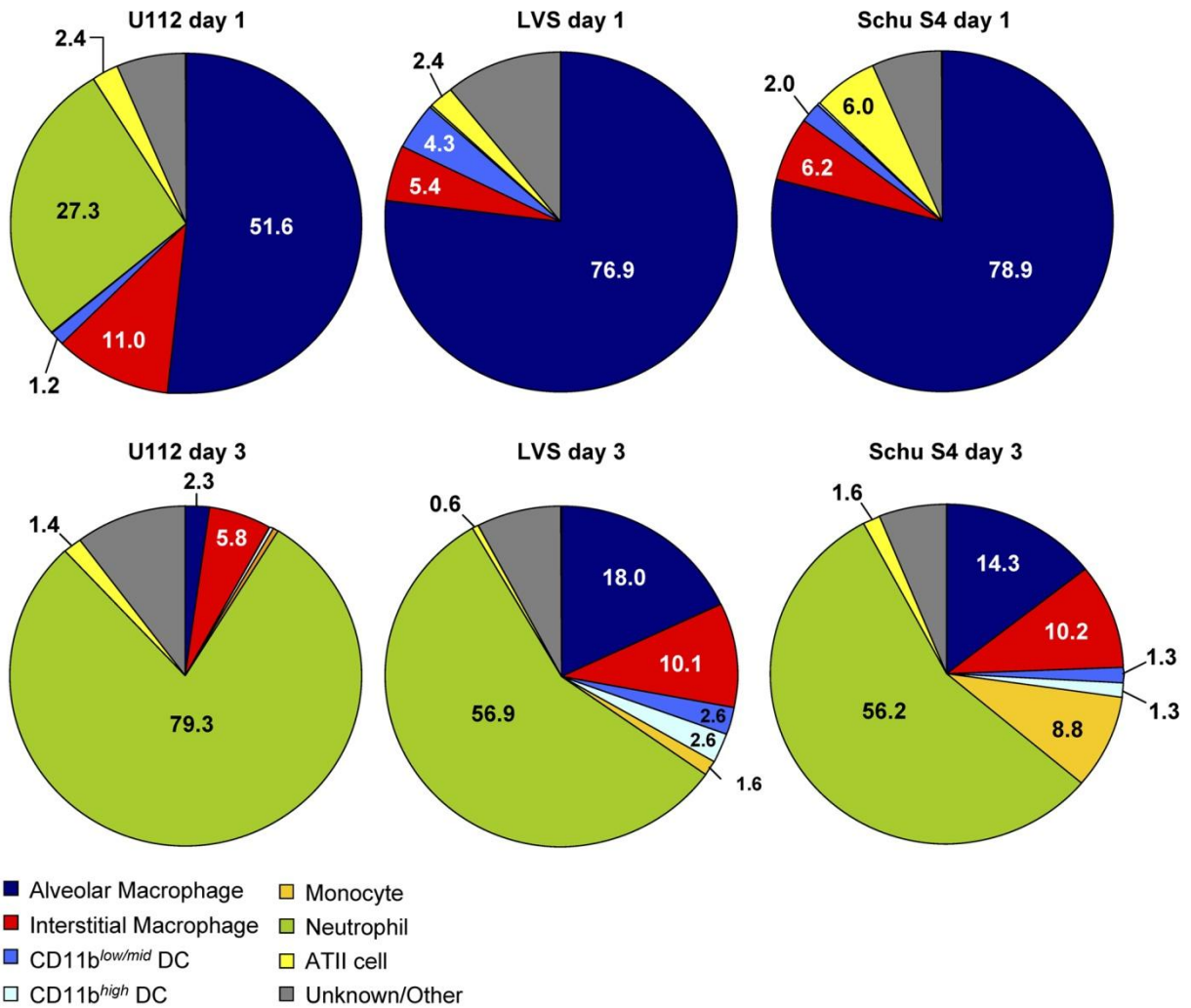
Figure 3.3. Lung cell types infected by *Francisella* following inhalation. Proportion of lung cell-types infected with *Francisella* strains U112, LVS, or Schu S4 on day 1 and day 3 post-inoculation.
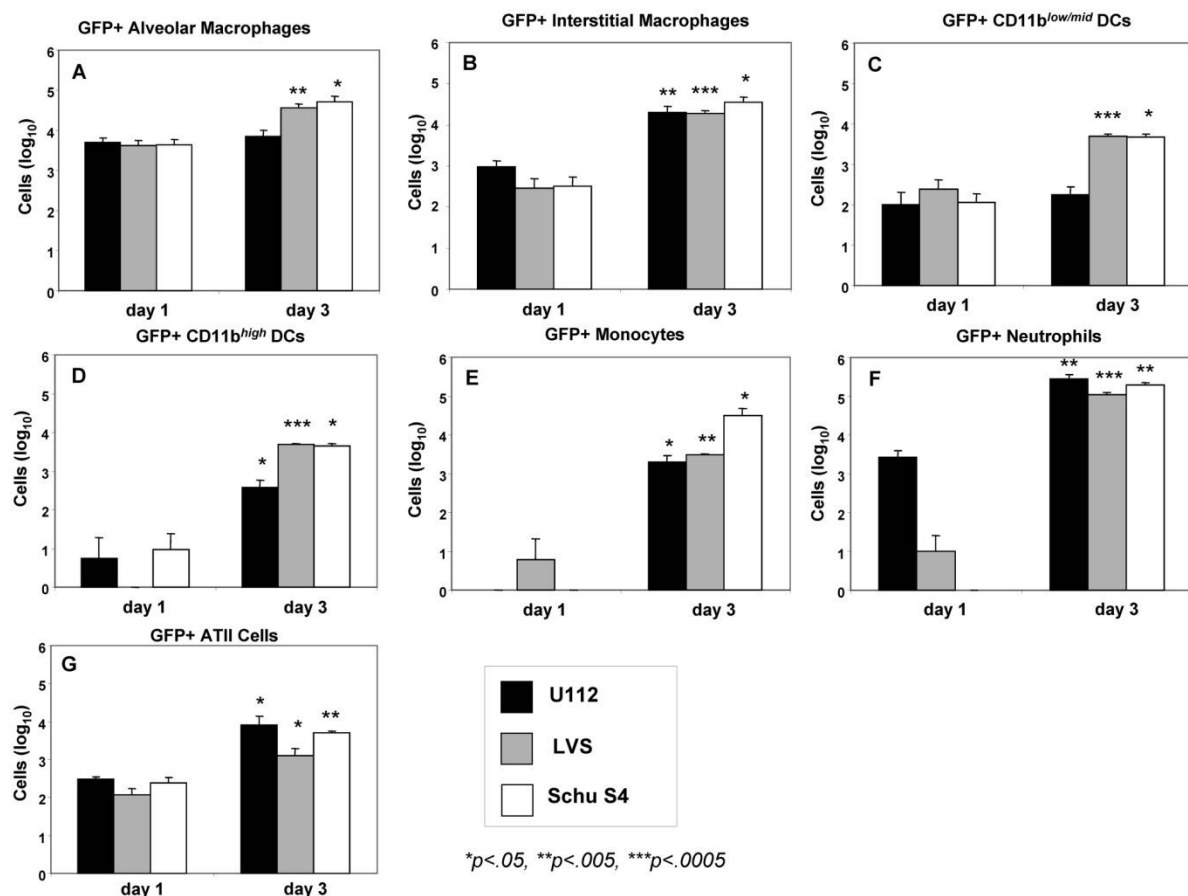
Figure 3.4. Number of *Francisella*-infected cells following inhalation. Absolute number of infected (A) alveolar macrophages, (B) interstitial macrophages, (C) CD11b$^{low/mid}$ DCs, (D) CD11b^{high} DCs, (E) monocytes, (F) neutrophils, and (G) ATII cells from mouse lungs on day1 and day 3 following 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*).
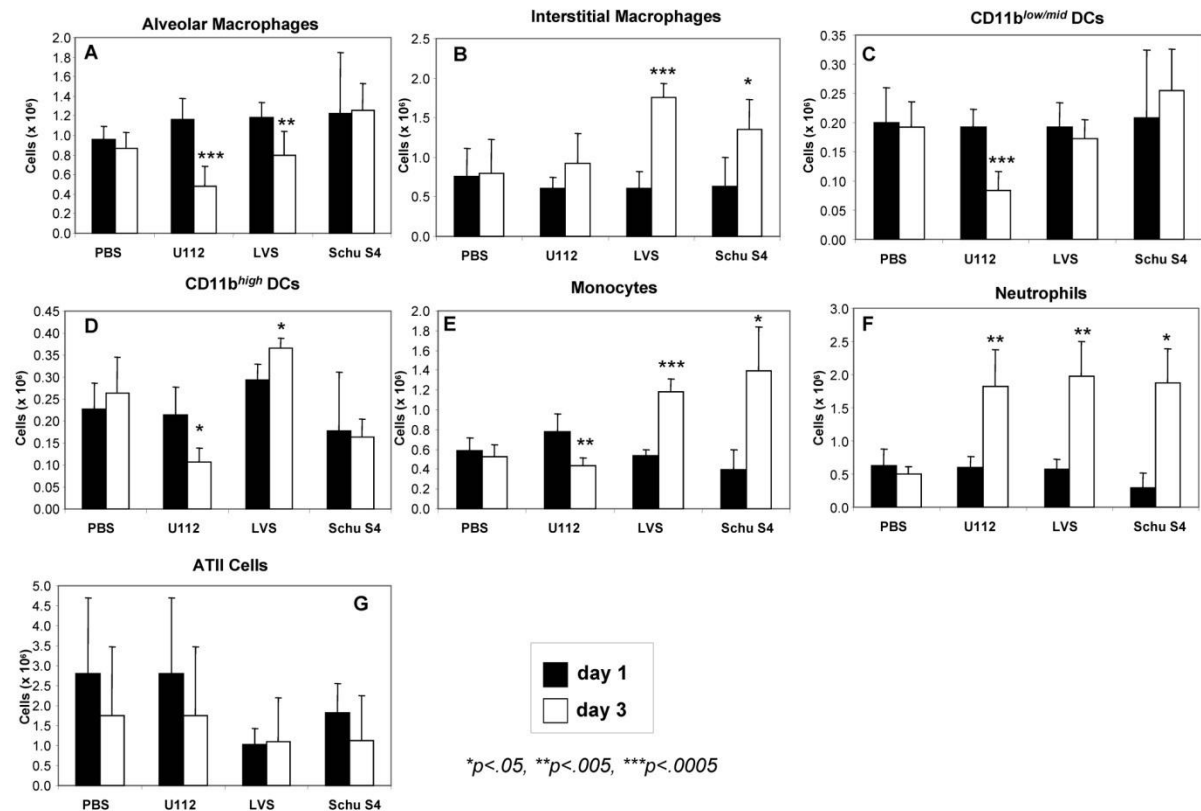
Figure 3.5. Effects of *Francisella* infection on cell number within lung populations. Absolute number of (A) alveolar macrophages, (B) interstitial macrophages, (C) CD11b$^{low/mid}$ DCs, (D) CD11b^{high} DCs, (E) monocytes, (F) neutrophils, and (G) ATII cells from mouse lungs on day1 and day 3 following 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

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 ℃. *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 (Figure 4.1A). 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 (Figure 4.1B). 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 4.1). 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 4.2A), 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 4.2B). 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% ($\pm 4.6\%$) and 84.1% ($\pm 1.8\%$) when cells were exposed to 100 and 200 nM concentrations, respectively (Figure 4.3A). 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% ($\pm 1.1\%$) and 77.1% ($\pm 1.1\%$) at 50 and 100 μM concentrations, respectively (Figure 4.3B). 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 4.4A and 4.4B). LVS association with EEA1 containing phagosomes increased from 1.4% ($\pm 2.4\%$) at 10 minutes to 40.4% ($\pm 5.3\%$) at 30 minutes, before decreasing (Figure 4.4C). LVS association with LAMP-1 containing vacuoles peaked two hours post-inoculation at 49.3% ($\pm 3.5\%$) (Figure 4.4C). 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 4.5A). The membrane was easily visualized, and in some cases was beginning to degrade (Figure 4.5A). By 24 hours all observed intracellular bacteria were no longer contained within a visible membrane, and appeared to be free in the cytoplasm (Figure 4.5B), 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 4.6A and 4.6B). 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 4.6C). This number increased to 70.0% (±5.5%) at 2 hours

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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 4.4C) 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.

Acknowledgements

Attributions

I performed all experiments described in this paper, except for TEM, which was done my Joshua Hall, who also assisted with inhibitor assays. This work has been previously published in the journal Infection and Immunity, Volume 76, epublished ahead of print April, 2008. Permission has been granted to reprint this material.

A

% TC-1s with associated LVS

B

% TC-1 associated LVS intracellular

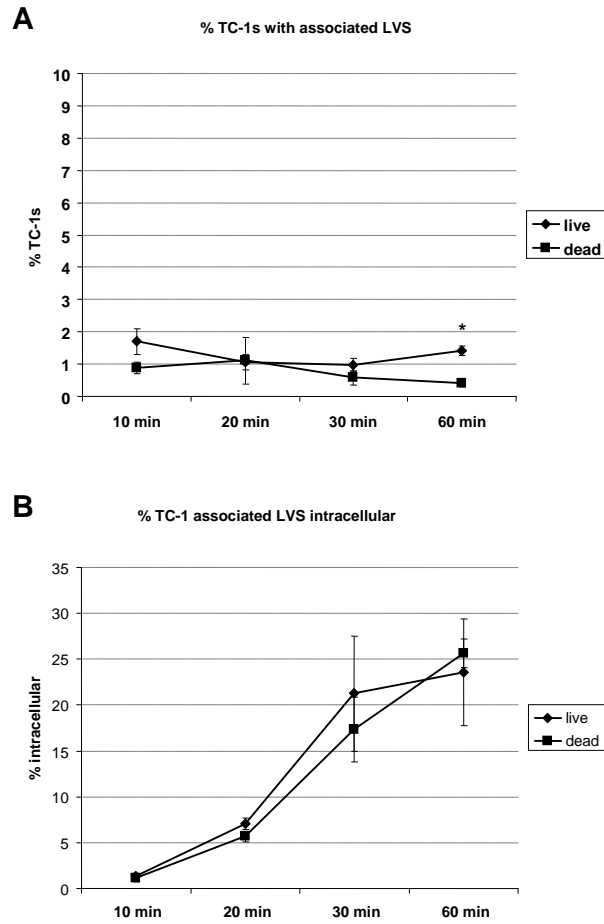**Figure 4.1.** *F. tularensis* LVS association with and internalization by TC-1 lung epithelial cells. (A) Percent of TC-1 cells with associated live (♦) or PFA-fixed (■) LVS at designated times post-inoculation. * Data are significantly different from untreated control, *P*<0.01 by paired two-tailed *t* test. (B) Percent of TC-1 cell-associated live (♦) or PFA-fixed (■) LVS that are intracellular at designated times post-inoculation.

A
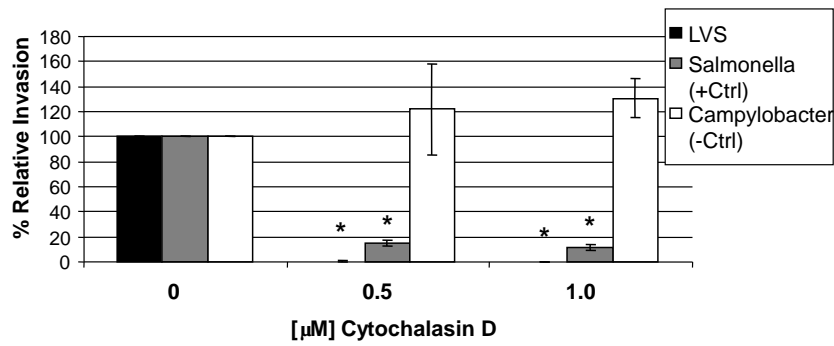
B
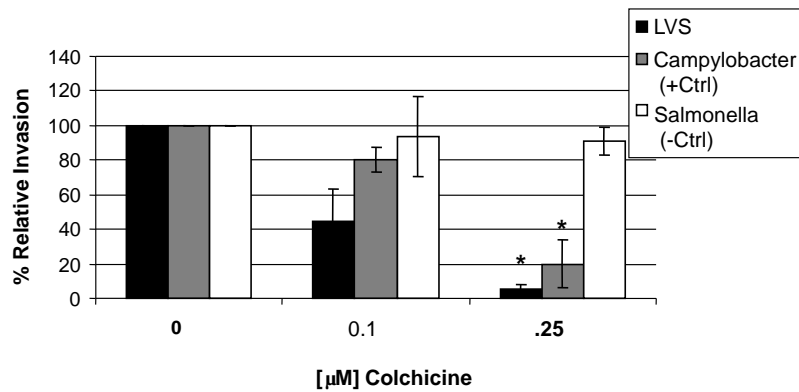
Figure 4.2. The effect of actin and microtubule polymerization on *F. tularensis* LVS invasion of lung epithelial cells. TC-1 cells were treated with designated concentrations of (A) cytochalasin D or (B) colchicine. Results are expressed as the percentage of LVS, *Salmonella* or *Campylobacter* that 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.

A

B

Figure 4.3. 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
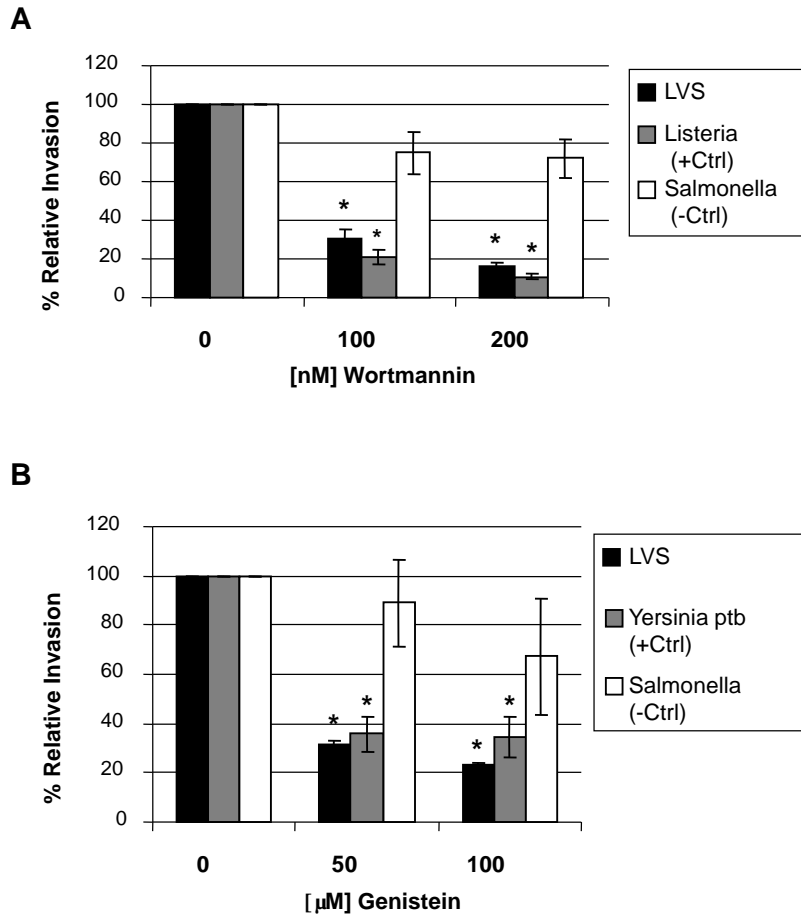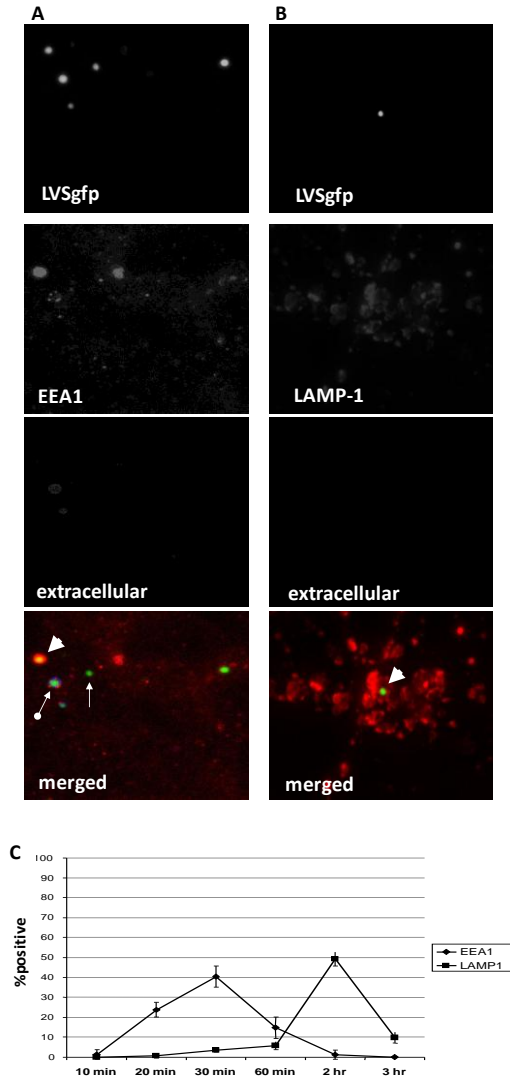
Figure 4.4. Representative fluorescence microscopy images demonstrating LVSgfp localization within EEA1 or LAMP-1containing endosomes in TC-1 cells. Images depict (A) TC-1 cells stained for EEA1 30 minutes post-inoculation with LVSgfp or (B) 2 hours post-inoculation stained for LAMP-1. Single color images: 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.
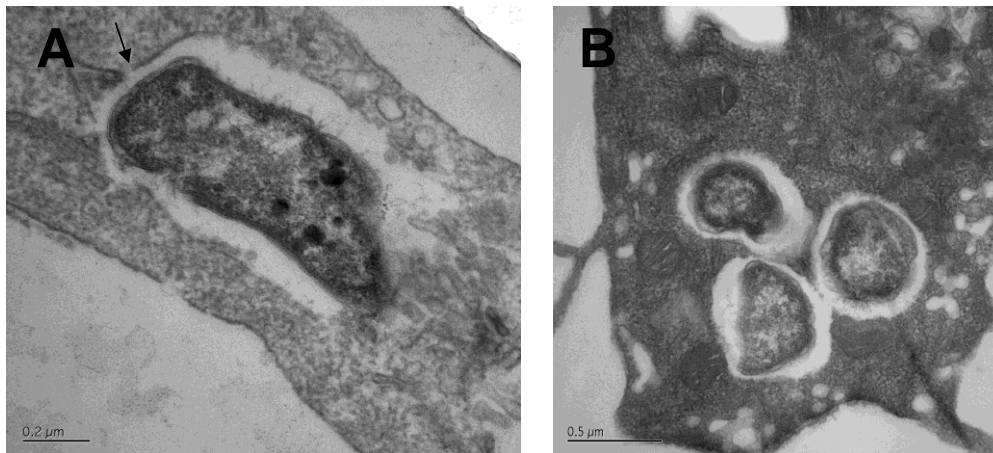
Figure 4.5. Transmission electron micrographs of TC-1 infected with *F. tularensis* LVS. (A) One hour post-inoculation TC-1 cells showing LVS in a membrane bound vacuole that in some cases appeared to be degrading (arrow). (B) 24 hours post-inoculation LVS were free in the cytoplasm.
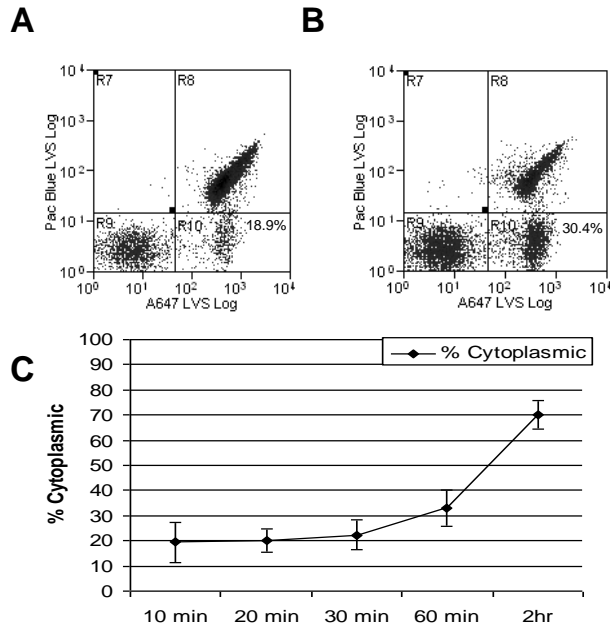
Figure 4.6. LVSgfp was analyzed by flow cytometry for escape from vacuoles into the cytoplasm of TC-1 cells. Extracellular bacteria were labeled with anti-*F. tularensis* LPS antibody conjugated to Pacific Blue (region R8). Cytoplasmic bacteria were identified by labeling with anti-*F. tularensis* LPS 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 5

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* Δ*ripA* 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* Δ*ripA* 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 its 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, 50 µg/ml hemoglobin, 15g/l agarose) supplemented with 1% IsoVitaleX (Becton-Dickson), complete BHI broth (35g/l BHI, 50 µg/ml hemin, 1% IsoVitalex), or Chamberlains Defined Media (12). All bacterial strains cultured on chocolate agar were grown at 37 ℃. Broth cultures were incubated in a shaking water bath at 37 ℃.

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 ℃ 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).

Gentamycin 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 200 μl prewarmed tissue culture media. Gentamycin 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 (10 μg/ml) was used to select for plasmid integration and 10% sucrose to counter select 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 ℃ 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 50 µl 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 according to 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.

<center>**Results**</center>

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). Gentamycin 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 5.1). 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

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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)* ($E = 1\times10^{-10}$), *Beggiatoa sp. PS* ($E = 2\times10^{-10}$), *Moritella sp. PE36* ($E = 1\times10^{-9}$), *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 5.2). 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, gentamycin 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 5.3). 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 $\Delta ripA$ was 1.23 x 10^7

versus 2.60 x 10^2 CFU (P<0.001), respectively, at 24 hours in TC-1 lung epithelial cells

(Figure 5.3A). Mean recovery for LVS and LVS $\Delta ripA$ was 1.04 x 10^8 versus 1.07 x 10^5

CFU (P<0.001), respectively, at 24 hours in J774 cells (Figure 5.3B). Intracellular

replication of LVS $\Delta ripA$ was restored by *trans* complementation with *ripA* (Figure 5.3).

The intracellular growth defect of LVS $\Delta ripA$ was not due to increased sensitivity to

gentamycin (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 Δ*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 5.4). 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 5.5).

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 5.5A). 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 5.6A). *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 5.6A).

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 5.6B). 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 5.6C), 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 5.7A). 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 5.7B). 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 *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.

Attributions

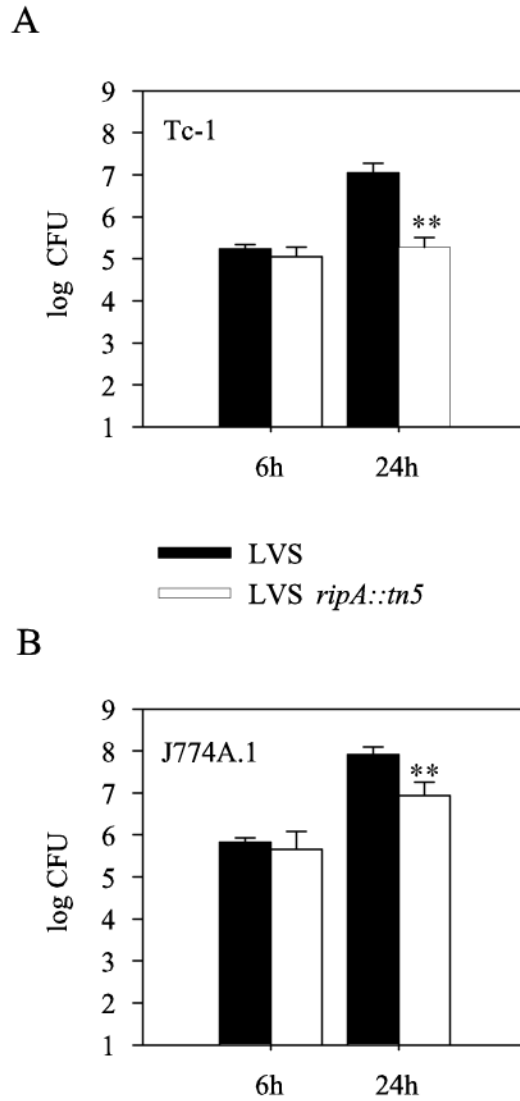I performed the vacuolar escape assays described in this chapter.

Figure 5.1. Gentamycin 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 **).
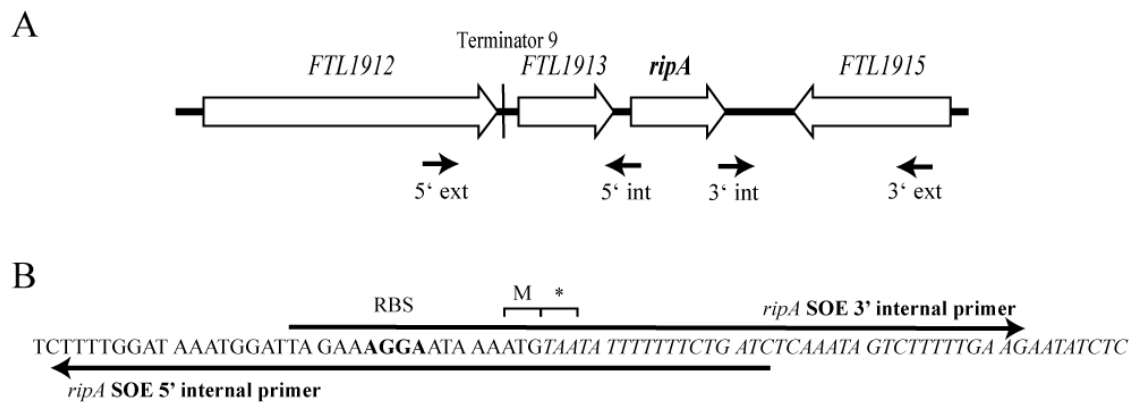
Figure 5.2. (A) Graphical representation of the genomic organization of the LVS *ripA* locus. Loci in close proximity to *ripA* (*FTL 1914*) on the chromosome are *FTL 1912* (30S ribosomal protein S1), *FTL 1913* (Sua5/YciO/YrdC family protein), and *FTL 1915* (Acetyltransferase). Primers utilized for 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.

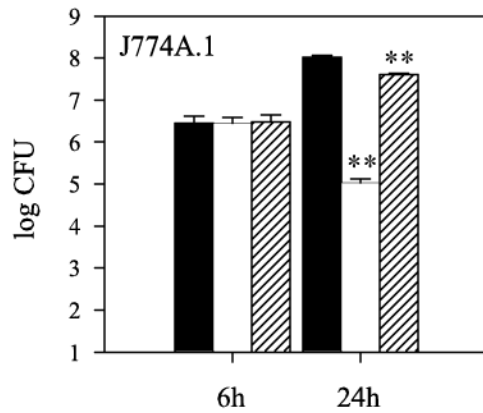125
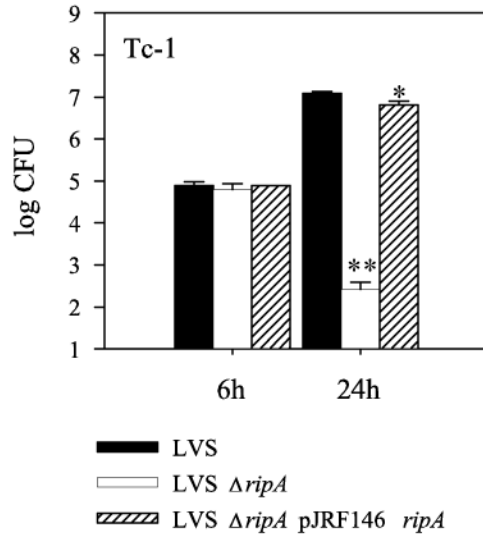
Figure 5.3. Gentamycin protection assays were conducted in TC-1 (A) lung epithelial cells or J774A.1 (B) mouse macrophage-like cells at an MOI of 100. Mean CFU (N=3) at 6 hours and 24 hours post inoculation with error bars ± one standard deviation. Student's t-tests were conducted comparing strains to wild type LVS at each time point (P<0.05 emarcated by * and P<0.01 demarcated by **).

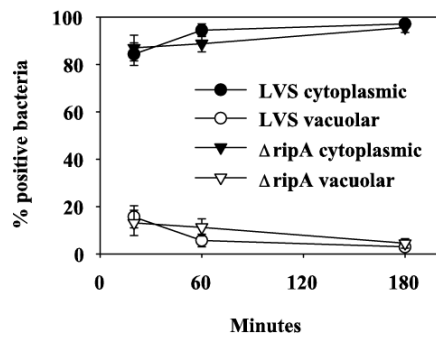**Figure 5.4.** Mean percentage (N=3) of intracellular bacteria in the cytoplasm or vacuole at 20 minutes, 1 hour, and 3 hours post invasion of J774A.1 mouse macrophage-like cells with error bars representing one standard deviation. Bacteria in the cytoplasm determined by accessibility to cytoplasmically delivered antibodies. Vacuolar bacteria defined as bacteria protected from cytoplasmically delivered antibodies.
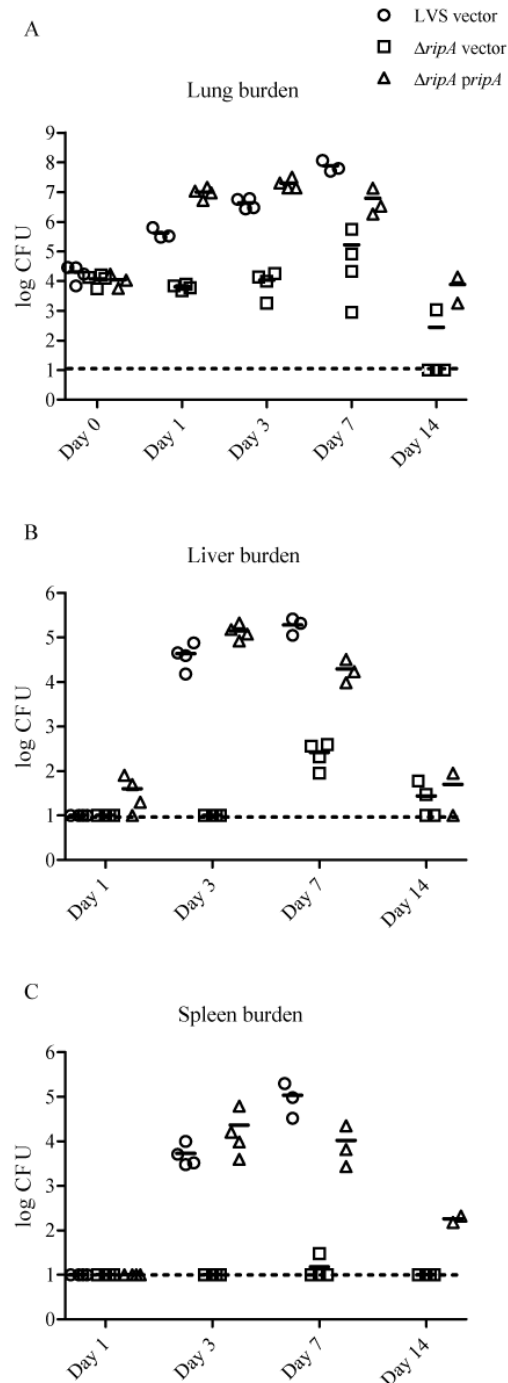
Figure 5.5. Bacterial organ burdens in infected mice (N=4) after intranasal inoculation quantitated as CFU and graphed as individual data points. Organ burdens in mice inoculated with LVS vector only (●), LVS Δ*ripA* vector only (○), and LVS Δ*ripA* pJRF146 *ripA* (▼) strains. Lung (A), liver (B), and 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.
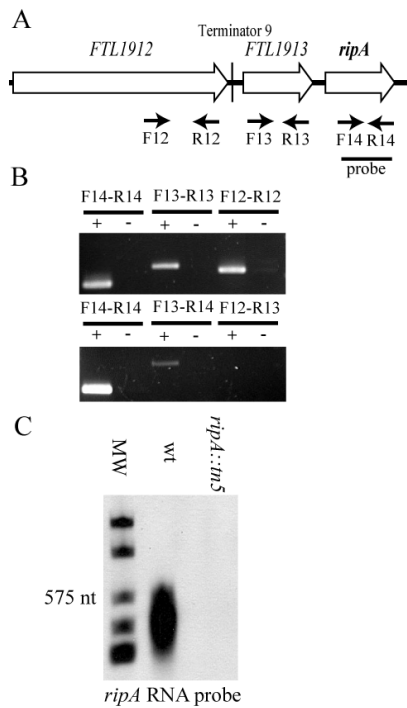
Figure 5.6. (A) Graphical representation of the *ripA* region. Primers utilized for RT-PCR marked are marked with arrows while the region complementary to the RNA probe used in the Northern analysis is demarcated by a solid line. (B) RT-PCR analysis of the expression of genes *FTL1912* (F14-R14), *FTL1913* (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*.
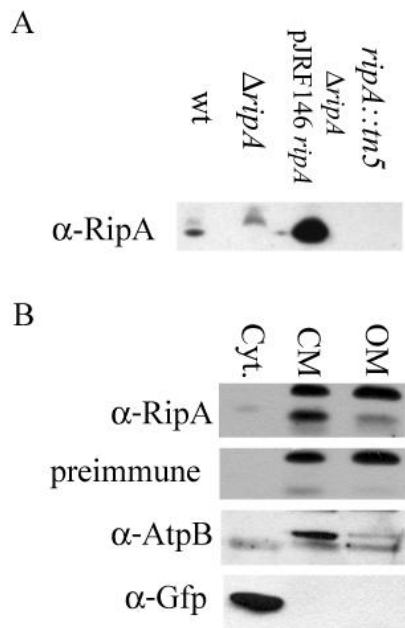
Figure 5.7. (A) Western blot analysis of cytoplasmic membrane enriched fractions of LVS, LVS Δ*ripA*, LVS Δ*ripA* pJRF146 *ripA*, and LVS *ripA::tn5*. Rabbit anti-RipA (1:1000) antiserum was used as the primary antibody. (B) Subcellular localization of RipA. Western analysis of cytosolic (Cyt.), cytoplasmic 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 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 x 10^{-8} ± 0.87 x 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*.

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.

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 ℃, and then plated on chocolate agar containing 10 μg of kanamycin/ml.

We consistently achieved an insertion frequency of 2.6 x 10^{-8} $\pm$ 0.87 x 10^{-8} (Table 6.1), 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 6.2 and Figure 6.1) was within a gene reported to encode a *Francisella* transposase (14). The insertions did not appear to cluster within a specific chromosomal segment (Figure 6.1).

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 6.2).

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

I assisted with the Southern Blots and the sequencing of mutants described in this paper. This work has been previously published in the journal Applied and Environmental Microbiology, Volume 70, pages 6901-6904. Permission has been granted to reprint this material.

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 6.1. Transposon insertion frequencies

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

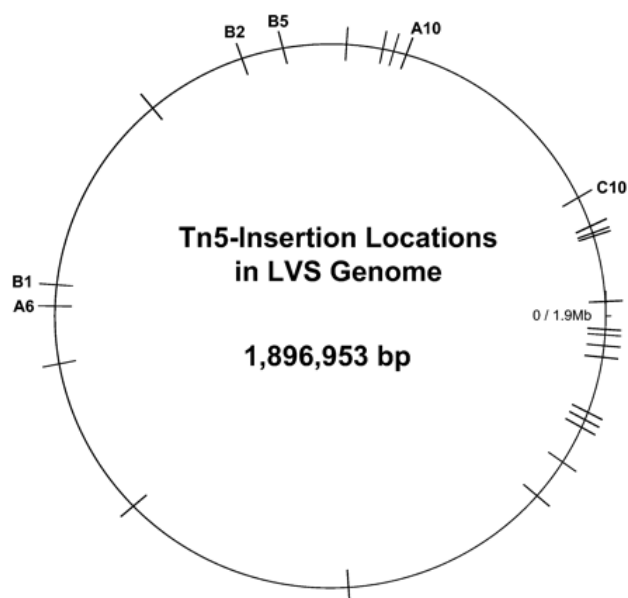**TABLE 6.2.** Identified transposon insertion sites

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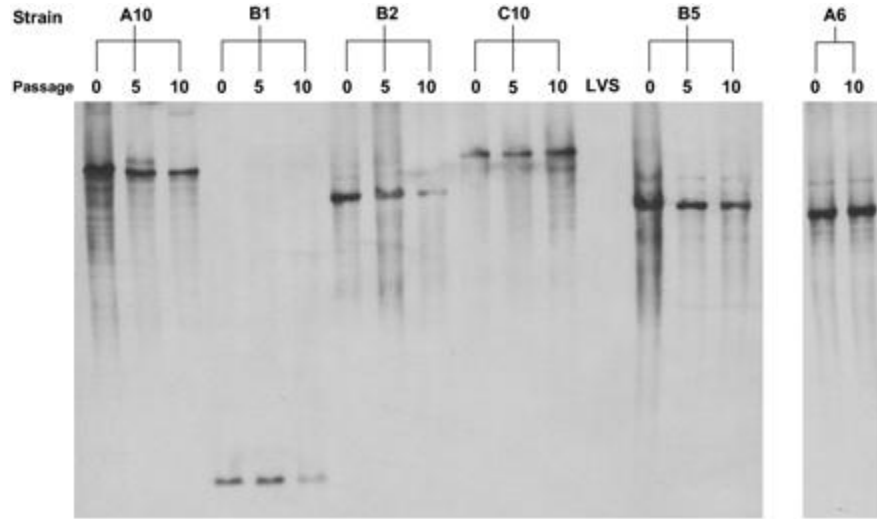
Figure 6.2. Southern blot of five insertion mutants (A6, A10, B1, B2, B5, and C10) and wild-type *F. tularensis* LVS probed with labeled Tn*5*. Chromosomal DNA was prepared from mutants after 0, 5, and 10 passages, digested with EcoRI, and probed. All insertions analyzed in this manner retained the transposon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

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CHAPTER 7

Discussion

Discussion

The research described here was directed toward understanding the interaction of

Francisella tularensis with the lung. Tularemia is contracted via a number of routes,

including respiratory, oral, ocular and skin contact (1, 3, 14, 17). We have chosen to focus on

interaction with the lung for several reasons. Untreated pneumonic and thyphoidal forms of

tularemia with a type A strain have a mortality rate 30-60%, compared to the 5-15% overall

fatality rate for untreated tularemia by all routes (6). A very small dose of as few as 10

organisms is necessary for human infection by the respiratory route (21). These facts, along

with a history of weaponization by the United States, the USSR and Japan, make *F.*

tularensis a biodefense concern (2, 4, 6, 15, 23). Therefore, understanding how *Francisella*

causes disease in the lung is relevant to developing vaccines that would protect people in the

event of an aerosol dispersal of *F. tularensis*, especially with organisms that may have been

intentionally modified to be resistant to standard treatments.

Chapters 2 and 3 describe our investigation of cell types infected in a mouse model of

inhalational tularemia. The existing pathogenesis literature focuses largely on the ability of *F.*

tularensis to replicate in macrophages, and the attenuation of those mutants in mice that are

unable to replicate in macrophages (10, 11, 18-20, 22, 25). In addition to the expected

phagocytic cells of the immune system, we also identify type II alveolar epithelial cells (ATII) containing *F. tularensis* in the mouse lung.

The alveoli are made up of type I and type II alveolar epithelial cells. Type I cells (ATI) are responsible for structure of the alveoli and gas exchange, and make up about 95% of the surface area of the alveolar epithelium (16). Type II cells (ATII), which make up about 5% of the alveolar surface area, have multiple functions in the lung, including the production, secretion and recycling of surfactant, proliferation to produce additional type II cells as well as transdifferentiation to produce additional type I cells, maintenance of alveolar fluid balance, and production of antimicrobial and anti-inflammatory substances (16). Both LVS and the type A strain, Schu S4, modulate cytokine production by ATII cells, stimulating immune cell chemotaxis *in vitro* (9). 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.

While the predominant cell type infected 24 hours post-inoculation with 10^2 to 10^3 CFU of LVS, Schu S4 or *F. novicida* U112 are alveolar macrophages, 2.4% to 6.0% of all infected cells are ATII cells. It should be noted that alveolar epithelial cells are fixed tissue cells, and as such cannot migrate to the site of bacteria, but can only take up bacteria that happen to come into contact with them. Given that ATII cells only account for 5% of the alveolar surface area, this contact is probably a rather low frequency occurrence. The number of infected ATII cells increases approximately one log by day 3 post-inoculation. This could indicate cell-to-cell spread of *Francisella*, or additional extracellular organisms coming into contact with and being internalized by ATII cells.

Using the mouse lung epithelial cell line TC-1, the mouse ATII cell line MLE12 and the human ATII cell line A549 as models we demonstrate that *F. tularensis* is able to invade these non-phagocytic cells and replicate *in vitro* as well (13). This ability to invade epithelial cells is particularly interesting given that the majority of naturally incurring infections are skin infections. As early as 1927 Edward Francis demonstrated that *F. tularensis* replicates in the epithelial cells of the wood tick's digestive tract, and that one of the unique features of tularemia was the ability of the organism to invade fixed tissue cells (24).

Some pathogenic bacteria have evolved strategies to invade cells that are not professional phagocytes (5, 7, 8). These strategies fall into two categories, termed trigger and zipper mechanisms of entry. The trigger mechanism is characterized by the use a bacterial type III secretion system to translocate effector proteins into the host cell. These bacterial proteins are able to manipulate host cell signaling, resulting in rearrangement of the cytoskeleton and engulfment of the bacterium. The zipper mechanism is characterized by the expression on the bacterial surface of a protein that is able to interact with a naturally occurring host cell receptor. This receptor-ligand binding leads to host cell signaling events that cause internalization of the receptor-bound bacterium. Chapter 4 describes our characterization of the internalization of *F. tularensis* by lung epithelial cells. We describe the necessity of host cell actin and microtubule polymerization, as well as PI3 kinase and tyrosine kinase signaling, to epithelial cell uptake of *F. tularensis*. The uptake of killed bacteria, along with the lack of a predicted type III secretion system, point toward a receptor mediated "zipper" mechanism of entry of *F. tularensis* into lung epithelial cells. It should be noted that we have observed *E. coli* taken up by lung epithelial cell lines in similar numbers to *F. tularensis*, but the *E. coli* do not replicate in these cells. This suggests that the ability to

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replicate in these cells, rather than a *F. tularensis*-specific induction of uptake, may be more relevant to disease.

Intracellular pathogens have also evolved strategies to avoid lysosomal degradation. Among these strategies are halting maturation of the endocytic vacuole, modification of the vacuole, and escape into the cytoplasm (12). *F. tularensis* that has been phagocytosed by macrophages survives intracellularly by escaping the endocytic pathway to replicate in the cytoplasm. In Chapter 4 we demonstrate that *F. tularensis* has a similar intracellular trafficking pattern in lung epithelial cells to that seen in macrophages. Intracellular bacteria are initially contained in an EEA1 containing vacuole, which rapidly matures into a LAMP-1 containing vacuole, followed by bacteria no longer being contained within a membrane bound vacuole as evidenced by both availability to cytoplasmically delivered antibodies and electron microscopy. It remains to be determined what role this initial interaction with the endocytic pathway plays in bacterial escape and whether blocking endosomal maturation would prevent escape into the cytoplasm and intracellular growth.

The early pathogenesis work in *Francisella* was primarily done in *F. novicida* due to the availability of genetic tools for use in this subspecies. However, the differences in disease outcome, as well as genetic differences at loci known to play a role in pathogenesis, point toward the necessity of studying the human pathogenic subspecies *tularensis* and *holarctica*. Chapter 6 describes our development of transposon mutagenesis in type B *F. tularensis*. This method allows the creation of mutant libraries that can be screened for phenotypes of interest. Chapter 5 describes a transposon insertion mutant in the gene *FTL1914* that does not replicate in epithelial cell lines, but does replicate (although to a lesser extent that wildtype) in macrophage-like cell lines. This mutant is also attenuated in a mouse respiratory model of

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tularemia, where it does not disseminate from the lungs to the liver and spleen, and does not cause death in mice. These findings, along with our discovery that ATII cells are infected in the mouse, lead us to further investigate the interaction of *F. tularensis* with lung epithelial cells. The inability to restore replication in epithelial cells by complementation of gene *FTL1914* lead to the creation of a deletion mutant of this gene. The deletion mutant does not replicate in epithelial cells or macrophages, leading to the name RipA (Required for Intracellular Persistence, Factor A), and is also attenuated in mice. The RipA mutant is taken up by both macrophage-like and lung epithelial cell lines similarly to wildtype LVS, and escapes the macrophage phagosome at the same rate and frequency as LVS.

Taken together this work demonstrates the discovery of ATII cells as a site of bacterial replication during respiratory tularemia, and describes the *F. tularensis* invasion of and trafficking within lung epithelial cells. Additionally we describe our contribution to the field of *F. tularensis* pathogenesis through the creation of stable transposon insertion mutants in LVS, and the potential to use these mutants to identify genes necessary for intracellular survival.

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