Consequences of immune activation during infection with *Francisella tularensis*

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Tularemia is a debilitating, febrile illness caused by *Francisella tularensis*. Delayed activation of host responses is associated with the virulence of *F. tularensis.* The bacterium can use several signals, including spermine, to adapt to the intracellular environment to limit its stimulation of host cells. However, the bacterial genes responsible for these responses remain unknown. Transposon mutagenesis in *F. tularensis* subsp. *holarctica* Live Vaccine Strain (LVS) identified FTL_0883 as important for spermine responsiveness. In-frame deletion mutants of FTL_0883 and FTT_0615c, the homolog of FTL_0883 in *F. tularensis* subsp. *tularensis* Schu S4 (Schu S4) were generated. These mutants demonstrated that the spermine response is associated with changes in capsule concentration on the surface of *F. tularensis*. Additionally, this locus was important for the virulence of LVS and Schu S4 *in vitro* and *in vivo*. FTL_0883 and FTT_0615c were needed in *F. tularensis* to limit the stimulation of host cells, but only LVS required FTL_0883 for its replication within macrophages. This attenuation depended on host responses to elevated levels of pro-inflammatory cytokines. The mutants were biochemically characterized to identify how they stimulated host cells more robustly than wild-type strains. There were not consistent changes in these mutants, which suggested there are divergent roles for the proteins in LVS and Schu S4. Collectively, these data define a novel function of FTL_0883/FTT_0615c in the virulence and evasion of the immune response of *F. tularensis.*

To further understand the requirements for the host response to virulent strains of *F. tularensis*, wild-type and MyD88 KO mice were infected with Schu S4. Schu S4 infected MyD88 KO had higher bacterial burdens, decreased pro-inflammatory cytokine responses, increased cell death in the lungs, and accelerated mortality compared to wild-type mice. These data highlight a beneficial role for the innate immune response generally and MyD88 signaling specifically against *F. tularensis.* Overall, the data presented in this thesis furthers our understanding of the genes required for *F. tularensis* adaptation to the host intracellular environment to avoid immune detection, a process dependent on the presence of MyD88.

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ABBREVIATIONS

ABSL-3	Animal Biosafety level 3
AIM2	Absent in myeloma 2
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BMDM	Bone marrow derived macrophages
BSL-3	Biosafety level 3
C3	Complement factor 3
C3bi	Complement factor 3b inhibitory
CBS	Cystathionine beta-synthase domain
CDC	Centers for Disease Control and Prevention
CDM	Chamberlain's chemically defined media
CFU	Colony Forming Units
CR3	Complement Receptor 3
DCHA	Dicyclohexylamine
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic Acid

EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent Assay
EM	Electron microscopy
FBS	Fetal bovine serum
FCP	*Francisella* containing phagosome
FCV	*Francisella* containing vacuole
FPI	*Francisella* pathogenicity island
g	Gravity
GFP	Green fluorescent protein
H&E	Hematoxylin and Eosin stain
HBSS	Hank's Balanced Salt Solution
HEK293	Human Embryonic Kidney 293 cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IL	Interleukin
KO	Knock-out
LAMP-1	Lysosome associated membrane protein-1,
LB	Luria Broth
LPS	Lipopolysaccharide
LVS	*Francisella tularensis* subspecies holarctica strain Live Vaccine Strain
MHB	Mueller Hinton Broth
MHB+Sp	Mueller Hinton Broth supplemented with spermine
MHCII	Major histocompatibility complex II
MMP-*9*	Matrix metalloprotease 9

MOI	Multiplicity of Infection
MTD	Median time to death
MyD88	Myeloid differentiation primary response gene (88)
NADPH oxidase	Nicotinamide adenine dinucleotide phosphatase oxidase)
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIAID	National Institute of Allergy and Infectious Disease
NIH	National Institutes of Health
NK cells	Natural Killer cells
NLR	Nod like receptor
NTHI	Non-typeable *Haemophilus influenzae*
OD_{600}	Optical density at 600nm
PAGE	polyacrylamide gel electrophoresis
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
RNA	Ribonucleic Acid
RND	Resistance-nodulation cell division superfamily
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
rRNA	Ribosomal RNA
Schu S4	*Francisella tularensis* subspecies *tularensis* strain Schu S4
SD	Standard deviation
SDS	Sodium dodecyl sulfate

SEM	Standard error of the mean
T6SS	Type six secretion system
TBS	Tris-buffered saline
TEM	Transmission electron microscopy
TIR	Toll-IL1 receptor domain
TLR	Toll like receptor
TMB substrate	3,3’,5,5’-Tetramethylbenzidine
TSB-c	Trypticase soy broth supplemented with cysteine
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
WT	Wild-type

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1.0 INTRODUCTION

1.1 *FRANCISELLA TULARENSIS*

A febrile illness was identified in ground squirrels in Tulare County, California in 1911 (Keim et al., 2007; McCoy, 1910; McCoy and Chapin, 1912). In the following year, a gram-negative bacterium, *Francisella tularensis* was isolated as the causative agent of the febrile illness (McCoy and Chapin, 1912). The first case of human tularemia was reported in 1914 (Wherry and Lamb, 1914), and it was realized that several febrile illnesses were all the result of infection with *F. tularensis* (Keim et al., 2007). Currently, there are between 100-200 cases of tularemia within the US each year.

F. tularensis is a non-motile bacterium (McCoy and Chapin, 1912). It is able to maintain both intracellular and extracellular phases within infected hosts (Forestal et al., 2007; White et al., 1964). The bacterium suppresses the host response, which delays the pro-inflammatory response compared to other gram-negative organisms (Bosio et al., 2007; Carlson et al., 2007; Hall et al., 2008). Host immune cells and epithelial cells are readily infected by *F. tularensis* (Hall et al., 2008; White et al., 1964). In addition to infecting a variety of cell types, *F. tularensis* has been isolated from over 250 species, ranging from amoeba to man (Oyston et al., 2004).

Tularemia in humans has a variety of presentations based on the route of exposure. Pulmonary tularemia is the most severe form of disease (Dennis et al., 2001; Francis, 1928). The bacterium is readily aerosolized, and disease can occur after exposure to as few as 10 colony forming units (Dennis et al., 2001). Infection with *F. tularensis* results in significant morbidity, and without effective treatment, mortality can be as high as 60% (Dennis et al., 2001). The ease of infection and the virulence of *F. tularensis* prompted governments to utilize the bacterium in their bioweapons programs during the 20th century (Oyston et al., 2004; World Health Organization., 1970). Following the anthrax releases in 2001, there has been renewed interest in *Francisella*.

1.1.1 Taxonomy

McCoy and Chapin identified a plague-like illness in ground squirrels in Tulare County in California in 1911, and in the following year, they isolated *Bacterium tularense* as the causative agent (Keim et al., 2007; McCoy, 1910; McCoy and Chapin, 1912; McLendon et al., 2006). The first case of tularemia in humans was identified in 1914 (Wherry and Lamb, 1914), and it soon became apparent that several diseases throughout North America, Europe, and Japan were the result of the same infectious agent (Keim et al., 2007). Between 1919 and 1922, Edward Francis named the disease tularemia, defined the clinical presentations, and identified transmission could occur through contact with contaminated meats or from biting insects (Francis, 1928; Kugeler et al., 2009). Continued investigation of *Bacterium tularense* revealed that it did not fit within the Bacterium genus, and a unique genus should be created. As a result of his pioneering work, *Bacterium tularense* was renamed *Francisella tularensis* in 1947 in

honor of Edward Francis (Keim et al., 2007; Kugeler et al., 2009; McLendon et al., 2006; Olsufiev et al., 1959).

The genus *Francisella* now contains six species: *tularensis, novicida, philomiragia, noantunensis, piscicida,* and *hispaniensis* (Huber et al., 2010; Ottem et al., 2007; Oyston et al., 2004). *F. philomiragia* is only associated with disease in humans in cases of near downing and is typically a pathogen of fish (Wenger et al., 1989). *F. novicida* is frequently only associated with disease of immunocompromised humans (Oyston et al., 2004). Less restrictive biocontainment is required for *F. novicida* than the virulent *F. tularensis* strains, and genetic manipulation has historically been easier with *F. novicida,* making it a useful model for the *Francisella* genus (McLendon et al., 2006). Analysis of the 16S rRNA of members of the *Francisella* genus initially characterized *F. novicida* as a subspecies of *F. tularensis*; however, due to the biochemical, genetic, and clinical differences, this is not an appropriate classification (Forsman et al., 1990; Forsman et al., 1994; Johansson et al., 2010). As a result *F. novicida* is now considered its own species within the *Francisella* genus (Johansson et al., 2010).

Francisella tularensis is the most clinically relevant species of *Francisella* (Dennis et al., 2001; Kugeler et al., 2009; Olsufiev et al., 1959). By 1959, two varieties of tularemia were identified and classified based upon geographic localization and disease severity (Olsufiev et al., 1959; Oyston et al., 2004). It was apparent that the Old World had less clinically severe form of tularemia than the New World, which suggested that there could be multiple *F. tularensis* subspecies causing disease (Keim et al., 2007; Kugeler et al., 2009; Olsufiev et al., 1959). In 1961, these observations were further clarified and *F. tularensis* was classified as either Type A or Type B (Keim et al., 2007). While only Type A is found in North America, Type B is found throughout the northern hemisphere (Keim et al., 2007; Kugeler et al., 2009). Currently, *F.*

tularensis can be divided into three subspecies, which differ based upon their geographic localization, virulence, and genetic makeup (Table 1). *F. tularensis* subsp. *medisiatica* is found in Central Asia and is not believed to cause disease in humans (Keim et al., 2007). *F. tularensis* subsp. *holarctica* (Type B) is found throughout the northern hemisphere. Infection with Type B results in a milder form of disease, which is almost never lethal (Keim et al., 2007; Olsufiev et al., 1959). *F. tularensis* subsp. *tularensis* (Type A) is found only in North America. Type A causes the most severe form of tularemia, with a mortality rate up to 60% in untreated individuals and 1-2% for individuals who do receive treatment (Keim et al., 2007). Type A can be broken down further, again by virulence and geographic localization, into Type AI and Type AII (Farlow et al., 2001). Type AII is found only in the Western half of North America and is a milder form of disease with a low fatality rate (Farlow et al., 2001; Molins et al., 2010; Petersen and Molins, 2010). Type AI is found across North America and is associated with the severe morbidity and high fatality rate described above for Type A (Farlow et al., 2001; Molins et al., 2010; Petersen and Molins, 2010).

1.1.2 Tularemia

Infection with *Francisella tularensis* causes the disease tularemia, which can present in several different forms: ocular, ulceroglandular, oropharyngeal, pneumonic, and typhoidal (Dennis et al., 2001; Francis, 1928). The presentation is based on the route of exposure, and the pneumonic form of disease is the most lethal in humans (Dennis et al., 2001; Evans et al., 1985; Francis, 1928; Sjostedt, 2007; Syrjala et al., 1984). In Europe, infection with *F. tularensis* occurs most frequently from biting insects (Eliasson et al., 2002). Mosquitos, ticks, and flies are capable of being infected with *Francisella* and transmitting it to humans (Dennis et al., 2001;

Olsuf'Ev et al., 1950; Schmid et al., 1983). In the United States, infection can also occur from contact with contaminated carcasses, ingestion of contaminated food and water, or inhalation of aerosolized bacteria (Keim et al., 2007).

Three to five days following infection, there is an abrupt appearance of flu-like symptoms; fever, chills, aches, coryza, and sore throat (Dennis et al., 2001; Evans et al., 1985; Francis, 1928; Syrjala et al., 1984). Often there is a cough associated with the illness independent of pneumonic form of disease. Clinical symptoms worsen with disease progression, and tularemia can rapidly incapacitate the afflicted individual (Dennis et al., 2001). Studies with human volunteers showed that within 24-48 hours of the start of symptoms, study participants were significantly impaired in their ability to perform daily activities, which could continue for several days after treatment (Dennis et al., 2001; Francis, 1928; McCrumb, 1961; Sawyer et al., 1966). The overall case fatality rate within the US is currently less than 2% (Dennis et al., 2001; Martone et al., 1979). However, prior to the advent of antibiotics, the overall case fatality rate was between 10-15% and pneumonic forms of tularemia had reported fatality rates up to 60% (Dennis et al., 2001; McCrumb, 1961).

Ulceroglandular tularemia is associated with a papule at the site of infection, which is often the result of a biting vector (Dennis et al., 2001). The papule fills with pus and eventually ruptures (Dennis et al., 2001; Francis, 1928). Regional lymph node involvement results in significant enlargement of the organ, which can become extensive enough to result in the rupture of the lymph node independent of antibiotic treatment (Dennis et al., 2001; Syrjala et al., 1984). Ocular tularemia occurs after direct contact of the eye with *F. tularensis*, most commonly occurring after handling contaminated meat (Hanna and Lyford, 1971; Pfunder, 1925). It is characterized by ulceration on the conjunctiva, chemosis, and regional lymphadenitis (Dennis et

al., 2001; Pfunder, 1925). Ingestion of food or water contaminated with *F. tularensis* leads to oropharyngeal tularemia. Symptoms can include vomiting, diarrhea, stomatitis, tonsillitis, and pharyngitis. Pulmonary tularemia develops following direct inhalation of *Francisella* or after dissemination from a primary location. The symptoms vary from those associated with systemic illness, but include evidence of pleuritis, pleuropneumonitis, bronchiolitis, and there may be bronchial infiltrates visible through radiologic examination (Dennis et al., 2001; McCrumb, 1961; Sawyer et al., 1966). Typhoidal tularemia is a form of disease when the origin of infection remains unknown (Evans et al., 1985).

1.1.3 *Francisella* as a bioweapon

The idea of biological weapons is not a new one. The first confirmed reported use of a biological weapon was in 400BC, when Scynthian archers dipped their arrow heads in feces or cadavers (Lesho et al., 1998). The long-term psychological impact of biological weapons is probably greater than the clinical harm they inflict (Beeching et al., 2002). Biological weapons are the most cost effective and probably one of the most potent weapons that can be produced, with conventional weapons costing about $2,000 to generate mass casualties on a 1 Km area and biological weapons costing about $1 (Lesho et al., 1998). The Biological Weapons Convention was signed by 165 countries in 1972 to ban their use, but the effectiveness of the treaty has been questioned. It is suspected that the Russian biological weapons program continued at least into the 1990's (Alibek and Handelman, 1999), and there is continued concern biological and chemical weapons could be used in the Middle East (N. A. J. Taylor, 2013). As Dr. Ishii, a General of the Japanese Army, said "If it is important enough to be included in a treaty, it must be worth having in your arsenal" (Lesho et al., 1998).

The intentional use of *F. tularensis* during combat has a long history. While unconfirmed, it is speculated that *F. tularensis* was first used as a biological weapon during the Antolian war in 1300 BC (Oyston et al., 2004; Trevisanato, 2007). There is also speculation that *F. tularensis* was intentionally released by the Soviet Union in the battle of Stalingrad during World War II (Dennis et al., 2001). Between 1932 and 1945, the Japanese tested *F. tularensis* as a bioweapon at secrete germ research units in Manchuria (Harris, 1992). The US developed bioweapons using *F. tularensis* in the 1950's and 1960's, but following executive order in 1970, the stockpiles were destroyed (Oyston et al., 2004). *F. tularensis* used in bioweapons created in Russia included strains that were resistant to both vaccines and antibiotics (Alibek and Handelman, 1999; Oyston et al., 2004), which would have made treating individuals infected by these strains nearly impossible.

Francisella tularensis can induce significant morbidity and mortality in humans. It is easily aerosolized allowing easy dissemination of the organism. It has a very low infectious dose compared to other pathogens, with less than 10 CFU required to induce pulmonary tularemia, compared to 8,000-50,000 spores to induce pulmonary anthrax (Dennis et al., 2001). In 1970, the World Health Organization modeled the release of 50Kg of *F. tularensis* over a population of five million (World Health Organization., 1970). They determined such a release would result in 250,000 cases of disease and 19,000 deaths, with one third of the population fleeing the city (World Health Organization., 1970). Due to its desirable attributes for intentional release, the CDC had listed *F. tularensis* as a Category A select agent. Now, under the revised classifications, *F. tularensis* is categorized as a Tier 1 pathogen with only thirteen other organisms such as *Yersinia pestis*, Ebola virus, and SARS coronavirus (2012).

1.1.4 Intracellular lifecycle

A hallmark of the virulence of *F. tularensis* is an ability to replicate within host cells. Historically, there has been an emphasis on its ability to replicate within macrophages, and they are one of the first cells infected by the pathogen within a mammal (Hall et al., 2008; White et al., 1964). The intracellular lifecycle is complex requiring the involvement of over 180 host genes and over 200 *Francisella* genes (Asare and Abu Kwaik, 2010). *F. tularensis* is able to enter the cell using the endocytic machinery, escape the phagosome, and replicate within the host cytosol (Figure 1)(Santic et al., 2006). How the bacterium egresses the host cell is still undefined (Santic et al., 2006).

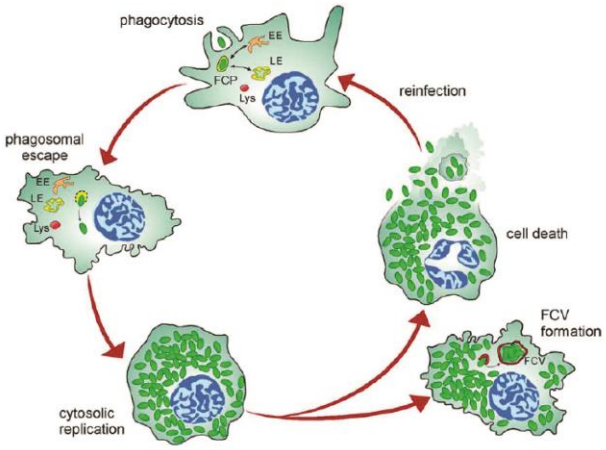

Figure 1: Intracellular lifecycle of *F. tularensis*

Graphical representation of the intracellular lifecycle of *Francisella tularensis.* Adapted from (Chong and Celli, 2010) with permission.

F. tularensis is capable of engaging the host cell using a variety of receptors (Santic et al., 2006). The mannose receptor and complement receptor 3 are the primary receptors employed by *F. tularensis* for entry (Asare and Kwaik, 2010; Geier and Celli, 2011; Schulert and Allen,

2006). However, *F. tularensis* can use FCR-γ, scavenger receptor class A, and nucleolin (Barel et al., 2010; Chong and Celli, 2010; Clemens and Horwitz, 2007). Interestingly, the receptor engaged determines the rate of escape from the phagosome as well as the strength of the pro-inflammatory response produced by the host cell in response to *F. tularensis* (Geier and Celli, 2011).

F. tularensis uses a novel mechanism for entry coined "looping phagocytosis", which is phenotypically different from membrane ruffling or coiling phagocytosis used by other bacteria (Chong and Celli, 2010; Clemens et al., 2005). This looping phagocytosis of *F. tularensis* is characterized by the formation of large pseudopod loops that engulf the bacteria (Clemens et al., 2005). The mechanism *F. tularensis* uses to induce the loops is still poorly defined; it is known that live bacteria are not required (Clemens et al., 2005). Additionally, about 1% of *F. tularensis* observed entering cells via looping phagocytosis have protrusions from their surface that contact the host membrane, suggesting some form of secretion system or pili might be present to coordinate the process (Clemens et al., 2005). For looping phagocytosis to occur, active C3 complement is needed as well as the expression of complement receptors on the surface of the macrophage (Clemens et al., 2005). The loops are believed to form through actin rearrangement as cytochalasin B blocks their formation (Clemens and Horwitz, 2007). Interestingly, C3 binds to the O-antigen of *F. tularensis*, but mutants lacking O-antigen are still competent for looping phagocytosis (Clemens and Horwitz, 2007). Further work is needed to mechanistically define the *F. tularensis* and host components required for looping phagocytosis and to delineate how the process changes when *F. tularensis* uses other receptors for bacterial entry.

Once inside the macrophage, *F. tularensis* is contained within a phagosome (Chong and Celli, 2010). The *Francisella*-containing phagosome (FCP) matures into a late endosome, and

then becomes positive for LAMP-1 (Chong and Celli, 2010). The colocalization of *F. tularensis* with LAMP-1 incorrectly lead to the conclusion that the FCP fused with the lysosome (Chong et al., 2008). It is now clear that the FCP never acquires cathepsin D, a lysosomal acid hydrolase, and it is now known that LAMP-1 is present on the late endosome as well as the lysosome (Clague, 1998). Independent of lysosomal fusion the acidification of the FCP is controversial. There are some reports that the v-ATPase is rapidly acquired on the FCP surface and acidification occurs (Santic et al., 2008). When acidification is blocked by ammonium chloride treatment, phagosomal escape is delayed, suggesting acidification is an important attribute of the FCP (Gavrilin et al., 2006). Conversely, there are reports that the pH within the FCP never goes below 6.7, indicating the FCP does not acidify (Clemens et al., 2004). Interestingly, the studies that conclude acidification occurs used opsonized bacteria for the infection, whereas a lack of acidification is observed when unopsonized bacteria are used (Chong and Celli, 2010).

After a period of time in the FCP, *Francisella* escapes into the cytosol where it is able to replicate exponentially (Chong and Celli, 2010). All reports agree that *Francisella* escapes, but the timing of escape varies from 1-4 hours post infection (Checroun et al., 2006; Clemens et al., 2004; Santic et al., 2008). It has been difficult to determine *Francisella* genes important for the cytosolic phase as many *Francisella* mutants never make it out of the phagosome. Still, at least 34 genes are required in the cytosol and not in the phagosome (Asare and Abu Kwaik, 2010). Many of the genes identified were required for amino acid metabolism and transport (Asare and Abu Kwaik, 2010). After approximately 16 hours post infection, a minority of *Francisella* can be found within *Francisella* containing vacuoles (FCV) (Checroun et al., 2006; Chong and Celli, 2010). The formation of the FCV coincides with the peak of intracellular replication (Cremer et al., 2009a). However, a replication defective mutant of *F. tularensis, ΔripA*, still forms the FCV,

which suggests intracellular replication is not needed for FCV formation (Cremer et al., 2009a). Activation of autophagy and formation of the autophagosome is needed for the formation of the FCV, which then fuses with a lysosome (Checroun et al., 2006). LC3 is detectable on the surface of the FCV, and knockdown of autophagy components will block the formation of FCV (Cremer et al., 2009a). MHCII is also present on the surface of FCV, but its role is unknown (Hrstka et al., 2007). The presence of FCV has only been observed within murine cells and their contribution to *Francisella* virulence is unknown particularly in the human host (Checroun et al., 2006). Finally, through a yet to be determined mechanism, *Francisella* escapes from the host cell.

1.1.5 Virulence factors

Francisella has been identified in greater than 250 host species, which is one of the most diverse host repertoires of any pathogen (Kugeler et al., 2009). *Francisella* thrives in diverse environmental conditions, suggesting the bacterium has numerous virulence attributes to thwart mechanisms toxic to its survival. Surprisingly, there are not Type III, IV, or V secretion systems that near ubiquitously contribute to the virulence of other gram-negative bacterial species, and there are no known secreted toxins (Larsson et al., 2005). A pathogenicity island was identified to be critical for the intracellular replication of *F. tularensis* (Nano and Schmerk, 2007). The LPS of *Francisella* contains a unique structure and is non-toxigenic, and the bacterium produces several capsules (Meibom and Charbit, 2010). With over 300 unique genes, it is becoming clear that *F. tularensis* uses alternative mechanisms for its extreme virulence (Larsson et al., 2005).

1.1.5.1 *Francisella* pathogenicity island

The *F. tularensis* pathogenicity island (FPI) comprises 17 genes, which are nearly conserved across the *Francisella* genus (Figure 2) (Nano and Schmerk, 2007). In the virulent strains, the FPI is present in two copies, while in *F. novicida* there is only one copy (Nano and Schmerk, 2007). *F. novicida* has become a model to study virulence of *F. tularensis* since there is only one copy of the FPI enabling detection of the FPI genes in large genetic screens (de Bruin et al., 2011). It is now know that 14 of the 18 genes are required for *Francisella* to replicate within a cell (de Bruin et al., 2011). Interestingly, the *pdpD* gene in the pathogenicity island differs among the members of the *Francisella* genus. The *pdpD* gene is truncated in Type B but not in *F. novicida* or Type A (Nano and Schmerk, 2007). The ability of *Francisella* to replicate in almost any cell type from an exceptionally diverse host range suggests that the pathogenicity island has evolved to target general eukaryotic mechanisms rather than a subset unique to any one organism or cell type.

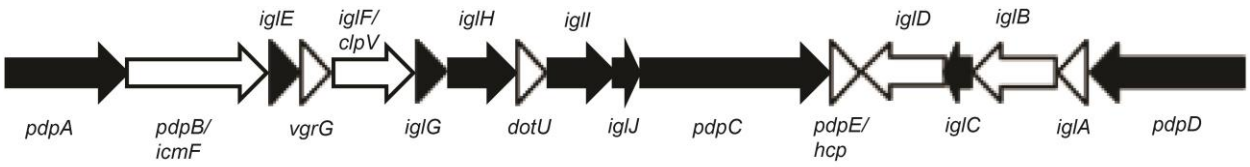

Figure 2: *Francisella* Pathogenicity Island

Schematic representation of the *Francisella* pathogenicity island from *F. tularensis* Schu S4. Adapted from (Barker et al., 2009) with permission.

iglC was the first of the FPI genes to be identified. It was discovered by comparing gene expression in *F. tularensis* after replication within a macrophage to cultivation in broth culture (Golovliov et al., 1997). The operon that contains *iglC* also encodes *iglA*, *iglB*, and *iglD*, and the

entire operon is required for virulence and phagosomal escape (Lai et al., 2004; Nano et al., 2004; Santic et al., 2005).

The FPI is homologous to Type VI secretion systems in other bacteria. *F. tularensis* has 13 of the 14 genes typically required for a functional Type VI secretion system (Barker et al., 2009; de Bruin et al., 2011). It is believed that IglA and IglB form a tube-like structure spanning the periplasm from the inner membrane to the outer membrane (de Bruin et al., 2011). Within the IglA/B tube there is a secondary tube comprised of IglC subunits (de Bruin et al., 2011). PdpB localizes to the inner membrane where it is thought to interact with DotU (de Bruin et al., 2011). However, there is quite a bit of evidence that T6SS would have to function through alternative mechanisms in *Francisella* compared to other bacteria (Barker et al., 2009; Bingle et al., 2008). The ClpV homolog in *Francisella* is predicted to lack the ATPase activity required for T6SS to function in other organisms (Barker et al., 2009). Additionally, there are two secreted components associated with T6SS, VgrG and Hcp (Bingle et al., 2008). There is not a homolog for Hcp in *Francisella,* and the homolog for VgrG that exists is a fragment of the full-length versions in other bacteria (Barker et al., 2009). Consistent with other organisms, VgrG is released from *F. tularensis*, but it is still secreted by a *ΔFPI* mutant demonstrating that VgrG secretion is independent of T6SS in *F. tularensis* (Broms et al., 2012). Despite the release of VgrG independent of the FPI and T6SS, it appears to contribute to the phagosomal escape and virulence of *F. tularensis* (Broms et al., 2012).

1.1.5.2 Surface structures

Lipopolysaccharide (LPS) is a carbohydrate found on the outer surface of gram-negative bacteria. The LPS from *Francisella* does not elicit a pro-inflammatory response from host cells

compared to the LPS from *E. coli*, which induces a robust pro-inflammatory response through TLR4 (Duenas et al., 2006; Kieffer et al., 2003; Medzhitov et al., 1997). In *Francisella*, there are six genes required for LPS biosynthesis (Gunn and Ernst, 2007). *lpxF* and *lpxE* are unique to *Francisella* and are responsible for removing phosphate from lipid A, which prevents signaling through TLR4 (Figure 3) (Gunn and Ernst, 2007; Vinogradov et al., 2002; Wang et al., 2006). *Francisella* LPS contains fatty acid chains that are 14-18 carbons long, which is longer than the 12-14 carbon long chains typically observed in other gram-negative bacteria (Duenas et al., 2006). Additionally, the carbon chains are hypoacylated, which is thought to inhibit proper engagement with receptors for pro-inflammatory signaling (Figure 3) (Duenas et al., 2006; Gunn and Ernst, 2007). The LPS also provides an important barrier to complement mediated killing. C3 attaches to the surface of *Francisella*, but it is rapidly inactivated to C3b*i*, which allows the bacterium to enter the host cell using the CR3 receptor (Clay et al., 2008).

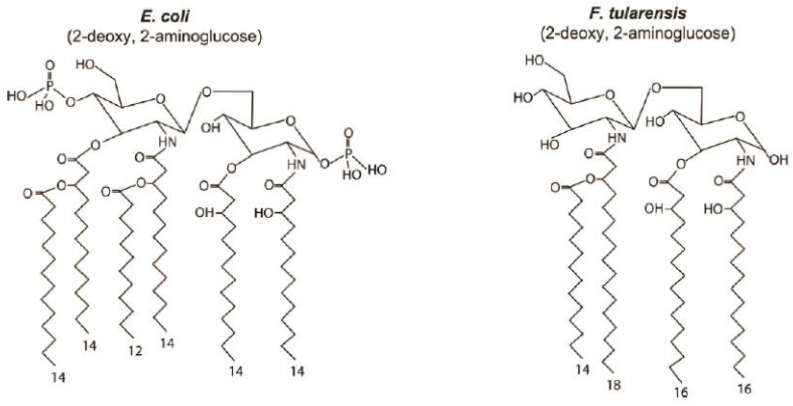

Figure 3: Lipid A of *E. coli* and *F. tularensis*

Graphical representation of the LPS of *E. coli* and *F. tularensis* depicting the longer acyl chain length and lack of phosphate modification of the LPS from *F. tularensis*. Image adapted from (Duenas et al., 2006) with permission.

Capsule polysaccharides are present on the surface of bacteria in addition to LPS and perform a variety of functions for virulence. There are two known types of capsules present on the surface of *Francisella*, an O-antigen capsule and a Capsule Like Complex (CLC), which is comprised of proteins and carbohydrates (Apicella et al., 2010; Bandara et al., 2011). Before genetic manipulation was routine in *Francisella*, spontaneous capsule deficient mutants suggested that the O-antigen capsule played a critical role for *F. tularensis* virulence (Sandstrom et al., 1988). The same chemical structure comprises the O-antigen capsule and the O-antigen modification of LPS in *F. tularensis*; however, the LPS and capsule biosynthetic pathways diverge allowing the blockade of a specific O-antigen modification. For instance, O-antigen modification of LPS can be blocked despite normal O-antigen capsule formation when *lpxL* is deleted in *F. tularensis* (Apicella et al., 2010). Targeted mutagenesis has revealed that the O-antigen capsule is needed for delayed activation of host cell death, replication within macrophages, and serum complement resistance (Lindemann et al., 2011). Recently, the CLC was isolated in *F. tularensis*; it is not important for serum resistance or intracellular replication *in vitro*, but is important for virulence *in vivo* (Bandara et al., 2011). In contrast to the O-antigen capsule, which is comprised almost entirely of carbohydrate, the CLC is a proteinase K resistant glycoprotein, and it is more physically similar to S-layer proteins (Bandara et al., 2011).

Pili are small macromolecular protrusions on the bacterial surface that contribute to bacterial adhesion, secretion, and pathogenesis (Sauer et al., 2000). The presence of pili on the surface of *F. tularensis* was initially described using EM (Gil et al., 2004). In support of visual observations, the genetic components required for pili biosynthesis are present in Type A strains (Larsson et al., 2005). Nonsense mutations in *pilE* and *pilV* occur in Type B strains, and *pilA* is truncated in some strains, but surprisingly, the genes are still needed for pili production

(Forsberg and Guina, 2007; Forslund et al., 2010). In *Francisella*, pili are important for virulence *in vivo* and for adhesion to cells *in vitro,* but they are not required for intracellular replication (Chakraborty et al., 2008; Forslund et al., 2010). In *F. novicida*, there is evidence that Type IV pilus contributes to secretion, but these findings have not been extended to Type A or Type B strains of *Francisella* (Hager et al., 2006).

1.1.5.3 Acid phosphatases

Acid phosphatases hydrolyze phosphomonoesters in prokaryotes and eukaryotes at an acidic pH (Cozzone, 2005). In bacteria, acid phosphatases can confer a survival advantage in neutrophils, because they are able to inhibit the respiratory burst (Aragon et al., 2001). There are four acid phosphatases (AcpA, AcpB, AcpC, and ApcH) in *Francisella,* and their contribution to virulence remains controversial. Initially, *F. novicida* was used to investigate the importance of acid phosphatases for *Francisella* (Reilly et al., 1996). In *F. novicida*, AcpA is required for phagosomal escape, replication in macrophages, and virulence *in vivo* (Mohapatra et al., 2007). Additionally, deletion of all four acid phosphates in *F. novicida* depleted greater than 95% of the phosphatase activity, and the quadruple deletion mutant was attenuated similar to AcpA alone (Mohapatra et al., 2008). AcpA from *F. novicida* is also important for inhibiting the oxidative burst in neutrophils by leading to the dephosphorylation of $p47^{Phox}$ and $p40^{Phox}$ (Mohapatra et al., 2010). In *F. tularensis*, a triple acid phosphatase deletion mutant, *ΔacpABC*, lost greater than 95% of its phosphatase activity, but unlike *F. novicida*, there was no impact on virulence *in vivo*, intracellular replication, or phagosomal escape (Child et al., 2010). More recently, it was determined AcpA is secreted into the macrophage cytosol from all strains of *Francisella* tested (Dai et al., 2012). However, the cytosolic target of AcpA is unknown, and the relevance of its secretion for the virulence of *F. tularensis* still needs to be determined (Dai et al., 2012),

especially considering the lack of importance of the gene in virulent *F. tularensis* (Child et al., 2010).

1.1.5.4 Efflux pumps

Efflux of toxic components is critical for the survival of any bacteria, and efflux systems often contribute to resistance to host antimicrobial defenses (Nikaido and Pages, 2012). *Francisella* encodes several efflux systems, such as RND efflux, TolC, and AcrAB (Larsson et al., 2005; Meibom and Charbit, 2010). TolC was not important for the intracellular replication of *F. tularensis*, but the *tolC* mutant was attenuated in a pulmonary model of pneumonic tularemia (Gil et al., 2006; Platz et al., 2010). *F. tularensis* strains lacking *tolC* are more stimulatory to macrophages and are more sensitive to detergents, dyes, and antibiotics (Gil et al., 2006; Platz et al., 2010). The *tolC* mutant was more cytotoxic to host cells, and its attenuation *in vivo* could have resulted from depleting the replicative niche (Platz et al., 2010). *acrAB* is an RND efflux system important for the resistance to antibiotics, detergents, and some bile acids (Bina et al., 2008). *In vivo*, a loss of *acrAB* led to an attenuation of virulence (Bina et al., 2008). Finally, TrkH regulates the concentration of intracellular potassium in *F. tularensis*, and it is needed *in vivo* for survival in the blood of the host independent of complement resistance (Alkhuder et al., 2010). This result suggests the regulation of ions *in vivo* is important for the survival of *Francisella*.

1.1.5.5 Other virulence determinants

Francisella also contains a variety of mechanisms to enhance its virulence at each stage of its lifecycle in addition to the ones discussed above. *Francisella* uses DeoB, in part, for invasion of macrophages through an unknown mechanism (Horzempa et al., 2008). During the

replication of *Francisella*, the pH is increased rather than decreased as occurs with most pathogens, and it is thought that the higher pH helps combat acidification of the phagosome (Meibom and Charbit, 2010). Additionally, ROS and RNS are targeted to the interior of the phagosome to protect the rest of the cell from their toxic effects. *Francisellae* encode a *katG* homolog that is important for the resistance to reactive oxygen and nitrogen species (Lindgren et al., 2007; Melillo et al., 2010). However, the virulent strains of *Francisella* remain more resistant to ROS and RNS than attenuated strains suggesting they have additional mechanisms to detoxify these compounds (Lindgren et al., 2011). Indeed, the virulent strains of *F. tularensis* have a lower intracellular concentration of iron, which prevents toxicity from oxidative stress (Lindgren et al., 2011). The cytosolic phase of the *Francisella* involves both replication and activation of the inflammasome. Several metabolic genes are important for intracellular replication including those involved in nucleotide synthesis, *purMCD* (Pechous et al., 2006). Activation of the inflammasome leads to pyroptosis and cell death (Fernandes-Alnemri et al., 2010). *Francisella* alters the timing of cell death induction (Schwartz et al., 2012). The regulation of cell death is thought be important for virulence of *Francisella*. Extending cell viability allows for intracellular replication, and then induction of cell death enhances spread of the *F. tularensis in vivo*. MviN, a lipid II flipase, is required for the activation of AIM2 in murine cells, and it contributes to the spread of *Francisella* within the liver (Ulland et al., 2010). Throughout the lifecycle, maintaining the activity of proteins present on the cell surface requires protein disulfide isomerase to alter the thiol redox state. Thus far, two lipoproteins with homology to DsbA and DsbB have been found, and they are required for virulence of *Francisella* (Qin et al., 2008; Qin et al., 2009). Thus, *F. tularensis* contains a repertoire of mechanisms to thwart normal cellular processes for its survival and virulence.

1.1.5.6 Regulation of *Francisella* virulence

The diverse environmental conditions in which *Francisella* survives suggests that it must have mechanisms to respond to environmental changes. Genetic regulation is tightly controlled by alternative sigma factors that interact with the RNA polymerase to determine gene expression (Kazmierczak et al., 2005). RpoH, an alternative sigma factor, negatively regulates FPI gene expression, while positively regulating general stress response genes (Grall et al., 2009). However, there are only two sigma factors encoded in *Francisella*, which is significantly fewer than typically found in pathogenic bacteria (Larsson et al., 2005). This suggests that there are alternative means of genetic regulation or much of the regulation occurs post transcriptionally (Larsson et al., 2005). Understanding genetic regulation in *Francisella* has focused on the FPI, due to its necessity *in vitro* and *in vivo* for virulence. MglA and MglB are homologs of stringent starvation response protein (Ssp) A and SspB in *E. coli* (Baron and Nano, 1998). MglA/B positively regulate FPI expression, and they negatively regulate genes important for the general stress response and the oxidative stress response such as KatG (Brotcke et al., 2006; Guina et al., 2007; Lauriano et al., 2004). In conjunction with MglA/B, MigR and FevR positively regulate FPI expression (Brotcke and Monack, 2008; Buchan et al., 2009). But they do not seem to be important for the regulation of oxidative stress responses. *Francisella* has both intracellular and extracellular phases *in vivo*. RipA is predicted to be important for sensing when *F. tularensis* is intracellular, by responding to changes in the pH (Fuller et al., 2008).

1.1.6 Host adaptation

Francisella tularensis is able to thrive in a plethora of environments inside and outside of its mammalian hosts, and it has mechanisms to detect environmental changes and adapt to them. Culture conditions significantly impact the phenotypes of *F. tularensis*, particularly, the ability to elicit cytokines from macrophages. There are a variety of culture media used to grow the bacterium. Chamberlain's chemically defined medium (CDM) was established to meet the fastidious requirements of *F. tularensis* for growth and to standardize the culture conditions for vaccine development (Chamberlain, 1965). CDM cultured bacteria elicit minimal amounts of cytokines from macrophages, whereas, Mueller Hinton Broth (MHB) cultured *F. tularensis* stimulate macrophages to produce cytokines (Figure 4) (Carlson et al., 2007). Polyamines present in CDM were found to suppress the stimulation of *F. tularensis* (Figure 4) (Carlson et al., 2009). The intracellular environment is replete with polyamines, particularly spermine. Polyamines are required for the viability of both eukaryotic and prokaryotic organisms. Bacterial and eukaryotic cells make putrescine and spermidine, but spermine is found only in eukaryotic cells (Tabor and Tabor, 1985). Using spermine as a cue for the intracellular environment, *F. tularensis* can adapt to the host (Carlson et al., 2009); however, the bacterial genes required for this response remain unknown. Aside from polyamine concentration, *F. tularensis* is sensitive to the concentration of amino acids in its environment (Hazlett et al., 2008). More broadly, recognition of the host environment is important. *F. tularensis* isolated from macrophages is less stimulatory to host cells than *F. tularensis* isolated from MHB (Loegering et al., 2006), which suggests *F. tularensis* is able to respond to the intracellular environment to become less stimulatory. Additionally, *F. tularensis* isolated from macrophages is more virulent in a mouse model of tularemia than *F. tularensis* cultured in broth (Loegering et al., 2006). Further,

transmission of *Francisella* to mammals, including humans, often requires a vector. The transition from a vector host to a mammalian host coincides with a temperature shift. TivA, a novel gene in *Francisella* is strongly up regulated by temperature and important for virulence (Horzempa et al., 2008). In conclusion, *F. tularensis* uses a variety of cues to adapt to the host environment to enhance its virulence and escape the host immune response.

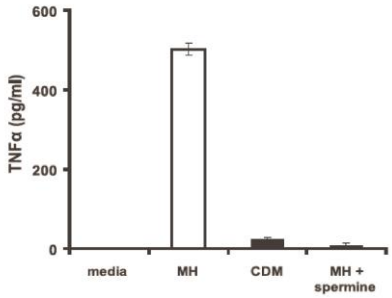

Figure 4: *Francisella* uses spermine as a signal to adapt to the host environment

Bacteria were cultured in broth overnight in the presence or absence of spermine. Human macrophages were cocultured with *F. tularensis* strains for 24 hours at an MOI of 10. The supernatants were collected and the concentration of TNF-α was quantified by ELISA. The figure was adapted from (Carlson et al., 2009) with permission.

1.2 IMMUNE RESPONSE TO *FRANCISELLA TULARENSIS*

The immune response to *Francisella tularensis* is both insufficient and delayed (Cowley, 2009). In contrast to infection with *F. tularensis*, recruited cells and cytokines are readily detectable within hours of infection with other pulmonary pathogens such as *Klebsiella* or *Pseudomonas* (Greenberger et al., 1995; Ye et al., 2001; Yu et al., 2000). Following infection with *F. tularensis*, an immune response is not detected for several days, and it is not until three

days post infection that cytokines and recruited cells are detected in the lung (Cowley, 2009; Hall et al., 2008; Russo et al., 2011; Schmitt et al., 2012). It is possible that the delayed activation of the host response contributes to the pathogenesis of *F. tularensis.* When the host immune response is activated earlier during infection, mice are able to survive challenge with *F. tularensis* longer than their untreated counterparts (Evans et al., 2010; Skyberg et al., 2012). Once the immune response becomes detectable, it rapidly develops into a septic-like state with hypercytokinemia and lymphocyte depletion (Mares et al., 2008; Mares et al., 2010). Organ pathology is observed at both the site of infection and at distal sites (Conlan et al., 2003; Parmely et al., 2009). It is still uncertain whether the bacteria itself, the host response, or a combination of the two leads to the observed organ pathology and the death of the host. Using attenuated strains of *F. tularensis* such as live vaccine strain (LVS), there has been significant progress in elucidating cell population, effector function, and cytokines important for the host response against *F. tularensis* (Cowley and Elkins, 2011). However, caution must be used when extrapolating the findings with the attenuated strains into the virulent model of tularemia. Direct assessment of these findings has failed to identify any cytokine or cell population that contributes to the host response against virulent *F. tularensis* strains (Chen et al., 2004b; KuoLee et al., 2011; Schmitt et al., 2012). Also, assessment of the responses of blood monocytes to *F. novicida* and *F. tularensis* SchuS4 has demonstrated that there are 13,000 genes differentially induced in monocytes by both pathogens. However, only 46% of the induced genes are shared between both strains of *Francisella*, demonstrating that the host response to virulent strains will be different than the response to the attenuated strains of *F. tularensis* (Butchar et al., 2008).

1.2.1 Cellular responses

The first cells that are infected following a pulmonary exposure to *F. tularensis* are alveolar macrophages and dendritic cells (Hall et al., 2008). *Francisella* is able to replicate within these cells and at the same time limit the pro-inflammatory response and production of cytokines, thus contributing to a delay in an effective host response (Bosio and Dow, 2005). What little response does occur is a result of signaling through TLR2 (Cole et al., 2007). Initially, it was thought that only human macrophages were able to produce cytokines in response to *F. tularensis*, but it is now apparent that both human and murine macrophages will produce cytokines (Bolger et al., 2005). *F. tularensis* is able to induce alternatively activated macrophages (Shirey et al., 2008). Alternatively activated macrophages suppress the immune response by producing IL-10 and TGF-β (Gordon, 2003). Additionally, macrophages clear cellular debris and infected cells at sites of infection (Korns et al., 2011). *F. tularensis* is able to block efferocytosis by macrophages contributing to accumulation of cell debris within the organs (Mares et al., 2011). Interestingly, cellular debris induces alternative activation of macrophages enhancing the suppressive effects of *F. tularensis* (Mares et al., 2011). Clodronate depletion of macrophages and dendritic cells in the lung prolonged survival of mice infected with LVS, suggesting that within the lung, these cells do not properly function during immune responses to *F. tularensis* (Bosio and Dow, 2005). More recently, replication within a macrophage was not required for the virulence of *F. tularensis* ΔpyrF mutant (Horzempa et al., 2010a). Further work is needed to define the role for the macrophage during the immune response to *F. tularensis.*

In addition to immune cells, epithelial and endothelial cells are present in the lung, but until recently the contribution of these cell populations to the host response to *Francisella* was largely ignored. *F. tularensis* is able to infect and replicate within type II alveolar epithelial cells

with kinetics that are similar to that of other cells (Craven et al., 2008). The importance of the *in vitro* observation has been confirmed *in vivo*, where *F. tularensis* is found replicating within type II alveolar epithelial cells by day four post infection (Hall et al., 2008). *F. tularensis* induces human umbilical vein endothelial cells to produce pro-inflammatory cytokines, demonstrating endothelial cells could have a role in the host response *in vivo* (Forestal et al., 2003). Further work is needed to understand the true importance of epithelial and endothelial cells to the host response to *F. tularensis in vivo*.

After a period of immune quiescence, cellular recruitment is detectable at the sites of bacterial replication (Cowley and Elkins, 2011). Neutrophils are the primary cell population recruited in both the lung and distal organs (Bokhari et al., 2008). It is suggested that they play an important role during an LVS infection; depletion of neutrophils enhances the virulence of LVS (Sjostedt et al., 1994). In contrast, neutrophil depletion in mice infected with *F. tularensis* Schu S4 does not change the median time to death compared to control mice, suggesting neutrophils do not contribute significantly to infection with virulent strains (KuoLee et al., 2011). It is known that both attenuated and virulent strains of *Francisella* infect and replicate within neutrophils (McCaffrey and Allen, 2006). Once inside the neutrophil, *Francisella* blocks the assembly and activity of the NADPH oxidase complex preventing a respiratory burst, thereby enabling *F. tularensis* to replicate unimpeded (McCaffrey and Allen, 2006).

IFN-γ is believed to be critically important to combat infection with *Francisella tularensis* (Green et al., 1993). Greater than 90% of IFN-γ is produced by Natural Killer (NK) cells during infection with *F. tularensis* (Schmitt et al., 2012). Cellular depletion of NK cells significantly reduces IFN-γ produced during pulmonary infection with *F. tularensis* (Schmitt et al., 2012). During infection with LVS, NK cells are important for formation of immune foci in

the liver, which contributes to containment of LVS and limits pathology in the liver (Bokhari et al., 2008). Additionally, NK cell depleted mice are more susceptible to infection with LVS than control mice (Lopez et al., 2004). Unfortunately, when Schu S4 is administered following NK cell depletion, there is no effect on survival (Schmitt et al., 2012). Still, a beneficial role for NK cells could exist within the context of an *F. tularensis* infection. Treatment of mice with immunological stimulants such as acai berry polysaccharide or non-typeable *Haemophilus* extract prior to infection with virulent *F. tularensis* prolongs survival (Evans et al., 2010; Skyberg et al., 2012). The beneficial effect from these stimulants is at least in part dependent on the production of IFN-γ by NK cells.

There is a role for mast cells during the immune response to *F. tularensis*. Mast cells are often described to play a role during allergic reactions (Urb and Sheppard, 2012). *In vitro*, mast cells restrict the replication of both LVS and Schu S4 demonstrating that they could play an important role during infection with both strains of *Francisella* (Rodriguez et al., 2011). However, only a role during infection with LVS has been experimentally confirmed *in vivo*, in which mast cells secrete IL-4 to function as a pro-survival signal for macrophages (Rodriguez et al., 2011). Their response to LVS is dependent on the presence of TLR2 (Rodriguez et al., 2012).

Attenuated strains of *F. tularensis* can be cleared in the murine model of tularemia. During infection with LVS, T cells are important for clearing the bacteria. Depletion of CD8 or CD4 T cells had no effect on clearance, but depletion of both cell populations in combination significantly prevented clearance (Conlan et al., 1994; Yee et al., 1996). These results demonstrated that either population is sufficient for clearance.

1.2.2 Cytokine responses

Signaling by cytokines and chemokines recruits immune cells to the sites of infection and induces the activation of antimicrobial effectors. TNF-α and IFN-γ are essential during infection with LVS, and mice cannot survive infection when either cytokine is depleted or knocked out. (Leiby et al., 1992) However, these cytokines seem to contribute only minimally during vaccination response, where they are only needed when high challenge doses are used (Sjostedt et al., 1996). In contrast to results with LVS, IFN-γ depleted or IFN-γ KO mice were not more susceptible to infection with virulent *F. tularensis* (Chen et al., 2004b). These findings led to the conclusion that IFN-γ does not have a role during infection with virulent *F. tularensis*. Conversely, a convalescent model has been developed, in which mice are administered virulent *F. tularensis* and treated with antibiotics to prolong infection (Crane et al., 2012). Within this model, only IFN-γ KO mice are unable to survive with antibiotic treatment, which suggests there is some role for IFN-γ during infection with *F. tularensis*, but the extent of this role still needs to be evaluated (Crane et al., 2012).

IL-17A has a role during pulmonary but not dermal infections with *F. tularensis* (Lin et al., 2009), and it is produced by double negative T cells in the lung (Cowley et al., 2010). IL-17A in combination with IFN-γ activates the alveolar epithelial cells leading to the control of LVS growth (Cowley et al., 2010). The process is dependent on the activation of NF-κB. Additionally, IL-17A is important for inducing the production of IL-12p40 and IFN-γ from macrophages, which leads to a Th1 polarization of T cells (Lin et al., 2009).

IL-6 is produced in high quantities during infection with *Francisella*, and it is a marker of the septic-like state that occurs in the latter stages of infection (Kurtz et al., 2012). Depletion of

IL-6 *in vivo* revealed it was important for restricting bacterial burdens in the organs and prolonging survival of LVS infected mice (Kurtz et al., 2012).

1.2.3 Pattern recognition receptors

Toll like receptors (TLRs) recognize common motifs present on pathogens; bacteria, fungi, and viruses. TLRs signal through myeloid differentiation primary response gene 88 (MyD88) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to induce the production of pro-inflammatory cytokines. Each TLR is specific for particular motifs. The LPS of bacteria typically activates TLR4, but the LPS of *F. tularensis* does not (Vinogradov et al., 2002). However, during infection with *F. tularensis*, activation of TLR4 does occur through the recognition of DnaK and elongation factor *Tu* (Ashtekar et al., 2008; Sharma et al., 2011b). Despite activating through TLR4, deletion of the receptor has no consequence on infection with *Francisella in vivo* (Chen et al., 2004a).

TLR2 is the primary receptor that is engaged following infection with *F. tularensis* (Cole et al., 2007). TLR2 is able to heterodimerize with TLR1 or TLR6, and both heterodimers contribute to TLR2 signaling in macrophages (Li et al., 2006). Interestingly, TNF-α and IL-6 are among many cytokines whose activation is increased following TLR2 activation, but IFN-γ is reduced (Malik et al., 2006). Two lipoproteins have been found on *F. tularensis* to activate TLR2, Tul4 and FTT_1103 (Thakran et al., 2008). Unexpectedly, a recent report suggests that bacterial DNA can also engage TLR2 to induce pro-inflammatory signaling in response to *F. tularensis* (Singh et al., 2013).

1.2.4 Suppression of the immune response

Francisella remains a formidable pathogen due to its ability to actively suppress activation of the host response through a variety of mechanisms. Macrophages are induced to produce PGE_2 by *F. tularensis* (Hunt et al., 2012). PGE_2 restricts production of IFN-γ by CD4 cells and induces the production of IL-10, an anti-inflammatory cytokine (Hunt et al., 2012). In the lung, Schu S4 is able to suppress activation of dendritic cells, and induce the production of the immunosuppressive cytokine TGF-β (Bosio et al., 2007). In contrast, infection with *F. novicida* induces the expression of miR155, a microRNA that targets SHIP for down regulation (Cremer et al., 2009b). SHIP down regulation enhances escape from the phagosome for *F. novicida* and increases cell death enhancing the virulence of *F. novicida* (Rajaram et al., 2009). Virulent *F. tularensis* does not induce the activation of miR155 as strongly as *F. novicida*, but the consequence of this is unknown (Cremer et al., 2009b). Additionally, *Francisella* activates PI3 kinase, which diminishes the effect of signaling through TLR2 and limits the response to *F. tularensis* (Medina et al., 2010).

There are a host of mutants known to induce more cell death leading to the production of more pro-inflammatory cytokines, suggesting that these genes are normally important for limiting cytokine production (Peng et al., 2011). An OmpA like protein in LVS and Schu S4 was found to be important for intramacrophage replication and limitation of cytokine production (Mahawar et al., 2012). In *F. novicida*, OmpC functions though an unknown mechanism to decrease STAT1 activation (Nallaparaju et al., 2011). Activation of STAT1 leads to increased nitric oxide production and restriction of bacterial burden (Nallaparaju et al., 2011). Further work is needed to identify additional genes in *F. tularensis* that restrict the host pro-inflammatory response.

1.3 CONTRIBUTION OF CORC TO BACTERIAL VIRULENCE AND PHYSIOLOGY

CorC is a member of the Cobalt Resistance (Cor) family in *Salmonella.* The first member of the family to be identified was CorA, which is one of the predominate magnesium transporters for bacteria in addition to MgtA and MgtB (Hmiel et al., 1986; Smith and Maguire, 1998). In addition to Mg^{2+} transport activity, a loss of CorA was associated with an increased resistance to cobalt. Preliminary screens to identify proteins in *S. typhimurium* that affected the activity of CorA identified CorB-D (Gibson et al., 1991). Little is known about the function and importance of CorB-D for bacterial virulence. There are homologs for CorB and CorC, but not CorA or CorD, in *F. tularensis* by BLAST analysis. Initial studies in *S. typhimurium*, identified a strain lacking *corC* had a decreased rate of cobalt efflux, which suggested that CorC has a role in regulating ion homeostasis and that it might function in conjunction with CorA (Gibson et al., 1991). Domain analysis of CorC reveals a tandem CBS domain in the N-terminus, and a CorC domain at the C-terminus. The function of CorC domain remains unknown, but CBS domains are considered to be regulatory elements responsive to Mg^{2+} and adenine derivatives such as ATP and cAMP (Scott et al., 2004). These findings have been extended to CorC, in which attempts to crystallize the protein demonstrate that the confirmation is altered by the binding of either ATP or Mg^{2+} (Zhang et al., 2010). Psort analysis localizes the protein to the cytoplasm or the inner membrane, which has been confirmed in *Flavobacterium* (Hunnicutt and McBride, 2001).

While there is evidence that the Mg^{2+} ion concentration is altered in *S. typhimurium,* it is unlikely that the protein is directly involved in the transport process itself. There are not membrane domains by bioinformatics analysis, and there is no evidence that it directly interacts

with the CorA protein to mediate these effects. A higher intracellular concentration of Mg^{2+} within *corC* mutants was thought to outcompete the toxic effects of cobalt allowing the mutant to have a higher resistance to cobalt (Gibson et al., 1991). Newer reports have connected a variety of phenotypes with the loss of *corC* in bacteria: chloramphenicol sensitivity in *E. coli* (Smith et al., 2007), a reduction in plasmid conjugation efficiency in *E. coli* (Perez-Mendoza and de la Cruz, 2009), and gliding motility in *Flavobacterium (Hunnicutt and McBride, 2001).* However, the role of CorC in gliding motility is not clear, disruption of the *corC* homolog in *Flavobacterium* was unsuccessful, and overexpression of *corC* was able to complement only one allelic mutant (Hunnicutt and McBride, 2001). Yet, it still remains to be determined how *corC* is able to mediate these seemingly unrelated phenotypes.

In *F. tularensis, corC* has been identified in three screens. In *F. novicida*, a negative selection screen identified *corC* was required for proliferation in the spleen, suggesting that it was important for the virulence of *F. tularensis in vivo* (Weiss et al., 2007). Additionally, *corC* was found in a screen conducted in drosophila cells to identify genes important for intracellular replication (Moule et al., 2010). There is some indication that *corC* could be relevant to the virulence of Type A strains. In a study set up to identify correlates of virulence from natural isolates, *corC* was disrupted in an attenuated Type A strain (Sjodin et al., 2010). However, this result was confounded by *pdpD* being disrupted in the same strain. Due to the already known role for *pdpD* in the virulence of *F. tularensis*, the attenuation of the Type A strain was attributed to disruption of *pdpD* and a role for *corC* was ignored. Thus, there is evidence that *corC* could play a role in the virulence of *F. tularensis*. Further work is needed to explore the contribution of *corC* to the bacterium's virulence.

1.4 STATEMENT OF THE PROBLEM

Infection with *Francisella tularensis* is associated with a delay in the host immune response and robust bacterial replication, which enables the organism to overtake its host. A key feature to escape the host immune response is an ability to detect the host environment and adapt. *F. tularensis* is capable of using multiple signals to adjust to the intracellular environment including, temperature, pH, iron concentration, amino acid concentration and polyamine concentration. However, the genes required for the process of adaption remain unknown. The work presented in this thesis, identifies *corC,* a ubiquitous gene with a poorly defined function, as a gene important for the bacterial response to spermine. In order to understand the importance of *corC* for the virulence and physiology of *F. tularensis,* deletion mutants were generated in the LVS and Schu S4 *corC* homologs, FTL_0883 and FTT_0615c respectively. These mutants were phenotypically characterized using *in vitro* and *in vivo* models. Understanding the role of *corC* in *F. tularensis* furthers our understanding of the virulence of this bacterium and how it escapes the host response to maintain its virulence.

Delayed innate immune activation contributes to the virulence of *F. tularensis*, as early activation extends survival in a murine model of tularemia. However, the role for the immune response is unclear in the context of virulent *F. tularensis* infections. Studies with attenuated strains of *F. tularensis* have identified important attributes of the host response; however, none of these correlates have translated to infections with virulent *F. tularensis* strains. Yet, during infection with virulent *F. tularensis,* the bacterial burden slows commensurate with immune activation suggesting the host response could play a role. To further define the role for the host response during primary pneumonic tularemia with virulent *F. tularensis*, MyD88 knock-out (KO) mice were used as an immunodeficient model. The pathogenesis of Schu S4 was

investigated in both wild-type and MyD88 deficient mice to define the contribution of the host response during infection. Overall, the work presented in this dissertation furthers our understanding of the interaction between *F. tularensis* and its host. Identifying that *F. tularensis* requires *corC* to maximize its virulence to avoid the host immune response, which is dependent on MyD88 signaling.

2.0 A *FRANCISELLA TULARENSIS* LOCUS REQUIRED FOR SPERMINE RESPONSIVENESS IS NECESSARY FOR VIRULENCE

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BC Russo designed the scope of the study, performed experiments, analyzed the data, and prepared the manuscript. GJ Nau designed the scope of the study and prepared the manuscript. J Horzempa performed experiments, assisted with cloning and the transposon screen. DM O'Dee performed experiments and analyzed data. PE Carlson designed and performed the transposon screen. DM Schmitt performed experiments. MJ Brown performed experiments. RJ Xavier provided reagents.

2.1 ABSTRACT

Tularemia is a debilitating febrile illness caused by the Category A Biodefense Agent, *Francisella tularensis*. This pathogen infects over 250 different hosts, has a low infectious dose, and causes high morbidity and mortality. Our understanding of the mechanisms by which *F. tularensis* senses and adapts to host environments is incomplete. Polyamines, including spermine, regulate *F. tularensis'* interactions with host cells. However, it is not known if responsiveness to polyamines is necessary for the virulence of the organism. Through transposon mutagenesis of *F. tularensis* subsp. *holarctica* Live Vaccine Strain (LVS), FTL_0883 was identified as a gene important for spermine responsiveness. In-frame deletion mutants of FTL_0883 and FTT_0615c, the homolog of FTL_0883 in *F. tularensis* subsp. *tularensis* Schu S4 (Schu S4), elicited higher levels of cytokines from human and murine macrophages compared to wild-type strains. Although deletion of FTL_0883 attenuated LVS replication within macrophages *in vitro*, the Schu S4 mutant with a deletion in FTT_0615c replicated similarly to wild-type Schu S4. Nevertheless, both the LVS and Schu S4 mutants were significantly attenuated *in vivo*. Growth and dissemination of the Schu S4 mutant was severely reduced in the murine model of pneumonic tularemia. This attenuation depended on host responses to elevated levels of pro-inflammatory cytokines. These data associate responsiveness to polyamines with tularemia pathogenesis and define FTL_0883/FTT_0615c as a *F. tularensis* gene important for virulence and evasion of the host immune response.

2.2 INTRODUCTION

Francisella tularensis, the causative agent of tularemia, is a small, non-motile, gram-negative bacterium (McLendon et al., 2006). The Centers for Disease Control and Prevention classified *F. tularensis* as a Category A Biodefense Agent because of the severity of tularemia and the ease of preparation and dissemination in an intentional release. There are two clinically relevant subspecies of *F. tularensis, F. tularensis* subsp. *tularensis* (Type A) and *F. tularensis* subsp. *holarctica* (Type B) (Petersen and Molins, 2010). Type A *Francisella* is capable of infecting a diverse host range and is highly infectious, as few as 10 bacteria can cause fatal disease in humans if left untreated (Dennis et al., 2001; McLendon et al., 2006). Type B *Francisella* results in a milder disease in humans and is rarely fatal (Petersen and Molins, 2010). The Live Vaccine Strain (LVS) was generated from a Type B isolate, which has become an important model for *F. tularensis* pathogenesis and biology (Petersen and Molins, 2010).

F. tularensis is a facultative, intracellular pathogen and mutations preventing invasion and intracellular replication typically result in severely attenuated strains (Baron and Nano, 1998; Charity et al., 2007; Qin et al., 2008; Qin et al., 2009). Once within the host, *F. tularensis* infects a variety of cell types, including phagocytes, neutrophils, alveolar epithelial cells, hepatocytes, and fibroblasts (Anthony et al., 1991; Carlson et al., 2007; Conlan and North, 1992; Hall et al., 2007; McCaffrey and Allen, 2006). Phagocytes, particularly macrophages, are thought to be an important replicative niche for *F. tularensis in vivo*. *F. tularensis* is detectable within host macrophages and dendritic cells within one hour after infection (Bosio et al., 2007; Craven et al., 2008; Hall et al., 2007; Hall et al., 2008). The pro-inflammatory response within these cells is blocked by *F. tularensis*, which prevents the production of cytokines and chemokines (Bosio et al., 2007; Carlson et al., 2007; Telepnev et al., 2003). Reduced levels of

cytokines early after infection have been correlated with an inability of the host to control replication of the bacterium (Elkins et al., 2003; Mares et al., 2008). The mechanism(s) by which *F. tularensis* manipulates the host immune response is poorly understood. In order to understand how the bacterium interacts with the host, it is critical to define the mechanisms of host immune evasion.

Successful evasion of the host immune response may be partly due to an adaptation of *F. tularensis* to the intracellular environment (Carlson et al., 2009; Loegering et al., 2006). Loegering *et al*. demonstrated that after replication within macrophages, *F. tularensis* is less stimulatory in subsequent macrophage infections compared to *F. tularensis* cultured in bacterial growth media (Loegering et al., 2006). These authors concluded that *F. tularensis* is able to adapt to the host environment to evade the immune response. However, the mechanism(s) enabling *F. tularensis* to sense and adapt to the host environment must be delineated. Among possible signals, polyamines, temperature, and amino acid concentration are known to be important environmental cues that alter bacterial virulence (Carlson et al., 2009; Hazlett et al., 2008; Horzempa et al., 2008).

Polyamines are small polycationic molecules. They are found ubiquitously in the cytosol of both prokaryotes and eukaryotes at milimolar concentrations (Wortham et al., 2007). These compounds have important roles in a variety of biological processes such as regulating transcription and translation, altering enzyme activity, and binding to DNA to neutralize its negative charge (Tabor and Tabor, 1985). For *F. tularensis*, polyamines signal the bacterium when it is within the host cytosol (Carlson et al., 2009). Macrophages infected with *F. tularensis* previously cultured in the presence of polyamines, including spermine, produce significantly less pro-inflammatory cytokines than macrophages infected with *F. tularensis* previously cultured in

media alone (Carlson et al., 2009). The *F. tularensis* proteins required to respond to spermine and adapt to the intracellular environment remain largely unknown. Moreover, the importance of this response remains undefined.

To understand the contribution of the spermine response to pathogenesis, a screen was developed in LVS to identify mutants unable to respond to extracellular spermine. We identified a gene in *F. tularensis* LVS, FTL_0883, which is necessary for spermine responsiveness. Mutants in FTL_0883 or its homolog in the Type A strain Schu S4, FTT_0615c, elicit increased levels of cytokines from macrophages, and are attenuated *in vivo*. Our results indicate that FTL_0883 and FTT_0615c are required for *F. tularensis* evasion of host defenses and virulence.

2.3 METHODS

Bacterial strains. *F. tularensis* subsp. *holarctica* LVS (a gift from Karen Elkins) and *F. tularensis* subsp. *tularensis* Schu S4 [obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: (FSC237), NR-643] were streaked onto chocolate II agar plates and cultured between one and three days at 37ºC with 5% CO_2. Liquid cultures of *F. tularensis* were grown at 37ºC at 250 rpm in either Trypticase Soy Broth supplemented with cysteine (TSB-c), Mueller Hinton Broth (MHB) supplemented with 0.1% glucose, 0.025% ferric pyrophosphate, and IsoVitaleX, or Chamberlain's defined media (CDM) (Chamberlain, 1965). *E. coli* strain EC100D was used for routine cloning and was cultured in Luria Broth (LB) or on LB agar. When antibiotics were required, kanamycin was used at 10 μg/mL for *Francisella* and 35 μg/mL for *E. coli*, and hygromycin was used at 200 μg/mL for LVS and *E. coli* and 400 μg/mL was used for Schu S4. All work with Schu S4 and strains

generated from Schu S4 was performed in BSL3 containment at the University of Pittsburgh with approval from the Centers for Disease Control and Prevention Select Agent Program.

Transposon screen for spermine response genes. To identify genes involved in responsiveness to spermine, a genetic screen was conducted. Plasmid pSD26 (a gift from Eric Rubin and Simon Dillon) was electroporated into LVS as previously described (Horzempa et al., 2008; Horzempa et al., 2010b). pSD26 encodes a C9 transposase and a *Himar1* transposon with a kanamycin resistance marker driven by the *F. tularensis groE* promoter (Rubin et al., 1999). After recovery in TSB-c, bacteria were plated on cysteine heart broth with 5% defibrinated rabbit blood containing kanamycin. Five thousand colonies were screened first for an inability to utilize extracellular spermine by failing to grow in CDM in the presence of a spermidine synthesis inhibitor, dicyclohexylamine (Becker et al., 2010; Chan and Chua, 2010; Hibasami et al., 1980; Mattila et al., 1984; Moritz et al., 2004). A positive in the primary screen was determined by an optical density at 600nm (OD_{600}) of less than 0.06 at 48 hours. To confirm that mutants from the primary screen were not CDM auxotrophs, the positive mutants were cultured in CDM without dicyclohexylamine. Mutants with an OD_{600} less than 0.06 were considered auxotrophic and removed from the pool of positive mutants. A secondary screen tested the production of TNF-α from host cells. Mutants were cultured in MHB alone or MHB supplemented with 200 μM spermine. Human macrophages were infected as described below, and the production of TNF-α was measured by ELISA.

Cloning and generation of in-frame deletion mutants. In frame deletions of FTL_0883 and FTT_0615c were performed using allelic replacement as described previously (Horzempa et

al., 2010a; Horzempa et al., 2010b). The 1kb upstream and downstream of each gene was amplified by PCR using two primer pairs, Primer 1 (gatcgcatgcaactaggtgatgcttattatatactcc) and Primer 2 (gatcctgcagttaaatttaattttagtcgaaaaatttaagaaataatgaagt) for the upstream region and Primer 3 (gatcctgcagttttataaaaggattattatctgccattttg) and Primer 4 (gatccccggggcagcttatgagaaaggcg) for the downstream region. The 1kb amplicons were cloned into pJH1 individually and then subcloned adjacent to each other into a single pJH1 vector to generate pJH1-ΔFTL_0883 and pJH1-ΔFTT_0615c. Because the genomic sequences of FTL_0883 and FTT_0615c loci and surrounding DNA are 99% similar based on sequence alignment on BLAST, we were able to use the same primers for Schu S4 and LVS for generating the deletion constructs. Confirmation of the deletion of either FTL_0883 (ΔFTL_0883) or FTT_0615c (ΔFTT_0615c) was performed by sequencing from amplicons from PCR reactions using genomic DNA as the template (Agencourt) and by analysis of PCR product size by electrophoresis (Figure 5).

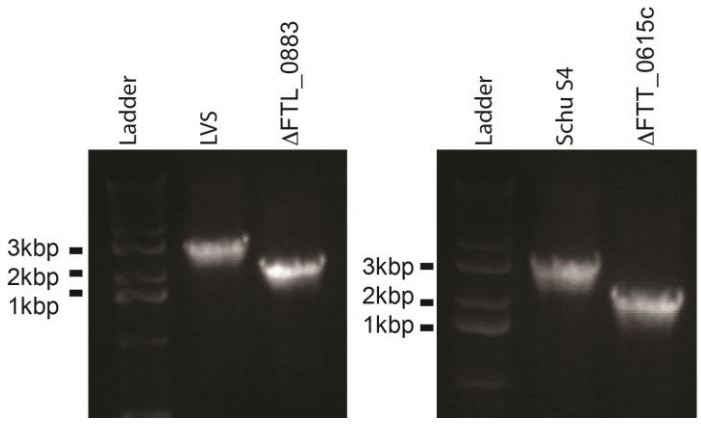

Figure 5: Confirmation of deletion mutants by PCR

Genomic DNA was isolated from *F. tularensis* strains. PCR was performed from 1,000bp upstream and downstream of the *corC* gene. The image is of an ethidium bromide stained agarose gel. Loss of *corC* results is a 850bp smaller PCR product.

A cis-complementing construct was generated utilizing the 1kb upstream of either FTL_0883 or FTT_0615c as the native promoter and to enable the vector to integrate into the genome. Amplicons were generated using Primer 4 (described above) and Primer 5 (catgctgcagttatttcttaaatttttcgactaaaatttaataatttttc) to include the entire open reading frame. The PCR amplicon was digested with PstI and XmaI and was ligated into pJH1 that had been digested with these same enzymes to generate pJH1-FTL_0883 or pJH1-FTT_0615c. To generate a vector control, 1 kb of DNA upstream of FTL_0883 or FTT_0615c was amplified using Primer 3 and 4. This amplicon and pJH1 were digested with PstI and XmaI and ligated together to generate pJH1-vector. The *E. coli* strains harboring the cis-complementing or vector controls were mobilized to *F. tularensis* ΔFTL_0883 and ΔFTT_0615c by conjugation as performed previously (Horzempa et al., 2010b). This generated the complementing (ΔFTL_0883/FTL_0883, "complement") and empty vector ("vector") strains in LVS and the complementing (ΔFTT_0615c/FTT_0615c, "complement") and empty vector ("vector") strains in Schu S4.

Determination of growth rates. LVS or ΔFTL_0883 were cultured overnight in either MHB or TSB-c. The cultures were diluted to an $OD_{600} < 0.1$, in the same media as used for the overnight culture. A growth curve was generated by measuring the OD_{600} of the culture over a 24 hour period using a M2 plate reader (Molecular Devices). Growth rates were calculated using measurements during the exponential phase of growth.

Generation of bone marrow derived macrophages (BMDM). Bone marrow was obtained from the healthy C57BL/6J mice (Jackson Laboratory) 6-8 weeks old. Femurs and

tibias were flushed with 10 mL of cold BMDM media (Dulbecco's Modified Eagle Medium (DMEM) supplemented with 25mM HEPES, 25% L-cell media, 1% sodium pyruvate, 10% FBS, 1% GlutaMAX, 1% non-essential amino acids). The cells were counted and seeded into 100 mm petri dishes at $7x10^6$ cells in 10mL of BMDM media. Bone marrow cells were allowed to differentiate into macrophages for seven days. The macrophages were used between 7 and 21 days of culture. The use of bone marrow was approved by the University of Pittsburgh's Department of Environmental Health and Safety and followed the Protocol for Tissue Transfer of the Institutional Animal Care and Use Committee.

Generation of human monocyte derived macrophages. Human macrophages were differentiated from human mononuclear cells as described previously (Carlson et al., 2007). Peripheral blood mononuclear cells were isolated from buffy coats of human blood donations (Central Blood Bank, Pittsburgh) using Ficoll gradients (GE Healthcare), and then monocytes were isolated using Optiprep gradients (Sigma). Finally, monocytes were further purified by panning. The resulting monocyte population is >95% pure (Carlson et al., 2007). Purified monocytes were then differentiated into macrophages in DMEM supplemented with 20% FBS, 10% human sera AB (complement replete Gem Cell Gemini Bio-Products), 25 mM HEPES, 1% GlutaMAX. The macrophages were cultured for seven days and used on day eight. All use of human cells was approved by the Institutional Review Board of the University of Pittsburgh.

Infection of macrophages. Macrophages were removed from culture dishes with PBS containing 8.5 mM lidocaine and 5 mM EDTA, resuspended in infection media (1% human sera AB, 25 mM HEPES, 1% GlutaMAX in DMEM) seeded into wells of a 96-well Primaria coated

culture dishes (BD Biosciences) at 5 x 10^4 cells/well, and infected with bacteria grown in MHB or TSB-c. The bacterial cultures were standardized to an OD_{600} of 0.3 and actual bacteria concentrations were determined by plating on chocolate agar plates. For experiments measuring cytokine production, bacteria were incubated with the macrophages at a multiplicity of infection (MOI) of 10 for 24 hours in 5% CO_2 at 37ºC. For experiments measuring the growth of *Francisella* within macrophages, a gentamicin protection assay was performed as described (Carlson et al., 2007). Briefly, macrophages were incubated with bacteria at an MOI of 500 for 2 hours, then washed with Hank's Balanced Salt Solution (HBSS) containing 20 μg/mL gentamicin for 20 min to kill extracellular bacteria. Wells were washed again with warm HBSS and then incubated at 37°C with 5% CO_2 in infection media. At 2 or 24 hours post-infection, macrophages were lysed with 0.02% sodium dodecyl sulfate. Serial dilutions of the lysates were plated on chocolate II agar plates for enumeration of viable bacteria. To ensure the replication of bacteria was not hampered by high levels of cell death, the integrity of the monolayer was monitored over the course of the experiment by microscopy. Independent experiments demonstrated similar rates of lactate dehydrogenase release from macrophages infected with *Francisella* to those previously observed (data not shown) (Carlson et al., 2007; Lai et al., 2004). Fold change in growth was calculated by dividing the number of viable bacteria at 24 hours by the number of viable bacteria at 2 hours.

Animal infections. Unless otherwise specified, female 6 to 8 week old C57BL/6J mice (Jackson Laboratory) were used. Experiments assessing the role of pro-inflammatory cytokines used 6-12 week old female B6129S-*Tnfrsf1a*^{tm1Imx}*Il1r1*^{tm1Imx}/J (TNFR1/IL-1R1 KO) and B6129SF2/J (control for KO mice) mice (Jackson Laboratory). All mice were housed in ABSL-

3 facilities at the University of Pittsburgh. The mice were infected intratracheally (i.t.) by oropharyngeal instillation as done previously (Horzempa et al., 2010a). A bacterial inoculum was deposited in the base of the oropharynx of anesthetized mice, allowing it to be aspirated. The mice were infected with approximately 10,000 CFU of LVS or 100 CFU of Schu S4. The mice were monitored twice a day following infection to assess morbidity, which was determined by a scale that incorporated the activity, appearance, and posture of the mice. Once a pre-determined score was achieved, the mice were euthanized.

For experiments requiring serial harvest, lungs, livers, spleens, and blood were removed from anesthetized mice. Blood was removed by cardiac puncture using a heparin coated needle and syringe. The lungs were homogenized in RPMI supplemented with 10% FBS, and the livers and spleens were homogenized in TSB-c. Samples were diluted and plated onto chocolate II agar plates. The process of homogenization, diluting and plating occurred in less than 30 minutes, which is less than the doubling time of the bacteria, approximately 2 hours, and any replication that occurred while the bacteria were in the culture media would be minimal. The bacteria were incubated at 37ºC with 5% CO_2 for 72 hours and individual colonies were counted to determine CFU. All animal infection studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

ELISA. The supernatants were harvested 24 hours post infection and cytokine levels were measured by ELISA. Murine TNF-α was measured using a matched antibody pair (eBiosciences). Human TNF-α, IL-12 p40, IL-6, and IL-1β were measured using DuoSets (R&D Systems). Cytokine concentrations were determined using a Molecular Dynamics M2 plate reader following addition of TMB substrate (Dako). The limit of detection was 15 pg/mL for

murine and human TNF-α. The limits of detection of human IL12-p40, IL-6 and IL-1β were 15, 9, and 4 pg/mL respectively.

Real-time PCR. Bacteria were cultured for 16-18 hours in MHB alone or containing 200 μM of spermine. RNA was harvested using TRI Reagent RT Liquid Samples (Molecular Research Center) according to the manufacturer's instructions and suspended in nuclease-free water. The samples were treated with DNase (Turbo DNA-*free*; Ambion) and the RNA quantity was measured by spectrophotometry. Quantitative real time-PCR was performed as previously described (Carlson et al., 2009). Data are depicted as the $\log_2$ fold change of transcripts levels in bacteria cultured in MHB with spermine divided by the transcript level in bacteria cultured in MHB alone using the ΔΔCt method.

Measurement of NF-κB activation using reporter cell line. RAW264.7 cells stably transfected with a NF-κB-GFP reporter were seeded into a 96-well plate as described for macrophage infections. The reporter contains GFP driven by the E-selectin promoter containing a NF-κB binding site (Stacey et al., 2003). The cells were infected with LVS at an MOI of 10. At 24 hours post infection, the cells were washed with HBSS and visualized on a Zeiss Axiovert 200M microscope. Images were collected using the Zeiss Axiovision software and brightness and contrast were adjusted consistently for all images in Adobe Photoshop.

SDS-PAGE analysis. For assessment of bacterial protein concentration, bacterial lysates were generated after overnight culture of the bacteria in the indicated media. The strains were normalized by OD_{600}, for western blot OD_{600}=2.0 and for emerald green analysis OD_{600}=20. For

western blotting, the bacterial lysates were sonicated and treated with lysis buffer (NuPAGE), heated at 95°C for 5 min, separated by polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes (Millipore), and blocked with 5% milk. Mouse anti-IglC, rabbit anti-IglB, rabbit anti-IglA, mouse anti-PdpD, rabbit anti-*Francisella*, anti-polyamine, mouse anti-*Francisella* LPS, mouse anti-potF, and mouse anti-O antigen capsule (11B7) were diluted 1:1000 in 5% milk. Blots were washed with PBS and the appropriate secondary antibodies were used diluted 1:2000. The blots were washed with PBS and incubated with chemoilluminescent substrate (GE healthcare). The blots were exposed to film and developed. For quatification of carbohydrates by emerald green staining. Bacterial lysates were sonicated and treated with proteinase K for 1 hour at 60°C, diluted into NuPAGE sample buffer (Invitrogen) and heated at 95°C for 5 min. The carbohydrates were separated with a 12-4% SDS-PAGE gel. Emerald green staining was performed using pro-Q emerald 488 according to manufacturers instructions (Molecular Probes) and the gels were visualized with a Typhoon 9400 flatbed fluorescent scanner.

For NF-κB immunoblotting, BMDM were cultured with LVS strains at an MOI of 500 for 2 hours. Macrophages were lysed using a lysis buffer (150 mM NaCl, 1% Triton-X100, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl; pH 7.4). The protein samples were separated by polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes (Millipore), blocked with 5% milk, and probed for total NF-κB p65 and phospho-p65 (#3987 and #3033 respectively, Cell Signaling). Membranes were washed and incubated with donkey anti-rabbit antibody coupled to horseradish peroxidase (A6154, Sigma). After washing again, signal was measured with SuperSignal West Femto chemiluminescent substrate (Pierce) and exposure

to film. Relative phospho-p65 was calculated by dividing phosph-p65 levels by total p65 levels using densitometry analysis performed with ImageJ 1.44P software (NIH).

Bacterial viability. *F. tularensis* strains were cultured in the indicated media overnight. They were normalized by OD_{600} and stained with the BacLight LIVE/DEAD bacterial stain (Molecular Probes) according to the manufacturers instructions. Briefly, bacteria were incubated in a 1:1000 dilution of propidium iodide and a 1:1000 dilution of Cyto9 in PBS for 15 min. Fluorescence was measured using a M2 plate reader with the emission at 530nm and 630nm with an excitation at 485nm.

Luminex. Lung homogenates from infected mice were centrifuged at 20,000 X *g* to remove cells and tissue debris. The resulting supernatants were filtered through a 0.2 μm filter and treated with gentamicin (100 μg/mL). Blood was collected from mice by cardiac puncture with heparinized syringes and plasma generated by centrifugation at 20,000 X *g* was then treated with gentamicin (300 μg/mL) and ciprofloxacin (25 μg/ml). These samples were tested using the Milliplex 32-plex Mouse Cytokine/Chemokine Panel (Millipore) on a Bio-Plex 200 system (Bio-Rad Laboratories). Analyte concentrations were calculated against the standards using Milliplex Analyst software version 3.5 (Millipore).

Statistics. For statistical analysis involving wild-type and mutant only, we used a two-tailed Student's t-test with an alpha=0.05. When greater than two strains were compared a one-way ANOVA was used to determine experimental significance and a Tukey post-hoc test was

used to make pair-wise comparisons. Statistical differences in survival were determined by a log-rank test. GraphPad Prism5 (GraphPad Software) was used for all statistical analysis.

2.4 RESULTS

2.4.1 Spermine responsiveness in *F. tularensis* requires the presence of FTL_0883.

Polyamines, particularly spermine, act as intracellular cues for *F. tularensis* (Carlson et al., 2009). A transposon screen was performed in LVS to identify mutants that were unable to respond to extracellular spermine. Bacteria can either make their own polyamines (putrescine and spermidine), or import polyamines (spermidine, putrescine, and spermine) from the extracellular environment to grow (Wortham et al., 2007). To identify mutants that were unable to utilize extracellular polyamines, we used an inhibitor of spermidine synthase, dicyclohexylamine (DCHA) (Becker et al., 2010; Chan and Chua, 2010; Hibasami et al., 1980; Mattila et al., 1984; Moritz et al., 2004). Isolates were selected from a library of mutants made with a Mariner-based transposon (Rubin et al., 1999) that were unable to replicate in the presence of the DCHA in CDM. Of the nearly 5000 transposon mutants screened, 257 mutants were initially identified in the primary screen as unable to replicate in CDM with the inhibitor. Of the 257 mutants, 32 were unable to replicate in the presence of spermine and the inhibitor, and were not simply auxotrophs in CDM (data not shown). These isolates were then subjected to a secondary screen for their ability to stimulate cytokine production from macrophages when they were cultured in the presence or absence of spermine. Of the 32 mutants identified from the primary screen, two mutants stimulated at least equivalent cytokines from macrophages whether

they were cultured in the presence or absence of spermine. One of the transposon mutants, 13B47 contained a transposon inserted in FTL_0883 and was selected for further investigation because its response to spermine was different from wild-type LVS, and it had been previously attributed to virulence in a negative selection screen in mice to identify mutants defective for dissemination to the spleen (Weiss et al., 2007).

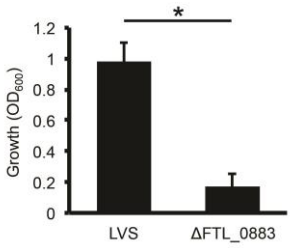

Figure 6. DCHA prevents replication of ΔFTL_0883 in CDM

Francisella was cultivated overnight in CDM in the presence of DCHA. Growth was determined by measuring the optical density of the culture at 600nm. The data are the mean ± SEM of three independent experiments. $p < 0.05$ is indicated by *.

An in-frame deletion of FTL_0883, ΔFTL_0883, was generated for further study. The ΔFTL_0883 mutant was assessed for its ability to grow in CDM containing DCHA to confirm it has a similar phenotype to 13B47. Consistent with the transposon mutant, the growth of ΔFTL_0883 was reduced compared to LVS when DCHA was added to CDM, which confirmed the deletion mutant would have been positive in the screen (Figure 6). As previously described (Carlson et al., 2009), wild-type LVS grown in MHB with spermine stimulates significantly less TNF-α than when it is grown in MHB alone (Figure 7A). Strain 13B47 stimulated more cytokines than wild-type LVS whether or not spermine was present in the MHB (Figure 7A), and it has been further characterized (Appendix A). Similar to the transposon mutant, ΔFTL_0883

grown in MHB or MHB with spermine stimulated more TNF-α production than wild-type (Figure 7A and 7B). Culturing 13B47 in MHB with spermine resulted in a significant increase in the induction of TNF-α in some experiments (Figure 7A). In contrast, the deletion mutant elicited equivalent amounts of cytokines from the macrophages whether it was cultured in the presence or absence of spermine (Figure 7A). These studies suggest that 13B47 and ΔFTL_0883 behave similarly, but due to the possibility of polar effects in the transposon mutant, subsequent experiments were performed with the in-frame deletion mutant, ΔFTL_0883. To confirm the responses were the result of the deletion, genetic complementation studies were performed to establish the role of FTL_0883 in these phenotypes. ΔFTL_0883 containing a cis-complementing construct cocultured with macrophages stimulated TNF-α levels comparable to wild-type when they were grown in the presence of spermine (Figure 7B). Integration of an empty vector did not alter the response of the macrophages to the mutant when cultured in the presence of spermine (Figure 7B). Therefore, FTL_0883 is needed for *F. tularensis* to become less stimulatory when exposed to extracellular spermine.

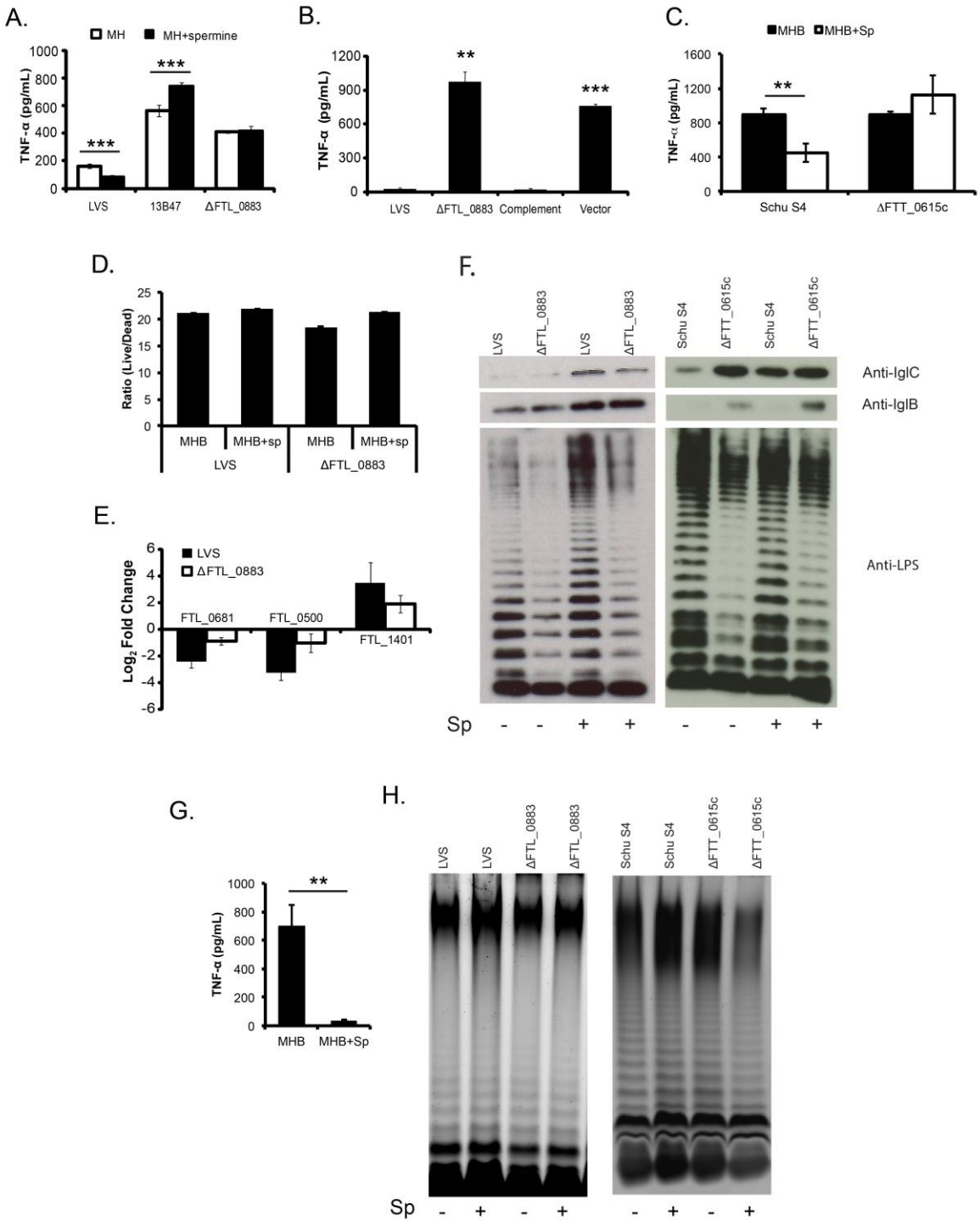

Figure 7: FTT_0883 and FTT_0615c are required for response to extracellular spermine

A and C) Cytokine response to bacteria grown with or without spermine. Bacterial strains were cultured in either MHB or MHB supplemented with spermine overnight. Human macrophages were cocultured with the strains at an MOI of 10. At 24 hours post infection, supernatants were harvested and TNF-α was measured by ELISA.

Macrophages incubated with media alone produced TNF-α levels that were below detectable limits. Statistical significance was determined using a Student's t-test by comparing the amount of TNF-α elicited from a strain cultured in MHB alone or supplemented with spermine. ** indicates $p < 0.01$, *** indicates a $p < 0.001$. B) Genetic complementation restores wild-type phenotypes. Bacteria were cultured in MHB supplemented with spermine. Human macrophages were infected at an MOI of 10, and TNF-α was measured from supernatants collected 24 hours post infection by ELISA. Macrophages incubated with media alone media produced undetectable levels of TNF-α. Data are presented as the mean ± standard deviation of triplicate wells within one experiment representative of two independent experiments using cells from different donors. Statistical significance was determined using a one-way ANOVA followed by a Tukey post-hoc test. ** indicates a $p < 0.01$, *** indicates a $p < 0.001$ compared to wild-type LVS. D) Bacteria were stained with BacLight Live/Dead stain to determine the effect of spermine on bacterial viability. The data is representative of three independent experiments. E) Real time PCR analysis of transcriptional response to spermine. LVS or ΔFTL_0883 were cultured in MHB with or without spermine overnight prior to RNA harvest. Data are presented as the mean $\log_2$ fold change (MHB+spermine/MHB) in transcript levels ± SEM of three independent experiments. F) Bacteria were cultured in MHB with or without spermine supplementation, and the changes in protein and carbohydrate expression were assessed by western blot. The data presented is representative of three independent experiments. G) Macrophages were cocultured with a *wbtA* transposon mutant at an MOI of 10 for 24 hours. The supernatants were collected and the production of TNF-α was assessed by ELISA. The data is representative of three independent experiments. Statistical significance was determined by Student's t-test. ** indicates $p < 0.01$. H) After culture in MHB with or without supplementation with spermine, the bacterial lysates were treated with proteinase K, and the carbohydrates were stained with Pro-Q Emerald 488. The data is representative of at least two independent experiments for each strain.

To extend our observations to the virulent strain of *F. tularensis,* an in-frame deletion mutant was generated in the Schu S4 homolog of FTL_0883, FTT_0615c (ΔFTT_0615c). Consistent with previous reports (Carlson et al., 2009), Schu S4 responded to the addition of spermine to the culture broth, and the bacterium elicited lower levels of cytokines from macrophages when it was cultured in the presence of spermine compared to MHB alone (Figure

7C). In contrast, ΔFTT_0615c elicited similar concentrations of cytokines from macrophages when cultured in the presence of MHB supplemented with spermine or MHB alone (Figure 7C). Importantly, culturing *F. tularensis* in the presence of spermine does not alter the viability of the bacteria. Greater than 90% of the bacteria remained alive in either MHB with or without spermine supplementation for both LVS and ΔFTL_0883 (Figure 7D). These data confirm the importance of the FTL_0883/FTT_0615c locus for the *Francisella* response to spermine.

The cultivation of *F. tularensis* in the presence of spermine changes the transcriptional profile of the bacteria compared to bacteria grown without spermine (Carlson et al., 2009). As an additional test of spermine responsiveness in the deletion mutant, expression of three genes of the spermine regulon in *Francisella* (Carlson et al., 2009), FTL_0500, FTL_1401, and FTL_0681, were analyzed. As expected for wild-type *Francisella*, there was increased transcription of FTL_1401 and decreased transcription of FTL_0500 and FTL_0681 when LVS was cultured in MHB with spermine compared to MHB alone (Figure 7E). The transcriptional changes by ΔFTL_0883, however, were substantially blunted compared to wild-type (Figure 7E), demonstrating the mutant had a diminished transcriptional response to spermine. Although the effects of spermine were not abolished, most likely because of multiple levels of transcriptional regulation, these observations demonstrate that FTL_0883 contributes to the transcriptional response to spermine in *F. tularensis.*

Host adaptation of *F. tularensis* is associated with an increase in the expression of IglC and IglB proteins (Zarrella et al., 2011), which is consistent with investigations of transcriptional changes in *F. tularensis* when it is cultured within a macrophage (Wehrly et al., 2009). In contrast, a response to spermine is associated with a decrease in FPI transcriptional expression (Carlson et al., 2009), but it is unknown if there is the same association in FPI protein expression

when spermine is added to the culture media. FPI protein expression was examined by immunoblot of bacterial lysates generated from *F. tularensis* cultured in either MHB or MHB supplemented with spermine. Consistent with previous reports (Hazlett et al., 2008; Zarrella et al., 2011), spermine increased the expression of IglC and IglB in LVS, but there was not increased HSP-70 expression when spermine was included in the culture media (Figure 7F and data not shown). Surprisingly, in ΔFTT_0883, there was an increase in the expression of IglC and IglB when spermine was added to the culture media despite the fact that this strain stimulates high levels of TNF-α from host macrophages (Figure 7F). Similar to the results with the LVS strains, when Schu S4 was cultured in MHB supplemented with spermine there was an increase in IglC protein expression compared to cultivation in MHB alone, but IglB was not readily detected (Figure 7F). Unexpectedly, there was no change in the expression of IglC or IglB when ΔFTT_0615c was cultured in the presence of spermine compared to MHB alone (Figure 7F). These data demonstrate that in LVS the spermine response is independent of changes in the pathogenicity island protein expression while in Schu S4 the spermine response correlated with changes in the pathogenicity island protein expression.

Carbohydrate quantity and modification are tightly controlled and are important for how bacteria interact with host cells (Finlay and McFadden, 2006; Zarrella et al., 2011). To explore the contribution of surface carbohydrates to *F. tularensis*'s response to extracellular spermine, LPS was assessed by western blot. The inclusion of spermine in MHB increased LPS on the surface of LVS strains, but there was not a change in the amount of LPS on the surface of the Schu S4 strains (Figure 7F). These data suggest that changes in the O-antigen expression are independent of the spermine response. A mutant in *wbtA*, dWbtA:, was used to test the hypothesis that LPS is not important for response to spermine for LVS. dWbtA was confirmed to

have the predicted phenotypes associated with a loss of *wbtA* function (Appendix B) When dWbtA was cultured in the presence of spermine, there was a reduction in the amount of cytokines elicited from macrophages (Figure 7G). These data demonstrate that there is not a role for the O-antigen during a response to spermine by *F. tularensis*.

The high molecular weight capsule is believed to function independent of the O-antigen in *F. tularensis* (Zarrella et al., 2011), and it could have a role during the spermine response. Changes in high molecular weight capsule of *F. tularensis* correlate with adaptation to the host environment, in which the concentration and size of the capsule increases on the bacterial surface as the stimulation of macrophages decreases. To assess the importance of carbohydrate modification when *F. tularensis* responds to spermine in the environment, carbohydrates were isolated from *F. tularensis* cultured in MHB or MHB supplemented with spermine and stained with pro-Q emerald for visualization. There is an increase in the high molecular weight capsule when LVS but not ΔFTL_0883 is cultured in the presence of spermine (Figure 7H). When Schu S4 was cultured in the presence of spermine there was an increase in the amount, but not the size, of the capsule present on the surface of the bacteria (Figure 7H). Surprisingly, there was a decrease in the amount of capsule present on the surface of ΔFTT_0615c when it was cultured in the presence of spermine compared to media alone (Figure 7H). These results reveal that the amount of capsule on the surface of *F. tularensis* correlated with the cytokines that were elicited from macrophages, and they suggest that alteration in the capsule could be the causal link between the presence of spermine in the environment and an increase in the virulence of *F. tularensis*.

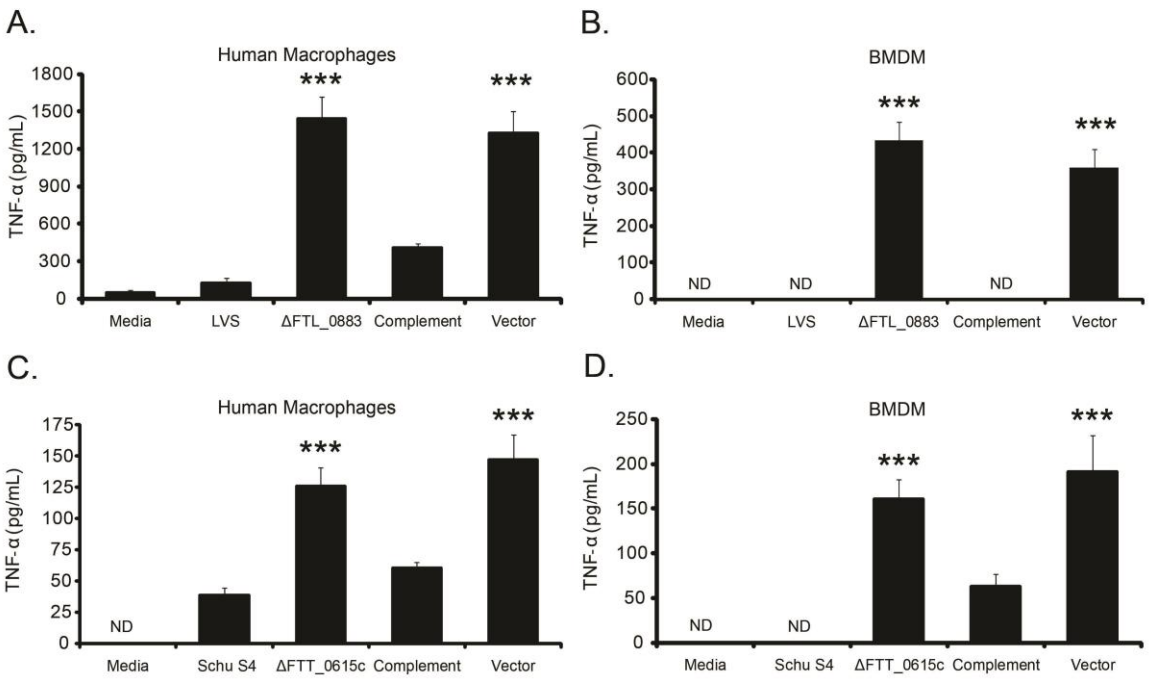

Figure 8: LVS ΔFTL_0883 and Schu S4 ΔFTT_0615c stimulate TNF-α

Bacteria cultivated overnight in TSB-c were used to infect either human macrophages or murine bone marrow derived macrophages (BMDM) at an MOI of 10. After 24 hours, the supernatants were removed, and an ELISA was performed to quantify the levels of TNF-α. The data are presented as the mean ± standard deviation of triplicate wells within one experiment representative of at least three independent experiments. A) Human macrophages infected with LVS strains. B) BMDM infected with LVS strains. C) Human macrophages infected with Schu S4 strains. D) BMDM infected with Schu S4 strains. TNF-α levels were compared by a one-way ANOVA followed by a Tukey post hoc test to allow for pair-wise comparisons. *** represents data with a $p < 0.001$ compared to wild-type strains. ND represents not detected.

2.4.2 Macrophages are activated by strains with mutations in FTL_0883 or FTT_0615c.

We extended our initial observations with the ΔFTL_0883 mutant by investigating other host cells. Human macrophages and murine bone marrow derived macrophages (BMDM) were infected at an MOI of 10 with wild-type bacteria, deletion mutants, or mutants that received their cognate gene or an empty vector *in cis*. After 24 hours, the supernatants were collected to analyze the amount of cytokines in the media. Significantly more TNF-α was produced by human and murine macrophages infected with the ΔFTL_0883 mutant compared to wild-type grown in TSB-c (Figure 8A and 8B), which was similar to results obtained with MHB supplemented with spermine (Fig. 7B). Restoration of FTL_0883 with the complementation construct, but not an empty vector, restored TNF-α levels close to those observed with wild-type (Figure 8A and 8B). Similar results were also obtained when RAW264.7 cells were used (data not shown). Again, enhanced activation was not the result of increased bacterial death leading to stimulation, as all strains were similarly viable prior to coculture with macrophages (Figure 9). A comparable pattern of cytokine production was observed with strains in the Schu S4 background. The ΔFTT_0615c mutant stimulated significantly more TNF-α from both human and murine macrophages than wild-type (Figure 8C and 8D). Strains of ΔFTT_0615c that received the complementing construct, but not the vector control, behaved similar to wild-type by stimulating lower levels of TNF-α than the deletion mutant.

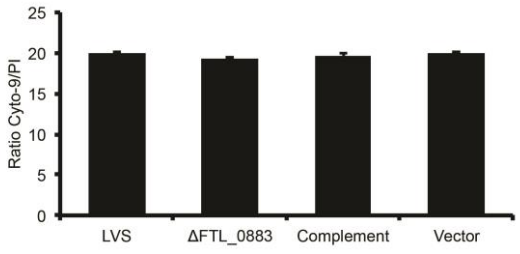

Figure 9: Bacterial viability of LVS strains

LVS strains were cultured overnight in TSB-c. They were diluted to equivalent OD_{600}, and then stained with the BacLight LIVE/DEAD kit according to manufacturers instructions. The data presented is the means ± standard deviation of one experiment and is representative of at least two independent experiments for each strain.

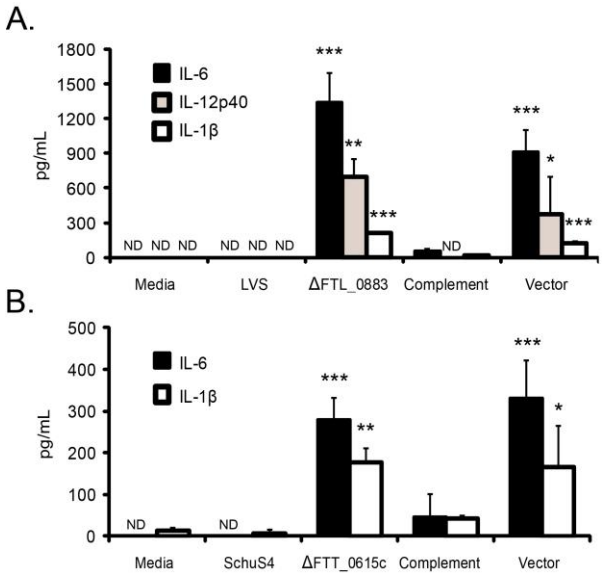

Figure 10: ΔFTL_0883 and ΔFTT_0615c stimulate pro-inflammatory cytokine production.

Human macrophages were infected at an MOI of 10 for 24 hours. After 24 hours, the supernatants were removed, and ELISAs were performed to quantify the levels of IL-6, IL-12p40 and IL-1β. A) Human macrophages infected with LVS strains. B) Human macrophages infected with Schu S4 strains. IL-12p40 was not detected in these samples. ND is not detected for values that were below the limit of detection for the ELISA. The data are presented as the mean ± standard deviation of triplicate wells within one experiment representative of at least three independent experiments. The induction of cytokines by mutant strains was compared to wild-type with a one-way ANOVA followed by a Tukey post-test. * indicates a $p < 0.05$, ** indicates a $p < 0.01$, and *** indicates a $p < 0.001$.

While TNF-α was used as a prototypical pro-inflammatory cytokine, similar trends in the production of other pro-inflammatory cytokines were observed. More IL-6, IL12-p40, and IL-1β

were produced by human macrophages cultured with ΔFTL_0883 (Figure 10A), and more IL-6 and IL-1β when human macrophages were cocultured ΔFTT_0615c (Figure 10B). Similar to TNF-α (Figure 8), stimulation of macrophages was reduced when FTL_0883 or FTT_0615c were returned *in cis*, but there was no effect when an empty vector was used (Figure 10A and 10B). These results show that FTL_0883 and FTT_0615c have a general role in limiting pro-inflammatory cytokine production by macrophages infected with *Francisella*.

F. tularensis actively inhibits intracellular signaling in macrophages, including NF-κB activation, thereby limiting pro-inflammatory cytokine production (Telepnev et al., 2005). We hypothesized that the heightened stimulation of macrophages by ΔFTL_0883 could correlate with an enhanced NF-κB activation. As a functional test of NF-κB activation, LVS strains were cocultured with RAW264.7 cells bearing a NF-κB GFP reporter in which the expression of GFP is under the regulation an NF-κB dependent promoter (Stacey et al., 2003). The NF-κB reporter cells showed minimal GFP expression when cultured with wild-type LVS, similar to the low levels observed with the media control (Figure 11A). In contrast, infection with the ΔFTL_0883 strain greatly enhanced GFP expression compared to LVS (Figure 11A). Similar to the results seen with TNF-α production (Figure 8), complementing the ΔFTL_0883 mutant with a wild-type copy of the gene, but not the empty vector, reduced the level of GFP expression (Figure 11A). Because the NF-κB reporter cells were insufficiently sensitive to detect changes at earlier time points and to assess activation on a molecular level, the activation of NF-κB was assessed by immunoblot (Figure 11B). Whole cell lysates were generated from BMDM 2 hours after infection to measure the phosphorylation of the p65 subunit of NF-κB (Figure 11B). Consistent with the results obtained with the NF-κB reporter strains, there was a four-fold increase in the phosphorylation of p65 in cells infected with ΔFTL_0883 compared to cells infected with LVS

(Figure 11B). This early NF-κB activation was reduced when the gene was restored *in cis* such that phosphorylated p65 was 2.5 times higher in BMDM cultured with vector compared to complement (Figure 11B). These data corroborate the results obtained by ELISA and demonstrate that macrophages are more activated by ΔFTL_0883. These findings indicate that FTL_0883 is needed by *Francisella* to minimize the activation of macrophages.

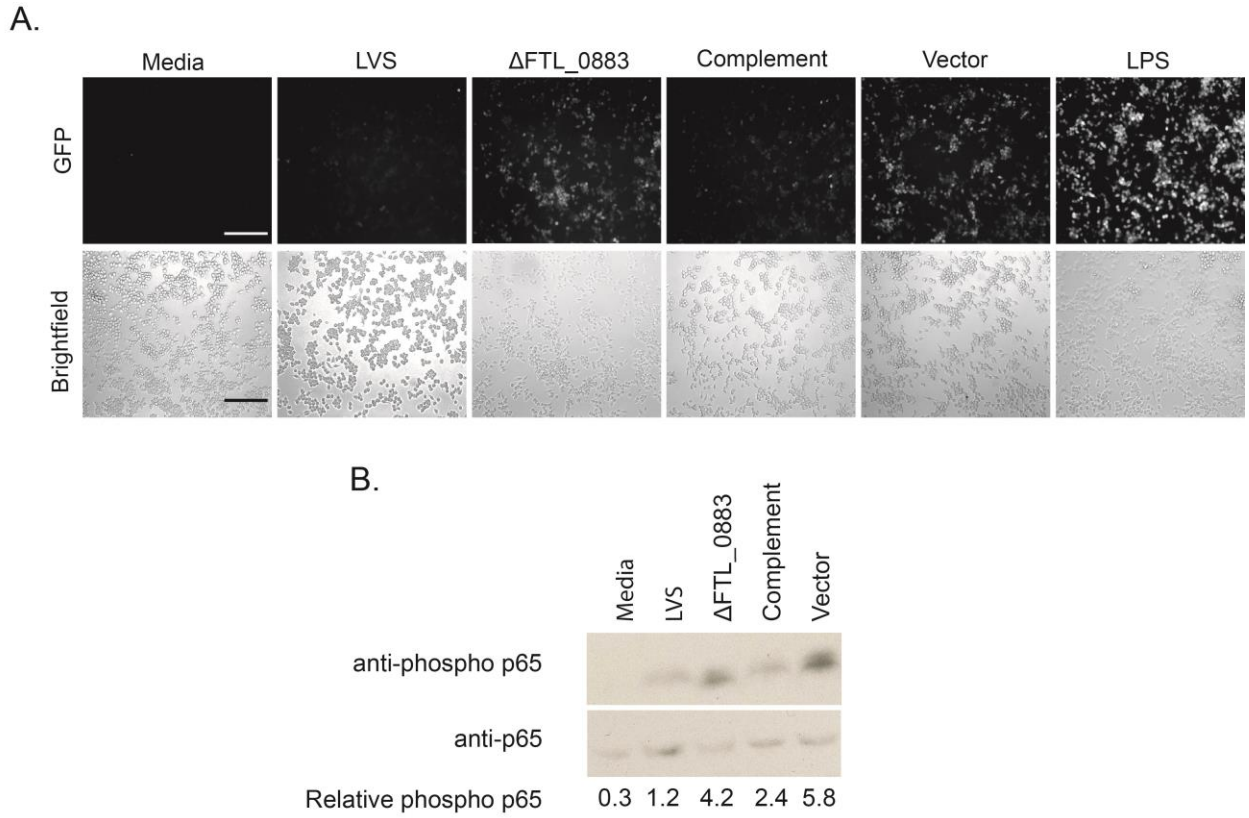

Figure 11: LVS ΔFTL_0883 stimulates activation of NF-κB

A) RAW264.7 cells containing GFP driven by a promoter whose activity is regulated by activation of NF-□B were infected at an MOI of 10 with *F. tularensis* grown overnight in TSB-c. After 24 hours, the cells were examined by fluorescence microscopy to assess the relative expression of GFP within the cells. Exposure times were constant across all fluorescent images. Images are from one experiment representative of three independent experiments. Scale bar represents a distance of 100 μm. B) NF-κB is activated early by ΔFTL_0883. BMDM were infected at an MOI of 500 for 2 hours and whole cell lysates were tested for NF-κB p65 and phospho-p65. Phospho-p65 levels

were normalized to total p65 levels, with the ratios displayed below the immunoblot images. Similar results were seen in two independent experiments.

2.4.3 Induction of TNF-α by Schu S4 does not correlate with replication within macrophages

Activated macrophages control *F. tularensis* replication *in vitro* (Edwards et al., 2010). Since we observed enhanced activation of macrophages infected with the deletion mutants as measured by pro-inflammatory cytokine production, we hypothesized that growth of these mutants within macrophages would be restricted. To test this, gentamicin protection assays were performed using human monocyte derived macrophages and murine BMDM infected with the LVS strains. Wild-type LVS replicated well in either host macrophage, increasing 10 to 100-fold over 24 hours (Figure 12A and 12B). Consistent with our hypothesis, the ΔFTL_0883 mutant was attenuated for growth in both human macrophages and BMDM, growing at least 10 fold less than wild-type (Figure 12A and 12B). As expected, returning the gene *in cis*, but not the empty vector, nearly restored growth to wild-type levels. The differences in growth rates were not attributable to differences of invasion between the mutant and wild-type as the number of intracellular bacteria two hours after infection differed less than two-fold between ΔFTL_0883 and LVS. These results show FTL_0883 is necessary for intracellular growth of LVS in macrophages.

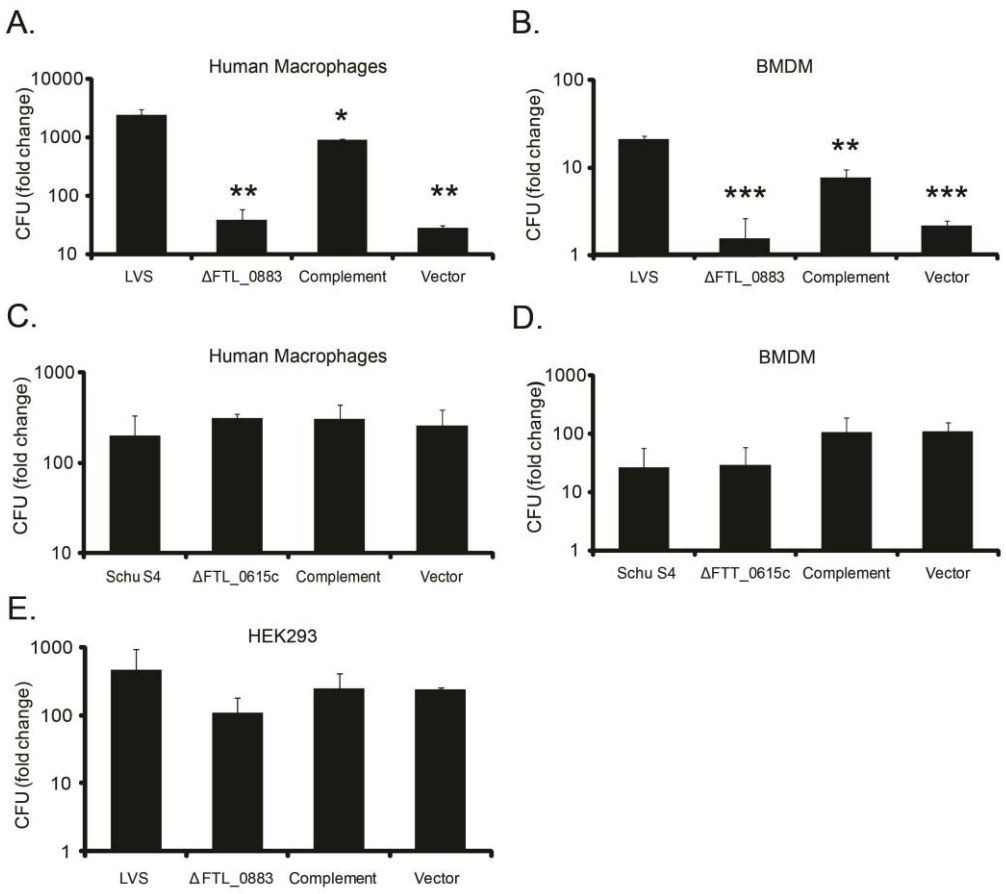

Figure 12: FTL_0883 but not FTT_0615c is needed for the replication of *F. tularensis* within macrophages *in vitro*

Bacteria cultivated overnight in TSB-c were used to infect human macrophages or bone marrow derived macrophages (BMDM) at an MOI of 500, followed by gentamicin treatment 2 hours post infection. At 2 hours and 24 hours, the macrophages were lysed and *F. tularensis* was diluted and plated to enumerate colony forming units (CFU). The data are presented as the mean fold change in CFU (CFU at 24 hours/ CFU at 2 hours) ± standard deviation of one experiment representative of at least three independent experiments. A) Human macrophages infected with LVS strains. B) BMDM infected with LVS strains. C) Human macrophages infected with Schu S4 strains. D) BMDM infected with Schu S4 strains. E) HEK293 cells infected with LVS strains. To assess differences in the fold change in CFU, a one-way ANOVA was performed followed by a Tukey's test to make pair-wise comparisons. * indicates a $p < 0.05$, ** indicates a $p < 0.01$, and *** indicates a $p < 0.001$ compared to wild-type strains.

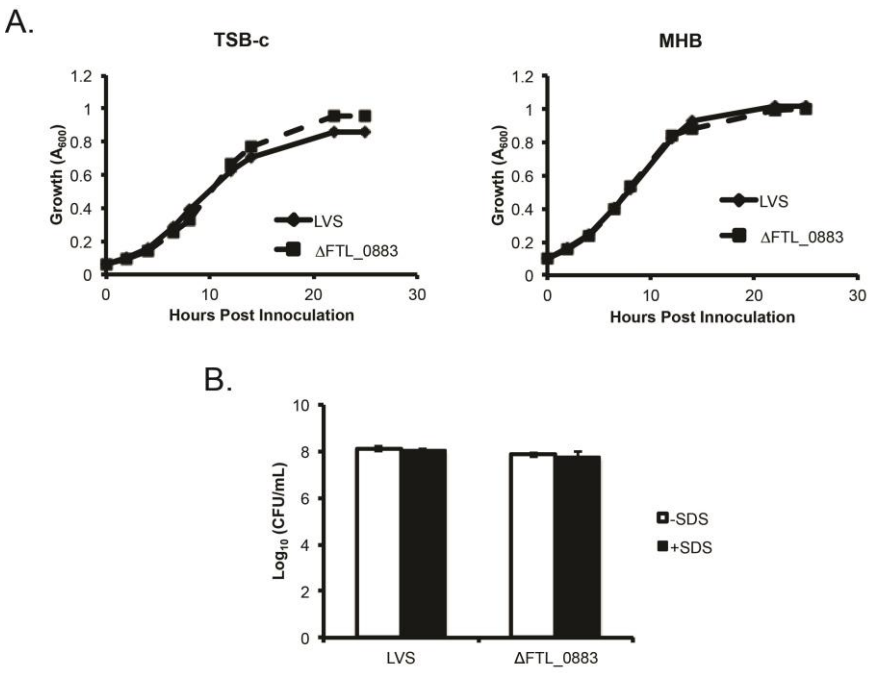

Figure 13: Intracellular growth defect of ΔFTL_0883 is not due to a general growth defect or sensitivity to SDS

A) LVS and ΔFTL_0883 were cultured in either TSB-c or MHB, and OD_{600} of the culture was monitored over a 24 hour period. The data presented is representative of two independent experiments. B) LVS and ΔFTL_0883 were incubated in the presence or absence of SDS, and the bacteria were diluted in PBS and plated to enumerate CFU. The data presented is representative of two independent experiments.

In contrast, the mutation in the Schu S4 background yielded unexpected results. Schu S4 and the ΔFTT_0615c mutant grew similarly in both human and murine macrophages (Figure 12C and 12D). Growth of the complemented strain or the empty vector strain was also indistinguishable. Again, similar rates of invasion were observed 2 hours post infection for Schu S4 and ΔFTT_0615c. While FTL_0883 was needed for wild-type rates of replication of LVS within macrophages, FTT_0615c, was dispensable for replication of Schu S4 within macrophages. These results were unexpected, but they are not unprecedented, as there are established differences in the ability of Schu S4 and LVS to withstand host defenses such as

oxidative stress (Lindgren et al., 2011), which could restrict replication of ΔFTL_0883 in activated macrophages but permit wild-type replication of ΔFTT_0615c.

The lack of intracellular replication for ΔFTL_0883 was not simply the result of a general growth defect. Similar doubling times of LVS and ΔFTL_0883 were observed in the media used for our studies: MHB (LVS: 2.63 ± 0.41 hours and ΔFTL_0883: 2.81 ± 0.32 hours) and TSB-c (LVS: 2.28 ± 0.05 hours and ΔFTL_0883: 2.23 ± 0.08 hours) (Figure 13A). The reduced growth of the ΔFTL_0883 mutant was also not the result of an increased sensitivity to SDS that was used to lyse the macrophages because the viability of the mutant and wild-type were unaffected by this concentration of SDS (Figure 13B). Therefore, the attenuation of intramacrophage replication of the mutant was not the result of an inherent difference in the rate of replication of ΔFTL_0883.

Although attenuated for growth in macrophages, the LVS mutant strain showed wild-type growth in non-macrophages. Wild-type LVS, ΔFTL_0883, complement, and vector all grew similarly in non-macrophage, HEK293 cells; there was not a significant difference in replication among the strains (Figure 12E). These results confirm observations in broth culture that ΔFTL_0883 does not suffer from a generalized growth defect.

The expression of FPI is important for the intracellular growth of *F. tularensis*. To determine if there was an altered expression of pathogenicity island genes that could explain the divergent patterns of intracellular replication for the LVS and Schu S4 strains, the expression of IglC was measured in the bacterial lysates by western blot. There was a reduction in the expression of IglC in ΔFTL_0883 compared to LVS (Figure 14). The expression of IglC was restored with complementation (Figure 14). Consistent with the results in the literature, the expression of FPI in LVS correlated with the ability of the strains to replicate intracellularly

within macrophages. In contrast, there was not a difference in the expression of IglC in the Schu S4 strains (Figure 14). These results suggest altered regulation of FPI in LVS and Schu S4 could contribute to the differences in intracellular replication for ΔFTL_0883 and ΔFTT_0615c in macrophages.

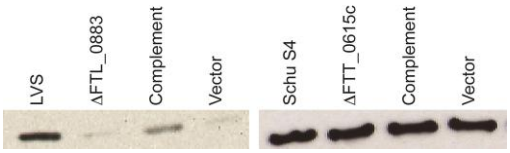

Figure 14: Altered IglC expression in LVS ΔFTL_0883 but not Schu S4 ΔFTT_0615c.

Bacteria were cultured overnight in TSB-c, bacterial lysates were generated, normalized to OD_{600}=2.0, separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-IglC antibody. The data presented is representative of at least two independent experiments for each strain.

2.4.4 Replication within a macrophage does not correlate with virulence *in vivo*.

The macrophage is believed to be an important, intracellular niche for *F. tularensis* within the host. Moreover, replication in macrophages has typically been associated with virulence (Buchan et al., 2009; Forslund et al., 2010; Golovliov et al., 2003; Lauriano et al., 2004; Meibom et al., 2009; Nano et al., 2004; Pechous et al., 2006). Based on the growth patterns in macrophages described above, it was hypothesized that the ΔFTL_0883 mutant would be attenuated but the ΔFTT_0615c mutant would retain virulence *in vivo*.

Virulence of LVS and ΔFTL_0883 was assessed first. Mice infected with wild-type LVS lost a significant amount of weight, a measure of morbidity, approaching 25% of their starting body weight by day 7 post infection (Figure 15A). In contrast, mice infected with ΔFTL_0883

lost less than 5% of their body weight over the course of the experiment, which was significantly different from wild-type from day 4 until day 12 post infection (Figure 15A). The weights of all the mice returned to their pre-infection values, and no mice died with the dose administered. Nevertheless, there was a significant decrease in morbidity for mice infected with ΔFTL_0883 compared to mice infected with LVS. This is consistent with the hypothesis that failed growth in macrophages *in vitro* predicts attenuation *in vivo.*

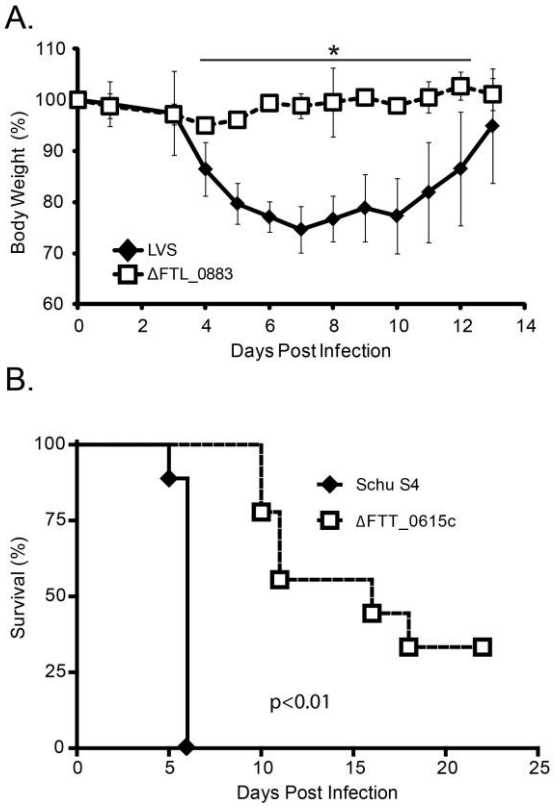

Figure 15: FTL_0883 and FTT_0615c are required for virulence *in vivo*.

A) Mice were infected intratracheally with 10,000 CFU of LVS or ΔFTL_0883 (n=4 per group). The weights of the mice were followed for 2 weeks post infection. The data are presented as mean ± standard deviation of a representative of two experiments performed similarly. Data under the line indicate days the weight of mice infected with the mutant were statistically different from wild-type infected mice. * indicates a $p < 0.05$ by Student's t-test. B) Mice were infected intratracheally with 100 CFU of either Schu S4 or ΔFTT_0615c. Data are combined from two independent experiments (n = 9 total per group). $p < 0.01$ by log rank test.

Once again, the ΔFTT_0615c mutant yielded unexpected results. It was anticipated that the mutant in the Schu S4 background would be fully virulent in mice because it replicated efficiently in macrophages *in vitro*. Surprisingly, there was a significant delay in time to death for mice that were infected with the ΔFTT_0615c mutant compared to wild-type Schu S4 (Figure 15B). The median time to death was 6 days and 15 days for wild-type and mutant, respectively, and one-third of the mice infected with the mutant survived until the end of the study 22 days post infection. Therefore, ΔFTT_0615c was attenuated *in vivo* compared to Schu S4, even though the mutant was able to replicate at wild-type rates in macrophages *in vitro*. These results contradict the hypothesis that replication in macrophages *in vitro* is a predictor of virulence, and suggest that the pro-inflammatory response *in vitro* is a better indicator of attenuation *in vivo*.

The kinetics of bacterial replication were next investigated in mice infected with ΔFTT_0615c and Schu S4 strains. We hypothesized that there would be reduced bacterial burden since there was an increase in the median time to death for mice infected with ΔFTT_0615c. Mice were sacrificed on days 2, 4, 6, or 12 post infection, and homogenates of their lungs, livers, spleens, and blood were tested for CFU. On day 2 post infection, organs of Schu S4 infected mice contained more CFU than organs from ΔFTT_0615c infected mice (Figure 16). While lower bacterial burdens were measured in all organs of mice infected with ΔFTT_0615c compared to Schu S4 infected mice on day 2 post infection, there was only a statistically significant difference in the blood where the mutant could not be detected (Figure 16). By day 4 post infection, there were statistically significant increases in CFU in all organs of Schu S4 infected mice compared to ΔFTT_0615c infected mice (Figure 16). Two to four logs fewer CFU were recovered from ΔFTT_0615c-infected organs compared to Schu S4 infected

organs (Figure 16). Similar to day 2, bacteria were not detected in the blood of mice infected with ΔFTT_0615c on day 4. On day 6 post infection, there were fewer CFU in the organs of ΔFTT_0615c infected mice compared to Schu S4 infected mice on day 4 post infection, and the blood of ΔFTT_0615c infected mice continued to have undetectable amounts of CFU (Figure 16). By day 12 post infection, CFU became detectable in the blood of ΔFTT_0615c infected mice, but there was still approximately 1000 fold fewer CFU than in the blood of a Schu S4 infected mouse on day 4 (Figure 16). Additionally, bacterial burden in the lungs, livers and spleens of ΔFTT_0615c infected mice on day 12 post infection was similar to the CFU recovered from organs of Schu S4 infected mice on day 4 (Figure 16). These data demonstrate that FTT_0615c is important for *F. tularensis* replication *in vivo* and its presence enhances dissemination to secondary sites of infection.

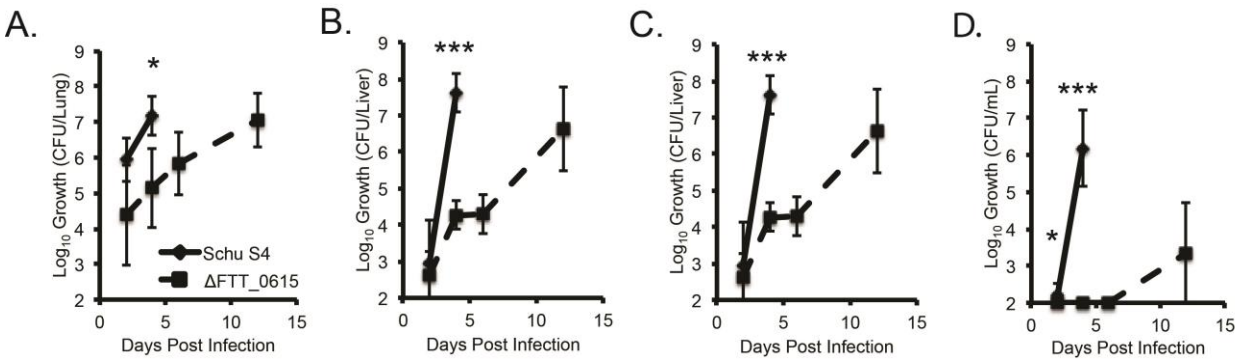

Figure 16: Mice infected with FTT_0615c have reduced bacterial burdens.

Mice were infected with 100 CFU of either Schu S4 or ΔFTT_0615c. On days 2, 4, 6 and 12 post infection, mice were sacrificed and CFU was determined. The limit of detection for spleens and lungs was 100 CFU, for blood it was 100 CFU/mL, and for livers it was 200 CFU. In total, 11 ΔFTT_0615c infected mice (three independent experiments) and 8 Schu S4 infected mice (two independent experiments) were sacrificed on day 2; 11 ΔFTT_0615c infected mice (three independent experiments) and 6 Schu S4 infected mice (two independent experiments) were sacrificed on day 4; 7 Schu S4 infected mice (two independent experiments) and 7 ΔFTT_0615c infected mice (two experiments independent experiments) were sacrificed on days 6 and 12 post infection. The data are presented as mean ± standard deviation. The bacterial burdens for mice infected with Schu S4 or ΔFTT_0615c were compared

for each organ on day 2 and 4 and differences were determined to be significant by Student's t-test. * indicates a $p < 0.05$ and *** indicates a $p < 0.001$.

2.4.5 Cytokine and chemokine responses to ΔFTT_0615c *in vivo.*

We next tested whether the enhanced cytokine stimulation observed with the ΔFTT_0615c mutant *in vitro* was recapitulated *in vivo*. The concentration of cytokines and chemokines in lung homogenates and plasma were analyzed by Luminex on days 2, 4, 6, and 12 post infection. There was no difference in the concentration of any of the 32 analytes tested in the lungs of mice infected with Schu S4 or ΔFTT_0615c on day 2 (Figure 17). On day 4, however, significantly higher concentrations of TNF-α, IL-1α, and IL-1β were present in the lungs of mice infected with the mutant (Figure 17). In contrast, significantly greater concentrations of G-CSF, MCP-1 (CCL2), and LIX (CXCL5) were found in the lungs of mice infected with Schu S4 (Figure 17). Interestingly, other analytes in mice infected with the ΔFTT_0615c strain were not elevated above the concentrations in Schu S4 infected mice, including IFN-γ (Figure 17). This was unexpected since IFN-γ is typically associated with effective host defenses against *F. tularensis* (Edwards et al., 2010; Elkins et al., 2009; Lopez et al., 2004). By day 12 post infection, there were increases in many proinflammatory cytokines over day 2 values in the ΔFTT_0615c infected mice with significant increases in IFN-γ, G-CSF, IL-1α, IL-1β, IP-10, KC, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, and TNF-α (Figure 17). In contrast to the expression of most cytokines, MCP-1 and G-CSF were significantly lower at day 6 post infection in ΔFTT_0615c infected mice compared to their levels in Schu S4 infected mice on day 4 (Figure 17). By day 12 post infection G-CSF was at a level similar to that in the lung of a Schu S4 infected mouse on

day 4, but MCP-1 remained significantly lower at the same level as on day 6 (Figure 17). Overall, these data demonstrate that pulmonary infection by ΔFTT_0615c was associated with elevated levels of prototypical pro-inflammatory cytokines, which correlated with slower replication of ΔFTT_0615c compared to Schu S4.

The striking lack of bacteremia after pulmonary infection by the ΔFTT_0615c strain suggested high concentrations of cytokines in the blood might contribute to restricting the growth of the mutant. Using Luminex technology, we measured cytokines in the blood of the same mice described above on days 2, 4, 6 and 12 post infection. In contrast to the lung measurements, mice infected with ΔFTT_0615c had significantly lower plasma concentrations of TNF-α, IL-1β, Eotaxin, G-CSF, GM-CSF, IL-6, IP-10, KC, MCP-1, MIP-1α, MIP-1β than mice infected with Schu S4 on day 4 post infection (Figure 18). The plasma concentrations of IL-1α were also 20-fold lower than those in the lungs of mice infected with the mutant, and were indistinguishable from plasma of mice infected with Schu S4 (Figures 17 and 18). Mice infected with wild-type Schu S4 had significantly greater plasma concentrations of G-CSF and MCP-1 than mice infected with ΔFTT_0615c, which was two to five-fold higher than levels in lung homogenates (Figure 17 and 18). There was not a difference in the expression of MIG on day 4 post infection in the serum of either Schu S4 or ΔFTT_0615c infected mice (Figure 18). While there were lower concentrations of many cytokines in the serum of mice infected with ΔFTT_0615c compared to the sera of Schu S4 infected mice on day 4, many of the cytokines were still elevated above their values on day 2 post infection in ΔFTT_0615c infected mice. Surprisingly, by day 6 post infection in ΔFTT_0615c infected mice, all of the elevated cytokines went back to their day 2 values except for IL-1α, IL-1β, TNF-α, and MIP-1α, which continued to increase through day 12 post infection (Figure 18). Only IL-1, TNF-α, GM-CSF, and MIP-1α

cytokines in the serum of ΔFTT_0615c infected mice correlated with the bacteremia in the mice, which suggested that these cytokines could be critical in combating infection with ΔFTT_0615c

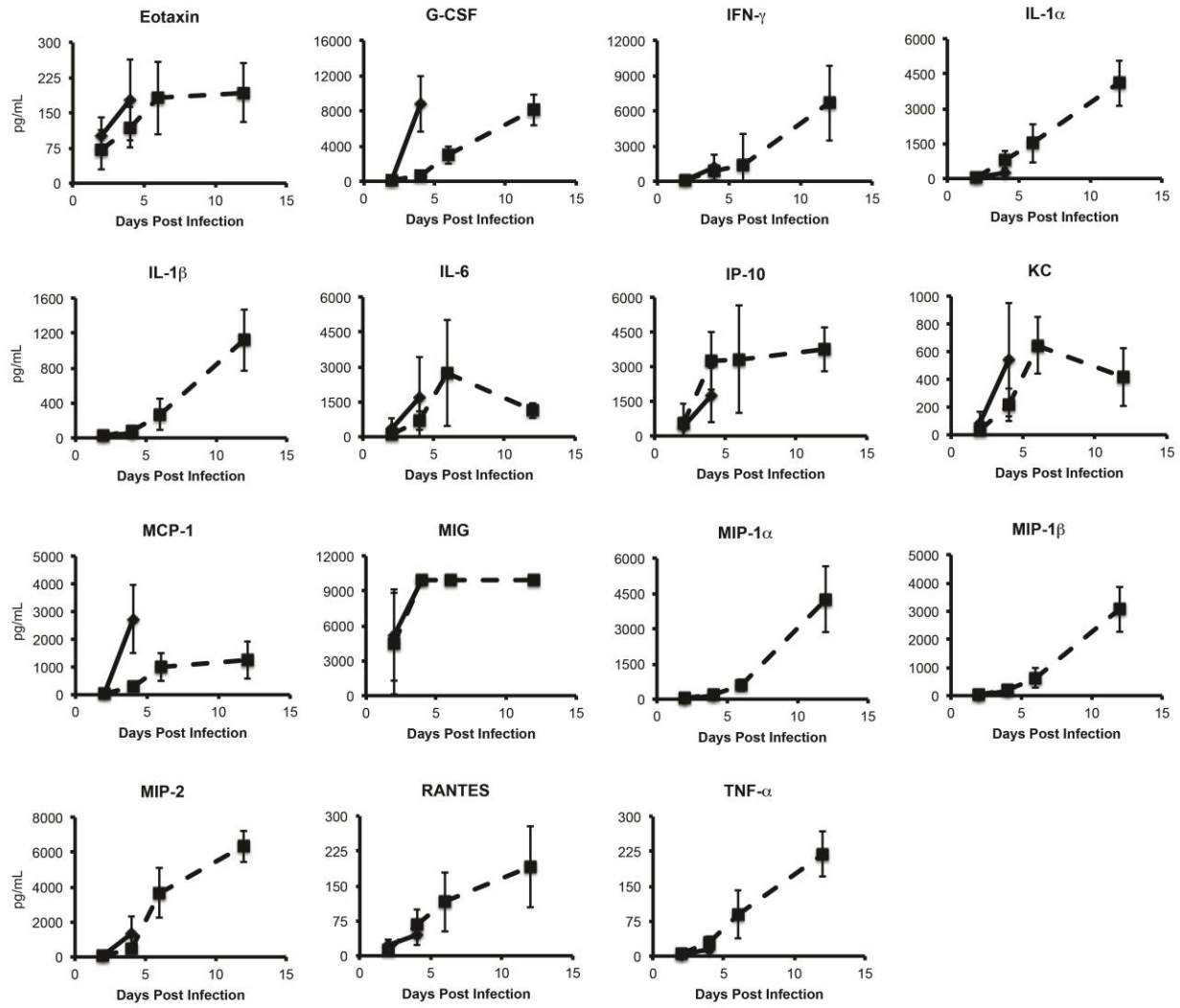

Figure 17: Wild-type and ΔFTT_0615c Schu S4 strains elicit different cytokine and chemokine profile within the lung *in vivo*.

Supernatants from lung homogenates were assayed by Luminex as described in Materials and Methods. A total of 11 samples (three independent experiments) from both Schu S4 and ΔFTT_0615c were tested on day 2. A total of 11 samples (three independent experiments) were tested on day 4 for ΔFTT_0615c and a total of 7 samples (two independent experiments) were tested for Schu S4. Data are presented as the mean ± standard deviation.

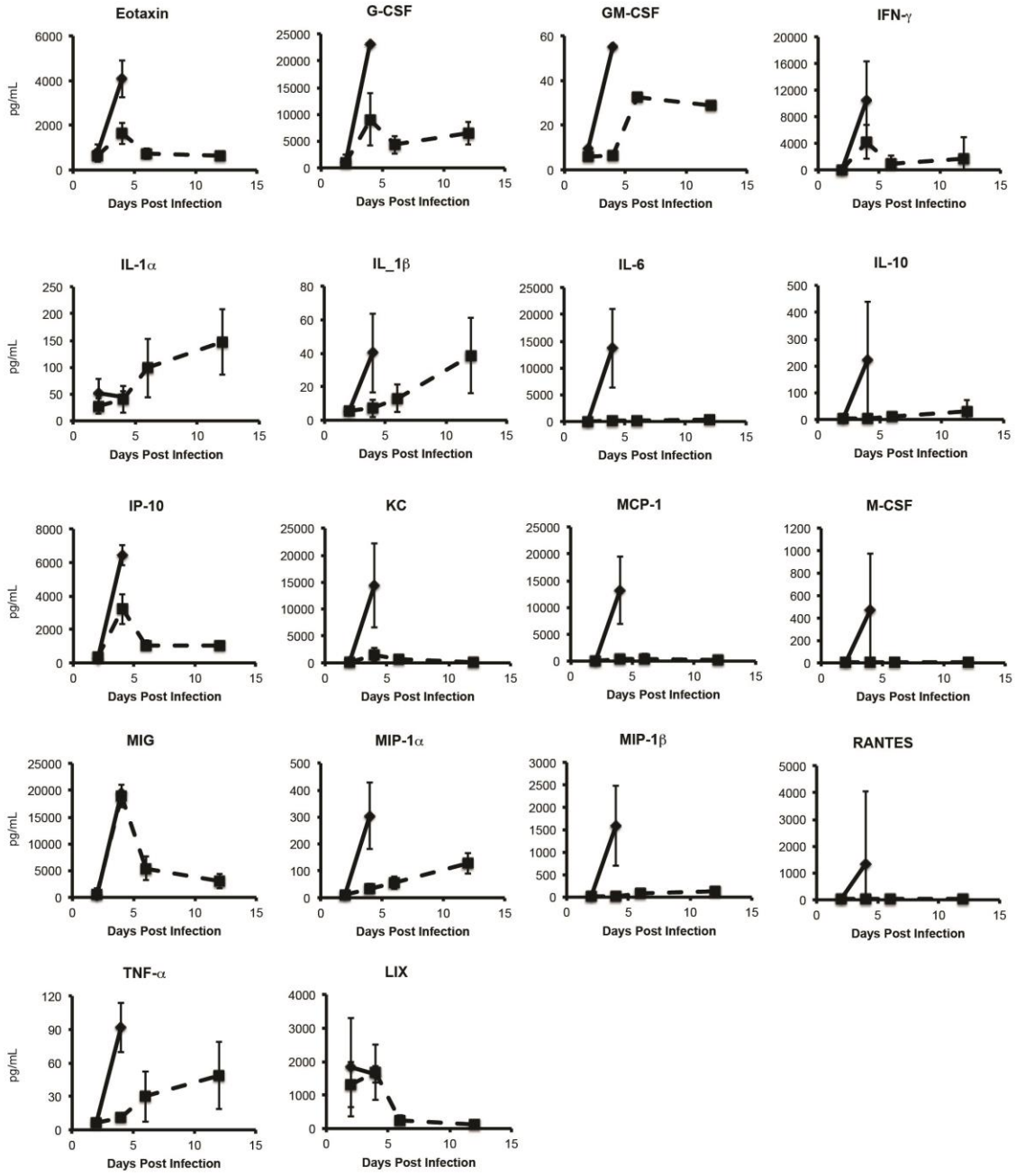

Figure 18: Mice infected with ΔFTT_0615c have lower pro-inflammatory response in the plasma.

Plasma was collected from heparinized blood and tested by Luminex as described in Materials and Methods. A total of 11 samples (three independent experiments) from both Schu S4 and ΔFTT_0615c were tested on day 2. A total of 11 samples (three independent experiments) were tested on day 4 for ΔFTT_0615c and a total of 7 samples (two independent experiments) were tested for Schu S4. Data are presented as the mean ± standard deviation.

2.4.6 Histopathologic changes within the organs.

There is not a detectable immune response to Schu S4 in mice until 3 days post infection. After three days, the presence of cytokines and immune cells can be detected within the organs of infected animals. The recruited immune cells form an immunological foci (Shen et al., 2010). These foci are sites of bacterial replication and immune cell death (Schmitt et al., 2013; Sharma et al., 2011a). It is unclear whether the bacteria or the immune response itself results in cell death. To determine if the more robust immune response with ΔFTT_0615c altered the pathologic changes during infection, histopathologic changes were assessed on days 2, 4, 6, and 12 post infection within the lungs, livers, and spleens of mice infected with Schu S4 and ΔFTT_0615c.

By day 4 post infection, Schu S4 elicited focal inflammation in the lung similar to previous reports (Figure 19) (Sharma et al., 2011a). Cell death occurred in the lungs of Schu S4 infected mice, which was determined by counting viable cells (Figure 20). In contrast to the rapid formation of immune foci and decline in viable cells in the lungs of Schu S4 infected mice, immune foci formation was delayed in the lungs of ΔFTT_0615c infected mice and more viable cells were present within the lung (Figure 19 and 20). Cellular recruitment was observed by day 4 post infection, but the amount of cellular material within the spaces of the lungs was reduced in the ΔFTT_0615c infected mice compared to Schu S4 infected mice. By day 12 post infection, there was a significant amount of immune cell recruitment to the immune foci, which was similar to Schu S4 infected mice on day 4 post infection (Figure 19). Similar to Schu S4, the immune foci in ΔFTT_0615c infected lungs were also TUNEL positive and predominately neutrophils (Personal communication Deanna Schmitt). The preservation of viable cells in the ΔFTT_0615c infected mice correlated with a reduction in the CFU in the lungs of mice compared to the Schu

S4 infected lungs. Overall, the delayed pathology and enhanced survival of immune cells within ΔFTT_0615c infected mice correlated with a prolonged survival.

The livers of Schu S4 and ΔFTT_0615c infected mice had a similar appearance on day 2 post infection, with no detectable cellular recruitment (Figure 21). On day 4, recruited cells were detected in livers of mice infected with Schu S4 and ΔFTT_0615c, but Schu S4 infected livers had more pronounced cellular recruitment (Figure 21 and 22). By day 6, there were similar numbers of foci present within the ΔFTT_0615c infected livers compared to Schu S4 infected livers on day 4 despite having lower bacterial burdens (Figure 21). On day 12 post infection, the number of immune cell foci that were visible continued to increase in the ΔFTT_0615c infected livers (Figure 21 and 22). Therefore, 10-fold fewer bacteria and 10-fold more sites of inflammation were found in the livers of mice infected with dFTT_0615c infection compared to WT infection. These data associate formation of the immune foci in the liver with better control of *F. tularensis* replication.

Similar to the liver, splenic architecture was preserved longer during infection with ΔFTT_0615c compared to Schu S4 (Figure 23). Schu S4 and ΔFTT_0615c infected spleens had a similar appearance on day 2 post infection (Figure 23). Consistent with the published reports (Sharma et al., 2011a), by day 4 post infection, there was robust recruitment of immune cells in the white pulp of the spleen, a loss of the red pulp, and a loss of follicle organization (Figure 23). In contrast, mice infected with ΔFTT_0615c had an altered pathological appearance compare to Schu S4 infected mice on day 4 post infection. ΔFTT_0615c infected spleens increased in size, there were some cellular infiltrates to the red pulp, and the architecture of the follicles was preserved (Figure 23 and 24). Splenomegaly in mice infected with ΔFTT_0615c plateaued by day 6 post infection (Figure 24). Additionally, on day 6 post infection the red pulp was

progressively replaced by leukocytes, which continued until the day 12 time point (Figure 23). By day 12 post infection, the splenic architecture of ΔFTT_0615c infected mice lost red pulp and follicle organization similar to the spleens of Schu S4 infected mice on day 4 (Figure 23). From the pathologic appearance, the splenic architecture was preserved longer in the mice infected with ΔFTT_0615c than the mice infected with Schu S4, which correlated with the increased survival time of the ΔFTT_0615c infected mice. Later during infection, the pathological changes in the spleen correlated with the mortality of the animals

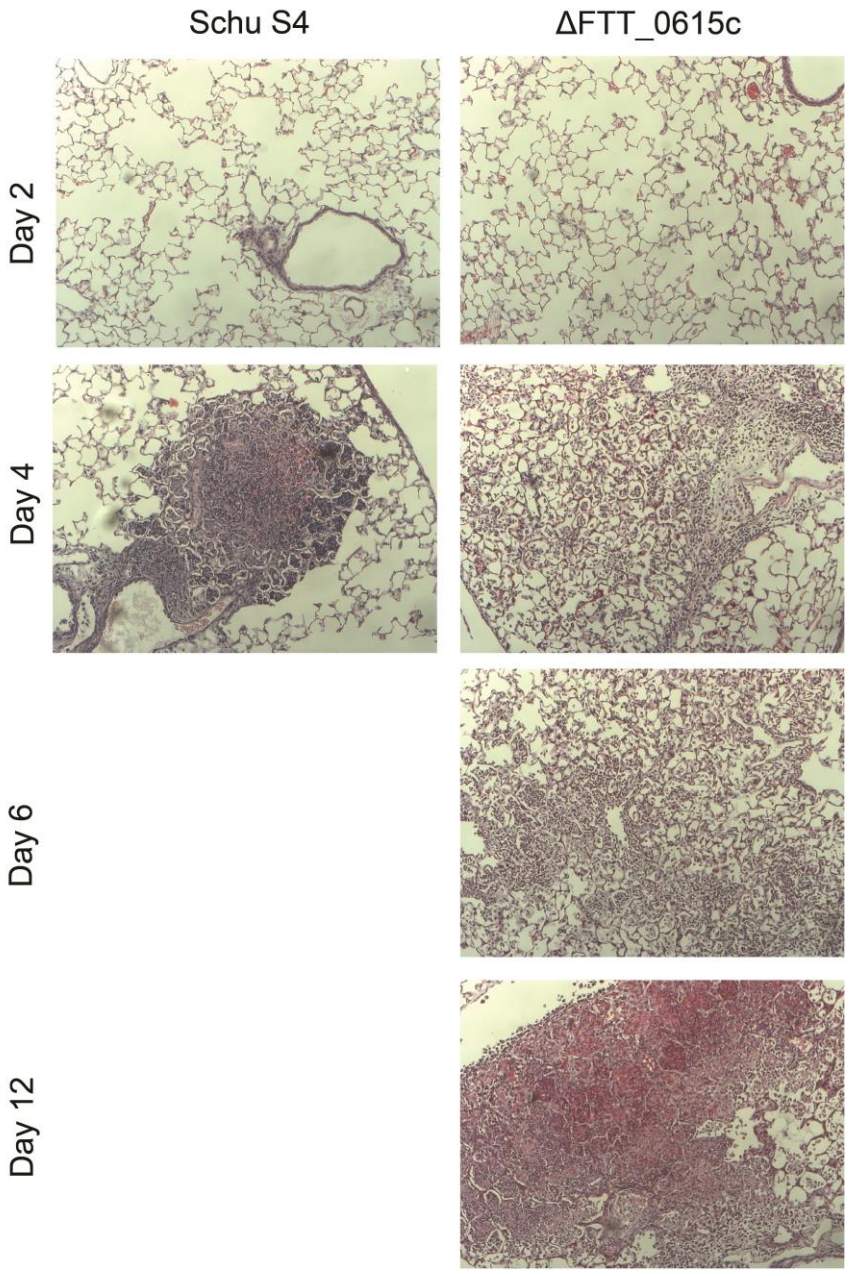

Figure 19: Reduced Lung pathology early during infection with ΔFTT_0615c

Lungs were harvested on day 2, 4, 6, and 12 post infection. The lungs were fixed with formalin, embedded in paraffin, and stained with H&E.

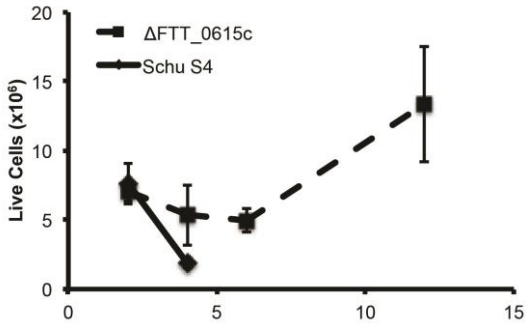

Figure 20: Viable cells in the lung during infection with Schu S4 and ΔFTT_0615c

Lungs were isolated on days 2, 4, 6, and 12 post infection from mice infected with Schu S4 or ΔFTT_0615c. The cell viability was determined by trypan staining.

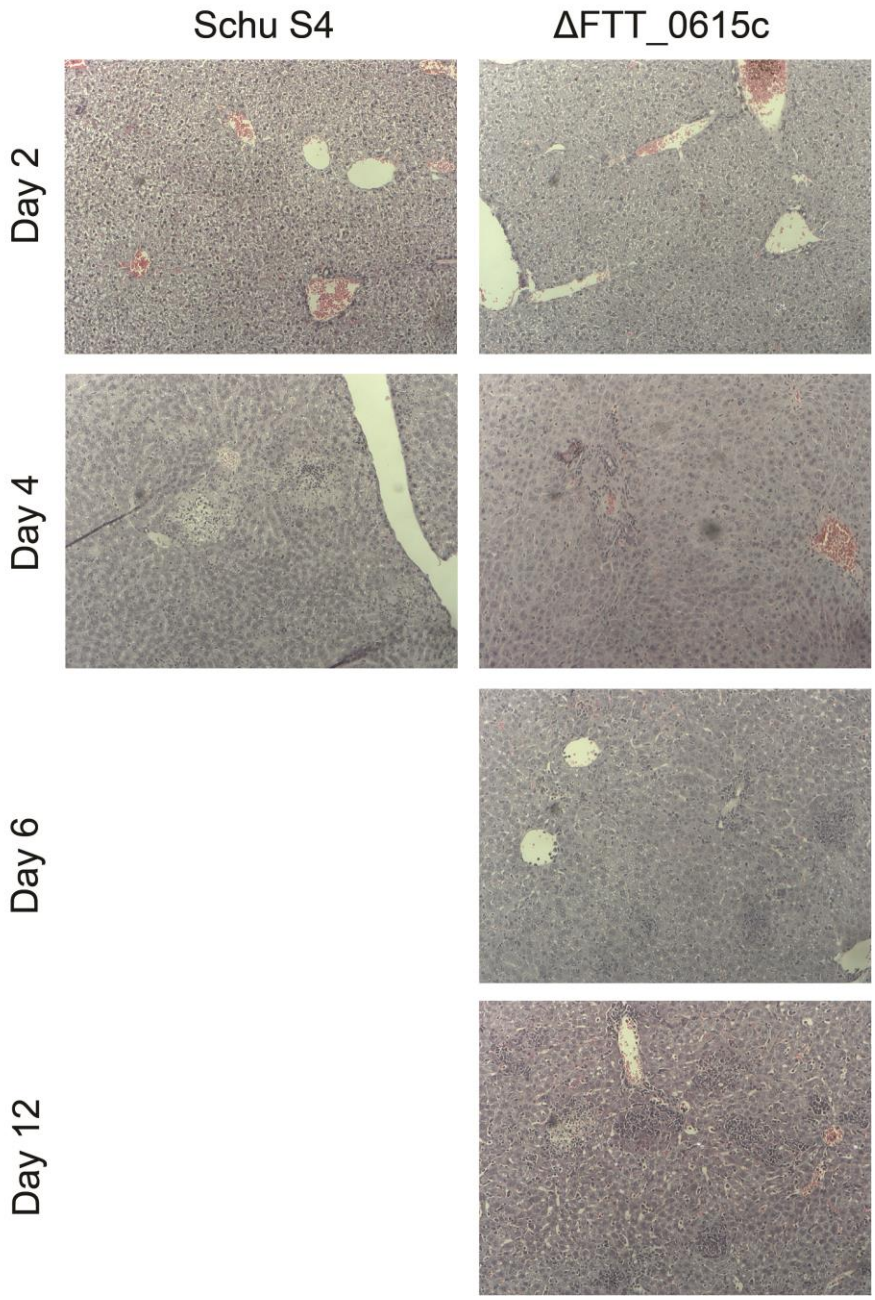

Figure 21: Reduced liver pathology early during infection with ΔFTT_0615c.

Livers were harvested on day 2, 4, 6, and 12 post infection. The livers were fixed with formalin, embedded in paraffin, and stained with H&E.

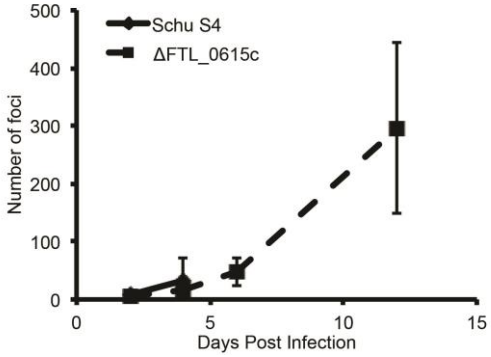

Figure 22: Number of inflammatory foci in the liver of Schu S4 and ΔFTT_0615c infected mice

Livers were isolated on days 2, 4, 6, and 12 post infection from mice infected with Schu S4 or ΔFTT_0615c. The livers were embedded in paraffin, sectioned, and stained with H&E. The number of inflammatory foci were counted in each section.

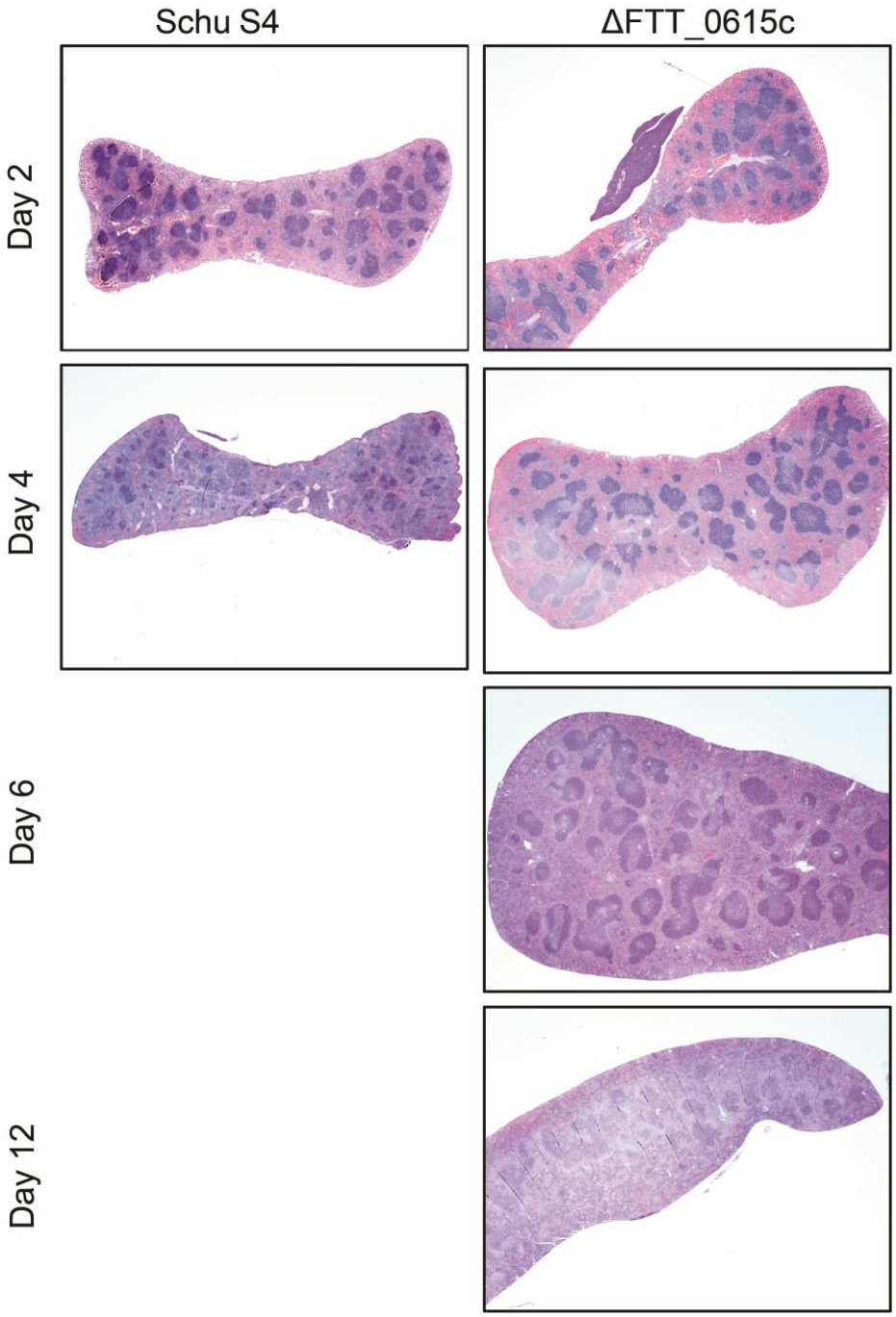

Figure 23: Reduced spleen pathology early during infection with ΔFTT_0615c.

Spleens were harvested on day 2, 4, 6, and 12 post infection. The spleens were fixed with formalin, embedded in paraffin, and stained with H&E.

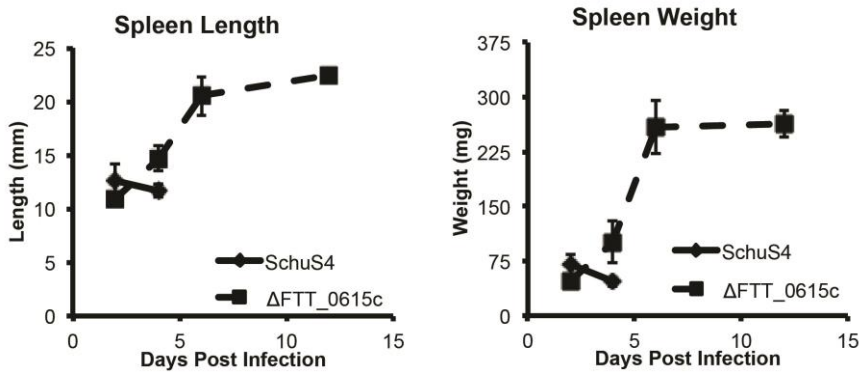

Figure 24: Splenomegaly occurs during infection with ΔFTT_0615c.

Spleens were harvested on day 2, 4, 6, and 12 post infection. The spleens were fixed with formalin, their lengths were measured and they were weighed.

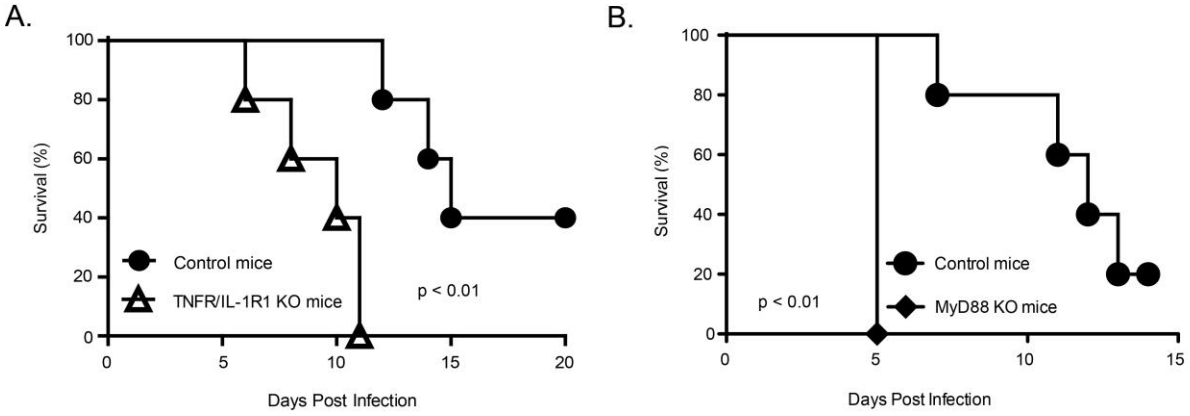

Figure 25: TNF-α receptor, IL-1β receptor, and MyD88 contribute to prolonged survival of mice infected with ΔFTT_0615c.

A) Control (B6129SF2/J) or knock-out (TNFR1/IL-1R1 KO) mice were infected intratracheally with 80 CFU of ΔFTT_0615c and survival was followed over time. B) Control (C57Bl/6) or MyD88 KO mice were infected with 100 CFU of ΔFTT_0615c intratracheally and survival was followed over time. The experiments were performed with an n=5 for each group. $p < 0.01$ by log rank test.

2.4.7 Contribution of TNFR/IL-1R and MyD88 to the host response to ΔFTT_0615c

While the enhanced cytokine production in the lungs of mice correlated with the attenuation of the mutant, it was unclear if these cytokines prolonged the survival of the mice infected with ΔFTT_0615c compared to mice infected with Schu S4. To investigate this possibility, mice lacking both TNF-α type 1 receptor and IL-1β type 1 receptor (TNFR1/IL-1R KO) or control mice were infected with ΔFTT_0615c. Mice were significantly more susceptible to the infection when lacking the TNF-α and IL-1β receptors than were wild-type mice (Figure 25A). The median time to death was 10 days for the knock-out mice compared to 15 days for wild-type mice, with two of five wild-type mice surviving the infection until the end of the study on day 20 (Figure 25A). These results define a role for TNF-α and IL-1 signaling in prolonging the survival of the mice infected with ΔFTT_0615c.

To further investigate the importance of the host response during infection with ΔFTT_0615c, the role of MyD88 was tested. MyD88 is a known adaptor for the IL-1R receptor and is needed for signaling from all TLRs except for TLR3, which is not involved in the host response to *F. tularensis*. Additionally, MyD88 signaling leads to the activation of NF-κB, which was associated with the enhanced pro-inflammatory responses to ΔFTL_0883 and ΔFTT_0165c *in vitro*. MyD88 KO mice and wild-type mice were administered 100 CFU of ΔFTT_0615c, and they were followed for survival. The MyD88 KO mice were significantly more susceptible to infection with ΔFTT_0615c than wild-type mice. MyD88 KO mice had a median time to death of 5 days whereas the wild-type had a median time to death 12 days post infection (Figure 25B). These results demonstrate that in addition to IL-1R and TNF-αR, MyD88 is required for the host immune response to ΔFTT_0615c.

To determine how MyD88 contributed to the host response, the interaction of macrophages deficient in MyD88 and *F. tularensis* was assessed. Coculture of wild-type BMDM with Schu S4 strains resulted in the production of TNF-α in 24 hours (Figure 26A). In contrast, there was no production of TNF-α by MyD88 KO BMDM when they were cocultured with Schu S4 strains (Figure 26A). These results suggest that MyD88 is required for the production of cytokines in response to *F. tularensis*. Finally, the ability of Schu S4 strains to replicate in WT and MyD88 KO BMDM was determined. There was not a difference in the intracellular replication of the Schu S4 strains irrespective of the presence of MyD88 (Figure 26B). There was not a difference in invasion of Schu S4 strains into WT or MyD88 KO BMDM at 2 hours (data not shown), suggesting that the ability of the macrophages to phagocytose *F. tularensis* is independent of MyD88. These data demonstrate that within a macrophage, MyD88 is needed for the cytokine production of Schu S4, but it does not affect the ability of *F. tularensis* to replicate within the macrophage.

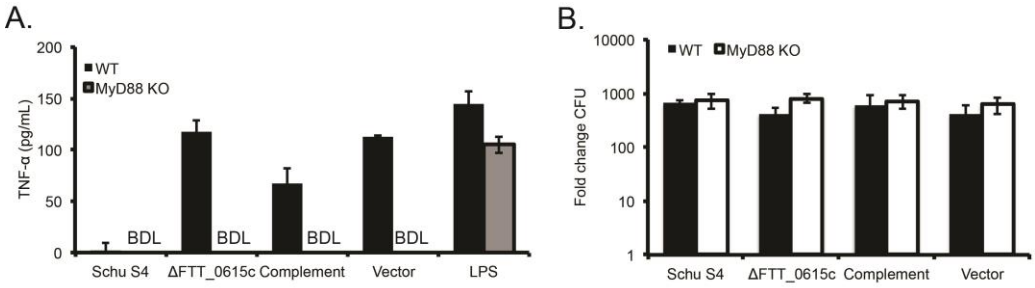

Figure 26: Requirement of MyD88 within macrophages

A) WT or MyD88 KO BMDM were cocultured with Schu S4 strains at an MOI of 10. After 24 hours the concentration of TNF-α in the culture supernatant was determined by ELISA. The data is means ± standard deviation of triplicate wells and is representative of three independent experiments. BDL indicates below the detectable limits of the assay. B) WT or MyD88 KO BMDM were infected with Schu S4 strains at an MOI of 500. At 2 hours a gentamicin protection assay was performed. At 2 and 24 hours the macrophages were lysed to quantify

intracellular CFU. The data depicts the mean fold change (CFU at 24 hour/CFU at 2 hour) ± standard deviation of triplicate wells and is representative of two experiments.

2.5 DISCUSSION

The pathogenesis of *F. tularensis* is associated with its ability to evade immune detection (Chase et al., 2009; Conlan et al., 2010; Fortier et al., 1992; Telepnev et al., 2003). Adaptation to the intracellular mammalian environment is likely a mechanism contributing to the stealth nature of this pathogen. Polyamines are abundant in the intracellular environment and cue *Francisella* to the host's cytosol (Carlson et al., 2009). The objectives of this study were to identify genes important for the polyamine response in *F. tularensis* and to determine if the genes contribute to the virulence of the organism. By performing a transposon screen within LVS, FTL_0883 was identified as a gene important for the spermine response. Unlike LVS, the FTL_0883 mutants stimulated high levels of cytokines from macrophages even if they were cultured in the presence of extracellular spermine (Figure 7A and 7B). The loss of spermine response in ΔFTL_0883 is not simply the result of stimulating higher amounts of cytokines than wild-type. Other transposon mutants from this screen and the Δ*pyrF* mutant in LVS (Horzempa et al., 2010a) stimulate more cytokines from macrophages than wild-type LVS. Nevertheless, they stimulate less cytokine production from macrophages after growing in MHB with spermine than MHB alone (data not shown). Further, FTL_0883 is needed for an optimal transcriptional and carbohydrate response to spermine (Figure 7E and 7H). Therefore, FTL_0883 contributes to *F. tularensis'* response to extracellular spermine.

Based on our data, an adequate response to spermine minimizes the activation of macrophages. Previously, it was proposed that a response to spermine could be an important determinant that enables host immune evasion (Carlson et al., 2009). Consistent with this proposal, a significant increase in the production of pro-inflammatory cytokines by macrophages infected with ΔFTL_0883 and ΔFTT_0615c compared to wild-type strains was observed (Figure 8 and 10). We also observed enhanced NF-κB activation and increased production of pro-inflammatory cytokines in macrophages infected with ΔFTL_0883, which both independently demonstrate that the macrophage is in an activated state (Figures 8, 10-11). Similar to the *in vitro* observations, ΔFTT_0615c stimulated more pro-inflammatory cytokines within the lung *in vivo* (Figure 17). The enhanced immune response within the lung correlated with a decrease in systemic bacterial numbers in mice infected with ΔFTT_0615c. Published data indicate that greater activation of the immune response controls the replication of *F. tularensis* Schu S4, and that the control correlated with increases in pro-inflammatory cytokine production (Evans et al., 2010). Similarly, our results demonstrate that there is a more robust pro-inflammatory response in the lungs of mice infected with ΔFTT_0615c compared to Schu S4, which correlated with control of the mutant. Moreover, the TNFR1/IL-1R KO and MyD88 KO mice are more susceptible to infection (Figure 25). This demonstrated that activation of immune responses by ΔFTT_0615c leading to the production of the pro-inflammatory cytokines TNF-α and IL-1β is required to control the infection.

Our results provide a significant step forward from previous studies that suggested a role for ΔFTL_0883 and ΔFTT_0615c in *Francisella* virulence. A negative selection screen *in vivo* identified the homolog in *F. novicida* was important for dissemination to the spleen in mice and another negative selection screen identified the homolog in *F. novicida* was important for

survival in *Drosophila* (Moule et al., 2010; Weiss et al., 2007). In addition, the presence of a functional FTT_0615c correlated with virulence within Type A strains, though a spontaneous deletion in a gene in the pathogenicity island, *pdpC*, confounded this study (Sjodin et al., 2010). Using gene specific deletions and complementation studies, our results provided direct evidence that FTL_0883 and FTT_0615c are important mediators of immune evasion in *F. tularensis*. To our knowledge, this study is the first to describe a *Francisella* gene important for sensing the host environment and its role during *in vivo* infections.

This study further questions the paradigm that intra-macrophage replication is a hallmark of *Francisella* pathogenesis (Santic et al., 2006). There have been multiple reports correlating the ability of mutants to replicate within macrophages to their fitness *in vivo* (Buchan et al., 2009; Forslund et al., 2010; Golovliov et al., 2003; Lauriano et al., 2004; Meibom et al., 2009; Nano et al., 2004; Pechous et al., 2006). While this correlation was true for ΔFTL_0883, it was not true for ΔFTT_0615c. The ΔFTT_0615c strain replicated in macrophages *in vitro* at rates similar to that of Schu S4. Unexpectedly, however, we found that there was a substantial increase in the median time to death for mice that were infected with ΔFTT_0615c compared to wild-type Schu S4. These results suggest that replication within a macrophage *in vitro* is not sufficient for virulence *in vivo*. We have also recently published that a Schu S4 *pyrF* mutant is unable to replicate in macrophages; it is, however, essentially fully virulent within a mouse (Horzempa et al., 2010a). Therefore, experiments with the *pyrF* mutant demonstrated that replication within a macrophage is not necessary for the virulence of *Francisella* (Horzempa et al., 2010a). In conjunction with the present study, intra-macrophage replication appears neither necessary nor sufficient to predict Schu S4 virulence in a mouse model of tularemia. However, these findings do not diminish the potential role(s) of the macrophage as a component of host

defenses since macrophage activation correlates with reduced virulence among the Schu S4 strains [this study and (Horzempa et al., 2010a)].

High levels of bacteria are typically found within the blood of mice infected with Schu S4. Surprisingly, there was a significant reduction in the amount of CFU in the blood of the mice infected with ΔFTT_0615c even at the latter time points of infection (Figure 16). This reduction was consistent with a significant reduction in the cytokine levels within the serum despite high levels of cytokines within the lungs (Figure 17 and 18). These results demonstrate that the levels of cytokines within the serum are not just a result of spill over from damaged tissues; rather, the high levels of cytokines within the serum seem to be a direct response to the bacteria present there. The controlled release of cytokines only where there is robust bacterial replication suggests that rather than an out of control sepsis-like syndrome, the high levels of cytokines observed later in infection may be tailored to the high levels of bacteria found within the organs.

Investigations of the immune response during infection with attenuated *F. tularensis* strains have revealed many cytokines and cell populations that are important for combating the bacterial infection (Bosio, 2011). When attempts have been made to translate that data to infections with virulent *F. tularensis* strains, there are no known cytokines or cell populations that have been identified to impact the pathogenesis of virulent strains (Chen et al., 2004b; Schmitt et al., 2012). These data have lead many to conclude that the immune response does not have a role during infection with the virulent *F. tularensis* strains (Cowley, 2009). The data that is presented in this chapter reveals that our attenuated ΔFTT_0615c mutant elicited a similar more robust immune response in the lung than Schu S4 (Figure 17). Additionally, this response was tailored to the locations of bacterial burden and required for the prolonged survival of the

mice. The more robust immune response was correlated with longer preservation of splenic architecture and was associated with an increase in immune foci, both of which correlated with a reduction in bacterial burden. These data suggest that although there is an immunological state that resembles sepsis within the mice, a greater immune response can be achieved, at least with our attenuated mutant, which allows for prolonged survival. It is tempting to suggest that the data generated investigating ΔFTT_0615c would suggest that the immune response plays a role, albeit limited, during infection with Schu S4.

The function of the proteins encoded by FTL_0883 and FTT_0615c remains undefined. Homologs were originally identified in *Salmonella* during a screen conducted to identify mutants that were resistant to cobalt (Gibson et al., 1991). From the screen, four mutants were identified that had increased resistance to cobalt, *corA-corD* (Gibson et al., 1991). *corA*, a magnesium transport protein, was demonstrated to be important for the virulence of *Salmonella* and other bacteria (Papp-Wallace et al., 2008; Pfeiffer et al., 2002; Zhu et al., 2009). *corC* has not been studied in any significant detail beyond the initial screen. Based on homology searches, there is a CorC domain and two tandem CBS domains in FTL_0883 and FTT_0615c proteins. CBS domains bind to adenine derivatives and magnesium; they are proposed to function as intracellular switches, which are sensitive to the energy and ion concentrations within the cell (Ignoul and Eggermont, 2005; Scott et al., 2004). The function of the CorC domain within the protein remains unknown. It is possible that within *Salmonella* where there is a functional CorA, CorC functions in conjunction with CorA to mediate divalent cation efflux (Gibson et al., 1991). However, a function in cation efflux within *F. tularensis* is unlikely since its genome does not encode a functional *corA*. Previous work with CorC has been performed in systems that contain a CorA protein. This is the first system to describe the importance of a putative CorC homolog

that is independent of CorA. *Francisella* provides a useful model to study the functions of the proteins encoded by FTL_0883/FTL_0615c that are independent of the activities of the CorA protein, which likely occur in a large number of bacteria as half of sequenced bacterial genomes lack a functional CorA protein (Moomaw and Maguire, 2008).

corC is genetically linked to *lnt*, an N-acyltransferase, which is required for the proper sorting of lipoproteins to the bacterial outer membrane (Tokuda and Matsuyama, 2004). It was possible that a loss of *corC* could affect the activity of *lnt*. The bacterial outer membrane lipoproteins were isolated and there were no apparent differences between ΔFTL_0883 or LVS, suggesting that CorC did not alter the activity of Lnt. There is additional support that *lnt* is not contributing to the phenotypes that are observed with our FTL_0883/FTT_0615c mutants. A transposon mutant in *lnt*, FTL_0882, was unable to replicate in CDM containing DCHA in the primary screen to identify mutants unable to respond to exogenous spermine. However, this mutant was able to respond to the addition of spermine in the culture media; eliciting less TNF-α from macrophages when cultured in the presence of spermine compared to MHB alone (Appendix C). Additionally, the *lnt* transposon mutant replicated within macrophages similar to wild-type LVS (Appendix C). These data demonstrate that mutation of FTL_0883 does not have polar effects on the downstream gene *lnt*, and mutation of *lnt* does not result in the same phenotypes as mutation of FTL_0883.

Based on available information, the proteins encoded by the FTL_0883/FTL_0615c loci could modulate the *Francisella* spermine response in one of three ways. First, CBS domains can regulate the activity of ABC transport systems, such as ClC channels (Estevez et al., 2004). In *Francisella*, it is plausible that the FTL_0883/FTL_0615c proteins interact with other ABC transport systems, such as the polyamine transport system, to regulate the transport of

polyamines. Altered polyamine transport in ΔFTL_0883/ΔFTL_0615c strains could affect the mutants' ability to respond to extracellular spermine. Second, bioinformatics analysis (psortb) localized the protein products of FTL_0883/FTL_0615c to the cytoplasmic membrane, and it was identified in the membrane fraction of *F. tularensis* subsp. *tularensis* FSC033 (Twine et al., 2005). The localization of the FTL_0883/FTL_0615c gene products to the membrane could enable it to function as a signal transducer, connecting the identification of extracellular spermine at the surface to transcriptional responses inside the bacteria. Third, the transcriptional response to spermine may result in an up- or down-regulation of proteins that interact with FTL_0883/FTL_0615c proteins within the membrane, resulting in bacteria that are less stimulatory for macrophages. The presence of spermine is likely to alter the activity of CorC rather than affect its expression to mediate the spermine response. Previous work demonstrated transcription of *corC* is not altered when spermine is included in the culture media (Carlson et al., 2009). The molecular function(s) of the FTL_0883/FTL_0615c gene products is currently under active investigation.

The spermine response by *F. tularensis* enables the bacteria to limit the pro-inflammatory cytokines produced by the immune cells. This response is critical for the rapid pathogenesis and the disease severity associated with *F. tularensis*. Bacterial adaptation in response to extracellular polyamines is not limited to *Francisella*, and is a more common theme in microbiology. The development of biofilms by *Vibrio cholera* and *Yersinia pestis* is regulated by the concentration of extracellular polyamines (McGinnis et al., 2009; Patel et al., 2006; Wortham et al., 2010). Additionally, efflux pump expression and quorum sensing in *Burkholderia pseudomallei* are regulated by the presence of spermidine (Chan and Chua, 2010). FTL_0883 is conserved among *Burkholderia*, *Vibrio*, and *Yersinia* species based on a BLAST

homology search, which suggests that FTL_0883 could serve as a model for the function of the homologous proteins. It is possible the homologs within these bacteria play important roles both in regulating their responses to polyamines specifically and their virulence more generally.

3.0 FUNCTIONAL IMPORTANCE OF CORC IN *FRANCISELLA TULARENSIS*

BC Russo designed the scope of the study, performed the experiments, analyzed the data and prepared the manuscript. GJ Nau designed the scope of the study and reviewed the manuscript. J Franks performed electron microscopy.

3.1 ABSTRACT

The FTL_0883/FTT_0615c loci are important for the spermine response and virulence in *F. tularensis*. These loci are homologous to the *corC* gene, which was initially described in *S. typhimurium*. In order to identify the importance of *corC* for the virulence and physiology of *F. tularensis*, ΔFTL_0883 and ΔFTT_0615c were phenotypically characterized in LVS and Schu S4 strains respectively. In contrast to *S. typhimurium*, mutation of FTL_0883/FTT_0615c did not change the sensitivity to cobalt or alter magnesium ion homeostasis. Deletion of FTL_0883/FTT_0615c enhanced stimulation of host cells, which was not attributable to a loss of active suppression. There was not a difference in lipoprotein expression or modification on the outer surface of the mutants to account for enhanced stimulation. Less carbohydrate, including LPS and O-antigen capsule, was expressed on ΔFTL_0883 compared to LVS, but surface carbohydrate expression was unchanged in ΔFTT_0615c strain compared to Schu S4. Finally, the release of stimulatory molecules was assessed. There was an increase in the release of pro-

inflammatory proteins in both ΔFTL_0883 and ΔFTT_0615c, but there was only a complementation of this phenotype in the LVS strain. Collectively, these data demonstrate a unique role for *corC* in *F. tularensis* compared to *S. typhimurium* and reveal divergent phenotypes associated with a loss of *corC* in Schu S4 and LVS strains.

3.2 INTRODUCTION

Francisella tularensis is the causative agent of tularemia, a febrile illness (Dennis et al., 2001). The bacterium has a low infectious dose of less than 10 colony forming units, is readily aerosolized, and causes significant morbidity and mortality, which has prompted the Centers for Disease Control and Prevention to list it as a Tier 1 pathogen (2012; Oyston et al., 2004). Even with antibiotic treatment, the case fatality rate remains at approximately 1%. *F. tularensis* is a gram-negative bacterium and thrives in the intracellular environment of its host. A key aspect of the virulence of the bacterium is an ability to adapt to the intracellular environment (Carlson et al., 2007; Hazlett et al., 2008; Loegering et al., 2006). A variety of signals can mediate adaptation; including amino acid concentration, temperature and polyamine concentration (Carlson et al., 2009; Hazlett et al., 2008; Horzempa et al., 2008). In order for *F. tularensis* to respond to spermine, it requires the presence of a *corC* homolog, FTL_0883 in LVS and FTT_0615c in Schu S4 (Russo et al., 2011). Additionally, *corC* has also been identified in genetic screens within *F. tularensis* designed to identify genes required for survival within the spleen (Weiss et al., 2007) and replication within drosophila cells (Moule et al., 2010). A final study identified the presence of a *corC* mutation in an attenuated natural isolate of Type A *F. tularensis* (Pandya et al., 2009).

corC was initially discovered during a screen to identify genes important for regulating the activity of the CorA magnesium transport system in *Salmonella typhimurium* (Gibson et al., 1991). Bioinformatic analysis of CorC shows there is a tandem CBS domain (Bateman domain) in the N-terminus and a CorC domain in the C-terminus. CBS domains are regulatory domains responsive to Mg^{2+} and adenine derivatives such as ATP and cAMP (Scott et al., 2004). Attempts to crystallize corC demonstrate that its confirmation changes by the binding of either ATP or Mg^{2+} (Zhang et al., 2010). The function of the corC domain remains undefined, and the domain lacks secondary structure homologous to other domains with known function. CorC was originally described to have an effect on ion homeostasis, increasing the rate of cobalt influx and magnesium efflux (Gibson et al., 1991). Direct complementation of CorC in *S. typhimurium* has not been done, and there is not evidence that CorC directly interacts with CorA to alter ion homeostasis.

A variety of phenotypes have been associated with a loss of *corC* homologs in bacteria other than *S. typhimurium*. These phenotypes include chloramphenicol sensitivity in *E. coli* (Smith et al., 2007), a reduction in plasmid conjugation efficiency in *E. coli* (Perez-Mendoza and de la Cruz, 2009), and defective gliding motility in *Flavobacterium* (Hunnicutt and McBride, 2001). However, the role of *corC* in gliding motility is not clear. Disruption of the *corC* homolog in *Flavobacterium* was unsuccessful, and overexpression of *corC* was only able to complement one allelic mutant of *gldB,* whose disruption resulted in a decreased gliding motility. Finally, evidence in *F. tularensis* demonstrates the *corC* homolog, FTL_0883/FTT_0615c, is required for the bacterium to adapt to the host environment using spermine as an environmental cue (Russo et al., 2011). Yet, how *corC* mediates these phenotypes is still unknown.

In this work, CorC mutants in *F. tularensis* were phenotypically characterized to understand how the protein encoded by *corC* contributes to the virulence and physiology of the bacterium. The results show that CorC does not have a role in Mg^{2+} ion homeostasis in LVS. There was a differential role for the CorC homolog in LVS and Schu S4. In LVS, FTL_0883 was required for proper expression of surface carbohydrate, while in Schu S4 it was needed for resistance to β-defensin-3. Finally, there was an enhanced release of pro-inflammatory molecules from both mutants, but the role for this in pathogenesis remains unclear. Thus, there is a different role for CorC in *F. tularensis* than *S. typhimurium.* Its loss has differential effects in LVS and Schu S4, but there was a common theme of altered surface phenotypes.

3.3 METHODS AND MATERIALS

Bacterial strains and culture

F. tularensis subsp. holarctica LVS and *F. tularensis* subsp. *tularensis* Schu S4 were maintained as frozen stocks. *F. tularensis* was streaked onto Chocolate II Agar plates and cultured for 72 hours at 37°C with 5% CO_2. Liquid cultures were grown in trypticase soy broth supplemented with cysteine (TSB-c) at 37°C at 250rpm for 12-18 hours.

Measurement of intracellular magnesium concentration

Bacteria were cultured overnight, the optical density of the culture was determined, and the bacteria were diluted to OD_{600}=2.0. The bacteria were pelleted at 21,000x*g* and resuspended in Buffer A (0.9% saline, 10mM HEPES, pH7.4). The bacteria were washed two more times in

Buffer A and suspended in indicator solution (5mM Mag-Fura 2 [Molecular Probes] and 0.025% Pluronic F-127 [Molecular Probes] in Buffer A). The bacteria were incubated with Mag-Fura 2 for 70 min. at room temperature. The bacteria were pelleted as above and washed two times with Buffer A, incubated an additional 30min. at room temperature to allow complete hydrolysis of the dye, and then washed one additional time. The fluorescence of Mag-Fura 2 was determined by monitoring the emission at 509nm with excitation at 340nm and 380nm. To determine total possible fluorescence of the bound form (Fmax), magnesium was added to a final concentration of 30mM, and the bacteria were lysed. Then, 50mM of EDTA was added to the lysed bacteria to determine to maximal fluorescence from unbound Mag-fura-2 (Fmin). Intracellular magnesium concentration was determined using the equation:

$$[\text{Magnesium}] = \text{Kd}\frac{\text{F} - \text{Fmin}}{\text{Fmax} - \text{F}}$$

Disc Diffusion assays

Bacteria were cultured overnight, and diluted to an OD_{600} = 0.3. 100µL of diluted culture was spread as a lawn onto Chocolate II Agar plates and 6mm discs were added to the plate. Chemicals were added to the disc at the indicated concentration. The plates were incubated for 72 hours at 37°C with 5% CO_2. The diameters of the zones were measured.

Cultivation of macrophages

Monocytes were isolated from peripheral blood mononuclear cells, and macrophages were differentiated from the monocytes as described previously (Chapter 2). *F. tularensis* was added to the macrophages at an MOI of 10 and they were allowed to coculture at 37°C with 5% CO_2 in infection media (1% human serum, 10mM HEPES, 1% GlutaMAX in DMEM). The

supernatants from LVS infected macrophages were collected to determine TNF-α concentration by ELISA (R&D systems). The supernatants from Schu S4 infected macrophages were collected, filtered, treated with gentamicin, and the concentration of TNF-α was determined by ELISA. Suppression of the macrophage pro-inflammatory response was assessed as done previously (Carlson et al., 2007). Briefly, macrophages were cocultured with *F. tularensis* at an MOI of 500 for 4 hours to ensure a high rate of invasion. The macrophages were washed three times with HBSS to remove extracellular bacteria. The macrophages were then incubated with 10μg/mL of LPS for 20 hours, and the supernatants were collected. TNF-α concentration in the supernatants was measured in the supernatants by ELISA.

Transmission Electron Microscopy

Bacteria were cultured overnight in TSB-c, pelleted and fixed in 2.5% gluteraldehyde in 0.1M PBS for 1 hour at room temperature. The bacteria were then washed 3 times in 0.1M PBS for 15 min. Post fixation occurred in 1% osmium tetroxide containing 1% potassium ferricyanide for 1 hour. The samples were then dehydrated in graded alcohols, 30%, 50%, 70%, 90% and 100% for 15 min each, and then washed two more times in 100% ethanol for 15 min each. This was further followed by 2 washes for 10 min. in propylene oxide. The bacteria were infiltrated with a 1:3 mixture of Epon:propylene oxide overnight, a 1:1 mixture of Epon: propylene oxide overnight, and then a 3:1 mixture of Epon:propylene oxide overnight. The infiltration continued with 3 changes of 100% Epon for 1 hour each under a vacuum. The bacteria were embedded in pure Epon at 37°C for 24 hours, and cured at 65°C for 48 hours. 70nm sections were cut with an ultra-microtome and imaged using a Joel JEM 1011. Specimen processing and imaging was performed at the Center for Biological Imaging at the University of Pittsburgh.

Triton X-114 phase extraction for isolation of outer membrane proteins

Phase extraction of membrane proteins was performed as done previously (Carroll, 2010). *F. tularensis* strains were cultured overnight in TSB-c and normalized to an OD_{600}=2.0. The normalized cultures were washed with PBS once, and pelleted at 15,000x*g* for 3min. at room temperature. The pellet was resuspended in cold Sample Solution (2% Triton X-114, 10mM Tris-HCl, 150mM NaCl), and incubated on at 4°C for 1 hour. An aliquot was removed for total protein, and the remaining sample was pelleted at 14,000rpm at 4°C for 30 min. The supernatant was decanted to a fresh 1.5 mL tube and the remaining pellet was stored on ice. The supernatant was incubated at 37°C for 3 min to allow for micelle formation, and pelleted at 21,000 x*g* for 15 min. The top, aqueous layer was removed. The remaining detergent pellet was washed three times: add 500uL of 1% Triton X-114, vortex to mix, heat for 3 min. at 37°C, and pellet at 21,000x*g* for 15 min. The proteins in the final pellet were separated from the detergent with a methanol-chloroform precipitation. Proteins were denatured in NuPAGE sample buffer and heated at 95°C for 5 min. The proteins were separated by SDS-PAGE and visualized by silver stain.

Staining carbohydrates

F. tularensis was cultured overnight, normalized to an OD600=2.0. 5mL of normalized bacterial culture was pelleted at 21,000*xg* and the pellet was resuspended in 500µL of TSB-c. The *F. tularensis* lysate was sonicated (MicrosonXL) at 14 for 10 pulses and treated with Proteinase K for 1 hour at 60°C. Sample loading buffer was added to the proteinase K treated

lysate and heated at 95°C for 5 min. The samples were resolved using a Novex 12-4% SDS-PAGE gel (Invitrogen). The carbohydrates were stained with pro-Q emerald 488 (Molecular Probes) following manufacturer's instructions. The gels were visualized using a Typhoon 9400 fluorescent flatbed scanner.

Congo red staining

F. tularensis was diluted to an OD_{600}=2.0, washed with PBS, and resuspended in congo red (0.002% (w/v) congo red in PBS) as described previously (Gophna et al., 2001). The bacteria were incubated for 2 hours at 37°C in the presence of the dye. The bacteria were pelleted, the supernatants were collected and the absorbance was determined at 500nm.

Western blotting

From an overnight broth culture, samples were normalized by OD_{600}, which was found to generate the similar protein concentrations. Samples were resolved through 12% SDS-PAGE gels, transferred to nitrocellulose, and blocked with 5% non-fat dry milk in PBS. Mouse anti-PdpD, rabbit anti-*Francisella*, anti-polyamine, mouse anti-*Francisella* LPS, mouse anti-PotF (kindly provided by Edwin Swiatlo), and mouse anti-O antigen capsule (11B7; kindly provided by Michael Apicella) were diluted 1:1000 in 5% milk and incubated with membranes. Blots were washed with PBS and the appropriate secondary antibodies were used at a final concentration of 1:2000. The blots were washed with PBS, incubated with chemoilluminescent substrate (GE healthcare), exposed to film, and developed using a Kodak .

3.4 RESULTS

3.4.1 Role of FTL_0883 and FTT_0615c in ion homeostasis

The *F. tularensis* genes FTL_0883 and FTT_0615c are homologs of *corC* in *Salmonella*. Mutation of genes in the Cor family enhances the resistance of *S. typhimurium* to the toxic effects of cobalt (Gibson et al., 1991). In *S. typhimurium,* loss of *corC* was hypothesized to increase intracellular magnesium concentration resulting in increased resistance to cobalt (Gibson et al., 1991). To determine if the *corC* homologs in *F. tularensis* function similarly to *corC* in *Salmonella,* the sensitivity of ΔFTL_0883 and ΔFTT_0615c to cobalt was assessed using a disc diffusion assay. To each disc, 20mM of $CoCl_2$ was added. There was not a difference in the sensitivity of *F. tularensis* mutants to cobalt compared to their wild-type counterparts (Figure 27A). The intracellular magnesium concentration was next assessed using Mag-Fura 2 (Froschauer et al., 2004). The intracellular magnesium concentration was similar in LVS and ΔFTL_0833 over a range of physiologically relevant concentrations of magnesium (Figure 27B). These data suggest that there is a different role for FTL_0883 and FTT_0615c in *F. tularensis* than *corC* in *Salmonella*.

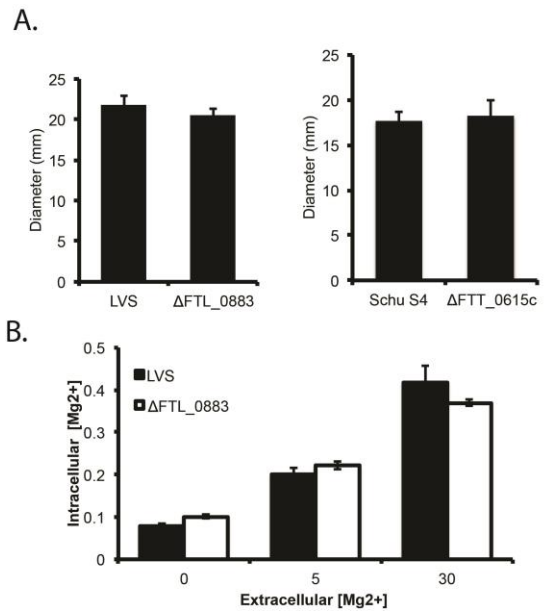

Figure 27: Assessment of cobalt sensitivity and intracellular Mg^{2+} concentration.

A) Bacteria were cultured overnight in TSB-c diluted and a lawn was spread onto Chocolate II agar plates. Three 6mm discs containing 10mg of $CoCl_2$were added to each plate. The plates were incubated for 72 hours at 37°C and the diameters of the zones were measured. The data presented are representative of three independent experiments B) LVS and ΔFTL_0883 were cultured overnight in TSB-c. The bacteria were resuspended in PBS and intracellular magnesium concentration was assessed using Mag Fura-2. The data presented are representative of three independent experiments.

3.4.2 Active suppression and pro-inflammatory response

ΔFTL_0883 and ΔFTT_0615c are more stimulatory to host cells by 24 hours post infection (Figure 8). *F. tularensis* actively suppresses macrophages and dendritic cells to limit the production of pro-inflammatory cytokines (Carlson et al., 2007; Chase et al., 2009). A loss of the ability to suppress the macrophage could account for the increased production of TNF-α that is observed in response to ΔFTL_0883. An *in vitro* assay to measure the ability of *F. tularensis* strains to suppress macrophages has been previously developed (Carlson et al., 2007).

In agreement with previous reports, macrophages infected with LVS did not produce as much TNF-α in response to LPS as macrophages that were treated with media alone (Figure 28). In the same assay, ΔFTL_0883 was able to suppress the production of TNF-α from macrophages in response to LPS equivalent to LVS (Figure 28). These data are consistent with the notion that a greater pro-inflammatory response by macrophages in response to ΔFTL_0883 is not due to a loss of active suppression.

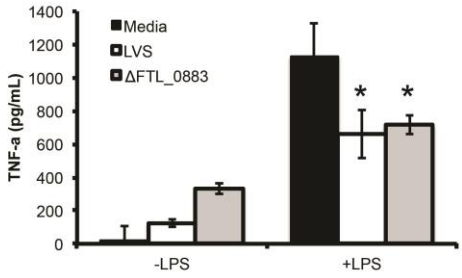

Figure 28: The enhanced activation of human macrophages by ΔFTL_0883 is not due to a loss of active suppression.

Macrophages were infected with LVS or ΔFTL_0883 at an MOI of 500 for 4 hours. A gentamicin protection assay was performed after 4 hours. LPS was added where indicated at a concentration of 10ug/mL for 20 hours. The supernatants were collected and TNF-α release was determined by ELISA. The data is representative of two independent experiments performed in triplicate. Student's t-test was used to compare LVS infected to uninfected. * indicates $p < 0.05$.

3.4.3 Assessment of the surface of ΔFTL_0883 by TEM

ΔFTL_0883 and ΔFTT_0615c are more stimulatory to macrophages shortly after they are added to the cells (Figure 11). There could be an altered surface of the bacteria resulting in enhanced binding of lipoproteins to pattern recognition receptors on the host cell. In order to

assess the differences in the mutants compared to the wild-type strains, TEM was performed on LVS and ΔFTL_0883 (Figure 29A). There was not a notable difference in the appearance of the bacterial surface of ΔFTL_0883 compared to LVS, but there was a significant increase in the size of ΔFTL_0883 compared to LVS (Figure 29B). The increase in size could be an explanation for the larger amount of TNF-α that is produced in response to ΔFTL_0883 compared to LVS. A larger bacterium could have an increased avidity with the receptors on the surface of the macrophage, which would in turn allow for an increase in the pro-inflammatory signaling within the cell and a greater production of TNF-α.

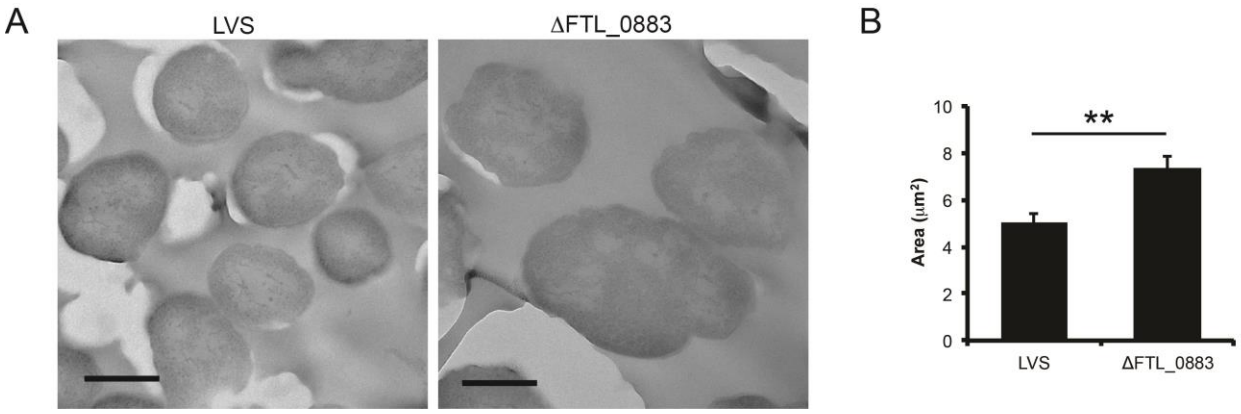

Figure 29: FTL_0883 mutants are larger than LVS

A. LVS and ΔFTL_0883 were cultured overnight in TSB-c and visualized by TEM. Magnification is 80,000X and scale bars indicate 100nm. B. The average area of LVS (n=33) and ΔFTL_0883 (n=26) was determined from images collected by TEM using MicroSuite software (Olympus). Statistical differences in bacterial area were assessed by Student's t-test. ** indicates $p < 0.01$.

3.4.4 Lipoproteins

Lipoproteins on the surface of *F. tularensis* are able to interact with TLRs and induce a pro-inflammatory response from host cell (Thakran et al., 2008). It is possible that there is an

altered expression of TLR ligands on the surface of ΔFTL_0883 that can account for the enhanced stimulation, such as Tul4 (Thakran et al., 2008). Lipoproteins were isolated from the surface of LVS and ΔFTL_0883 using a triton X-114 phase extraction, which enriches outer membrane lipoproteins in a detergent phase. Silver staining did not reveal differences in the expression of lipoproteins in the outer membrane fraction (Figure 30), suggesting that there was not a gross difference in the expression of lipoproteins to account for the enhanced stimulation.

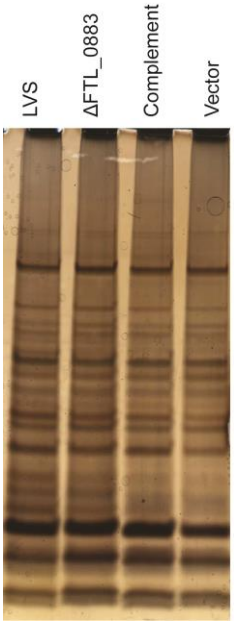

Figure 30: Distribution of lipoproteins on the surface of ΔFTL_0883

Outer membrane lipoproteins were isolated from LVS strains using a Triton X-114 phase extraction method. The lipoproteins were separated by SDS-PAGE and visualized with silver stain. The data presented is representative of three independent experiments.

There is a layer of carbohydrates on the surface of bacteria. The carbohydrates function as a shield to protect the bacterium from the effects of antimicrobials and limit the access of host receptors to the proteins and other ligands on the bacterial surface. Recently, changes in the

amount of carbohydrate on the surface of *F. tularensis* were correlated with the ability of the bacterium to elicit cytokines from macrophages (Zarrella et al., 2011). Therefore, we tested the hypothesis that changes in carbohydrate accounted for the increased stimulation of host cells by *corC* mutants. To test this hypothesis, we first assessed the amount of carbohydrate using a congo red binding assay. Congo red binds to proteins and lipids on the surface of the bacteria, but its binding is blocked by the presence of carbohydrates. Therefore, the amount of carbohydrates present on the surface negatively correlates with the amount of congo red that will bind to the bacteria (Raynaud et al., 2007). The mutant and vector absorbed more congo red from the buffer, causing a greater drop in congo red concentration measured by absorbance (Figure 31A). Therefore, ΔFTL_0883 had significantly less carbohydrate on the surface compared to LVS. This finding was further investigates using pro-Q emerald to stain for carbohydrates. There was a reduction of both capsular, high-molecular weight material and LPS on the surface of the ΔFTL_0883, but there did not appear to be a reduction in the amount of lipid A (Figure 31B). Western blotting was performed as a more sensitive measure of carbohydrate on *F. tularensis*. By western blot, there was less LPS present on the surface of the ΔFTL_0883 compared to LVS (Figure 31C and 31D). To assess the amount of O-antigen capsule present on the surface of the *F. tularensis* strains an O-antigen specific antibody (11B7 clone) was used (Apicella et al., 2010). There was less O-antigen capsule on ΔFTL_0883 compared to LVS (Figure 31C and 31D). Overall, these data demonstrate that there is less surface carbohydrate present on ΔFTL_0883.

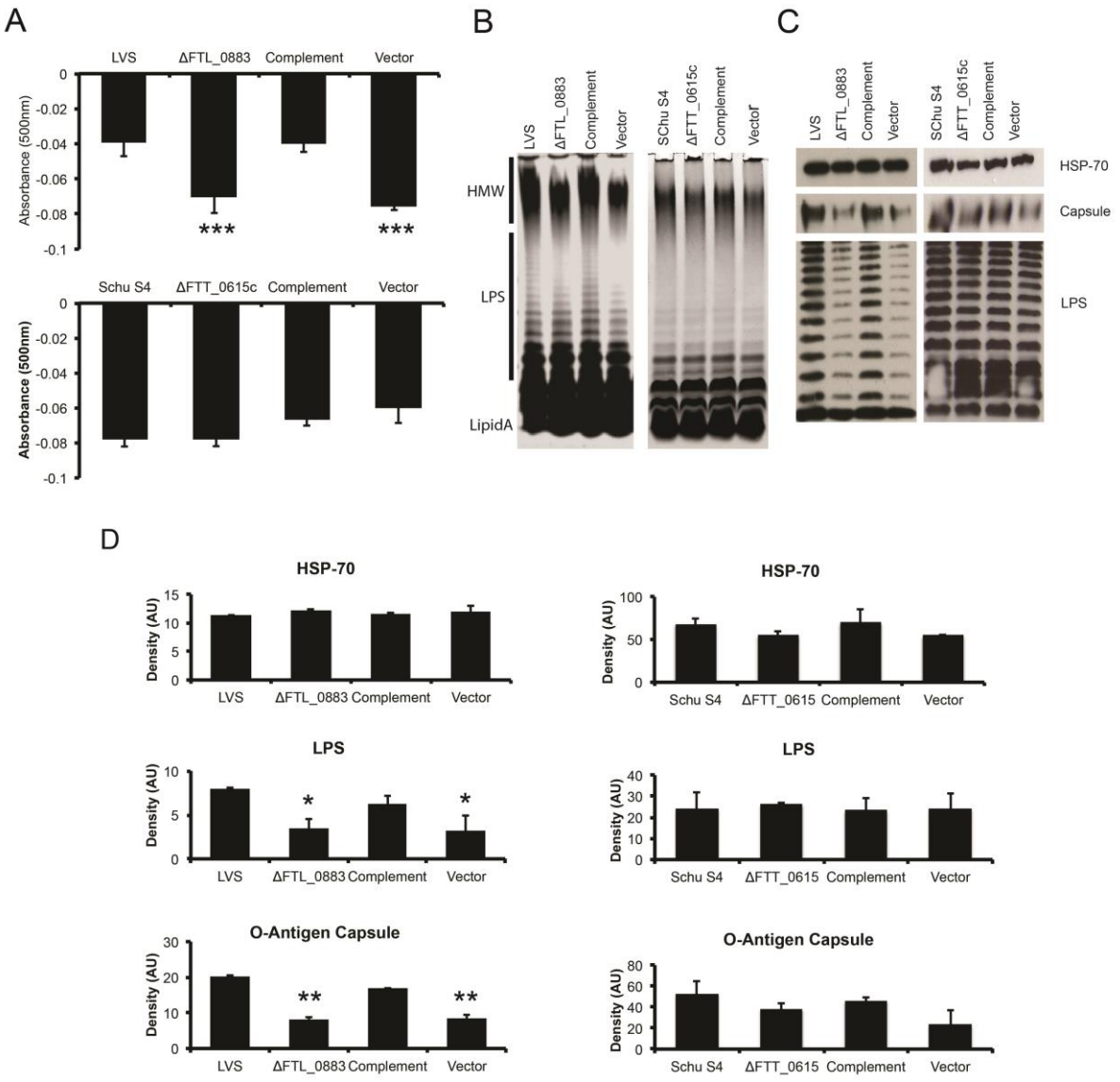

Figure 31: ΔFTL_0883 but not ΔFTT_0615c has a reduction in surface carbohydrates.

A) Bacteria were cultured overnight in TSB-c, and stained with congo red. The data presented is representative of three independent experiments for LVS strains and two independent experiments for Schu S4 strains B) Bacteria were cultured overnight, proteinase K treated, the remaining carbohydrates were separated by SDS-PAGE and visualized with Pro-Q Emerald Green. The data is representative of at least two independent experiments for each strain. C) Bacteria were cultured overnight in TSB-c; the presence of LPS and O-antigen capsule was assessed by western blot. The data is representative of three independent experiments for LVS strains and two independent experiments for Schu S4 strains. D) Quantification of the results by western blot. The data displays the means ±

SEM of three independent experiments for LVS strains and the means ± SEM of two independent experiments for Schu S4 strains. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

To extend our observations with the LVS mutant to Schu S4, the amount of extracellular carbohydrate on ΔFTT_0615c was assessed. In contrast to the results with LVS, there was a similar amount of congo red binding for ΔFTT_0615c and Schu S4 (Figure 31A), suggesting that there is a similar amount of carbohydrate on the surface of the mutant and wild-type strains. Using pro-Q emerald to look at all of the carbohydrates in more detail, there was not a difference in the amount of lipid A or the LPS in ΔFTL_0615c and Schu S4 (Figure 31B). There was a slight reduction in the amount of high-molecular weight capsule material by pro-Q emerald staining, but the reduction was not statistically significant (Figure 31B). Again, by western blotting, there was not a difference in the amount of LPS present on the surface of ΔFTT_0615c and Schu S4 (Figure 31C and 31D). Similar to the results with pro-Q emerald, there was a reduction in the amount of O-antigen capsule on ΔFTT_0615c, but the reduction did not achieve significance (Figure 31C and 31D). Collectively, these results suggest that there is a differential role for corC in LVS and Schu S4, in which FTL_0883 is important for the regulation of O-antigen carbohydrates in LVS, but FTT_0615c is dispensable in Schu S4.

3.4.5 Release of bacterial components to stimulate host cells

The enhanced stimulation of host cells early during an *in vitro* infection could result from an altered surface or from releasing more bacterial components. Analysis of *F. tularensis* culture filtrates reveals that there is not a large amount material released from the bacterium, but the components released are known TLR ligands (Konecna et al., 2010). To investigate if there was

a difference in the amount of material released from ΔFTL_0883, overnight culture supernatants were collected. Components present in the bacterial culture supernatants were visualized by western blot using an anti-*Francisella* antibody. LVS and ΔFTL_0883 released both proteins and carbohydrates, but ΔFTL_0883 released more (Figure 32A and 32B). More HSP-70 was released from ΔFTL_0883 than LVS (Figure 32C). In contrast to the amount of HSP-70 released, there was a similar amount in the bacterial lysate of LVS and ΔFTL_0883 (Figure 31C). The culture supernatants were next added to macrophages to determine if they could elicit cytokines. Indeed, there was an enhanced production of TNF-α from macrophages treated with culture supernatants from ΔFTL_0883 compared to LVS (Figure 32D). These results suggest that the release of material from the mutants could account for at least part of the enhanced stimulation of ΔFTL_0883 compared to LVS.

To extend our observations in LVS to the virulent strain of *F. tularensis*, the amount of bacterial components released by ΔFTT_0615c was first assessed. There was an increase in the amount of material released by ΔFTT_0615c compared to wild-type Schu S4 (Figure 32E). However, the enhanced release of components did not complement, which was particularly evident when the stimulation of macrophages by the bacterial culture supernatants was assessed. Macrophages were treated for 24 hours with bacterial culture media from the Schu S4 strains. ΔFTT_0615c culture supernatants stimulated more cytokine production than culture supernatants from Schu S4 (Figure 32F). However, there was not a difference in the amount of TNF-α produced in response to the culture supernatants from ΔFTT_0615c, complement, or vector strains (Figure 32F). These results suggest that the stimulation of macrophages could be partly attributable to the enhanced release of components from ΔFTL_0883 and ΔFTT_0615c.

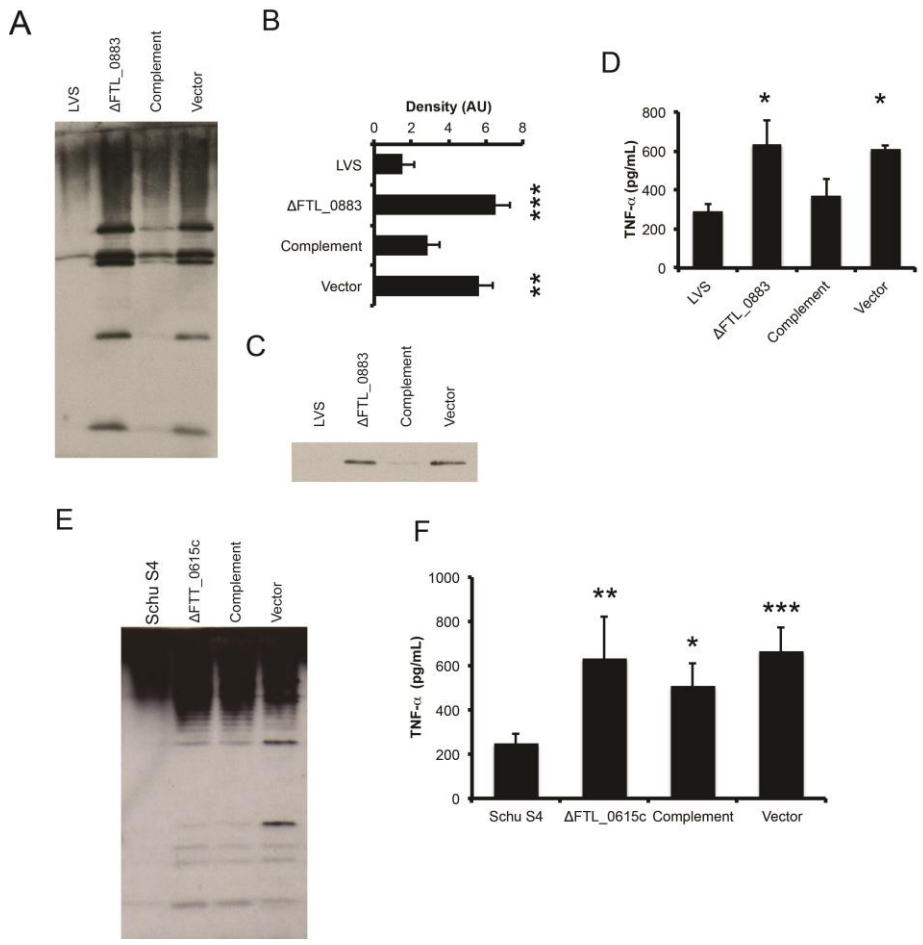

Figure 32: Release of bacterial components from ΔFTL_0883 and ΔFTT_0615c

Bacterial supernatants were collected from overnight culture of bacteria grown in TSB-c and normalized by OD_{600}. A) The amount of material released into the supernatant was determined by western blotting with an anti-*Francisella* antibody. B) Densitometry analysis of A. C) Demonstration HSP-70 is released from LVS strains by western blot analysis. D) The bacterial culture supernatants were added to macrophages and incubated for 24 hours. The supernatants from the macrophages were collected and TNF-α concentration was determined by ELISA. E) Release of material from Schu S4 strains was assessed using anti-*Francisella* antibody. F) Schu S4 supernatants were incubated with macrophages for 24 hours and the concentration of TNF-α was quantified by ELISA. * indicates $p<0.05$, ** indicates $p<0.01$, and *** indicates $p<0.001$.

3.4.6 Attenuation *in vivo* not due to increased susceptibility to host effectors

F. tularensis ΔFTL_0883 and ΔFTT_0615c are attenuated *in vivo* (Russo et al., 2011). The attenuation is attributable to TNF-α and IL-1 signaling; however, it is unclear if the attenuation is the result of increased stimulation of these pathways or the result of enhanced sensitivity to the antimicrobial effector mechanisms these pathways activate. Additionally, there was a reduction of carbohydrate on the surface of ΔFTL_0883 (Figure 31), which could enhance the sensitivity of this strain to antimicrobials. Thus, sensitivity of ΔFTL_0883 to a variety of antibiotics and host antibacterial mechanisms was assessed to determine if there was an increased sensitivity compared to LVS. Assessment of the antibiotic sensitivity of ΔFTL_0883 demonstrated that there was not a statistically significant increase in the sensitivity to a variety of antibiotics; including gentamicin, chloramphenicol, streptomycin, ciprofloxacin, moxifloxacin, and vancomycin (Table 1). Importantly, we did not observe a difference in the sensitivity to polymixin B, which is an analog to antimicrobial peptides. Typically only gram-positive bacteria are sensitive to vancomycin, but if there are large lesions in the outer membrane of gram-negative bacteria, they can also become sensitive to vancomycin. A lack of sensitivity to vancomycin demonstrates that the outer membrane is not largely defective in our mutants.

Table 1: Antibiotic susceptibility of LVS and FTL_0883

Antimicrobial	µg/disc	LVS	ΔFTL_0883
Gentamicin	10	27±1	31±1
Streptomycin	10	25±1	28±2
Moxyfloxacin	20	34±4	38±1
Ciprofloxicin	20	38±1	39±2
Chloramphenicol	5	26±2	29±3
Vanocmycin	30	6±0	6±0
Polymixin B	100	6±0	6±0

A lawn was spread onto agar plates from overnight cultures of bacteria grown in TSB-c. The lawn was allowed to dry and three 6mm discs were placed onto each plate containing antibiotics. The bacteria were cultivated for 72 hours at 37°C with 5% CO_2, and the diameter of the zones was measured. The data depicts the results from one experiment and is representative of each antibiotic tested in at least two independent experiments.

The susceptibility of ΔFTL_0883 and ΔFTT_0615c to host defenses was next assessed. By disc diffusion assay, ΔFTL_0883 and ΔFTT_0615c remained resistant to oxidative stress induced by hydrogen peroxide similar to the wild-type strains (Figure 33A). We observed a significant reduction in bacterial burden in the blood of infected animals (Russo et al., 2011). *F. tularensis* is resistant to the effects of serum complement (Raynaud et al., 2007); however, enhanced sensitivity of the mutants to complement could account for the low levels of bacteremia. *F. tularensis* strains were exposed to active complement for one hour, ΔFTL_0883 or ΔFTT_0615c were resistant to 80% guinea pig serum, the highest concentration tested (Figure 33B). In addition, ΔFTL_0883 and ΔFTT_0615c were also resistant to human and mouse serum, suggesting that there is not a species-specific effect (data not shown). The sensitivity of the mutants to the antimicrobial peptide β-defensin 3 was next assessed. This peptide is expressed in the lung in response to TNF-α and IL-1 signaling, and LVS and *F. novicida* are known to be sensitive to its effects (Amer et al., 2010; Han et al., 2008). Consistent with previous reports, LVS was sensitive to the effects of the peptide at a concentration of 100pg/mL (Figure 33C). ΔFTL_0883 and LVS were similarly resistant to this peptide. Interestingly, Schu S4 was resistant to the β-defensin 3, and ΔFTT_0615c was significantly more sensitive to the peptide than Schu S4 (Figure 33C). Finally, the sensitivity of LVS and Schu S4 strains to ethidium bromide was tested. We observed that there was an increased sensitivity of ΔFTL_0883 to

ethidium bromide compared to LVS, but ΔFTT_0615c and Schu S4 were similarly resistant to ethidium bromide (Figure 33D). Collectively, these data suggest that there is altered sensitivity of LVS and Schu S4 strains to antimicrobial agents. However, there are differences to which antimicrobial agents ΔFTL_0883 and ΔFTT_0615c are sensitive.

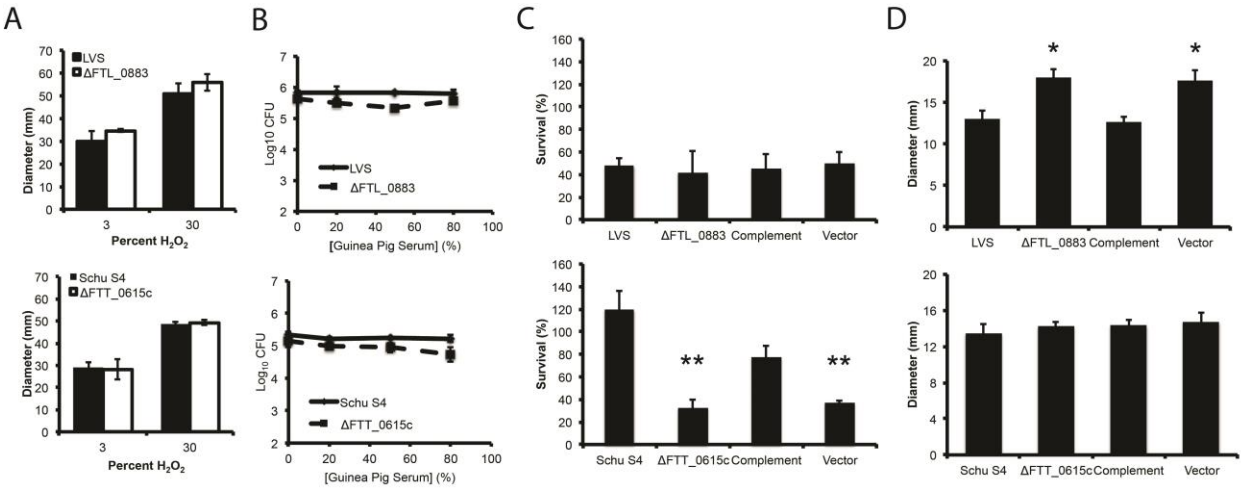

Figure 33: Assessment of sensitivity to host defense mechanisms.

A) LVS and Schu S4 strains were cultured overnight in TSB-c, diluted and a lawn was spread on Mueller Hinton Agar. Discs containing 10uL of either 3% or 30% H_2O_2 were placed on the lawn. The plates were incubated for 72 hours and the diameter of the zone of inhibition was measured. The data presented are representative of three independent experiments. B) LVS and Schu S4 strains were cultured overnight in TSB-c, diluted to approximated 10^5 CFU/mL and incubated for two hours in the presence of guinea pig serum. The bacteria were then diluted and plated for enumeration of CFU. The data presented is representative of 2 independent experiments. C) LVS and Schu S4 strains were cultured in TSB-c, diluted, and incubated with β-defensin 3 for 2 hours. The bacteria were then diluted in TSB-c and plated to enumerate CFU. The data are representative of at least 3 independent experiments for each strain. ** indicates $p < 0.01$. D) LVS and Schu S4 strains were cultured in TSB-c, diluted and a lawn was spread onto Chocolate II Agar plates. Three 6mm discs containing 5ug of ethidium bromide were added to each plate. The plates were incubated for 72 hours and the diameters were measured. The data is representative for at least 3 independent experiments for LVS strains and two independent experiments for Schu S4 strains. * indicates $p < 0.05$

3.5 DISCUSSION

CorC is a ubiquitous, bacterial protein whose function remains unclear. In *F. tularensis*, a homolog of CorC, FTL_0883 and FTT_0165c, was identified to be important for the virulence of the bacterium. Here, an investigation was undertaken to identify the contribution of the CorC protein to the virulence and physiology of *F. tularensis*.

Characterization of CorC in *Salmonella* showed that it was important for the resistance of the bacterium to the toxic effects of cobalt. In this model, CorC was predicted to affect the activity of the CorA magnesium transporter. A loss of *corC* was thought to cause an increase in the intracellular magnesium concentration, which would result in enhanced sensitivity to cobalt (Gibson et al., 1991). However, in *F. tularensis*, the presence of CorC did not affect the resistance of the bacterium to cobalt (Figure 27A). Additionally, there was not a difference in intracellular Mg^{2+} concentration when *corC* was deleted in *F. tularensis* (Figure 27B). These data suggest that there is a unique role for CorC in *F. tularensis* independent of ion homeostasis.

CorC was initially identified as a protein that affected the activity of the magnesium transporter CorA in *S. typhimurium*. However, there is not a CorA homolog present in *F. tularensis*. Therefore, it is plausible that the phenotypic differences that exist between *S. typhimurium* and *F. tularensis corC* mutants could be attributable to the missing CorA transport system in *F. tularensis*. Thus, *F. tularensis* makes a good model to study the activities of this protein independent of the CorA transport system. Further studies will need to occur to identify other protein-protein interactions that contribute to the activities of the protein encoded by FTL_0883 and FTT_0615c. It would be important to perform these studies in parallel in both LVS and Schu S4 as there are phenotypic differences in the expression of carbohydrates when the homolog is deleted in the two *F. tularensis* strains (Figure 31). It remains undetermined if

this is a result of different protein interactions within the strains or if there is a differential regulation of downstream proteins in the corC pathway(s).

In *F. tularensis*, the attenuated virulence of strains lacking *corC* can be attributed at least in part to the enhanced stimulation of the host cells by the *F. tularensis* mutants leading to a more adequate immune response. A more robust host response could be due to enhanced stimulation of the host cells or arise from a failure of the bacterium to suppress the host cell pro-inflammatory response. It was determined that ΔFTL_0883 was capable of actively suppressing host cells (Figure 28). Demonstrating that the mechanism for stimulation was a result of enhanced stimulation and not due to a failure to suppress the host cells.

To determine how the bacteria was altered to activate a more robust host response to the ΔFTL_0883/ ΔFTT_0615c mutants, the bacteria were visualized by TEM microscopy. There was not a gross difference in the surface of the *F. tularensis* mutants that could be detected, but there was a difference in the size of the mutants. Bacterial size is an important attribute for the host cell–pathogen interaction (Justice et al., 2008). Small bacteria are more resistant to phagocytosis, and similarly larger bacteria are also resistant to phagocytosis (Doshi and Mitragotri, 2010). However, the change in the size didn't affect the rate of phagocytosis of the mutants (Chapter 2, Figure 12). Alternatively, an increase in size could also allow for a larger platform for assembly of host receptors on the pathogen surface increasing the avidity of the interaction and amplifying the pro-inflammatory response. The results suggest that an increase in the size of our mutant could provide an explanation for the increased pro-inflammatory signaling.

Further phenotypic classification of the surface of the bacterium revealed that there was not a difference in the lipoprotein expression in the outer membrane fraction of the *F. tularensis*

FTL_0883 mutant, but there was a reduction in the amount of surface carbohydrate expression in this strain (Figure 31). The reduced carbohydrate could allow for better access to PAMPs on the bacterial surface allowing for greater stimulation. This process has been observed in the adaption of *F. tularensis* to the host environment (Zarrella et al., 2011). However, there was not a difference in the amount of surface carbohydrates in ΔFTT_0615c or wild-type Schu S4 (Figure 31). These data suggest that a reduction in carbohydrate on the surface of the bacterium is unlikely to be contributing to the differences that are observed in the pro-inflammatory response to the mutant. The differences in the expression of carbohydrate on the surface of the LVS and Schu S4 mutant also highlights that Schu S4 has a different mechanism to regulate the surface carbohydrate than LVS, which could contribute to the enhanced virulence of the strains.

There was an increased release of stimulatory material including HSP-70 from both ΔFTL_0883 and ΔFTT_0615c, however, this phenotype was only complemented in the LVS strain (Figure 32). While, this suggested that there could be some contribution of the released components to the stimulation of host cells, it is unlikely that this is the sole contributor as the complement of both strains is significantly less stimulatory to host macrophages than either the mutant or vector control strains. It is interesting to speculate that the enhanced release of bacterial components suggests that there is a defective membrane. A destabilization in the membrane integrity could explain why there is an enhanced sensitivity of ΔFTT_0615c to the antimicrobial peptide β-defensin 3 (Figure 33). However, unlike the release of bacterial components, the sensitivity to β-defensin 3 did complement. Additionally, there was not a difference in the sensitivity of the LVS mutant to β-defensin 3, but this could be simply due to the fact that LVS is already rather sensitive to this antimicrobial peptide. Thus, it is unlikely that the release of pro-inflammatory molecules from the either ΔFTL_0883 or ΔFTT_0615c is

significantly contributing to the stimulation of host cells by these mutants, but it could indicate that there is an altered membrane stability in these strains.

In conclusion, there is a novel role for the *corC* homolog, FTL_0883/FTT_0615c, in *Francisella tularensis*, which is independent of ion homeostasis. There was not a significant increase in the sensitivity of FTL_0883 to antimicrobials, but there was an increase in the sensitivity of FTT_0615c to β-defensin 3. The enhanced stimulation of host cells by ΔFTL_0883 and ΔFTT_0615c was not a result of a loss of active suppression; rather, these mutants are more stimulatory. Further work is needed to identify the nature of how this stimulation arises.

4.0 ROLE OF THE INNATE IMMUNE RESPONSE DURING THE PATHOGENESIS OF *FRANCISELLA TULARENSIS*

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Russo BC, Brown MJ, Nau GJ (2013) MyD88 prolongs survival and reduces bacterial burden during pulmonary infection with virulent *Francisella tularensis.* The American Journal of Pathology

BC Russo designed the scope of the study, performed experiments, analyzed data, and prepared the manuscript. MJ Brown performed experiments. GJ Nau prepared the manuscript.

4.1 ABSTRACT

Francisella tularensis is the causative agent of the debilitating, febrile illness tularemia. The severe morbidity associated with *F. tularensis* infections is attributed to its ability to evade the host immune response. Innate immune activation is undetectable until more than 48 hours after infection. The ensuing inflammatory response is considered pathologic, eliciting a septic like state characterized by hypercytokinemia and cell death. To investigate potential pathological consequences of the innate immune response, mice deficient in a key innate immune signaling

molecule, MyD88, were studied. MyD88 knock-out (KO) mice were infected with the prototypical virulent *F. tularensis* strain, Schu S4. MyD88 KO mice succumbed to infection more rapidly than WT mice, the first KO mouse described to do so. The enhanced pathogenicity of Schu S4 in MyD88 KO mice was associated with greater bacterial burdens in lungs and distal organs, and the absence of IFN-γ in the lungs, spleens, and sera. Cellular infiltrates were not observed on histologic evaluation of the lungs, livers, or spleens of MyD88 KO mice. Despite the absence of cellular infiltration, there was more cell death in the lungs of MyD88 KO mice. Thus, the host pro-inflammatory response is beneficial, and MyD88 signaling is required to limit bacterial burden and prolong survival during pulmonary infection by virulent *F. tularensis*.

4.2 INTRODUCTION

Inhalation of *Francisella tularensis* results in pneumonic tularemia, which is the most severe form of disease and associated with mortality rates up to 30-60% among untreated individuals (Dennis et al., 2001; McLendon et al., 2006). Additionally, its low infectious dose of <10 colony-forming units (CFU) and high morbidity have lead to its incorporation into previous governmental bioweapons programs and prompted the Centers for Disease Control and Prevention to list *F. tularensis* as a Tier 1 select agent (Dennis et al., 2001; McLendon et al., 2006; Pohanka and Skladal, 2009). There are two clinically relevant species of *F. tularensis*, *F. tularensis* subsp. *tularensis (*Type A) and *F. tularensis* subsp. *holarctica* (Type B) with the former being the most infectious and causing the most severe form of disease (Petersen and Molins, 2010). Due to the restrictive biocontainment needed to work with these strains,

attenuated strains such as *F. tularensis* subsp. *holarctica* Live Vaccine Strain (LVS) are often used to model the more virulent strains of *F. tularensis.*

F. tularensis is considered to be a "stealth pathogen" due to its ability to evade immune detection (Sjostedt, 2006). Following pulmonary exposure, *F. tularensis* replicates within macrophages and dendritic cells while simultaneously suppressing their activation and thereby limiting the production of pro-inflammatory cytokines (Bosio et al., 2007; Carlson et al., 2007; Hall et al., 2008; Telepnev et al., 2003). Robust bacterial replication and dissemination to distal organs occurs during the early stages of infection while there is not a detectable host response (Cowley, 2009). The lack of an innate immune response early during infection is important for the extreme virulence of *F. tularensis*. Administration of nontypeable *Haemophilus influenzae* (NTHi) or acai berry polysaccharide prior to infection with *F. tularensis* activates the innate immune response and prolongs survival of mice (Evans et al., 2010; Skyberg et al., 2012). Additionally, mutants of *F. tularensis* that stimulate the host immune response more robustly are less virulent in a pulmonary model of tularemia (Russo et al., 2011). Taken together, *F. tularensis* is able to evade the host immune response for at least 48 hours after infection. However, early induction of the host response decreases the morbidity of mice infected with *F. tularensis*, suggesting that the bacterium is susceptible to an activated host response.

Cytokines and recruited immune cells are readily detected in the lungs by 72 hours post infection, but it is uncertain if this host response is beneficial or detrimental (Cowley, 2009; Hall et al., 2008; Schmitt et al., 2012). The immune response has been characterized as a septic like state with hypercytokinemia in the organs and blood, bacteremia, and depletion of immune cells (Mares et al., 2008; Mares et al., 2010; Sharma et al., 2011a). To control LVS infection, a clear role has been established for a variety of cell populations, cytokines, and signaling pathways of

the innate immune response, including TNF-α, IFN-γ, TLR2 and MyD88 (Collazo et al., 2006; Leiby et al., 1992). However, direct assessments of neutrophils, natural killer cells, IFN-γ, and TNF-α during primary tularemia with virulent *F. tularensis* strains suggest they do not have a comparable role (Chen et al., 2004b; KuoLee et al., 2011; Schmitt et al., 2013). Production of host matrix metalloprotease 9, which is important for the recruitment of neutrophils, also exacerbates the pathogenesis of *F. tularensis* (Malik et al., 2007). However, the septic like state also occurs in mice infected with attenuated *Francisella* strains at non-lethal doses of bacteria (Schmitt et al., 2012), suggesting that the correlation with death is not causal. There is also a plateau in the bacterial burden subsequent to immune activation, consistent with the host response restricting bacterial growth (Schmitt et al., 2012). Using a convalescent model in which antibiotic administration extends the life of the mice infected with *F. tularensis*, beneficial contributions of IFN-γ, IL-12, T cells, and B cells were established that could not otherwise be observed in a native infection (Crane et al., 2012). Investigations of the convalescent model suggest that there is a role for the immune response in combating virulent strains of *F. tularensis*, which contrasts with previous studies investigating the cytokines and cell populations during *F. tularensis* infection in naïve mice (Chen et al., 2004b). Therefore, additional studies investigating the innate host response against virulent *F. tularensis* are required to clarify the role for this response and to define its contribution to *F. tularensis* pathogenesis.

The innate immune response in conjunction with the epithelial barrier provides the first line of defense against pathogens. The TLR/IL-1R receptor (TIR) domain containing proteins play a crucial role in innate immune responses (Kumar et al., 2009). The TIR family is comprised of Toll Like Receptors (TLRs) and IL-1 family receptors as well as adaptor proteins required for signal transduction from the receptors to induce pro-inflammatory cytokines (Chong

et al., 2012). Myeloid differentiation primary response gene 88 (MyD88) is a TIR domain containing adaptor protein for signaling from IL-1 family receptors and all TLRs except for TLR3, which makes MyD88 an important signaling molecule of the initial host response.(Janssens and Beyaert, 2002). For many bacterial pathogens, the host requires MyD88 signaling to successfully control and clear the infection. However, the importance of MyD88 is specific for each infection model. MyD88 dependent signaling is often required to prolong survival (Biondo et al., 2005; Sukhumavasi et al., 2008; Villamon et al., 2004), for cellular recruitment and inflammation (Power et al., 2004; Skerrett et al., 2004; Sukhumavasi et al., 2008; Villamon et al., 2004), and restriction of bacterial burden (Biondo et al., 2005; Power et al., 2004). In circumstances where MyD88 signaling is required, it is almost universally needed for cytokine and chemokine responses (Biondo et al., 2005; Koedel et al., 2004; Power et al., 2004; Sporri et al., 2006; Sukhumavasi et al., 2008; Villamon et al., 2004). In contrast, there is at least one scenario when MyD88 signaling is actually detrimental to the host response (Weighardt et al., 2002). Thus, while MyD88 may be important generally, the phenotypic requirement for MyD88 is specific for each pathogen.

The near ubiquitous need for MyD88 signaling during the innate host response to microbial infections makes it a good model to explore the contribution of host activation to *F. tularensis* pathogenesis. Microbiological, immunological, and natural history outcomes were assessed by comparing wild-type (WT) and MyD88 KO mice infected with the prototypical type A strain of *F. tularensis*, Schu S4, to identify the possible beneficial and detrimental roles of the innate host response. MyD88 KO mice had reduced survival, greater bacterial burdens, and more cell death in the lungs. Collectively, these data demonstrate a beneficial role for the innate immune response during primary pneumonic tularemia with virulent *F. tularensis.*

4.3 MATERIALS AND METHODS

Bacterial culture:

Francisella tularensis subsp. *tularensis* Schu S4 was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH: (FSC237), NR-643. Schu S4 was cultured on Chocolate II Agar plates at 37°C with 5% CO_2. Overnight cultures were incubated at 37°C at 250rpm using Mueller Hinton Broth supplemented with IsoVitaleX, 0.025% ferric pyrophosphate, and 0.1% glucose.

Animal Infections:

C57BL/6 mice and mice with a MyD88-deficient allele encoding a deletion of the locus' third exon were purchased from Jackson Laboratory or kindly provided by Dr. Timothy Billiar, and they were infected as described previously (Russo et al., 2011). Briefly, approximately 100 colony-forming units (CFU) in 50µL of PBS were administered intratracheally by oropharyngeal instillation. Blood was removed from the mice by cardiac puncture using a heparinized needle and syringe. The lungs were removed and crushed in RPMI supplemented with 10% FBS. The livers and spleens were removed and homogenized in trypticase soy broth supplemented with 0.1% cysteine (TSB-c). To quantify CFU, serial dilutions were performed of the organs and blood in TSB-c and the bacteria were plated on Chocolate II Agar plates. Morbidity of the mice was followed using a clinical score incorporating both the appearance and the activity of the mice. The activity of the mice was determined as follows: 1, mild reduction in activity; 2, moderate reduction in activity; 3, severe reduction in activity. The appearance of the mice was scored as: 1, mild piloerection; 2, moderate piloerection; 3, severe piloerection. The sum of both scores was used with 0 being a healthy mouse and 4 requiring the mouse to be euthanized. Mice

were monitored daily, but once the mice showed signs of illness, they were assessed every 2-8 hours based upon the severity of illness.

Cytokine analysis:

To measure cytokine production, lungs were manually homogenized through a cell strainer in RPMI supplemented with 10% FBS, centrifuged at 21,000x*g* for 3 min filtered and treated with gentamicin (100μg/mL). Plasma was generated from collected blood by centrifugation at 21,000x*g* for 3 min and then treated with gentamicin (100μg/mL) and ciprofloxacin (200μg/mL). After spleens were homogenized for enumeration of CFU, they were centrifuged at 21,000x*g* for 3 min to remove cellular debris. The resulting supernatants were filtered and treated with genatimicin (100μg/mL). Cytokines from the lungs, plasma, and spleens were measured by ELISA following manufacturer's instructions; IFN-γ (Duoset, R&D systems), IL-12 and TNF-α (matched antibody pairs, eBiosciences), IL-6 (BD Biosciences).

Histologic analysis:

After sacrifice of the animal, lungs were inflated with 37.5% formaldehyde for 10 min. and spleens and livers were placed directly into the fixative. The organs were embedded into paraffin blocks and sectioned by the University of Pittsburgh, School of Medicine Histology Core in the Department of Pathology Development Laboratory. The sections were deparaffinized and stained with hematoxylin and eosin (H&E) or immunohistochemistry was performed on the tissues. TUNEL staining was performed following the manufacturer's protocol (Roche).

4.4 RESULTS

4.4.1 MyD88 signaling prolongs survival and delays morbidity

There is robust activation of the innate host immune response during infection with *F. tularensis* after 72 hours, but the impact of the response on pathogenesis remains uncertain.(Sharma et al., 2011a) To investigate the importance of this innate immune response, wild-type C57BL/6 (WT) and MyD88 knock out (KO) mice were administered 100 CFU of virulent *F. tularensis* Schu S4 intratracheally to model pulmonary tularemia. The median time to death (MTD) for WT mice was 5.7 days post infection with 100% of the mice succumbing to infection by day six post infection (Figure 34A). In contrast, the MTD for MyD88 KO mice was 4.7 days post infection with 100% of these mice succumbing to infection by 5.2 days post infection, prior to the MTD of the WT mice (Figure 34A). Thus, there was a one day decrease in the MTD of the MyD88 KO mice demonstrating a beneficial role for MyD88 during pulmonary tularemia with virulent *F. tularensis.*

The morbidity of mice was used as an additional measure to determine the impact of the innate host response. Mice were monitored over the course of the infection using weight loss and a clinical score, which included both the appearance and activity of the mice. The clinical score for WT or MyD88 KO mice did not change for the first three days of the infection. By the fourth day, there was a slight increase in the average clinical score of the WT mice to 0.8 ± 0.2 (Figure 34B). In contrast, there was a significant increase in the morbidity of the MyD88 KO mice by day four post infection resulting in an average clinical score of 2.2 ± 0.3 (Figure 34B). Because of the increased morbidity, 7/9 MyD88 KO mice needed to be removed from the study by day four, but 0/8 WT mice needed to be removed before day five post infection. In contrast

to the clinical score, the weight of MyD88 KO mice did not change (1.7% ± 2.3%) whereas the WT mice lost weight (11% ± 1.7%) by day four post infection compared to their starting weights (Figure 34C). Overall, these results demonstrate that MyD88 signaling delays the onset of clinical symptoms measured by the clinical score and contributes to weight loss.

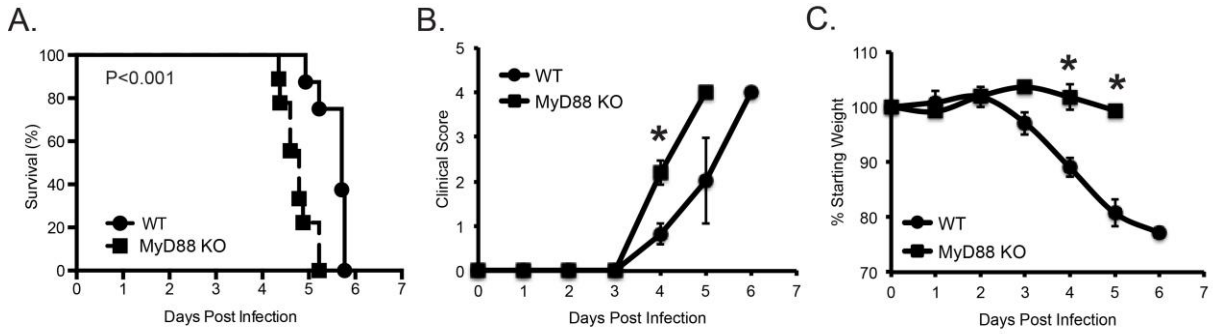

Figure 34: MyD88 signaling prolongs survival during pulmonary tularemia

WT and MyD88 KO mice were infected intratracheally with 100 CFU of Schu S4. Survival of the mice was assessed as described in Methods (A). There are nine mice in the MyD88 KO group and eight mice in the WT group, combined from two independent experiments. Statistical significance was determined using a log rank test. The morbidity of the mice over the course of the infection was monitored using a clinical score (B) and weight loss (C). These data represent the mean ± SEM for two independent experiments. Significance was determined using a two-tailed Students t-test to compare clinical score and weight loss (* represents $p<0.05$).

4.4.2 The presence of MyD88 restricts bacterial replication

Activation of the host immune response correlates with stabilization of bacterial burden within the organs of mice (Schmitt et al., 2012). To assess the impact of MyD88 dependent processes on bacterial burden, WT and MyD88 KO mice were sacrificed two and four days after infection for enumeration of CFU. Two days after infection, there was not a statistical difference in CFU found in the lungs, livers, spleens, or blood of infected MyD88 KO or WT mice (data not

shown). By day four post infection, there was over one log more CFU in all organs of MyD88 KO mice compared to WT mice (Figure 35). The largest difference was observed in the blood, in which MyD88 KO mice contained 3.5 logs more CFU than WT mice (Figure 35). These data demonstrate that the previously described plateau in bacterial burden is the result of the MyD88 dependent immune responses.

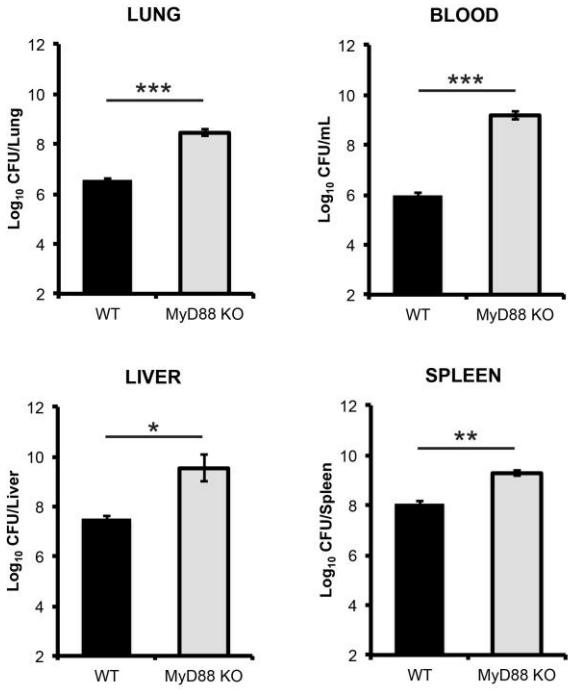

Figure 35: MyD88 dependent processes restrict bacterial burden

On day fourpost infection the lungs, livers, spleens, and blood were harvested from WT and MyD88 KO mice and bacterial burdens were determined. The data are the mean ± SEM for three independent experiments. Significance was determined using a two-tailed Students t-test (* represents $p<0.05$, ** represents $p<0.01$, *** represents $p<0.001$).

4.4.3 MyD88 deficient mice have an impaired cytokine response

Due to the critical importance of MyD88 for initiating and maintaining a pro-inflammatory cytokine response, the production of several cytokines was analyzed in the lungs, sera, and spleens of the infected mice. Cytokine concentrations in MyD88 KO and WT mice treated with PBS were not different (data not shown). Additionally, cytokine production on day two post infection in Schu S4 infected WT or MyD88 KO mice was similar to PBS treated WT and MyD88 KO mice (data not shown), which is consistent with previous observations that the inflammatory response is delayed with a Schu S4 infection (Cowley, 2009; Schmitt et al., 2012; Sharma et al., 2011a). Four days after infection, however, there were statistically significant differences in expression of cytokines between WT and MyD88 KO mice (Figure 36). IFN-γ and IL-12 concentrations were at least 1000-fold higher in the lungs, sera, and spleens of WT mice infected with Schu S4 than in uninfected mice or in MyD88 KO mice infected with Schu S4 (Figure 36). IL-6 was readily measured in the lungs, sera, and spleens of both strains of mice after infection, but levels were still 3-5 fold lower in the MyD88 KO mice (Figure 36). TNF-α was consistently elevated in the sera of WT mice compared to MyD88 KO mice, but there was not a consistent statistical difference in the spleens or lungs of infected WT or MyD88 KO mice (Figure 36). Overall, cytokine concentrations were higher in the organs and serum of infected WT mice than infected MyD88 KO mice. These data correlate the strength of the pro-inflammatory response with an increase in the MTD of the mice and a decrease in the bacterial burden during pulmonary tularemia.

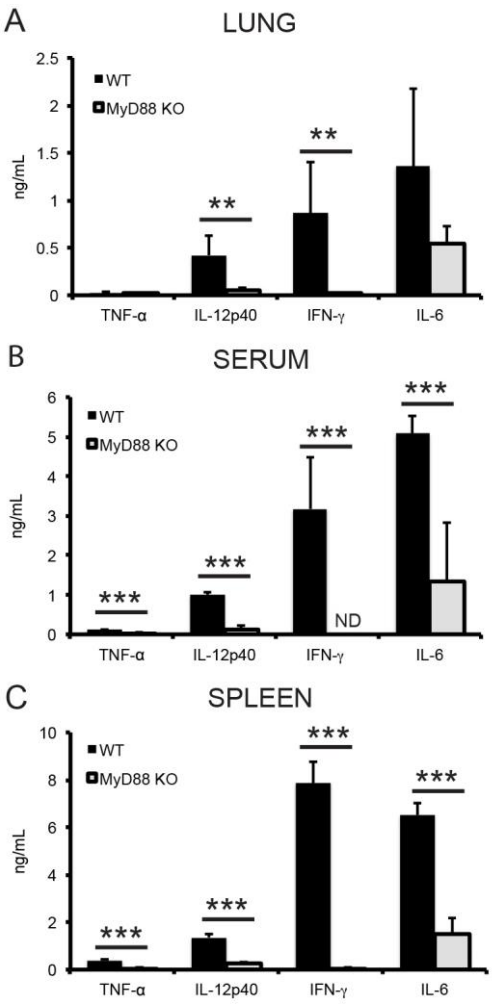

Figure 36: Loss of MyD88 reduces cytokine production in response to *F. tularensis*

The cytokine response was assessed in the lungs (A), sera (B), and spleens (C) of WT and MyD88 KO mice on day four post infection. The data are representative of three independent experiments with four to five mice per group for the lung and plasma, and two independent experiments with three mice per group for the spleens. Significance was determined using a two-tailed Students t-test (** represents $p<0.01$, *** represents $p<0.001$, ND indicates value below detectable limits).

4.4.4 MyD88 is required for the recruitment of inflammatory cells into the lungs after infection

A histopathological hallmark of infection with *Francisella* is the formation of inflammatory foci within the lungs and livers of infected mice (Bokhari et al., 2008; Conlan and North, 1992; Sharma et al., 2011a). To assess the formation of these foci and the recruitment of immune cells into the lungs, livers, and spleens of infected mice, H&E staining was performed on tissue sections collected four days after infection. There was not a difference in the appearance of lungs, spleens, or livers from mice treated with PBS (Figure 37A-F). Robust recruitment of immune cells to the alveolar spaces was observed in the lungs of WT mice infected with *F. tularensis* (Figure 37G), which is in agreement with previous studies (Sharma et al., 2011a). In contrast to the WT mice, MyD88 KO mice did not have cells visible within the alveolar spaces of the lung (Figure 37J). The lack of cells recruited to the alveolar spaces was consistent with the lower cytokine levels measured in these lungs (Figure 36A). Similar to previous observations (Conlan et al., 2003), the spleens of WT mice were pale on gross pathology, and depletion of the red pulp was visible by H&E staining (Figure 37H). Additionally, there was a loss of white pulp organization, in which there was deterioration in the architecture associated with the marginal zone and follicle (Figure 37H). The regions of red pulp in the spleens of infected WT mice showed the presence of white cells (Figure 37H). In contrast, the spleens of the MyD88 KO mice looked normal in color on gross inspection (data not shown). Despite the normal gross appearance, there was a loss of the marginal zones, but the centers of the follicles appeared mostly intact (Figure 37K). Additionally, there did not appear to be white cells present throughout the red pulp as was observed for WT infected mice; rather, there were more intact erythrocytes present in the MyD88 KO mice compared to WT mice (Figure 37K).

Inflammation in the livers of WT mice was consistent with previous reports in which the livers of WT infected mice had small inflammatory foci scattered throughout (Conlan et al., 2003), but MyD88 KO mice did not have similar infiltrates (Figure 37I and 37L). In MyD88 KO mice there were large lesions readily visible throughout the livers of infected MyD88 KO mice (Figure 37L). These lesions were comprised of particles that stained with hematoxylin, consistent with the size of *F. tularensis.* These data suggest that the recruitment of inflammatory cells to organs during infection with *F. tularensis* required MyD88, and without MyD88 there was an altered pathology associated with increased mortality in MyD88 KO mice.

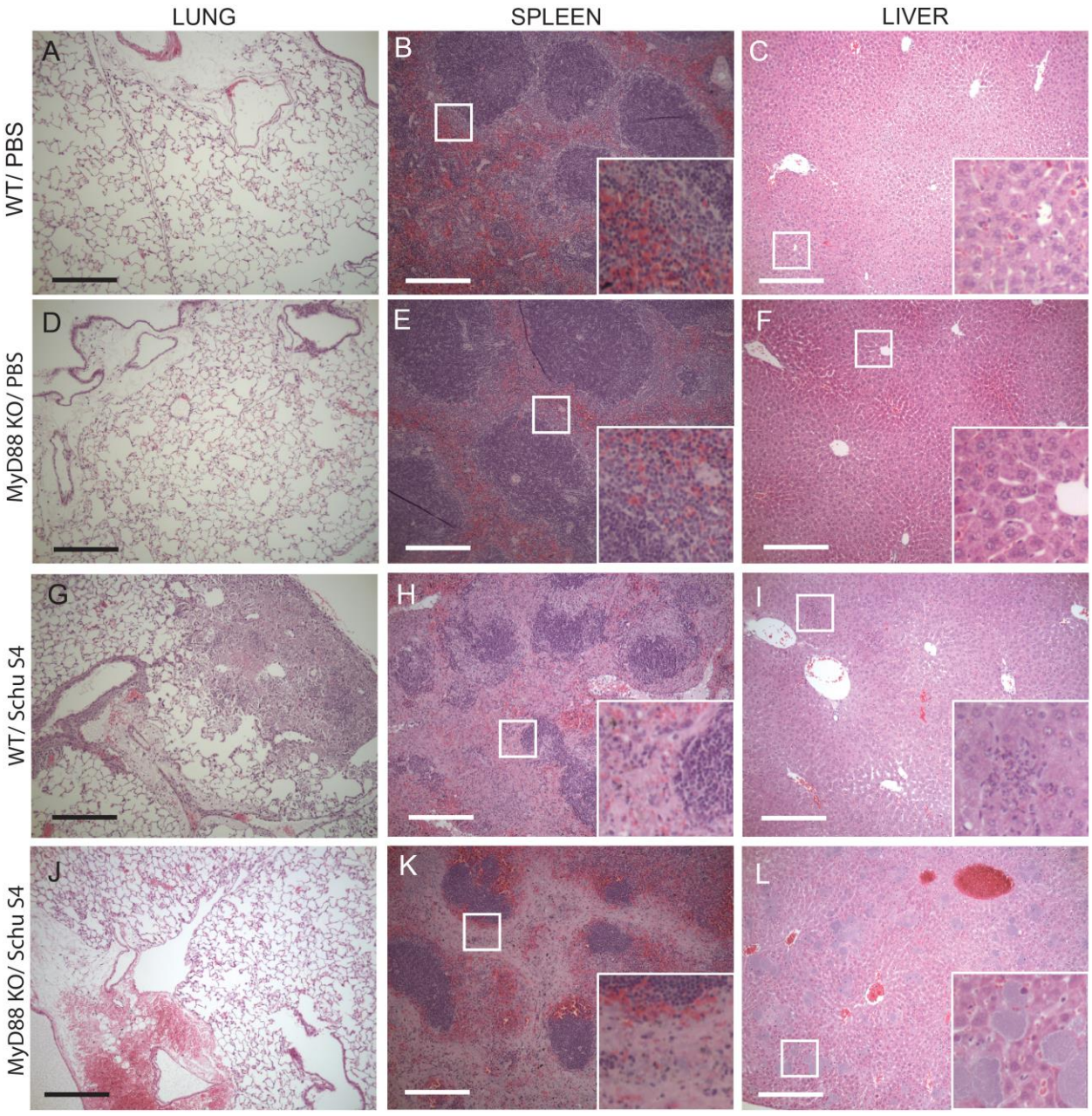

Figure 37: Altered organ pathology in MyD88 KO mice during infection with *F. tularensis*

Lungs (A,D,G, and J), spleens (B, E, H, and K), and livers (C, F, I, and L) were harvested from MyD88 KO and WT mice on day four post infection, fixed in formalin, and embedded in paraffin. Hematoxylin and eosin staining was performed to assess histopathological changes within the organs. The images shown are representative of two lungs, two spleens and two livers for PBS treated mice. Six lungs, five spleens, and five livers were collected over three independent experiments for Schu S4 treated WT and MyD88 KO mice. Scale bars indicate 100µm. Inserts are digital magnifications measuring 50 µm square.

4.4.5 MyD88 signaling reduces cell death in the lung

Within the mouse model of pulmonary tularemia, there is depletion of inflammatory cells particularly NK cells and T cells (Schmitt et al., 2012; Sharma et al., 2011a), but it is unknown if the bacteria cause the cell death directly or if it is a result of bystander effects from the host response. To delineate the contribution of the host response to cell death (Schmitt et al., 2012; Sharma et al., 2011a) and the histopathology seen in MyD88 KO mice (Figure 37), tissue sections from mice infected for four days with Schu S4 were investigated using a TUNEL assay. Similar to previous findings (Sharma et al., 2011a), cell death was observed in the lungs of WT mice infected with *F. tularensis* (Figure 38A-D). Both TUNEL staining and *F. tularensis* were in the same regions of inflammation in the lungs of the WT mice (Figure 38A-D). Unlike WT mice, *F. tularensis* antigen was found throughout the lungs of MyD88 KO mice consistent with the greater bacterial burden (Figure 38E-H). TUNEL positive cells were also broadly present throughout the lungs of MyD88 KO mice, including areas near the large airways and structural components of alveoli (Figure 38E-H). Like WT, the *Francisella* antigen staining was associated with regions of TUNEL positivity, though not necessarily co-localized with one another. (Figure 38D and 38H inserts). Some TUNEL staining in the lungs of both WT and KO mice appeared more diffuse than isolated eukaryotic nuclei (Figure 38D and 38H). This staining pattern has been observed previously and has been associated with cell death (Sharma et al., 2011a).

In contrast to the lungs of MyD88 KO mice, the livers and spleens of MyD88 KO mice showed levels of cell death similar to WT mice after infection. The spleens of WT mice showed TUNEL positivity in areas where there was *F. tularensis* antigen (Figure 38I-L), consistent with previous reports (Sharma et al., 2011a). Similar to the WT mice, TUNEL positive cells also

localized to areas with *F. tularensis* antigen in the spleens of MyD88 KO mice (Figure 38M-P). Scattered TUNEL positive inflammatory cells were found in the livers of WT mice infected with Schu S4 (Figure 38Q-T). *F. tularensis* antigen staining was limited in these mice, and it was localized to areas of inflammation and TUNEL staining (Figure 38Q-T). In contrast, the amorphous lesions seen on H&E staining of MyD88 KO liver sections (Figure 37L) stained intensely with *F. tularensis* antigen (Figure 38U-X). Similar to WT mice, some cells were positive for both TUNEL and *F. tularensis* antigen, but the vast majority of *F. tularensis* staining in the livers of MyD88 KO mice did not co-localize with TUNEL positive cells, including bacteria comprising the amorphous lesions described above (Figure 38U-X). Overall, discrete *Francisella* antigen staining did not co-localize with TUNEL in lungs, spleens, and especially livers, which indicated the TUNEL staining was not from bacterial DNA. Together, these data highlight the organ specific contributions of MyD88 during infection with *F. tularensis*, and demonstrate the presence of MyD88 limits the areas where *F. tularensis* antigen can be found in the lungs and the livers of infected mice. Finally, these data highlight that within the lung, MyD88 has an additional role of limiting cell death associated with *F. tularensis* pathogenesis.

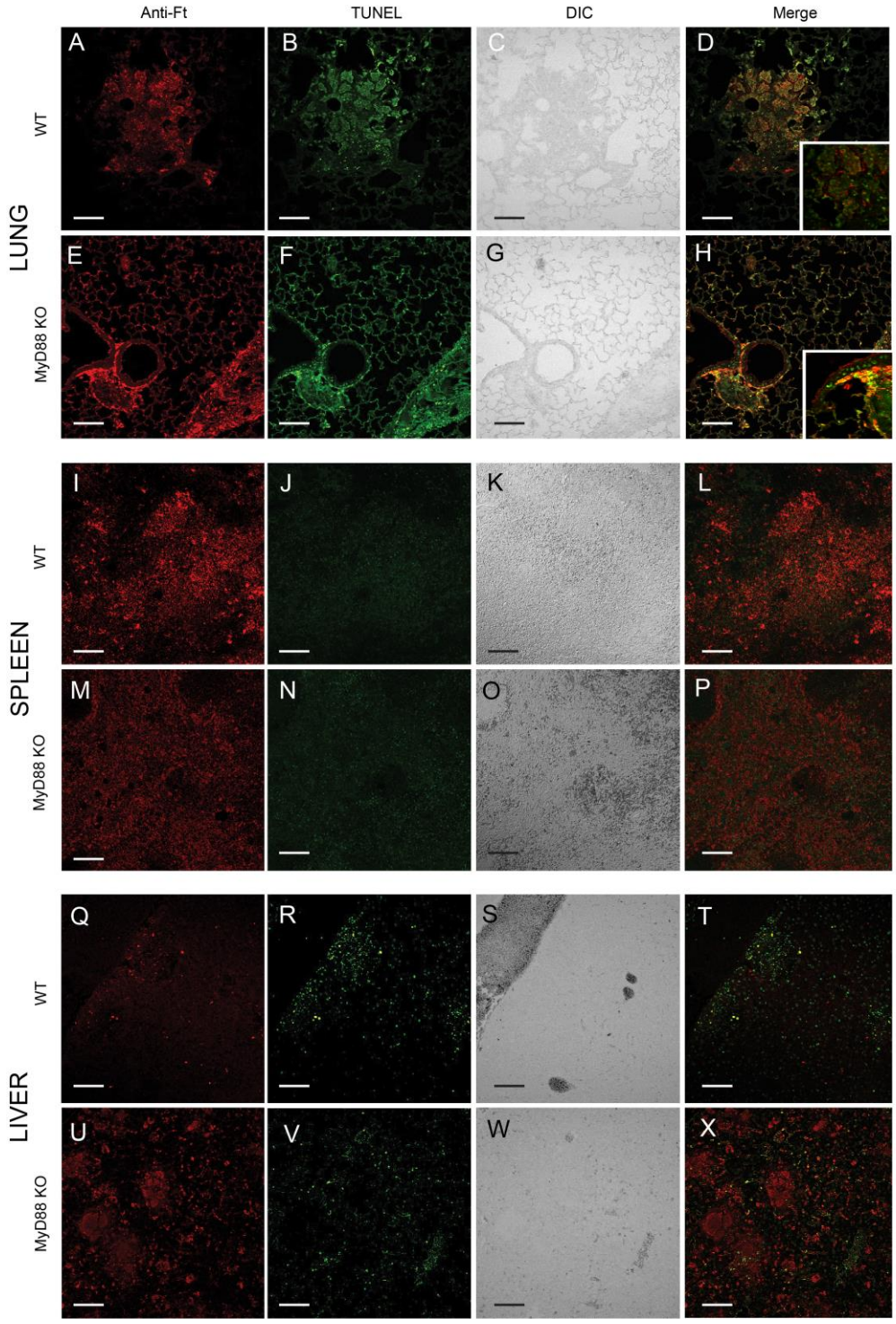

Figure 38: MyD88 restricts cell death in the lung of MyD88 KO mice infected with *F. tularensis*

The lungs (A-H), spleens (I-P) and livers (Q-X) were harvested from MyD88 KO and WT mice on day four post infection and fixed in formalin and paraffin embedded. Immunohistochemistry was performed to identify the presence of *F. tularensis* (red) and cell death by TUNEL staining (green). The images shown are representative of

six lungs, five spleens, and five livers collected over three independent experiments. Scale bars indicate 100μm. Inserts in panels D and H are digital magnification measuring 125μm square.

4.5 DISCUSSION

Francisella tularensis is a formidable pathogen owing to its ability to infect a diverse host range and evade the immune response (Cowley and Elkins, 2011). Immunological studies using the attenuated strains of *F. tularensis* recapitulate the organ pathology observed with virulent strains suggesting they are appropriate models (Bosio, 2011; Conlan et al., 2011; Cowley and Elkins, 2011). The induction of pulmonary tularemia in mice using LVS has revealed TNF-α, IFN-γ, NK cells and neutrophils are required for early control of LVS, but clearance and resolution of the infection requires the presence of either CD4 or CD8 T cells (Bosio, 2011; Cowley and Elkins, 2011). These host defenses, however, have not translated to the virulent Type A or B strains of *F. tularensis*. SCID, B cell KO, IFNγ KO, NK cell depleted, and neutrophil depleted mice infected with virulent strains of *F. tularensis* do not have altered survival compared to WT controls (Chen et al., 2004b; KuoLee et al., 2011). These studies have lead to the conclusion that the innate immune response does not contribute to the control of virulent *F. tularensis* (Chen et al., 2004b). However, recent evidence demonstrates that following immune activation and immune cell infiltration there is a plateau of bacterial burden in the lung, liver, and spleen suggesting an immune response curtails bacterial replication (Schmitt et al., 2012). Here, we demonstrate for the first time using virulent *F. tularensis* in a murine model of pulmonary tularemia that early host responses depend on MyD88 to limit bacterial replication and prolong survival of infected mice.

Phenotypes associated with a loss of MyD88 are not apparent until the innate immune response can be detected experimentally. Recent investigations of the immune response to *F. tularensis* have demonstrated that there are not cytokines or recruited cells detected until three days post infection. Although a host response could function below the limits of detection of standard assays, assessment of *F. tularensis* infection two days after infection in MyD88 KO and WT mice did not reveal differences in CFU, cytokines, or lung pathology (data not shown). Therefore, any response that might be occurring below the limits of detection did not have an impact on CFU. These data are consistent with previous publications demonstrating that the immune response is suppressed until at least 48 hours post infection (Cowley, 2009; Schmitt et al., 2012; Sharma et al., 2011a). The similarities between WT and MyD88 KO mice on day two post infection substantiate the conclusion that *F. tularensis* is a stealth pathogen until days after infection.

Weight loss can be an indicator of morbidity during infection (Russo et al., 2011), but results reported here suggest it may be an unreliable measure. MyD88 KO mice maintained their weight over the course of infection, despite significant bacterial burdens. In contrast, WT mice lost >20% of their body weight on average (Figure 34C). These results suggest that the weight loss observed during infection with *F. tularensis* is dependent on the presence of MyD88 and is likely a result of the greater inflammatory response observed during infection of WT mice with *F. tularensis.* These results are in agreement with a previous report in which WT mice infected with *Clostridium difficile* lost more weight than MyD88 KO mice (Jarchum et al., 2012). In patients with HIV, weight loss is also correlated with activation of the inflammatory response (Rimaniol et al., 1996). Finally, LPS induced anorexia is dependent on MyD88 signaling from myeloid cells (Ruud et al., 2013). Thus, weight loss as a clinical indicator of disease during

tularemia is associated with the inflammatory response itself rather than direct effects of *F. tularensis.*

Immunological mechanisms responsible for cellular recruitment and the role of these cells during virulent *F. tularensis* infection are not completely understood. Inflammation develops in the lung and liver commensurate with immune activation, and is typically detectable by 72 hours post infection (Bokhari et al., 2008; Conlan et al., 2003; Schmitt et al., 2012; Sharma et al., 2011a). However, methodological differences such as, administering a lower dose of a different type A strain by aerosol, could affect the amount of pulmonary inflammation observed (Conlan et al., 2003). Matrix metalloprotease-9 (MMP-9) KO mice infected with LVS have reduced neutrophil recruitment in the lung compared to WT controls (Malik et al., 2007). In the same study, MMP-9 deficient mice were less susceptible to infection with either LVS or Schu S4 (Malik et al., 2007), demonstrating that neutrophil recruitment could be detrimental during infection with both attenuated and virulent *F. tularensis* strains. IFN-γ mediates formation of focal inflammation in the livers of mice infected with LVS (Bokhari et al., 2008). However, opposing results have been obtained with virulent *F. tularensis*. Different studies have concluded that IFN-γ does (Chen et al., 2004b) and does not (Wickstrum et al., 2009) contribute to focal hepatic inflammation. In our experiments, focal inflammation was consistently present in the lungs and livers of WT mice but not in MyD88 KO mice four days after infection (Figure 37). These data demonstrate that MyD88 signaling is necessary for the recruitment of inflammatory cells to the lungs and livers of mice infected with virulent *F. tularensis*. In contrast, focal inflammation was observed in the livers of MyD88 KO mice infected with the attenuated LVS strain, suggesting different immunological mechanisms contribute to the host response to these closely related bacteria (Collazo et al., 2006). In conclusion, these studies

indicate that while neutrophils specifically may be detrimental, the recruitment of inflammatory cells overall is beneficial, and may be causal to controlling replication of virulent *F. tularensis*.

The MyD88 KO mice also provide insight into how the innate immune response restricts the cell range and location of bacterial replication. *F. tularensis* infections typically culminate with high bacterial loads in the lungs and distal organs. There is a plateau in the bacterial burden coinciding with activation of the host response (Schmitt et al., 2012), but the reason for the limit in bacterial replication could be from depleting the replicative niche or activities of the immune system. In MyD88 KO mice there were significantly higher bacterial burdens reached in the organs, and as high as 10^{10} CFU in the liver (Figure 35). These high bacterial burdens were achieved in the absence of recruited cells (Figure 37), which have been hypothesized to expand the available replicative niche for *F. tularensis* (Hall et al., 2008). Therefore, these data demonstrate that bacterial replication in WT mice is inhibited by activities of the host response rather than depletion of the niche.

The enhanced cell death seen in *F. tularensis* infected MyD88 KO mice indicates the host response is beneficial rather than detrimental. Previous work concluded that over activation and misdirection of the immune response stimulates cell death (Cowley and Elkins, 2011; Mares et al., 2008; Sharma et al., 2011a). Here, however, cell death was the same (spleen and liver) or greater (lung) in MyD88 KO mice compared to WT (Figure 38). While these results do not rule out the possibility of a pathologic role for inflammation later during infection of WT mice, they support the idea that the host response is beneficial. They corroborate findings using the convalescent model of *F. tularensis* in which beneficial contributions of the host response were identified (Crane et al., 2012), but also show that MyD88 signaling actually contributes to host cell survival within the lung.

A definitive mechanism of MyD88 dependent protection during pulmonary tularemia remains to be defined. MyD88 functions as an adaptor for multiple TIR containing receptors including IL-1 family receptors and TLRs (Janssens and Beyaert, 2002). Therefore, MyD88 dependent signaling can drive a variety of host responses. *F. tularensis* is resistant to a variety of host antimicrobials, including oxidative stress, bile salts, and host complement, which suggests that this bacterium must contend with a variety of host defenses (Bina et al., 2008; Gil et al., 2006; Raynaud et al., 2007). Recently, it was found that NADPH oxidase contributes to resistance against *F. tularensis* Schu S4 *in vivo* (KuoLee et al., 2011). MyD88 signaling is important for the assembly of the NADPH oxidase complex (Laroux et al., 2005). In this study, however, the phenotypes associated with a loss of MyD88 were greater than in previous studies looking at the pathogenesis of *F. tularensis* in $gp91^{phox-/-}$ KO mice (KuoLee et al., 2011). The general importance of MyD88 (Janssens and Beyaert, 2002) and the overall suppression of cytokines (Figure 36) suggests that the requirement for MyD88 is likely going to be multifaceted and could provide an explanation for why attempts to target specific cytokines or immune cell populations have been unsuccessful in altering the course of infection with virulent *F. tularensis*.

Despite the beneficial role observed for MyD88 during primary tularemia with virulent *F. tularensis* strains, the innate immune response is still inadequate to combat infection. This is in part due to bacterial effects that restrict MyD88-dependent, pro-inflammatory responses. Monocytes infected with Schu S4 down regulate MyD88 gene expression (Butchar et al., 2008), which would limit the pro-inflammatory response. There is activation of MKP-1 during LVS infection, which restricts the pro-inflammatory response through TLR2 (Medina et al., 2010). Bacterial inhibition of MyD88 would exacerbate infection, accelerate mortality, and could explain in part why the median time to death decreased by only one day in the KO mice.

In conclusion, the work presented here demonstrates for the first time a positive role for the host immune response during a pulmonary infection with virulent *F. tularensis.* The activities of MyD88 signaling are needed for the production of IFN-γ and IL-12p40, WT levels of IL-6 and TNF, maintaining the survival of host cells, and the recruitment of immune cells to the sites of *F. tularensis* replication. The immunological functions that depend on MyD88 restrict the replication of *F. tularensis* and prolong survival of infected mice. These results highlight a previously unappreciated role for the host response during infection with virulent *F. tularensis*.

5.0 DISCUSSION AND SUMMARY

Francisella tularensis is a gram-negative bacterium of significant importance as a result of its low infectious dose, significant morbidity and mortality, and potential for use in biological weapons (Dennis et al., 2001; Oyston et al., 2004). The bacterium has over 300 unique genes, lacks classical virulence determinants such as secreted toxins, and can be found both intracellularly and extracellularly within its host (Larsson et al., 2005). The interaction of *F. tularensis* with its mammalian host is different from other gram-negative pathogens in that the host immune response is delayed until several days after infection (Cowley, 2009; Greenberger et al., 1995; Yu et al., 2000). This delayed activation is critical for the extreme virulence of *F. tularensis*, and attempts to activate the immune response earlier extend survival during a murine model of tularemia (Evans et al., 2010; Skyberg et al., 2012). Thus, to understand the virulence of *F. tularensis*, it is critical to define the mechanisms the bacteria uses to avoid and delay the host response as well as to determine the role of the host response once activated.

Adaptation to the host environment is a process *F. tularensis* uses to avoid activating the immune response. As with many bacteria, *F. tularensis* is able to respond to environmental conditions, altering the expression of virulence-associated genes (Carlson et al., 2009; Hazlett et al., 2008; Horzempa et al., 2008). When *F. tularensis* responds to the host environment it becomes less stimulatory to host cells, allowing the bacterium to escape immune detection (Carlson et al., 2007; Loegering et al., 2006). One mechanism *F. tularensis* uses to respond to the

intracellular environment is the recognition of exogenous spermine, a host-specific molecule (Carlson et al., 2009). It is still unknown how *F. tularensis* responds to spermine.

In chapter two of this dissertation, a greater understanding of how *F. tularensis* responds to spermine was achieved. Initially, a screen was conducted in LVS which identified *corC* was important for the response to spermine. To further understand the contribution of this gene to the spermine response of *F. tularensis*, deletion mutants were generated in LVS, FTL_0883, and its homolog in Schu S4, FTT_0615c. These mutants confirmed the results of the screen, and demonstrated that *corC* is required in *F. tularensis* for the bacterium's response to exogenous spermine (Figures 6 and 7). Further phenotypic characterization of these mutants demonstrated that the spermine response is associated with transcriptional changes and an increase in the amount of capsule carbohydrate in *F. tularensis* (Figure 7). However, only in Schu S4 did changes in pathogenicity island protein expression correlate with the response to spermine (Figure 7). Collectively, these data are the first to identify a gene required for the spermine response in *F. tularensis*, and they establish that LVS and Schu S4 respond to spermine in different ways.

There are still a number of questions that need to be addressed to completely understand how *F. tularensis* responds to spermine. It is unknown if *F. tularensis* senses spermine at its surface, or if it transports spermine to the bacterial cytoplasm. Additionally, the receptor responsible for sensing spermine needs to be determined either on the bacterial surface or in the cytosol. Spermine can bind to many of the same sites as magnesium (Athwal and Huber, 2002; Taglialatela et al., 1995). CorC contains CBS domains, which bind magnesium and adenine derivatives (Scott et al., 2004). Therefore, it is possible that spermine could interact with the CBS domains in the N-terminus of CorC, but it is unknown if the protein is spatially in the same

location as spermine in *F. tularensis*. Transposon mutagenesis in chapter two was able to identify two genes required for the response to spermine. However, other genes have been identified in *F. tularensis* for adapting to the host environment using other environmental signals, such as the transcriptional regulator MglA (Zarrella et al., 2011). To identify additional genes involved in the spermine response an *F. tularensis* ordered library could be screened using just the secondary screen from chapter two (the primary screen assessed growth in the presence of DCHA, and the secondary screen assessed the elicitation of cytokines after cultivation in the presence of spermine). This approach would only look for mutants that elicit similar amounts of TNF-α from macrophages when cultured in the presence or absence of spermine. Interestingly, the results with the *corC* mutants in LVS and Schu S4 suggest that there is a divergent mechanism for how the bacteria are able to reduce their stimulation (Figure 7). There is little evidence in Schu S4 about the transcriptional changes that occur in response to spermine. However, the initial report on the transcriptional changes associated with a response to spermine noted that some of the genes in Schu S4 and LVS were differentially regulated (Carlson et al., 2009). CorC is not predicted to have a DNA binding domain, so it is unlikely that it is directly altering gene expression in response to spermine. A possible explanation for the differential transcriptional regulation could be due to the transcriptional regulator MglA. MglA regulates different genes in *F. novicida* and LVS (Brotcke et al., 2006; Charity et al., 2007). It could be possible that upon recognition of exogenous spermine, CorC could interact with MglA, altering its activity, which would result in transcriptional changes to drive the response to spermine. Since it is likely that MglA regulates different genes in LVS and Schu S4, if CorC interacts with MglA, different genes would be activated in the two strains resulting in different phenotypes. However, further work is needed to confirm this mechanism is occurring. Thus, the work here

has identified a gene that is important for the response to spermine in LVS and Schu S4, but there is significantly more work needed to fully understand how either of these strains respond to the presence of exogenous spermine.

Additional work in chapter two demonstrates that there is a role for *corC* in the virulence of *F. tularensis*. Prior to this thesis adaptation to the host environment through spermine was hypothesized to contribute to the virulence of the *F. tularensis*, but no direct assessment had been made. The importance of FTL_0883 and FTT_0615c was assessed *in vitro* and *in vivo* in order to establish the contribution of these genes to the virulence of *F. tularensis* and to demonstrate the importance of the spermine response for the pathogenesis of this bacterium. These mutants were more stimulatory to macrophages *in vitro* inducing the activation of NF-κB and the production of TNF-α, IL-6, and IL-1β (Figures 8, 10-11). Similarly, there were elevated levels of these cytokines *in vivo* (Figure 17 and 18). In a murine model of pneumonic tularemia, these mutants were attenuated for virulence (Figure 15). Assessment of the importance of the inflammatory response *in vivo* revealed that MyD88 and TNFR/IL1R were required for control of ΔFTT_0615c (Figure 25), which demonstrated that the attenuation of ΔFTT_0615c was the result of the mutants' elicitation of pro-inflammatory cytokines. These results are consistent with other observations in the *F. tularensis* literature, in which replication within macrophages rather than broth culture enhanced the virulence of LVS (Loegering et al., 2006). These data establish that *corC* is required for the full virulence of *F. tularensis*. Additionally, they further establish the spermine response contributes to the virulence of the bacterium.

A screen in *S. typhimurium* designed to identify mutations that convey cobalt resistance initially discovered *corC*, as member of the cobalt resistance (Cor) family (Gibson et al., 1991). Loss of corC affected the activity of the CorA magnesium transport system in addition to

enhancing the resistance of *S. typhimurium* to cobalt (Gibson et al., 1991). *F. tularensis* lacks a homolog for CorA, suggesting that CorC would not function similarly this bacterium as it does in *S. typhimurium*. In support of this argument, there was not a defect in magnesium ion homeostasis in ΔFTL_0883 and the resistance to cobalt was not increased in either ΔFTL_0883 or ΔFTT_0615c (Figure 27). Thus, chapter 3 of this thesis established that there is a novel role for *corC* in the pathogenesis of *F. tularensis*

Further work is needed to understand the full contribution of this protein to the physiology of *F. tularensis* specifically and bacteria generally. The protein is highly conserved among gram-negative bacteria suggesting that there is some basic bacterial function it is fulfilling. Studies should focus on identifying other proteins that interact with CorC in *F. tularensis*. Due to the divergent phenotypes that have been established in chapters two and three (Figure 7, 12, 31-33), it will be important to look at interacting proteins in Schu S4 and LVS as well as other bacterial genera. It is possible that there are different interacting proteins that contribute to the divergent phenotypes. Antibodies for CorC need to be developed. An antibody will facilitate studying the subcellular localization of CorC as well as perform investigations without tags. While FTL_0883 and FTT_0615c do not have recognized DNA binding domains, a microarray study investigating transcriptional changes with the loss of these genes could be revealing. They may allow for the identification of a pathway that CorC is important for and suggest a functional role for this protein in *F. tularensis.* From the experiments in chapter three (Figure 31), carbohydrate biosynthesis pathways would be an anticipated result for LVS. Work identifying binding partners and a functional role for CorC will provide needed insight into the role of this protein generally in bacterial pathogenesis and specifically for the spermine response and virulence of *F. tularensis*.

Chapters two and four provide a unique perspective of the innate immune response during infection with virulent strains of *F. tularensis.* As stated above, the host response to *F. tularensis* is delayed compared to other pulmonary infections such as *Klebsiella* or *Pseudomonas* (Greenberger et al., 1995; Hall et al., 2008; Schmitt et al., 2012; Ye et al., 2001; Yu et al., 2000). After a delay, the host response is activated, but the consequences of the immune activation for the pathogenesis of *F. tularensis* remain undefined. Evidence in the literature suggests that the host response directly contributes to the pathology associated with the infection (Mares et al., 2008; Sharma et al., 2011a). Further understanding of the host response to *F. tularensis* occurs through studying the pathogenesis of ΔFTT_0615c in chapter two. Interestingly, investigation of the inflammatory cytokine response during infection with Schu S4 and ΔFTT_0615c showed that there were elevated cytokines in the lungs of infected mice by day 4 post infection, but cytokines were only elevated in the serum of Schu S4 infected mice (Figures 17 and 18). Thus, the concentration and localization of cytokine production correlated with the CFU detected in Schu S4 and ΔFTT_0615c infected mice in the lungs and sera (Figure 16-18), which suggested that the host pro-inflammatory response is tailored to the bacterial burden rather than just an out of control septic state. These results also suggest that the cytokines present in the sera of infected mice is not simply the result of spill over from the organs. Finally, there was an enhanced number of inflammatory foci in the liver of ΔFTT_0615c infected mice (Figures 21-22). The increased number of foci correlated with the enhanced host pro-inflammatory cytokine response and the greater containment of ΔFTT_0615c replication. Therefore, the results with our attenuated mutant suggest that greater activation of the host response is beneficial and associated with delayed organ pathology and prolonged survival. These data provide the initial evidence

that the host response to virulent *F. tularensis* could be a controlled response tailored to the level of burden despite its resemblance to a septic state.

Further support for a beneficial role for the host response arises in the literature. Mice treated with acai berry polysaccharide or non-typeable *Haemophilus* extract prior to infection with Schu S4 survive infection longer than their untreated counterparts (Evans et al., 2010; Skyberg et al., 2012). In these studies, the prolonged survival is dependent on activation of the host immune response and production of IFN-γ (Skyberg et al., 2012). Recently, a robust time course assessing bacterial burden during infection with Schu S4, revealed that shortly after immune activation, there is a plateau in the bacterial burden (Schmitt et al., 2012). These results, in conjunction with the observations in chapter two, further the argument that there are likely beneficial contributions of the host response during infection with virulent strains of *F. tularensis*.

Previous work has demonstrated that the host response to virulent *F. tularensis* is likely multifaceted and not dependent on a particular cytokine or cell population. While there is a significant understanding of the host factors required to combat infection with LVS (Cowley and Elkins, 2011), these findings have not translated to the virulent strains of *F. tularensis*. In chapter four of this dissertation, a role for the innate host response to *F. tularensis* was defined. The contribution of the innate host response during primary pneumonic tularemia was studied in mice deficient in MyD88. These mice allow for investigation of *F. tularensis* pathogenesis in the context of a broadly deficient host response, as MyD88 is required for a variety of host responses including; activation of effector mechanisms such as ROS, production of pro-inflammatory cytokines, and recruitment of immune cells (Ichikawa et al., 2012; Janssens and Beyaert, 2002). Importantly, host recognition of *F. tularensis* is predicted to occur through TLR2, which requires

the activity of MyD88 for downstream signaling. A loss of MyD88 was associated with an increase in the bacterial burdens and a decrease in pro-inflammatory cytokine production (Figures 35 and 36). Wild-type mice survive infection with Schu S4 longer than MyD88 KO mice (Figure 34). These results demonstrate that MyD88 specifically and the host response generally play a beneficial role in prolonging survival during infection with *F. tularensis*. Additionally, they confirm the prediction from studies with ΔFTT_0615c and mounting evidence in the literature that the host response has a beneficial role during infection with virulent *F. tularensis*.

Infection with *F. tularensis* is associated with pathologic changes in the lungs and distal organs. Histological assessment of the MyD88 KO mice was performed to determine if the host response was directly responsible for the pathology observed during infection with *F. tularensis* Schu S4. Spleens of WT and MyD88 KO mice were not significantly different, but there was altered pathology in the lungs and livers (Figure 37). In the lung, *F. tularensis* and TUNEL positive cells were found throughout, which was associated with a reduction in the recruitment of immune cells to the alveolar spaces (Figure 37 and 38). In the liver, the immune foci did not form in MyD88 KO mice, and *F. tularensis* antigen was readily detected within hepatocytes and as large extracellular lesions (Figure 37 and 38). Control of the bacterial burden was associated with increased production of pro-inflammatory cytokines and the formation of immune foci in the lungs and liver of wild-type mice (Figures 35-38). Thus, significant impairment of the host response does not alleviate the organ pathology during infection with *F. tularensis*. These studies suggest that the bacteria, rather than the host response, contribute to much of the deterioration of organ appearance.

There are a variety of known immunological regulators of the host response to attenuated *F. tularensis* strains. Studies in this thesis demonstrate the first known signaling protein important for the host response against virulent *F. tularensis,* MyD88. Future work will need to explore which MyD88 dependent signaling pathways are required for the host response. A good starting point would be TLR2, which is required *in vitro* for the production of pro-inflammatory cytokines in response to *F. tularensis* (Rodriguez et al., 2011; Singh et al., 2013). TLR4 KO mice have already been assessed during infection of virulent *F. tularensis,* and there is not a role for this receptor (Chen et al., 2004a). Additional studies should investigate the contribution of the immune foci to controlling *F. tularensis*. There is a limited knowledge of these structures, which is mainly focused on the cell populations within them. There is a lack of understanding of the signaling required for their formation outside of the contribution of MyD88 presented within this dissertation. Finally, IFN-γ is lacking in the MyD88 KO mice infected with Schu S4 (Figure 34). Researchers have suggested that this cytokine is important for the host response to *F. tularensis* (Crane et al., 2012). However, depletion of IFN-γ during infection with *F. tularensis* does not impact disease progression (Chen et al., 2004b). This finding suggests there is either not a role for IFN-γ or the previous studies could not adequately detect a role. MyD88 KO mice provide a model in which immunological components, such as cell populations and cytokines, can be reconstituted to assess their role during infection with virulent *F. tularensis*. Further work is needed to delineate the cell populations and cytokines that depend on MyD88 signaling to contribute to the host response against *F. tularensis.*

In conclusion, the work presented within this dissertation investigated the interaction of *Francisella tularensis* and the host immune response. *corC* was identified to be required for the spermine response by *F. tularensis,* and it is needed for the full virulence of the bacterium.

Further investigation of the role of *corC* in LVS and Schu S4 showed that these strains regulate their virulence differently. Additionally, MyD88 signaling mediates a beneficial host response against virulent *F. tularensis* during primary pneumonic tularemia. Thus, this thesis furthers our understanding of the pathogenesis of *F. tularensis* identifying both how the host mounts a response to the bacterium and demonstrating a mechanism the bacterium uses to subvert the host response.

APPENDIX A

ATTENUATION OF 13B47

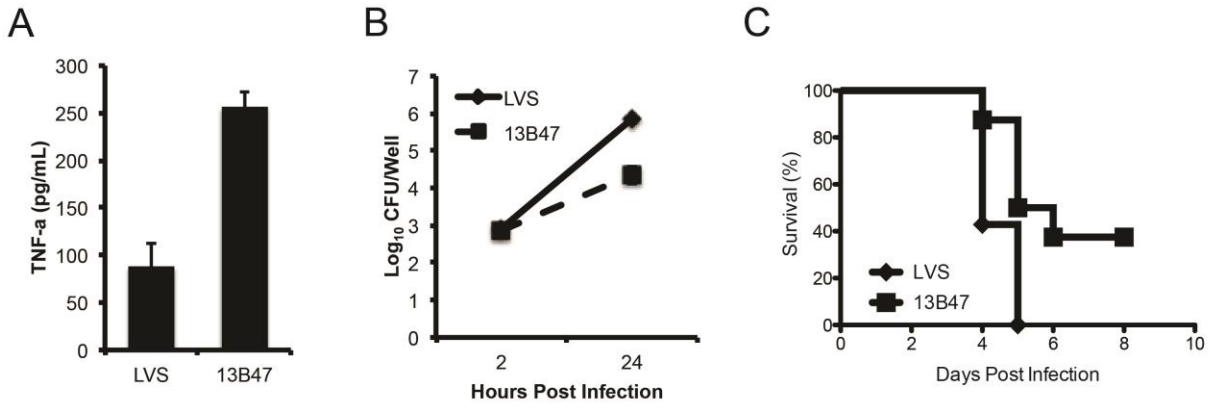

Figure 39: 13B47 is attenuated

A) Macrophages were infected at an MOI of 10 with either LVS or 13B47. After 24 hours of coculture the supernatant was collected. TNF-α concentration released into the culture supernatant was quantified by ELISA. Means ± standard deviation of triplicate wells are depicted, and the data is representative of at least three independent experiments B) Macrophages were infected at an MOI of 500 with either LVS or 13B47. After 2 hours a gentamicin protection assay was performed to kill extracellular bacteria. At the indicated time points, the macrophages were lysed with SDS and the bacteria were serial diluted and plated to enumerate CFU. Means ± standard deviation of triplicate wells are depicted the data is representative of at least three independent experiments C) Chicken embryos were infected with 10^4 CFU of either LVS or 13B47. For LVS n=7 and for 13B47 n=8 . The data shown is representative of at least two similarly performed experiments.

APPENDIX B

PHENOTYPIC CHARACTERIZATION OF WBTA

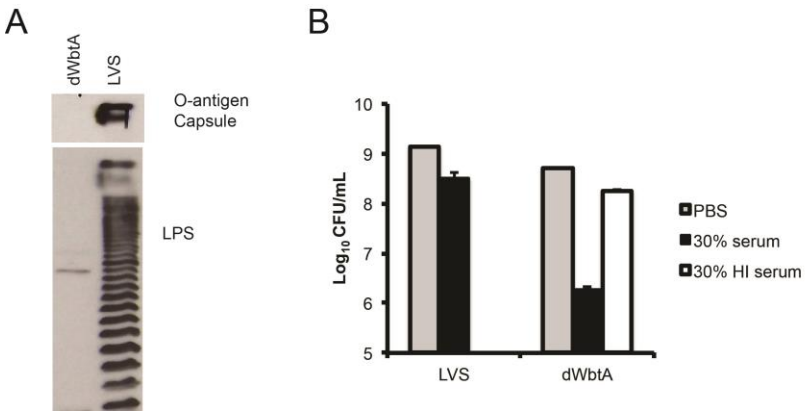

Figure 40: Disruption of WbtA has predicted phenotypes

A) LVS and the WbtA disruption was cultured overnight in TSB-c and bacterial lysates were generated. The lysates was separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-LPS and anti-O-antigen capsule antibodies. B) *F. tularensis* strains were cultured overnight, diluted to equivalent OD_{600} and incubated in the presence of active complement for 2 hours at 37°C at 250rpm. The bacteria were serial diluted and plated to enumerate CFU.

APPENDIX C

PHENOTYPES ASSOCIATED WITH LOSS OF *LNT*

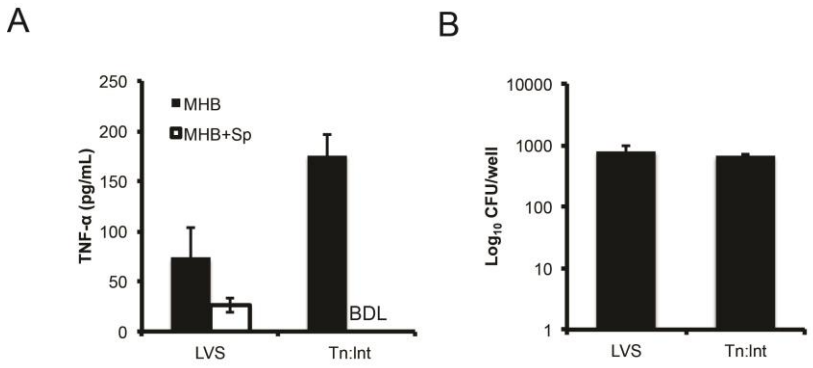

Figure 41: Phenotypes associated with loss of *lnt*

A) LVS strains were cultured overnight in MHB with or without spermine. Human macrophages were cocultured with LVS and Tn:*lnt* for 24 hours at an MOI of 10. The supernatants were collected and the concentration of TNF-α was determined by ELISA. Data depicts the mean± standard deviation of triplicate wells. B) LVS strains were cultured in TSB-c and RAW264.7 cells were infected at an MOI of 500. A gentamicin protection assay was performed 2 hours post infection to kill extracellular bacteria. Macrophages were lysed at 2 and 24 hours post infection to enumerate CFU. The data are presented as the mean fold change in CFU (CFU at 24 hours/ CFU at 2 hours) ± standard deviation of one experiment. BDL indicates a value below the limits of detection of the assay.

APPENDIX D

BACTERIAL GENES

Table 2: Gene names and functions

Gene	Name/Function	Reference
acpA	Acyl carrier protein phosphodiesterase A; acid phosphatase	(Mohapatra et al., 2008)
acpB	Acyl carrier protein phosphodiesterase B; acid phosphatase	(Mohapatra et al., 2008)
acpC	Acyl carrier protein phosphodiesterase C; acid phosphatase	(Mohapatra et al., 2008)
acpH	Acyl carrier protein phosphodiesterase H; acid phosphatase	(Mohapatra et al., 2008)
acrAB	Acridine efflux pump protein A and Protein B, functions to efflux small molecules	(Bina et al., 2008)
clpV	Gene in the FPI; HSP100/ ring forming ATPase, energy for T6SS has ATPase activity.	(Barker et al., 2009; Bingle et al., 2008)
cor	Cobalt resistance gene family	(Gibson et al., 1991)
corA	Cobalt resistance protein A; functions in magnesium import and export	(Hmiel et al., 1986)
corB	Cobalt resistance protein B; affects magnesium efflux	(Gibson et al., 1991)
corC	Cobalt resistance protein C; affects magnesium efflux	(Gibson et al., 1991)
corD	Cobalt resistance protein D; affects magnesium efflux	(Gibson et al., 1991)
deoB	Phosphopentomutase, required for invasion and replication of *F. tularensis*	(Horzempa et al., 2008)
dnaK	Molecular chaperone, member of the HSP70 family	(Bardwell and Craig, 1984)

dotU	Gene in the FPI; stabilized pdpB for T6SS	(Broms et al., 2012)
dsbA	Disulfide bond Protein A, thioredoxin	(Pavkova et al., 2006)
dsbB	Disulfide Bond Protein B, thioredoxin	(Qin et al., 2008)
elongation factor Tu	Elongation factor thermo unstable; functions to move aminocyl-tRNA into free site in ribosome	(Sharma et al., 2011b)
fevR	FTN_0480; required for the expression of FPI genes in conjunction with MglA	(Brotcke and Monack, 2008)
FTL_0500	SpeE, spermidine synthase	(Larsson et al., 2005)
FTL_0681	PotG, polyamine transporter	(Larsson et al., 2005)
FTL_0883	Homolog of CorC in LVS; functions in spermine response and virulence	(Russo et al., 2011)
FTL_1401	Fsr1, *Francisella* spermine response gene 1, hypothetical gene with unknown function	(Carlson et al., 2009)
FTT_0615c	Homolog of corC in Schu S4; functions in spermine response and virulence	(Russo et al., 2011)
FTT_1103	*Francisella* infectivity potentiator protein B; thioredoxin	(Qin et al., 2009)
groE	Molecular chaperone, member of the HSP60 family	(Zeilstra-Ryalls et al., 1991)
hcp	Hybrid cluster protein, hydroxylamine reductase; secreted effector of T6SS	(Bingle et al., 2008)
HSP-70	Heat Shock Protein 70kD, molecular chaperone	
iglA	Intracellular growth locus A; gene in the FPI; forms outer tube of T6SS	(de Bruin et al., 2011; Gray et al., 2002)
iglB	Intracellular growth locus B; gene in the FPI; forms outer tube of T6SS	(de Bruin et al., 2011; Gray et al., 2002)
iglC	Intracellular growth locus C; gene in the FPI; forms inner tube of T6SS	(de Bruin et al., 2011; Gray et al., 2002)
iglD	Intracellular growth locus D; gene in the FPI	(de Bruin et al., 2011; Gray et al., 2002)
katG	Catalase, functions in resistance to ROS and RNS	(Lindgren et al., 2007; Melillo et al., 2010)
lnt	Apoliprotein N-acyltransferase; important for sorting lipoproteins to the outer membrane	(Gupta et al., 1993)

lpxE	Phosphatase; functions to remove the 1-phosphate group from lipid A of *F. tularensis*	(Wang et al., 2004)
lpxF	Phosphotase; functions to remove the 4-phosphate group from lipid A of *F. tularensis*	(Wang et al., 2006)
lpxL	Lauryl-acyl carrier protein (ACP)-dependent acyltransferase; functions in lipid A biosynthesis	(Apicella et al., 2010)
mglA	Homolog to SspA in E. coli; positive regulator of the FPI	(Baron and Nano, 1998)
mglB	Homolog to SspB in E.coli; positive regulator of the FPI	(Baron and Nano, 1998)
mgtA	Magnesium transporter, mediates magnesium influx	(Snavely et al., 1989)
mgtB	Magnesium transporter	(Hmiel et al., 1989)
migR	Macrophage intracellular growth regulator; *Francisella* specific gene, regulates expression of Igl operon in FPI	(Buchan et al., 2009)
mviN	Lipid II flipase	(Ulland et al., 2010)
ompA	Outer membrane protein A; porin	(Haller and Henning, 1974)
ompC	Outer membrane protein C; porin	(Haller and Henning, 1974)
pdpD	Pathogenicity determinant protein D	(Ludu et al., 2008)
pilA	Major type 1 subunit fimbrin (pillin)	(Forslund et al., 2010)
pilE	Minor type 1 subunit fimbrin (pilin)	(Ark and Mann, 2011)
pilV	Adhesin for pili	(Winther-Larsen et al., 2001)
purMCD	Operon involved in purine synthesis	(Pechous et al., 2008)
pyrF	Orotidine-5'-phosphate decarboxylase; involved in pyrimidine synthesis	(Horzempa et al., 2010a)
ripA	Required for intracellular proliferation factor A; *Francisella* specific gene	(Fuller et al., 2008)
rpoH	Functions as an alternative sigma factor in *F. tularensis*	(Grall et al., 2009)
sspA	Stringent Starvation response protein A	(Williams et al., 1994)
sspB	Stringent starvation response protein B	(Williams et al., 1994)

tolC	TolC outer membrane channel, involved in efflux	(Gil et al., 2006; Koronakis et al., 2004)
trkH	TrkH potassium ion transporter	(Alkhuder et al., 2010; Schlosser et al., 1995)
tul4	Outer membrane lipoprotein in *Francisella tularensis*	(Sjostedt et al., 1991)
vgrG	Gene in the FPI; secreted effector of T6SS	(Bingle et al., 2008; Broms et al., 2012)
wbtA	Member of LPS biosynthetic operon; required for the addition of O-antigen onto lipid A	(Raynaud et al., 2007)

BIBLIOGRAPHY

(2012). Department of Health and Human Services, 42 CFR part 73: Possession, Use, and Transfer of Select Agents and Toxins. Federal Registrar *77*, 61083-61115.

Alibek, K., and Handelman, S. (1999). Biohazard : the chilling true story of the largest covert biological weapons program in the world, told from the inside by the man who ran it, 1st edn (New York: Random House).

Alkhuder, K., Meibom, K.L., Dubail, I., Dupuis, M., and Charbit, A. (2010). Identification of *trkH*, encoding a potassium uptake protein required for *Francisella tularensis* systemic dissemination in mice. PloS one *5*, e8966.

Amer, L.S., Bishop, B.M., and van Hoek, M.L. (2010). Antimicrobial and antibiofilm activity of cathelicidins and short, synthetic peptides against *Francisella*. Biochemical and biophysical research communications *396*, 246-251.

Anthony, L.D., Burke, R.D., and Nano, F.E. (1991). Growth of *Francisella* spp. in rodent macrophages. Infection and immunity *59*, 3291-3296.

Apicella, M.A., Post, D.M., Fowler, A.C., Jones, B.D., Rasmussen, J.A., Hunt, J.R., Imagawa, S., Choudhury, B., Inzana, T.J., Maier, T.M., *et al.* (2010). Identification, characterization and immunogenicity of an O-antigen capsular polysaccharide of *Francisella tularensis*. PloS one *5*, e11060.

Aragon, V., Kurtz, S., and Cianciotto, N.P. (2001). *Legionella pneumophila* major acid phosphatase and its role in intracellular infection. Infection and immunity *69*, 177-185.

Ark, N.M., and Mann, B.J. (2011). Impact of *Francisella tularensis* pilin homologs on pilus formation and virulence. Microbial pathogenesis *51*, 110-120.

Asare, R., and Abu Kwaik, Y. (2010). Molecular complexity orchestrates modulation of phagosome biogenesis and escape to the cytosol of macrophages by *Francisella tularensis*. Environmental microbiology *12*, 2559-2586.

Asare, R., and Kwaik, Y.A. (2010). Exploitation of host cell biology and evasion of immunity by *Francisella tularensis*. Frontiers in microbiology *1*, 145.

Ashtekar, A.R., Zhang, P., Katz, J., Deivanayagam, C.C., Rallabhandi, P., Vogel, S.N., and Michalek, S.M. (2008). TLR4-mediated activation of dendritic cells by the heat shock protein DnaK from *Francisella tularensis*. Journal of leukocyte biology *84*, 1434-1446.

Athwal, G.S., and Huber, S.C. (2002). Divalent cations and polyamines bind to loop 8 of 14-3-3 proteins, modulating their interaction with phosphorylated nitrate reductase. The Plant journal : for cell and molecular biology *29*, 119-129.

Bandara, A.B., Champion, A.E., Wang, X., Berg, G., Apicella, M.A., McLendon, M., Azadi, P., Snyder, D.S., and Inzana, T.J. (2011). Isolation and mutagenesis of a capsule-like complex (CLC) from *Francisella tularensis*, and contribution of the CLC to *F. tularensis* virulence in mice. PloS one *6*, e19003.

Bardwell, J.C., and Craig, E.A. (1984). Major heat shock gene of *Drosophila* and the *Escherichia coli* heat-inducible *dnaK* gene are homologous. Proceedings of the National Academy of Sciences of the United States of America *81*, 848-852.

Barel, M., Meibom, K., and Charbit, A. (2010). Nucleolin, a shuttle protein promoting infection of human monocytes by *Francisella tularensis*. PloS one *5*, e14193.

Barker, J.R., Chong, A., Wehrly, T.D., Yu, J.J., Rodriguez, S.A., Liu, J., Celli, J., Arulanandam, B.P., and Klose, K.E. (2009). The *Francisella tularensis* pathogenicity island encodes a secretion system that is required for phagosome escape and virulence. Mol Microbiol *74*, 1459-1470.

Baron, G.S., and Nano, F.E. (1998). MglA and MglB are required for the intramacrophage growth of *Francisella novicida*. Molecular microbiology *29*, 247-259.

Becker, J.V., Mtwisha, L., Crampton, B.G., Stoychev, S., van Brummelen, A.C., Reeksting, S., Louw, A.I., Birkholtz, L.M., and Mancama, D.T. (2010). *Plasmodium falciparum* spermidine synthase inhibition results in unique perturbation-specific effects observed on transcript, protein and metabolite levels. BMC Genomics *11*, 235.

Beeching, N.J., Dance, D.A., Miller, A.R., and Spencer, R.C. (2002). Biological warfare and bioterrorism. BMJ *324*, 336-339.

Bina, X.R., Lavine, C.L., Miller, M.A., and Bina, J.E. (2008). The AcrAB RND efflux system from the live vaccine strain of *Francisella tularensis* is a multiple drug efflux system that is required for virulence in mice. FEMS microbiology letters *279*, 226-233.

Bingle, L.E., Bailey, C.M., and Pallen, M.J. (2008). Type VI secretion: a beginner's guide. Current opinion in microbiology *11*, 3-8.

Biondo, C., Midiri, A., Messina, L., Tomasello, F., Garufi, G., Catania, M.R., Bombaci, M., Beninati, C., Teti, G., and Mancuso, G. (2005). MyD88 and TLR2, but not TLR4, are required for host defense against *Cryptococcus neoformans*. European journal of immunology *35*, 870-878.

Bokhari, S.M., Kim, K.J., Pinson, D.M., Slusser, J., Yeh, H.W., and Parmely, M.J. (2008). NK cells and gamma interferon coordinate the formation and function of hepatic granulomas in mice infected with the *Francisella tularensis* live vaccine strain. Infection and immunity *76*, 1379-1389.

Bolger, C.E., Forestal, C.A., Italo, J.K., Benach, J.L., and Furie, M.B. (2005). The live vaccine strain of *Francisella tularensis* replicates in human and murine macrophages but induces only the human cells to secrete proinflammatory cytokines. Journal of leukocyte biology *77*, 893-897.

Bosio, C.M. (2011). The subversion of the immune system by *Francisella tularensis*. Frontiers in microbiology *2*, 9.

Bosio, C.M., Bielefeldt-Ohmann, H., and Belisle, J.T. (2007). Active suppression of the pulmonary immune response by *Francisella tularensis* Schu4. J Immunol *178*, 4538-4547.

Bosio, C.M., and Dow, S.W. (2005). *Francisella tularensis* induces aberrant activation of pulmonary dendritic cells. J Immunol *175*, 6792-6801.

Broms, J.E., Meyer, L., Lavander, M., Larsson, P., and Sjostedt, A. (2012). DotU and VgrG, core components of type VI secretion systems, are essential for *Francisella* LVS pathogenicity. PloS one *7*, e34639.

Brotcke, A., and Monack, D.M. (2008). Identification of *fevR*, a novel regulator of virulence gene expression in *Francisella novicida*. Infection and immunity *76*, 3473-3480.

Brotcke, A., Weiss, D.S., Kim, C.C., Chain, P., Malfatti, S., Garcia, E., and Monack, D.M. (2006). Identification of MglA-regulated genes reveals novel virulence factors in *Francisella tularensis*. Infection and immunity *74*, 6642-6655.

Buchan, B.W., McCaffrey, R.L., Lindemann, S.R., Allen, L.A., and Jones, B.D. (2009). Identification of *migR*, a regulatory element of the *Francisella tularensis* live vaccine strain *iglABCD* virulence operon required for normal replication and trafficking in macrophages. Infect Immun *77*, 2517-2529.

Butchar, J.P., Cremer, T.J., Clay, C.D., Gavrilin, M.A., Wewers, M.D., Marsh, C.B., Schlesinger, L.S., and Tridandapani, S. (2008). Microarray analysis of human monocytes infected with *Francisella tularensis* identifies new targets of host response subversion. PloS one *3*, e2924.

Carlson, P.E., Jr., Carroll, J.A., O'Dee, D.M., and Nau, G.J. (2007). Modulation of virulence factors in *Francisella tularensis* determines human macrophage responses. Microbial pathogenesis *42*, 204-214.

Carlson, P.E., Jr., Horzempa, J., O'Dee, D.M., Robinson, C.M., Neophytou, P., Labrinidis, A., and Nau, G.J. (2009). Global transcriptional response to spermine, a component of the intramacrophage environment, reveals regulation of *Francisella* gene expression through insertion sequence elements. J Bacteriol *191*, 6855-6864.

Carroll, J.A. (2010). Methods of identifying membrane proteins in spirochetes. Current protocols in microbiology *Chapter 12*, Unit12C 12.

Chakraborty, S., Monfett, M., Maier, T.M., Benach, J.L., Frank, D.W., and Thanassi, D.G. (2008). Type IV pili in *Francisella tularensis*: roles of *pilF* and *pilT* in fiber assembly, host cell adherence, and virulence. Infection and immunity *76*, 2852-2861.

Chamberlain, R.E. (1965). Evaluation of Live Tularemia Vaccine Prepared in a Chemically Defined Medium. Applied microbiology *13*, 232-235.

Chan, Y.Y., and Chua, K.L. (2010). Growth-related changes in intracellular spermidine and its effect on efflux pump expression and quorum sensing in *Burkholderia pseudomallei*. Microbiology *156*, 1144-1154.

Charity, J.C., Costante-Hamm, M.M., Balon, E.L., Boyd, D.H., Rubin, E.J., and Dove, S.L. (2007). Twin RNA polymerase-associated proteins control virulence gene expression in *Francisella tularensis*. PLoS pathogens *3*, e84.

Chase, J.C., Celli, J., and Bosio, C.M. (2009). Direct and indirect impairment of human dendritic cell function by virulent *Francisella tularensis* Schu S4. Infect Immun *77*, 180-195.

Checroun, C., Wehrly, T.D., Fischer, E.R., Hayes, S.F., and Celli, J. (2006). Autophagy-mediated reentry of *Francisella tularensis* into the endocytic compartment after cytoplasmic replication. Proceedings of the National Academy of Sciences of the United States of America *103*, 14578-14583.

Chen, W., KuoLee, R., Shen, H., Busa, M., and Conlan, J.W. (2004a). Toll-like receptor 4 (TLR4) does not confer a resistance advantage on mice against low-dose aerosol infection with virulent type A *Francisella tularensis*. Microbial pathogenesis *37*, 185-191.

Chen, W., KuoLee, R., Shen, H., and Conlan, J.W. (2004b). Susceptibility of immunodeficient mice to aerosol and systemic infection with virulent strains of *Francisella tularensis*. Microbial pathogenesis *36*, 311-318.

Child, R., Wehrly, T.D., Rockx-Brouwer, D., Dorward, D.W., and Celli, J. (2010). Acid phosphatases do not contribute to the pathogenesis of type A *Francisella tularensis*. Infect Immun *78*, 59-67.

Chong, A., and Celli, J. (2010). The *Francisella* intracellular life cycle: toward molecular mechanisms of intracellular survival and proliferation. Front Microbiol *1*, 138.

Chong, A., Wehrly, T.D., Child, R., Hansen, B., Hwang, S., Virgin, H.W., and Celli, J. (2012). Cytosolic clearance of replication-deficient mutants reveals *Francisella tularensis* interactions with the autophagic pathway. Autophagy *8*, 1342-1356.

Chong, A., Wehrly, T.D., Nair, V., Fischer, E.R., Barker, J.R., Klose, K.E., and Celli, J. (2008). The early phagosomal stage of *Francisella tularensis* determines optimal phagosomal escape and *Francisella* pathogenicity island protein expression. Infection and immunity *76*, 5488-5499.

Clague, M.J. (1998). Molecular aspects of the endocytic pathway. The Biochemical journal *336 (Pt 2)*, 271-282.

Clay, C.D., Soni, S., Gunn, J.S., and Schlesinger, L.S. (2008). Evasion of complement-mediated lysis and complement C3 deposition are regulated by *Francisella tularensis* lipopolysaccharide O antigen. J Immunol *181*, 5568-5578.

Clemens, D.L., and Horwitz, M.A. (2007). Uptake and intracellular fate of *Francisella tularensis* in human macrophages. Annals of the New York Academy of Sciences *1105*, 160-186.

Clemens, D.L., Lee, B.Y., and Horwitz, M.A. (2004). Virulent and avirulent strains of *Francisella tularensis* prevent acidification and maturation of their phagosomes and escape into the cytoplasm in human macrophages. Infection and immunity *72*, 3204-3217.

Clemens, D.L., Lee, B.Y., and Horwitz, M.A. (2005). *Francisella tularensis* enters macrophages via a novel process involving pseudopod loops. Infection and immunity *73*, 5892-5902.

Cole, L.E., Shirey, K.A., Barry, E., Santiago, A., Rallabhandi, P., Elkins, K.L., Puche, A.C., Michalek, S.M., and Vogel, S.N. (2007). Toll-like receptor 2-mediated signaling requirements for *Francisella tularensis* live vaccine strain infection of murine macrophages. Infection and immunity *75*, 4127-4137.

Collazo, C.M., Sher, A., Meierovics, A.I., and Elkins, K.L. (2006). Myeloid differentiation factor-88 (MyD88) is essential for control of primary in vivo *Francisella tularensis* LVS infection, but not for control of intra-macrophage bacterial replication. Microbes and infection / Institut Pasteur *8*, 779-790.

Conlan, J.W., Chen, W., Bosio, C.M., Cowley, S.C., and Elkins, K.L. (2011). Infection of mice with *Francisella* as an immunological model. Current protocols in immunology / edited by John E Coligan [et al] *Chapter 19*, Unit 19 14.

Conlan, J.W., Chen, W., Shen, H., Webb, A., and KuoLee, R. (2003). Experimental tularemia in mice challenged by aerosol or intradermally with virulent strains of *Francisella tularensis*: bacteriologic and histopathologic studies. Microbial pathogenesis *34*, 239-248.

Conlan, J.W., and North, R.J. (1992). Early pathogenesis of infection in the liver with the facultative intracellular bacteria *Listeria monocytogenes*, *Francisella tularensis*, and *Salmonella typhimurium* involves lysis of infected hepatocytes by leukocytes. Infection and immunity *60*, 5164-5171.

Conlan, J.W., Shen, H., Golovliov, I., Zingmark, C., Oyston, P.C., Chen, W., House, R.V., and Sjostedt, A. (2010). Differential ability of novel attenuated targeted deletion mutants of *Francisella tularensis* subspecies *tularensis* strain SCHU S4 to protect mice against aerosol challenge with virulent bacteria: effects of host background and route of immunization. Vaccine *28*, 1824-1831.

Conlan, J.W., Sjostedt, A., and North, R.J. (1994). CD4+ and CD8+ T-cell-dependent and -independent host defense mechanisms can operate to control and resolve primary and secondary *Francisella tularensis* LVS infection in mice. Infection and immunity *62*, 5603-5607.

Cowley, S.C. (2009). Editorial: Proinflammatory cytokines in pneumonic tularemia: too much too late? Journal of leukocyte biology *86*, 469-470.

Cowley, S.C., and Elkins, K.L. (2011). Immunity to *Francisella*. Frontiers in microbiology *2*, 26.

Cowley, S.C., Meierovics, A.I., Frelinger, J.A., Iwakura, Y., and Elkins, K.L. (2010). Lung CD4-CD8- double-negative T cells are prominent producers of IL-17A and IFN-gamma during primary respiratory murine infection with *Francisella tularensis* live vaccine strain. J Immunol *184*, 5791-5801.

Cozzone, A.J. (2005). Role of protein phosphorylation on serine/threonine and tyrosine in the virulence of bacterial pathogens. Journal of molecular microbiology and biotechnology *9*, 198-213.

Crane, D.D., Scott, D.P., and Bosio, C.M. (2012). Generation of a convalescent model of virulent *Francisella tularensis* infection for assessment of host requirements for survival of tularemia. PloS one *7*, e33349.

Craven, R.R., Hall, J.D., Fuller, J.R., Taft-Benz, S., and Kawula, T.H. (2008). *Francisella tularensis* invasion of lung epithelial cells. Infection and immunity *76*, 2833-2842.

Cremer, T.J., Amer, A., Tridandapani, S., and Butchar, J.P. (2009a). *Francisella tularensis* regulates autophagy-related host cell signaling pathways. Autophagy *5*, 125-128.

Cremer, T.J., Ravneberg, D.H., Clay, C.D., Piper-Hunter, M.G., Marsh, C.B., Elton, T.S., Gunn, J.S., Amer, A., Kanneganti, T.D., Schlesinger, L.S., *et al.* (2009b). MiR-155 induction by *F. novicida* but not the virulent *F. tularensis* results in SHIP down-regulation and enhanced pro-inflammatory cytokine response. PloS one *4*, e8508.

Dai, S., Mohapatra, N.P., Schlesinger, L.S., and Gunn, J.S. (2012). The acid phosphatase AcpA is secreted in vitro and in macrophages by *Francisella* spp. Infection and immunity *80*, 1088-1097.

de Bruin, O.M., Duplantis, B.N., Ludu, J.S., Hare, R.F., Nix, E.B., Schmerk, C.L., Robb, C.S., Boraston, A.B., Hueffer, K., and Nano, F.E. (2011). The biochemical properties of the *Francisella* pathogenicity island (FPI)-encoded proteins IglA, IglB, IglC, PdpB and DotU suggest roles in type VI secretion. Microbiology *157*, 3483-3491.

Dennis, D.T., Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Friedlander, A.M., Hauer, J., Layton, M., *et al.* (2001). Tularemia as a biological weapon: medical and public health management. JAMA : the Journal of the American Medical Association *285*, 2763-2773.

Doshi, N., and Mitragotri, S. (2010). Macrophages recognize size and shape of their targets. PloS one *5*, e10051.

Duenas, A.I., Aceves, M., Orduna, A., Diaz, R., Sanchez Crespo, M., and Garcia-Rodriguez, C. (2006). *Francisella tularensis* LPS induces the production of cytokines in human monocytes and signals via Toll-like receptor 4 with much lower potency than *E. coli* LPS. International immunology *18*, 785-795.

Edwards, J.A., Rockx-Brouwer, D., Nair, V., and Celli, J. (2010). Restricted cytosolic growth of *Francisella tularensis* subsp. *tularensis* by IFN-gamma activation of macrophages. Microbiology *156*, 327-339.

Eliasson, H., Lindback, J., Nuorti, J.P., Arneborn, M., Giesecke, J., and Tegnell, A. (2002). The 2000 tularemia outbreak: a case-control study of risk factors in disease-endemic and emergent areas, Sweden. Emerging infectious diseases *8*, 956-960.

Elkins, K.L., Colombini, S.M., Krieg, A.M., and De Pascalis, R. (2009). NK cells activated *in vivo* by bacterial DNA control the intracellular growth of *Francisella tularensis* LVS. Microbes Infect *11*, 49-56.

Elkins, K.L., Cowley, S.C., and Bosio, C.M. (2003). Innate and adaptive immune responses to an intracellular bacterium, *Francisella tularensis* live vaccine strain. Microbes Infect *5*, 135-142.

Estevez, R., Pusch, M., Ferrer-Costa, C., Orozco, M., and Jentsch, T.J. (2004). Functional and structural conservation of CBS domains from CLC chloride channels. J Physiol *557*, 363-378.

Evans, M.E., Gregory, D.W., Schaffner, W., and McGee, Z.A. (1985). Tularemia: a 30-year experience with 88 cases. Medicine *64*, 251-269.

Evans, S.E., Scott, B.L., Clement, C.G., Larson, D.T., Kontoyiannis, D., Lewis, R.E., Lasala, P.R., Pawlik, J., Peterson, J.W., Chopra, A.K., *et al.* (2010). Stimulated innate resistance of lung epithelium protects mice broadly against bacteria and fungi. American journal of respiratory cell and molecular biology *42*, 40-50.

Farlow, J., Smith, K.L., Wong, J., Abrams, M., Lytle, M., and Keim, P. (2001). *Francisella tularensis* strain typing using multiple-locus, variable-number tandem repeat analysis. Journal of clinical microbiology *39*, 3186-3192.

Fernandes-Alnemri, T., Yu, J.W., Juliana, C., Solorzano, L., Kang, S., Wu, J., Datta, P., McCormick, M., Huang, L., McDermott, E., *et al.* (2010). The AIM2 inflammasome is critical for innate immunity to *Francisella tularensis*. Nature immunology *11*, 385-393.

Finlay, B.B., and McFadden, G. (2006). Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. Cell *124*, 767-782.

Forestal, C.A., Benach, J.L., Carbonara, C., Italo, J.K., Lisinski, T.J., and Furie, M.B. (2003). *Francisella tularensis* selectively induces proinflammatory changes in endothelial cells. J Immunol *171*, 2563-2570.

Forestal, C.A., Malik, M., Catlett, S.V., Savitt, A.G., Benach, J.L., Sellati, T.J., and Furie, M.B. (2007). *Francisella tularensis* has a significant extracellular phase in infected mice. The Journal of infectious diseases *196*, 134-137.

Forsberg, A., and Guina, T. (2007). Type II secretion and type IV pili of *Francisella*. Annals of the New York Academy of Sciences *1105*, 187-201.

Forslund, A.L., Salomonsson, E.N., Golovliov, I., Kuoppa, K., Michell, S., Titball, R., Oyston, P., Noppa, L., Sjostedt, A., and Forsberg, A. (2010). The type IV pilin, PilA, is required for full virulence of *Francisella tularensis* subspecies *tularensis*. BMC Microbiol *10*, 227.

Forsman, M., Sandstrom, G., and Jaurin, B. (1990). Identification of *Francisella* species and discrimination of type A and type B strains of *F. tularensis* by 16S rRNA analysis. Applied and environmental microbiology *56*, 949-955.

Forsman, M., Sandstrom, G., and Sjostedt, A. (1994). Analysis of 16S ribosomal DNA sequences of *Francisella* strains and utilization for determination of the phylogeny of the genus and for identification of strains by PCR. International journal of systematic bacteriology *44*, 38-46.

Fortier, A.H., Polsinelli, T., Green, S.J., and Nacy, C.A. (1992). Activation of macrophages for destruction of *Francisella tularensis*: identification of cytokines, effector cells, and effector molecules. Infect Immun *60*, 817-825.

Francis, E. (1928). Symptoms, diagnosis and pathology of tularemia. JAMA : the Journal of the American Medical Association *91*, 1155-1161.

Froschauer, E.M., Kolisek, M., Dieterich, F., Schweigel, M., and Schweyen, R.J. (2004). Fluorescence measurements of free [Mg2+] by use of mag-fura 2 in *Salmonella enterica*. FEMS microbiology letters *237*, 49-55.

Fuller, J.R., Craven, R.R., Hall, J.D., Kijek, T.M., Taft-Benz, S., and Kawula, T.H. (2008). RipA, a cytoplasmic membrane protein conserved among *Francisella* species, is required for intracellular survival. Infection and immunity *76*, 4934-4943.

Gavrilin, M.A., Bouakl, I.J., Knatz, N.L., Duncan, M.D., Hall, M.W., Gunn, J.S., and Wewers, M.D. (2006). Internalization and phagosome escape required for *Francisella* to induce human monocyte IL-1beta processing and release. Proceedings of the National Academy of Sciences of the United States of America *103*, 141-146.

Geier, H., and Celli, J. (2011). Phagocytic receptors dictate phagosomal escape and intracellular proliferation of *Francisella tularensis*. Infection and immunity *79*, 2204-2214.

Gibson, M.M., Bagga, D.A., Miller, C.G., and Maguire, M.E. (1991). Magnesium transport in *Salmonella typhimurium*: the influence of new mutations conferring Co2+ resistance on the CorA Mg2+ transport system. Molecular microbiology *5*, 2753-2762.

Gil, H., Benach, J.L., and Thanassi, D.G. (2004). Presence of pili on the surface of *Francisella tularensis*. Infection and immunity *72*, 3042-3047.

Gil, H., Platz, G.J., Forestal, C.A., Monfett, M., Bakshi, C.S., Sellati, T.J., Furie, M.B., Benach, J.L., and Thanassi, D.G. (2006). Deletion of TolC orthologs in *Francisella tularensis* identifies roles in multidrug resistance and virulence. Proceedings of the National Academy of Sciences of the United States of America *103*, 12897-12902.

Golovliov, I., Ericsson, M., Sandstrom, G., Tarnvik, A., and Sjostedt, A. (1997). Identification of proteins of *Francisella tularensis* induced during growth in macrophages and cloning of the gene encoding a prominently induced 23-kilodalton protein. Infection and immunity *65*, 2183-2189.

Golovliov, I., Sjostedt, A., Mokrievich, A., and Pavlov, V. (2003). A method for allelic replacement in *Francisella tularensis*. FEMS microbiology letters *222*, 273-280.

Gophna, U., Barlev, M., Seijffers, R., Oelschlager, T.A., Hacker, J., and Ron, E.Z. (2001). Curli fibers mediate internalization of *Escherichia coli* by eukaryotic cells. Infection and immunity *69*, 2659-2665.

Gordon, S. (2003). Alternative activation of macrophages. Nature reviews Immunology *3*, 23-35.

Grall, N., Livny, J., Waldor, M., Barel, M., Charbit, A., and Meibom, K.L. (2009). Pivotal role of the *Francisella tularensis* heat-shock sigma factor RpoH. Microbiology *155*, 2560-2572.

Gray, C.G., Cowley, S.C., Cheung, K.K., and Nano, F.E. (2002). The identification of five genetic loci of *Francisella novicida* associated with intracellular growth. FEMS microbiology letters *215*, 53-56.

Green, S.J., Nacy, C.A., Schreiber, R.D., Granger, D.L., Crawford, R.M., Meltzer, M.S., and Fortier, A.H. (1993). Neutralization of gamma interferon and tumor necrosis factor alpha blocks in vivo synthesis of nitrogen oxides from L-arginine and protection against *Francisella tularensis* infection in Mycobacterium bovis BCG-treated mice. Infection and immunity *61*, 689-698.

Greenberger, M.J., Strieter, R.M., Kunkel, S.L., Danforth, J.M., Goodman, R.E., and Standiford, T.J. (1995). Neutralization of IL-10 increases survival in a murine model of *Klebsiella pneumonia*. J Immunol *155*, 722-729.

Guina, T., Radulovic, D., Bahrami, A.J., Bolton, D.L., Rohmer, L., Jones-Isaac, K.A., Chen, J., Gallagher, L.A., Gallis, B., Ryu, S., *et al.* (2007). MglA regulates *Francisella tularensis*

subsp. *novicida* (*Francisella* novicida) response to starvation and oxidative stress. Journal of bacteriology *189*, 6580-6586.

Gunn, J.S., and Ernst, R.K. (2007). The structure and function of *Francisella* lipopolysaccharide. Annals of the New York Academy of Sciences *1105*, 202-218.

Gupta, S.D., Gan, K., Schmid, M.B., and Wu, H.C. (1993). Characterization of a temperature-sensitive mutant of *Salmonella typhimurium* defective in apolipoprotein N-acyltransferase. The Journal of biological chemistry *268*, 16551-16556.

Hager, A.J., Bolton, D.L., Pelletier, M.R., Brittnacher, M.J., Gallagher, L.A., Kaul, R., Skerrett, S.J., Miller, S.I., and Guina, T. (2006). Type IV pili-mediated secretion modulates *Francisella* virulence. Molecular microbiology *62*, 227-237.

Hall, J.D., Craven, R.R., Fuller, J.R., Pickles, R.J., and Kawula, T.H. (2007). *Francisella tularensis* replicates within alveolar type II epithelial cells i*n vitro* and *in vivo* following inhalation. Infection and immunity *75*, 1034-1039.

Hall, J.D., Woolard, M.D., Gunn, B.M., Craven, R.R., Taft-Benz, S., Frelinger, J.A., and Kawula, T.H. (2008). Infected-host-cell repertoire and cellular response in the lung following inhalation of *Francisella tularensis* Schu S4, LVS, or U112. Infect Immun *76*, 5843-5852.

Haller, I., and Henning, U. (1974). Cell envelope and shape of *Escherichia coli* K12. Crosslinking with dimethyl imidoesters of the whole cell wall. Proceedings of the National Academy of Sciences of the United States of America *71*, 2018-2021.

Han, S., Bishop, B.M., and van Hoek, M.L. (2008). Antimicrobial activity of human beta-defensins and induction by *Francisella*. Biochemical and biophysical research communications *371*, 670-674.

Hanna, C., and Lyford, J.H. (1971). Tularemia infection of the eye. Annals of ophthalmology *3*, 1321 passim.

Harris, S. (1992). Japanese biological warfare research on humans: a case study of microbiology and ethics. Annals of the New York Academy of Sciences *666*, 21-52.

Hazlett, K.R., Caldon, S.D., McArthur, D.G., Cirillo, K.A., Kirimanjeswara, G.S., Magguilli, M.L., Malik, M., Shah, A., Broderick, S., Golovliov, I., *et al.* (2008). Adaptation of

Francisella tularensis to the mammalian environment is governed by cues which can be mimicked *in vitro*. Infection and immunity *76*, 4479-4488.

Hibasami, H., Tanaka, M., Nagai, J., and Ikeda, T. (1980). Dicyclohexylamine, a potent inhibitor of spermidine synthase in mammalian cells. FEBS Lett *116*, 99-101.

Hmiel, S.P., Snavely, M.D., Florer, J.B., Maguire, M.E., and Miller, C.G. (1989). Magnesium transport in *Salmonella typhimurium:* genetic characterization and cloning of three magnesium transport loci. Journal of bacteriology *171*, 4742-4751.

Hmiel, S.P., Snavely, M.D., Miller, C.G., and Maguire, M.E. (1986). Magnesium transport in *Salmonella typhimurium*: characterization of magnesium influx and cloning of a transport gene. Journal of bacteriology *168*, 1444-1450.

Horzempa, J., Carlson, P.E., Jr., O'Dee, D.M., Shanks, R.M., and Nau, G.J. (2008). Global transcriptional response to mammalian temperature provides new insight into *Francisella tularensis* pathogenesis. BMC microbiology *8*, 172.

Horzempa, J., O'Dee, D.M., Shanks, R.M., and Nau, G.J. (2010a). *Francisella tularensis* DeltapyrF mutants show that replication in nonmacrophages is sufficient for pathogenesis *in vivo*. Infect Immun *78*, 2607-2619.

Horzempa, J., Shanks, R.M., Brown, M.J., Russo, B.C., O'Dee, D.M., and Nau, G.J. (2010b). Utilization of an unstable plasmid and the I-SceI endonuclease to generate routine markerless deletion mutants in *Francisella tularensis*. Journal of microbiological methods *80*, 106-108.

Hrstka, R., Krocova, Z., Cerny, J., Vojtesek, B., Macela, A., and Stulik, J. (2007). *Francisella tularensis* strain LVS resides in MHC II-positive autophagic vacuoles in macrophages. Folia microbiologica *52*, 631-636.

Huber, B., Escudero, R., Busse, H.J., Seibold, E., Scholz, H.C., Anda, P., Kampfer, P., and Splettstoesser, W.D. (2010). Description of *Francisella* hispaniensis sp. nov., isolated from human blood, reclassification of *Francisella* novicida (Larson et al. 1955) Olsufiev et al. 1959 as *Francisella tularensis* subsp. *novicida* comb. *nov.* and emended description of the genus *Francisella*. International journal of systematic and evolutionary microbiology *60*, 1887-1896.

Hunnicutt, D.W., and McBride, M.J. (2001). Cloning and characterization of the *Flavobacterium johnsoniae* gliding motility genes *gldD* and *gldE*. Journal of bacteriology *183*, 4167-4175.

Hunt, D., Wilson, J.E., Weih, K.A., Ishido, S., Harton, J.A., Roche, P.A., and Drake, J.R. (2012). *Francisella tularensis* elicits IL-10 via a PGE(2)-inducible factor, to drive macrophage MARCH1 expression and class II down-regulation. PloS one *7*, e37330.

Ichikawa, S., Miyake, M., Fujii, R., and Konishi, Y. (2012). MyD88 associated ROS generation is crucial for Lactobacillus induced IL-12 production in macrophage. PloS one *7*, e35880.

Ignoul, S., and Eggermont, J. (2005). CBS domains: structure, function, and pathology in human proteins. Am J Physiol Cell Physiol *289*, C1369-1378.

Janssens, S., and Beyaert, R. (2002). A universal role for MyD88 in TLR/IL-1R-mediated signaling. Trends in biochemical sciences *27*, 474-482.

Jarchum, I., Liu, M., Shi, C., Equinda, M., and Pamer, E.G. (2012). Critical role for MyD88-mediated neutrophil recruitment during *Clostridium difficile* colitis. Infection and immunity *80*, 2989-2996.

Johansson, A., Celli, J., Conlan, W., Elkins, K.L., Forsman, M., Keim, P.S., Larsson, P., Manoil, C., Nano, F.E., Petersen, J.M., *et al.* (2010). Objections to the transfer of *Francisella novicida* to the subspecies rank of *Francisella tularensis*. International journal of systematic and evolutionary microbiology *60*, 1717-1718; author reply 1718-1720.

Justice, S.S., Hunstad, D.A., Cegelski, L., and Hultgren, S.J. (2008). Morphological plasticity as a bacterial survival strategy. Nature reviews Microbiology *6*, 162-168.

Kazmierczak, M.J., Wiedmann, M., and Boor, K.J. (2005). Alternative sigma factors and their roles in bacterial virulence. Microbiology and molecular biology reviews : MMBR *69*, 527-543.

Keim, P., Johansson, A., and Wagner, D.M. (2007). Molecular epidemiology, evolution, and ecology of *Francisella*. Annals of the New York Academy of Sciences *1105*, 30-66.

Kieffer, T.L., Cowley, S., Nano, F.E., and Elkins, K.L. (2003). *Francisella novicida* LPS has greater immunobiological activity in mice than *F. tularensis* LPS, and contributes to *F. novicida* murine pathogenesis. Microbes and infection / Institut Pasteur *5*, 397-403.

Koedel, U., Rupprecht, T., Angele, B., Heesemann, J., Wagner, H., Pfister, H.W., and Kirschning, C.J. (2004). MyD88 is required for mounting a robust host immune response to *Streptococcus pneumoniae* in the CNS. Brain : a journal of neurology *127*, 1437-1445.

Konecna, K., Hernychova, L., Reichelova, M., Lenco, J., Klimentova, J., Stulik, J., Macela, A., Alefantis, T., and Delvecchio, V.G. (2010). Comparative proteomic profiling of culture filtrate proteins of less and highly virulent *Francisella tularensis* strains. Proteomics *10*, 4501-4511.

Korns, D., Frasch, S.C., Fernandez-Boyanapalli, R., Henson, P.M., and Bratton, D.L. (2011). Modulation of macrophage efferocytosis in inflammation. Frontiers in immunology *2*, 57.

Koronakis, V., Eswaran, J., and Hughes, C. (2004). Structure and function of TolC: the bacterial exit duct for proteins and drugs. Annual review of biochemistry *73*, 467-489.

Kugeler, K.J., Mead, P.S., Janusz, A.M., Staples, J.E., Kubota, K.A., Chalcraft, L.G., and Petersen, J.M. (2009). Molecular Epidemiology of *Francisella tularensis* in the United States. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America *48*, 863-870.

Kumar, H., Kawai, T., and Akira, S. (2009). Pathogen recognition in the innate immune response. The Biochemical journal *420*, 1-16.

KuoLee, R., Harris, G., Conlan, J.W., and Chen, W. (2011). Role of neutrophils and NADPH phagocyte oxidase in host defense against respiratory infection with virulent *Francisella tularensis* in mice. Microbes and infection / Institut Pasteur *13*, 447-456.

Kurtz, S., Foreman, O., Bosio, C.M., Anver, M.R., and Elkins, K.L. (2012). IL-6 is essential for primary resistance to *Francisella tularensis* LVS infection. Infection and immunity.

Lai, X.H., Golovliov, I., and Sjostedt, A. (2004). Expression of IglC is necessary for intracellular growth and induction of apoptosis in murine macrophages by *Francisella tularensis*. Microb Pathog *37*, 225-230.

Laroux, F.S., Romero, X., Wetzler, L., Engel, P., and Terhorst, C. (2005). Cutting edge: MyD88 controls phagocyte NADPH oxidase function and killing of gram-negative bacteria. J Immunol *175*, 5596-5600.

Larsson, P., Oyston, P.C., Chain, P., Chu, M.C., Duffield, M., Fuxelius, H.H., Garcia, E., Halltorp, G., Johansson, D., Isherwood, K.E., *et al.* (2005). The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. Nature genetics *37*, 153-159.

Lauriano, C.M., Barker, J.R., Yoon, S.S., Nano, F.E., Arulanandam, B.P., Hassett, D.J., and Klose, K.E. (2004). MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intraamoebae and intramacrophage survival. Proceedings of the National Academy of Sciences of the United States of America *101*, 4246-4249.

Leiby, D.A., Fortier, A.H., Crawford, R.M., Schreiber, R.D., and Nacy, C.A. (1992). *In vivo* modulation of the murine immune response to *Francisella tularensis* LVS by administration of anticytokine antibodies. Infection and immunity *60*, 84-89.

Lesho, M.E., Dorsey, M.D., and Bunner, D. (1998). Feces, dead horses, and fleas. Evolution of the hostile use of biological agents. The Western journal of medicine *168*, 512-516.

Li, H., Nookala, S., Bina, X.R., Bina, J.E., and Re, F. (2006). Innate immune response to *Francisella tularensis* is mediated by TLR2 and caspase-1 activation. Journal of leukocyte biology *80*, 766-773.

Lin, Y., Ritchea, S., Logar, A., Slight, S., Messmer, M., Rangel-Moreno, J., Guglani, L., Alcorn, J.F., Strawbridge, H., Park, S.M., *et al.* (2009). Interleukin-17 is required for T helper 1 cell immunity and host resistance to the intracellular pathogen *Francisella tularensis*. Immunity *31*, 799-810.

Lindemann, S.R., Peng, K., Long, M.E., Hunt, J.R., Apicella, M.A., Monack, D.M., Allen, L.A., and Jones, B.D. (2011). *Francisella tularensis* Schu S4 O-antigen and capsule biosynthesis gene mutants induce early cell death in human macrophages. Infection and immunity *79*, 581-594.

Lindgren, H., Honn, M., Salomonsson, E., Kuoppa, K., Forsberg, A., and Sjostedt, A. (2011). Iron content differs between *Francisella tularensis* subspecies *tularensis* and subspecies holarctica strains and correlates to their susceptibility to H(2)O(2)-induced killing. Infect Immun *79*, 1218-1224.

Lindgren, H., Shen, H., Zingmark, C., Golovliov, I., Conlan, W., and Sjostedt, A. (2007). Resistance of *Francisella tularensis* strains against reactive nitrogen and oxygen species with special reference to the role of KatG. Infection and immunity *75*, 1303-1309.

Loegering, D.J., Drake, J.R., Banas, J.A., McNealy, T.L., Mc Arthur, D.G., Webster, L.M., and Lennartz, M.R. (2006). *Francisella tularensis* LVS grown in macrophages has reduced ability to stimulate the secretion of inflammatory cytokines by macrophages *in vitro*. Microb Pathog *41*, 218-225.

Lopez, M.C., Duckett, N.S., Baron, S.D., and Metzger, D.W. (2004). Early activation of NK cells after lung infection with the intracellular bacterium, *Francisella tularensis* LVS. Cellular immunology *232*, 75-85.

Ludu, J.S., de Bruin, O.M., Duplantis, B.N., Schmerk, C.L., Chou, A.Y., Elkins, K.L., and Nano, F.E. (2008). The *Francisella* pathogenicity island protein PdpD is required for full virulence and associates with homologues of the type VI secretion system. Journal of bacteriology *190*, 4584-4595.

Mahawar, M., Atianand, M.K., Dotson, R.J., Mora, V., Rabadi, S.M., Metzger, D.W., Huntley, J.F., Harton, J.A., Malik, M., and Bakshi, C.S. (2012). Identification of a novel *Francisella tularensis* factor required for intramacrophage survival and subversion of innate immune response. The Journal of biological chemistry *287*, 25216-25229.

Malik, M., Bakshi, C.S., McCabe, K., Catlett, S.V., Shah, A., Singh, R., Jackson, P.L., Gaggar, A., Metzger, D.W., Melendez, J.A., *et al.* (2007). Matrix metalloproteinase 9 activity enhances host susceptibility to pulmonary infection with type A and B strains of *Francisella tularensis*. J Immunol *178*, 1013-1020.

Malik, M., Bakshi, C.S., Sahay, B., Shah, A., Lotz, S.A., and Sellati, T.J. (2006). Toll-like receptor 2 is required for control of pulmonary infection with *Francisella tularensis*. Infection and immunity *74*, 3657-3662.

Mares, C.A., Ojeda, S.S., Morris, E.G., Li, Q., and Teale, J.M. (2008). Initial delay in the immune response to *Francisella tularensis* is followed by hypercytokinemia characteristic of severe sepsis and correlating with upregulation and release of damage-associated molecular patterns. Infect Immun *76*, 3001-3010.

Mares, C.A., Sharma, J., Li, Q., Rangel, E.L., Morris, E.G., Enriquez, M.I., and Teale, J.M. (2011). Defect in efferocytosis leads to alternative activation of macrophages in *Francisella* infections. Immunology and cell biology *89*, 167-172.

Mares, C.A., Sharma, J., Ojeda, S.S., Li, Q., Campos, J.A., Morris, E.G., Coalson, J.J., and Teale, J.M. (2010). Attenuated response of aged mice to respiratory *Francisella* novicida is characterized by reduced cell death and absence of subsequent hypercytokinemia. PloS one *5*, e14088.

Martone, W.J., Marshall, L.W., Kaufmann, A.F., Hobbs, J.H., and Levy, M.E. (1979). Tularemia pneumonia in Washington, DC. A report of three cases with possible common-source exposures. JAMA : the journal of the American Medical Association *242*, 2315-2317.

Mattila, T., Honkanen-Buzalski, T., and Poso, H. (1984). Reversible inhibition of bacterial growth after specific inhibition of spermidine synthase by dicyclohexylamine. Biochem J *223*, 823-830.

McCaffrey, R.L., and Allen, L.A. (2006). *Francisella tularensis* LVS evades killing by human neutrophils via inhibition of the respiratory burst and phagosome escape. Journal of leukocyte biology *80*, 1224-1230.

McCoy, G.W. (1910). Plague among Ground Squirrels in America. The Journal of hygiene *10*, 589-601.

McCoy, G.W., and Chapin, C.W. (1912). Further observations on a plague-like disease of rodents with a preliminary note on the causative agent, *Bacterium tularense.* Journal of Infectious Disease *10*.

McCrumb, F.R. (1961). Aerosol Infection of Man with *Pasteurella tularensis*. Bacteriological reviews *25*, 262-267.

McGinnis, M.W., Parker, Z.M., Walter, N.E., Rutkovsky, A.C., Cartaya-Marin, C., and Karatan, E. (2009). Spermidine regulates *Vibrio cholerae* biofilm formation via transport and signaling pathways. FEMS Microbiol Lett *299*, 166-174.

McLendon, M.K., Apicella, M.A., and Allen, L.A. (2006). *Francisella tularensis*: taxonomy, genetics, and Immunopathogenesis of a potential agent of biowarfare. Annual review of microbiology *60*, 167-185.

Medina, E.A., Morris, I.R., and Berton, M.T. (2010). Phosphatidylinositol 3-kinase activation attenuates the TLR2-mediated macrophage proinflammatory cytokine response to *Francisella tularensis* live vaccine strain. J Immunol *185*, 7562-7572.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A., Jr. (1997). A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature *388*, 394-397.

Meibom, K.L., and Charbit, A. (2010). The unraveling panoply of *Francisella tularensis* virulence attributes. Current opinion in microbiology *13*, 11-17.

Meibom, K.L., Forslund, A.L., Kuoppa, K., Alkhuder, K., Dubail, I., Dupuis, M., Forsberg, A., and Charbit, A. (2009). Hfq, a novel pleiotropic regulator of virulence-associated genes in *Francisella tularensis*. Infect Immun *77*, 1866-1880.

Melillo, A.A., Bakshi, C.S., and Melendez, J.A. (2010). *Francisella tularensis* antioxidants harness reactive oxygen species to restrict macrophage signaling and cytokine production. The Journal of biological chemistry *285*, 27553-27560.

Mohapatra, N.P., Balagopal, A., Soni, S., Schlesinger, L.S., and Gunn, J.S. (2007). AcpA is a *Francisella* acid phosphatase that affects intramacrophage survival and virulence. Infection and immunity *75*, 390-396.

Mohapatra, N.P., Soni, S., Rajaram, M.V., Dang, P.M., Reilly, T.J., El-Benna, J., Clay, C.D., Schlesinger, L.S., and Gunn, J.S. (2010). *Francisella* acid phosphatases inactivate the NADPH oxidase in human phagocytes. J Immunol *184*, 5141-5150.

Mohapatra, N.P., Soni, S., Reilly, T.J., Liu, J., Klose, K.E., and Gunn, J.S. (2008). Combined deletion of four *Francisella novicida* acid phosphatases attenuates virulence and macrophage vacuolar escape. Infection and immunity *76*, 3690-3699.

Molins, C.R., Delorey, M.J., Yockey, B.M., Young, J.W., Sheldon, S.W., Reese, S.M., Schriefer, M.E., and Petersen, J.M. (2010). Virulence differences among *Francisella tularensis* subsp. *tularensis* clades in mice. PloS one *5*, e10205.

Moomaw, A.S., and Maguire, M.E. (2008). The unique nature of mg2+ channels. Physiology (Bethesda) *23*, 275-285.

Moritz, E., Seidensticker, S., Gottwald, A., Maier, W., Hoerauf, A., Njuguna, J.T., and Kaiser, A. (2004). The efficacy of inhibitors involved in spermidine metabolism in *Plasmodium falciparum*, *Anopheles stephensi* and *Trypanosoma evansi*. Parasitol Res *94*, 37-48.

Moule, M.G., Monack, D.M., and Schneider, D.S. (2010). Reciprocal analysis of *Francisella novicida* infections of a *Drosophila melanogaster* model reveal host-pathogen conflicts mediated by reactive oxygen and imd-regulated innate immune response. PLoS Pathog *6*.

N. A. J. Taylor, J.A.C., Michael Hamel-Green (2013). Dialogue on Middle East Biological, Nuclear, and Chemical Weapons Disarmament: Constraints and Opportunities. Alternatives: Global, Local, Political *38*, 78-98.

Nallaparaju, K.C., Yu, J.J., Rodriguez, S.A., Zogaj, X., Manam, S., Guentzel, M.N., Seshu, J., Murthy, A.K., Chambers, J.P., Klose, K.E., *et al.* (2011). Evasion of IFN-gamma signaling by *Francisella novicida* is dependent upon *Francisella* outer membrane protein C. PloS one *6*, e18201.

Nano, F.E., and Schmerk, C. (2007). The *Francisella* pathogenicity island. Annals of the New York Academy of Sciences *1105*, 122-137.

Nano, F.E., Zhang, N., Cowley, S.C., Klose, K.E., Cheung, K.K., Roberts, M.J., Ludu, J.S., Letendre, G.W., Meierovics, A.I., Stephens, G., *et al.* (2004). A *Francisella tularensis* pathogenicity island required for intramacrophage growth. J Bacteriol *186*, 6430-6436.

Nikaido, H., and Pages, J.M. (2012). Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. FEMS microbiology reviews *36*, 340-363.

Olsuf'Ev, N.G., Dunaeva, T.N., Emel'Ianova, O.S., and Petrov, V.G. (1950). [Study of the properties of *Bacterium tularense* and its biological correlation to host animals and tick-transmitters]. Vestnik Akademii meditsinskikh nauk SSSR *3*, 20-29.

Olsufiev, N.G., Emelyanova, O.S., and Dunayeva, T.N. (1959). Comparative study of strains of *B. tularense* in the old and new world and their taxonomy. Journal of hygiene, epidemiology, microbiology, and immunology *3*, 138-149.

Ottem, K.F., Nylund, A., Karlsbakk, E., Friis-Moller, A., Krossoy, B., and Knappskog, D. (2007). New species in the genus *Francisella* (Gammaproteobacteria; *Francisella*ceae); *Francisella piscicida* sp. *nov*. isolated from cod (Gadus morhua). Archives of microbiology *188*, 547-550.

Oyston, P.C., Sjostedt, A., and Titball, R.W. (2004). Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*. Nature reviews Microbiology *2*, 967-978.

Pandya, G.A., Holmes, M.H., Petersen, J.M., Pradhan, S., Karamycheva, S.A., Wolcott, M.J., Molins, C., Jones, M., Schriefer, M.E., Fleischmann, R.D., *et al.* (2009). Whole genome single nucleotide polymorphism based phylogeny of *Francisella tularensis* and its application to the development of a strain typing assay. BMC microbiology *9*, 213.

Papp-Wallace, K.M., Nartea, M., Kehres, D.G., Porwollik, S., McClelland, M., Libby, S.J., Fang, F.C., and Maguire, M.E. (2008). The CorA Mg2+ channel is required for the virulence of *Salmonella enterica* serovar *typhimurium*. Journal of bacteriology *190*, 6517-6523.

Parmely, M.J., Fischer, J.L., and Pinson, D.M. (2009). Programmed cell death and the pathogenesis of tissue injury induced by type A *Francisella tularensis*. FEMS microbiology letters *301*, 1-11.

Patel, C.N., Wortham, B.W., Lines, J.L., Fetherston, J.D., Perry, R.D., and Oliveira, M.A. (2006). Polyamines are essential for the formation of plague biofilm. Journal of bacteriology *188*, 2355-2363.

Pavkova, I., Reichelova, M., Larsson, P., Hubalek, M., Vackova, J., Forsberg, A., and Stulik, J. (2006). Comparative proteome analysis of fractions enriched for membrane-associated proteins from *Francisella tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* strains. Journal of proteome research *5*, 3125-3134.

Pechous, R., Celli, J., Penoske, R., Hayes, S.F., Frank, D.W., and Zahrt, T.C. (2006). Construction and characterization of an attenuated purine auxotroph in a *Francisella tularensis* live vaccine strain. Infect Immun *74*, 4452-4461.

Pechous, R.D., McCarthy, T.R., Mohapatra, N.P., Soni, S., Penoske, R.M., Salzman, N.H., Frank, D.W., Gunn, J.S., and Zahrt, T.C. (2008). A *Francisella tularensis* Schu S4 purine auxotroph is highly attenuated in mice but offers limited protection against homologous intranasal challenge. PloS one *3*, e2487.

Peng, K., Broz, P., Jones, J., Joubert, L.M., and Monack, D. (2011). Elevated AIM2-mediated pyroptosis triggered by hypercytotoxic *Francisella* mutant strains is attributed to increased intracellular bacteriolysis. Cellular microbiology *13*, 1586-1600.

Perez-Mendoza, D., and de la Cruz, F. (2009). *Escherichia coli* genes affecting recipient ability in plasmid conjugation: are there any? BMC genomics *10*, 71.

Petersen, J.M., and Molins, C.R. (2010). Subpopulations of *Francisella tularensis* ssp. *tularensis* and holarctica: identification and associated epidemiology. Future microbiology *5*, 649-661.

Pfeiffer, J., Guhl, J., Waidner, B., Kist, M., and Bereswill, S. (2002). Magnesium uptake by CorA is essential for viability of the gastric pathogen *Helicobacter pylori*. Infection and immunity *70*, 3930-3934.

Pfunder, M.C. (1925). Primary tularemia of the eye. The Journal of the AmericanMedical Assocation *85*, 1061.

Platz, G.J., Bublitz, D.C., Mena, P., Benach, J.L., Furie, M.B., and Thanassi, D.G. (2010). A *tolC* mutant of *Francisella tularensis* is hypercytotoxic compared to the wild type and elicits increased proinflammatory responses from host cells. Infection and immunity *78*, 1022-1031.

Pohanka, M., and Skladal, P. (2009). Bacillus anthracis, *Francisella tularensis* and *Yersinia pestis*. The most important bacterial warfare agents - review. Folia microbiologica *54*, 263-272.

Power, M.R., Peng, Y., Maydanski, E., Marshall, J.S., and Lin, T.J. (2004). The development of early host response to *Pseudomonas aeruginosa* lung infection is critically dependent on myeloid differentiation factor 88 in mice. The Journal of biological chemistry *279*, 49315-49322.

Qin, A., Scott, D.W., and Mann, B.J. (2008). *Francisella tularensis* subsp. *tularensis* Schu S4 disulfide bond formation protein B, but not an RND-type efflux pump, is required for virulence. Infection and immunity *76*, 3086-3092.

Qin, A., Scott, D.W., Thompson, J.A., and Mann, B.J. (2009). Identification of an essential *Francisella tularensis* subsp. *tularensis* virulence factor. Infection and immunity *77*, 152-161.

Rajaram, M.V., Butchar, J.P., Parsa, K.V., Cremer, T.J., Amer, A., Schlesinger, L.S., and Tridandapani, S. (2009). Akt and SHIP modulate *Francisella* escape from the phagosome and induction of the Fas-mediated death pathway. PloS one *4*, e7919.

Raynaud, C., Meibom, K.L., Lety, M.A., Dubail, I., Candela, T., Frapy, E., and Charbit, A. (2007). Role of the *wbt* locus of *Francisella tularensis* in lipopolysaccharide O-antigen biogenesis and pathogenicity. Infection and immunity *75*, 536-541.

Reilly, T.J., Baron, G.S., Nano, F.E., and Kuhlenschmidt, M.S. (1996). Characterization and sequencing of a respiratory burst-inhibiting acid phosphatase from *Francisella tularensis*. The Journal of biological chemistry *271*, 10973-10983.

Rimaniol, A.C., Zylberberg, H., Zavala, F., and Viard, J.P. (1996). Inflammatory cytokines and inhibitors in HIV infection: correlation between interleukin-1 receptor antagonist and weight loss. AIDS *10*, 1349-1356.

Rodriguez, A.R., Yu, J.J., Guentzel, M.N., Navara, C.S., Klose, K.E., Forsthuber, T.G., Chambers, J.P., Berton, M.T., and Arulanandam, B.P. (2012). Mast cell TLR2 signaling is crucial for effective killing of *Francisella tularensis*. J Immunol *188*, 5604-5611.

Rodriguez, A.R., Yu, J.J., Murthy, A.K., Guentzel, M.N., Klose, K.E., Forsthuber, T.G., Chambers, J.P., Berton, M.T., and Arulanandam, B.P. (2011). Mast cell/IL-4 control of *Francisella tularensis* replication and host cell death is associated with increased ATP production and phagosomal acidification. Mucosal immunology *4*, 217-226.

Rubin, E.J., Akerley, B.J., Novik, V.N., Lampe, D.J., Husson, R.N., and Mekalanos, J.J. (1999). In vivo transposition of mariner-based elements in enteric bacteria and *mycobacteria*. Proceedings of the National Academy of Sciences of the United States of America *96*, 1645-1650.

Russo, B.C., Horzempa, J., O'Dee, D.M., Schmitt, D.M., Brown, M.J., Carlson, P.E., Jr., Xavier, R.J., and Nau, G.J. (2011). A *Francisella tularensis* locus required for spermine responsiveness is necessary for virulence. Infection and immunity *79*, 3665-3676.

Ruud, J., Wilhelms, D.B., Nilsson, A., Eskilsson, A., Tang, Y.J., Strohle, P., Caesar, R., Schwaninger, M., Wunderlich, T., Backhed, F., *et al.* (2013). Inflammation- and tumor-induced anorexia and weight loss require MyD88 in hematopoietic/myeloid cells but not in brain endothelial or neural cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *27*, 1973-1980.

Sandstrom, G., Lofgren, S., and Tarnvik, A. (1988). A capsule-deficient mutant of *Francisella tularensis* LVS exhibits enhanced sensitivity to killing by serum but diminished sensitivity to killing by polymorphonuclear leukocytes. Infection and immunity *56*, 1194-1202.

Santic, M., Asare, R., Skrobonja, I., Jones, S., and Abu Kwaik, Y. (2008). Acquisition of the vacuolar ATPase proton pump and phagosome acidification are essential for escape of *Francisella tularensis* into the macrophage cytosol. Infection and immunity *76*, 2671-2677.

Santic, M., Molmeret, M., Klose, K.E., and Abu Kwaik, Y. (2006). *Francisella tularensis* travels a novel, twisted road within macrophages. Trends in microbiology *14*, 37-44.

Santic, M., Molmeret, M., Klose, K.E., Jones, S., and Kwaik, Y.A. (2005). The *Francisella tularensis* pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. Cellular microbiology *7*, 969-979.

Sauer, F.G., Mulvey, M.A., Schilling, J.D., Martinez, J.J., and Hultgren, S.J. (2000). Bacterial pili: molecular mechanisms of pathogenesis. Current opinion in microbiology *3*, 65-72.

Sawyer, W.D., Jemski, J.V., Hogge, A.L., Jr., Eigelsbach, H.T., Wolfe, E.K., Dangerfield, H.G., Gochenour, W.S., Jr., and Crozier, D. (1966). Effect of aerosol age on the infectivity of airborne *Pasteurella tularensis* for *Macaca mulatta* and man. Journal of bacteriology *91*, 2180-2184.

Schlosser, A., Meldorf, M., Stumpe, S., Bakker, E.P., and Epstein, W. (1995). TrkH and its homolog, TrkG, determine the specificity and kinetics of cation transport by the Trk system of *Escherichia coli*. Journal of bacteriology *177*, 1908-1910.

Schmid, G.P., Kornblatt, A.N., Connors, C.A., Patton, C., Carney, J., Hobbs, J., and Kaufmann, A.F. (1983). Clinically mild tularemia associated with tick-borne *Francisella tularensis*. The Journal of infectious diseases *148*, 63-67.

Schmitt, D.M., O'Dee, D.M., Brown, M.J., Horzempa, J., Russo, B.C., Morel, P.A., and Nau, G.J. (2012). Role of NK cells in host defense against pulmonary type A *Francisella tularensis* infection. Microbes and infection / Institut Pasteur.

Schmitt, D.M., O'Dee, D.M., Brown, M.J., Horzempa, J., Russo, B.C., Morel, P.A., and Nau, G.J. (2013). Role of NK cells in host defense against pulmonary type A *Francisella tularensis* infection. Microbes and infection / Institut Pasteur *15*, 201-211.

Schulert, G.S., and Allen, L.A. (2006). Differential infection of mononuclear phagocytes by *Francisella tularensis*: role of the macrophage mannose receptor. Journal of leukocyte biology *80*, 563-571.

Schwartz, J.T., Barker, J.H., Kaufman, J., Fayram, D.C., McCracken, J.M., and Allen, L.A. (2012). *Francisella tularensis* inhibits the intrinsic and extrinsic pathways to delay constitutive apoptosis and prolong human neutrophil lifespan. J Immunol *188*, 3351-3363.

Scott, J.W., Hawley, S.A., Green, K.A., Anis, M., Stewart, G., Scullion, G.A., Norman, D.G., and Hardie, D.G. (2004). CBS domains form energy-sensing modules whose binding of adenosine ligands is disrupted by disease mutations. The Journal of clinical investigation *113*, 274-284.

Sharma, J., Mares, C.A., Li, Q., Morris, E.G., and Teale, J.M. (2011a). Features of sepsis caused by pulmonary infection with *Francisella tularensis* Type A strain. Microb Pathog *51*, 39-47.

Sharma, J., Mishra, B.B., Li, Q., and Teale, J.M. (2011b). TLR4-dependent activation of inflammatory cytokine response in macrophages by *Francisella* elongation factor *Tu*. Cellular immunology *269*, 69-73.

Shen, H., Harris, G., Chen, W., Sjostedt, A., Ryden, P., and Conlan, W. (2010). Molecular immune responses to aerosol challenge with *Francisella tularensis* in mice inoculated with live vaccine candidates of varying efficacy. PloS one *5*, e13349.

Shirey, K.A., Cole, L.E., Keegan, A.D., and Vogel, S.N. (2008). *Francisella tularensis* live vaccine strain induces macrophage alternative activation as a survival mechanism. J Immunol *181*, 4159-4167.

Singh, A., Rahman, T., Malik, M., Hickey, A.J., Leifer, C.A., Hazlett, K.R., and Sellati, T.J. (2013). Discordant Results Obtained with *Francisella tularensis* during *In Vitro* and *In Vivo* Immunological Studies Are Attributable to Compromised Bacterial Structural Integrity. PloS one *8*, e58513.

Sjodin, A., Svensson, K., Lindgren, M., Forsman, M., and Larsson, P. (2010). Whole-genome sequencing reveals distinct mutational patterns in closely related laboratory and naturally propagated *Francisella tularensis* strains. PLoS One *5*, e11556.

Sjostedt, A. (2006). Intracellular survival mechanisms of *Francisella tularensis*, a stealth pathogen. Microbes and infection / Institut Pasteur *8*, 561-567.

Sjostedt, A. (2007). Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. Annals of the New York Academy of Sciences *1105*, 1-29.

Sjostedt, A., Conlan, J.W., and North, R.J. (1994). Neutrophils are critical for host defense against primary infection with the facultative intracellular bacterium *Francisella tularensis* in mice and participate in defense against reinfection. Infection and immunity *62*, 2779-2783.

Sjostedt, A., North, R.J., and Conlan, J.W. (1996). The requirement of tumour necrosis factor-alpha and interferon-gamma for the expression of protective immunity to secondary murine tularaemia depends on the size of the challenge inoculum. Microbiology *142 (Pt 6)*, 1369-1374.

Sjostedt, A., Tarnvik, A., and Sandstrom, G. (1991). The T-cell-stimulating 17-kilodalton protein of *Francisella tularensis* LVS is a lipoprotein. Infection and immunity *59*, 3163-3168.

Skerrett, S.J., Liggitt, H.D., Hajjar, A.M., and Wilson, C.B. (2004). Cutting edge: myeloid differentiation factor 88 is essential for pulmonary host defense against *Pseudomonas aeruginosa* but not *Staphylococcus aureus*. J Immunol *172*, 3377-3381.

Skyberg, J.A., Rollins, M.F., Holderness, J.S., Marlenee, N.L., Schepetkin, I.A., Goodyear, A., Dow, S.W., Jutila, M.A., and Pascual, D.W. (2012). Nasal Acai polysaccharides potentiate innate immunity to protect against pulmonary *Francisella tularensis* and *Burkholderia pseudomallei* Infections. PLoS pathogens *8*, e1002587.

Smith, L.K., Gomez, M.J., Shatalin, K.Y., Lee, H., and Neyfakh, A.A. (2007). Monitoring of gene knockouts: genome-wide profiling of conditionally essential genes. Genome biology *8*, R87.

Smith, R.L., and Maguire, M.E. (1998). Microbial magnesium transport: unusual transporters searching for identity. Molecular microbiology *28*, 217-226.

Snavely, M.D., Florer, J.B., Miller, C.G., and Maguire, M.E. (1989). Magnesium transport in *Salmonella typhimurium:* expression of cloned genes for three distinct Mg2+ transport systems. Journal of bacteriology *171*, 4752-4760.

Sporri, R., Joller, N., Albers, U., Hilbi, H., and Oxenius, A. (2006). MyD88-dependent IFN-gamma production by NK cells is key for control of *Legionella pneumophila* infection. J Immunol *176*, 6162-6171.

Stacey, K.J., Young, G.R., Clark, F., Sester, D.P., Roberts, T.L., Naik, S., Sweet, M.J., and Hume, D.A. (2003). The molecular basis for the lack of immunostimulatory activity of vertebrate DNA. J Immunol *170*, 3614-3620.

Sukhumavasi, W., Egan, C.E., Warren, A.L., Taylor, G.A., Fox, B.A., Bzik, D.J., and Denkers, E.Y. (2008). TLR adaptor MyD88 is essential for pathogen control during oral *toxoplasma gondii* infection but not adaptive immunity induced by a vaccine strain of the parasite. J Immunol *181*, 3464-3473.

Syrjala, H., Karvonen, J., and Salminen, A. (1984). Skin manifestations of tularemia: a study of 88 cases in northern Finland during 16 years (1967-1983). Acta dermato-venereologica *64*, 513-516.

Tabor, C.W., and Tabor, H. (1985). Polyamines in microorganisms. Microbiol Rev *49*, 81-99.

Taglialatela, M., Ficker, E., Wible, B.A., and Brown, A.M. (1995). C-terminus determinants for Mg2+ and polyamine block of the inward rectifier K+ channel IRK1. The EMBO journal *14*, 5532-5541.

Telepnev, M., Golovliov, I., Grundstrom, T., Tarnvik, A., and Sjostedt, A. (2003). *Francisella tularensis* inhibits Toll-like receptor-mediated activation of intracellular signalling and secretion of TNF-alpha and IL-1 from murine macrophages. Cellular microbiology *5*, 41-51.

Telepnev, M., Golovliov, I., and Sjostedt, A. (2005). *Francisella tularensis* LVS initially activates but subsequently down-regulates intracellular signaling and cytokine secretion in mouse monocytic and human peripheral blood mononuclear cells. Microbial pathogenesis *38*, 239-247.

Thakran, S., Li, H., Lavine, C.L., Miller, M.A., Bina, J.E., Bina, X.R., and Re, F. (2008). Identification of *Francisella tularensis* lipoproteins that stimulate the toll-like receptor (TLR) 2/TLR1 heterodimer. J Biol Chem *283*, 3751-3760.

Tokuda, H., and Matsuyama, S. (2004). Sorting of lipoproteins to the outer membrane in *E. coli*. Biochimica et biophysica acta *1694*, IN1-9.

Trevisanato, S.I. (2007). The 'Hittite plague', an epidemic of tularemia and the first record of biological warfare. Medical hypotheses *69*, 1371-1374.

Twine, S.M., Mykytczuk, N.C., Petit, M., Tremblay, T.L., Lanthier, P., Conlan, J.W., and Kelly, J.F. (2005). *Francisella tularensis* proteome: low levels of ASB-14 facilitate the visualization of membrane proteins in total protein extracts. Journal of proteome research *4*, 1848-1854.

Ulland, T.K., Buchan, B.W., Ketterer, M.R., Fernandes-Alnemri, T., Meyerholz, D.K., Apicella, M.A., Alnemri, E.S., Jones, B.D., Nauseef, W.M., and Sutterwala, F.S. (2010). Cutting edge: mutation of *Francisella tularensis mviN* leads to increased macrophage absent in melanoma 2 inflammasome activation and a loss of virulence. J Immunol *185*, 2670-2674.

Urb, M., and Sheppard, D.C. (2012). The role of mast cells in the defence against pathogens. PLoS pathogens *8*, e1002619.

Villamon, E., Gozalbo, D., Roig, P., Murciano, C., O'Connor, J.E., Fradelizi, D., and Gil, M.L. (2004). Myeloid differentiation factor 88 (MyD88) is required for murine resistance to *Candida albicans* and is critically involved in *Candida* -induced production of cytokines. European cytokine network *15*, 263-271.

Vinogradov, E., Perry, M.B., and Conlan, J.W. (2002). Structural analysis of *Francisella tularensis* lipopolysaccharide. European journal of biochemistry / FEBS *269*, 6112-6118.

Wang, X., Karbarz, M.J., McGrath, S.C., Cotter, R.J., and Raetz, C.R. (2004). MsbA transporter-dependent lipid A 1-dephosphorylation on the periplasmic surface of the inner membrane: topography of *Francisella* novicida LpxE expressed in *Escherichia coli*. The Journal of biological chemistry *279*, 49470-49478.

Wang, X., McGrath, S.C., Cotter, R.J., and Raetz, C.R. (2006). Expression cloning and periplasmic orientation of the *Francisella* novicida lipid A 4'-phosphatase LpxF. The Journal of biological chemistry *281*, 9321-9330.

Wehrly, T.D., Chong, A., Virtaneva, K., Sturdevant, D.E., Child, R., Edwards, J.A., Brouwer, D., Nair, V., Fischer, E.R., Wicke, L., *et al.* (2009). Intracellular biology and virulence determinants of *Francisella tularensis* revealed by transcriptional profiling inside macrophages. Cellular microbiology *11*, 1128-1150.

Weighardt, H., Kaiser-Moore, S., Vabulas, R.M., Kirschning, C.J., Wagner, H., and Holzmann, B. (2002). Cutting edge: myeloid differentiation factor 88 deficiency improves resistance against sepsis caused by polymicrobial infection. J Immunol *169*, 2823-2827.

Weiss, D.S., Brotcke, A., Henry, T., Margolis, J.J., Chan, K., and Monack, D.M. (2007). *In vivo* negative selection screen identifies genes required for *Francisella* virulence. Proc Natl Acad Sci U S A *104*, 6037-6042.

Wenger, J.D., Hollis, D.G., Weaver, R.E., Baker, C.N., Brown, G.R., Brenner, D.J., and Broome, C.V. (1989). Infection caused by *Francisella philomiragia* (formerly *Yersinia philomiragia*). A newly recognized human pathogen. Annals of internal medicine *110*, 888-892.

Wherry, W.B., and Lamb, B.H. (1914). infection of man with *Bacterium tularense*. Journal of Infectious Disease *15*.

White, J.D., Rooney, J.R., Prickett, P.A., Derrenbacher, E.B., Beard, C.W., and Griffith, W.R. (1964). Pathogenesis of Experimental Respiratory Tularemia in Monkeys. The Journal of infectious diseases *114*, 277-283.

Wickstrum, J.R., Bokhari, S.M., Fischer, J.L., Pinson, D.M., Yeh, H.W., Horvat, R.T., and Parmely, M.J. (2009). *Francisella tularensis* Induces Extensive Caspase-3 Activation and Apoptotic Cell Death in the Tissues of Infected Mice. Infection and immunity *77*, 4827-4836.

Williams, M.D., Ouyang, T.X., and Flickinger, M.C. (1994). Starvation-induced expression of SspA and SspB: the effects of a null mutation in *sspA* on *Escherichia coli* protein synthesis and survival during growth and prolonged starvation. Molecular microbiology *11*, 1029-1043.

Winther-Larsen, H.C., Hegge, F.T., Wolfgang, M., Hayes, S.F., van Putten, J.P., and Koomey, M. (2001). *Neisseria gonorrhoeae* PilV, a type IV pilus-associated protein essential to human epithelial cell adherence. Proceedings of the National Academy of Sciences of the United States of America *98*, 15276-15281.

World Health Organization. (1970). Health aspects of chemical and biological weapons : report of a WHO group of consultants (Geneva: World Health Organization).

Wortham, B.W., Oliveira, M.A., Fetherston, J.D., and Perry, R.D. (2010). Polyamines are required for the expression of key Hms proteins important for *Yersinia pestis* biofilm formation. Environ Microbiol *12*, 2034-2047.

Wortham, B.W., Patel, C.N., and Oliveira, M.A. (2007). Polyamines in bacteria: pleiotropic effects yet specific mechanisms. Advances in experimental medicine and biology *603*, 106-115.

Ye, P., Garvey, P.B., Zhang, P., Nelson, S., Bagby, G., Summer, W.R., Schwarzenberger, P., Shellito, J.E., and Kolls, J.K. (2001). Interleukin-17 and lung host defense against *Klebsiella pneumoniae* infection. American journal of respiratory cell and molecular biology *25*, 335-340.

Yee, D., Rhinehart-Jones, T.R., and Elkins, K.L. (1996). Loss of either CD4+ or CD8+ T cells does not affect the magnitude of protective immunity to an intracellular pathogen, *Francisella tularensis* strain LVS. J Immunol *157*, 5042-5048.

Yu, H., Nasr, S.Z., and Deretic, V. (2000). Innate lung defenses and compromised *Pseudomonas aeruginosa* clearance in the malnourished mouse model of respiratory infections in cystic fibrosis. Infection and immunity *68*, 2142-2147.

Zarrella, T.M., Singh, A., Bitsaktsis, C., Rahman, T., Sahay, B., Feustel, P.J., Gosselin, E.J., Sellati, T.J., and Hazlett, K.R. (2011). Host-adaptation of *Francisella tularensis* alters the bacterium's surface-carbohydrates to hinder effectors of innate and adaptive immunity. PLoS One *6*, e22335.

Zeilstra-Ryalls, J., Fayet, O., and Georgopoulos, C. (1991). The universally conserved GroE (Hsp60) chaperonins. Annual review of microbiology *45*, 301-325.

Zhang, N., Ren, X., Zhu, D., Li, D., and Wang, D. (2010). Crystallization and preliminary crystallographic studies of CorC, a magnesium-ion transporter. Acta crystallographica Section F, Structural biology and crystallization communications *66*, 681-683.

Zhu, Y., Davis, A., Smith, B.J., Curtis, J., and Handman, E. (2009). *Leishmania major* CorA-like magnesium transporters play a critical role in parasite development and virulence. International journal for parasitology *39*, 713-723.