DEVELOPMENT OF

OF A NOVEL FRANCISELLA TULARENSIS SCHU S4

HTPG DELETION MUTANT

by

Jason Clements

M.S. Biomedical Science (Hood College) 2020

MOCK GRANT PROPOSAL

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE

in the

GRADUATE SCHOOL

of

HOOD COLLEGE

May 2020

Accepted:

Ann Boyd, Ph.D. Committee Member	Ann Boyd, Ph.D. Director, Biomedical Science Program
Craig Laufer, Ph.D. Committee Member	
Ronald Cobb, Ph.D. Project Adviser	April Boulton, Ph.D. Dean of the Graduate School

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ACKNOWLEDGEMENTS

I would like to thank Dr. Rachel Beyer, Dr. Ann Boyd, Dr. Oney Smith, and Traci Holland for their continual assistance with helping me push this project forward through the years in its various iterations. Further, I would like to express my deep gratitude to Dr. Ron Cobb for agreeing to serve as my advisor on this project, and for his invaluable assistance for providing input, advise, and support.

Mock Grant Application

Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

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Type	Activity	Version – HCBMS.011712
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*
In Vitro and *In Vivo* Evaluation of a Novel *Francisella tularensis* htpG Deletion Mutant

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR — **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME (Last, first, middle): Clements, Jason, Adam

3b. DEGREE(S): B.S.

3h. eRA Commons User Name: N/A

3c. POSITION TITLE: Graduate Student

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3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT: Department of Biology

3f. MAJOR SUBDIVISION: Biomedical Science Program

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4. HUMAN SUBJECTS RESEARCH ☒ No ☐ Yes

4a. Research Exempt: N/A

4b. Federal-Wide Assurance No.: N/A

4c. Clinical Trial ☒ No ☐ Yes

4d. NIH-defined Phase III Clinical Trial ☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☐ No ☒ Yes

5a. Animal Welfare Assurance No.: N/A

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month. day. year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
08/01/20	06/01/22				

9. APPLICANT ORGANIZATION
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10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
DUNS NO.: N/A Cong. District: N/A

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14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*

DATE: 04/22/2020

PROJECT SUMMARY (See instructions):

Development of a live attenuated vaccine for *F. tularensis* has been challenged by the difficulty of balancing attenuation with efficacy. To date, all vaccine candidates have failed to achieve licensure due to residual virulence and non-optimal protection against pneumonic tularemia, leaving a gap in biopreparedness. One recent mutant of *F. tularensis* subsp. *tularensis* SCHU S4 with a deletion of the *clpB* gene has been shown to be a highly attenuated and effective vaccine but attempts to add additional attenuating deletions have resulted in loss of efficacy. The need for additional attenuation may be required for licensure. Therefore, there is a critical need to identify other SCHU S4 mutants which may share similar properties to SCHU S4 Δ*clpB* and may be more permitting of additional attenuation. We propose development and characterization of a SCHU S4 Δ*htpG* mutant *in vitro* and *in vivo* in BALB/c mice for safety and efficacy upon aerosol challenge by the virulent SCHU S4 strain. In addition, we propose to assess course of infection upon administration and molecular immune responses to determine if SCHU S4 Δ*htpG* may present a unique opportunity to develop a new tularemia vaccine platform which is more permitting of additional attenuating deletions.

RELEVANCE (See instructions):

F. tularensis is a potential bioterror weapon and discovery of a safe and efficacious vaccine has been a significant challenge. This study will evaluate the potential for a SCHU S4 strain Δ*htpG* mutant to display attenuation and efficacy superior to that of the Live Vaccine Stain and elucidate the role of *htpG* in contributing to *F. tularensis* subsp. *tularensis* virulence.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location			
Organizational Name: Department of Biology, Hood College			
DUNS:			
Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick	State: MD	
Province:	Country: United States	Zip/Postal Code: 21702	
Project/Performance Site Congressional Districts: 6th Congressional District			

Additional Project/Performance Site Location			
Organizational Name:			
DUNS:			
Street 1:		Street 2:	
City:	County:	State:	
Province:	Country:	Zip/Postal Code:	
Project/Performance Site Congressional Districts:			

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

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eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

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University of Maryland, College Park	B.S.	2010	Biological Sciences
Hood College, Frederick, MD	M.S.	2020 (anticipated)	Biomedical Science

A. Positions and Honors

N/A

B. Selected peer-reviewed publications (in chronological order)

N/A

C. Research Support

N/A

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

MRI Global, Kansas City, Missouri: Fully available, Select Agents registered BSL-3/ABSL-3 laboratories compliant with CDC and NIH guidelines for containment of risk group 3 Select Agents and housing of animals, prior to and during work with infectious materials.

Clinical:

N/A

Animal:

MRI Global, Kansas City, Missouri: Fully available, Select Agents registered BSL-3/ABSL-3 laboratories compliant with CDC and NIH guidelines for containment of risk group 3 Select Agents and housing of animals, prior to and during work with infectious materials.

Computer:

Hood College Computer Laboratories: Equipped with necessary statistical software for evaluation of data.

Office:

Hood College Offices: For general use.

Other:

N/A

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

- Centrifuges – Clarification and concentration
- PCR machines – For colony PCR
- Electroporator (transformation)
- bCon Biocontainment System (animal housing)
- Lovelace nebulizer – for aerosol challenge
- Nose Only Exposure Chamber – for aerosol challenge
- Aerosol-proof homogenizers (tissue homogenization)
- MILLIPLEX MAP Cytokine/Chemokine Magnetic Bead Panel
- Luminex 200 system – For MILLIPLEX MAP assay
- LDH assay kit – for cytotoxicity analysis
- Computers with pre-loaded software – For data analysis

The following consumables will be purchased with grant funding:

- pJC84 vector (design and synthesis)
- Live Vaccine Strain (from ATCC)
- BALB/c mice
- Multi-well plates
- Pipette consumables
- Agar plates, broth, and media

SPECIFIC AIMS

The highly virulent facultatively intracellular pathogen *Francisella tularensis* subspecies *tularensis* is a biothreat for which development of a safe and effective vaccine for protection against aerosolized *Francisella* has been a considerable challenge. Over the past 70 years, various vaccine candidates have been developed, including the Live Vaccine Strain (LVS), but none have yet attained licensure as a human vaccine. Licensure of LVS has been hampered by significant residual toxicity and poor understanding of its mechanisms of attenuation, and there has only been preliminary development of other potential candidates. This despite the ample amount of safety data that have been accumulated in humans (McCrumb *et al.* 1961; El Sahly *et al.* 2009; Saslaw *et al.* 1961). These other candidates have either failed to be as protective as LVS or significant difficulty has been encountered in attenuating them in a manner which would meet regulatory requirements for licensure (Jia and Horwitz 2018). Others require prime-boost or intranasal administration, while the ideal vaccine candidate would be a single-dose vaccine administered through the intradermal route. There is a significant need for a vaccine candidate which is at least as effective in protecting against fulminant pneumonic tularemia, of greatest concern for preparedness for use of disseminated *F. tularensis* aerosols as a biological weapon. Such a candidate must also be highly attenuated, ideally with multiple attenuating mutations.

To address this need, we will investigate the use of a novel mutant vaccine candidate based on the *F. tularensis* subsp. *tularensis* SCHU S4 (SCHU S4) strain in which the high-temperature protein G (HtpG) gene (*htpG*) has been deleted. We will perform an

initial screening study to analyze the capability of the new candidate vaccine to replicate *in vitro*, as well as evaluate any potential cytotoxicity. Additionally, we will evaluate the ability of the new vaccine to protect mice against SCHU S4 aerosol challenge, course of infection, and molecular immune responses. The feasibility of this approach is supported by the similarity of previously studied *F. tularensis* Δ*htpG* deletion mutants with existing deletion mutants of *F. tularensis* Δ*clpB* and preliminary data suggesting that *F. tularensis* Δ*htpG* deletion mutants are attenuated both *in vitro* and *in vivo* (Asare *et al*. 2010; Ireland *et al*. 2019; Tempel *et al*. 2006; Weiss *et al*. 2007). Based on this data, we hypothesize that SCHU S4 Δ*htpG* will show an attenuated phenotype *in vitro* and *in vivo* upon aerosol challenge by SCHU S4, and this phenotype will be demonstrated by sublethal dissemination and molecular immune responses indicative of protection. Using this mutant, we will further elucidate the contribution of *Francisella* heat-shock proteins to virulence and determine whether SCHU S4 Δ*htpG* may hold promise for further investigation as a new vaccine candidate and as a platform on which additional attenuating mutations may be added while retaining efficacy.

- **Aim 1: Develop and characterize a SCHU S4 Δ*htpG* mutant *in vitro***. We will develop a SCHU S4 Δ*htpG* mutant based on established methods and analyze this vaccine candidate for growth in murine bone marrow-derived macrophages (BMMs) for intracellular growth and cytotoxicity in comparison to wild-type SCHU S4. We hypothesize that like Δ*htpG* mutants of other subspecies of *F. tularensis*, SCHU S4 Δ*htpG* will show a significant intracellular growth defect and

significantly reduced cytotoxicity. LVS and SCHU S4 Δ*clpB* will be included as positive controls. Uninfected cells will serve as negative controls.

- **Aim 2: Establish the safety and efficacy of the SCHU S4 Δ*htpG* mutant against aerosol challenge by wild-type SCHU S4 *in vivo***. We will inoculate BALB/c mice intradermally (i.d.) and intranasally (i.n.) using two doses of 10^5 and 10^8 colony-forming units (CFU) of SCHU S4 Δ*htpG* and use LVS as the comparator strain. For both groups of mice, LD_{50} will be calculated to determine attenuation. Mice which survive immunization will be challenged with 20 CFU of wild-type SCHU S4 via aerosol and further monitored for survival. We hypothesize that SCHU S4 Δ*htpG* will be significantly more attenuated pre-challenge (LD_{50}) and at least as protective post-challenge (median days of survival) compared to LVS. SCHU S4 Δ*clpB* (i.d.) and naive mice will be included as controls.

- **Aim 3: Assess course of sublethal infection and molecular immune responses in SCHU S4 Δ*htpG* immunized mice**. Using the most effective dosing scheme identified in Aim 2, we will immunize BALB/c mice with SCHU S4 Δ*htpG* and LVS. Blood and organ samples will be collected from mice to assess bacteremia and bacterial load at in serum, skin, lungs, and spleen. Organ homogenates and blood will be homogenized or lysed (respectively) and plated for microbial enumeration. Cytokine and chemokine concentrations will be measured from the serum, skin, and organ sites. IgG and IgM concentrations will be measured from serum. We hypothesize that SCHU S4 Δ*htpG* will elicit an effective immune

response, indicated by dissemination from the inoculation site as measured by CFU enumeration and cytokine and chemokine concentrations at least equivalent to those measured from mice immunized with LVS. Controls will include cytokine/chemokine kit controls and mice immunized with SCHU S4 Δ*clpB*.

BACKGROUND AND SIGNIFICANCE

Francisella tularensis as Public Health Threat and Intracellular Lifecycle

Francisella tularensis subsp. *tularensis*, a pathogenic, facultative intracellular bacterium, is the causative agent of tularemia and a significant public health and bioterrorism threat. In its pneumonic, fulminant form, tularemia has a high mortality rate. Despite its treatability, the extreme virulence and low infectious dose of *F. tularensis* subsp. *tularensis* has led to its classification as a Tier 1 Select Agent – as little as 1 CFU is capable of infecting humans (Jones *et al.* 2005). Aerosol dissemination of *F. tularensis* would constitute a potent biological weapon, resulting in pleuropneumonitis which would progress to respiratory failure, shock, and death without appropriate antibiotic treatment. Due to its infectivity and capability for aerosol transmission, *F. tularensis* was stockpiled by the United States and Soviet Union in the middle of the 20th century as a part of their biological weapons programs, including engineered antibiotic and vaccine resistant strains (Dennis *et al.* 2001). The Amerithrax anthrax attacks in 2001 lead to increased interest in development of vaccines against potential biowarfare agents such as *F. tularensis* (Jia and Horwitz 2018).

Francisella tularensis can infect most cell types, but primarily infects macrophages. The bacteria are internalized by macrophages via phagocytosis. In macrophages, it transiently resides in within an acidic vacuole prior to escape into the cytosol by effectors released through a type VI secretion system (T6SS) encoded by the Francisella Pathogenicity Island (FPI). In the cytosol, the bacterium replicates resulting in

apoptosis of the cell and subsequent release of the bacteria, and the infection cycle repeats (Clemens *et al.* 2018).

History of *F. tularensis* Vaccine Development

Historically, live attenuated homologous vaccines have demonstrated the greatest potential for single dose, i.d. protection against respiratory challenge by virulent subsp. *tularensis*, the route of most concern with respect to use of the agent as a biological weapon. Live attenuated vaccine candidates based on *F. tularensis* subsp. *novicida*, *holarctica*, and *tularensis* have been developed. Of these, only mutants of subsp. *holartica* and *tularensis* have shown the potential to protect against aerosol challenge of subsp. *tularensis* (Jia and Horwitz 2018). Since subsp. *novicida* differs from subsp. *holartica* and *tularensis* in the mechanism of pathogenicity, cell surface structure, mechanism of cellular entry, types of cells infected *in vivo*, and ability to evade host responses, mutants of this subspecies have not shown such efficacy (Kingry and Petersen 2014).

Developed in the 1950s, the Live Vaccine Strain (LVS) derived from *F. tularensis* subsp. *holarctica* is unique in that it has been extensively evaluated in both animal models and in humans. LVS retains significant virulence in animals and toxicity in humans and provides incomplete protection to against SCHU S4 aerosol challenge. Phase I study participants vaccinated with LVS frequently experienced systemic effects including headache, fatigue, injection site effects, and other adverse reactions which interfered with activity (El Sahly *et al.* 2009). In humans, LVS administered i.d. or i.n. is only partially protective against subsequent aerosol challenge with SCHU S4. LVS administered by scarification was found to be 25 – 100% effective upon aerosol challenge (Hornick and

Eigelsbach 1966; Saslaw *et al.* 1961). Historically, the mechanism of attenuation of LVS has been poorly understood, though recent work has demonstrated that its attenuation is attributable to two gene deletions (Salomonsson *et al.* 2009). Due to these factors, LVS has not been licensed in the United States or European Union. Despite the recent knowledge gained regarding its mechanism of attenuation, LVS still faces significant challenges to achieve licensure due to its residual toxicity. Since LVS is the only vaccine candidate which has demonstrated partial efficacy in humans, any alternative vaccine candidate would require superior attenuation and efficacy at least equivalent to that of LVS in animal models.

In general, deletion mutants of LVS can only offer efficacy against pneumonic tularemia caused by *F. tularensis* subsp. *tularensis* aerosols equivalent to that of the parental strain. Of 24 LVS mutants studied, three recombinant strains have shown promise for demonstrating superior attenuation and equivalent efficacy to LVS upon SCHU S4 aerosol challenge with single dose i.d. administration (Jia and Horwitz 2018). However, these still only provide approximately 50% survival post-challenge to immunized BALB/c mice and require either intranasal administration or a prime-boost strategy to achieve greater efficacy. Due to the safety concerns associated with the i.n. route, i.d. administration is preferred for a *F. tularensis* vaccine candidate. Single dose administration is preferred due to the scenarios in which such a licensed vaccine is most likely to be needed – as post-exposure prophylaxis in response to a bioterrorism incident or use in the armed forces where prime-boost administration may be logistically unfeasible.

Other approaches for developing a *F. tularensis* vaccine have had poor or limited success. Subunit vaccines comprising *F. tularensis* proteins or lipoproteins have failed to

demonstrate strong protective immunity against *Francisella* (Conlan *et al.* 2002; Fulop *et al.* 2001; Golovliov *et al.* 1995; Sjostedt *et al.* 1992). Several live attenuated heterologous vaccines administered i.d. have shown some promise for protection against SCHU S4 aerosol but require a prime-boost immunization approach with recombinant LVS to achieve efficacy superior to LVS (Jia and Horwitz 2018). The need to find an effective single dose, i.d. vaccine candidate remains unmet.

Due to the inadequacy of live attenuated heterologous vaccines derived from subsp. *novicida* and *holarctica*, there have been attempts at developing mutants based on subsp. *tularensis* which demonstrate superior attenuation, superior efficacy, and single dose administration via the i.d. route. The advantage of this approach is that genetic background is important for immunogenicity, as demonstrated by the development of mutants with identical gene deletions in different *F. tularensis* subspecies (i.e., *holarctica*, *novicida*, and *tularensis*) which exhibit greatest efficacy on the subsp. *tularensis* genetic background. For example, *clpB* deletional mutants of subsp. *tularensis* provide full protection against respiratory challenge of SCHU S4, while an LVS mutant with the same deletion fails to provide protection. This difference is likely due to immune response to antigens specific to subspecies *tularensis* (Golovliov *et al.* 2013; Twine *et al*. 2006). Recently, a *F. tularensis* subsp. *tularensis* SCHU S4 strain possessing a deletion of the heat shock gene *clpB* (SCHU S4 Δ*clpB*) has shown promise, demonstrating superior attenuation and efficacy for immunization against aerosolized wild-type SCHU S4 challenge in mice compared to LVS (Conlan *et al.* 2010; Golovliov *et al.* 2013). After SCHU S4 aerosol challenge, the median time to death in BALB/c mice immunized with SCHU S4 Δ*clpB* was >28 d (i.d.) and 16 d (oral) compared to 8 d (i.d.) and 5 d (oral) for mice immunized with

LVS (Conlan *et al.* 2010; Shen *et al.* 2010). Difficulty has been encountered in adding an additional attenuating deletion to this strain while retaining efficacy, widely considered necessary for achieving licensure due to the concern that an SCHU S4 mutant with a single attenuating deletion is one mutation away from reversion to virulence. Investigators have attempted adding multiple additional attenuating deletions to SCHU S4 Δ*clpB*, but these double deletion mutants lose virtually all efficacy for protecting against aerosol challenge. Double-deletion SCHU S4 Δ*clpB*Δ*pmrA*, Δ*clpB*Δ*relA*, Δ*clpB*Δ*capB*, Δ*clpB*Δ*wbtC*, Δ*clpB*Δ*fupA* mutants have all been evaluated and lose efficacy compared to LVS upon i.d. or i.n. inoculation and subsequent SCHU S4 aerosol challenge. Several of these additional deletions (Δ*pmrA*, Δ*relA*, Δ*capB*) do not demonstrate significant attenuation when administered as single-deletion mutants (LD_{50} $<10^1$ CFU) (J. Conlan, personal communication; Golovliov *et al.* 2013). This suggests that SCHU S4 Δ*clpB* has reached the limit of attenuation while retaining efficacy. It is apparent that developing an effective vaccine for *F. tularensis* requires a careful balance of attenuation to ensure safety while avoiding loss of efficacy, requiring a vaccine candidate that is neither hypo-attenuated nor hyper-attenuated (Jia and Horwitz 2018).

Vaccine Dissemination and Molecular Immune Response

Natural infection by *F. tularensis* is characterized by bacterial dissemination to and multiplication in spleen, liver, lung, lymph nodes, and bone marrow upon i.d. or aerosol exposure (Conlan *et al.* 2003; Fritz *et al.* 2014). Due to the outsized contribution of cell-mediated immunity in eliciting protection against challenge by *F. tularensis* subsp. *tularensis*, vaccine efficacy is intrinsically linked to the capacity to persist *in vivo*,

disseminating from the inoculation site to distant organ sites without causing overt disease. For example, LVS is capable of dissemination upon i.d. inoculation in BALB/c mice to blood, lungs, liver, and spleen prior to clearance by the host immune system (Chen *et al.* 2003). Completely avirulent *F. tularensis* mutants have failed to disseminate to host organ sites, demonstrating poor efficacy in mice upon subsequent SCHU S4 challenge (Pechous *et al.* 2008; Rockx-Brouwer *et al.* 2012; Twine *et al.* 2005).

In humans and mice, LVS vaccination or natural infection with *F. tularensis* induces protective immunity through both humoral and cellular immune responses. Upon LVS inoculation, BALB/c mice show marked increase in tumor necrosis factor alpha (TNF-α), interferon gamma (INF-γ), and interleukin-6 (IL-6) expression (De Pascalis *et al.* 2012; Kim *et al.* 2008; Ryden *et al.* 2013). Upon inoculation by SCHU S4 single deletion mutants, protection is correlated with elevated serum and splenic levels of TNF-α, INF-γ, IL-6, keratinocyte chemoattractant (KC), macrophage inflammatory protein 1 beta (MIP-1β), monocyte chemoattractant protein 1 (MCP-1), and elevated interleukin-17 (IL-17) in the lungs (Ryden *et al.* 2013). Protective immunity in humans against *F. tularensis* primarily relies on a Th1-type immune response, while humoral immunity plays a secondary role (Karttunen *et al.* 1987). Th1-type cytokines tend to produce the proinflammatory responses responsible for killing intracellular pathogens. Others have demonstrated an IL-17 recall response in humans vaccinated with LVS. Gene expression analysis has shown that IL-17 transcripts were induced in peripheral blood mononuclear cells from previous LVS vaccinees stimulated with *F. tularensis* antigens (Paranavitana *et al.* 2010).

Aerosol Challenge of SCHU S4 and Comparison to LVS

Protective immunity by *F. tularensis* vaccines is best indicated by direct aerosol challenge by virulent subsp. *tularensis*, because efficacy against non-respiratory challenge with subsp. *tularensis* or respiratory challenge with other less virulent *F. tularensis* subspecies is not predictive of aerosol protection against subsp. *tularensis* (Conlan 2011; Marohn and Barry 2013). Since LVS is currently the gold standard vaccine strain and any superior candidate must be more attenuated than LVS and at least as effective, development of these candidates must consider direct comparison to LVS upon aerosol challenge by subsp. *tularensis*. In mice, LVS exhibits an LD_{50} of $>10^8$ CFU when administered i.d. and the median time to death upon aerosol challenge by SCHU S4 is approximately 8 d (Conlan *et al.* 2010; Shen *et al.* 2004).

Characteristics of *F. tularensis* subps. *tularensis* SCHU S4 Δ*clpB*

Identification in Screens to Identify F. tularensis *Virulence Factors*

One of the most promising live attenuated vaccine candidates recently evaluated is a mutant of the *F. tularensis* subsp. *tularensis* strain SCHU S4 with a deletion of the *clpB* heat-shock chaperone (SCHU S4 Δ*clpB*). *Francisella tularensis* subsp. *tularensis* FSC033 and SCHU S4 mutants possessing this deletion have been associated with impaired response to oxidative stress, low pH, and heat shock (Alam *et al.* 2018; Lenco *et al.* 2005; Meibom *et al.* 2008; Twine *et al.* 2006). The ClpB gene has also been identified in screening of LVS genes essential for lung infection in mice (Su *et al.* 2007).

Characterization in vitro

In vitro, *clpB* has been associated with contributing to intracellular replication and cytopathogenicity of LVS and SCHU S4 in mouse BMMs, mouse peritoneal macrophages, human (THP-1) macrophages, and J774 macrophage-like cells (Alam *et al.* 2018; Gray *et al.* 2002; Meibom *et al.* 2008). One investigator did not find an intracellular growth defect of LVS Δ*clpB* in BMMs, J774 macrophage-like cells, mouse alveolar macrophage cells, or the human alveolar type II epithelial cell line A549 (Barrigan *et al.* 2013). This may be due to method, as the investigators removed medium from the macrophage growth plates prior to removing macrophages for lysis and enumeration of intracellular bacteria. Since Alam *et al.* (2018) found that the *clpB* mutants of both SCHU S4 and LVS were significantly less cytopathic than their wild-type counterparts, the method used by Barrigan *et al.* (2013) may have inadvertently removed viable bacteria that had escaped lysed macrophages into the cell culture medium, leading to reduced counts for the wild-type control in comparison to the *clpB* mutant. Indeed, Alam *et al.* (2018) found that after 18 hours, the wild-type LVS strain lysed approximately 65% of infected macrophages in comparison to the positive lysis control in BMM cells (Figure 1). This indicates the importance of accounting for the free bacteria in media as part of the enumeration assay.

Characterization in vivo

In vivo, an LVS Δ*clpB* mutant has been shown to be entirely avirulent in mice, with up to 10^7 CFU causing no death among experimental subjects (Meibom *et al.* 2008). Analysis of the course of infection in mice has also shown the capability for LVS and SCHU S4 Δ*clpB* mutants to disseminate from the site of immunization to distant organ

sites, such as the spleen and liver, prior to clearance by the host's immune system (Figures 2 and 3) (Alam *et al.* 2018; Meibom *et al.* 2008; Ryden *et al.* 2013). For SCHU S4 Δ*clpB*, this capacity to disseminate was associated with effective protection against subsequent i.d. or i.n. SCHU S4 challenge (Figure 4) (Ryden *et al.* 2013). Intranasal LVS Δ*clpB* immunization has been found to protect C57BL/6J mice against subsequent lethal i.n. challenge of wild-type LVS and i.d. SCHU S4 Δ*clpB* has been found to protect BALB/c mice against subsequent lethal i.d., i.n., or aerosol challenge of wild-type SCHU S4 (Barrigan *et al.* 2013; Conlan *et al.* 2010; Ryden *et al.* 2013; Twine *et al.* 2012). Intradermal or oral immunization of the SCHU S4 Δ*clpB* strain has been shown to protect BALB/c mice against subsequent i.d. or aerosol challenge with wild-type SCHU S4 superior to protection provided by immunization with LVS, with no advantage conferred by a boosting regimen in comparison to single-dose immunization. Specifically, the median time to death in BALB/c mice immunized with SCHU S4 Δ*clpB* was >28 d (i.d.) and 16 d (oral) compared to 8 d (i.d.) and 5 d (oral) for mice immunized with LVS (Conlan *et al.* 2010; Shen *et al.* 2010). Protection for mice immunized i.d. was associated with moderate IgG/IgM titers (1175 ±283 / 428 ±80) compared to LVS (1814 ±764 / 411 ±142) (Conlan *et al.* 2010). A study of correlates of protection for various SCHU S4 mutant strains found that elevated levels of pulmonary IL-17 was associated with enhanced protection provided by SCHU S4 Δ*clpB* versus LVS (Figure 5) (Ryden *et al.* 2013; Shen *et al.* 2010). A comparison of the *F. tularensis* subsp. *holarctica* FSC200 Δ*clpB* strain with SCHU S4 Δ*clpB* found that while both provided superior protection to LVS following challenge with wild-type SCHU S4, the protection provided by SCHU S4 Δ*clpB* was superior to that of FSC200 Δ*clpB* (Golovliov *et al.* 2013). Throughout studies using

various Δ*clpB* mutants, except for a mutant of *F. tularensis* subsp. *novicida* administered interperitoneally, fewer than 1% of mice have died upon immunization, prior to challenge by SCHU S4 (J. Conlan, personal communication; Tempel *et al.* 2006). In contrast, some mice immunized i.d. with doses $\geq 10^2$ CFU of LVS routinely die (Anderson *et al.* 2010; Eigelsbach and Downs 1961; Fortier *et al.* 1991). The significant attenuation of the SCHU S4 Δ*clpB* strain led to the Federal Select Agent Program excluding the strain as a Select Agent in 2014.

Contribution of clpB *to F. tularensis Virulence*

Recently, investigation into the mechanism by which *clpB* contributes to *F. tularensis* virulence has been partially elucidated. ClpB is necessary for disassembly of the *Francisella* type VI secretion system (T6SS) which delivers effectors for vacuole escape inside macrophages (Alam *et al.* 2018; Brodmann *et al.* 2016). However, this disassembly function is not essential for cytoplasmic replication, and the explanation for compromised intracellular growth of Δ*clpB* mutants may be susceptibility to low pH (Alam *et al.* 2018). ClpB has also been found to be upregulated in the late stages of intracellular infection in macrophages (Figure 6) (Wehrly *et al.* 2009). Combined, the role of ClpB in contributing to *Francisella* pathogenicity with respect to low pH, its upregulation late in intracellular replication, the compromised intracellular growth of Δ*clpB* mutants, and its *in vivo* attenuation point to a series of characteristics which are indicative of a promising *F. tularensis* deletion target. Specifically, the primary role of ClpB appears to be that of a chaperone responsible for responding to the low pH encountered during intracellular replication in macrophages, especially late during infection. This raises the possibility that

other *Francisella* stress response genes may serve as promising targets for attenuating deletions.

Characteristics of *F. tularensis* Δ*htpG* Mutants

The HtpG protein encoded by the *htpG* gene of *F. tularensis* is a member of the heat shock protein 90 (hsp90) family of proteins and has been identified as a virulence factor in other bacterial species (King *et al.* 2014). As heat shock proteins with chaperone activity, both *htpG* and *clpB* have been identified to upregulate upon exposure to oxidative stress (Lenco *et al.* 2005). Preliminary evidence has shown that *htpG* shares many of the other characteristics of *clpB* that make it a potential candidate for investigation as an attenuating mutation to *F. tularensis*.

Identification in Screens to Identify F. tularensis *Virulence Factors*

Along with *clpB*, *htpG* was identified during evaluation of differential expression profiles of *F. tularensis* SCHU S4 strain general stress response genes at multiple timepoints post-infection in BMMs, showing significant upregulation at 1-, 16-, and 24-hours post-infection (Wehrly *et al.* 2009). These timepoints correspond to the early phagosomal and the late vacular stages of the *Francisella* intracellular lifecycle, indicating the role of these genes in responding to the intracellular stressors in the macrophage during replication (Figure 6). Furthermore, both *clpB* and *htpG* were identified by a global analysis of SCHU S4 genes for fitness during competitive infection of Fisher 344 rats. After intravenous challenge of an SCHU S4 himar1 transposon library, the investigators used a transposon-directed insertion site sequencing method to evaluate gene fitness in

bacteria isolated from rat spleens at 24 h post-challenge (Ireland *et al.* 2019). The data indicate that genes associated with bacterial fitness included *clpB* and *htpG*. These results indicate that these genes both impose moderate reductions in bacterial fitness during colonization of the rat spleens and may be potential targets as attenuating deletions. This possibility has already been substantiated by *in vivo* evaluation of *clpB* mutants, as previously discussed.

Characterization in vitro

Like *clpB* mutants, mutants with the deletion of *htpG* have also been associated with compromised intracellular growth *in vitro* in macrophages and other cell lines. An *F. tularensis* subsp. *novicida* U112 strain Δ*htpG* mutant showed compromised intracellular growth and did not induce cell death when pre-stimulated murine BMMs were infected with an MOI of 10 (Figure 7) (Weiss *et al.* 2007). A similar defect in intracellular growth has been demonstrated in *Drosophila melanogaster* S2 cells. An *F. tularensis* subsp. *novicida* mutant library was constructed and infected into S2 cells at an MOI of 10 for 1 h and at 24 hrs post-infection, cells were lysed, serially diluted, and plated on agar plates for enumeration. The *htpG* mutant showed approximately a 2-$\log_{10}$ reduction in growth relative to the wild-type (Asare *et al.* 2010).

Characterization in vivo

In vivo, *htpG* has also shown attenuation in BALB/c mice upon intraperitoneal inoculation with 6 x 10^3 CFU of an *F. tularensis* subsp. *novicida* Δ*htpG* strain with 100% survival. In this study, all mice died upon subsequent intraperitoneal challenge by the wild-type U112 strain of *F. tularensis* subsp. *novicida*, indicating that the Δ*htpG* strain was not protective. However, it is notable that a separate group of mice were also inoculated with the same 6 x 10^3 CFU of a *F. tularensis* subsp. *novicida* Δ*clpB* strain, and all mice died prior to challenge by the U112 strain (Tempel *et al.* 2006). This is in contrast with data showing near 100% survival of mice inoculated i.d. with SCHU S4 Δ*clpB* – based on the more virulent subsp. *tularensis* SCHU S4 strain – through non-peritoneal routes of administration. The intraperitoneal route of administration and challenge is neither a realistic route of administration for a human vaccine nor an expected route of exposure for wild-type *F. tularensis*, including in a bioterrorism scenario. This route bypasses skin and mucosal immune defenses, likely leading to a lower infectious dose and altered virulence characteristics in comparison to the i.d. route. For example, for LVS the intraperitoneal LD_{50} in mice is less than 10 CFU; in contrast, the i.d. LD_{50} is 10^5 – 10^8 CFU, depending on host (Fortier *et al.* 1991; KuoLee *et al.* 2007). Therefore, while this study verifies attenuation of *F. tularensis* subsp. *novicida* Δ*htpG* via intraperitoneal administration at a low dose (10^3 CFU), the degree to which the strain may offer protection against wild-type *F. tularensis* cannot be relied upon. Another set of investigators constructed an *F. tularensis* subsp. *novicida* transposon insertion library, performed a negative selection screen of mutants in the spleens of C57BL/6 mice after intraperitoneal infection, and found that both *htpG* and *clpB* were negative selected indicating that they are required for

Francisella growth and survival *in vivo*. Subsequently, they conducted *in vivo* validation of negatively selected genes, including *htpG*, by performing competition experiments in which mice were infected subcutaneously and intraperitoneally. These investigators found that the mutant was moderately attenuated in the spleen in comparison with the other negatively selected genes, including the entire FPI, for which mutants were severely attenuated (Figure 8) (Weiss *et al.* 2007). This result also indicates the capability for *F. tularensis* Δ*htpG* mutants to disseminate from the i.d. inoculation site to organs in mice.

Significance

As with many pathogenic intracellular bacteria, the search for an effective and safe *F. tularensis* vaccine for preparedness against a potential bioterror event has been fraught with difficulty. Potential candidates such as LVS retain virulence and offer only partial protection against pneumonic tularemia caused by subsp. *tularensis*. Recently, there has been significant promise in the *F. tularensis* subsp. *tularensis* SCHU S4 Δ*clpB* strain which has shown to be highly attenuated and effective in providing at least partial protection to aerosol challenge of wild-type SCHU S4 in mice. Unfortunately, there has been difficulty in adding additional attenuating mutations to the strain that would likely be a requirement for eventual licensure as a commercial vaccine. The mechanisms of its attenuation and efficacy are still being elucidated, but the attributes of the strain that are indicative of a safe and effective vaccine candidate are now well-understood and can be used in the search of alternate candidates. A deletion mutation of *htpG* matches these attributes: a gene with a similar expression profile to *clpB*, impaired intracellular growth, moderate attenuation *in vitro* and *in vivo*, and the potential to disseminate from the site of inoculation to induce a

robust immune response. We propose to conduct an initial investigation of a Δ*htpG* mutant with the SCHU S4 genetic background *in vitro* and *in vivo* to assess the potential of this strain as a promising vaccine candidate. Our goals are to verify compromised intracellular growth and cytotoxicity in comparison to SCHU S4, determine the safety and efficacy of the strain by comparison to LVS, investigate molecular immune responses which correlate with protection, and elucidate the role of this known *F. tularensis* virulence factor in tularemia pathogenesis.

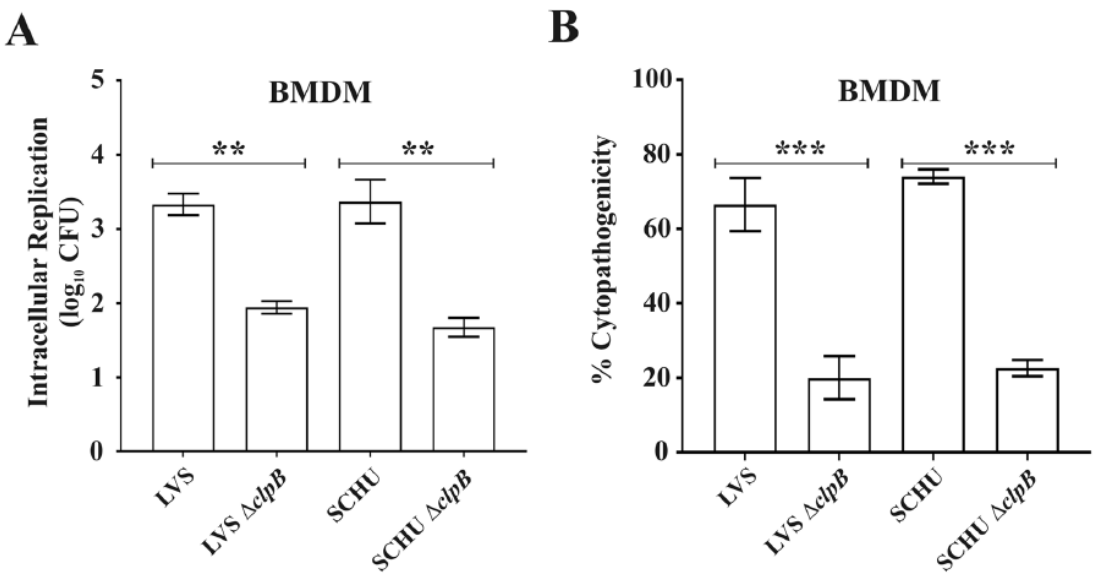

Figure 1. Intracellular growth and lactate dehydrogenase release from *F. tularensis*-infected murine bone-marrow derived macrophages. Growth of the LVS and SCHU S4 Δ*clpB* mutants and the corresponding wild-type strains were analyzed by lysis of the infected cells at 0 h and 18 h and the number of CFU enumerated. For assaying cytopathogenicity, supernatants of infected cultures were harvested at 18 h and the activity was expressed as a percentage of the level of uninfected lysed cells. Statistical significance as compared to the wild-type strain: ** $P < 0.01$; *** $P < 0.001$. Image adapted from Alam *et al.* 2018.

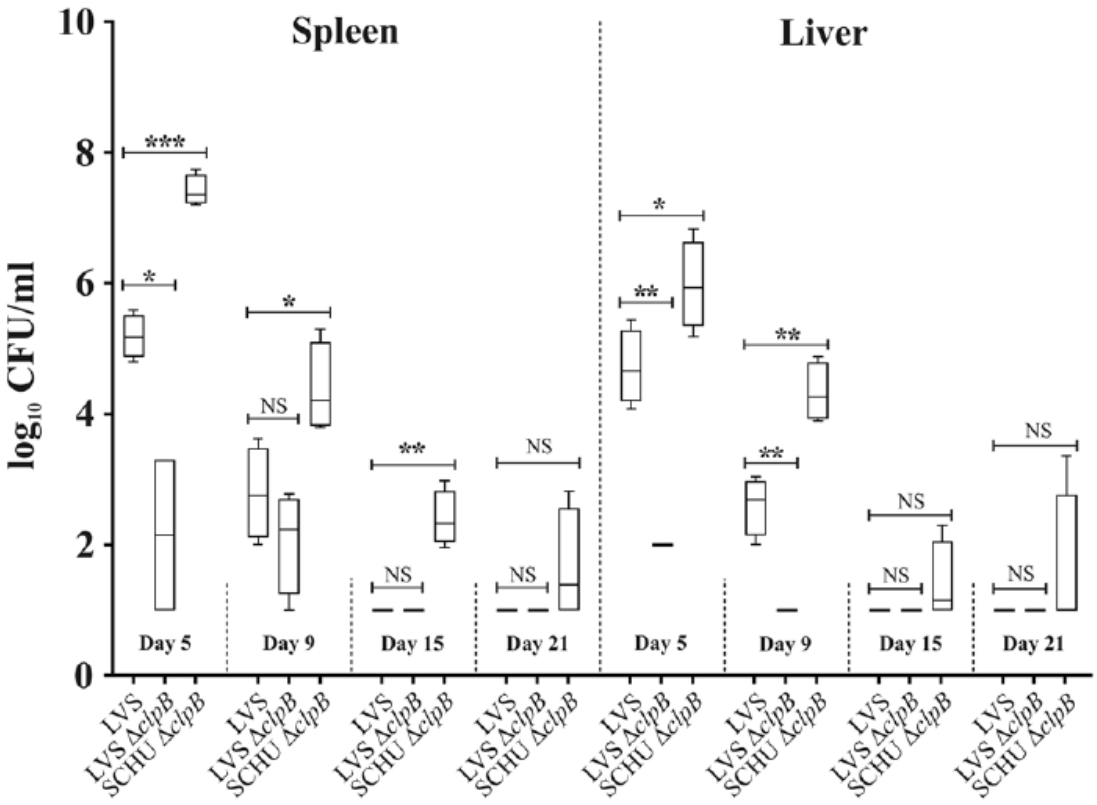

Figure 2. Replication of LVS, LVS Δ*clpB*, and SCHU S4 Δ*clpB* as determined by enumeration of bacterial burden in spleens and livers of mice inoculated with 10^4 CFU of each strain. SCHU Δ*clpB* replicated to higher numbers and remained in infected tissues longer than LVS or the LVS Δ*clpB* strain. ** $P < 0.01$; *** $P < 0.001$; NS = $P > 0.05$. Image adapted from Alam *et al*. 2018.

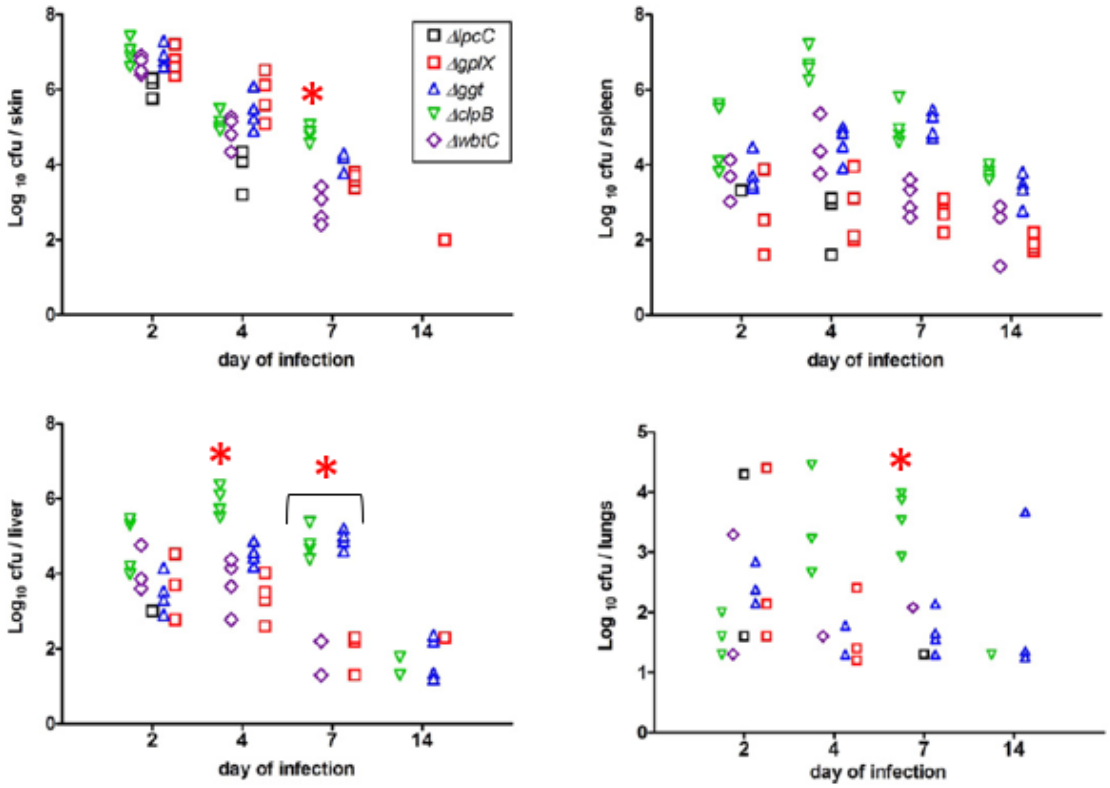

Figure 3. *In vivo* growth of single-deletion mutant strains of SCHU S4. BALB/c mice were immunized intradermally with 10^5 CFU of one or another mutant. Mice were killed on the indicated days after vaccination and bacterial burdens were determined in skin, spleen, liver, and lung by enumeration. Mice immunized with SCHU S4 Δ*clpB* are indicated by green triangles. Red asterisks indicate timepoints in which SCHU S4 Δ*clpB* burdens were significantly higher than all other strains, except as indicated on day 7, in which SCHU S4 Δ*ggt* and Δ*clpB* were both significantly higher than others. Image adapted from Ryden *et al.* 2013.

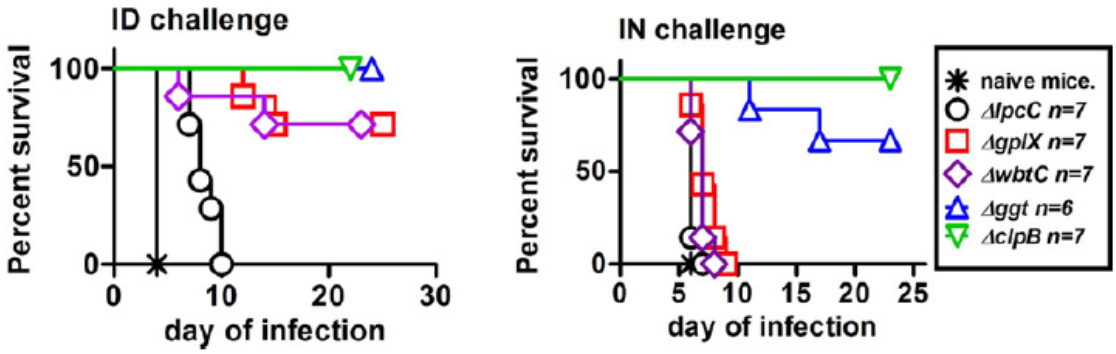

Figure 4. Protection against i.d. or i.n. challenge with SCHU S4 upon immunization i.d. with 10^5 CFU or one or another SCHU S4 single-deletion mutant. Six weeks after vaccination, mice were challenged i.d. with 2000 CFU or i.n. with 75 CFU of SCHU S4 and monitored for survival. Mutants which were capable of robust dissemination to host organ sites (Δ*ggt* and Δ*clpB*, indicated in blue and green, respectively) show greater protection against SCHU S4 challenge (refer to Figure 3). Image adapted from Ryden *et al.* 2013.

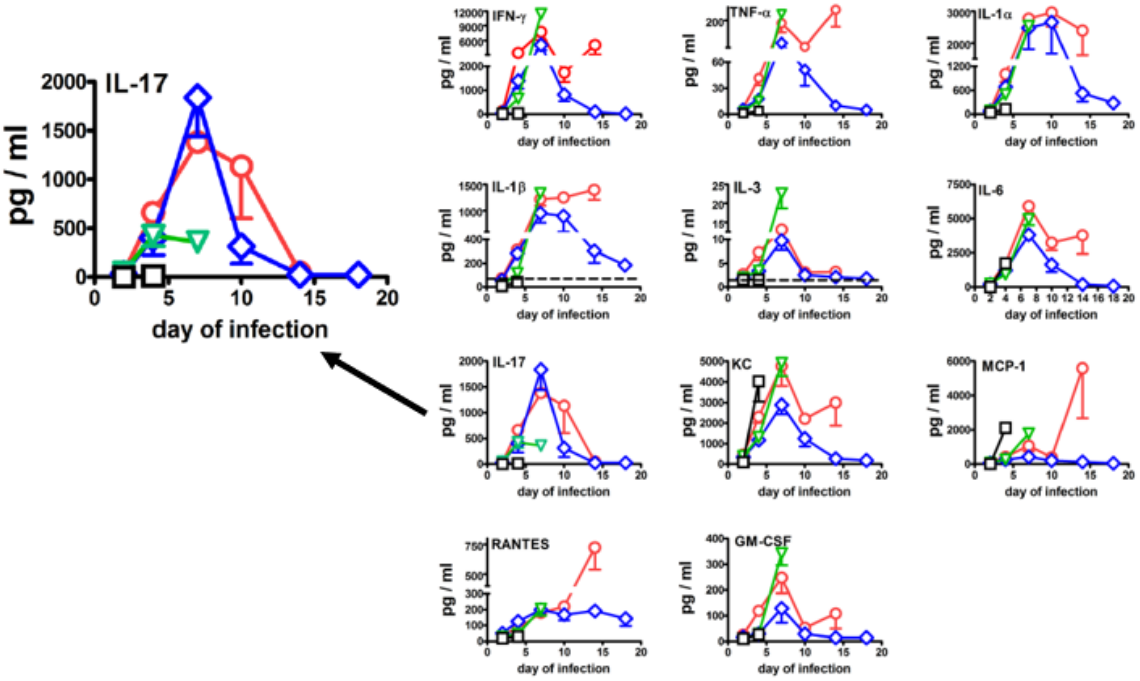

Figure 5. Changes in pulmonary cytokine and chemokine levels in naive mice (black) and mice immunized i.d. with 10^5 CFU of LVS (green), Δ*clpB* (blue), or Δ*fupA*Δ*capA* (red) and then challenged by aerosol six weeks later with SCHU S4. Image adapted from Shen *et al.* 2010.

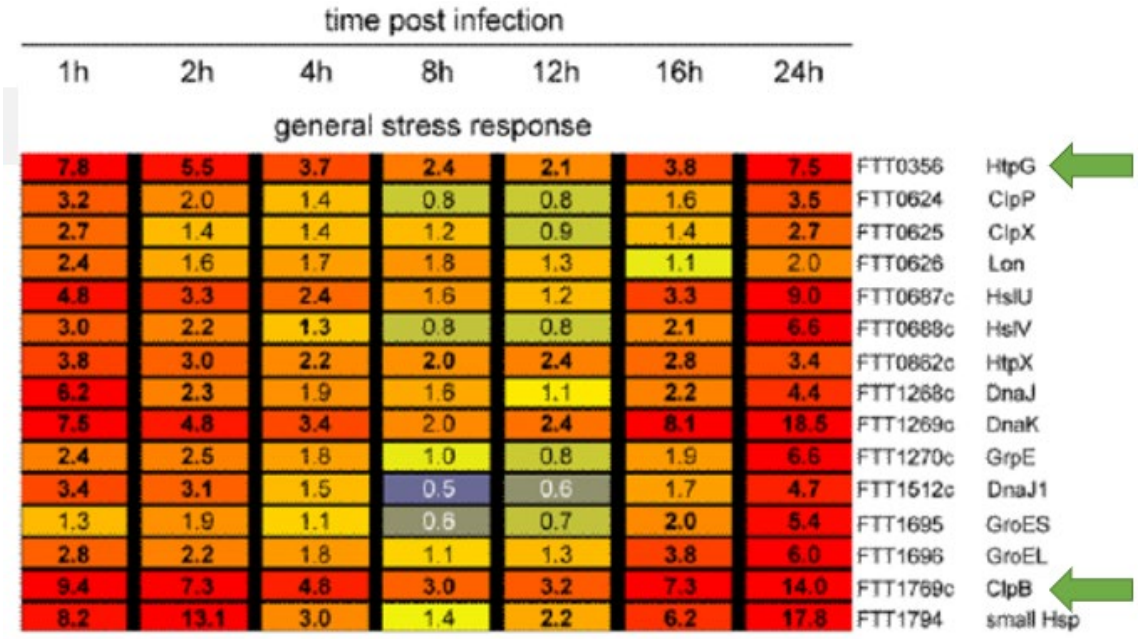

Figure 6. Correlation of differential expression profiles of SCHU S4 genes with specific stages of intracellular replication. Representations of mRNA are color-coded at all time-points analyzed, with orange-red indicating increased expression. The numbers indicate fold changes in mRNA levels relative to time zero, and bold numbers correspond to significant changes in mRNA levels as determined by statistical analysis. The green arrows indicate HtpG and ClpB. Image adapted from Wehrly *et al.* 2009.

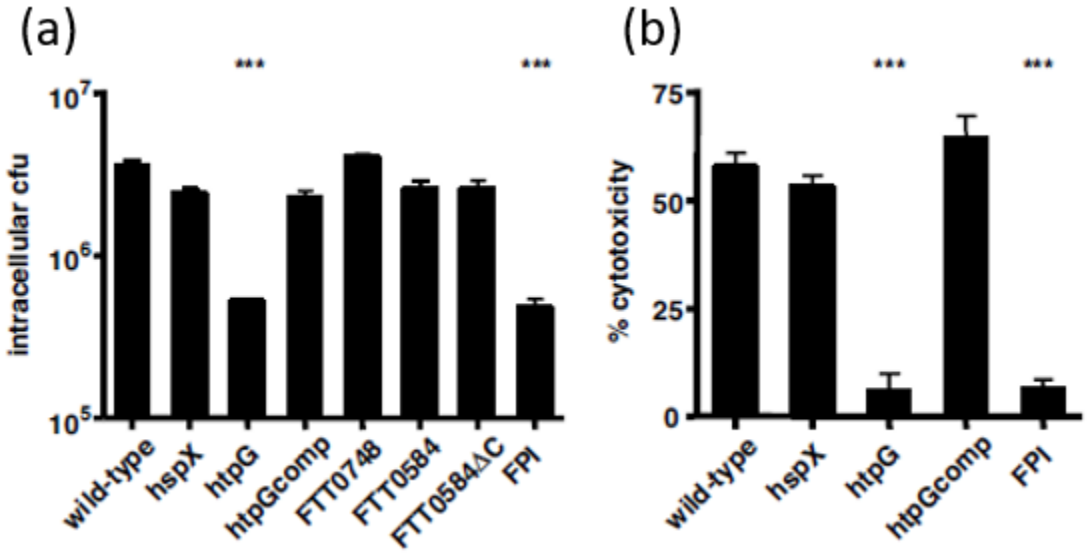

Figure 7. Bone-marrow derived macrophages were infected with *F. tularensis* subsp. *novicida* wild-type (U112 strain) and U112 transposon mutants at a multiplicity of infection of 10:1 for the indicated bacterial strains. (a) Cells were plated for enumeration 10-hours after infection, post-lysis. (b) Pre-activated macrophages were infected with the indicated strain and cell death was quantified by lactate dehydrogenase (LDH) release assay. Data are representative of three independent experiments. Statistical significance as compared with wild-type: ***, $P < 0.0005$. Image adapted from Weiss *et al.* (2007).

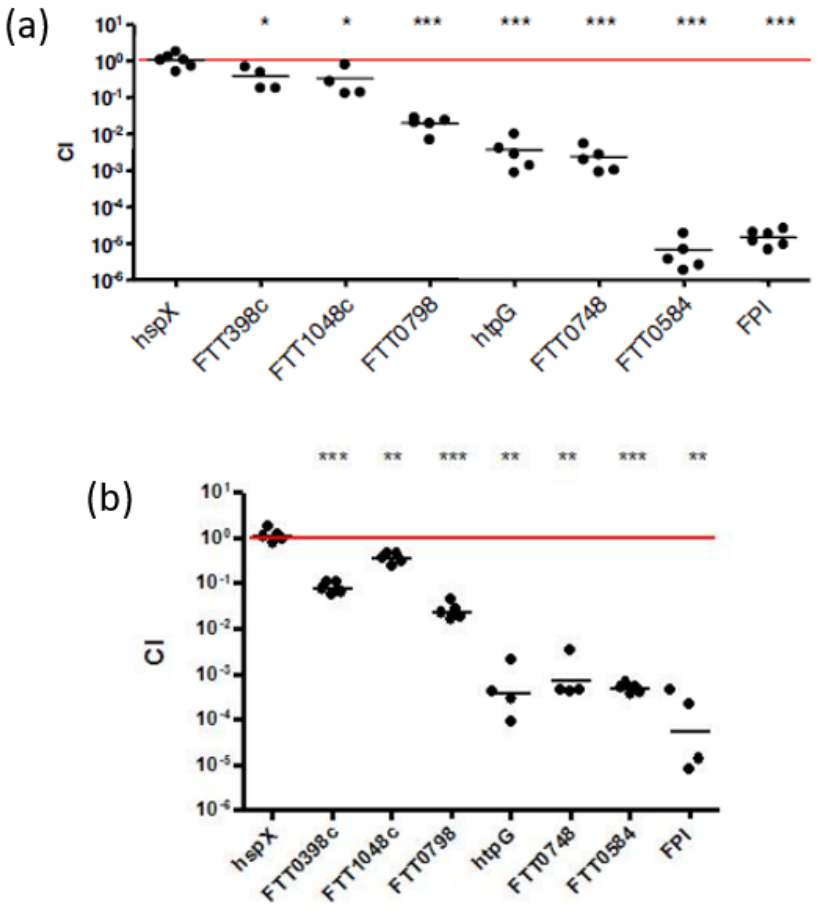

Figure 8. *F. tularensis* subsp. *novicida* transposon mutants after inoculation in mice. Groups of four or five mice were infected (a) subcutaneously or (b) intraperitoneally with a 1:1 mixture of wild-type *F. tularensis* subsp. *novicida* and the indicated mutant strain. The data represent the competitive index value for CFU of mutant/wild-type in the spleen 48-hours after infection. Bars represent the geometric mean CI value for each group of mice. $P < 0.005$ (**), $P < 0.0005$ (***). Image adapted from Weiss *et al.* (2007).

RESEACH DESIGN / METHODS

Animal Husbandry and Care

Female BALB/c mice will be purchased from Charles River Laboratories (St. Constant, Quebec, Canada) and will be acclimated prior to experimental procedures. Research will be conducted in compliance with the Animal Welfare Act (AWA, 7 U.S.C. §2131, 2002, 2007 and 2008) and other federal statutes and regulations relating to animals and experiments involving animals. Mice will be maintained and used in accordance with Institutional Animal Care and Use Committee (IACUC) requirements and guidelines. General procedures for animal care and housing will be in accord with the Association for Assessment and Accreditation of Laboratory Animal Care International recommendations. Veterinary care will be provided by an institutional on-call veterinarian (or designee).

Methods for anesthesia and euthanization follow IUCUC mandates for limiting discomfort, distress, and pain of animals in the research facility. For experimental procedures, mice will be anesthetized with a single intraperitoneal injection of 12.5 mg/ml ketamine + 3.8 mg/ml xylazine immediately prior to immunization or infection. All work with infectious bacteria will be performed in animal biosafety level 3 holding and experimental rooms. Throughout these studies, mice inoculated with vaccine strains or post-challenge with SCHU S4 will be examined daily for signs of infection and euthanized via CO_2 asphyxiation followed by cervical dislocation when they display signs of irreversible morbidity as indicated by abnormal appearance or activity.

Aim 1: Develop and characterize a SCHU S4 Δ*htpG* mutant *in vitro* (Months 01 – 09)

Experimental Outline

All work with infectious bacteria will be performed in a licensed, Select Agents approved biosafety level 3 facility. The SCHU S4 Δ*htpG* mutant will be generated by an in-frame deletion through use of the pJC84 suicide vector (Wehrly *et al.* 2009). A vector design and synthesis service (ATUM, Newark, CA) will be used to synthesize the pJC84-Δ*htpG* vector, using the pJC84 sequence (GenBank accession number FJ155667) with *htpG* inserted into the multiple cloning site. Through the pJC84-Δ*htpG* vector, in-frame *htpG* gene deletion will be accomplished through sacB-assisted allelic replacement and selection will be accomplished by a kanamycin resistance gene (Golovliov *et al.* 2003). Muller-Hinton broth cultures of SCHU S4 will be grown to the mid-exponential phase, washed with sucrose, and suspended in sucrose to obtain an approximate concentration 10^{10} CFU per ml according to optical density. pJC84-Δ*htpG* will be mixed with SCHU S4 and electroporated using an Eporator electroporator (Eppendorf, Hamburg, Germany) (Maier *et al.* 2004). After electroporation, cells will be briefly incubated at room temperature and plated on Muller-Hinton agar containing kanamycin for selection. Correct integration of the allelic replacement plasmid will be verified by assaying kanamycin-resistant clones with colony PCR using suitable primers for amplification of *sacB* and an internal fragment of the pJC84 backbone. Sucrose counterselection will be used to select against kanamycin-resistant clones. Cultures will be incubated with 5% sucrose before plating serial dilutions on Muller-Hinton agar supplemented with 8% sucrose and subsequent incubation for 2 days. Sucrose-resistant clones will then be plated on Muller-

Hinton agar supplemented with kanamycin to verify loss of the kanamycin-resistance gene. For verification, the final clone selected will be sequenced to detect correct allelic replacement, loss of the *sacB* gene, and loss of the kanamycin resistance gene.

The ATCC 29684 isolate of LVS will be used as a control throughout these studies. Wild-type SCHU S4 and SCHU S4 Δ*clpB* will be obtained from collaborating laboratories (USAMRIID, Frederick, MD; and National Research Council of Canada, Ottawa, Canada, respectively).

Stock cultures of all strains will be grown on blood cysteine glucose agar (BCGA) at 37°C for 24 h, harvested into sterile phosphate buffered saline (PBS), and stored at -80°C as single-use aliquots. For *in vivo* inoculation and challenge materials, stock cultures will be expanded in modified cysteine partial hydrolysate (MCPH) broth and incubated for 48 h at 37°C, shaking at 180 rpm. Bacteria will be centrifuged (4000 x *g*, 10 min), resuspended in PBS, and vialed. Target concentration will be a minimum of 10^{10} CFU per ml. Bacterial titer will be quantitated by serial dilution and plating on BCGA.

For *in vitro* analysis, murine BMMs will be generated by flushing BALB/c mice femurs with cation-free Dulbecco's PBS using a 23-gauge needle. Cells will be grown in DMEM supplemented with L929 cell-conditioned medium, heat-inactivated fetal bovine serum, L-glutamine, penicillin G, and streptomycin. Cells will be incubated at 37°C in 5% CO2 for 5 – 7 d until uniform monolayers of macrophages are established. Cells will be re-plated in either 6-, 12-, or 24-well culture-treated plates and primed with heat-killed SCHU S4 for 12 h prior to infection. BMMs will be infected in triplicate with SCHU S4 Δ*htpG*, LVS, SCHU S4 Δ*clpB*, and SCHU S4 at an MOI of 50 and washed after 1 h to remove extracellular bacteria. Uninfected cells will be included as controls. At 3-h

timepoints up to 24 h, intracellular growth will be quantified by lysis of BMMs with 0.5% saponin and removal of cell lysate for dilution and enumeration on BCGA. Separately, after 24 h, culture supernatants will be assayed for lactate dehydrogenase using an LDH assay kit (Promega, Madison, WI) according to the manufacturer's instructions to determine cytotoxicity. Uninfected cells will be lysed with 0.5% saponin to serve as a positive control and arbitrarily considered as 100% cell lysis. Sample absorbance will be expressed as a percentage of the positive control value. Both the intracellular growth and cytotoxicity assays will be run in triplicate and repeated independently three times. Results will be analyzed using a two-tailed unpaired Student's *t* test.

Expected Results and Potential Problems

Previous findings have shown a deficiency in intracellular replication and cytotoxicity for *F. tularensis* subsp. *novicida* Δ*htpG* in BMMs and S2 cells from *Drosophila melanogaster* (Weiss *et al.* 2007, Asare *et al.* 2010). We hypothesize that our SCHU S4 Δ*htpG* mutant will show a similar intracellular growth defect in BMMs and expect to see a significant reduction of intracellular CFU compared to wild-type SCHU S4. We also expect to see significantly reduced cytotoxicity in comparison to SCHU S4. If this is shown experimentally, it will indicate that SCHU S4 Δ*htpG* may be attenuated *in vivo*.

For controls, we expect to see significantly impaired intracellular growth and cytotoxicity in both LVS and SCHU S4 Δ*clpB* in comparison to SCHU S4 in alignment with previously reported results.

We could fail to confirm our hypothesis and not see impaired intracellular growth and/or cytotoxicity of SCHU S4 Δ*htpG*. This would contradict previous findings for *F. tularensis* subsp. *novicida* Δ*htpG* mutants. Since *htpG* has been identified as a *Francisella* virulence factor, including in SCHU S4, this result would point to a different role of *htpG* in contributing to SCHU S4 virulence which may be further elucidated by study *in vivo*. Any failure to see a deficiency in intracellular growth and cytotoxicity in positive controls (LVS and SCHU S4 Δ*clpB*) would indicate a problem with the assay, since these characteristics are well-established for these strains. Conversely, we expect to see normal growth and sterility of uninfected cells.

Aim 2: Establish the safety and efficacy of the SCHU S4 Δ*htpG* mutant against aerosol challenge by wild-type SCHU S4 *in vivo* (Months 09 – 18)

Experimental Outline

All work with infectious bacteria will be performed in a licensed, Select Agents approved biosafety level 3 facility. Immunization and challenge stocks will be prepared as previously described. Inocula will be prepared by diluting stocks to the appropriate concentration by serial dilution in sterile PBS.

Separate groups of mice (Table 1) will be challenged with 10^5 or 10^8 CFU of SCHU S4 Δ*htpG* and LVS via the i.d. and i.n. routes, and SCHU S4 Δ*clpB* via the i.d. route only. SCHU S4 Δ*clpB* will serve as a positive control based on known LD_{50} and median time to death for the i.d. route, and LVS will also serve as a control strain. Naive mice inoculated with saline will serve as the negative control. For i.d. immunization, 0.05 mL of saline

inoculum containing the given strain will be injected into a fold of skin in the mid-belly. For i.n. immunization, mice will be inoculated with 0.02 mL of saline inoculum containing the given strain and chased with 0.02 mL of saline. After six weeks, surviving mice dosed with 10^5 or 10^8 CFU will be challenged with ~20 CFU of SCHU S4 via aerosol. Aerosols will be generated with a Lovelace nebulizer operating at 40 psi to produce particles in the 4 – 6 μm range. Mice will be exposed using a Nose Only Exposure Chamber (In-Tox Products, Albuquerque, NM). Equipment parameters for exposure will follow published standards for delivery of < 20 CFU (Shen *et al.* 2004). Inocula concentration will be verified by plating on BCGA at the time of immunization or challenge.

Survival analysis will be performed using the log-rank test using Prism software for mice inoculated with SCHU S4 Δ*htpG*, SCHU S4 Δ*clpB*, and LVS survival curves, as well as pre-challenge with SCHU S4. Differences will be considered significant at $P < 0.05$. Median survival days post-challenge will be compared to determine comparative efficacy of SCHU S4 Δ*htpG* against LVS and SCHU S4 Δ*clpB* and compared to previously published results.

Expected Results and Potential Problems

Based on pre-existing evidence for the attenuation of *F. tularensis* Δ*htpG* strains, our hypothesis is that mice immunized with SCHU S4 Δ*htpG* (prior to SCHU S4 challenge) will exhibit greater survival data than mice immunized with LVS with statistical significance as measured by LD_{50}. Furthermore, we hypothesize that mice immunized with SCHU S4 Δ*htpG* will demonstrate median days of survival at least equivalent to those immunized with LVS after SCHU S4 challenge. This hypothesis is based on data from *F.*

tularensis subsp. *novicida* Δ*htpG* mutants demonstrating the capability for disseminated subclinical infection, which is associated with the ability to elicit a protective immune response. To validate results, comparisons will be made to published median time to death in mice immunized with i.d. LVS and SCHU S4 Δ*clpB* upon subsequent aerosol challenge of SCHU S4. The median time to death in BALB/c mice immunized i.d. with 10^5 CFU is approximately 8 d for LVS and >28 d for SCHU S4 Δ*clpB* (Conlan *et al.* 2010; Shen *et al.* 2004). We expect all naive mice challenged with SCHU S4 will succumb within approximately 5 d without any survivors.

It is possible that SCHU S4 Δ*htpG* will retain virulence in BALB/c mice. This could result in an inadequate number of surviving mice for subsequent challenge with wild-type SCHU S4, and would render the mutant strain inadequate as a single deletion vaccine candidate since it would not exceed the gold standard comparator strain (LVS) in safety. However, it still may hold promise for additional attenuating deletions to evaluate in a future study for a double deletion mutant. If SCHU S4 Δ*htpG* retains virulence at a dose of 10^5 CFU, we will inoculate an additional group of mice i.d. with a lower dose of 10^3 CFU, monitor for survival, and challenge again with SCHU S4. Conversely, it is possible that SCHU S4 Δ*htpG* will be significantly over-attenuated, resulting in inadequate protection upon subsequent SCHU S4 challenge in comparison to LVS. This would also render it inadequate as a vaccine candidate. In this case, we will use the higher dose (10^8 CFU) and attempt prime-boost immunization to see if efficacy can be obtained.

Table 1. Experimental design for Aim 2 animal groups, route of immunization, treatment, dose, animals per dose, and total animals.

Cohort	Route	Treatment	Dose (CFU)	Animals Per Dose	Total Animals
1	i.d.	Control	0	6	6
		SCHU S4 Δ*htpG*	10^5, 10^8	6	12
		LVS	10^5, 10^8	6	12
		SCHU S4 ΔclpB	10^5, 10^8	6	12
2	i.n.	Control	0	6	6
		SCHU S4 Δ*htpG*	10^5, 10^8	6	12
		LVS	10^5, 10^8	6	12

Aim 3: Assess course of sublethal infection and molecular immune responses in SCHU S4 Δ*htpG* immunized mice (Months 18 – 26)

Experimental Outline

All work with infectious bacteria will be performed in a licensed, Select Agents approved biosafety level 3 facility. Based on the results from the Aim 2 experiment, an additional 20 mice will be immunized with SCHU S4 Δ*htpG* for the dosing scheme which was at least as effective as LVS for protecting against SCHU S4 challenge, including LVS and SCHU S4 Δ*clpB* as controls. Following, mice will be sacrificed on each day 2, 4, 7, and 14 timepoint (Table 2).

At each timepoint, course of infection will be assessed by removal of a 1 cm^2 piece of skin at the inoculation site along with spleens and lungs. Tissues will be homogenized using aerosol-proof homogenizers and diluted in sterile saline. To determine bacteremia, cardiac puncture will be performed at the time of necropsy for collection of whole blood. Blood will be diluted 1:10 in sterile water for lysis and further diluted in sterile saline. Dilutions of tissue homogenate and blood will be split for quantitation of bacterial burden and molecular immune response analysis. For quantitation of cytokine, chemokine, and antibody concentrations, blood and homogenates will be clarified via centrifugation. Serum and organ homogenates will be sterilized through a 0.22 μm membrane and stored at -20°C until needed.

For quantitation of bacterial burden, dilutions of pre-clarified blood and tissue homogenates will be serially diluted in sterile saline, plated on BCGA, and bacterial colonies will be enumerated. Bacterial burdens will be analyzed using a two-tailed Mann-

Whitney U test using Prism software. Values of $P < 0.05$ will be considered statistically significant.

Cytokine and chemokine concentration will be determined using MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel Immunology Multiplex Assay (MilliporeSigma, Burlington, MA) on the Luminex® 200 system (Luminex, Austin, TX). This kit will be used for analysis of TNF-α, INF-γ, IL-6, IL-17, KC, MIP-1β, and MCP-1. Additional cytokines and chemokines included in this 25-plex kit will be analyzed for information only. Cytokine/chemokine concentrations for SCHU S4 Δ*htpG,* SCHU S4 Δ*clpB*, and LVS will be calculated against the standards using BeadView software and compared.

For quantitation of antibody response, IgG and IgM ELISA titers against killed SCHU S4 will be determined as previously described (Pasetti *et al.* 2008). Briefly, heat and formalin-killed SCHU S4 antigens will be obtained from ATCC (Manassas, VA). The SCHU S4 antigens will be bound to 96-well plates. Non-specific binding sites on the plates will be blocked by incubating the plates with solutions containing BSA. Serial dilutions of the mouse serum samples will then be added to each well and allowed to incubate. Non-bound materials will be removed by washing. SCHU S4 specific antibodies will be detected using goat anti-mouse IgG or goat anti-mouse IgM horseradish peroxidase-labeled antibodies. Titers will be calculated from linear regression curves and expressed as ELISA Units per ml. Titers between SCHU S4 Δ*htpG,* SCHU S4 Δ*clpB*, and LVS will be compared by one-way ANOVA. Results for SCHU S4 Δ*clpB* and LVS will be validated by comparison to published values.

Expected Results and Potential Problems

Our hypothesis states that SCHU S4 Δ*htpG* infection in BALB/c mice will be characterized by dissemination from the inoculation site which is required for a robust immune response, and this is consistent with previous findings with respect to *htpG* deletion mutants of *F. tularensis* subsp. *novicida*. Therefore, we expect that SCHU S4 Δ*htpG* immunized mice will exhibit bacteremia (in blood) and bacterial load (in organs) which is at least statistically equivalent to that of LVS. For the SCHU S4 Δ*clpB* control, we expect that bacteremia and bacterial load in immunized mice will be significantly greater than that of LVS immunized mice, in alignment with previously published results.

For chemokine and cytokine response, we hypothesize that SCHU S4 Δ*htpG* efficacy will be correlated with elevated pulmonary IL-17 in alignment with results for other SCHU S4 single-deletion mutants (Ryden *et al.* 2013). Further, we hypothesize that protection will correlate with significantly elevated serum and splenic levels of TNF-α, INF-γ, IL-6, KC, MIP-1β, and MCP-1. For SCHU S4 Δ*htpG* immunized mice, we expect that these levels will be at least equivalent to LVS immunized mice. This hypothesis is based on the expectation that SCHU S4 Δ*htpG* will be capable of measurable, robust immune response. For SCHU S4 Δ*clpB* immunized mice (controls), we expect that these levels will be significantly elevated over LVS immunized mice in alignment with previously published results (Ryden *et al.* 2013; Shen *et al.* 2010). We offer no hypothesis for IgM/IgG titer results – these are analyzed for information only.

A potential problem is that we will fail to confirm our hypothesis that SCHU S4 Δ*htpG* immunized mice will show blood bacteremia and organ bacterial load at least equivalent to that seen with LVS immunized mice. Since the conduct of Aim 3 is

dependent on efficacy seen as a result of at least one dosing scheme used in Aim 2, this is an unlikely result given the correlation between dissemination of vaccine strains and efficacy. We further expect that dissemination and efficacy will be correlated with a robust immune response as measured by elevated cytokine and chemokine levels. Therefore, a negative result here would more likely indicate a problem with conduct of the experiment than a true negative result. If SCHU S4 Δ*htpG* is protective against SCHU S4 aerosol challenge, we expect to see elevated IL-17 in the lungs in alignment with results seen for SCHU S4 Δ*clpB* and LVS.

Similarly, for our SCHU S4 Δ*clpB* control, we expect to see elevated cytokine and chemokine levels as previously described. If we fail to see such levels, this would again indicate a problem with the conduct of the experiment. If we did see such elevated levels in SCHU S4 Δ*clpB* immunized mice, but not SCHU S4 Δ*htpG* immunized mice, this may indicate a problem with the Aim 2 experiment since it would verify the cytokine/chemokine assay, but would cast doubt that SCHU S4 Δ*htpG* is protective against aerosol challenge of SCHU S4.

Table 2. Experimental design for Aim 3 animal groups, route of immunization, treatment, dose, animals per dose, and total animals.

Cohort	Route[1]	Treatment	Dose (CFU)[1]	Animals Per Timepoint[2]	Total Animals
3	i.d. or i.n	SCHU S4 Δ*htpG*	10^5 or 10^8	5	20
		SCHU S4 Δ*clpB*	10^5 or 10^8	5	20
		LVS	10^5 or 10^8	5	20

[1]Most effective route and dose as evaluated in Aim 2 experiment for SCHU S4 Δ*htpG* immunization (one route and dose).
[2]Day 2, 3, 4, and 14.

Future Work

If our study shows that SCHU S4 Δ*htpG* is more attenuated than and at least as protective as LVS against SCHU S4 aerosol challenge, we would propose further work to evaluate double deletion mutants with additional gene deletions to assess if SCHU S4 Δ*htpG* may be capable of additional attenuating mutations while retaining efficacy. This is the overall goal of our effort. We would also propose to further elucidate the mechanism of attenuation for SCHU S4 Δ*htpG*, determining how deletion of this gene contributes to *F. tularensis* virulence. This would be necessary to pursue SCHU S4 Δ*htpG* mutants as potential human vaccine candidates to meet regulatory requirements.

If our study shows that SCHU S4 Δ*htpG* is protective, but less attenuated than LVS, we would repeat our Aim 2 *in vivo* analysis using a lower dose (e.g., 10^3 CFU). This would determine whether SCHU S4 Δ*htpG* virulence is dose dependent. We would also pursue double deletion mutants to determine if we can identify an attenuated phenotype that retains efficacy. If SCHU S4 Δ*htpG* retained its virulence such that protection could not be analyzed, this pursuit of a double mutant would represent the most reasonable approach.

Finally, if our study shows that SCHU S4 Δ*htpG* is not protective, but highly attenuated, we could attempt a prime-boost dosing scheme to see if it is capable of protection with the addition of booster doses. However, if prime-boost administration were effective, this mutant would likely be less tolerant of multiple attenuation deletions while retaining efficacy since it would theoretically be near or at maximum attenuation. We would attempt to confirm this alongside any additional study using prime-boost administration by introducing several double-deletion mutants into the study.

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