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Tularemia vaccine development: paralysis or progress?

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Abstract

Francisella tularensis (*Ft*) is a gram-negative intercellular pathogen and category A biothreat agent. However, despite 15 years of strong government investment and intense research focused on the development of a US Food and Drug Administration-approved vaccine against *Ft*, the primary goal remains elusive. This article reviews research efforts focused on developing an *Ft* vaccine, as well as a number of important factors, some only recently recognized as such, which can significantly impact the development and evaluation of *Ft* vaccine efficacy. Finally, an assessment is provided as to whether a US Food and Drug Administration-approved *Ft* vaccine is likely to be forthcoming and the potential means by which this might be achieved.

Keywords

Sex bias; media impact; differential protection; cellular immunity; humoral immunity

Introduction

Fifteen years after the 2001 attacks on the World Trade Center in New York City, when it was recognized that organisms such as *Francisella tularensis* (*Ft*) could be utilized as a biothreat agent, a US Food and Drug Administration (FDA)-approved vaccine for tularemia remains an elusive goal. This is despite extensive financial investment since 2001 in research and development of such a vaccine. The question thus remains as to whether an *Ft* vaccine is still possible. This review discusses what we have learned since 2001, the confounding factors that may have helped to produce for some a sense of paralysis in the tularemia vaccine field, and whether an FDA-approved tularemia vaccine remains plausible.

Microbiology and etiology of *Ft*

Based on DNA similarity and fatty acid composition, the genus *Francisella* has been classified into three species: *F. tularensis* (*Ft*), *F. philomiragia*, and *F. hispaniensis*.[1] *Ft* is further classified into five subspecies of *Ft tularensis* (also called *Ft* type A), *Ft holarctica* (*Ft*

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type B), *Ft novicida*, *Ft mediasiatica*, and a variant of *Ft holarctica* found in Japan.[2] In the case of *F. novicida*, it should also be noted that based on the high degree of genetic relatedness between *Ft* and *F. novicida*, *F. novicida* was assigned as a subspecies of *Ft* in 2006. However, there was a formal objection in favor of *F. novicida* being designated as its own species in 2010,[3] in which it was suggested that the original assignment was based solely on genetic relatedness and did not take into consideration the phenotypic and genomic difference between *Ft* and *F. novicida*. However, despite this objection, the original assignment of *F. novicida* as a subspecies of *Ft* was reaffirmed.[4] More importantly, of the aforementioned species, only types A and B are the major causes of human disease, whereas *F. novicida* is virulent in mice but avirulent in humans.[5] *F. philomiragia* is a muskrat pathogen. *Ft* type A is a highly virulent organism exclusively found in North America and is associated with rabbits and a wide range of arthropod vectors.[6] *Ft* type A is also more genetically diverse and evolutionarily older than the moderately virulent *Ft* type B.[2] Furthermore, molecular characterizations have identified two distinct clades or genotypes of *Ft* type A that differ in their geographic location and virulence.[7,8] In contrast, *Ft* type B is generally less virulent and is associated with semiaquatic rodents, hares, ticks, and mosquitoes. It is widely distributed throughout much of the northern hemisphere and is the only species found in Europe.[6] In addition, molecular typing studies have identified three distinct biovariants of *Ft* type B that differ in antibiotic resistance pattern and geographic locations in Europe.[9] The live vaccine strain (LVS) is an attenuated variant of the *Ft* subspecies *holarctica*. However, *Ft* type A represents the greatest concern in terms of bioterrorism and human disease in that it is highly virulent and intradermal (ID) or inhaled exposure to just ten to 50 bacteria can cause severe infection and death.[10,11]

The immune response to *Ft* and correlates of protection

It is generally believed that immune responses to *Ft* are induced through traditional mechanisms of immune response induction, which include *Ft* antigen (Ag) uptake, processing, and presentation by B-cells, dendritic cells (DCs), macrophages (MØs), and subsequent *Ft*-specific T- and B-cell activation. Thus, the key to developing an effective vaccine against *Ft* is a clear understanding of those immune components required for protection. Furthermore, the route of infection, as well as bacterial virulence, will ultimately determine the degree of protection achieved by a given mode of vaccination. *Ft* can infect the host through multiple routes: ulceroglandular (through skin scratch), pneumonic (through lungs), oropharyngeal (through gastrointestinal tract), oculoglandular (infection through conjunctiva), and typhoidal (ingestion may be the mode of transmission).[12–14] It is also important to note that although all subspecies of *Ft* have been demonstrated to infect humans, most studies focused on understanding the immune response to *Francisella* have utilized mice. In addition, it is important to note that the type A strain is highly virulent in both humans and mice.[2,15] Similarly, type B *Ft holarctica* strain, which includes *Ft* LVS, is virulent in both mice and humans, with mice, however, being much more susceptible.[5]

Humoral immunity

The role of humoral immunity in the resolution of infection and protection against *Ft* remains controversial, in part because of the conventional wisdom asserting that cellular

immune responses are more important for protection against intracellular pathogens.[16] However, a number of investigations have demonstrated that humoral immunity can play a role in protection against tularemia, consistent with the observation that *Ft* has been shown to have an extracellular phase.[16–18] Furthermore, studies have demonstrated that both mouse and human antibody (Ab) responses are similar in terms of Ag recognition, with the Ab being predominantly directed against bacterial lipopolysaccharides (LPS). In the case of humans, a robust Ab response is generated within 2 weeks of immunization or infection, while the peak Ab response in mice is 7 weeks after infection.[16,19–22] Furthermore, studies have clearly shown a role for both immunoglobulin (Ig) A and IgG in protection.[16,19,22–31] Specifically, passive immunization of naive mice with immune sera from *Ft* LPS, heat-killed *Ft* LVS, or live *Ft* LVS-immunized animals affords protection against a subsequent *Ft* LVS infection. Nevertheless, *Ft* SchuS4-challenged mice are not protected.[16,22,30,31] Moreover, passive transfer of *Ft*-specific IgM or IgG provided protection against *Ft* LVS infection.[25] Furthermore, serum isolated from humans immunized with *Ft* LVS induced significant protection in mice against *Ft* LVS infection.[29] Most significantly, however, passive immunization of naive mice with immune serum from the mice that survived *Ft* SchuS4 infection following levofloxacin treatment exhibited protection against *Ft* SchuS4 challenge in recipient mice.[27] In other studies, passive transfer of Ab specific for the membrane protein fraction of *Ft* LVS alone could augment low-dose gentamicin treatment and provide protection against an *Ft* SchuS4 respiratory challenge, when administered on days 1 and 4 post-challenge.[32] Additional studies supporting a role for Abs demonstrated that vaccine-induced immunity against pulmonary tularemia is lost in IgA-deficient mice.[16,23,24,28] In the case of IgG-mediated protection, it is also important to note that Fcγ receptors (FcγRs) are required.[16,22] Specifically, Kirimanjeswara et al[22] demonstrated that intraperitoneal inoculation of naive recipient mice with immune sera from *Ft* LVS-immunized animals could successfully protect recipient wild-type (WT) mice against an IN *Ft* LVS challenge. The protective capability of the *Ft* LVS-specific immune sera was, however, lost when FcγR common γ-chain knockout (KO) mice were used as naive recipients.[22]

In conclusion, while it is generally accepted that Abs do mediate protection in the case of *Ft* LVS (type B) challenge, in the case of *Ft* SchuS4 (type A) challenge, the importance of Ab is more controversial. Specifically, it remains unclear that generation of Ab alone via vaccination will be sufficient to provide full and consistent protection against *Ft* type A challenge.

Cell-mediated immunity

It has been believed for more than 2 decades that cell-mediated immunity (CMI) plays a critical role in protection against tularemia.[16] This thinking was due, in part, to the intracellular nature of *Ft* infection. Thus, early studies investigating the role of CMI have focused on CD4+ and CD8+ T-cells,[33] although emerging evidence is also showing critical roles for DCs, MØs,[34] polymorphonuclear neutrophils (PMNs), and natural killer (NK) cells.[35]

With regard to T-cells, it has also been suggested that T-cells are the primary cell population responsible for mediating immunity against *Ft*.[16] Specifically, both CD4 and CD8 T-cells

can proliferate and produce interferon-γ (IFN-γ) in response to a number of *Ft* proteins.[33] Additionally, depletion of CD4 T-cells, CD8 T-cells, or IFN-γ abolishes vaccine-induced immunity against type A *Ft* SchuS4 infection.[36,37] In addition, studies have demonstrated that passive protection observed when administering *Ft*-specific Ab to naive recipient mice is dependent on IFN-γ and mature T-cells, in that mice depleted of IFN-γ or athymic nude mice were not protected against *Ft* LVS infection following adoptive transfer of mouse immune sera.[16,25]

Infected MØs are the predominant site of bacterial replication within the host, somewhat surprisingly, depletion of alveolar MØs using liposomal clodronate does not impede disease progression and death in mice infected IN with *Ft* LVS.[22] This result is likely due, in part, to *Ft*'s ability to replicate in other host cells, including epithelial cells and DCs.[35,38–40] Specifically, multiple studies have found that *Ft* can infect DCs, interfere with DC maturation, and thereby dampen the immune response during the first 72 hours of infection, leading to unhampered growth and spread to systemic organs.[16,35,39,41] Nevertheless, it has also been demonstrated in the clodronate study that alveolar MØs are critical for passive Ab-mediated protection, since when these cells are depleted, protection is lost.[22] In this regard, it has also been shown that alveolar MØs do internalize and kill *Ft*, when treated with IFN-γ and immune sera.[22] Thus, MØs play a role in pathogen clearance, which is optimal when *Ft*-specific Ab and IFN-γ are present.

In the case of PMN, their role in resolving *Ft* infection, similar to that of Ab's role in resolving *Ft* type A infection, is controversial. While Ab-mediated depletion of PMN suggests these cells are essential for surviving a primary intradermal (ID) or intravenous (IV) *Ft* infection, this is not the case for intranasal (IN) challenge.[35,42,43] It has been demonstrated that neither depletion nor recruitment of PMN to the lungs of *Ft* SchuS4 IN-infected mice impacts bacterial burden or survival time.[43] Interestingly, IFN-γ-producing PMNs are detectable at the site of infection within 72 hours, suggesting a potentially protective role for cytokines released by these cells.[16,35,43,44] It has also been demonstrated that Ab-mediated protection of passively transferred *Ft* LVS-specific immune sera is lost when PMNs are depleted and mice are subsequently challenged IN with *Ft* LVS.[22]

NK cells are an early responder to *Ft* infection and thus thought to be an early source of IFN-γ.[45,46] Furthermore, NK cells are key for regulating hepatic granuloma formation, which helps in controlling bacterial spread.[47] Interestingly, NK cell depletion decreases mean survival time following primary infection but does not affect vaccine-induced immunity, since fewer NK cells are recruited to the lung of immunized and challenged mice compared to unimmunized controls.[23,45] This suggests that while NK cells are early responders to infection and produce IFN-γ, they are only necessary following a primary exposure of naive individuals.

In conclusion, with regard to the aforementioned cells and vaccine development, it appears clear that the induction of memory T-cells, and in particular IFN-γ-producing T helper 1 (Th1) cells, is likely to be key to developing an effective vaccine strategy against *Ft*. In support of this contention, studies have also shown that while both Ab and IFN-γ can be critical for vaccine-induced protection,[24] the need for Ab can be overcome, when IFN-γ

levels are sufficiently high.[28] Nevertheless, evidence also suggests that Ab can play a protective role by supplementing the protective impact of IFN-γ in vaccine-induced protection against *Ft* type A infection.

The immune response in human *Ft* infection and vaccination

The human immune response to *Ft* infection and vaccination has been reviewed elsewhere.[48] Briefly, in the case of natural infection, *Ft*-specific IgM, IgG, and IgA Abs are detectable ~2 weeks after infection. Similar to *Ft* infection in mice, the majority of the Ab response is directed to *Ft* LPS.[21] Also similar to that observed in mice, ex vivo production of Th1-type cytokines such as IFN-γ, TNF-α, and IL-2 by CD4 and CD8 T-cells is observed by restimulated lymphocytes obtained from tularemia-infected individuals.[49,50] Similar to natural infection, in the case of vaccination utilizing *Ft* LVS administered via scarification, *Ft*-specific IgM, IgA, and IgG Abs are detected in serum 2 weeks post vaccination, while lymphocytes from vaccinated individuals restimulated ex vivo produce Th1-type cytokines, in particular IFN-γ.[20,51] However, it is also important to note that in the case of Ab responses, similar to mice infected with *Ft*, the generation of anti-*Ft* Abs is not necessarily predictive of protection against subsequent infection with virulent *Ft* type A organisms.

Ft vaccine strategies

Due to its high infectivity, high mortality rate at a very low infectious dose (ten to 50 organisms), and its ability to be aerosolized, *Ft* has been designated a category A biothreat agent by the Centers for Disease Control and Prevention (CDC). The need for a vaccine is further emphasized by the fact that although WT strains of *Ft* do respond to antibiotic treatment, which includes fluoroquinolones, tetracyclines, and the aminoglycosides,[52] *Ft* strains have been engineered to be antibiotic resistant.[14,15] Furthermore, despite extensive research and investment over the last 15 years, there remains no FDA-approved vaccine. Thus, there remains a critical need for an effective *Ft* vaccine. Various strategies, which have been used to accomplish this, are discussed subsequently.

Live attenuated vaccines

Live attenuated vaccines have shown the greatest promise thus far, although concerns about reversion remain a significant roadblock to their use as an *Ft* vaccine. A number of live attenuated vaccine candidates were made from *Ft holarctica* by the Soviets in the 1940s and 1950s.[53] However, *Ft* LVS is the only vaccine available in the West to combat tularemia.[54] However, although *Ft* LVS does provide partial protection against a type A challenge in humans,[54] it is not licensed in the USA, primarily due to the uncertainty regarding its source of attenuation and it instability in culture.[53,55] However, despite the fact *Ft* LVS is not approved as a vaccine, considerable additional money and effort have been devoted to the development of a safe and efficacious attenuated *Ft* vaccine using *Ft* LVS (Table 1), *F. novicida* (Table 2), and *Ft* SchuS4 (Table 3). Our own studies (unpublished data) and that of others[56] using a *SodB* mutant of *Ft* LVS have clearly demonstrated the potential for generating protection against a primary infection, as well as high-dose secondary exposure to *Ft* SchuS4, when immunizing with this attenuated organism (Figure 1). Thus, should a fully protective attenuated vaccine be developed, in which safety concerns are eliminated or

further minimized, possibly via multiple targeted/well-defined mutations, this approach could still produce a strong *Ft* vaccine candidate.

Inactivated Ft vaccines

Over 70 years ago, Foshay and his research group attempted to develop the first killed vaccine for tularemia.[57,58] Although Foshay's vaccine preparations were able to protect nonhuman primates against *Ft* SchuS4,[53] they exhibited significant toxicity, including the generation of necrotic lesions. In addition, there was no significant protection observed in laboratory workers or in subsequent controlled animal trials.[57,58] Consistent with the latter study, more recent attempts at developing a killed *Ft* vaccine have also met with mixed success.

While incorporation of Freund's adjuvant into killed (phenol–merthiolate treated) *Ft* LVS or *Ft* SchuS4 did not augment *Ft* vaccine efficacy,[59] a heat-killed *Ft* LVS vaccine formulated with IL-12 expressed in a vasicular stomatitis virus-based vector generated enhanced *Ft* LVS clearance versus nonadjuvanted vaccine.[31] In another study, mucosal vaccination with inactivated *Ft* (i*Ft*) LVS (paraformaldehyde or UV treated) in combination with IL-12 conferred >90% protection against lethal *Ft* LVS challenge. This protection was correlated with enhanced bacterial clearance, reduced tissue inflammation, and increased *Ft*-specific serum IgG and IgA Ab responses. However, this strategy proved ineffective at protecting against an *Ft* SchuS4 challenge.[23] Similarly, while Eyles et al[60] showed that intramuscular immunization of BALB/c mice with i*Ft* adjuvanted with immune-stimulating complexes (ISCOMS) or preformed ISCOMS admixed with immunostimulatory CpG oligonucleotides provided robust protection against respiratory challenges with *Ft holarctica* HN63, the same vaccine formulation did not protect against a low-dose aerosol challenge with *Ft* SchuS4.

An alternative approach to the use of adjuvants involved the use of FcγR-targeted monoclonal antibody (mAb)-i*Ft* immune complexes (ICs). Such ICs, when administered IN, induced full protection against *Ft* LVS challenge and up to 50% protection against *Ft* SchuS4 challenge.[24] Consistent with this increased protection, enhanced humoral and cellular immune responses were also observed, as compared to i*Ft* administered alone.[24] Bitsaktsis et al[28] also demonstrated that the addition of CTB adjuvant to i*Ft* could similarly induce complete protection of mice challenged with *Ft* LVS and partial protection of *Ft* SchuS4-challenged mice. The observed protection also correlated with enhanced production of IFN-γ, as was also the case in the studies using mAb-i*Ft* ICs as immunogen.[24] Thus, while killed vaccines are less likely to generate a strong cellular immune response, as apposed to attenuated vaccines,[23] successful protection against an *Ft* type A strain was observed. Importantly, it is also believed that killed vaccines provide a significant safety advantage versus attenuated vaccines.

Subunit vaccines

From the perspective of manufacture, safety, and FDA approval, an ideal vaccine against tularemia would use a recombinant subunit approach, which would eliminate the potential for reversion that could occur with live attenuated vaccines and significantly reduce the potential for toxicity, which could occur with killed vaccines. However, to date, no *Ft*

proteins capable of generating strong protective immunity against an *Ft* type A challenge have been identified.[15,33,61–63] Additionally, while LPS purified from *Ft* LVS, or as a part of a crude membrane fraction, has been utilized as a vaccine candidate and offers some protection against *Ft* LVS infection, LPS has proven ineffective as a protective immunogen against *Ft* SchuS4 challenge, making the development of a subunit vaccine against *Ft* difficult at this time.[5,19,30,64–67] Additional efforts to develop such a vaccine have included vaccination of mice with O-Ag capsular polysaccharide in the presence of adjuvant or chemically conjugated to bovine serum albumin, which enhanced protection against *Ft* LVS but failed to protect mice against aerosol challenge with more virulent strains of *Ft*.[64,68] Additionally, while immunization of mice with *Ft* LVS LPS in the presence of PorB, a porin produced by *Neisseria meningitidis* and a TLR2 ligand, enhanced the survival of mice challenged with *Ft* LVS, additional studies are still required to determine whether this approach is effective against the more virulent subspecies of *Ft*.[69] Furthermore, an LPS immunogen derived from *Ft* SchuS4 did not generate protective immunity against a subsequent *Ft* SchuS4 challenge, although it did provide protection when mice were challenged with *Ft holarctica*.[67,70]

Other bacterial components have also been investigated for use in a subunit vaccine but with limited success. Tul4, an *Ft* surface lipoprotein, when administered alone did not generate immune responses capable of controlling *Ft* LVS bacterial replication following IV challenge.[71] In addition, immunizing mice with Tul4 and DnaK, an *Ft* heat shock protein, in the presence of GPI (a semi-synthetic triterpene glycoside adjuvant) could also induce significant protection of mice against a respiratory challenge with *Ft* LVS. However, the effectiveness of this approach in protecting against an *Ft* SchuS4 challenge was not reported.[72] Other studies utilized intraperitoneal immunization with *Ft* outer membrane proteins emulsified in Freund's adjuvant, which did protect ~50% of mice challenged IN with *Ft* SchuS4, although the specific protein responsible for this protection was not identified.[66] Because of the abundance of the *Ft* outer membrane protein A (FopA) and the knowledge that FopA-specific Abs are found in sera of recovering patients, Hickey et al[73] sought to determine whether FopA would provide protection against *Ft* challenge. Although FopA immunization in the presence of IL-12 and aluminum hydroxide did protect mice against IN or ID *Ft* LVS challenge, it did not provide protection against an ID *Ft* SchuS4 challenge.[73] Thus, while numerous studies have focused on utilizing/identifying *Ft*-Ag that could be incorporated into an *Ft* subunit vaccine, the key requirement for a subunit vaccine, identification of a single Ag that confers effective protection against type A *Ft* strain, still has not been met.

Bacterial and viral vector vaccines

Attenuated microorganisms such as bacteria and viruses have been successfully used as vehicles to deliver vaccine Ags. Furthermore, the advent of genetic engineering has facilitated the alteration of pathogenic microorganisms, thereby attenuating them and allowing them to serve as vehicles for heterologous Ags. In addition, intrinsic characteristics of microorganisms, such as LPS and other pathogen-associated molecular pattern molecules, enable such vehicles to evoke strong innate immune responses, which can in turn guide a robust adaptive immune response against the target Ag(s)/organism.[74,75] A number of

microbes have been developed for this purpose: *Salmonella*, *Listeria monocytogenes*,[76] *Vibrio cholerae*, lactic acid bacteria,[77] *Bordetella pertussis*,[78] and *Mycobacterium bovis*,[79] and viruses, such as adenovirus, retrovirus, lentivirus, cytomegalovirus, and Sendai virus.[75] However, to date, only a few attempts have been made to develop a tularemia vaccine using microbial vectors. Jia et al used *L. monocytogenes* to deliver a number of *Ft* proteins. However, only the expression of IglC by this organism led to 100% protection against lethal *Ft* LVS challenge. However, the results of the type A challenge are open to interpretation, in that although immunization with the vector-expressing IglC provided 80%–100% protection, immunization with the vector control, which lacked the Ags, generated 40%–50% protection.[80] In another study, Fulop et al[81] used *Salmonella enterica* serovar Typhimurium to deliver *Ft* FopA protein. However, this vaccine failed to induce significant protection against *Ft* LVS challenge. More recently, Banik et al used TMV as a vaccine vehicle for OmpA, DnaK, and Tul4 Ags. They incorporated these Ags into the TMV vector either together in a single virion (monoconjugate vaccine) or in separate virions (multiconjugate vaccine), which were then mixed to introduce all three Ags into the host. Both strategies elicited moderate levels of protection against a high-dose challenge with *Ft* LVS.[82] Thus, despite some promising results with *Ft* LVS challenge, this approach has also failed to provide an effective vaccine strategy against *Ft* type A strains. As with subunit vaccines, this failure may also primarily stem from the lack of identified *Ft* Ags capable of inducing protection against the highly virulent type A *Ft* strains.

FcγR-targeted vaccines

Targeted vaccines direct an immunogen to a specific immunological target, such as a specific cell type or receptor, in order to stimulate an enhanced host immune response. One of the primary functions of FcγR is to mediate internalization (phagocytosis), processing, and presentation of Ag.[24,83,84] Consistent with this function, Rawool et al[24] demonstrated that paraformaldehyde i*Ft*, when administered IN in the form of mAb-i*Ft* IC, induces full protection against *Ft* LVS challenge and partial protection against *Ft* SchuS4 challenge, as apposed to that of i*Ft* alone, which provided 50% and 0% protection, respectively. Consistent with the increased protection, humoral and cellular immune responses were also enhanced, and the use of traditional adjuvant was not required.[24] In regard to mechanisms involved in the FcγR-enhanced protection against *Ft* challenge, Iglesias et al demonstrated that when administered IN, the transport of i*Ft* from the nasal passage to the nasal associated lymphoid tissue is significantly enhanced, when in the form of mAb-i*Ft*. In addition, the rate of i*Ft* binding and internalization by antigen-presenting cells (APCs) is also significantly enhanced, as well as the length of time over which the i*Ft* is presented by APCs to T-cells is extended.[85] These studies were also followed up by more extensive mechanistic studies focused on in vivo responses to IN immunization with mAb-i*Ft* versus i*Ft*. Specifically, Bitsaktsis et al[86] demonstrated that as apposed to IN administration of i*Ft* alone, direct targeting of i*Ft* to FcγR via mAb-i*Ft* IC elicits a higher frequency of activated DCs within the lung of mAb-iFt-immunized mice following *Ft* challenge. The number of IFN-γ producing effector memory CD4 T-cells is also increased in this case, via an IL-12-dependent mechanism.[86] Finally, studies by Suresh et al[87] also indicate that similar FcγR targeting of a live attenuated *Ft* vaccine can result in improved vaccine efficacy when utilizing a live attenuated mAb-*Ft* IC vaccine followed by *Ft* SchuS4 challenge. Specifically,

the authors demonstrated that an oxidant-sensitive *Ft* LVS mutant (emrA1) administered IN could extend median time to death following a subsequent *Ft* SchuS4 challenge, as compared to unvaccinated controls.[87] They went on to show that time to death was further extended when the emrA1 mutant bacteria was delivered in the form of mAb-emrA1 *Ft* IC, providing additional evidence for the benefits of FcγR-targeted vaccines in the generation of enhanced immunity against *Ft*.[87] Nevertheless, it is also important to note that mAb-i*Ft* IC can engage both activating FcγR and the inhibitory FcγR (FcγRIIB). Importantly, the latter could thus limit the level of immune enhancement/protection, generated by mAb-i*Ft* immunogen. In this regard, using FcγRIIB KO mice, Franz et al[88] demonstrated this was in fact the case, suggesting that if an FcγR-targeted vaccine could be developed, which engages the activating FcγR, but not FcγRIIB, the enhanced immunity and protection observed with mAb-i*Ft* IC could be significantly improved.

DNA vaccines

The primary advantages of DNA vaccines are that they are simple and relatively cheap to manufacture, as compared to conventional vaccines (whole cell or protein based). In addition, DNA has a higher shelf life and can be stored at room temperature, making its transport and storage more cost effective.[89] More importantly, DNA vaccines induce both Ab-mediated immunity and CMI,[89] the latter being critical for protection against tularemia.[56] However, despite the apparent advantages of DNA vaccines over conventional vaccines, efforts to develop a DNA vaccine against tularemia are limited. In one such study, a DNA vaccine using T-cell epitopes (identified by their reactivity to T-cells of previously infected humans) induced proinflammatory cytokines and protection against *Ft* LVS challenge. However, protection against type A *Ft* infection by this vaccine was not determined.[90] A similar study using another set of epitopes also generated protection against lethal *Ft* LVS challenge but failed to protect mice against a type A *Ft* challenge. The latter vaccine included CD8 T-cell epitopes, which induced a strong CD8 T-cell response but only limited CD4 T-cell responses.[91]

Major factors influencing *Ft* vaccine efficacy and vaccination studies

In addition to the Ag/immunogen utilized, a number of other key factors influence *Ft* vaccine efficacy, which include bacterial strain, growth conditions of the attenuated or killed vaccine and/or challenge strain, the genetic background of the animal model, and sex. Furthermore, the lack of experimental consistency and consideration of such factors (Tables 1–3) have only served to muddy the water in terms of the successful development of a tularemia vaccine.

Impact of bacterial strain

Bacterial strain dictates not only virulence but also, when used as an attenuated or killed vaccine, the level of protection generated. The best example in regard to strain differences impacting virulence is *Ft* LVS (type B) versus *Ft* SchuS4 (type A). While *Ft* LVS is lethal in mice, it is not lethal in humans and has thus been used as an attenuated vaccine for humans.[54] In contrast, *Ft* SchuS4 is highly virulent in mice and humans.[8] However, despite extensive investigations over the last 15 years, the precise reasons for this difference remain

unknown. More recent studies have also revealed differing levels of virulence between the type A subpopulations A1a, A1b, and A2. Human infections due to A1b resulted in significantly higher mortality (24%) than those caused by A1a (4%) and A2 (0%).[8] These observations are further supported by primary infection studies using C57BL/6 mice, in which mice infected with A1b died significantly earlier than those infected with strains A1a or A2.[92,93] A similar tendency has been noticed following vaccination in which mice infected with two distinct type A strains, *Ft* FSC033 and *Ft* SchuS4, exhibited increased susceptibility of both naive and *Ft* LVS-immunized mice (BALB/c and C57BL/6) by *Ft* FSC033 versus *Ft* SchuS4.[94] Furthermore, a more recent study showed that subcutaneous vaccination with a sublethal dose of a highly virulent *Ft* LVS strain is capable of protecting BALB/c mice against respiratory challenge with a virulent type A strain.[95,96] Similar results were observed using C57BL/6 mice vaccinated with two different strains of *Ft* LVS, which differed in their median lethal dose (LD_{50}). Specifically, 100% of mice vaccinated with the highly virulent strain of *Ft* LVS survived *Ft* SchuS4 challenge, whereas mice vaccinated with a less virulent strain of *Ft* LVS strain all succumb to *Ft* SchuS4 infection. Consistent with the latter observation, earlier studies by Eigelsbach et al reported the existence of two different colony variants of the prototypical virulent type A *Ft* SchuS4 and type B *Ft* LVS strains. These variants were identified on the basis of colony morphology (rough colonies versus smooth colonies) and their appearance (blue versus gray).[53,55,97] In the latter case, WT SchuS4 and *Ft* LVS appear blue and the variants gray. These phenotypic differences were also linked to differences in virulence, as well as immunologic properties. In regard to virulence, the *Ft* LVS gray variants exhibited less virulence, as well as being less efficacious in protection against the virulent *Ft* type A strain challenge studies compared to blue variants.[53,55,98] However, as noted throughout this review, the most important aspect of these strain differences is that the majority of protective *Ft* vaccines using an *Ft* LVS type B challenge fail to generate similar protection, when using an *Ft* type A challenge. Nevertheless, current evidence tends to indicate that differences in virulence are largely due to intrinsic properties of the bacterial strains and are not directly related to host sex, susceptibility, genetics, or otherwise failed immune responses.[8] However, regardless of the cause of the strain differences in virulence, the use of an *Ft* type A challenge to accurately identify potential vaccine candidates and evaluate *Ft* vaccine efficacy is generally required.

Impact of bacterial growth medium

Immunogens used as attenuated or killed vaccines must first be grown in vitro. However, culture medium has been shown to have a profound impact on the set of proteins expressed by microbes.[99] Thus, the choice of medium can significantly alter the antigenic composition and efficacy of whole cell-based attenuated and killed vaccines. For example, *M. bovis* (BCG) used in human vaccination is grown in Sauton medium.[100] However, research laboratories use Middlebrook 7H9 medium.[101] BCG grown in Middlebrook 7H9 and Sauton media exhibits different protein expression profiles and different levels of sensitivity to reactive nitrogen intermediates.[102] This difference is also reflected in its protective efficacy, as BCG grown in Middlebrook 7H9 medium confers better protection compared to BCG grown in Sauton medium. Moreover, the elevated protection generated by BCG grown in Middlebrook 7H9 medium is also associated with higher numbers of Mycobacteria-specific TH17 cells and higher Ab levels.[101] Similarly, a number of other microbes have been

reported to differentially express immunogenic molecules, depending on the growth medium.[103–107] *Ft* grown in vitro in Mueller Hinton Broth (MHB) expresses a distinct set of genes as compared to those obtained from tissues or MØs following *Ft* infection.[108] In addition, MHB-grown *Ft* (*Ft*-MHB) can induce production of select proinflammatory cytokines, while *Ft* obtained from *Ft*-infected animals or MØs exhibit a reduced ability to do so.[109,110] Importantly, *Ft* grown in brain heart infusion (BHI) medium in vitro (*Ft*-BHI) exhibits a protein expression and proinflammatory cytokine pattern more closely resembling that of *Ft* obtained from DCs or MØs in vivo.[99,108] *Ft*-BHI and *Ft*-MHB also differ in their ability to interact with complement and *Ft* LPS-specific Abs, with *Ft*-MHB being more reactive. The altered immune responses to *Ft*-MHB versus *Ft*-BHI can be attributed to differential protein expression, surface carbohydrates expression, and structural integrity.[99] With this in mind, we have investigated the efficacy of *Ft* LVS-based vaccines generated in MHB versus BHI and have found that while *Ft*-MHB is more protective in mice challenged with *Ft* LVS (manuscript in preparation), *Ft*-BHI is a more protective immunogen following an *Ft* SchuS4 challenge (Figure 2). These findings once again emphasize the importance of challenge strain, as well as the growth medium, when evaluating *Ft* vaccine efficacy.

Impact of animal model

The murine model—The murine model is of particular interest in this regard, since the majority of the studies focused on *Ft* vaccination and infection are done using the mouse model, and in particular C57BL/6 or BALB/c mice. The genetic background of the individual strains of mice can have a significant impact on the outcome of both the immune response and survival, in murine models of infectious disease and vaccination.[111–113] More specifically, it has been demonstrated that C57BL/6 mice are more susceptible to *Ft* infection and less easily protected against challenge with the highly virulent type A *Ft*, as apposed to BALB/c mice. Specifically, ID immunization of BALB/c mice with *Ft* LVS generates protective immunity against a successive ID challenge but not a respiratory challenge with type A *Ft*.[96] In contrast, similarly, immunized C57BL/6 mice are not protected against either ID or respiratory challenge with the same *Ft* challenge organism.[114] Similarly, ID vaccination with SchuS4-clpB (a heat shock protein mutant) protects BALB/c but not C57BL/6 mice from a subsequent respiratory challenge with *Ft* SchuS4. The increased susceptibility of C57BL/6 mice to tularemia compared to that of BALB/c mice has been attributed to the increased IFNγ and pulmonary IL-17 levels observed in the lungs of C57BL/6 mice.[114] There are numerous factors, in addition to increased IFNγ and pulmonary IL-17 levels, observed in the lungs of C57BL/6 mice that may also explain the differences in susceptibility of vaccinated C57BL/6 versus BALB/c mice. More severe tissue damage is observed in C57BL/6 mice than BALB/c mice, following pulmonary infection.[115] It has also been demonstrated that C57BL/6 mice favor the development of a Th2 phenotype in the lung versus the more protective Th1 response.[111,116] It is also possible that *Ft* LVS vaccination fails to induce and maintain sufficient numbers of Ag-specific memory T-cells in the lungs of C57BL/6 mice.[36,117] Collectively, this suggests that BALB/c mice, following vaccination, develop a more protective immune response to subsequent *Ft* infection as compared to that of C57BL/6 mice. An additional example of this finding has also been observed with C3H/HeN versus BALB/c mice. Intradermal immunization with a sublethal dose of *Ft* LVS produced reduced survival in C3H/HeN versus BALB/c mice, receiving an aerosol *Ft*

SchuS4 challenge. Consistent with the latter, BALB/c mice immunized ID with the SchuS4-clpB mutant also exhibited increased survival as compared to C3H/HeN mice. However, in contrast to the aforementioned observation, oral priming and boosting of C3H/HeN mice with SchuS4-clpB mutant resulted in significantly longer survival than that of BALB/c mice following an aerosol *Ft* SchuS4 challenge.[118] Whether such differences help or hinder *Ft* vaccine development is likely to depend on the approach. By studying such differences, one may more easily identify correlates of protection. However, the more limited the genetic diversity of the animal model being used, in particular as it applies to major histocompatibility complex Class I and Class II expression, the more likely one may fail to identify the vaccines that are most efficacious in an outbred population, such as humans.[119]

Additional animal models—The majority of *Ft* research has been, and continues to be, carried out in mice. However, vaccine approval will ultimately require studies be verified in additional animal models. In this regard, an extensive review of such animal models for tularemia has been written.[120] These animal models include monkey, rat, rabbit, guinea pig, and marmoset.[120,121] In the aforementioned review, it was concluded that significantly more information on how species, including the rat, rabbit, and guinea pig, respond to *Ft* infection was needed, including a database containing clinical, pathological, and microbiological information, in order to effectively assess strengths and weaknesses of each animal model. In addition, each animal model has specific advantages and disadvantages, which must be considered in the context of the specific goals of animal studies being conducted.

Impact of sex

It is well established that sex-dependent host factors can significantly impact susceptibility to infection. Multiple studies by various research groups have reported sex-based susceptibility to numerous pathogens and infectious diseases. In general, males of many species are more susceptible than females to bacterial, viral, and fungal infections.[122–124] However, studies on sex bias in tularemia infection have not been published. Nevertheless, clinical incidence and progression of tularemia in endemic areas has been shown to be significantly higher in males than in females in all age-groups except children (aged 5–9 years). While this may reflect, in part, differences in pathogen exposure through hunting and outdoor professional activities (CDC, http://www.cdc.gov/tularemia/statistics/agesex.html),[125] in male versus female susceptibility could also be a contributing factor. We have observed for the first time that while both naive male and female C57BL6 mice are equally susceptible to *Ft* LVS infection, prior immunization with i*Ft* or live *Ft* vaccine results in a sex-based immune response and protection in the case of both *Ft* LVS [126] and *Ft* SchuS4 challenge (Figure 3). Specifically, vaccinated male mice develop severe clinical disease and exhibit a significantly higher mortality rate, which correlates with increased tissue destruction, a higher bacterial burden, and weight loss, as compared to immunized female mice. Importantly, this implies that tularemia vaccine efficacy will vary based on sex, which has been observed in clinical trials involving other infectious agents.[127–130] Thus, development of a successful vaccine against tularemia will require an understanding of the impact sex has on vaccine-induced protection against this organism, with sex differences necessarily being a serious consideration in any future tularemia vaccine development studies.

Paralysis or progress: what does the future hold for *Ft* vaccine development?

Despite 15 years of intense research focused on the development of an effective vaccine against the highly virulent type A *Ft*, a fully protective, FDA-approved vaccine remains elusive. While attenuated vaccines have provided the most promising results, with a relatively large selection of potential candidates, concerns over safety and in particular reversion, represent significant impediments to the licensure of an attenuated *Ft* vaccine. Some promising results have also been obtained with killed vaccines, in particular when targeting i*Ft* to FcγR IN. However, a number of limitations remain to be overcome in this regard as well. First, 100% protection against the type A strain of *Ft* (SchuS4) has not been achieved in this case. Furthermore, the formation of mAb-i*Ft* IC can vary significantly from batch to batch, and, as a result, the degree of protection observed can also vary significantly, also leading to significant difficulties with regard to vaccine reproducibility and consequently FDA approval. Thus, in this case, it will be necessary to devise an FcγR-targeting vaccine strategy, which can be more easily produced, is more well defined, and in addition engages activating FcγR without engaging the inhibitory FcγR (FcγRIIB). In fact, such a vaccine for *Ft* is currently being developed in our laboratory. In regard to subunit vaccines, this represents the ideal approach in terms of cost, safety, and production and could be accomplished using either a protein or DNA vaccine approach. However, the primary limiting factor in both cases is the lack of an identified protective *Ft* Ag to incorporate into such a vaccine. Given the lack of progress over the last 15 years in this regard, the incorporation of multiple *Ft* Ags may provide an alternative means of generating an effective subunit vaccine. Thus, despite the absence of success thus far, a number of viable options still exist to produce a fully protective *Ft* vaccine. Furthermore, a number of published studies, many of which are listed in Tables 1–3, and Figures 1–3 presented in this review demonstrate protection against *Ft* type A challenge following vaccination is possible.

With regard to future studies focused on *Ft* vaccine development, it is also clear that a number of important factors, such as bacterial strain, growth medium, the genetics of the animal model being used, and sex, can impact protection and must be considered. As indicated in Tables 1–3, these factors vary widely between studies and laboratories and may explain inconsistencies in protection studies observed between laboratories. Thus, it will ultimately be necessary to identify the optimal conditions in each of these cases and consistently use those conditions in evaluating *Ft* vaccine efficacy.

Conclusion

Given the large number of studies that have generated some level of protection against the type A strain of *Ft* and the many options still available to improve *Ft* vaccine efficacy, progress is being made and it would appear likely that an effective vaccine against *Ft* will be forthcoming, although additional money, time, and research effort will be required.

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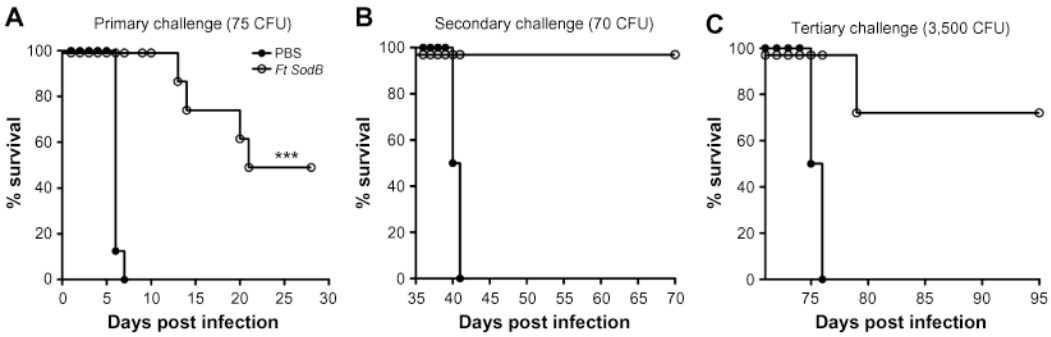

Figure 1.

Acute and convalescent protection of C57BL/6 mice vaccinated with a live attenuated *Ft* vaccine (*SodB* mutant) and subsequently challenged with high-dose *Ft* SchuS4.

Notes: C57BL/6 female mice were immunized ID with either PBS or ~ 1×10^3 CFU of attenuated *Ft* LVS *SodB* mutant grown in BHI medium in 50 μL on day 0 and boosted IN on day 21 with either 20 μL of PBS or ~ 1×10^3 CFU of attenuated *Ft* LVS *SodB* mutant. Mice were then challenged IN on day 42 with 75 CFU of *Ft* SchuS4 (~60–70× LD_{50}) and subsequently monitored for 30 days for survival (**A**). 35 days after primary challenge survivors were rechallenged IN with 70 CFU of SchuS4 and subsequently monitored for 30 days for survival (**B**). 35 days after secondary challenge survivors were again rechallenged IN with 3,500 CFU of *Ft* SchuS4 and subsequently monitored again for 30 days for survival (**C**). *** $P \leq 0.001$.

Abbreviations: *Ft, Francisella tularensis*; ID, intradermal; PBS, phosphate-buffered saline; IN, intranasal; LVS, live vaccine strain; LD_{50}, median lethal dose; CFU, colony forming unit; BHI, brain heart infusion.

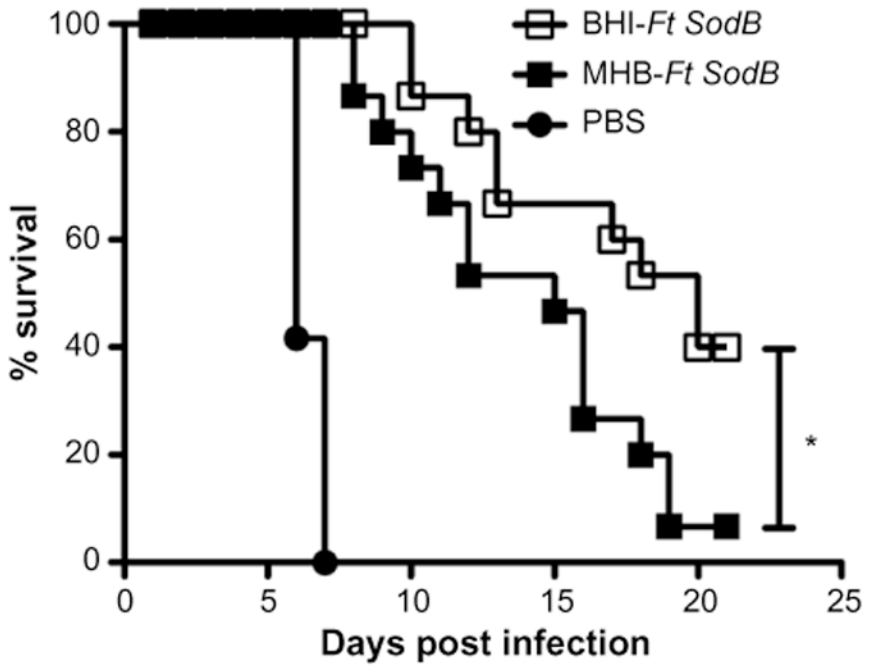

Figure 2.

Impact of growth medium on *Ft* vaccine efficacy.

Notes: Challenge studies were conducted as follows: C57BL/6 male and female mice were immunized IN with *Ft SodB* mutant of *Ft* LVS grown in either BHI or MHB media. Mice were immunized on day 0 and boosted on day 21, then challenged IN on day 42 with 33 CFUs of *Ft* SchuS4 and subsequently monitored for 25 days for survival. *$P \leq 0.05$.

Abbreviations: *Ft*, *Francisella tularensis*; IN, intranasal; LVS, live vaccine strain; LD_{50}, median lethal dose; PBS, phosphate-buffered saline; BHI, brain heart infusion; MHB, Mueller Hinton Broth; CFU, colony forming unit.

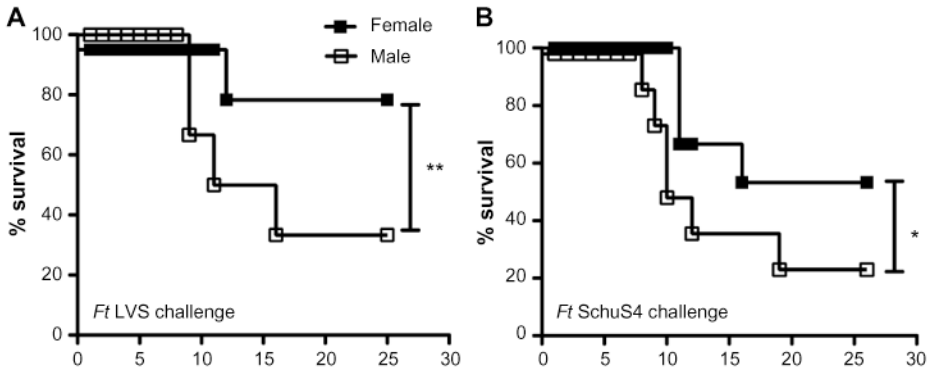

Figure 3.

Impact of sex on *Ft* vaccine efficacy.

Notes: Challenge studies were conducted as follows: C57BL/6 male and female mice were immunized IN with either 20 μL of vehicle (PBS) or 20 μL of 75 ng of i*Ft* on day 0 and boosted on day 21. Mice were then challenged IN on day 35 with 1,500 CFU (2× LD_{50}) of *Ft* LVS and subsequently monitored for 25 days for survival (**A**). C57BL/6 male and female mice were immunized ID with either PBS or ~ 1×10^3 CFU of attenuated *Ft* LVS *SodB* mutant in 50 μL day 0 and boosted IN on day 21 with either 20 μL of PBS or ~1×10^3 CFU of attenuated *Ft* LVS *SodB* mutant. Mice were then challenged IN on day 42 with 33 CFU of *Ft* SchuS4 and subsequently monitored for 30 days for survival (**B**). **P*≤0.05. ***P*≤0.01.

Abbreviations: *Ft, Francisella tularensis*; IN, intranasal; PBS, phosphate-buffered saline; i*Ft*, inactivated Ft; LD_{50}, median lethal dose; LVS, live vaccine strain; ID, intradermal; CFU, colony forming unit.

Table 1

***Ft LVS*-based live attenuated vaccines**

Vaccine strain (*Ft* LVS)	Growth medium	Animal model	Sex	Vaccine dose (route)	*Ft* LVS challenge % protection (dose, route)	*Ft* SchuS4 challenge % protection (dose, route)	References
Ft LVS	MHB	C57BL/6	F	100 CFU (IN)	ND	100% (25 CFU, IN)	131
SodB	MHB	C57BL/6	M/F	1,200 CFU (IN)	100% (1.2×10^6 CFU, IN)	40% (103 CFU, IN)	56
ClpB	BHI CDM	C57BL/6	F	5×10^4 CFU (IN)	100% (5×10^3 CFU, IN)	10% (30 CFU, IN)	132
emrA1	MHB	C57BL/6	F	10^6 CFU (IN)	100% (10^7 CFU, IN)	15% (17 CFU, IN)	87
CapB	MHB	BALB/c	F	10^6 CFU (IN)	ND	100% (10 LD_{100}, aerosol)	133
clpB	NA	BALB/c	NA	5×10^4 CFU (IN)	ND	30% (86 CFU, IN)	134
dsbA	McLeod	BALB/c	F	10^6 CFU (SC)	ND	100% (100 CFU, SC) 50% (100 CFU, IN)	135
wbtA	CHAH	BALB/c	M	1.5×10^7 CFU (IN)	100% (25 LD, IN)	25% (10 CFU, IN)	136
Wzy	MHB	BALB/c	M	2.4×10^7 CFU (IN)	100% (1.2×10^5 CFU, IN)	84% (8 CFU, IN)	137

Abbreviations: *Ft, Francisella tularensis*; LVS, live vaccine strain; MHB, Mueller Hinton Broth; F, female; ND, not determined; IN, intranasal; M, male; BHI, brain heart infusion; CDM, Chamberlain's defined medium; NA, not available; SC, subcutaneous; CHAH, cysteine heart agar containing 2% hemoglobin; LD_{50}, median lethal dose; LD_{100}, absolute lethal dose.

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Table 2

***F. novicida*-based live attenuated vaccines**

Vaccine strain (*F. novicida*)	Growth medium	Animal model	Sex	Vaccine dose (route)	*Ft* LVS challenge % protection (dose, route)	*Ft* SchuS4 challenge % protection (dose, route)	References
iglB: fopC	TSB	C57BL/6	NA	10^3 CFU (oral)	80% (3.5×10^4 CFU, IN)	ND	138
IglD	TSB CDM	BALB/c	NA	9.7×10^8 CFU (IN)	ND	0% (10^3 CFU, IN)	139
		Fischer rats (344)	F	10^5 CFU (IT)	ND	100% (10^4 CFU, IT)	
		NHP	M/F	10^8 CFU (BR)	ND	83% (10^3 CFU, aerosol)	
iglB	TSB	Fischer rats (344)	F	10^7 CFU (oral or IT)	ND	50% ($\sim10^4$ CFU, oral or IT)	140
iglB:fljB	TSB	BALB/c	NA	10^3 CFU (oral)	83% (8.5×10^4 CFU, IN)	ND	141
		Fischer rats (344)	NA	10^7 CFU (oral)	ND	83% (10^4, IT)	

Abbreviations: *F. novicida*, *Francisella novicida*; *Ft*, *Francisella tularensis*; LVS, live vaccine strain; TSB, tryptic soy broth medium; NA: not available; IN, intranasal; ND, not determined; CDM, Chamberlain's defined medium; F, female; IT, intrathecal; NHP, nonhuman primate (cynomolgus macaque); M, male; BR, bonchoscopy route.

Table 3

***Ft* SchuS4-based live attenuated vaccines**

Vaccine strain (*Ft* SchuS4)	Growth medium	Animal model	Sex	Vaccine dose (route)	*Ft* LVS challenge % protection (dose, route)	*Ft* SchuS4 challenge % protection (dose, route)	References
FTT1103	MHA TSB CDM	C57BL/6	NA	10^7–10^8 CFU (IN)	ND	100% (37–68 CFU, IN)	142
		BALB/c	NA	10^7–10^8 CFU (IN)	ND	75% (37–68 CFU, IN)	
clpB	CHAH MHB	C57BL/6	F	10^5 CFU (ID)	ND	0% (100 CFU, IN)	114
		BALB/c	F	10^5 CFU (ID)	ND	80% (100 CFU, IN)	
iglD	TSB CDM	BALB/c	NA	4.8×10^6 CFU (IN)	0% (IN)	ND	139
		Fischer rats (344)		10^7 CFU (IT)	ND	50% (10^4 CFU, IT)	
FTT0369 FTT1676	MHB	BALB/c	F	50 CFU (IN or ID)	ND ND	90% (50 CFU, IN) 100% (50 CFU, ID)	143
capB	CDM BCGA	BALB/c	F	10^4 CFU (SC)	ND	60% (10^3, SC)	144
FTT0918	CHAH MHB	BALB/c	F	10^5 CFU (ID)	ND	100% (500, SC)	145
ggt	CDM BCGA	BALB/c	F	8.75×10^5 CFU (SC)	ND	100% (10^2, SC)	146
*gua*BA *aro*D	BHI	NZW rabbit	F	10^9 CFU (scarification)	ND	27%–36% (10^4, aerosol)	147

Note: Data from Reed et al.[147]

Abbreviations: *Ft*, *Francisella tularensis*; LVS, live vaccine strain; TSB, tryptic soy broth; CDM, Chamberlain's defined medium; NA: not available; IN, intranasal; ND, not determined; CHAH, cysteine heart agar containing 2% hemoglobin; MHB, Mueller Hinton Broth; F, female; ID, intradermal; IT, intrathecal; BCGA, blood cysteine glucose agar; SC, subcutaneous; BHI, brain heart infusion; NZw, New Zealand white; MHA, Mueller Hinton Agar.