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Review

Current vaccine strategies and novel approaches to combatting *Francisella* infection

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ABSTRACT

Tularemia is caused by subspecies of *Francisella tularensis* and can manifest in a variety of disease states, with the pneumonic presentation resulting in the greatest mortality. Despite decades of research, there are no approved vaccines against *F. tularensis* in the United States. Traditional vaccination strategies, such as live-attenuated or subunit vaccines, are not favorable due to inadequate protection or safety concerns. Because of this, novel vaccination strategies are needed to combat tularemia. Here we discuss the current state of and challenges to the tularemia vaccine field and suggest novel vaccine approaches going forward that might be better suited for protecting against *F. tularensis* infection.

1. Immune response to *Francisella* infection

Tularemia is caused by the gram-negative, facultative-intracellular bacterium *Francisella tularensis*. Infection by *F. tularensis* can occur via insect bite, exposure to infected animals, such as lagomorphs or other rodents, or inhaled as an aerosol; the bacteria can also be found in soil or water as depicted in Figure 1. These different transmission routes can lead to a variety of disease presentations which include ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic and typhoidal, with pneumonic and typhoidal displaying the highest mortality rates [1]. *F. tularensis* has two subtypes – Type A, *F. tularensis subsp. Tularensis* (*F. tularensis*) and Type B, *F. tularensis* subsp. *Holarctica* (*F. holarctica*). Type A is the more virulent form of the bacterium and is more commonly associated with severe disease in humans, while Type B does not typically cause severe disease. In 2019, there were 274 reported cases of tularemia in the United States, and 1,463 cases in the European Union with Type A being more common in North America and Type B in Europe [2,3]. Aerosol transmission and inhalation results in the most serious form of tularemia: pneumonic. Theoretically as few as 25 colony forming units (CFU) can cause pulmonary disease making it one of the most infectious bacterial pathogens by the aerosol route [4]. Due to its low infectious dose and high mortality in the pneumonic form, *F. tularensis* is a CDC-designated Tier 1 select agent governing possession and research activities with the microorganism. *F. tularensis* also has a sordid history as a biological threat agent and has been previously weaponized by several countries [5].

The immune response to *F. tularensis* has been well characterized, and comprehensive reviews have been written by others and therefore that response will be only briefly covered here [6-9]. Immunity to *F. tularensis* is remarkably sophisticated and includes both a cellular and antibody-based response. Both B and T cells are elicited following *F. tularensis* infection. Antibodies play an important role and are useful in recognizing LPS or outer membrane proteins; however, serum transfer from mice immunized with *F. holarctica* live vaccine strain (LVS) cannot protect against pulmonary challenge with *F. tularensis*, suggesting that a cross-reactive antibody response is not sufficient to confer protection, at least against pulmonary disease [10]. The LPS of *Francisella* is tetraacylated, weakly activates TLR4, and therefore does not induce inflammatory cytokines [11]. When sera from mice pretreated with *F. tularensis* is passively transferred, recipient mice can be protected from subsequent challenge, but only if the T cells are intact, highlighting the role that the cell-mediated response plays in protection [12]. B cells may play a more important role in secondary infection by potentially contributing to antigen presentation and by producing important cytokines and chemokines during infection [13]. *Francisella* can infect B cells causing apoptosis and potentially hampering their function, which could explain their less defined role during infection [14]. Antibody titers do not appear to be a reliable marker of vaccine efficacy, and an intact T cell

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response appears critical for infection control [15]. αβ, γδ, and mucosal invariant T cells (MAIT) have all been shown to contribute to clearance and protective immunity in animal models [9]. IFNγ and TNF producing Th1 T cells are required to control infection, although roles for IL-17, IL-2, and IL-12 have likewise been described [7]. Innate immunity also contributes to protection, where NK cells likely produce IFNγ before a T cell response is engaged [16]. Like other intracellular pathogens, *Francisella* can exploit host cells like macrophages and dendritic cells by replicating intracellularly, but IFNγ can inhibit this growth through reactive oxygen species [7]. Neutrophils have also been implicated in the initial response to *Francisella* as mice lacking neutrophils succumb to infection rapidly; however, this may be dependent on infection route as a lack of neutrophils has less of an impact on respiratory infection [17]. The effector mechanisms for neutrophils in human infection are less clear, especially as *Francisella*-infected neutrophils do not undergo respiratory burst [18]. In addition to IFNγ, TNF and IL-12 are critical for initial infection control, although how quickly they are detected is route dependent [17]. Despite some understanding as to what roles are played by the innate and adaptive immune response to *Francisella*, how these responses come to together to elicit long term protection remains unclear. Figure 2 briefly depicts the immunology of the lung and nasal cavity during infection.

2. Vaccine correlates of protection against *Francisella* infection

The most notable achievement in developing an efficacious *Francisella* vaccine is the development of the Live Vaccine Strain (LVS). This strain was created in the 1960s in the Soviet Union by attenuating *F. holarctica* [19]. This vaccine is not currently licensed in the US due to its inability to provide full protection against human-virulent *F. tularensis* and concerns over the possibility that it might revert to a more virulent form of the bacteria [8]. The original LVS strain has been highly passaged, making it less efficacious against fully virulent strains. The LVS can also undergo spontaneous phase shifts to a more attenuated form, which also fails to be protective [20]. Natural infection with *F. tularensis* results in protection from infection, indicating that immunity is possible and that basing a vaccine off the natural infection should yield protection. In humans, the LVS vaccine induces expansion of CD4+ and CD8+ T cells, γδ T cells, NK cells, and monocytes, eliciting a host response pattern emulating infection with *F. tularensis* [21]. Despite this expansion of immune cells in human tularemia aerosol challenge studies, only 54% of all LVS vaccinated volunteers were protected by the vaccine and as a result did not require additional treatment with antibiotics to clear the bacteria [22]. As such, nearly half (46%) of the challenged volunteers were left unprotected by the LVS vaccine highlighting the low efficacy of this vaccine against the most lethal form of disease. While this protection was dependent on vaccination dose and challenge route, this emphasizes that LVS vaccination is not sufficient to fully protect against virulent *Francisella* exposure in most cases. In more recent years, studies have sought to identify potential correlates of protection in both mice and rats following *F. tularensis* respiratory infection to better understand necessary vaccine attributes. Roles for B cells and antibodies, T cells, and control of intramacrophage bacterial growth have all been identified as important, but the exact balance of these for vaccine mediated protection against subsequent infection remains unclear [23,24]. Notably, a very recent study examining human immune correlates to LVS vaccination showed that the cytokines IFNγ, TNF, IL-17 and IL-2 produced by T cells were most correlated with monocyte derived macrophage killing of the highly virulent SchuS4 strain *in vitro* [25]. Additionally, this study showed a range of both CD4 and CD8 memory T cell responses that lasted over a year after vaccination.

It has been established that T cells are correlated with and in some cases absolutely required for vaccine-mediated protection [26,27]. However, until recently, it wasn't clear whether circulating or tissue resident T cells were required for protection. Recently, Roberts et al., using both parabiosis and intravascular staining techniques, showed that both circulating and resident T cells are essential; however, neither cell type on their own was sufficient for protection [28,29]. This indicated an important role for both T cell types, especially because *Francisella* easily disseminates from the initial infection site to distal tissues [28,29]. Historically, antibody response has been used as the

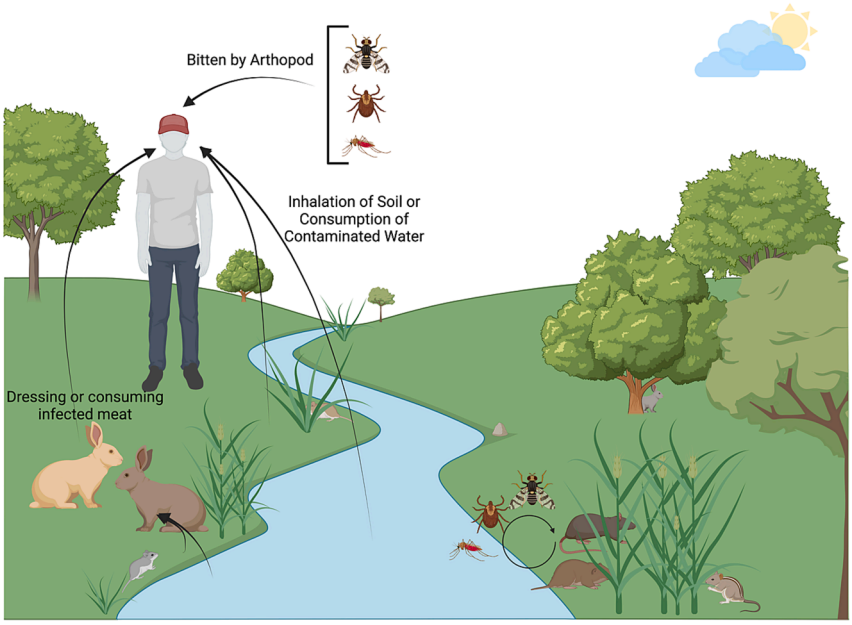

Figure 1. Transmission of *Francisella tularensis* in the environment and within animal species. Humans can acquire *Francisella tularensis* from a variety of both environmental and zoonotic sources. *Francisella* has two lifecycles – one terrestrial and one aquatic. In the terrestrial lifecycle, small rodents and lagomorphs are the main reservoirs, with flies and ticks being capable of spreading disease to other animals; soil can also contain bacteria, and inhalation or consumption of contaminated soil can lead to disease. During the aquatic lifecycle, semiaquatic animals and mosquitos are the main reservoirs, but *Francisella* can also be found in water, and thus consuming contaminated water could result in infection. Having two distinct lifecycles allows *Francisella* to be transmitted to humans in a variety of ways including through broken skin or via conjunctival, oral, or respiratory routes. Created with BioRender.

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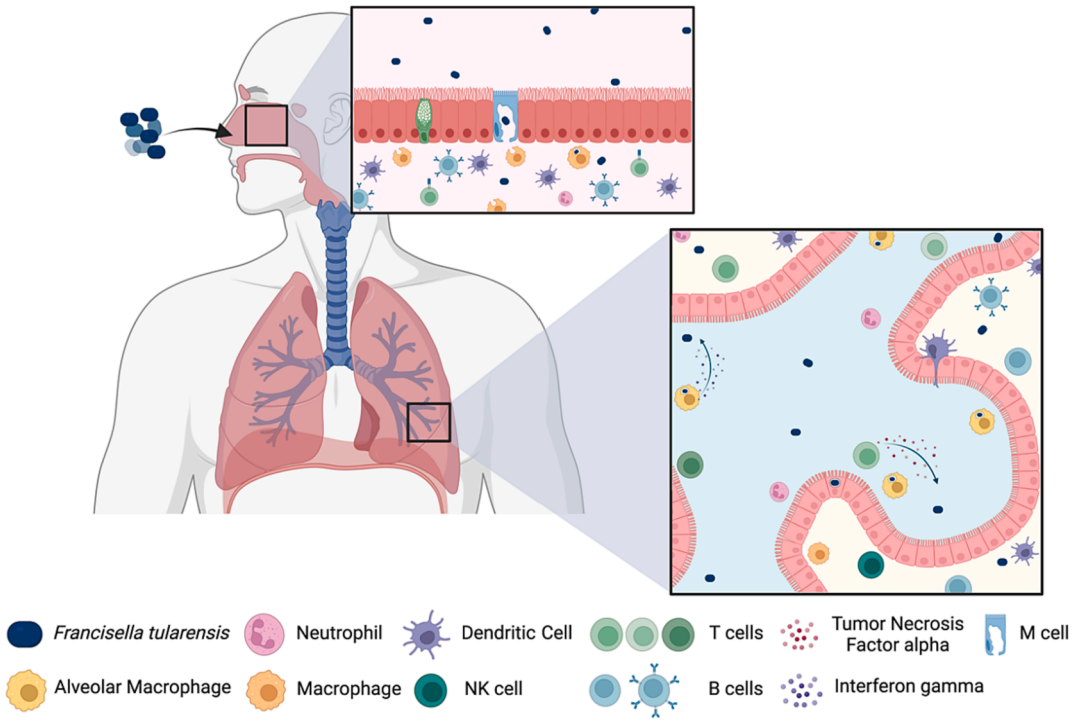

Figure 2. Proposed immune response to pulmonary infection of *Francisella tularensis*. *F. tularensis* can contracted through the respiratory route where it may first encounter the nasal mucosa that is rich with immune cells. While not much is known about the host-pathogen interaction of Francisella in the nasal mucosa, it is likely that the pathogen is taken up in the nasal-associated lymphoid tissue where both innate and adaptive immune cells mount an immune response. Once past the nasal mucosa, bacteria can be further inhaled into the lungs where in the luminal space, *Francisella* has adapted to preferentially infect alveolar macrophages. In the lungs, dendritic cells are important for uptake and presentation of the bacterial antigens to adaptive immune cells, particularly T cells. Proinflammatory cytokines like IFNγ and TNF are necessary to help combat *Francisella* infection. B cells and antibodies, particularly IgA in the lungs, play an important role in *Francisella* immunity, but this immunity relies primarily on cell mediated immunity and is not enough alone to confer protection. Due to the highly vascular nature of the lungs, *F. tularensis* can easily disseminate from the respiratory tract into the circulatory system and induce systemic infection, making it critically important to understand the appropriate immune response to elicit when creating new vaccines. Created with BioRender.

measurement of vaccine responses, but for intracellular pathogens, antibodies may be less relevant. Correlates of cell-mediated protection are better suited for protecting against these types of organisms. For *Francisella*, clear differences in antibody responses and repertoire are not always present when comparing the protective capacity of vaccine strains [30]. Vaccination strategies that primarily drive a humoral response cannot elicit protection alone; cell-mediated immunity is also a requirement [31]. A panel of upregulated genes identified as associated with protection from severe disease include NOS2, IL-21, CCL5, LTA, FasL, IL-2RA, IFNγ and CXCL9 [24]. Recently, this panel of genes was validated using the Type A strain-derived vaccine candidate *ΔclpB,* which showed partial protection against aerosol challenge with high doses of the homologous strain [32]. While this panel is promising for identifying cell-mediated correlates of protection, the studies from Roberts et al. also highlight that creating a vaccine to protect against disseminated infection can be challenging. Thus, identifying the correlates is essential but it is also critical to thoroughly assess immunogenicity and protective efficacy using the appropriate infectious challenge modality and vaccine delivery route needed to achieve optimal protection.

3. Animal models to study *Francisella* vaccine responses

Francisella species have multiple hosts and a variety of clinical manifestations during infection. Accordingly, the establishment of appropriate animal models to study immune responses and thus develop appropriate vaccines continues to be a complex and biologically sophisticated work in progress. A large share of past research has focused on the pneumonic form due to its high mortality rate and possible bioterrorism threat. Nonhuman primates (NHP), rabbits, and various rodent species (mice, rats, and guinea pigs) have all been employed as models to study *Francisella* infection and immunology with NHPs most closely mimicking human disease. Several species of NHP have been employed to study tularemia including *Chlorocebus atheiops* (African green monkeys), *Macaca fascicularis* (cynomolgus macaques), and *Macaca mulatta* (rhesus macaques) [9]. Pneumonic tularemia in the cynomolgus macaque has been developed to the point of being qualified as an acceptable disease model for medical countermeasures development [33]. Rodent species, including mice, either BALB/c or C57Bl/6 strains, have commonly been utilized as disease models as susceptibility to low doses of *F. tularensis* results in systemic disease somewhat correlative with human host response. Popular murine models have demonstrated varied susceptibility to LVS based on both strain of mouse chosen, strain of *Francisella* used, and route of inoculation employed [9,34]. Mice are particularly susceptible to intraperitoneal infection with LVS, with a theoretical dose of one (1) CFU required to induce disease [35]; however, the implications for this route and model are unclear as intraperitoneal infection is not a natural route for tularemia. For natural routes of infection which include intradermal injection or inhalation by aerosol, mouse strains require differing doses for induction of disease which may result in lethality, making it difficult to fully translate host responses in this species compared to other models [36]. Mice are also susceptible to *F. novicida*, a species closely related to *F. tularensis*, which has been investigated for use as a surrogate bacterium in place of either LVS or the Type A strain of *Francisella*, as it causes a tularemia-like disease in mice. Despite some dose and route differences observed, *F. novicida* can be a valuable tool to evaluate novel vaccine candidates and for mechanistic studies. Fischer 344 rats can also mimic human susceptibility, but typically require higher doses than what is necessary for disease induction in humans, potentially calling into

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question their utility in modelling human disease [37]. Nonetheless, the ability of rats to tolerate higher doses makes them a good model of lethal aerosol challenge, which is highly relevant to human disease [38]. Rabbits, a natural *Francisella* host, can recapitulate human pathology and disease. The guinea pig, while a less defined model, have primarily been used to test LVS protection against challenge, and it is not clear how closely disease in this model matches human disease [19]. A further discussion on each of these animal models for *Francisella* can be found in Roberts et al. and Lyons and Wu [9,34]. Animal models are critical for the development of the new *Francisella* vaccines both due to rarity of infection and its associated lethality [32,39]. New vaccine candidates are likely to be evaluated under the FDA Animal Rule and therefore understanding the advantages and disadvantages of each model is necessary to achieve a desirable protective response and determining the correlates of protection needed to properly test and evaluate immunogenic and efficacious vaccines.

4. Current Approaches to *Francisella* Vaccination

4.1. Live Attenuated and Inactivated Whole Cell Vaccines

Since the development of the LVS, work has proceeded in developing a more effective vaccine. As mentioned previously, the current LVS lacks efficacy against fully virulent strains; this strain also displays a mixed colony morphology, making it difficult to regulate immunogenicity between batches [20]. It also lacks type IV pili, normally found in *F. tularensis*, due to a spontaneous mutation that occurred during the attenuation process. The type IV pili is a potent antigen that may account for the decreased immunogenicity of LVS [40]. In clinical practice LVS as an attenuated live vaccine holds an unacceptably high risk of reactogenicity during immunization, especially in sensitive populations. Since the development of LVS, there have been numerous groups working to create novel *F. tularensis* vaccine candidates, many of which are listed in Table 1. While not exhaustive, the list highlights the varied approaches that have been taken in vaccine design and the subsequent outcomes. Since the original LVS showed moderate efficacy, others have constructed a modified LVS using modern good manufacturing processes to meet current standards for human testing. One new strain of LVS was well tolerated in rabbits and produced high antibody titers, with no organisms colonizing the liver or spleen [41]. This new lot of LVS was recently tested in phase 2 clinical trials and vaccine administration to human volunteers displayed mild to moderate adverse clinical events (e.g., malaise, headache, muscle aches, sore throat) considered mildly reactogenic but resulting in robust seroconversion [42]. It is unclear which antibody isotypes (IgG, IgM, or IgA) were produced following vaccination or what cellular immune responses were induced as no data was available on T cell activation, making it difficult to compare the efficacy of this new LVS candidate to the original formulation, particularly considering, as discussed earlier, there is an absolute T cell requirement as a correlate of protection. Other clinical trials have shown that vaccination with these newer generated strains induced changes in the lipidome, proteome, metabolome, and transcriptome that are linked to increases in inflammation, antigen presentation, and protein processing which can give insight into what is necessary to achieve the appropriate immune response to tularemia [43-46]. Knockout strains of LVS have been generated to determine their efficacy compared to wild-type LVS. *ΔclpB* mutants of LVS have shown protection against challenge with LVS with minimal weight loss and increased bacterial clearance [47]. Another study using the *ΔclpB* mutant of LVS showed a 40% survival rate following challenge with SchuS4 [48]. A subsequent study by another group, was able to show that vaccination with a knockout strain of the Type A SchuS4, *ΔclpB*, resulted in robust homologous and heterologous protection against LVS and SchuS4 infection, but protection waned against high doses of SchuS4 [32]. The *ΔclpB* candidate shows great promise, and potential evaluation of other vaccination routes, like intranasal inoculation, could offer better protection at higher doses. Like *ΔclpB* mutants, *aro* mutants of *Francisella* strains have also been explored as live attenuated vaccine candidates. Specifically, the *ΔaroD* mutant of SchuS4 has shown strong protection in mice and induced both cellular and humoral immune responses [49]. A different study that used rabbits as the model instead of mice showed decreased protection with the *ΔclpB* strain compared to *ΔaroD* and that the development of O-antigen antibodies correlated with protection, although it appears that different models may have different requirements for these antibodies [50]. Additional studies have shown that it is likely the production of prostaglandin E(2) by *ΔclpB* infected macrophages that is altering the immune response and leading to better outcomes, indicating this could be an important pathway to target in vaccine development [51-53]. There have been a variety of attempts to create mutant strains of LVS and SchuS4 to develop better vaccine candidates, a few of which are highlighted below. Further discussion of this rational vaccine approach can be found in a review by Marohn and Barry [54], extensively describing different mutations in *Francisella* and their vaccine efficacy. More recently, a SchuS4 mutant, ΔFTT0369c, or double mutant combined with ΔFTT1676, also shown great protection against intradermal or intranasal challenge with SchuS4 [55]. Other groups have explored knockout or mutant strains of LVS in attempts to protect against SchuS4. Jia et al. created a *capB* mutant strain of LVS that, when used in a prime boost strategy with a recombinant *Listeria monocytogenes* strain expressing the *F. tularensis* immunoprotective antigen IgIC, showed 75% protection against aerosol challenge with SchuS4 [56]. Mutations in other LVS genes including FTL_0291, FTL_0325, and FTL_0057 have been tested as intranasal vaccines and showed 100% protection against intranasal challenge [57]. These mutant strains show that there is promise in live-attenuated vaccines using either Type A or Type B strains, but that it is a matter of identifying the right combination of genes to achieve proper immunogenicity for protection.

Other studies have sought to use *F. novicida* as a live-attenuated vaccine instead of the LVS. *F. novicida* is not traditionally a human pathogen (only causing disease in the severely immunocompromised), making it safer to manufacture and administer. *F. novicida* is also more genetically tractable than the *F. holarctica*-derived LVS. Studies using live-attenuated *F. novicida* vaccination in macaques displayed pulmonary protection against *F. tularensis* in addition to higher antibody titers and IFNγ T cell responses compared to animals receiving LVS, highlighting that *F. novicida* could be a viable platform on which to build a novel tularemia vaccine [58]. *F. novicida* shares ~97% of its genome with *F. tularensis* and is also capable of causing tularemia-like disease in mice, which has led researchers to use it as a surrogate for the study of *F. tularensis* in a mouse model of infection [7,59-62]. However, while there is high homology between the genomes of these *Francisella* species, it has been shown that there are enough genomic and protein function differences to pose a challenge to the use of *F. novicida* as a surrogate for *F. tularensis* [63-65].

To elicit both humoral and cellular responses, inactivated whole cell vaccines have also been developed and tested. These vaccines are attractive as they expose the host to the entirety of pathogen related antigens without the risk of infection or reversion to more virulent forms. Early attempts by Foshay using formalin-inactivated or heat-killed bacteria resulted in highly reactogenic formulations [66]. Alternatively, phenol preservation used to inactivate the bacteria offered some protection from disease in macaques; however, some animals still developed necrotic legions and lymphadenopathy [67]. More recently, studies reevaluated inactivated LVS to assess formulations that do not exhibit the same reactogenicity observed in Foshay's original formulations. Animals vaccinated with paraformaldehyde-inactivated LVS plus IL-12 displayed enhanced bacterial clearance, reduced tissue inflammation, and increased antibody responses when challenged with live LVS; however, the animals were not challenged with fully virulent *F. tularensis,* so it is not clear if this preparation would be effective against the most virulent subspecies [68]. The addition of IL-12 to the formulation was necessary for protection, perhaps in part due to its

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Table 1

Vaccine platforms developed for *Francisella tularensis* in the 21st century. Vaccine platforms are not prioritized and are listed in the order that they occur in the literature. Control groups used in each study are not detailed in the table but should be considered when interpreting survival outcomes. i.d. = intradermal, s.c. = subcutaneous, i.n. = intranasal, CFU = colony forming unit, PFU = plaque forming unit, PFA = paraformaldehyde, i.m. = intramuscular, i.t. = intratracheal, i.p. = intraperitoneal, CFA or IFA = complete or incomplete Freund's Adjuvant, AAV = adeno-associated virus, TMV = tobacco mosaic virus, CpG = cytosine-phosphate-guanine, OMVs = outer membrane vesicles

Immunization Vaccine Platform	Antigens	Adjuvants	Route	Animal Model	Challenge Strain	Route	Dose (CFU)	Survival	Ref
Live Attenuated	Live attenuated LVS $1x10^5$ - $1x10^9$ CFU	n/a	i.d, s.c., scarification	New Zealand white rabbit	Unchallenged				[41]
	Live attenuated SchuS4, ΔFTT0369c, 50 or 10 CFU	n/a	i.n. i.d.	BALB/c	SchuS4	i.n. i.d.	50 10	80% at d30 100% at d30	[55]
	Live attenuated LVS Δ*capB*, 10^6 CFU, rLm/igIC, 10^6 CFU	n/a	i.d.	BALB/c	SchuS4	Aerosol	10 LD_{50}	75% at d21	[56]
	Live attenuated LVS, FTL_0291, FTL_0325, or FTL_0057, $1x10^7$ CFU	n/a	i.n.	BALB/c	SchuS4	i.n.	100	100% at d21	[57]
	Live attenuated *F. novicida* (IgID), 10^7 CFU	n/a	Oral i.t. Pulmonary	Fischer 344 Rats Cynomolgus macaques	SchuS4	Pulmonary Pulmonary Aerosol	$1x10^4$ 2500-5000	83% at d30 100% at d30 83% at d30	[58]
	Live attenuated LVS Δ*clpB*, $5x10^4$ CFU	n/a	i.n.	C57Bl/6 or BALB/c	LVS	i.n.	$5x10^3$	100% at d14	[47]
	Live attenuated SchuS4 Δ*aroD*, 10^5 CFU, 10^8 CFU	n/a	i.n.	C57Bl/6	SchuS4	i.n.	100	80% at d23	[49]
	Live attentuated SchuS4 Δ*clpB*, 1-1.4 x 10^8 CFU	n/a	Aerosol	New Zealand White Rabbits	SchuS4	Aerosol	2.1 x 10^3	30% at d30	[50]
	Live attentuated SchuS4 Δ*clpB*, 1 $x10^4$ or $5x10^6$-$1x10^7$ CFU	n/a	i.d. s.c.	C57Bl/6 Fischer 344 Rats	LVS SchuS4	i.p. Aerosol	$1x10^6$ $1x10^4$ or $5x10^4$	85% at d30 100% at d20 for low dose, 50% at d20 for high dose	[32]
Immunization Vaccine Platform	**Antigens**	**Adjuvants**	**Route**	**Animal Model**	**Challenge Strain**	**Route**	**Dose (CFU)**	**Survival**	**Ref**
Killed Whole Cell	LVS, UV, paraformaldehyde or heat-killed; $1x10^8$	IL-12 for 3 days following immunization	i.n.	C57Bl/6	LVS	i.n.	$1x10^4$	UV and PFA, 100% at day 21; heat-killed 87.5% on d21	[68]
	Irradiated LVS, $7x10^8$	ISCOMs, ISCOMs + CpG, or alum	i.m.	BALB/c	SchuS4 *F. holarctica* HN63	Aerosol Aerosol	6 or 20 900	ISCOMs + CpG 20% at d21 ISCOMs, 67% at d25, ISCOMs + CpG 100%, alum 0% at d12	[70]
	paraformaldehyde-inactivated LVS	Cholera toxin B (CTB)	i.n.	BALB/c C57Bl/6	LVS SchuS4	i.n. i.n.	$2-8x10^4$ 20	100% at d21 50% at d21	[71]
Immunization Vaccine Platform	**Antigens**	**Adjuvants**	**Route**	**Animal Model**	**Challenge Strain**	**Route**	**Dose (CFU)**	**Survival**	**Ref**
Subunit	OMPs from Ftt	CFA	i.n.	C3H/HeN	SchuS4	i.n.	40	50% at d20	[105]
	O-antigen capsular polysaccharide from FTT	TiterMax Gold Adjuvant	i.p.	BALB/c	LVS	i.p.	$1x10^4$-$1x10^5$	100% at d14	[72]
	Liposomal FopA from LVS	aluminum hydroxide, IL-12	s.c. or i.p./ i.n. (booster)	BALB/c C57Bl/6	LVS	i.d. or i.n.	"lethal dose"	80% at d21 100% at d21	[73]
					SchuS4	i.d.		0% at d9	
	Tul4 in AAV, 10^5 PFU	n/a	i.m. or i.d.	BALB/c	LVS	i.p.	210-400	60% at d10	[74]
	DnaK and Tul4	GPI-0100	i.n.	C57Bl/6	LVS	i.n.	$1.5x10^6$ $8x10^6$	86% at d16 36% at d16	[75]
	Synthetic Tul4 and FopA peptides	CpG	s.c.	C57Bl/6	Unchallenged				[79]
	Whole cell lysate from LVS in cataionic surfactant vesicles	MPL	i.p./i.p i.p./i.n	C57Bl/6J	LVS SchuS4	i.n. i.n.	5000 20	100% at d14 60% at d15	[88]
	FTT0438, FTT1043, FTT0814, LPS in glucan particles	n/a	s.c.	Fischer 344 Rats	SchuS4	Aerosol	$1.6x10^3$	100% at d14	[86]
	FTT0438, FTT1043, FTT0814, LPS, GltA, Mip, IgIC, PilA in glucan particles		i.m. or s.c.	C57Bl/6					

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Table 1 (*continued*)

Immunization Vaccine Platform	Antigens	Adjuvants	Route	Animal Model	Challenge Strain	Route	Dose (CFU)	Survival	Ref
	Tul4 in Ad5 vector, $1x10^8$ CFU	$Al(OH)_3$ + CpG	i.n. or i.m.	BALB/c	LVS	i.p.	$2x10^4$	90% at d21 for i.m. immunized, 40% at d21 for i.n. immunized	[76]
						i.n.	$4x10^4$	90% at d21 for i.n. and i.m. immunized	
Immunization Vaccine Platform	**Antigens**	**Adjuvants**	**Route**	**Animal Model**	**Challenge Strain**	**Route**	**Dose (CFU)**	**Survival**	**Ref**
Conjugate	OAg-BSA	IFA	s.c.	BALB/c	Ftt #33,Type A	i.d.	60	0% at d9	[81]
						Aerosol	50	0% at d6	
					Fth #108,Type B	i.d.	80	100% at d20	
						Aerosol	30	0% at d10	
	inactivated LVS conjugated to mAb against LPS	n/a	i.n.	BALB/c C57Bl/6	LVS	i.n.	$2x10^4$	100% at d25	[84]
					SchuS4	i.n.	18	50% at d32	
	O-antigen conjugated to ExoA from P. aeruginosa	Sigma Adjuvant System	i.p.	BALB/c	F. holarctica HN63	i.p.	100	50% at d25	[82]
	OAg-ExoA	MF59	s.c. or i.p.	Fischer 344Rats	SchuS4	Aerosol	$5.48x10^2$	100% at d21	[83]
	very high molecular weight OAg-TT	Alhydrogel	i.p.	BALB/c	LVS	i.n.	$1x10^4$	80% at d25	[80]
Immunization Vaccine Platform	**Antigens**	**Adjuvants**	**Route**	**Animal Model**	**Challenge Strain**	**Route**	**Dose (CFU)**	**Survival**	**Ref**
F. novicida subunit	*F. novicida* OMVs	n/a	i.n.	BALB/c	*F. novicida*	i.n.	43	75% at d9	[94]
	F. novicida OMVs	n/a	i.n.	BALB/c	*F. novicida*	i.n.	620	100% at d21	[95]
							960	0% at d18	
	OmpA, DnaK, Tul4, SucB in TMV	CpG	i.n.	C57Bl/6	LVS	i.n.	$1x10^5$	100% at d21	[87]

ability to drive IFNγ production and activation of Th1 cell responses [69]. Irradiated LVS administered intramuscularly with immunostimulating complexes (ISCOMS) in BALB/c mice prolonged median time to death (MTD) but failed to protect against mortality in a low dose pulmonary challenge with *F. tularensis* [70]. Paraformaldehyde inactivated LVS has also been co-administered with cholera toxin B as the adjuvant and showed 100% protection against intranasal challenge with LVS and 50% protection against intranasal challenge with *F. tularensis* strain SchuS4 [71]. Collectively, these findings indicate that live attenuated or inactivated whole cell vaccines with an added adjuvant could comprise promising platforms to developing a successful vaccine, but that the current LVS strain may not be an ideal base to protect against infections with Type A *F. tularensis*.

4.2. Subunit and Conjugate Vaccines

Both subunit and conjugate vaccine approaches to combating *F. tularensis* infection have been explored. These methods have showed variable efficacy, but none have been as successful as the live attenuated vaccines discussed above. Development of subunit vaccines typically relies on identifying protective antigens that elicit humoral and/or cellular immune responses. Lipopolysaccharide (LPS) and proteins like Tul4, FopA, DnaK, GroEL, KatG, and SucB have all been identified as common vaccine targets following *F. tularensis* infection, but all have failed to induce a protective response against pulmonary *F. tularensis* in studies [72-76]. Each of these subunit vaccines require the addition of an adjuvant to elicit a strong immune response, and various adjuvants were evaluated in these studies. To optimize the efficacy of *Francisella* vaccines, the selection of an adjuvant should be strategically targeted to elicit a robust cell-mediated immune response. In several of the subunit approaches, combination adjuvants were used that would allow for induction of both arms of the adaptive immune response, or solo adjuvants that could drive a cell-mediated response. Adjuvant choice in these approaches is critical, but it is the combination of the adjuvant/antigen pair that truly dictates protection. The inability of these subunit vaccines to confer complete protection could be due to a lack of known immunodominant epitopes seen in *Francisella*, thus making it more difficult to determine the best protein or polysaccharide combination to use [8,20]. How antigens are displayed may also play a role in vaccine efficacy. For example, *Francisella* has been shown to cause aberrant induction of proinflammatory cytokines when infecting dendritic cells in the airway and can cause downregulation of MHC on macrophages following infection [77,78]. These findings highlight that single epitopes or targets may not be the best candidates as they may fail to closely mimic what is seen during infection. Prior studies have used peptides derived from outer membrane proteins of *F. tularensis* known to be recognized by CD4 T cells following infection. These synthetic peptides from Tul4 and FopA were delivered with the adjuvant CpG and drove dendritic cell maturation and secretion of inflammatory cytokines in addition to antigen-specific IgG production [79]. While this formulation was not tested against *F. tularensis* challenge, it could be promising as it elicited both cellular and humoral responses.

Tetanus-toxoid conjugated to portions of the O antigen of LPS from LVS showed moderate protection against low dose *F. tularensis* intranasal challenge and demonstrated high antibody titers in BALB/c mice. The response was specific to the molecular weight of the O antigen used in the formulation, with higher molecular weight O antigen conferring greater protection [80]. The O antigen has also been conjugated to bovine serum albumin, and mice immunized with this formulation were protected against intradermal challenge with LVS, but not aerosolized LVS or *F. tularensis* [81]. A glycoconjugate-based vaccine using the *F. tularensis* O antigen linked to P. *aeruginosa* exotoxin A (ExoA) induced high antibody titers, and prolonged mean time to death following LVS challenge, with approximately half of immunized mice surviving more than 20 days post challenge, which was roughly 15 days longer than unvaccinated controls [82]. In a follow-up investigational study, a rat

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model of infection combined with the same glycoconjugate-based vaccine and the adjuvant MF59 was used, showing strong protection against pulmonary challenge with low dose *F. tularensis* [83]. It was shown that immunizations using this vaccine adjuvant combination elicited an IFNγ response from splenocytes, which was driven by ExoA. Rawool et al. sought to target their vaccine candidate directly to APCs by using inactivated *F. tularensis* bound by *F. tularensis* LPS specific IgG2a monoclonal antibodies [84]. This allowed the vaccine to be directly taken up by APCs via their Fc receptors and showed protective efficacy against LVS challenge but only moderate protection against virulent *F. tularensis* SchuS4. Another intriguing possibility would be to directly target complement receptor on APCs using engineered vaccine strains specifically designed for this purpose. This has already been shown to be effective in a *Francisella* model that targets macrophage complement receptors and could be broadened to other APCs [85]. Collectively, these studies showed variable efficacy but are suggestive that a combination of *Francisella* antigens and other components like adjuvants are ultimately needed to elicit the right balance of protective cellular and humoral immunity.

4.3. Nanoparticle Vaccines

Other approaches have sought to conjugate *Francisella* proteins to novel nanoparticle delivery systems. One method used glucan particles that were loaded with *F. tularensis* antigens combined with LVS-derived LPS conjugated to the particle surface. This approach induced protection against aerosol challenge with *F. tularensis* SchuS4 and reduced signs of clinical disease severity in both C57Bl/6 mice and Fisher 344 rats, however they did not identify correlates of protection [86]. Using a similar approach (proteins conjugated to a carrier system), OmpA, DnaK, Tul4, and SucB were coupled to the surface of a tobacco mosaic virus (TMV) to create a quadrivalent particle vaccine formulation. Intranasal immunization with this protein-TMV conjugate elicited 100% protection against lethal challenge with LVS up to 21 days post challenge and 80% protection up to 163 days post challenge [87]. This formulation was also able to produce recall IFNγ responses 84 days later in splenocytes in addition to IgG titers emulating antibodies elicited from infectious challenge [87]. This study represents a promising approach to *Francisella* vaccination. While protection was only assessed against the LVS, the recall response for splenocytes was also measured against virulent *F. tularensis* which still showed high production of IFNγ. Another approach used self-assembling nanostructures: catanionic surfactant vesicles that were incorporated with a whole-cell lysate from *F. tularensis* [88]. These vesicles, adjuvanted with monophosphoryl lipid A (MPL-A) showed complete protection against LVS and moderate protection against *F. tularensis* SchuS4. It was highlighted that while this formulation strongly increased antibody responses, the T cell response was not well characterized and could be the key to understanding and creating a strong protective vaccine against *F. tularensis* SchuS4.

Bacterial outer membrane vesicles (OMVs) represent a unique subset of nanoparticles as they are naturally shed from gram-negative bacteria and thus can simulate live bacteria [89]. OMVs are being explored in a variety of contexts as nanoparticle vaccines, including in the FDA-approved vaccine for meningococcal group B disease, Bexsero [90]. Other work has shown that the same OMVs can be both a vaccine and an adjuvant, thus potentially behaving as a self-adjuvanting antigen delivery system [89,91-93]. OMVs from the closely related *F. novicida* may be one novel vaccine approach for combatting tularemia. While it is known that *F. novicida* OMVs are protective against homologous infection with *F. novicida*, investigational studies are needed to assess whether this addition will elicit protection against *F. tularensis* [94,95]. The OMV vaccine approach is complicated by the fact that *F. tularensis* itself doesn't produce OMVs in sufficient quantity to be of use as a vaccine. Therefore, another possible approach would be to use *F. novicida* OMVs that express, or are conjugated to, known protective antigens from *F. tularensis* thereby combining the inherent adjuvanticity and immune stimulating aspects of OMVs with the most promising antigens that offer protection against *F. tularensis* infection.

Nanoparticles as delivery systems or adjuvants are becoming more desirable, especially considering the success of the Pfizer and Moderna SARS-CoV-2 mRNA vaccines that are encapsulated in lipid nanoparticles [96]. For nucleic acid-based vaccines, like the mRNA vaccines, this delivery system is critical in delivering antigen to the right host compartment to achieve protective immunity. Nanoparticles themselves display inherent adjuvanticity due to their size and composition which would also allow formulations using them to benefit from this effect [97]. When considering the development of vaccines against intracellular bacteria like *Francisella*, it may also be sensible to use a nanoparticle-based system that could deliver target antigens into a host cell, where they are likely to be contextually encountered to achieve immune responses mimicking natural infection.

5. Conclusions and future vaccine efforts

Over the past decade, new knowledge has been gained regarding immunity to *Francisella* infection and vaccine correlates of protection against a variety of infection routes. Despite this work, these advances have not yielded a FDA approved human vaccine for use in military or civilian populations. Several candidates that have been highlighted here have shown preclinical promise, but only one has entered clinical trials and is not yet approved for humans in all reaches of the globe. Traditional approaches including live-attenuated, subunit, and conjugate vaccines have all been attempted with varying degrees of success. This could be due to the failure of newer vaccine formulations in engaging an immune response that is like infection, or it could be their inability to engage both the innate and adaptive arms of the immune system. A recent study showed that depending on the route of infection, *F. tularensis* has different, specific metabolic niches [98]. This could mean that there is no one answer to vaccinating against *F. tularensis* and that having multiple types of vaccines available is ideal for combatting different routes of infection. Some newer vaccine approaches included an adjuvant to stimulate the immune response, which often yielded better results compared to non-adjuvanted groups [68,70,83,99]. The addition of an appropriate adjuvant could be the key to unlocking a successful vaccine approach. Adjuvants are known to boost the immune system in a variety of ways and to act as depots, pattern recognition receptor activators, while having the capacity to engage the inflammasome [100]. Protective immunity to *Francisella* requires T cells, with a less defined role for B cells; innate responses also contribute to immunity leading to production of cytokines and ultimately control of intracellular replication [7,9]. Vaccination against *Francisella* should take all of these into consideration and engage the immune system in the appropriate manner with the proper combination of antigens and adjuvants. It is possible that new, untested adjuvants could have a potent effect on protective immunity against *F. tularensis* infection. This is particularly true in the case of adjuvants that can target mucosal tissues with appropriate immune responses. For example, it is known that the ADP-ribosylating adjuvant double mutant heat labile toxin (dmLT) can drive both antigen specific T and B cells into mucosal tissues such as the lung and gut [101,102]. This is true even when the dmLT adjuvanted vaccine was delivered non-mucosally. Further, a variety of newer adjuvants, including dmLT, can be delivered directly via mucosal routes and it is known that mucosal vaccination can induce robust tissue resident immunity at sites of delivery, so this is perhaps another strategy to protect against pulmonary infection [103]. It may also be possible to combine different adjuvants to generate an even more robust immune response that engages the innate immune system and subsequently induces synergistically increased cellular immunity. This has been shown with the adjuvants dmLT combined with the lipopolysaccharide-derived monophosporyl lipid A that activate the non-pyroptotic inflammasome to increase antigen specific CD4 T cell numbers more than what would be expected from either adjuvant alone [104].

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It is becoming clear that novel vaccine approaches are needed against *F. tularensis*, especially for infections that are acquired via the respiratory tract. Some of these strategies may require a combination of novel adjuvants and formulations such as nanoparticle delivery systems. Other strategies could be the use of alternate mucosal routes of vaccination, such as an intranasal or oral, inhaled spray vaccine that directly targets immunity in the lung. Combining preclinical animal models with novel vaccine approaches that can lead to clinical trials is the essential next step for developing tularemia vaccines that can protect against infection and represents the next step in vaccine development for this lethal bacterial pathogen.

CRediT authorship contribution statement

Jaikin E. Harrell: Writing – original draft, Writing – review & editing, Conceptualization. **Chad J. Roy:** Writing – original draft, Writing – review & editing. **John S. Gunn:** Writing – original draft, Writing – review & editing. **James B. McLachlan:** Conceptualization, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of competing interest

Tulane holds a patent on which JBM is an inventor.

Data availability

No data was used for the research described in the article.

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