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NEW ANTI-INFECTIVE STRATEGIES FOR TREATMENT OF TULAREMIA

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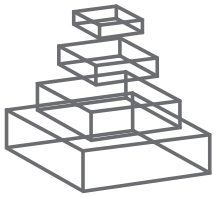

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NEW ANTI-INFECTIVE STRATEGIES FOR TREATMENT OF TULAREMIA

Topic Editor:
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Francisella tularensis, the causative agent of tularemia, is a paradigm among human pathogens. This Gram-negative bacterium has an intracellular lifestyle, which probably reflects an adaptation to its natural animal and protozoa reservoirs. This is one of the most infectious agents in humans and animals; only a few bacteria are needed to induce a severe infection in both types of hosts. The clinical presentation and severity of human tularemia varies according to the portal of entry of bacteria, the bacterial inoculum, the virulence of the infecting strain, and the immune response of the host. Although most infections occur after direct inoculation of bacteria through the skin (through skin wounds or bites of arthropods), pneumonia due to inhalation of infected aerosols is the most feared of the clinical forms of the disease, particularly in the context of biological threat. Two subspecies are responsible for tularemia (subsp. tularensis and subsp. holarctica), and several clades have been described for each, which might be associated with changes in disease severity in humans. Tularemia is also more severe in people with an impaired immune response. No safe vaccine is currently available for prophylaxis of tularemia in humans. On the other hand, control of proliferation of *F. tularensis* in wildlife is not feasible. Thus, only the anti-infective agents are used for treatment and prophylaxis of human tularemia. The standard options include aminoglycosides (gentamicin), tetracyclines (eg, doxycycline) and fluoroquinolones (eg, ciprofloxacin). The selection of acquired resistance to these antibiotics in *F. tularensis*, especially in the context of a biological threat, may quickly limit the therapeutic options. New prophylactic and therapeutic alternatives must be developed rapidly. The present Research Topic focuses on potential new strategies for treatment of tularemia, including the development and evaluation of new compounds having proper antibacterial activity, reducing the virulence of *F. tularensis* or enhancing the immune host response.

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EDITORIAL
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New anti-infective strategies for treatment of tularemia

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Keywords: *Francisella tularensis*, tularemia, antiinfective agents, immunomodulators, virulence

This collection of 10 articles in *Frontiers and Cellular Microbiology* aims to present the current opportunities for development of innovative therapeutic strategies for tularemia, a zoonotic disease caused by *Francisella tularensis*. This Gram-negative, facultative intracellular bacterium is a class 3 human pathogen and a CDC category A bioterrorism agent. Two *F. tularensis* subspecies are responsible for tularemia in humans, subsp. *holarctica* (type B) in the northern hemisphere and subsp. *tularensis* (type A) in North America, which themselves are split into genotypes of variable virulence. *F. tularensis* is transmitted to humans via the dermal, oral, conjunctival or respiratory routes, through direct contact with infected animals (specifically lagomorphs), ingestion of contaminated food or water, inhalation of contaminated aerosols, or arthropod bites (mainly ticks). The disease usually manifests as a regional lymphadenopathy (ulceroglandular, glandular, oculoglandular, and pharyngeal forms), or as systemic typhoid-like (typhoidal form) or pneumonic (pneumonic form) diseases. This latter form is the most feared in the context of bioterrorism. There is no vaccine for tularemia, and few antibiotics are effective in treating tularemia patients, including the aminoglycosides (streptomycin and gentamicin), the fluoroquinolones and the tetracyclines. No acquired resistance to these drugs has been reported in clinical situations, but antibiotics resistant strains could be genetically engineered for biothreat purposes. Thus, tularemia is a relevant model for development of modern anti-infective alternatives.

Because tularemia is a rare disease, clinical trials in infected humans for comparing different therapeutic strategies are not feasible. A number of *in vitro* and *in vivo* models have been developed for evaluation of new treatment alternatives. Importantly, the most relevant models are those using the highly virulent A1b genotype strains of *F. tularensis*, especially the Schu S4 strain. However, experiments using this select agent must be performed in biological safety level 3 laboratories and are submitted to drastic regulation in most countries. Thus, *F. tularensis* strains with attenuated virulence have been used for decades in research laboratories, especially the *F. novicida* Utah 112 strain. In a comparative review, Kingry and Petersen (2014) clearly demonstrate that *F. novicida* should no longer be used as a surrogate of *F. tularensis* for therapeutic efficacy testing in animal models. These two bacteria strongly differ in their natural life cycle, behavior in phagocytes, immunomodulatory properties, and pathogenicity to humans.

Potential new therapeutic strategies for tularemia include the development of new antibiotics or new ways of using existing antibiotics, the reduction of *F. tularensis* virulence and the enhancement of the host innate and/or adaptive immune response (Boisset et al., 2014). Hamblin et al. (2014) report here the high *in vivo* efficacy of a commercial formulation of liposome-encapsulated ciprofloxacin (Lipoquin®, Aradigm Corporation) in mice challenged with the Schu S4 strain. While developing a new dye uptake assay to test the activity of antibiotics against intracellular *F. tularensis*, Sutera et al. (2014) found a significant activity of linezolid (whose activity is currently restricted to Gram-positive bacteria) against intracellular *F. tularensis*. Schmitt et al. (2013) report the intracellular bactericidal activity of resazurin, a compound used for a long time to evaluate the bacterial and eukaryotic cell viability, against *F. tularensis* and *Neisseria gonorrhoeae* (gonorrhea agent). Caspar et al. (2014) present the activity of novel synthetic bis-indole derivatives against *F. tularensis*, although their cytotoxicity precluded evaluation of their activity against the intracellular form of the bacterium.

Opportunities to develop anti-infective agents inhibiting *F. tularensis* virulence or modulating the immune host response are then discussed. Jones et al. (2014) summarize the mechanisms of virulence of *F. tularensis*, including: (1) a capsule-related resistance of *F. tularensis* to opsonization and killing by the complement; (2) lack of activation of TLR4 cytokine response pathway by the lipid A of *F. tularensis* LPS leading to a poor inflammatory response of the phagocytic cells; (3) *F. tularensis* escape of phagolysosomal pathway of phagocytic cells thanks to effectors encoded by a number of genes gathered in the *Francisella* pathogenicity island (FPI); (4) alteration of several adaptive immune response pathways precluding development of an efficient host response. The intracellular multiplication of *F. tularensis* may first be altered by blocking nutrient retrieval of this bacterium within its eukaryotic host cell. As an example, Abu Kwaik (2013) report that, to increase intracellular cysteine concentration, *F. tularensis* cleaves intracellular host glutathione using a gamma-glutamyl transpeptidase, an enzyme that could represent a useful therapeutic target. *F. tularensis* is able to infect and multiply in polymorphonuclears (PMN), a rare property among pathogenic bacteria. Allen (2013) describes how *F. tularensis* turns the intrinsic properties of the PMNs to increase its pathogenicity, including PMNs longevity enhancing due to apoptosis blockade, accumulation of PMNs in infected tissues, granuloma

formation and finally extensive tissue damages. Gillette et al. (2014) summarize how *F. tularensis* perturbs the host immune response by altering the intracellular signaling pathways of monocytes/macrophages. Each step of *F. tularensis*-induced dysregulation of the inflammatory process may represent a target for development of molecules capable of restoring the host immune response. Gillette et al. (2014) mention the potential clinical usefulness of available immunomodulatory compounds for treatment of severely diseased tularemia patients, including type I and type II interferons, celecoxib, imiquimod, and the granulocyte-macrophage colony-stimulating factor (GM-CSF).

In conclusion, promising opportunities exist to improve the treatment of severe forms of tularemia. However, it is probable that none of them taken separately may solve all clinical situations. The combination of one or several immunomodulatory strategies with the administration of drugs inhibiting growth and/or virulence of *F. tularensis* may represent new and interesting therapeutic alternatives, which justifies having simultaneously addressed these different aspects in the present research topic. As an editor of this research topic, I would like to greatly thank all coauthors for their valuable and interesting contributions. We wish the readers of this e-book a productive and enjoyable reading, and we hope that new ideas will emerge on this topic.

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Comparative review of *Francisella tularensis* and *Francisella novicida*

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Francisella tularensis is the causative agent of the acute disease tularemia. Due to its extreme infectivity and ability to cause disease upon inhalation, *F. tularensis* has been classified as a biothreat agent. Two subspecies of *F. tularensis*, *tularensis* and *holarctica,* are responsible for tularemia in humans. In comparison, the closely related species *F. novicida* very rarely causes human illness and cases that do occur are associated with patients who are immune compromised or have other underlying health problems. Virulence between *F. tularensis* and *F. novicida* also differs in laboratory animals. Despite this varying capacity to cause disease, the two species share ~97% nucleotide identity, with *F. novicida* commonly used as a laboratory surrogate for *F. tularensis.* As the *F. novicida* U112 strain is exempt from U.S. select agent regulations, research studies can be carried out in non-registered laboratories lacking specialized containment facilities required for work with virulent *F. tularensis* strains. This review is designed to highlight phenotypic (clinical, ecological, virulence, and pathogenic) and genomic differences between *F. tularensis* and *F. novicida* that warrant maintaining *F. novicida* and *F. tularensis* as separate species. Standardized nomenclature for *F. novicida* is critical for accurate interpretation of experimental results, limiting clinical confusion between *F. novicida* and *F. tularensis* and ensuring treatment efficacy studies utilize virulent *F. tularensis* strains.

Keywords: tularemia, *Francisella tularensis, Francisella novicida,* intracellular pathogen, virulence

INTRODUCTION AND OVERVIEW

F. tularensis was first isolated in 1912 as the causative agent of a plague-like disease affecting squirrels in Tulare county, California (McCoy and Chapin, 1912). Subsequently, Edward Francis, for whom the genus is named, established that several clinical syndromes in humans were caused by *F. tularensis* and proposed the name "tularemia" to describe the illness (Francis, 1925). In 1950 researchers isolated a bacterium from salt water collected from Ogden Bay, Great Salt Lake, Utah (Larson et al., 1955). Initial experiments revealed the bacterium resembled *F. tularensis* morphologically, but fermented sucrose, was of lesser virulence than *F. tularensis* and did not cross-react with serum from rabbits inoculated with heat, ether, formalin, or phenol killed *F. tularensis* (Larson et al., 1955). Based on these phenotypic differences, the isolate was given the unique species name *F. novicida* (Larson et al., 1955). The classification of *F. novicida* as a distinct species was further substantiated in 1964 as a result of its less fastidious growth requirements as compared to *F. tularensis* and the lack of heterologous vaccine protection in mice immunized with killed *F. novicida* and challenged with several *F. tularensis* strains (Owen et al., 1964). Additionally, guinea pigs injected subcutaneously with rabbit immune serum against either *F. tularensis* or *F. novicida* followed by challenge with antigen preparations from both *F. tularensis* and *F. novicida* showed antibody adsorption to homologous but not heterologous antigen at the sight of serum injections (i.e., passive cutaneous anaphylaxis assay) (Owen et al., 1964).

DNA-DNA hybridization experiments performed with *F. tularensis* and *F. novicida* in the 1980s indicated a high degree of genetic relatedness between the two (≥92% at 50°C; ≥86% at 65°C) (Hollis et al., 1989). On this basis, it was proposed, though not validly published that *F. novicida* be reassigned as a subspecies of *F. tularensis* (*F. tularensis* subsp. *novicida*) (Hollis et al., 1989; Lapage, 1992; Tindall et al., 2006). To meet Bacteriological Code requirements, a proposal to transfer *F. novicida* to the subspecies rank of *F. tularensis* (i.e., *F. tularensis* subsp. *novicida*) was validly published in 2010 in the International Journal of Systematic and Evolutionary Microbiology (IJSEM) (Tindall et al., 2006; Huber et al., 2010). This proposal was met with formal objection in IJSEM, as it was based solely on genetic relatedness and did not take into consideration the documented phenotypic and genomic differences between *F. tularensis* and *F. novicida* (Johansson et al., 2010). Indeed, in the 2010 publication proposing reclassification of *F. novicida*, 11 metabolic traits unique to *F. novicida* as compared to *F. tularensis* were identified but not considered with respect to its suggested reassignment as *F. tularensis* subsp. *novicida* (Huber et al., 2010).

In recent decades, an explosion in the amount of basic research focused on understanding and treating tularemia has occurred due to concern about the intentional misuse of *F. tularensis* as a bioweapon (Dennis et al., 2001; Cowley and Elkins, 2011). Research studies using virulent *F. tularensis* strains can be particularly challenging, as they can only be handled under BSL-3 conditions and, in the U.S., within laboratories that are Tier 1 select agent approved (Federal

Register, 2012). In contrast, the *F. novicida* type strain U112 is exempt from select agent regulations in the U.S. and can be handled under standard BSL-2 laboratory conditions (Federal Register, 2012). Information about select agent exemptions for *F. tularensis* can be found at the following website www.selectagents.gov/Select%20Agents%20and%20Toxins%20Exclusions.html#francisella. The less stringent containment requirement for *F. novicida* U112, its high genetic identity to *F. tularensis*, its ability to infect macrophages *in vitro,* to cause illness in laboratory mice, and ease of genetic manipulation as compared to *F. tularensis* have all contributed to widespread use of *F. novicida* U112 as a surrogate for *F. tularensis* (Anthony et al., 1991; Mdluli et al., 1994; Schmerk et al., 2009; Cowley and Elkins, 2011).

To date, the appropriate nomenclature for *F. novicida* remains controversial and non-standardized. While *F. novicida* is recognized on the Approved List of Bacterial Names (Skerman et al., 1980), *F. tularensis* subsp. *novicida* is validly published (Huber et al., 2010), resulting in two different names and no clear decision on the correct nomenclature. As a result, a variety of names including, but not limited to, *F. tularensis, F. tularensis* subsp. *novicida*, *Ft novicida, Ftn, Ftt, Fn,* and *F. novicida*, have all been used in the published literature. This lack of standardized terminology is further complicated by non-enforcement of consistent nomenclature by journals and editors. It is particularly problematic when trying to interpret published experimental results obtained using *F. novicida* U112, but described only as *F. tularensis* with no strain information included. Another negative outcome of the proposed classification of *F. novicida* as a subspecies of *F. tularensis* is that other *F. novicida* strains, excluding the exempt U112 strain, are considered select agents in the US, despite the fact they do not cause tularemia. In order to support maintaining separate species designations for *F. novicida* and *F. tularensis,* genomic as well as clinical, virulence, ecologic and pathogenic differences between the two organisms are reviewed here (**Table 1**). We also discuss the utility of *F. novicida* as a laboratory surrogate for *F. tularensis* with respect to treatment of tularemia.

HUMAN DISEASE AND TRANSMISSION

F. tularensis is one of the most infectious bacterial pathogens known. Studies in human volunteers in the 1960s demonstrated that infection was established with as few as 25 organisms when aerogenically exposed to *F. tularensis* subsp. *tularensis*, with clinically overt disease occurring 3–5 days post exposure (McCrumb, 1961). Two subspecies of *F. tularensis*, *tularensis* (also called type A) and *holarctica* (also called type B), cause human tularemia (Petersen and Molins, 2010). Between these two subspecies, disease outcome and geographic distribution differs (Olsufiev et al., 1959). *F. tularensis* subsp. *tularensis* causes disease only in North America and is associated with higher mortality in humans as compared to *F. tularensis* subsp. *holarctica,* which causes less severe illness throughout the Northern Hemisphere (Olsufiev et al., 1959). Human infection due to a third subspecies, *F. tularensis* subsp. *mediasiatica,* has never been documented in the published literature.

Within both *F. tularensis* subsp. *tularensis* and subsp. *holarctica*, distinct subpopulations have been delineated by a number of different genotyping methods (Petersen and Molins, 2010). In the case of *F. tularensis* subsp. *tularensis,* pulsed field gel electrophoresis defined three subpopulations, A1a, A1b, and A2, which differ with respect to clinical outcome (Kugeler et al., 2009). Among patients infected with A1b strains, significantly higher fatality rates were observed as compared to those patients infected with A1a or A2 strains (Kugeler et al., 2009). The higher mortality rate for infection with an A1b strain was not associated with host factors (age, sex, underlying illness), indicating an intrinsic characteristic of A1b strains (i.e., virulence) is responsible for the observed difference (Kugeler et al., 2009).

F. tularensis causes the zoonotic, vector-borne disease tularemia. Clinical expression of tularemia in humans depends primarily on the route of transmission (Tärnvik and Berglund, 2003; WHO, 2007). Humans acquire infections by a variety of different mechanisms, including arthropod bites (ticks, flies, mosquitoes), direct contact with infected animals (e.g., skinning animals after hunting), ingestion of water or food contaminated by infected animals, and inhalation of infective aerosols (Dennis et al., 2001; WHO, 2007). For all forms, fever and acute symptoms are hallmarks of tularemia in healthy individuals. Arthropod transmission of *F. tularensis* causes glandular and ulceroglandular forms of tularemia, with the latter form of disease presenting as an ulcer at the site of the arthropod bite. Skinning infected animals also leads to ulceroglandular tularemia. Other forms of tularemia include oculoglandular tularemia, acquired via direct inoculation of the eye; oropharyngeal tularemia, acquired through ingestion of water or food contaminated by infected animals; and pneumonic (respiratory) tularemia, acquired through inhalation of infective aerosols during landscaping, farming, or laboratory activities. It is the pneumonic form of tularemia that is the most severe and of highest concern with respect to an intentional aerosol event (Dennis et al., 2001).

In comparison to *F. tularensis, F. novicida* infection is not associated with healthy individuals. *F. novicida* infection in humans is exceedingly rare and therefore often difficult to diagnose accurately (Brett et al., 2012; Birdsell et al., 2009). Only 12 cases have been documented (Hollis et al., 1989; Clarridge et al., 1996; Leelaporn et al., 2008; Birdsell et al., 2009; Brett et al., 2012; Respicio-Kingry et al., 2012; Sjödin et al., 2012; Whitehouse et al., 2012). An *F. novicida*-like infection was reported in an Australian patient, however, genome comparisons indicate the strain is more similar to *F. hispanensis* (Whipp et al., 2003; Sjödin et al., 2012). Illness caused by *F. novicida* does not resemble tularemia. Clinical information available for 11 reported cases indicate that 9 of the *F. novicida* cases occurred in patients who were immunocompromised or had underlying health problems (Hollis et al., 1989; Clarridge et al., 1996; Leelaporn et al., 2008; Birdsell et al., 2009; Brett et al., 2012; Respicio-Kingry et al., 2012; Whitehouse et al., 2012). Fever and acute disease, hallmarks of tularemia in healthy individuals, were only observed for *F. novicida* infections in compromised patients (Hollis et al., 1989; Clarridge et al., 1996; Leelaporn et al., 2008; Brett et al., 2012; Respicio-Kingry et al., 2012; Whitehouse et al., 2012). In the two healthy individuals with *F. novicida* infection, regional lymphadenopathy,

Table 1 | Genetic and phenotypic differences between *F. tularensis* and *F. novicida*.

		F. tularensis[a]	*F. novicida*[b]	References
Genome	Size	1,892,819 bp	1,910,031 bp	Larsson et al., 2005; Rohmer et al., 2007
	Protein coding genes	1445	1731	Larsson et al., 2005; Rohmer et al., 2007
	Pseudogenes	254	14	Rohmer et al., 2007
	FPI	2 copies	1 copy	Nano et al., 2004; Larsson et al., 2005
	Restriction modification systems	1 gene	4 functional systems/6 genes	Gallagher et al., 2008
	CRISPR/Cas	No	Yes	Sampson et al., 2013; Schunder et al., 2013
	O-antigen	15 genes	12 genes; aa identity to Ft 98% to 20%	Thomas et al., 2007; Sjödin et al., 2012
	Recombination	No	Yes	Larsson et al., 2009
	IS element proliferation	Yes	No	Larsson et al., 2009
Clinical	Tularemia	Yes	No	Francis, 1925
	Transmission	Vector-borne, animal contact, inhalation of aerosols	Salt water; brackish water	Larson et al., 1955; Dennis et al., 2001; Brett et al., 2012; Whitehouse et al., 2012
Ecology	Animal hosts	Zoonotic: small mammals, lagomorphs	No	Hopla, 1974; Jellison, 1974
	Arthropod hosts	Ticks, flies, mosquitoes	No	Jellison, 1974; Petersen et al., 2009b
Virulence (LD_{50} by subcutaneous or intradermal route of infection)	Mice	1 CFU	Range from 10 to $>10^7$ CFU	Bell et al., 1955; Larson et al., 1955; Olsufiev et al., 1959; Owen et al., 1964; Meshcheriakova et al., 1995; Kieffer et al., 2003
	Guinea pig	1 CFU	Range from 10 to $>10^5$ CFU	Bell et al., 1955; Olsufiev et al., 1959; Meshcheriakova et al., 1995
	Rabbit	1 CFU	$>10^8$ CFU	Olsufiev et al., 1959; Meshcheriakova et al., 1995
(LD_{50} by intranasal or intratracheal route of infection)	Mice	<10 CFU	Approximate LD_{50} of 10 CFU	Lauriano et al., 2004; Pechous et al., 2008
	Fischer 344 rats	5×10^2CFU	Approximate LD_{50} of 5×10^6 CFU	Ray et al., 2010
Mechanisms of pathogenicity	Cytokine induction upon cellular uptake	No	Yes	Butchar et al., 2008; Dai et al., 2013
	Inflammasome activation	Delayed	Yes	Mariathasan et al., 2006; Weiss et al., 2007b; Fernandes-Alnemri et al., 2010; Dotson et al., 2013
	CRISPR/cas mediated TLR2 evasion	No	Yes	Dai et al., 2013; Sampson and Weiss, 2013a,b
	PI3K/Akt signaling	No; miR-155 suppressed	Yes; miR-155 induced	Cremer et al., 2009
	Pulmonary cell association	Alveolar MΦ/dendritic cells	Alveolar MΦ/neutrophils	Hall et al., 2008
	O-antigen role	Intracellular replication	Complement resistance	Thomas et al., 2007; Case et al., 2014
	Knockout of *iclR* or *galE* genes attenuates virulence in mice	No	Yes	Mortensen et al., 2010; Thomas et al., 2011

[a] *Specific numbers given are in reference to F. tularensis subsp. tularensis Schu S4, except for virulence which is from (Olsufiev et al., 1959) strain Schu.*

[b] *Specific numbers given are in reference to F. novicida U112.*

lacking fever or other symptoms, was reported (Hollis et al., 1989; Birdsell et al., 2009). Classic forms of tularemia, including ulceroglandular, pneumonic, oropharyngeal, and oculoglandular, have not been observed for *F. novicida* infection in healthy individuals.

Given the rarity of *F. novicida* infection in humans, little is known with regards to how the organism is transmitted. Reported human infections are associated with uncertain routes of exposure. For those cases where the mode of infection was ascertained, two cases were due to near-drowning events in salt water and three cases were associated with environmental contamination of outdoor ice machines (Brett et al., 2012; Respicio-Kingry et al., 2012; Whitehouse et al., 2012). No evidence exists to suggest that *F. novicida* is transmitted by animals or arthropod vectors (see Ecology section).

ECOLOGY

In nature, *F. tularensis* and *F. novicida* occupy distinct ecological niches; *F. tularensis* is a classic vector-borne zoonotic pathogen, whereas *F. novicida* is not. As an intracellular pathogen, *F. tularensis* (both subsp. *tularensis* and subsp. *holarctica*) infects and causes disease and mortality in a large number of animal hosts (Hopla, 1974; Jellison, 1974). The bacterium is most often associated with lagomorphs and rodents, including voles, squirrels, and beavers. *F. tularensis* is also found in nature in a number of arthropod vectors, including ticks, flies, and mosquitoes, which bite both animal and human hosts and thereby transmit the organism (Petersen et al., 2009b). Maintenance of *F. tularensis* in nature involves a cycle in which mammals serve as the amplifying hosts and arthropod vectors feed on these bacteremic hosts to disseminate the bacterium to other animals (Petersen et al., 2009b).

In contrast to *F. tularensis,* the identification of *F. novicida* has never been reported in wild animals (healthy or moribund), indicating that in nature *F. novicida* is not a zoonotic bacterium. *F. novicida* has also never been identified in arthropod vectors in nature. Moreover, in the case of arthropods, the lack of identification of *F. novicida* does not appear to be due to inadequate testing methods as numerous *Francisella*-like endosymbionts have been identified in ticks via PCR and sequencing (Scoles, 2004; Goethert and Telford, 2005; Kugeler et al., 2005; Machado-Ferreira et al., 2009; De Carvalho et al., 2011; Ivanov et al., 2011; Kreizinger et al., 2013). As arthropod vectors characteristically acquire infection from bacteremic animal hosts, the lack of identification of *F. novicida* in arthropods is consistent with the presumed inability *F. novicida* to cause bacteremia in wild animals. It is likely that *F. novicida* resides in an environmental niche and is propagated in nature via a mechanism that does not involve mammalian or arthropod hosts. Indeed, the sole source of *F. novicida* isolates to date has been salt water. This includes the *F. novicida* U112 type strain as well as 9 other *F. novicida* isolates (Larson et al., 1955; Petersen et al., 2009a; Whitehouse et al., 2012). Additional environmental sources of *F. novicida*, including brackish water and soil, have been implicated based on PCR detection and sequencing analysis (Barns et al., 2005; Kuske et al., 2006; Berrada and Telford, 2010).

GENOMICS

Genome sequencing has been performed on several *F. tularensis* and *F. novicida* strains, with a limited number of genomes fully assembled and annotated (Larsson et al., 2005; Beckstrom-Sternberg et al., 2007; Chaudhuri et al., 2007; Rohmer et al., 2007; Barabote et al., 2009; Champion et al., 2009; Larsson et al., 2009; Modise et al., 2012; Sjödin et al., 2012; Svensson et al., 2012; Antwerpen et al., 2013). Consistent with the high degree of genetic similarity previously determined via DNA-DNA reassociation (Hollis et al., 1989), the average nucleotide identity observed across 1.1 MB of genomic sequence from 3 *F. novicida* strains and 13 *F. tularensis* strains (8 subsp. *holarctica*, 1 subsp. *mediasiatica*, 2 subsp. *tularensis* A1, and 2 subsp. *tularensis* A2) is $\geq$97.7% (Larsson et al., 2009). Despite this high degree of nucleotide identity, differences are apparent between their respective genomes. *F. novicida* U112 has a larger genome of 1,910,031 bases with more protein coding genes (1731) as compared to *F. tularensis*. The genome size of *F. tularensis* subsp *holarctica* LVS and *F. tularensis* subsp. *tularensis* Schu S4 is 1,895,998 and 1,892,819 bases, with 1380 and 1145 protein coding genes, respectively (Rohmer et al., 2007; Larsson et al., 2009).

Selective genome reduction in the intracellular pathogen *F. tularensis* is clear; the *F. tularensis* subsp. *tularensis* Schu S4 and *F. tularensis* subsp. *holarctica* LVS genomes contain 254 and 303 pseudogenes, respectively (Rohmer et al., 2007). In contrast, only 14 pseudogenes are evident in the *F. novicida* U112 genome (Rohmer et al., 2007). Larsson et al. identified a total of 279 gene losses present in six *F. tularensis* genomes (3 subsp. *holarctica*, 1 subsp. *mediasiatica*, and 2 subsp. *tularensis* strains) as compared to the *F. novicida* U112 genome (Larsson et al., 2009). Frequently it is components of metabolic pathways that are deleted during the transition to an intracellular pathogen, as the nutrients can be acquired from the host. Indeed, metabolic differences between *F. novicida* and *F. tularensis* date back to early characterization of *F. novicida*, when it was found to be less fastidious compared to *F. tularensis* (Owen et al., 1964). More recently, Huber *et al.* identified 11 different metabolic traits present only in *F. novicida* as compared to *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, and *F. tularensis* subsp. *mediasiatica* (Huber et al., 2010). Genomic analyses of *F. novicida* U112, *F. tularensis* subsp. *tularensis* Schu S4, and *F. tularensis* subsp. *holarctica* LVS indicate 41.2 percent of the genes predicted to be involved in amino acid biosynthesis in *F. novicida* U112 are inactivated in one or both *F. tularensis* strains (Rohmer et al., 2007). *F. novicida* U112 appears to have 3 incomplete amino acid synthesis pathways (lysine, histidine, and methionine) whereas in *F. tularensis* subsp. *tularensis* Schu S4 there are 9 incomplete pathways (arginine, histidine, lysine, tyrosine, methionine, cysteine, threonine, valine, and isoleucine) (Larsson et al., 2005; Rohmer et al., 2007; Meibom and Charbit, 2010; KEGG, 2014).

The *F. novicida* U112 genome encodes 84 genes (including those involved in amino acid biosynthesis) that are inactivated in both *F. tularensis* subsp. *tularensis* Schu S4 and *F. tularensis* subsp. *holarctica* LVS (Rohmer et al., 2007). The predicted function of these genes (carbohydrate metabolism, amino acid biosynthesis, metabolite transport, energy metabolism, transport, and DNA restriction/modification) is consistent with *F. novicida*

maintaining the ability to exist in the environment, outside animal hosts. For example, *F. novicida* U112 encodes 4 intact restriction barrier systems in its genome that impair acquisition of foreign methylated DNA by as much as 10^6 fold over native *F. novicida* U112 DNA, suggesting *F. novicida* resides in a niche where it encounters foreign DNA (Maier et al., 2004; Gallagher et al., 2008). The majority of genes encoding restriction barrier systems in *F. tularensis* genomes (subsp. *tularensis* Schu S4 and WY96-3418, subsp. *holarctica* LVS, FTA, and OSU18, and subsp. *mediasiatica*) are present in the form of pseudogenes, suggesting that with its transition to an intracellular pathogen, restriction barrier systems were no longer necessary for survival (Gallagher et al., 2008). This evolutionary phenomenon is also present in strains of increasing virulence in both *Yersinia* and *Burkholderia* (Ong et al., 2004; Kim et al., 2005; Gallagher et al., 2008). Another example of *F. novicida* retaining functions for environmental survival and persistence is the identification of 5 genes (FTN_0451-0456) encoded in the *F. novicida* U112 genome that are responsible for the synthesis and breakdown of the secondary messenger, bis—(3′–5′)—cyclic dimeric GMP (cdGMP) (Zogaj et al., 2012). Overproduction of cdGMP in *F. novicida* U112 initiates biofilm formation as well as attenuates its ability replicate within mouse macrophages. The absence of these genes in *F. tularensis* suggests their elimination provided a selective advantage to its pathogenic intracellular life-cycle (Zogaj et al., 2012).

Gene amplification is evidenced in *F. tularensis* genomes as compared to *F. novicida* genomes. Most notably, genomic analyses of *F. tularensis* (6 subsp. tularensis, 12 subsp. holarctica, and 2 subsp. mediasiatica strains) and *F. novicida* (9 strains) indicate a duplication of the 30 kbp Francisella Pathogenicity Island (FPI) in *F. tularensis* as compared to *F. novicida* which contains only a single copy (Nano et al., 2004; Larsson et al., 2005, 2009; Rohmer et al., 2007). The FPI consists of 16–19 genes comprising a Type VI secretion system (T6SS) (Nano et al., 2004; Nano and Schmerk, 2007; De Bruin et al., 2011). Deletion of most genes within the FPI of both *F. tularensis* and *F. novicida* generates mutants that are defective for intra-macrophage growth and severely attenuated for virulence in mice (Tempel et al., 2006; Maier et al., 2007; Nano and Schmerk, 2007; Bröms et al., 2010; De Bruin et al., 2011; Chou et al., 2013). Given the importance of the FPI for intracellular replication and virulence, it seems likely duplication in *F. tularensis* represents a unique adaptation to its intracellular niche.

Only 7 genes unique to *F. tularensis* were identified via comparative genomic analysis of 20 *F. tularensis* strains (6 subsp. *tularensis*, 12 subsp. *holarctica*, and 2 subsp. *mediasiatica*); counterparts to these genes are absent in 9 *F. novicida* strains (Sjödin et al., 2012). All 7 genes are predicted to encode components necessary for the outer surface of *F. tularensis* cells (Sjödin et al., 2012). FTT0794, FTT0795, and FTT0796 are part of 12.5 kb locus important for formation of a capsule-like complex on the surface of *F. tularensis* (Bandara et al., 2011; Zarrella et al., 2011). The proteins encoded by these genes contain conserved domains for methyltransferase (FTT0795) and phosphocholine metabolism (FTT0794 and FTT0796) (Thomas et al., 2011). FTT1453c (wzx), FTT1454c (wbtJ), and FTT1458 (wzy) encode proteins involved in lipopolysaccharide O-antigen synthesis (Sjödin et al., 2012). The wbtJ gene of *F. tularensis* encodes an N-formyltransferase which converts the O-antigen sugar, dTDP-4,6-dideoxy-4-amino-D-glucose to dTDP-4,6-dideoxy-4-formamido-D—glucose, while the wzy gene product is an O-antigen polymerase whose function is to catalyze addition of newly synthesized O-antigen repeat units (Kim et al., 2010; Zimmer et al., 2013). FTT1188 encodes a hypothetical membrane protein lacking significant homology to known proteins (Sjödin et al., 2012).

Genomic analyses indicate that *F. tularensis* and *F. novicida* evolved as two distinct populations (Larsson et al., 2009). *F. tularensis* strains are highly clonal, differentiating them from *F. novicida* strains, which are characterized by a propensity for recombination. Recombination was noted in 10% of the 742 *Francisella* core genes tested in seven *F. novicida* genomes, whereas there was no evidence of recombination in these same genes when 20 *F. tularensis* genomes were examined (Larsson et al., 2009; Sjödin et al., 2012). Additionally, the *F. tularensis* Schu S4 genome shows evidence of 79 IS element insertions compared to only 26 IS element insertions in the *F. novicida* U112 genome (Rohmer et al., 2007). Genome decay due to IS element proliferation is clear in *F. tularensis;* IS elements in *F. tularensis* are responsible for at least 22 percent of inactivated genes (Larsson et al., 2009). IS element proliferation in *F. tularensis* is also proposed to be responsible for duplication of the FPI in *F. tularensis* (Rohmer et al., 2007; Larsson et al., 2009). Between *F. novicida* and *F. tularensis,* substantial differences are also observed in the ratio of substitution rates at non-synonymous and synonymous sites (dN/dS), with high dN/dS ratios for all *F. tularensis* branches, and considerably lower ratios for *F. novicida* (Larsson et al., 2009). Overall, these findings are consistent with the idea that niche restricted bacteria, such as intracellular pathogens, tend to have monomorphic genomes, whereas environmental bacteria are under weaker purifying selection and therefore retain the capacity to adapt to differing conditions by undergoing genomic changes (Moran, 2002; Achtman, 2008; Larsson et al., 2009).

VIRULENCE

The differing virulence between *F. tularensis* subspecies was classically determined by measuring the number of organisms required to kill 50–100% of infected mice, guinea pigs, and rabbits (Francis and Felton, 1953; Bell et al., 1955; Olsufiev et al., 1959). Variation in the time-to-death of *F. tularensis*-infected animals was also linked to virulence differences between *F. tularensis* subspecies (Olsufiev et al., 1959). Summarized in this section are results of virulence testing for *F. novicida* and *F. tularensis* by two routes of infection (subcutaneous and pulmonary) in mice, guinea pigs, rabbits, and rats. We note that the intent of this section is not to discuss the merits of using one animal model over another for tularemia research.

Both mice and guinea pigs are highly susceptible to *F. tularensis* (both subsp. *tularensis* and subsp. *holarctica*) when introduced via routes that mimic infection due to arthropod bite, with an observed LD_{100} of only 1 organism for subcutaneous inoculation (Bell et al., 1955; Olsufiev et al., 1959). The differing virulence

between *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* in guinea pigs and mice manifests as a shortened time to death; *F. tularensis* subsp. *tularensis* infected mice and guinea pigs (<1000 organisms) die markedly earlier as compared to those infected with *F. tularensis* subsp. *holarctica* (Bell et al., 1955; Olsufiev et al., 1959). Differences in time to death of infected mice are also detected between subpopulations of *F. tularensis* subsp. *tularensis*. Intradermal infection of C57BL/6 mice with 10–20 CFUs results in significantly shortened survival times for those mice infected with A1b strains as compared to those infected with either A1a or A2 strains (Molins et al., 2010), consistent with human epidemiologic data indicating A1b strains have higher virulence than other *F. tularensis* subsp. *tularensis* strains (Kugeler et al., 2009).

In contrast to mice and guinea pigs, virulence is markedly different in rabbits between *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica*. When introduced subcutaneously, an LD_{100} of 1 organism is observed for *F. tularensis* subsp. *tularensis* opposed to 10^9 organisms for *F. tularensis* subsp. *holarctica* (Bell et al., 1955; Olsufiev et al., 1959). White rats are less susceptible to *F. tularensis* subsp. *tularensis* infection as compared to rabbits; an LD_{100} of 10^8–10^9 was reported for subcutaneous infection by either *F. tularensis* subsp. *tularensis* or *F. tularensis* subsp. *holarctica* (Olsufiev et al., 1959).

The virulence of *F. novicida* upon subcutaneous introduction appears to be less than *F. tularensis* in mice, guinea pigs and rabbits, although the exact extent of the difference is difficult to quantify. There is limited data in the literature with respect to the number of *F. novicida* organisms required to kill animals as determined by LD_{50} or LD_{100} studies. Similarly, there is a lack of published data comparing time to death of animals infected with *F. tularensis* vs. *F. novicida*. Initial experiments performed with *F. novicida* U112 indicated 50 organisms introduced subcutaneously was sufficient to kill 100% (4 of 4) of infected mice and guinea pigs (Larson et al., 1955). Owen et al. subsequently reported that 10–100 cells of *F. novicida* U112 were required to kill a mouse and 10–1000 cells required to kill a guinea pig, although no primary data or route of infection was provided (Owen et al., 1964). Experiments using BALB/cByJ mice infected intradermally with *F. novicida* U112 determined an LD_{50} of 2400 CFU (Kieffer et al., 2003). Much higher lethal doses for *F. novicida* introduced subcutaneously were reported in a study published in the Russian literature (Meshcheriakova et al., 1995). An LD_{50} of 1.3×10^4 organisms and LD_{100} of $\sim 10^7$, $>10^8$, $>10^8$ organisms was determined upon subcutaneous infection of outbred mice with *F. novicida* U112, *F. novicida* F6168, and *F. novicida* D9876, respectively (Meshcheriakova et al., 1995). In the same study, an $LD_{100} > 10^5$ organisms was identified for all three *F. novicida* strains (U112, F6168, D9876) via subcutaneous infection of guinea pigs, and in rabbits no mortality was observed with 10^8 organisms of each strain (Meshcheriakova et al., 1995).

In recent years, the intranasal route of infection has been used to induce respiratory illness in mice, given the severity of pneumonic tularemia and the potential impact of an intentional aerosol release of *F. tularensis*. Published studies are consistent with a difference in virulence between *F. tularensis* subsp. *tularensis* Schu S4 and *F. novicida* U112 via this route of infection. An LD_{50} of <10 CFUs was determined for *F. tularensis* subsp. *tularensis* Schu S4 in BALB/c mice (Pechous et al., 2008) and 100% mortality is reported for infection of C57BL/6, BALB/c, and BALB/cByJ with 13-25 CFU (Qin et al., 2008; Cong et al., 2009; Child et al., 2010; Okan et al., 2013; Richard et al., 2014). For *F. novicida* U112, an approximate LD_{50} of 10 CFU was determined in inbred mice by intranasal inoculation, with two of five BALB/c mice surviving an inoculum of 30 CFU, and one of five surviving an inoculum of 300 CFU (Lauriano et al., 2004). In C57BL/6 and BALB/c mice, 100% mortality is reported using doses ranging from 100 to 445 CFU of *F. novicida* U112 (Pammit et al., 2004; Mares et al., 2008; Sharma et al., 2009).

Significant virulence differences between *F. tularensis* and *F. novicida* are evident upon pulmonary infection of Fischer 344 rats via intratracheal instillation (Ray et al., 2010). Fischer 344 rats show the highest sensitivity to *F. tularensis* subsp. *tularensis* Schu S4 (approximate LD_{50} of 5×10^2 CFU) as compared to *F. tularensis* subsp. *holarctica* OR96-0246 (approximate LD_{50} of 1×10^5 CFU) (Ray et al., 2010). In contrast, Fischer 344 rats are highly resistant to *F. novicida* U112 infection, with an approximate LD_{50} of 5×10^6 CFU (Ray et al., 2010). Of note, a rapid time to death (MTD = 3 days) was observed in the rats which succumbed to infection with *F. novicida*, as compared to rats which died due to infection with *F. tularensis* (MTD = 10 days), suggesting death due to *F. novicida* was likely a toxic effect from the large number of organisms rather than from a productive infection (Ray et al., 2010).

MECHANISMS OF PATHOGENESIS

As described in this review, the genomes of *F. novicida* and *F. tularensis* are highly similar, with the vast majority of genes in *F. tularensis* also found in *F. novicida*. Despite this overall genetic similarity, evidence indicates differential regulation of and distinct roles for homologous genes in *F. tularensis* and *F. novicida* as pertains to pathogenesis. Moreover, *F. tularensis* has developed strategies distinct from *F. novicida* to evade host immune responses. This section will focus on some of the differences that have been described to date.

Evidence that the same genes in *F. novicida* and *F. tularensis* play distinct roles in pathogenesis comes from knockout studies of homologous genes. For example, inactivation of the genes encoding the transcriptional regulator IclR or the UDP-glucose-4-epimerase GalE resulted in attenuation of *F. novicida* U112, but not *F. tularensis* subsp. *tularensis* Schu S4 in a mouse model of infection (Weiss et al., 2007a; Mortensen et al., 2010; Thomas et al., 2011). In the case of the *dsbB* gene, which encodes disulfide bond formation B protein, deletion mutants were attenuated in both *F. tularensis* subsp. *tularensis* Schu S4 and *F. novicida* U112. However, *F. novicida* U112 knockouts provided protection from challenge with *F. novicida* U112, while *F. tularensis* subsp. *tularensis* Schu S4 mutants provided no homologous protection (Tempel et al., 2006; Qin et al., 2008). Intramacrophage secretion of FPI proteins also differs between *F. tularensis* and *F. novicida*. Upon infection of macrophages, 8 FPI proteins (IglE, IglC, IglI, IglJ, IglF, VgrF, PdpE, and PdpA) were secreted by *F. tularensis* subsp. *holarctica* LVS, whereas only 4 (IglE, IglC, PdpE, and PdpA) were secreted by *F. novicida,* suggesting fundamental differences may

exist between the two species with respect to the Type VI secretion mechanism (Bröms et al., 2012).

The cell surface, a critical pathogenicity determinant, differs between *F. tularensis* and *F. novicida*. Early studies indicated a lack of serum cross-reactivity between *F. tularensis* and *F. novicida*. More recently all genes unique to *F. tularensis* as compared to *F. novicida* were predicted to encode outer surface components (see Genomics section) (Larson et al., 1955; Owen et al., 1964; Sjödin et al., 2012). Indeed, distinct structures for the core oligosaccharide and O-antigen of *F. tularensis* and *F. novicida* LPS have been described. The core oligosaccharide of *F. tularensis* lacks a glucose residue attached to the β-glucose branch as compared to *F. novicida* (Vinogradov et al., 2002; Vinogradov and Perry, 2004; Gunn and Ernst, 2007; Okan and Kasper, 2013), while the O-antigen of *F. tularensis* contains two distinct sugar moieties at either end of the tetra-saccharide repeat and is present in longer oligomer chains as compared to *F. novicida* (Vinogradov et al., 2004; Thomas et al., 2007; Barker et al., 2014). Reflecting the observed structural variation, three of the genes in the O-antigen encoding locus are unique to *F. tularensis* (see Genomics section) and among the other 12 genes, amino acid identity ranges from 98% to as low as 20% (Thomas et al., 2007; Sjödin et al., 2012). The structurally and antigentically unique O-antigens from *F. tularensis* and *F. novicida* appear to play different roles in the pathogenicity of each strain. In *F. tularensis,* the O-antigen is critical for intracellular survival as an O-antigen mutant (*wbtDEF*) (Thomas et al., 2007; Jones et al., 2012) is significantly attenuated for intracellular growth as compared to a similar *wbtDEF* mutant in *F. novicida,* which replicates normally in macrophages (Thomas et al., 2007). Recent evidence indicates that the O-antigen of *F. tularensis* subsp. *tularensis* Schu S4 protects it from autophagic detection once it reaches the cytosol (Case et al., 2014).

The cell surface of *F. tularensis* also plays an important role in cell entry and evasion of the host innate immune response. Within host serum, the function of complement proteins is to recognize pathogens and protect the host by direct lysis of the pathogen or opsonization leading to phagocytosis. Both *F. tularensis* and *F. novicida* have been shown to fix human complement protein C3 on their surface, but are resistant to complement mediated lysis due to rapid conversion of C3b to C3bi (Clay et al., 2008). This conversion of C3 leads to the interaction of C3bi with complement receptor protein C3R on host cells and cellular uptake by phagocytosis (Clemens et al., 2005; Ben Nasr and Klimpel, 2008; Clay et al., 2008; Dai et al., 2013). Deposition of C3 on *F. novicida* has been shown to increase both the production of reactive oxygen species (ROS) by human neutrophils and the production of TNFα, IL-6, and IL-1β by human monocytes (Barker et al., 2009; Dai et al., 2013). In stark contrast, C3 deposition on *F. tularensis* subsp. *holarctica* LVS resulted in significantly less ROS production by human neutrophils, and C3 deposition on *F. tularensis* subsp. *tularensis* Schu S4 was directly linked to suppression of the host immune response as monitored by the decreased production of the proinflammatory cytokines, TNFα, IL-6, and IL-1β, during uptake by human monocytes (Barker et al., 2009; Dai et al., 2013). Taken together, these results suggest different means of cellular entry for *F. tularensis* and *F. novicida* and also differential effects on the early host immune response.

A side-by-side comparison of pulmonary infection by *F. tularensis* or *F. novicida* in C57BL/6 mice demonstrated dissimilar cell types were infected *in vivo*. One day post-infection, via the intranasal route, *F. tularensis* subsp. *tularensis* Schu S4, *F. tularensis* subsp. *holarctica* LVS, and *F. novicida* U112 were preferentially associated with alveolar macrophages, although this proportion differed at 78.9, 70.3, and 51.6%, respectively (Hall et al., 2008). Strikingly, 27.3% of *F. novicida* infected cells on day 1 were neutrophils as compared to only 0 and 0.4% for *F. tularensis* subsp. *tularensis* Schu S4 and *F. tularensis* subsp. *holarctica* LVS, respectively (1000 fold difference in the number of neutrophils), indicating that neutrophils responded to and phagocytosed *F. novicida* U112 to a significantly greater extent than they did *F. tularensis* (Hall et al., 2008). Moreover, increasing numbers of alveolar macrophages and dendritic cells were infected from day 1 to 3 following inhalation with either *F. tularensis* subsp. *tularensis* Schu S4 or *F. tularensis* subsp. *holarctica* LVS, but not for *F. novicida* U112, suggesting more rapid killing of *F. novicida* infected cells (Hall et al., 2008).

Within host cells, *F. tularensis* and *F. novicida* display distinct abilities to evade the host immune response. The formation of the inflammasome, a multi-protein complex present in the host cell cytoplasm, is activated by microbial components to induce maturation of the inflammatory cytokines, interleukin IL-1β and IL-18, thereby leading to death of infected cells (Bauernfeind and Hornung, 2013). *F. novicida* is unable to efficiently evade this host innate immune response, and is recognized by the inflammasome upon escape from the phagosome and entry into the host cell cytoplasm (Mariathasan et al., 2006; Weiss et al., 2007b; Fernandes-Alnemri et al., 2010; Jones et al., 2012; Dotson et al., 2013). In contrast, *F. tularensis* successfully escapes inflammasome activation early in infection (∼12 h) via a mechanism involving suppression of TLR2 signaling (Dotson et al., 2013). Presumably, this early suppression of the inflammasome allows *F. tularensis* time to successfully replicate to high levels in the cytoplasm prior to host cell death (Dotson et al., 2013).

Toll-like receptors (TLRs) play a central role in initiating innate cellular immune responses (Lim and Staudt, 2013). Evasion of TLR2 signaling has been shown to be involved in the intracellular replication of both *F. tularensis* and *F. novicida*, although the mechanism utilized diverges between the two bacteria (Telepnev et al., 2003; Katz et al., 2006; Malik et al., 2006; Abplanalp et al., 2009; Dai et al., 2013). Within the phagosome, *F. novicida* down-regulates the production of an endogenous transcript (FTN_1103), encoding a TLR2 stimulating lipoprotein, in a CRISPR/Cas system dependent manner (Sampson et al., 2013; Sampson and Weiss, 2013a,b). In contrast, *F. tularensis* lacks both the functional CRISPR/Cas system as well as the FTN_1103 homolog; genomic analyses indicate significant disruption/degradation of these genes (Schunder et al., 2013; Sampson and Weiss, 2013b). Rather, *F. tularensis* appears to evade TLR2 activation via a mechanism that involves the PI3K/Akt pathway, which when activated leads to production of the pro-inflammatory cytokines IL-6, IL-8, and IL-1β (Butchar et al., 2008; Cremer et al., 2009, 2011; Medina et al., 2010). The

P13K/Act pathway is subject to negative regulation by the enzyme SHIP and a cellular micro-RNA, miR-155 (Cremer et al., 2009, 2011). Induction of miR-155 down-regulates SHIP to promote activation of the P13/Act pathway and inflammatory cytokine production. *F. tularensis* subverts or suppresses the induction of miR-155, thereby repressing the PI3K/Akt pathway. In contrast, *F. novicida* strongly induces miR-155, leading to activation of the P13K/Act pathway and the production of TNFα and IL-6 by human monocytes (Cremer et al., 2009, 2011).

TREATMENT

Development of novel therapeutics for the treatment of tularemia is an area of active research given concern regarding the potential misuse of *F. tularensis* as a bioweapon. Standard antimicrobial therapy is effective for the treatment of tularemia, with aminoglycosides, tetracyclines, and chloramphenicol approved for treatment of tularemia by the U.S. Food and Drug Administration. Although ciprofloxacin and other fluoroquinolones are not currently FDA-approved for treatment of tularemia, they show very good efficacy against *F. tularensis in vitro*, in animals, and in humans (Johansson et al., 2000, 2002; Steward et al., 2006; Klimpel et al., 2008; Meric et al., 2008; Urich and Petersen, 2008; Nelson et al., 2010; Weber et al., 2012).

Antibiotic resistance to frontline therapeutics recommended for treatment of tularemia has never been identified in naturally occurring strains of *F. tularensis* or *F. novicida* (Ikäheimo et al., 2000; Garcia Del Blanco et al., 2004; Tomaso et al., 2005; Urich and Petersen, 2008; Valade et al., 2008; Georgi et al., 2012). Although treatment failure has been documented for human cases of tularemia, it is not associated with spontaneous antibiotic resistance, but rather a delay in antibiotic initiation (Celebi et al., 2006; Meric et al., 2008; Kaya et al., 2011). Nonetheless, antibiotic resistance remains a concern, whether spontaneous or intentionally engineered. *In vitro* experiments demonstrate that both *F. tularensis* and *F. novicida* have the ability to rapidly acquire resistance to quinolones. Passage of either *F. tularensis* subsp. *holarctica* LVS or *F. novicida* U112 on increasing concentrations of ciprofloxacin resulted in resistance to homologous classes of drugs (Sutera et al., 2014). Of note, in the case of *F. novicida*, but not *F. tularensis* subsp. *holarctica,* cross-resistance to heterologous classes of antimicrobials, including doxycycline and erythromycin, was observed (Sutera et al., 2014). This suggests *F. novicida* U112 encodes other genes not present in *F. tularensis* that confer multidrug resistance and is consistent with genomic comparisons indicating more transporters are present in the genome of *F. novicida* U112 (Rohmer et al., 2007; Sutera et al., 2014).

New therapeutic approaches for tularemia range from targeting the organism itself to modulating the host response in order to mount a protective response. These therapeutic approaches are covered in other chapters of this series. For approval and licensure of new therapeutics for tularemia, direct evaluation of the product's efficacy in a clinical setting is needed. Because therapeutic efficacy testing is not always feasible in a clinical setting (e.g., limited numbers of cases), the U.S. Food and Drug Administration developed the "Animal Rule" (21 CFR 314.610 and 21 CFR 601.91) to allow animal efficacy data to support product licensure or approval. Of note with respect to *F. tularensis* and *F. novicida*, the "Animal Rule" states that the etiological agent used in animal studies generally should be identical to the one that causes human disease. As discussed above, *F. novicida* does not cause tularemia in humans and differences between *F. novicida* and *F. tularensis* are also apparent in animals, indicating that *F. novicida* should not substitute for *F. tularensis* in efficacy testing of therapeutics. As recent studies indicate virulence differs among *F. tularensis* subsp. *tularensis* strains in humans (Kugeler et al., 2009; Molins et al., 2010), the use of more virulent A1b strains should be considered for therapeutic efficacy testing in animals.

CONCLUSIONS AND PERSPECTIVE

Bacterial species have traditionally been defined on the basis of DNA-DNA hybridization values (Lapage, 1992; Stackebrandt et al., 2002). The importance of phenotypic differences, however, cannot be understated with respect to classification of bacterial species. In 2002, the *ad-hoc* committee for the re-evaluation of bacterial species definition stated: "Phenotype, including chemotaxonomic markers, will remain important diagnostic properties in a species description. The ecological role can, in certain cases, decide on the species status. For example, medical organisms with defined clinical symptoms may continue to bear names that may not necessarily agree with their genomic relatedness so as to avoid unnecessary confusion among microbiologists and non-microbiologists ['*nomen periculosum*' according to Rule 56a(5) of the International Code of Nomenclature of Bacteria (Lapage, 1992)]" (Stackebrandt et al., 2002). A classic example of the value in utilizing phenotypic data to maintain distinct species designations comes from the bacteria *Yersinia pestis* and *Yersinia pseudotuberculosis*. Although these two bacteria share >97% nucleotide identity across 75% of their genes, they retain individual species names, due to their striking clinical and ecological differences (Chain et al., 2004; Carniel et al., 2006). *Y. pestis* causes the highly fatal vector-borne disease, plague, whereas *Y. pseudotuberculosis* is transmitted by the fecal-oral route and infection rarely leads to death.

In this review, we have highlighted clinical, ecological, genomic, virulence, and pathogenic differences between *F. novicida* and *F. tularensis* that when considered in conjunction with genetic identity clearly warrants maintaining *F. novicida* and *F. tularensis* as separate species (**Table 1**). *F. tularensis* causes the zoonotic vector-borne disease tularemia, whereas *F. novicida* does not. As determined by whole genome comparisons, *F. tularensis* evolved independently of *F. novicida,* which is consistent with its completely distinct ecological niche (*F. tularensis* is a zoonotic pathogen whereas *F. novicida* is not) and mechanisms of transmission (*F. tularensis* is transmitted by arthropod vectors whereas *F. novicida* is not). Moreover, as part of *F. tularensis*' pathogenic intracellular lifestyle, it has developed strategies distinct from *F. novicida* to evade host immune responses and successfully propagate in animal hosts.

F. novicida and its mutants have clearly contributed to our understanding of the biology of *F. tularensis*. A classic example was the discovery of the 30 kbp FPI in *F. novicida* (Gray et al., 2002; Nano et al., 2004). In more recent years, side-by-side

experiments including both *F. novicida* and *F. tularensis* have highlighted the value of direct comparison between the two as pertains to understanding the unique pathogenic mechanisms *F. tularensis* has evolved to elicit its extreme virulence (Vinogradov et al., 2002, 2004; Vinogradov and Perry, 2004; Thomas et al., 2007; Butchar et al., 2008; Hall et al., 2008; Cremer et al., 2009; Mortensen et al., 2010; Bröms et al., 2012; Dai et al., 2013; Dotson et al., 2013; Sutera et al., 2014). As we move forward, findings utilizing the select agent exempt *F. novicida* U112 strain will no doubt continue to provide novel insight into the closely related species, *F. tularensis*. It is essential, however, to keep the two species separate and utilize standardized nomenclature for *F. novicida*. The recognition of *F. novicida* as a separate species via consistent and accepted nomenclature will limit misinterpretation of experimental results as pertains to the human disease tularemia caused by *F. tularensis,* avoid confusion between *F. tularensis* and *F. novicida* in clinical settings and ensure *F. tularensis* strains are used for treatment efficacy studies.

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New therapeutic approaches for treatment of tularaemia: a review

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Antibiotic treatment of tularaemia is based on a few drugs, including the fluoroquinolones (e.g., ciprofloxacin), the tetracyclines (e.g., doxycycline), and the aminoglycosides (streptomycin and gentamicin). Because no effective and safe vaccine is currently available, tularaemia prophylaxis following proven exposure to *F. tularensis* also relies on administration of antibiotics. A number of reasons make it necessary to search for new therapeutic alternatives: the potential toxicity of first-line drugs, especially in children and pregnant women; a high rate of treatment relapses and failures, especially for severe and/or suppurated forms of the disease; and the possible use of antibiotic-resistant strains in the context of a biological threat. This review presents novel therapeutic approaches that have been explored in recent years to improve tularaemia patients' management and prognosis. These new strategies have been evaluated *in vitro*, in axenic media and cell culture systems and/or in animal models. First, the activities of newly available antibiotic compounds were evaluated against *F. tularensis*, including tigecycline (a glycylcycline), ketolides (telithromycin and cethromycin), and fluoroquinolones (moxifloxacin, gatifloxacin, trovafloxacin and grepafloxacin). The liposome delivery of some antibiotics was evaluated. The effect of antimicrobial peptides against *F. tularensis* was also considered. Other drugs were evaluated for their ability to suppress the intracellular multiplication of *F. tularensis*. The effects of the modulation of the innate immune response (especially via TLR receptors) on the course of *F. tularensis* infection was characterized. Another approach was the administration of specific antibodies to induce passive resistance to *F. tularensis* infection. All of these studies highlight the need to develop new therapeutic strategies to improve the management of patients with tularaemia. Many possibilities exist, some unexplored. Moreover, it is likely that new therapeutic alternatives that are effective against this intracellular pathogen could be, at least partially, extrapolated to other human pathogens.

Keywords: tularaemia, treatment, antibiotics, immunotherapy, antibodies

INTRODUCTION

Francisella tularensis, the agent of tularaemia, is a facultative intracellular pathogen of humans and hundreds of animal species (including mammals, insects, arthropods, and protozoa). It is highly infectious, with only 10 bacteria able to cause a lethal infection. Two *F. tularensis* subspecies are virulent for humans: subsp. *tularensis* (type A), found mainly in North America, and subsp. *holarctica* (type B), found throughout the northern hemisphere. Both subspecies are classified as category A agents of biological threat by the Centers for Disease Control (CDC, Atlanta, Georgia, USA).

Tularaemia is considered a re-emerging disease, and recent outbreaks have been reported worldwide (Chitadze et al., 2009; Hauri et al., 2010). The mean incubation period of tularaemia is only 3–5 days (Tarnvik and Chu, 2007) and even less in case of aerosol contamination. Most clinical cases involve chronic lymphadenopathy associated with a skin, mucosa or conjunctival inoculation lesion. Less frequently, patients suffer from systemic diseases, and inhalation of infected aerosols may lead to severe pneumonia with mortality rates up to 60% (Gill and Cunha, 1997). This latter clinical form is the most feared in the context of bioterrorism. No vaccine is currently available and only a few antibiotic classes are effective to treat tularaemia patients, including the fluoroquinolones, the tetracyclines and the aminoglycosides (only streptomycin and gentamicin) (KuoLee and Chen, 2007; Hepburn and Simpson, 2008). Although no acquired resistance to these antibiotics has been described in natural strains of *F. tularensis*, many therapeutic failures and relapses have been reported (Perez-Castrillon et al., 2001; Kosker et al., 2013). Even with antibiotic treatment, tularaemia may be associated with 2% mortality (Evans et al., 1985). It is therefore essential to develop novel and effective preventive and curative treatments.

We will review the current therapeutic approaches and new therapeutic strategies (**Table 1**) published in the English literature.

Table 1 | New therapeutic alternatives evaluated against *F. tularensis* infection.

Drug	Mechanism of action/target	Activity on *F. tularensis*	References
NEW ANTIBIOTICS			
Ketolides	Inhibit protein synthesis by interacting with the peptidyl-transferase site of the bacterial 50S ribosomal subunit.	Effective *in vitro* against French isolates of *F. tularensis* subsp. *holarctica*	Gestin et al., 2010
Tigecycline	Inhibits protein translation by binding to the 30S ribosomal subunit and blocking entry of amino-acyl tRNA molecules into the A site of the ribosome	Effective *in vitro* against Hungarian *F. tularensis* subsp. *holarctica*	Kreizinger et al., 2013b
Grepafloxacin Trovafloxacin Sparfloxacin Gatifloxacin	Inhibit DNA synthesis by targeting DNA gyrase and topoisomerases	Highly active *in vitro* against American and Austrian *F. tularensis* subsp. *tularensis* and subsp. *holarctica* strains Gatifloxacin effective *in vivo* in a BALB/c mouse model of *F. tularensis* Schu S4 infection	Ikaheimo et al., 2000 Johansson et al., 2002 Tomaso et al., 2005 Piercy et al., 2005; Steward et al., 2006
Linezolide	Inhibits the initiation process of protein synthesis	Effective *in vitro* against Turkish strains, Not effective against *F. tularensis* strains isolated in Hungary or in North America	Yesilyurt et al., 2011 Johansson et al., 2002
ANTIMICROBIAL PEPTIDE			
LL-37	Stimulates the innate immune response, increases in the production of IL-6, Il-12, IFN-gamma, and MCP-1	Moderately and transiently effective in a murine model of LVS-induced pneumonia	Flick-Smith et al., 2013
OTHER MODULATOR OF INNATE IMMUNE RESPONSE			
IL-12	Activates Th1 and NK cells and induces the production of IFN-gamma	Improves the clinical outcome and survival of animals infected with *F. novicida*, when co-administrated with gentamicin.	Pammit et al., 2004 Kirimanjeswara et al., 2008
AGP	Synthetic TLR4 agonist, increases the amount of intrapulmonary cytokines and chemokines	Reduced bacterial replication in the lungs, liver and spleen, and increased survival of animals infected with *F. novicida*	Lembo et al., 2008
CpG	TLR9 activator, indirectly activates NK cells resulting in cytokines and NO production	Better survival of mice infected with *F. tularensis* LVS, but no effect in mice with *F. tularensis* SCHU4 pneumonia	Elkins et al., 2009 Rozak et al., 2010
poly(I:C)	Synthetic double stranded RNA analog, TLR3 activator, induces an early and effective innate immune response	Better survival of mice infected with *F. tularensis* LVS or Schu S4 Reduction in replication of *F. tularensis* within human monocyte-derived macrophages	Pyles et al., 2010
Galantamine	Influences the immune response via the cholinergic anti-inflammatory pathway by up-regulating IFN-gamma production and down-regulating IL-6 production	Reduced mortality rates in mice infected with *F. tularensis* LVS	Pohanka et al., 2012
Acai PS	Enhances Th1 cell-related immunity	Increased survival of mice infected with *F. tularensis* Schu S4	Skyberg et al., 2012
SPECIFIC ANTIBODIES			
Monoclonal antibodies	Monoclonal antibodies against the LPS of *F. tularensis* LVS	Successfully used to treat LVS-induced pneumonia; No effect on mice infected with *F. tularensis* Schu S4	Lu et al., 2007 Kirimanjeswara et al., 2008 Savitt et al., 2009

(Continued)

Table 1 | Continued

Drug	Mechanism of action/target	Activity on *F. tularensis*	References
Immune sera	Immune sera from mice infected via intra-nasal Schu S4 challenge, and then treated with levofloxacin Sera containing abundant immunoglobulin IgG2a	Protective in mice infected with *F. tularensis* Schu S4, when administered 24 h post-infection	Klimpel et al., 2008
Anti-MPF IgM and IgG antibodies	Antibodies directed against the membrane protein fraction (MFP) of *F. tularensis* Schu S4	Successfully used to treat mice infected with *F. tularensis* Schu S4 when combined with gentamicin	Sutherland et al., 2012

RECOMMENDED ANTIBIOTICS FOR TULARAEMIA TREATMENT (WHO GUIDELINES)

According to the WHO guidelines (WHO, 2007), patients with severe tularaemia requiring hospitalization should receive parenteral administration of streptomycin or gentamicin. Both aminoglycosides display *in vitro* bactericidal activity against *F. tularensis* types A and B (Enderlin et al., 1994; Johansson et al., 2002; Kreizinger et al., 2013b). Aminoglycosides are associated with better outcomes and lower relapse rates (Enderlin et al., 1994). However, they can only be used parenterally, and they are occasionally associated with severe toxicity (especially ototoxicity and nephrotoxicity). Streptomycin is no longer available in many countries.

Fluoroquinolones are now advocated as first-line drugs in patients with clinical manifestations of mild to moderate severity (Ellis et al., 2002; Johansson et al., 2002). Ciprofloxacin may be given intravenously or by oral administration. *In vitro*, fluoroquinolones are highly active against *F. tularensis* subsp. *holarctica* (Syrjala et al., 1991; Scheel et al., 1993; Ikaheimo et al., 2000; Kreizinger et al., 2013b) and *F. tularensis* subsp t*ularensis* (Johansson et al., 2002). Most data are restricted to ciprofloxacin, but levofloxacin, with an oral bioavailability of over 99% and daily administration, is an attractive alternative. Murine models have shown that the intraperitoneal administration of levofloxacin is 100% effective in preventing tularaemia after *F. tularensis* challenge (Klimpel et al., 2008). Intravenous administration of levofloxacin was also successful in treating human tularaemia cases, without relapse (Limaye and Hooper, 1999; Aranda, 2001). The bioavailability and efficacy of levofloxacin against *F. tularensis* was also studied in a common marmoset model of inhalational tularaemia that more faithfully mimics human tularaemia pneumonia (Nelson et al., 2010). Orally administered levofloxacin was highly effective in preventing the onset of acute inhalation tularaemia. Meric et al. also reported that moxifloxacin was as successful as ciprofloxacin for treatment of oropharyngeal tularaemia (Meric et al., 2008). Recently, Sutera et al. obtained *in vitro* mutants highly resistant to fluoroquinolones in different *Francisella* species (*F. tularensis* subsp. *holarctica* LVS, *F. novicida*, and *F. philomiragia*) by propagating strains with increasing concentration of ciprofloxacin. All high-level resistant mutants shared cross-resistance to other fluoroquinolones tested (moxifloxacine and levofloxacine), while some also revealed striking levels of cross-resistance to other clinically relevant antibiotic classes (the aminoglycoside gentamicin and tetracyclines) (Sutera et al., 2014).

Tetracyclines, especially doxycycline, are considered a potential alternative. However, due to its bacteriostatic activity, treatment must be administrated for a minimum of 2 weeks, and higher relapse rates have been reported as compared to fluoroquinolones (Enderlin et al., 1994). Tetracyclines are classically contraindicated in children less than 8 years old because of the risk of teeth discoloration, and in pregnant women because of foetal bone toxicity.

Whatever drug is administered, failures and relapses are often associated with delayed and/or insufficiently long treatment. Suppurated lymph nodes often need to be removed surgically to obtain clinical cure (Penn and Kinasewitz, 1987; Celebi et al., 2006).

OTHER ANTIBIOTICS

Most beta-lactams have no bacteriostatic activity against *F. tularensis in vitro* (Tarnvik and Chu, 2007). Although third-generation cephalosporins (e.g., ceftriaxone) may be active *in vitro* against specific *F. tularensis* strains (Tomaso et al., 2005), therapeutic failures with these antibiotics are common (Cross and Jacobs, 1993). The ineffective activity of the beta-lactams against *F. tularensis* may be related both to the secretion of a class A beta-lactamase by this bacterium (Antunes et al., 2012) and to the poor penetration of these antibiotics in eukaryotic cells (Maurin et al., 2000). Thus, the beta-lactams are considered unreliable for tularaemia treatment.

It was first assumed that North American isolates of *F. tularensis* were naturally susceptible to macrolides, whereas most European strains were naturally resistant, including the vaccine strain *F. tularensis* LVS (Kudelina and Olsufiev, 1980). Later, Georgi et al. showed that *F. tularensis* subsp. *holarctica* isolates could be split into two biovars according to natural susceptibility or resistance to erythromycin. Strains with high-level resistance to erythromycin (i.e., biovar II) have spread to the northern and eastern parts of Europe (including Scandinavia, the Balkans, etc.), but also to Asia. The macrolide-susceptible strains (i.e., biovar I) predominate in western and southern Europe (France, Switzerland, Spain) and in North America. There is an overlap in the geographical distributions of both types of biovars, such as in Germany (Georgi et al., 2012). Nevertheless, failures and relapses in tularaemia patients treated with a macrolide have been

reported worldwide, including in France (Maurin et al., 2011). Note that all isolates tested by Garcia del Blanco et al. during an outbreak in Spain were resistant to erythromycin (Garcia del Blanco et al., 2004). Thus, erythromycin is not a reliable option for treatment of tularaemia. Azithromycin is more active *in vitro* and has recently been proposed as a possible alternative in pregnant women in areas where the susceptible type B biovar I strains predominate (Dentan et al., 2013).

Although *F. tularensis* strains are susceptible to rifampicin *in vitro* (Tomaso et al., 2005), this antibiotic is not recommended for treatment of tularaemia because of a high risk of emergence of resistance during therapy. Chloramphenicol is less effective *in vitro* against *F. tularensis*, and it is associated with high relapse rates as well as bone marrow toxicity (Enderlin et al., 1994; Ikaheimo et al., 2000).

NEW ANTIBIOTICS

Ketolides

The ketolide compounds (telithromycin and cethromycin) are a subclass of macrolide antibiotics, which have been designed to address the problem of macrolide resistance in respiratory pathogens such as *Streptococcus pneumoniae*. Telithromycin was more effective *in vitro* against French isolates of *F. tularensis* subsp. *holarctica* than erythromycin (Gestin et al., 2010). Cethromycin has comparable tissue penetration, pharmacokinetics and *in vitro* activity to telithromycin, especially against bacterial species responsible for community-acquired pneumonia (Hammerschlag and Sharma, 2008). Compared with other macrolides, these compounds have different advantages, including a higher affinity for the 50S ribosomal subunit and a better stability at acidic pH (Hammerschlag and Sharma, 2008), which may partly explain the better activity in the intracellular environment of phagocytic cells where *F. tularensis* multiplies. Both telithromycin and cethromycin are potential alternatives for treatment of *F. tularensis* infection, but their clinical use in tularaemia patients has never been reported. Some authors have emphasized that cross-resistances between macrolide compounds may rapidly occur (Georgi et al., 2012).

Glycylcyclines

Tigecycline reaches high intracellular concentrations in tissues, macrophages and neutrophils, which makes this agent an interesting alternative for the treatment of intracellular pathogens (Pankey, 2005). Two papers report that *F. tularensis* strains isolated in Hungary and in Turkey were susceptible to tigecycline (Yesilyurt et al., 2011; Kreizinger et al., 2013a). These *in vitro* data suggest the potential usefulness of this antibiotic in tularaemia patients, but further *in vivo* experiments are needed for confirmation.

New fluoroquinolones

Ikäheimo et al. evaluated the *in vitro* antimicrobial susceptibility of 38 type B strains of *F. tularensis* isolated from human samples and dead wild animals. Ciprofloxacin, levofloxacin, grepafloxacin, and trovafloxacin had similar low MICs (Ikaheimo et al., 2000). Johansson et al. (Johansson et al., 2002) showed that gatifloxacin, grepafloxacin and sparfloxacin, were highly active against types A and B strains of *F. tularensis* isolated in different regions of the US. An Austrian study tested the susceptibility of 50 *F. tularensis* strains mainly isolated from hares to a wide range of antibiotics. The results showed low MIC values for all six fluoroquinolones tested (ciprofloxacin, levofloxacin, moxifloxacin, gatifloxacin, sparfloxacin) (Tomaso et al., 2005). The *in vivo* efficacy of gatifloxacin compared with ciprofloxacin and moxifloxacin was assessed in a BALB/c mouse model of *F. tularensis* Schu S4 infection. Lower mortality rates were found for gatifloxacin and moxifloxacin as compared to ciprofloxacin (Piercy et al., 2005; Steward et al., 2006).

Linezolid

Although linezolid is used for treatment of infections caused by Gram-positive bacteria, a Turkish study showed that *F. tularensis* could be susceptible to linezolid *in vitro* (Yesilyurt et al., 2011). However, linezolid was not effective against *F. tularensis* strains isolated in Hungary and North America (Johansson et al., 2002).

Liposome delivery of antibiotics

Treatment failures and relapses may also be related to the poor penetration and accumulation of antibiotics in tissues, especially in the subcellular compartment where *F. tularensis* multiplies. The liposome formulation of antibiotics is usually more effective than the conventional formulations against intracellular bacteria (Conley et al., 1997). Two studies have evaluated the liposome delivery of fluoroquinolones in order to reach high therapeutic doses of antibiotics within the intracellular compartment. One study demonstrated the efficacy of liposome-encapsulated ciprofloxacin, delivered to the lungs through aerosol inhalation, in mice with *F. tularensis* pneumonia (Conley et al., 1997). Another study showed that IV injection of liposome-encapsulated ciprofloxacin resulted in higher serum levels of this antibiotic, as well as increased drug retention in lungs, liver and spleen compared with the free drug. They also confirmed that aerosol inhalation of liposome-encapsulated ciprofloxacin, given for prophylactic or therapeutic purposes, provided mice with complete protection against a lethal *F. tularensis* pulmonary infection (Wong et al., 2003). The enhanced therapeutic efficacy can be attributable to the increased retention of ciprofloxacin in the lungs and to better intracellular delivery of ciprofloxacin by liposomes (Wong et al., 2003). The major drawback of this new therapeutic strategy is the need for more efficient techniques for preparing liposomal nanoparticles with great potential in targeting of antibiotics to bacteria and with high safety for humans (Hallaj-Nezhadi and Hassan, 2013).

ANTIMICROBIAL PEPTIDES

The human cathelicidin antimicrobial peptide LL-37 provides protection through host immunomodulation. It selectively regulates gene activation for expression of cytokines, chemokines and their receptor to control the recruitment of leukocytes to the infection site (Scott et al., 2002). It also activates or blocks TLR signaling (Into et al., 2010) and modulates apoptosis in neutrophils and epithelial cells (Barlow et al., 2011). Flick et al. evaluated the protective effect of LL-37 against *F. tularensis* LVS infection in cell culture and murine models. The objective was

to overcome the immunosuppressive effects of *F. tularensis* infection by stimulating the innate immune response. Following LVS intranasal challenge in mice, the administration of LL-37 resulted in an increased production of IL-6, Il-12, IFN-gamma, and MCP-1, and thereby reduced proliferation of bacteria in the lungs 48–72 h post-challenge. However, LL-37 was only moderately and transiently effective, and mice ultimately succumbed to infection. The immune potentiating activity of LL-37 suggests it could be used as an adjunct therapy in the treatment of persistent forms of tularaemia (Flick-Smith et al., 2013).

MODULATION OF THE INNATE IMMUNE RESPONSE AND SUPPRESSION OF *F. TULARENSIS* REPLICATION

The pathogenicity of *F. tularensis* is correlated with its ability to evade or suppress the activation of the host immune system, and to replicate in phagocytic cells (Bosio et al., 2007). The rapid induction of the innate immune response is critical in controlling the early spread of intracellular pathogens. IFN-gamma and TNF-alpha can activate macrophages *in vitro* to restrict the replication of *F. tularensis* (Skyberg, 2013). IL-12 and Toll-like receptor (TLR) signaling are also involved in IFN-gamma and TNF-alpha production. One possible way to improve the efficacy of tularaemia treatment, and reduce the dosage and duration of the antibiotic treatment, is to combine cytokines with first-line antibiotic drugs. Indeed, different studies support the hypothesis that increasing the production of pro-inflammatory cytokines at the early stage of infection can be beneficial. This review focuses on immune agonists that, when administered after *F. tularensis* challenges, prevent or attenuate the consequences of infection and thus could be used as a curative therapeutic adjuvant in tularaemia patients. Prophylactic aspects have been presented in other reviews (KuoLee and Chen, 2007; Hepburn and Simpson, 2008; Skyberg, 2013). This approach has been used successfully by Pammit et al. (2004), who showed that although IL-12 alone was not able to reduce bacterial burdens in mice infected with *F. novicida*, intranasal IL-12 treatment decreased spleen and liver bacterial burdens more than 100-fold and lung bacterial burdens 50-fold when co-administrated with gentamicin. This combination improved clinical outcome and survival of animals when administrated early (8–24 h) after infection (Pammit et al., 2004). It should be mentioned that results obtained with *F. novicida,* a highly virulent bacterium in mice but non-virulent in humans, perhaps cannot be extrapolated to human infections caused by *F. tularensis*. Treatment of mice with IL-12 resulted in an early IFN-gamma response that allows the recruitment of neutrophils to the lungs during the first 24 h of infection. Neutrophils contribute to the rapid clearance of bacteria (Kirimanjeswara et al., 2008).

The activation of TLR signaling pathways in phagocytic cells has been reported to be a major defence mechanism against *Francisella* infection (Cowley and Elkins, 2011). Lembo et al. demonstrated that intranasal administration of a synthetic TLR4 agonist (aminoalkyl glucosaminide phosphatase, AGP) increased intrapulmonary cytokine and chemokine production. Mice treated with AGP after *F. novicida* inhalation exhibited reduced bacterial replication in the lung, liver and spleen, and increased survival (Lembo et al., 2008).

Administration of bacterial or oligonucleotide DNA containing CpG motifs to mice induced an immune activation and effective protection against a lethal parenteral challenge of these animals with *F. tularensis* LVS. This protection was highly dependent on B lymphocyte cells and INF-gamma production. Furthermore, animals surviving this lethal challenge developed a pathogen-specific secondary immunity (Elkins et al., 1999). More recently, these authors showed that DNA-related protection against LVS infection was dependent on TLR9, which is expressed intracellularly in murine macrophages, dendritic cells and B cells. Surprisingly, their data indicated that the role of B cells was not related to the action of CpG DNA motifs but to the anti-LPS antibody production by these cells in the peritoneal cavity. Instead, the production of soluble mediators by NK cells primed with CpG DNA *in vivo* contributed to DNA-mediated protection. Because murine NK cells do not express TLR9, *in vivo* activation was indirect and may have involved dendritic cells. In conclusion, this study suggested that indirect activation of NK cells by CpG DNA, via a TLR9-dependent pathway, resulted in the production of cytokines and NO, which controlled the intracellular growth of *F. tularensis* LVS in infected mice (Elkins et al., 2009). More recently, Rozak et al. conducted the same experiment with the more virulent SCHU4 strain. Their results revealed that CpG DNA failed to positively affect the course of *F. tularensis* SCHU4 pneumonic infection in mice (Rozak et al., 2010). These contradictory data emphasize the need for evaluation of both type A and type B *F. tularensis* strains in animal models.

Pyles et al. (2010) evaluated the effect of poly(I:C) (polyinosine polycytosine), a synthetic double-stranded RNA analog that stimulates TLR3, as a novel treatment for *F. tularensis* respiratory infection. TLR3 is expressed by respiratory epithelial cells and macrophages and can trigger the induction of the host innate immune response, including TNF-alpha, IFN-gamma, IL-8, and IL-6 secretion. Intranasal administration of poly(I:C) in mice induced an early and effective innate immune response by increasing the secretion of cytokines and the neutrophil influx in the lungs. This effect was transient but prolonged the survival of animals. Furthermore, poly(I:C) increased cytokine secretion by human monocyte-derived macrophages and significantly reduced intracellular replication of *F. tularensis* in these cells. Thus, Poly(I:C) may be a useful additive therapeutic agent in patients infected by *F. tularensis* aerosols.

Galantamine is an inhibitor of acetyl-cholinesterase used for treatment of Alzheimer's disease. Galantamine has also been reported to modify TNF-alpha levels through stimulation of the cholinergic anti-inflammatory pathway (Liu et al., 2010). The resolution of tularaemia infection is mediated by the production of IFN-gamma and the activation of macrophages, resulting in an increased production of nitric oxide synthase and killing of *F. tularensis* by these phagocytic cells. Pohanka et al. (2012) showed that galantamine could significantly influence the immune response via the cholinergic anti-inflammatory pathway by up-regulating IFN-gamma production and down-regulating IL-6 production. Mice treated with galantamine showed lower mortality rates when infected with *F. tularensis*.

Skyberg et al. (2012) reported that a natural polysaccharide extract isolated from Acai berry (Acai PS, derived from

the berry of the palm tree *Euterpe oleracea* in South America) enhanced the clearance of *F. tularensis* LVS and Schu4 from human macrophages when co-cultured with autologous natural killer cells. Impaired replication of *F. tularensis* in human macrophages was related to increased production of IFN-gamma by NK cells. Intranasal administration of Acai PS to mice immediately, 24 or 48 h after a Schu4 aerosol challenge resulted in survival rates of 73, 60, and 33%, respectively. In both human and murine cells, Acai PS enhanced Th1 cell-mediated immunity. Acai PS is currently the most active immunotherapeutic agent reported in the literature for treatment of experimental pneumonia caused by type A *F. tularensis*.

The activation of the innate immunity may help antibiotherapy to eradicate *F. tularensis* in infected patients. However, activation of the innate immunity may lead to higher inflammatory response and injury of the lung tissue. The timing of administration of proinflammatory treatments requires careful management, since there is a danger that their use may exacerbate the symptoms of disease in infected patients (D'Elia et al., 2013). If treatment is initiated either just before or the same time as infection, then the induction of proinflammatroy cytokines by stimulatory molecules has beneficial effects on host survival as shown with CpG oligonucleotides (Elkins et al., 1999). Moreover, the mode of administration of such combination in humans remains to be determined. The small window for such treatment greatly limits application of this adjunctive therapy in clinical practice. It has been observed that the use of proinflammatory cytokines themselves such as IL-1β cause side effects, including generalized fatigue, headache, nausea, vomiting, myalgia, and arthralgia (Crown et al., 1991). By modulating rather than up-regulating the immune response via mechanisms such as cholinergic anti-inflammatory pathways (example of galantamine, Pohanka et al., 2012), the damaging cytokine storms can be prevented. This may allow a longer window for diagnosis and treatment (D'Elia et al., 2013) but these strategies require more in *vivo* investigation for further evaluation of their efficacy and tolerance.

ADMINISTRATION OF SPECIFIC ANTIBODIES

Before the antibiotic era, patients suffering from tularaemia were successfully treated with xenogeneic immune sera (Elkins et al., 2007). The role of antibodies for protection against infections caused by intracellular pathogens has long been controversial, but recent studies have shown that they contribute to protection against *F. tularensis* infection (Kirimanjeswara et al., 2008; Skyberg, 2013). Kirimanjeswara et al. showed that passive intraperitoneal transfer of immune serum in mice provided complete protection against intranasal challenge with lethal doses of LVS, even when administrated 24–48 h post-infection. These results indicate that serum antibodies may be used for both prophylactic and therapeutic purposes (Kirimanjeswara et al., 2007). Another study showed that monoclonal antibodies against the LPS of *F. tularensis* LVS could be successfully used to treat LVS-induced pneumonia (Lu et al., 2007). Unfortunately, these promising results could not be reproduced when using a *F. tularensis* type A strain. Anti-LVS antibodies failed to protect mice challenged with *F. tularensis* Schu S4. One explanation could be that the Schu S4 strain completely abolished the inflammatory responses that are required for efficient antibody-mediated bacterial clearance (Kirimanjeswara et al., 2008). Another study also showed relative efficacy for immunotherapy using monoclonal antibodies (MAbs). Most particularly, MAbs directed against LVS components could confer protection in mice challenged with this strain. However, MAbs were much less active in protecting mice challenged with the type A *F. tularensis* Schu S4 strain (Savitt et al., 2009). Klimpel et al. showed that mice infected via intra-nasal Schu S4 challenge and then treated with levofloxacin developed protective immunity. Interestingly, sera from these mice were protective when passively transferred to naive mice, even when administered 24 h post-infection. In these protective sera, the most abundant immunoglobulin class was IgG2a, suggesting that a Th1-type immune response was predominant (Klimpel et al., 2008). Sutherland et al. showed that the passive transfer of antibodies directed against the membrane protein fraction (MFP) of *F. tularensis* Schu S4, to mice infected with the same strain, resulted in complete protection when combined with gentamicin treatment. These sera contained high titers of anti-MPF IgM and IgG antibodies, comparable to those observed during natural infection. Post-exposure immunization with MPF antigens was an effective means of enhancing the activity of conventional antimicrobial therapy for pneumonic tularaemia (Sutherland et al., 2012). Immune sera are probably not effective enough to treat severe pulmonary tularaemia cases, but could be used in combination with antibiotics to obtain a more rapid response to treatment.

CONCLUSION

The fear of a bioterrorism attack scenario after the September 11, 2001, incident has renewed the medical and scientific interest in *F. tularensis*, a class A bioterrorism agent. However, only limited progress has been made in the development of new tularaemia therapies. Due to the lack of an effective and safe vaccine, antibiotics remain the only strategies available for prophylaxis and treatment of tularaemia. New therapeutic alternatives are needed because of the potential toxicity of first-line drugs, especially in children and pregnant women; high rates of treatment relapses and failures, especially for severe and/or suppurated forms of the disease; and the possible use of antibiotic-resistant strains in the context of biological threat. Among the new therapeutic strategies, no published study present large-scale screening of new molecules for inhibitory potential of *F. tularensis* growth. There is no more data in the literature concerning the use of bacteriophage against *Francisella*. Activation of the innate immune system can enhance resistance of the host to *F. tularensis* infection and could help clearance of the bacteria and disease cure when combined with the administration of antibiotics. Promising results have been obtained in animal models, but this strategy only applies if the involved *F. tularensis* strain is still susceptible to antibiotics, which may not be the case in the context of bioterrorism. Several virulence factors have been identified such as the pathogenicity island but also genes encoding type IV pili, a type II secretion system, a type VI secretion system, iron acquisition systems (Larsson et al., 2005). Bacterial virulence factors have been increasingly regarded as attractive targets for development of

novel antibacterial agents. This type of approach has for example been used in *Salmonella enterica* serovar Typhimurium and led to the identification of Cytosporone B, an inhibitor of the Type III Secretion System of *Salmonella* as novel antibacterial agent (Li et al., 2013). Finally, it should be remembered that new anti-infective strategy effective against type B strains may not apply to the more severe infections caused by type A strain.

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The potential of liposome–encapsulated ciprofloxacin as a tularemia therapy

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Liposome-encapsulation has been suggested as method to improve the efficacy of ciprofloxacin against the intracellular pathogen, *Francisella tularensis*. Early work with a prototype formulation, evaluated for use against the *F. tularensis* live vaccine strain, showed that a single dose of liposomal ciprofloxacin given by the intranasal or inhalational route could provide protection in a mouse model of pneumonic tularemia. Liposomal ciprofloxacin offered better protection than ciprofloxacin given by the same routes. Liposomal ciprofloxacin has been further developed by Aradigm Corporation for *Pseudomonas aeruginosa* infections in patients with cystic fibrosis and non-cystic fibrosis bronchiectasis. This advanced development formulation is safe, effective and well tolerated in human clinical trials. Further evaluation of the advanced liposomal ciprofloxacin formulation against the highly virulent *F. tularensis* Schu S4 strain has shown that aerosolized CFI (Ciprofloxacin encapsulated in liposomes for inhalation) provides significantly better protection than oral ciprofloxacin. Thus, liposomal ciprofloxacin is a promising treatment for tularemia and further research with the aim of enabling licensure under the animal rule is warranted.

Keywords: tularemia, liposomal, ciprofloxacin, *Francisella tularensis*

INTRODUCTION

The extremely low infectious dose and severe disease following inhalation previously led to the development of *Francisella tularensis* as a biological weapon by Russia, Japan, and the USA (Dennis et al., 2001). To combat the threat of a deliberate release, there is a need for an easily administered therapy which can be used in the event of a mass casualty situation. The latest consensus statement on tularemia as a biological weapon suggested ciprofloxacin or doxycycline should be given in the event of a mass casualty situation (Dennis et al., 2001).

Ciprofloxacin, a broad spectrum second generation fluoroquinolone, has been used to treat infections for over 20 years. Tularemia outbreaks in Spain (Perez-Castrillon et al., 2001) and Turkey (Meric et al., 2008) have been successfully treated with ciprofloxacin. However, the outbreaks in Spain and Turkey were likely caused by the less virulent type B form of *Francisella tularensis* (Hepburn and Simpson, 2008). The efficacy of ciprofloxacin against the more virulent type A strains in humans is less well understood. Animal models of infection with the type A strain Schu S4 suggest that orally delivered ciprofloxacin is only effective against type A systemic infections if administered within 24 h of challenge (Piercy et al., 2005). Furthermore, studies using an animal model of pneumonic infection with a type A strain, the most fatal form of the disease, suggest that orally delivered ciprofloxacin offers poor protection (Steward et al., 2006).

Enhancing cellular delivery of antibiotics by encapsulation in liposomes has been suggested as a method to improve antibiotic efficacy against intracellular pathogens (Pinto-Alphandary et al., 2000). Macrophages which are infected by *F. tularensis* can take up liposomes containing antibiotics, enabling intracellular delivery and a close proximity between the drug and bacteria. In addition, the inhalation of encapsulated drugs, such as ciprofloxacin, enables high sustained concentrations of drugs to be achieved in the lungs. For the pneumonic form of tularemia, this approach has an obvious advantage and, if administered soon after infection, encapsulated antibiotics could reduce or prevent the spread of disease and reduce mortality. Without encapsulation, small drugs such as ciprofloxacin are rapidly cleared after administration into the lung (Wong et al., 1995, 2003). This review describes early studies evaluating the efficacy of a prototype liposomal ciprofloxacin against *F. tularensis* and further development of the novel antibiotic preparation.

PRELIMINARY STUDIES

An initial liposomal ciprofloxacin preparation comprised negatively charged liposomes prepared from phosphatidylcholine: cholesterol: phosphatidyl serine (in a ratio of 7:3:1) with 45% of the ciprofloxacin encapsulated (Di Ninno et al., 1993). Even with this very early formulation, results were very promising. Efficacy was evaluated in a murine model of tularemia in which mice were infected with *F. tularensis* live vaccine strain (LVS). The *F. tularensis* LVS strain is relatively avirulent in humans (and hence has been used historically as a live vaccine for humans) yet causes a lethal infection in mice and therefore is commonly used as a surrogate for more virulent strains.

In mice challenged intravenously with 10^3 CFU of *F. tularensis* LVS, a single intravenous dose of liposomal ciprofloxacin (50 mg/kg) provided a high level of protection if administered within 48 h of the challenge. Conversely, a single intravenous dose of unencapsulated ciprofloxacin (50 mg/kg) offered no measurable protection even when administered 24 h post-challenge (Di Ninno et al., 1993).

To model pneumonic tularemia, mice were challenged with 100 CFU of *F. tularensis* LVS via intranasal instillation. A single dose of intranasal liposomal ciprofloxacin (50 mg/kg) provided better protection than a single dose of intranasal ciprofloxacin (50 mg/kg) when therapy was initiated at 2 or 3 days post-challenge (Di Ninno et al., 1993; Wong et al., 1995) (**Figure 1** shows 3 day data). The improved efficacy of intranasal liposomal ciprofloxacin compared to intranasal ciprofloxacin may be explained by the quick elimination of unencapsulated ciprofloxacin from the lung. Following intranasal instillation, unencapsulated ciprofloxacin is almost completely eliminated from the lungs within 2 h (Wong et al., 1995). Liposome encapsulation increased the half-life of ciprofloxacin in the mouse model from 1 to 10 h, and ciprofloxacin could still be detected in the lung of mice 24 h after dosing (Wong et al., 1995).

An alternative liposomal ciprofloxacin formulation, which encapsulated 90% of the ciprofloxacin, was developed using egg phosphatidylcholine and cholesterol in a 1:1 ratio (Conley et al., 1997). The pneumonic tularemia mouse model was used to evaluate the efficacy of this formulation delivered by the inhalational route. This delivery route is more representative, when compared to intranasal instillation, of the expected human pulmonary administration using a nebulizer or inhaler. In the pneumonic tularemia mouse model, a single dose of aerosolized ciprofloxacin (1 mg/kg lung dose) provided little or no protection whereas a single dose of aerosolized liposomal ciprofloxacin (1 mg/kg lung dose) offered 100% protection even when administered as late as 72 h post-challenge (Wong et al., 2003) (**Figure 1** shows 72 h data). In addition, a single dose of aerosolized liposomal ciprofloxacin still offered a high level of protection (87%) when administered at 96 h post-challenge (Wong et al., 2003).

The efficacy of liposomal ciprofloxacin administered at such late time points after challenge is encouraging as late initiation of therapy increases the risk of a negative treatment outcome (Celebi et al., 2006). The use of liposomal ciprofloxacin may widen the window of opportunity for initiating therapy, providing more time for a *F. tularensis* deliberate release to be detected and for those affected to be successfully treated.

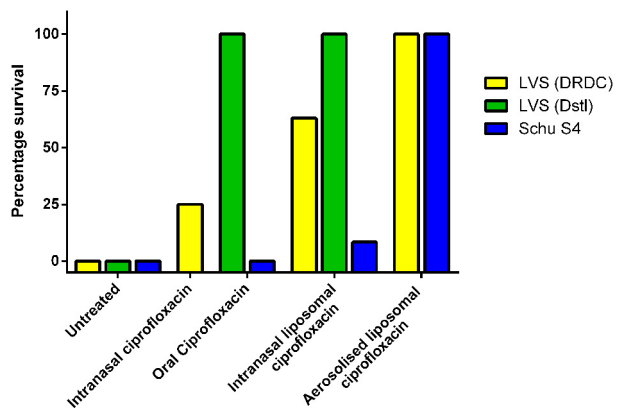

FIGURE 1 | Therapeutic efficacy of a single dose of ciprofloxacin or liposomal ciprofloxacin against murine inhalational *F. tularensis* LVS and Schu S4 infection. Mice were challenged with *F. tularensis* LVS by the intranasal route (approximately 1×10^2 CFU at DRDC or 6×10^4 CFU at DstI) or *F. tularensis* Schu S4 by the aerosol route (10 CFU retained dose). Treatment was initiated at 72 h post-challenge for LVS infections and 24 h post-challenge in the Schu S4 study. Treatment included 50 mg/kg of oral ciprofloxacin, 50 mg/kg of intranasal ciprofloxacin, 50 mg/kg liposomal ciprofloxacin, or 1 mg/kg lung dose of aerosolized liposomal ciprofloxacin. Graph shows percentage survival at the end of the experiment. LVS (DRDC) data is adapted from Di Ninno et al. (1993) (Intranasal ciprofloxacin and liposomal ciprofloxacin) and Wong et al. (2003) (aerosolized liposomal ciprofloxacin). Schu S4 and LVS (DstI) data is adapted from Hamblin et al. (2014).

LIPOSOMAL CIPROFLOXACIN FORMULATION DEVELOPMENT AND HUMAN CLINICAL TRIALS

Liposomal ciprofloxacin has been further developed by Aradigm Corporation to improve encapsulation efficacy and increase the shelf life. More than 99% of the ciprofloxacin is encapsulated in this improved formulation, which has an extended shelf life of 24 months at 5°C (Cipolla et al., 2010). The human clinical development is summarized in **Table 1**. Aradigm Corporation initially conducted a Phase 1 study in healthy volunteers and Phase 2a studies in cystic fibrosis (CF) and non-cystic fibrosis bronchiectasis (BE) patients with the liposomal formulation CFI (Lipoquin®, also known as ARD-3100). The Phase 1 trials in healthy volunteers demonstrated the safety and tolerability of 300 mg CFI daily for 7 days (Bruinenberg et al., 2010). A Phase 2a study in patients with CF also demonstrated the potential to considerably increase lung ciprofloxacin concentrations by aerosol administration of CFI compared to orally administered ciprofloxacin. At steady state, a 500 mg oral dose of ciprofloxacin results in peak sputum concentrations of 1.86 μg/g in patients with CF (LeBel et al., 1986). In contrast, following administration of CFI the mean sputum concentration at day 7 was 88.4 μg/g (Bruinenberg et al., 2010).

The Phase 1 study with CFI also demonstrated the low systemic exposure of ciprofloxacin; following the 300 mg CFI dose, the average peak plasma concentration was only 0.1 μg/ml and an area under the curve (AUC) was 0.9 h·μg/ml (Bruinenberg et al., 2010). These values are considerably less than those for oral ciprofloxacin, where a 750 mg dose results in a peak concentration of 3.8 μg/ml and an AUC of 16.8 h·μg/ml (Lettieri et al., 1992). This reduction in systemic exposure may reduce ciprofloxacin associated side effects. It is believed that side effects or fear of side effects was responsible for 4% of US postal workers ceasing their ciprofloxacin medication during the 2001 USA anthrax attack (Brechner et al., 2001). Therefore, the use of inhaled CFI rather than oral ciprofloxacin may improve compliance with a prophylaxis regimen.

Prior to entry into Phase 2b studies, Aradigm Corporation evaluated an additional formulation, dual release ciprofloxacin

Table 1 | Clinical phase 1 and 2 trials evaluating once daily dosing with CFI or DRCFI for treatment of patients with chronic *Pseudomonas aeruginosa* infection.

Purpose/indication	Formulation	Trial design and subjects	Main results	References
Safety/tolerability/ pharmacokinetics	CFI	Phase 1, open-label, 20 healthy subjects. Treatment: doses of 150, 300, or 450 mg[a] for 1 day; 300 mg[a] for 7 days	Incidence of any adverse events was low; no serious or severe adverse events. Only three potential drug-related adverse-events, all mild, not dose-related	Bruinenberg et al., 2010
Safety/tolerability/ pharmacokinetics	CFI and DRCFI	Phase 1/2a, open-label, nine healthy and six BE[b] subjects. Treatment: dose ranging for CFI and DRCFI	No clinically relevant reduction in FEV_1^c. All adverse events were mild (except for a urinary tract infection, not treatment-related)	Serisier, 2012
Cystic fibrosis	CFI	Phase 2a, open-label, single-arm, 22 well-treated adults with CF[d]. Treatment: 14 days with 300 mg[a]	Significant reduction in *P. aeruginosa* in sputum; 6.9% absolute increase in FEV_1^c vs. baseline; exceptionally good pulmonary safety and tolerability compared to historical controls	Bruinenberg et al., 2010
Non-CF bronchiectasis	CFI	Phase 2a, open-label, single-arm, 36 BE[b] adult patients. Treatment: 28 days of either 150 or 300 mg[a]	Significant reduction in *P. aeruginosa* in sputum; no difference between the two doses; no decline in lung function	Bruinenberg et al., 2010
Non-CF bronchiectasis	CFI	Phase 2b, randomized, double-blind, placebo-controlled, 95 adult BE[b] patients. Treatment: 28 days of either 100 or 150 mg[a] vs. matching placebos (ORBIT-1)	Both doses significantly reduced *P. aeruginosa* in sputum vs. placebo; treatment well tolerated—no need for pre-treatment or rescue with bronchodilators	Serisier, 2012
Non-CF bronchiectasis	DRCFI	Phase 2b, randomized, double -blind, placebo-controlled, 42 BE[b] adult patients. Treatment: 150 mg[a] vs. matching placebo for six cycles of 28 days on/28 days off (ORBIT-2)	Significant reduction in *P. aeruginosa* in sputum; median time to first pulmonary exacerbation more than doubled with DRCFI vs. placebo; DRCFI has better respiratory adverse effects profile than placebo	Serisier et al., 2013

[a] *Expressed in terms of ciprofloxacin hydrochloride dose loaded in the nebulizer, of which approximately 15–20% is delivered to the lung.*

[b] *Bronchiectasis.*

[c] *Forced expired volume in 1 s.*

[d] *Cystic fibrosis.*

for inhalation (DRCFI, Pulmaquin®, also known as ARD-3150) in a second Phase 1 study, with the view that a mixed pharmacokinetic profile combining the benefits of slow release with a transient initial peak of ciprofloxacin could provide incremental benefits for patients. This formulation was the result of mixing equal volumes of CFI and a solution of free ciprofloxacin. The DRCFI treatment was well tolerated in the Phase 1 study (Serisier, 2012).

Subsequently, both CFI and DRCFI have been investigated in patients with non-cystic fibrosis BE against placebo into two Phase 2b trials (ORBIT-1 and 2). In ORBIT-1, both doses of CFI were well tolerated and significantly reduced *P. aeruginosa* in sputum vs. placebo (Serisier, 2012). In ORBIT-2, DRCFI treatment also significantly reduced *P. aeruginosa* in sputum (Serisier et al., 2013). Notably, the median time to the first pulmonary exacerbation was more than two times longer in the patients treated with DRCFI vs. those treated with placebo. The DRCFI therapy was also well tolerated with the respiratory adverse effects profile of the patients treated with DRCFI being better than that for patients treated with placebo (Serisier et al., 2013).

FURTHER EVALUATION OF LIPOSOMAL CIPROFLOXACIN AGAINST *F. TULARENSIS*

Utilizing the advanced product, CFI (Lipoquin®), the efficacy of liposomal ciprofloxacin against *F. tularensis* has been further evaluated. As ciprofloxacin prophylaxis is generally given orally, inhaled CFI was compared to oral ciprofloxacin. In mice challenged intranasally with approximately 6×10^4 CFU of *F. tularensis* LVS, the efficacy of oral ciprofloxacin (50 mg/kg) and intranasally instilled CFI (50 mg/kg) could not be distinguished as a single dose of either formulation offered full protection against a lethal challenge, even when therapy was delayed until 72 or 96 h post-challenge (Hamblin et al., 2014) (see **Figure 1** for 72 h data). This clearly highlights the limitations of using reduced virulence *F. tularensis* strains for evaluating the efficacy of antibiotics against tularemia.

To discriminate between the two formulations, a mouse model of infection with the more virulent *F. tularensis* Schu S4 was used. In these studies, mice were challenged with *F. tularensis* Schu S4 via the aerosol route, with each mouse exposed to approximately

10 CFU. A single dose of aerosolized CFI (1 mg/kg lung dose) provided full protection against a lethal aerosol challenge. In contrast, a single dose, or 3 or 5 days of twice daily oral ciprofloxacin treatment (50 mg/kg) did not prevent mortality, with all mice succumbing to the infection (**Figure 1** shows the single dose data). However, the 3 or 5 days course of twice daily oral ciprofloxacin treatment did increase the time to death of infected mice when compared to PBS treatment (Hamblin et al., 2014). Ciprofloxacin, administered by the oral route, does enter the lungs and maximal concentrations are similar to those achieved after an aerosol dose of CFI. However, the clearance rate of ciprofloxacin from the lung is 4000-fold higher than aerosolized CFI. Therefore, the superior efficacy of CFI, compared to ciprofloxacin, against aerosolized *F. tularensis* may be due to the persistence of CFI in the lungs (Hamblin et al., 2014).

Interestingly, aerosolized CFI was found to be more effective than intranasally instilled CFI, as a single dose of intranasally instilled CFI (50 mg/kg) did not prevent mortality (>10% of the mice survived) (see **Figure 1**). This may be due to different lung distribution following the two routes of administration, since aerosolized CFI is distributed more uniformly throughout the lung. The high level of protection offered by CFI against a highly virulent strain of *F. tularensis* is encouraging and suggests further in-depth studies are warranted.

FUTURE WORK

Further evaluation of the efficacy of liposomal ciprofloxacin using mouse and non-human primate models of tularemia could support an application for licensure by the FDA under the animal rule. Such studies could determine the maximum window of opportunity for initiating therapy and the shortest regimens that are effective. Dose ranging studies could inform device selection by determining if an effective dose can be administered using a small portable hand-held inhaler or if a higher dose requiring delivery by a nebulizer is needed. For example, the AERx® inhaler developed by Aradigm Corporation can deliver 50 µl in each metered dose. In comparison, nebulizers can deliver more drug (6 ml of DRCFI in Aradigm Corporation's clinical trials) but are larger and require a power source. These studies could also enable a comparison of the two liposomal ciprofloxacin formulations, CFI and DRCFI, to determine which is more effective and therefore more appropriate as a tularemia therapy.

In addition, studies to date have not investigated the efficacy of inhaled liposomal ciprofloxacin against systemic tularemia, which can develop from the pneumonic form. Co-treatment of liposomal ciprofloxacin with an orally or intravenously delivered antibiotic may warrant investigation as this therapy regimen could enable successful treatment of pneumonic infections that have spread systemically.

CONCLUSION

CFI is a promising therapy for pneumonic tularemia, having the potential to shorten the current prophylactic regimen used in the event of a deliberate release of *F. tularensis*. CFI also offers potential for enhanced therapeutic outcomes for the treatment of naturally occurring tularemia caused by highly virulent strains of *F. tularensis*. Further study of liposomal ciprofloxacin is warranted to fully determine the utility of this formulation as a tularemia therapy.

AUTHOR CONTRIBUTIONS

Karleigh A. Hamblin, Jonathan P. Wong, James D. Blanchard, and Helen S. Atkins drafted the manuscript. The final manuscript was approved by Karleigh A. Hamblin, Jonathan P. Wong, James D. Blanchard, and Helen S. Atkins.

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A new dye uptake assay to test the activity of antibiotics against intracellular *Francisella tularensis*

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Francisella tularensis, a facultative intracellular bacterium, is the aetiological agent of tularaemia. Antibiotic treatment of this zoonosis is based on the administration of a fluoroquinolone or a tetracycline for cases with mild to moderate severity, whereas an aminoglycoside (streptomycin or gentamicin) is advocated for severe cases. However, treatment failures and relapses remain frequent, especially in patients suffering from chronic lymph node suppuration. Therefore, new treatment alternatives are needed. We have developed a dye uptake assay for determination of minimal inhibitory extracellular concentrations (MIECs) of antibiotics against intracellular *F. tularensis*, and validated the method by comparing the results obtained using a CFU-enumerating method. We also compared MIECs with MICs of the same compounds determined using a CLSI broth microdilution method. We tested the activity of 11 antibiotics against two clinical strains of *F. tularensis* subsp. *holarctica* isolated in France. Both strains displayed low MICs ($\leq$1 μg/mL) to fluoroquinolones (ciprofloxacin, levofloxacin and moxifloxacin), gentamicin, doxycycline and rifampicin. Higher MICs ($\geq$8 μg/mL) were found for carbapenems (imipenem and meropenem), daptomycin and linezolid. Erythromycin MICs were 4.0 and 16.0 μg/mL, respectively, for the two clinical strains. MIECs were almost the same with the two methods used. They were concordant with MICs, except for erythromycin and linezolid (respectively, four and eight times more active against intracellular *F. tularensis*) and gentamicin (four to eight times less active against intracellular *F. tularensis*). This study validated the dye uptake assay as a new tool for determination of the activity of a large panel of antibiotics against intracellular *F. tularensis*. This test confirmed the intracellular activity of first-line antibiotics used for tularaemia treatment, but also revealed significant activity of linezolid against intracellular *F. tularensis*.

Keywords: tularaemia, *Francisella tularensis*, dye uptake assay, antibiotic activity, intracellular infection

INTRODUCTION

Tularaemia is a zoonotic disease caused by the Gram-negative bacterium *Francisella tularensis*. Two subspecies are responsible for the majority of human infections, including *F. tularensis* subsp. *tularensis* in North America and *F. tularensis* subsp. *holarctica* throughout the Northern hemisphere. The latter subspecies is split into two biovars. Biovar I, naturally susceptible to erythromycin, is found in North America and Western Europe. Biovar II, naturally resistant to erythromycin, is found in Eastern Europe and Asia (Keim et al., 2007). *F. tularensis* is highly infectious for humans and has been classified as a category A bioterrorism agent by the CDC (Bossi et al., 2004).

Only a few antibiotic classes are effective to treat tularaemia patients. The aminoglycosides (streptomycin and gentamicin) are considered the reference treatment for severe forms of the disease (Hepburn and Simon, 2008). For mild to moderate tularaemia cases, fluoroquinolones (ciprofloxacin, levofloxacin) and tetracyclines (doxycycline) are advocated as first-line drugs (Tärnvik and Chu, 2007). All these antibiotics have side effects and their use should be restricted especially in pregnant women (Dentan et al., 2013). Moreover, antibiotic treatments using a tetracycline or a fluoroquinolone are associated with high rates of failure and relapse (Johansson et al., 2001; Perez-Castrillon et al., 2001). Finally, high-level resistance to macrolides, tetracyclines and fluoroquinolones was easily selected *in vitro* in *F. tularensis* (La Scola et al., 2008; Gestin et al., 2010; Loveless et al., 2010; Sutera et al., 2014), which raises some concern about the misuse of resistant strains in the bioterrorism context or the possibility of *in vivo* selection of such resistance in tularaemia patients.

F. tularensis is a slow growing, facultative intracellular bacterium. It replicates in the cytoplasm of macrophages (Anthony et al., 1991; Chong and Celli, 2010) and non-phagocytic cells (Hall et al., 2007). Both cell types are involved in tularaemia patients, especially in the lower airways in patients suffering from pneumonia (Horzempa et al., 2010). A number of techniques have been developed to test the activity of antibiotics against intracellular pathogens. In most studies, the intracellular activity of the tested antibiotic is evaluated by measuring the viable

bacterial counts (VBCs) after antibiotic exposure compared to an untreated control. VBCs are usually determined using the colony forming unit (CFU)-enumeration methodology (Segreti et al., 1996; Wright Valderas and Barrow, 2008). Because this technique is fastidious and time consuming and not adapted for microorganisms growing exclusively in eukaryotic cells, methods based on DNA quantification using quantitative real-time PCR technology (Boulos et al., 2004) or immunofluorescent-antibody testing (Ives et al., 1997) have been proposed. The intracellular growth of bacteria can also be deduced from their cytotoxic effect in eukaryotic cell culture systems (Edouard and Raoult, 2013), especially using a simple dye uptake assay, as previously described for strict intracellular bacteria such as *Rickettsia* species (Rolain et al., 1998). This technique is based on the capacity for live cells to internalize a vital dye such as neutral red (Borenfreund and Puerner, 1985). In this system, the activity of an antibiotic is deduced from its potential to prevent cytotoxic effects by inhibiting bacterial multiplication.

In this study, we adapted the dye uptake assay to evaluate the activity of several antibiotics against two clinical strains of *F. tularensis* subsp. *holarctica*. Our first goal was to demonstrate that this simple technique gives equivalent results compared to the VBCs method. Because the dye uptake assay is much easier to perform, it allowed us to screen the intracellular activity of a large number of antibiotic compounds against several strains of *F. tularensis*. This work could facilitate the search for new treatment alternatives for tularaemia, as well as the detection of acquired resistances to available antibiotics.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CELL LINE

Two clinical strains of *F. tularensis* subsp. *holarctica* were used: Ft6 isolated in 2007 from a blood culture and Ft24 isolated in 2009 from an axillary lymphadenopathy. Both strains were identified to the subspecies level by sequencing the intergenic region located between 16S and 23S RNA encoding genes (Maurin et al., 2011). They were kept frozen in cryotubes (MastDiagnostic, Amiens, France) at −80°C. They were grown in a biosafety level 3 laboratory, using chocolate agar supplemented with Polyvitex®(CHA-PVX medium, bioMérieux, Marcy l'Etoile, France) incubated at 37°C in a 5% CO_2-enriched atmosphere.

We used three control strains for MIC determination: *Escherichia coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853 and *Staphylococcus aureus* ATCC29213. They were grown on Columbia medium supplemented with 5% sheep blood (COS medium, bioMérieux) incubated 24 h at 37°C with 5% CO_2.

For the cell system, we used the human pulmonary diploid fibroblastic cells MRC-5 (RD Biotech, Besançon, France). Cell monolayers were grown in Minimum Essential Medium (MEM, Gibco®, Life Technologies, Saint Aubin, France) supplemented with 10% decomplemented foetal calf serum (FCS, Gibco), at 37°C, in a 5%CO_2-enriched atmosphere. These fibroblastic cells are strictly adhesive and stop their multiplication when at confluence, which enables cells proliferation control. Moreover, this model has already been used for susceptibility testing of other pathogens such as *Legionella pneumophila* (Segreti et al., 1996) or *Tropheryma whipplei* (Boulos et al., 2004). Finally, *F. tularensis* subsp. *holarctica* strains were able to efficiently infect and proliferate in this model (*cf.* Result section).

ANTIBIOTICS

We used ciprofloxacin (Panpharma, Fougères, France), levofloxacin (Fresenius kabi, Sèvres, France), moxifloxacin (Bayer, Puteaux, France), imipenem (Panpharma), meropenem (Panpharma), daptomycin (Novartis, Rueil-Malmaison, France), doxycycline (Sigma-Aldrich, Lyon, France), rifampicin (Sanofi-Aventis, Paris, France), gentamicin (Panpharma), linezolid (Pfizer, Paris, France) and erythromycin (Fluka, Lausanne, Switzerland). Stock solutions of these 11 antibiotics were prepared in sterile distilled water for gentamicin, ciprofloxacin and erythromycin, and in 0.45% sodium chloride solution for the other antibiotics, and kept frozen at −80°C until used.

MIC DETERMINATION

MICs were determined using a microdilution method in Mueller-Hinton (MH) broth (bioMérieux) supplemented with 2% PolyViteX®, using a CLSI methodology (Clinical and Laboratory Standards Institute, 2009).

Briefly, each antibiotic was diluted in MH-2%PVX to obtain twofold serial concentrations (**Table 1**), and 75 µL of each suspension was dispensed in one well of 96-well microtiter plates. An equal volume of a 10^6 bacterial suspension was added to each well. After incubation of the plates for 48 h at 37°C, the lowest antibiotic concentration inhibiting visible bacterial growth was recorded as the MIC. All experiments were run in duplicate. The reference strains *E. coli* ATCC25922, *P. aeruginosa* ATCC27853 and *S. aureus* ATCC29213 were used as positive controls. Wells receiving only MH broth were used as negative controls.

MIEC DETERMINATION

The MRC-5 cells were prepared in MEM-10% FCS at a concentration of 6×10^5 cells/mL. This cell suspension was dispensed (100 µL per well) in flat-bottom 96-well microtiter plates, and incubated 16 h at 37°C in a 5% CO_2-enriched atmosphere to

Table 1 | Ranges of antibiotic concentrations tested for determination of MICs (broth microdilution method) and MIECs (dye uptake assay) for 11 antibiotics against *Francisella tularensis* subsp. *holarctica*.

Antibiotics	Antibiotic targets	MIC (µg/mL)	MIEC (µg/mL)
Gentamicin	Ribosome	0.032–16	0.064–32
Ciprofloxacin	Type II topoisomerases	0.001–0.5	0.004–2.0
Levofloxacin	Type II topoisomerases	0.001–0.5	0.004–2.0
Moxifloxacin	Type II topoisomerases	0.001–0.5	0.004–2.0
Doxycycline	Ribosome	0.032–16	0.032–16
Erythromycin	Ribosome	0.25–128	0.25–128
Imipenem	Cell wall	0.25–128	0.25–128
Meropenem	Cell wall	0.25–128	0.25–128
Linezolid	Ribosome	0.064–32	0.064–32
Rifampicin	RNA polymerase	0.004–2.0	0.016–8.0
Daptomycin	Cytoplasmic membrane	0.5–256	0.5–256

MIC, minimum inhibitory concentration; MIEC, minimum inhibitory extracellular concentration.

obtain confluent cell monolayers. In parallel, a 1-McFd standard suspension of each *F. tularensis* strain tested was grown for 24 h in brain-heart infusion broth (BHI, bioMérieux) supplemented with 2% PVX, at 37°C, in 5%CO_2. For each strain, the bacterial suspension obtained was adjusted to 0.5 McF standard by adding BHI-2%PVX, and further diluted in MEM-10% FCS to achieve a bacterial inoculum of 1.2×10^5 bacteria/50 µL of medium (i.e., 2.4×10^6 bacteria/mL). The cell monolayers were infected by replacing the supernatant with 50 µL of the previously prepared bacterial suspension. The plates were incubated at 37°C, in 5% CO_2 for 3 h to allow internalization of bacteria into MRC-5 cells. Cell monolayers were then washed in pH 7.2 sterile phosphate buffer saline (PBS, Gibco) and re-incubated for 1 h (37°C, 5% CO_2,)in MEM-10% FCS medium containing 5 µg/mL of gentamicin in order to eliminate extracellular bacteria. At that time, the intracellular bacterium inoculum (referred to as the primary intracellular inoculum) was determined using the CFU-enumeration method (see below). After 3 additional washes with PBS, infected MRC-5 monolayers were incubated (37°C, 5% CO_2) in MEM-10% FCS medium containing twofold serial concentrations of the tested antibiotic (**Table 1**). MIECs were read after 1 or 5 days incubation of the plates at 37°C, in 5% $CO_2$for the CFU method and the dye uptake assay, respectively. All assays included a *F. tularensis* positive growth control (infected MRC-5 cells with no antibiotic) and two negative controls (uninfected MRC-5 with no antibiotic or with the antibiotic at the maximum concentration tested). The activity of antibiotics against intracellular *F. tularensis* was then evaluated in parallel using two methods: the dye uptake assay and CFU counting. All experiments were run in duplicates to confirm results.

Dye uptake assay

After incubation of the plates, the cell supernatants were removed and replaced with 50 µL of 0.15% neutral red dye (Sigma-Aldrich) in PBS, pH 5.5. The plates were incubated 1 h at 37°C in 5% CO_2 to allow penetration of the dye into the cells. The excess dye was then removed by three washes in PBS, pH 6.5. The red staining of cell monolayers was visually evaluated in comparison to positive (T+, MRC-5 monolayer infected at MOI 200:1 without antibiotics) and negative controls (T-ATB, uninfected MRC-5 monolayer incubated with the highest concentration of the antibiotic tested). A staining score of 1 corresponded to complete lysis of the cell monolayer, i.e., T+ control. A staining score of 4 corresponded to full preservation of the cell monolayer, i.e., T-ATB control. Scores 2 and 3 corresponded to intermediate color intensities (**Figure 1**). The MIEC was defined as the minimum extracellular concentration of the antibiotic tested allowing prevention of a *F. tularensis* cytotoxic effect (staining score of 4).

CFU counts

After incubation of the plates, the cell supernatants were removed and replaced with 200 µL of 1% saponin solution (ProLabo®, Leuven, Belgium). The plates were incubated 15 min at room temperature to allow disruption of the eukaryotic cell membranes and release of intracellular bacteria into the cell supernatant. After homogenisation, 1, 10, and 100 µL of the bacterial suspension of each well were plated on CHA-PVX media. CFU counts were determined after 48 h incubation of the CHA-PVX plates at 37°C, in 5% CO_2. The same procedure was used to determine the primary intracellular bacterial inoculum, as mentioned above. Thus

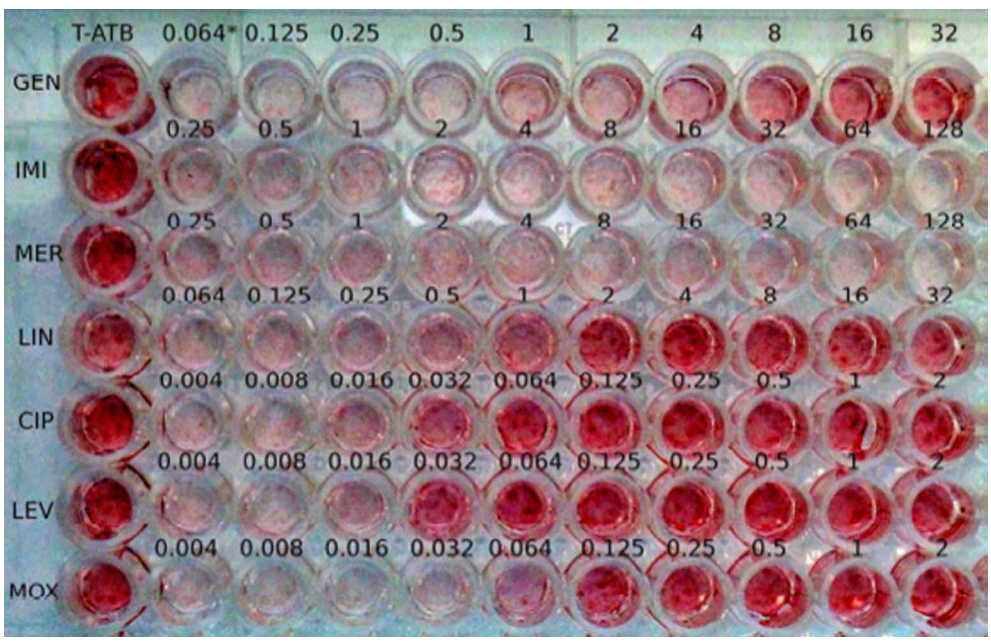

FIGURE 1 | Image of a dye uptake assay for the Ft24 strain. *, antibiotic concentrations in the well (µg/mL); T-ATB, uninfected MRC-5 monolayer incubated with the highest concentration of the antibiotic tested; GEN, gentamicin; IMI, imipenem; MER, meropenem; LIN, linezolid; CIP, ciprofloxacin; LEV, levofloxacin; MOX, moxifloxacin.

the activity of antibiotics was deduced from their capacity to completely inhibit bacterial growth, i.e., CFU counts after antibiotic exposure ≥ CFU counts of the primary intracellular bacterial inoculum.

RESULTS

DYE UPTAKE ASSAY VALIDATION

We first tested the activity of ciprofloxacin and doxycycline against intracellular *F. tularensis* Ft6 strain, using both the CFU and dye uptake assays (**Figure 2**). For drug-free controls, the mean increase in intracellular bacterial loads after 24 h incubation of cultures was between 2.41 and 3.43 log CFU/well (data not shown). As for ciprofloxacin (**Figure 2A**), the CFU method determined the MIEC to be 0.125 μg/mL. The same method also revealed that ciprofloxacin induced a two-log reduction of bacterial loads at concentrations above the MIEC. The dye uptake assay showed complete destruction of the cell monolayers after 5 days incubation of cultures (score = 1) at ciprofloxacin concentrations up to 0.032 μg/mL, a score of 3 at 0.064 μg/mL, and a score of 4 for concentrations ≥ 0.125 μg/mL. Thus, ciprofloxacin MIEC was the same for the dye uptake and CFU count assays. For doxycycline (**Figure 2B**), the MIECs were 0.25 μg/mL and 0.5 μg/mL using the CFU and dye uptake assays, respectively. Doxycycline only induced a lower (<1 log) reduction in bacterial counts after 24 h incubation. Also, a reduction in the dye uptake scores was observed for doxycycline concentrations ≥ 2 μg/mL, suggesting a toxic effect of this compound against MRC-5 cells at these concentrations.

MICs AND MIECs OF 11 ANTIBIOTICS AGAINST *F. TULARENSIS* FT6 AND FT24 STRAINS

Both strains displayed low MICs for gentamicin, fluoroquinolones (ciprofloxacin, levofloxacin and moxifloxacin), doxycycline and rifampicin (**Table 2**). Erythromycin was less effective, with four times higher MIC for the Ft24 strain compared to the Ft6 strain. The carbapenems (imipenem, meropenem) and daptomycin had no inhibitory activity against *F. tularensis*. Similar MIC and MIEC values were obtained for fluoroquinolones, doxycycline and rifampicin. Gentamicin was less effective against intracellular *F. tularensis*, with MIECs four to eight times higher than MICs. In contrast, erythromycin and linezolid displayed improved activity against intracellular *F. tularensis*. MIECs were four to eight times lower than MICs for erythromycin and eight times lower than MICs for linezolid.

DISCUSSION

We adapted a dye uptake assay previously used to test the antibiotic susceptibilities of strict intracellular pathogens (Rolain et al., 1998) to investigate the activity of antibiotics against the intracellular form of the facultative intracellular bacterium *F. tularensis*. This test is safer than traditional CFU-based assays because it does not need manipulation of large quantities of culture dishes of this highly infectious agent. Although the test should be performed in a biosafety level 3 laboratory, the intracellular activity of several

Table 2 | Activities of 11 antibiotics against two clinical strains (Ft6 and Ft24) of *F. tularensis* subsp. *holarctica* determined using a broth microdilution method and a dye uptake assay, respectively.

	MIC (μg/mL)		MIEC (μg/mL)	
	Ft6	Ft24	Ft6	Ft24
Gentamicin	0.25	0.5	2	2
Ciprofloxacin	0.032	0.032	0.064	0.064
Levofloxacin	0.064	0.064	0.064	0.064
Moxifloxacin	0.125	0.125	0.125	0.125
Doxycycline	0.5	1	0.5	0.5
Erythromycin	4	16	1	2
Imipenem	>128	>128	>128	>128
Meropenem	>128	>128	>128	>128
Linezolid	8	8	1	1
Rifampicin	0.5	0.5	0.5	0.5
Daptomycin	>256	>256	>256	>256

MIC, minimum inhibitory concentration; MIEC, minimum inhibitory extracellular concentration.

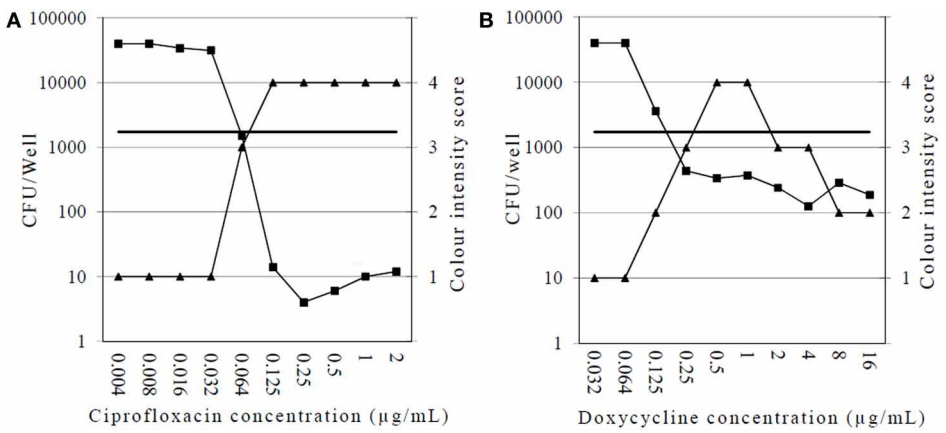

FIGURE 2 | Comparison of results obtained with the CFU enumeration and dye uptake assays for intracellular activity of ciprofloxacin (A) and doxycycline (B) against *F. tularensis* subsp. *holarctica* stain Ft6. Squares, CFU counts 24 h post-infection (CFU/well); triangles, dye uptake assay (score 1–4); horizontal line, 1 h post-infection intracellular bacterial load (CFU/well) as determined by the CFU-enumeration method (results of one experiment).

compounds against a large panel of type A and type B *F. tularensis* strains could be easily achieved. We first validated this new assay by comparing the results obtained for two antibiotics against two clinical strains of *F. tularensis* subsp. *holarctica* when using either the dye uptake assay or a traditional method of VBC determination using CFU methodology. We found a high correlation between MIECs determined using both methods. This was not unexpected since, in our model, *F. tularensis* multiplication led to complete lysis of cell monolayers. In contrast, inhibition of intracellular bacterial growth prevented *F. tularensis* cytolytic effects. Complete monolayer lysis was revealed using the live stain neutral red. This molecule is commonly used for cytotoxicity (Borenfreund and Puerner, 1985) or vacuolation assays (Cover et al., 1991), as it rapidly accumulates in lysosomes after penetration in viable cells by diffusion through the cytoplasmic membrane. It has been previously shown that the presence of weak bases (Ohkuma and Poole, 1981) or bacterial compound (Cover et al., 1991) influences vacuoles production level. Crystal violet has been used for similar experiments with the advantage that this stain binds to chromatin and thus color intensity may not vary according to the antibiotic used (Brasaemle and Attie, 1988). However, we observed an increase in neutral red uptake in cells exposed to antibiotics facilitating interpretation of results due to higher contrast between lysed and unlysed monolayers (**Figure 1**). For standardization, we framed each assay with controls used as visual cut-off for independent interpretation for each antibiotic (i.e., Materials and Methods section).

We then evaluated the extracellular and intracellular activity of 11 antibiotic compounds against the same two clinical strains of *F. tularensis*, respectively, using a CLSI broth microdilution method and the dye uptake assay developed. The broth microdilution method for antibiotic susceptibility testing of *F. tularensis* remains fastidious and the results are poorly predictive of the clinical situation for some antibiotics (Valade et al., 2008). Numerous controls are also needed, such as the use of non-fastidious control strains to check antibiotic activity in more standardized conditions. MIC results were consistent with previous studies. As expected, the carbapenems, which belong to the beta-lactams family, were not effective against *F. tularensis* (Georgi et al., 2012). Both strains were susceptible to antibiotics used as first-line treatment of tularaemia, including three fluoroquinolone compounds, doxycycline and gentamicin (Urich and Petersen, 2008; Valade et al., 2008). Rifampicin was also highly effective against *F. tularensis in vitro*. Erythromycin was less effective, although we tested type B biovar I strains of *F. tularensis*, which was consistent with previous studies (Gestin et al., 2010; Georgi et al., 2012). The new compounds daptomycin (a lipopeptide) and linezolid (an oxazolidinone) were either ineffective or poorly effective against extracellular *F. tularensis*. To our knowledge, linezolid has only been tested against *Francisella* sp. strains using MIC test strips, with inconsistent results between studies. MIC ranges were 2–16 μg/mL for North American strains tested using Mueller-Hinton supplemented with 1% IsovitaleX (Johansson et al., 2002) and 0.5–2.0 μg/mL for Turkish strains from central Anatolia tested using glucose/cysteine blood agar (GCBA) plates supplemented with 9% sheep blood (Yeşilyurt et al., 2011), whereas the MIC_{90} was 32 μg/mL for Hungarian strains tested using modified Francis agar plates (Kreizinger et al., 2012). These discrepancies may represent true differences in linezolid susceptibilities among *F. tularensis* strains of different geographic origin, but may also reflect poor standardization of the methods used. Valade et al. (2008) previously demonstrated that MIC test strips gave different results when using different agar media, and that the results obtained with this method were poorly correlated to those obtained using the reference agar dilution method, especially for nalidixic acid and rifampicin.

We found ciprofloxacin and doxycycline had similar inhibitory activity against extracellular and intracellular *F. tularensis*. These two antibiotic classes are concentrated within eukaryotic cells (Hof, 2003) and are used as reference treatments for infectious diseases caused by intracellular pathogens (Rolain et al., 1998; Wright Valderas and Barrow, 2008). However, using the CFU method, we found a more pronounced bactericidal effect of ciprofloxacin as compared to doxycycline against *F. tularensis* strains grown in MRC-5 cells, which confirms previously published data using macrophage-like cells (Maurin et al., 2000). These findings are consistent with the current recommendation of the use of ciprofloxacin and doxycycline as first-line treatment of tularaemia (Johansson et al., 2000; Scheftel et al., 2010; Maurin et al., 2011). In our model, the aminoglycoside gentamicin was four to eight times less effective against the intracellular form of *F. tularensis*. Previous experiments have shown that gentamicin displays a bactericidal activity against intracellular *F. tularensis*, but prolonged exposure of infected cells to this antibiotic is needed because of its slow penetration and concentration within eukaryotic cells (Maurin and Raoult, 2001). Whereas streptomycin was considered the referenced treatment of tularaemia (Tärnvik and Chu, 2007), gentamicin has been recently associated with treatment failures and relapses (Kaya et al., 2012).

Regarding antibiotics not currently recommended for treatment of tularaemia, three situations were observed. Rifampicin displayed similar extracellular and intracellular activities. This antibiotic is not used for treatment of tularaemia because of concern about selection of resistant mutants (Bhatnagar et al., 1994). Daptomycin and the carbapenems were not effective against the extra- and intracellular forms of the two *F. tularensis* strains tested. The beta-lactams are usually considered unreliable for treatment of tularaemia (Cross and Jacobs, 1993). Although *F. tularensis* may harbor a class A beta-lactamase (Antunes et al., 2012), mechanisms of resistance to carbapenems in this species need further investigation. To our knowledge, susceptibility to imipenem has been reported only for three biovar II strains of *F. tularensis* subsp. *holarctica* (including the LVS strain) (Tomaso et al., 2005). Lee et al. (1991) reported a case of tularaemia with favorable progression after 14-day treatment with imipenem. Finally, erythromycin and linezolid were more active when tested in the MRC-5 cell system. MIECs were eight times lower than MICs, suggesting that these compounds could concentrate in the intracellular compartment of *F. tularensis* multiplication. The same observation was previously reported for azithromycin and the LVS strain of *F. tularensis* (Ahmad et al., 2010). Erythromycin, like other macrolides, can concentrate within acidic compartments of eukaryotic cells (especially lysosomes) because of their

low base nature (Carlier et al., 1987). However, their intracellular activity may be reduced owing to their protonation at acidic pH (Goldman et al., 1990). The macrolides are not considered a safe alternative for tularaemia patients (Enderlin et al., 1994), but recent case reports indicate that azithromycin may be useful in pregnant women with mild disease caused by type B biovar I strains of *F. tularensis* (Dentan et al., 2013). More surprisingly, linezolid displayed significant activity against intracellular *F. tularensis in vitro*. This oxazolidinone is currently used for treatment of infections caused by multi-drug-resistant Gram-positive bacterial species, such as *S. aureus*, *Streptococcus pneumoniae* and *Enterococcus* sp. Interestingly, an additive effect of the combination of linezolid and gentamicin was reported against *S. aureus* (Grohs et al., 2003). Linezolid is not active against aerobic Gram-negative bacteria such as enterobacterial and *Pseudomonas* sp. (Leclercq, 2010). On the other hand, linezolid did not accumulate in THP-1 human macrophage cells (Lemaire et al., 2011). Thus, the mechanism of action of linezolid against intracellular *F. tularensis* should be further investigated.

In conclusion, we adapted a dye uptake assay in order to evaluate the activity of antibiotics against intracellular *F. tularensis*. This test would facilitate screening of the activity of new compounds against this fastidious, facultative intracellular bacterium in the search for new therapeutic alternatives for tularaemia. Also, because the proposed technique is much easier to perform than the traditional CFU methodology, it may help standardize antibiotic susceptibility testing for *F. tularensis* strains. Finally, this study highlights the potential usefulness of linezolid as a therapeutic alternative for tularaemia patients, especially in case of failure or relapses after administration of current first-line antibiotics. *In vitro* results obtained with this drug warrant further investigation in animal models.

AUTHOR CONTRIBUTION

Research project design: Vivien Sutera and Max Maurin. Experiments: Vivien Sutera, Yvan Caspar and Sandrine Boisset. Writing: Vivien Sutera and Max Maurin.

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The use of resazurin as a novel antimicrobial agent against *Francisella tularensis*

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The highly infectious and deadly pathogen, *Francisella tularensis*, is classified by the CDC as a Category A bioterrorism agent. Inhalation of a single bacterium results in an acute pneumonia with a 30–60% mortality rate without treatment. Due to the prevalence of antibiotic resistance, there is a strong need for new types of antibacterial drugs. Resazurin is commonly used to measure bacterial and eukaryotic cell viability through its reduction to the fluorescent product resorufin. When tested on various bacterial taxa at the recommended concentration of 44 μM, a potent bactericidal effect was observed against various *Francisella* and *Neisseria* species, including the human pathogens type A *F. tularensis* (Schu S4) and *N. gonorrhoeae*. As low as 4.4 μM resazurin was sufficient for a 10-fold reduction in *F. tularensis* growth. In broth culture, resazurin was reduced to resorufin by *F. tularensis*. Resorufin also suppressed the growth of *F. tularensis* suggesting that this compound is the biologically active form responsible for decreasing the viability of *F. tularensis* LVS bacteria. Replication of *F. tularensis* in primary human macrophages and non-phagocytic cells was abolished following treatment with 44 μM resazurin indicating this compound could be an effective therapy for tularemia *in vivo*.

Keywords: *Francisella*, resazurin, antibiotic, *Neisseria*, resorufin, tularemia, antibacterial, macrophages

INTRODUCTION

Francisella tularensis is the causative agent of the zoonotic disease tularemia (Oyston et al., 2004). This disease is endemic in North America, Europe, and Asia with outbreaks often associated with the handling of infected animals or transmission by arthropod vectors (Sjostedt, 2007; Oyston, 2008). The Centers for Disease Control and Prevention has categorized *F. tularensis* as a Category A bioterrorism agent due to its ease of aerosolization, low infectious dose, and high mortality rate (McLendon et al., 2006). Inhalation of fewer than 10 bacteria results in an acute pneumonia that is lethal in 30–60% of individuals if left untreated (Dennis et al., 2001; McLendon et al., 2006).

When implemented early in infection, antibiotics are effective at reducing the case fatality rate for tularemia (Dennis et al., 2001; Barry et al., 2009). Aminoglycosides are commonly prescribed, specifically streptomycin or gentamicin, although tetracyclines and fluoroquinolones also have antimicrobial activity against *F. tularensis* (Nigrovic and Wingerter, 2008; Oyston, 2009). Tetracyclines, however, are associated with high relapse rates in tularemia patients (Thomas and Schaffner, 2010). Since this disease is often misdiagnosed due to its generic symptoms, antibiotic treatment may be delayed resulting in reduced survival (Barry et al., 2009). There is also a potential for the introduction of antibiotic-resistant strains (Oyston, 2009). While a tularemia vaccine is available (live vaccine strain, LVS), it is not currently licensed for use in the United States (Conlan and Oyston, 2007). Due to these concerns, there is an increased interest in developing alternative therapies for tularemia.

Resazurin, the active compound in alamarBlue®, has been used for decades to measure proliferation and cytotoxicity in prokaryotic and eukaryotic cells (Page et al., 1993; Ahmed et al., 1994; O'Brien et al., 2000). In metabolically active cells, this blue, non-fluorescent dye is reduced to the pink and highly fluorescent compound resorufin allowing for a quantitative measurement of cell viability (**Figure 1**) (O'Brien et al., 2000). Upon use of resazurin to monitor *F. tularensis* viability in culture at the recommended concentration of 44 μM, we discovered a novel antibacterial activity for this compound. Resazurin, and its reduced derivative resorufin, decreased the number of viable *F. tularensis* bacteria in broth culture by 100-fold after 1 day of cultivation. Growth of other bacterial genera was unaffected by this compound with the exception of *Neisseria* species, particularly the human pathogen *N. gonorrhoaea*. Resazurin also limited replication of *F. tularensis* in human macrophages and non-phagocytic cells highlighting the potential use of this compound as a novel antibacterial therapy *in vivo*.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

Bacterial strains used in this study are listed in **Table 1**. Bacteria grown on solid media were used to inoculate chocolate II agar plates or TSBc [trypticase soy broth (BD Biosciences) containing

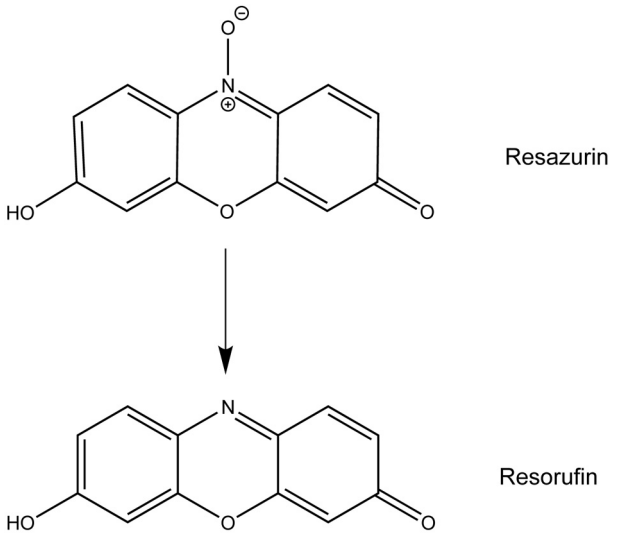

FIGURE 1 | The reduction of resazurin to resorufin.

Table 1 | Bacterial strains used in this study.

Bacterial strain	Source
Francisella tularensis subsp. *holarctica* live vaccine strain	Karen Elkins
F. tularensis subsp. *tularensis* Schu S4 (NR-643)	NIH BEI Resources Repository[a]
F. novicida U112	Karen Elkins
F. philomiragia (ATCC 25018)	ATCC
Neisseria gonorrhoeae (ATCC 9793)	ATCC
N. polysaccharea (ATCC 43768)	ATCC
N. sicca (ATCC 9913)	ATCC
Acinetobacter baumannii (ATCC 19606)	ATCC
Pseudomonas aeruginosa 1244	Peter Castric
Escherichia coli	WLU-MCC[b]
Salmonella typhimurium	WLU-MCC[b]
Staphylococcus aureus	WLU-MCC[b]
Listeria monocytogenes EGD	Douglas Drevets
Klebsiella pneumoniae	WLU-MCC[b]
Streptococcus pneumoniae, clinical isolate	Robert Shanks

[a] *National Institutes of Health Biodefense and Emerging Infections (NIH BEI) Research Resources Repository, National Institute of Allergy and Infectious Diseases.*

[b] *WLU-MCC = West Liberty University Microbiology Culture Collection, bacterial species routinely verified by standard metabolic and physiological tests.*

0.1% L-cysteine hydrochloride monohydrate (Fisher)] supplemented with or without various concentrations of resazurin sodium salt (Acros Organics, dissolved in water) or resorufin (Tokyo Chemical Industries, dissolved in dimethyl sulfoxide). Chocolate II agar plates were incubated at 37°C with 5% CO_2 (*Francisella* species, *Neisseria* species, and *Listeria monocytogenes*) or without CO_2 (all other bacteria) for 1–3 days while broth cultures were incubated at 37°C with shaking. All work with Schu S4 was conducted under BSL-3 conditions at the University of Pittsburgh with approval from the CDC Select Agent Program.

REDUCTION OF RESAZURIN TO RESORUFIN BY *F. tularensis*

F. tularensis was cultured in TSBc supplemented with 44 μM resazurin at 37°C with shaking for 24 h. At select timepoints, a Spectronic 200 Spectrophotometer was used to measure the absorbance at 600 nm and 570 nm to detect the presence of resazurin and resorufin, respectively. The ratio of these two optical densities was used to evaluate reduction of resazurin to resorufin over time.

GROWTH OF *F. tularensis* IN HUMAN MACROPHAGES AND HEK293 CELLS

Human monocytes purified from buffy coats from blood donations (New York Blood Center, Long Island City, NY and the Central Blood Bank, Pittsburgh, PA) were differentiated into macrophages as described previously (Carlson et al., 2007, 2009; Horzempa et al., 2008a,b, 2010; Robinson and Nau, 2008; Robinson et al., 2010, 2012; Russo et al., 2011; Schmitt et al., 2012). Macrophages were then washed and resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% human serum AB (Gemini Bio-Products), 25 mM HEPES (Cellgro), and 1% glutamine dipeptide (Fisher Scientific). HEK293 cells (ATCC CRL-1573), a non-phagocytic kidney epithelial cell line (Tachado et al., 2007), were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco), 25 mM HEPES, and 1% glutamine dipeptide with 100 U/ml penicillin-streptomycin (Cellgro). HEK293 cells were passaged at least once without antibiotics prior to use. To assess intracellular growth, gentamicin protection assays were performed (Small et al., 1987). Macrophages and HEK293 cells were seeded in Primaria 96-well culture dishes (BD Biosciences) at a density of 5×10^4 cells/well. *F. tularensis* bacteria recovered from broth cultures described above were adjusted to an OD_{600} of 0.3 (approximately 1.5×10^9 CFU/ml) and diluted to achieve a multiplicity of infection (MOI) of 500. The actual MOI was measured by plating serial dilutions of the inoculum on chocolate II agar plates. Cells were incubated with this MOI for 2 h yielding an infection rate of >80% (Carlson et al., 2007; Horzempa et al., 2008a) either in the absence or presence of 44 μM resazurin. After this time period, cells were incubated with gentamicin (100 μg/ml) for 30 min to kill extracellular bacteria and then washed twice with warm Hanks balanced salt solution (Cellgro). Fresh culture media with or without resazurin (44 μM) was then added and cells were incubated for another 22 h at 37°C with 5% CO_2. At the indicated timepoints, cells were lysed with 0.02% sodium dodecyl sulfate and viable CFU were measured as described below.

ENUMERATION OF BACTERIA

At the indicated timepoints, a portion of the *F. tularensis* broth cultures or human cell lysates were serially diluted and plated onto chocolate II agar plates. Plates were incubated at 37°C at 5% CO_2 for 2–3 days and individual colonies were enumerated. The limit of detection was 100 CFU per ml for broth culture or per 5×10^4 cells for intracellular growth assays.

ANALYTICAL METHODS

Statistically significant differences in bacterial number were determined by a Student's *t*-test or ANOVA followed by a Dunnett's or Bonferroni *post-hoc* test (GraphPad Prism 5). The chemcial structures of resazurin, resorufin, and acridine were drawn using ChemDraw Pro 13.0 for comparative analysis.

RESULTS

RESAZURIN SELECTIVELY INHIBITS GROWTH OF FRANCISELLA AND NEISSERIA SPECIES

Resazurin has been used previously as an indicator of cell growth for various bacterial species (Mendoza-Aguilar et al., 2012; Bassett et al., 2013; Bauer et al., 2013; Lall et al., 2013). We were interested in using this compound to monitor viability of *F. tularensis* in broth culture over time. Unexpectedly, no viable bacteria were detected 24 h post-inoculation following inclusion of resazurin in TSBc cultures of *F. tularensis* LVS at the concentration recommended by the manufacturer (44 μM) (data not shown). This concentration of resazurin had no effect on the growth of *E. coli* or *P. aeruginosa* cultivated in the same medium (data not shown). The antimicrobial activity of resazurin on *F. tularensis* LVS was not specific to TSBc as these bacteria were also unable to grow on chocolate II agar plates containing this compound (**Table 2**) as well as a chemically defined medium (data not shown). Lowering the resazurin concentration to as little as 4.4 μM still resulted in a 10-fold reduction in viable *F. tularensis* LVS compared to growth medium alone (**Figure 2**). These data suggest that resazurin exhibits bactericidal activity against *F. tularensis*.

To determine whether the antibacterial effect of resazurin was specific to this organism, an assortment of bacteria from diverse taxa were plated on chocolate II agar plates supplemented with resazurin. All bacterial species tested were able to grow in the presence of 44 μM resazurin except *F. tularensis* and *Neisseria* species (**Table 2**). These data suggest that resazurin is an antimicrobial compound with specificity for *F. tularensis* and *Neisseria* species bacteria.

Table 2 | Resazurin inhibits the growth of *Francisella* and *Neisseria* species on chocolate II agar.

	Resazurin Concentration	
Bacterial strain	**0 μM**	**44 μM**
Francisella tularensis Schu S4	+	−
F. tularensis LVS	+	−
F. novicida	+	+
F. philomiragia	+	+
Neisseria gonorrhoeae	+	−
N. polysaccharea	+	−
N. sicca	+	−
Acinetobacter baumannii	+	+
Pseudomonas aeruginosa 1244	+	+
Escherichia coli	+	+
Salmonella typhimurium	+	+
Staphylococcus aureus	+	+
Listeria monocytogenes	+	+
Klebsiella pneumoniae	+	+
Streptococcus pneumoniae	+	+

+, *robust growth.*

−, *no growth.*

REDUCTION OF RESAZURIN TO RESORUFIN DOES NOT ALTER ITS ANTIBACTERIAL ACTIVITY

As previously mentioned, viable cells are capable of converting resazurin to resorufin. To determine whether this reduction was occurring in *F. tularensis* LVS cultures, the ratio of resorufin to resazurin was measured over time using the optical densities at 570 nm (resorufin) and 600 nm (resazurin). The ratio of resorufin to resazurin increased 3-fold within 2 h of inoculation with *F. tularensis* LVS, reaching a maximum ratio of 5 four hours post-inoculation which was maintained for the remainder of the 24 h period (**Figure 3A**). This suggested *F. tularensis* LVS was reducing resazurin to resorufin. Therefore, we determined if resorufin also exhibited antibacterial activity against *F. tularensis*. Following 24 h of culture in the presence of resorufin, the number of *F. tularensis* LVS bacteria was significantly reduced compared to growth medium supplemented with vehicle alone (**Figure 3B**). A similar decrease in bacterial number was observed following incubation of *F. tularensis* LVS with resazurin (**Figure 3B**). These data suggest resorufin is also bactericidal against *F. tularensis*. To evaluate whether resazurin must first be converted to resorufin to exhibit antibacterial activity, we measured viable *F. tularensis* LVS bacteria over time grown in the presence of resazurin and resorufin. In cultures treated with resazurin, reduction of this compound to resorufin was observed as early as

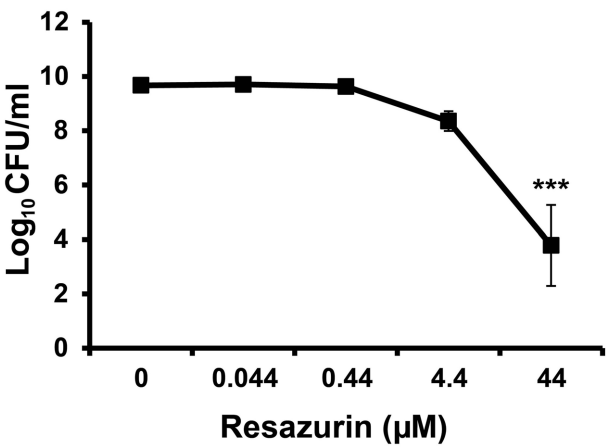

FIGURE 2 | Resazurin has an antimicrobial effect on *F. tularensis* LVS. Bacteria were cultivated in tryptic soy broth supplemented with 0.1% cysteine HCl (TSBc) in the presence or absence of resazurin at the designated concentrations for 24 h. Cultures were then diluted and plated to determine the number of viable *F. tularensis* LVS bacteria 24 h post inoculation. Data shown are mean ± s.e.m. from three individual experiments. The limit of detection was 100 CFU per ml. Statistically significant differences in growth post-inoculation were determined by One-Way ANOVA followed by Dunnett's *post-hoc* test (***$p < 0.001$ compared to 0 μM resazurin).

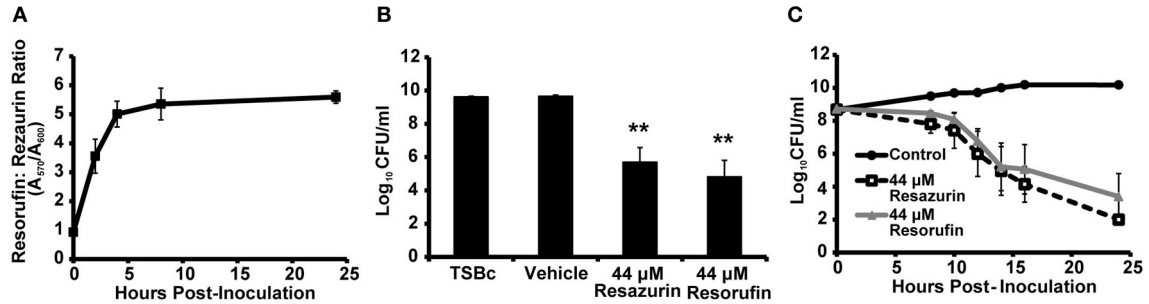

FIGURE 3 | *F. tularensis* LVS reduces resazurin to resorufin which also inhibits bacterial growth. *F. tularensis* LVS bacteria were cultivated in TSBc that was treated with resazurin, resorufin, or the solvent vehicle (DMSO). **(A)** Conversion of resazurin (absorbance at 600 nm) to resorufin (absorbance at 570 nm) in *F. tularensis* LVS cultures over 24 h. **(B,C)** *F. tularensis* LVS cultures were diluted and plated to determine the number of viable bacteria at 24 h **(B)** or the indicated timepoints **(C)**. Data shown are mean ± s.e.m. from three individual experiments. The limit of detection was 100 CFU per ml. Statistically significant differences in growth post-inoculation were determined by **(B)** One-Way ANOVA followed by Dunnett's *post-hoc* test (** $p < 0.01$ compared to TSBc) or **(C)** Two-Way ANOVA followed by Bonferroni comparison of means ($p < 0.05$ for Control vs. Resazurin and Control vs. Resorufin for 12–24 h).

2 h post-inoculation (**Figure 3A**). However, a decline in viable bacteria occurred 8 h post-inoculation, reaching statistical significance by 12 h (**Figure 3C**). Moreover, a similar decrease in bacterial viability was observed in cultures initially treated with resorufin (**Figure 3C**). No significant differences in viable bacteria were observed between *F. tularensis* cultures treated with either resazurin or resorufin at any of the time points (**Figure 3C**). Because resazurin and resorufin both exhibit a similar antimicrobial effect on *F. tularensis*, this suggests that the redox reaction itself is not responsible for the observed bactericidal activity. However, the data indicating that resazurin is rapidly converted to resorufin, while the drop in viability occurs most drastically after 8 h, may suggest that resorufin is the biologically active form responsible for decreasing the viability of *F. tularensis* LVS bacteria.

RESAZURIN LIMITS INTRACELLULAR REPLICATION OF *F. tularensis* IN PHAGOCYTIC AND NON-PHAGOCYTIC CELLS

In an infected host, *F. tularensis* resides and replicates inside macrophages (Elkins et al., 2007). Therefore, resazurin must be able to limit intracellular growth of this bacterium in these cells in order to be an effective therapeutic. To test this, primary human macrophages were infected with *F. tularensis* LVS, and viable intracellular bacteria were quantified using a gentamicin protection assay as described previously (Horzempa et al., 2008a, 2010). Cells were treated with 44 µM resazurin during the entire assay (+Rz), beginning 2 h post-infection (+2 h Rz), or left untreated. Both resazurin treatments resulted in a significant decrease in viable *F. tularensis* LVS bacteria over 22 h (**Figure 4A**). Visible observation of the macrophages 24 h post-infection indicated resazurin was reduced to resorufin, although this phenomenon was not quantified (data not shown). This suggested that the macrophages were still viable, and that the combination of resazurin and *F. tularensis* LVS did not culminate in undesirable toxicity. *F. tularensis* is also capable of infecting and replicating in non-phagocytic cells like epithelial cells and hepatocytes which is sufficient for pathogenesis (Horzempa et al., 2010). We next determined whether resazurin was also capable

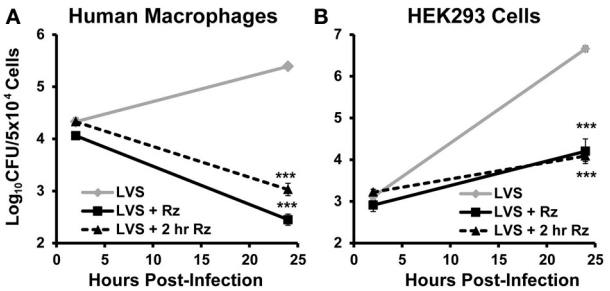

FIGURE 4 | Resazurin exhibits antimicrobial activity on intracellular *F. tularensis* LVS. Primary human macrophages **(A)** or HEK293 **(B)** cells were infected with *F. tularensis* LVS bacteria using a gentamicin protection assay. Cells were treated with 44 µM resazurin during the entire assay (+ Rz), at 2 h post-infection (+ 2 h Rz), or were untreated. Data shown are mean ± s.e.m. and representative of three independent experiments. The limit of detection was 100 CFU. Statistically significant differences in growth at 24 h post-infection were determined by Two-Way ANOVA with Dunnett's *post-hoc* test (*** $p < 0.001$).

of inhibiting growth of *F. tularensis* LVS in non-phagocytic cells. To test this, a human kidney epithelial cell line HEK293 was used as a model for non-phagocytic cells. Similar to the results obtained with macrophages, treatment with resazurin significantly reduced the number of viable *F. tularensis* LVS bacteria in HEK293 cells 22 h post-infection (**Figure 4B**). In an additional experiment, no viable *F. tularensis* LVS bacteria were detected from HEK293 cells at 72 h post infection following treatment with 44 µM resazurin (data not shown). Based on these data, resazurin exhibits antimicrobial activity against intracellular *F. tularensis* bacteria.

DISCUSSION

The prevalence of antibiotic resistance in today's society highlights the need for new classes of antibiotics (Bassetti et al., 2013). Here, we identified an unanticipated bactericidal activity for a compound commonly used to measure cellular viability. Resazurin inhibited growth of only *F. tularensis* and *Neisseria*

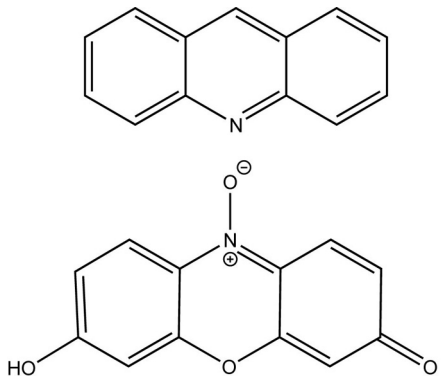

FIGURE 5 | Chemical structures of resazurin and acridine.

species *in vitro*, notably the human pathogens, type A *F. tularensis* (Schu S4) and *N. gonorrhoeae*. The fact that resazurin targets such a limited array of pathogenic organisms is extremely desirable from the standpoint of limiting the potential of drug resistance in the future.

Most antibiotics target pathways that are conserved by numerous bacterial species like cell wall or protein synthesis (Lewis, 2013). Resazurin is unique in that it only exhibits antimicrobial activity against two types of bacteria tested in this work, *F. tularensis* and *Neisseria* (**Table 2**). Aside from their fastidious nature, there are no apparent similarities between these two groups of bacteria that distinguish them from the other bacterial genera tested to suggest a mechanism of action. In culture, resazurin is reduced to resorufin by *F. tularensis* LVS, however, this chemical reaction is not responsible for the decline in viability since both compounds are equivalently bactericidal (**Figure 3**). Examination of the chemical structure of resazurin elucidated similarities to acridine (**Figure 5**). Many acridine derivatives were used as antibacterial agents during World War II (Wainwright, 2001). The planar area of the tricyclic acridine nucleus allows for intercalation of DNA resulting in its bactericidal activity (Wainwright, 2001). The possibility that resazurin functions in a similar fashion is currently being investigated.

In the human host, *F. tularensis* and *N. gonorrhoeae* reside inside host cells (Post et al., 2002; Oyston, 2008; Horzempa et al., 2011). Therefore, resazurin must be able to penetrate infected host cells to maintain its antibacterial activity *in vivo*. During the *in vitro* infection assays, resazurin was capable of limiting intracellular replication of *F. tularensis* in both phagocytic and non-phagocytic cells (**Figure 4**). While resazurin was converted to resorufin in these assays, the reduction of this compound by the host cells did not decrease the potency of the drug, which is consistent with the data indicating that both derivatives are bactericidal (**Figures 3**, **4**). Resazurin has been tested for toxicity in mice at a dose 100-fold higher than that used in this study and shown to be relatively well-tolerated (Lutty, 1978). Another recently developed antibacterial compound, fosmidomycin that was effective at killing intracellular *Francisella* bacteria *in vitro*, also prolonged survival following a lethal challenge in an animal model (McKenney et al., 2012). The data presented here strongly suggest that resazurin will be an effective therapeutic for use during an *in vivo F. tularensis* or *Neisseria* infection, and substantiate the need for pre-clinical animal trials using mouse models of infection.

AUTHOR CONTRIBUTIONS

Deanna M. Schmitt, Gerard J. Nau, and Joseph Horzempa conceived and designed the experiments. Joseph Horzempa, Deanna M. Schmitt, Dawn M. O'Dee, Brianna N. Cowan, James W.-M. Birch, and Leanne K. Mazzella performed the experiments. Deanna M. Schmitt, Joseph Horzempa, Gerard J. Nau, and Dawn M. O'Dee analyzed the data. Deanna M. Schmitt and Joseph Horzempa wrote the paper.

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Bis-indolic compounds as potential new therapeutic alternatives for tularaemia

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Francisella tularensis is the etiological agent of tularaemia and a CDC class A biological threat agent. Few antibiotic classes are currently useful in treating tularaemia, including the aminoglycosides gentamicin and streptomycin, fluoroquinolones, and tetracyclines. However, treatment failures and relapses remain frequent and *F. tularensis* strains resistant to antibiotics have been easily selected *in vitro*. In this study, we evaluated the activity of new synthetic bis-indole derivatives against this pathogen. Minimum inhibitory concentrations (MICs) of four compounds (dcm01 to dcm04) were determined for the reference strains *F. tularensis* subsp. *holarctica* LVS NCTC10857, *F. tularensis* subsp. *novicida* CIP56.12 and *F. philomiragia* ATCC25015, and for 41 clinical strains of *F. tularensis* subsp. *holarctica* isolated in France. Minimal bactericidal concentrations (MBCs) were determined for the dcm02 and dcm04 compounds for the LVS and two clinical strains. Killing curves were also determined for the same three strains exposed to dcm04. All tested bis-indole compounds were bacteriostatic against *F. tularensis* subsp. *holarctica* strains, with a MIC_{90} of 8 μg/mL for dcm01, dcm02, and dcm03, and 2 μg/mL for dcm04. Only one strain was resistant to both dcm01 and dcm03, with MICs > 32 μg/mL. In contrast, *F. tularensis* subsp. *novicida* was resistant to all derivatives and *F. philomiragia* was only susceptible to dcm02 and dcm04, with MICs of 16 and 4 μg/mL, respectively. MBC and killing curve experiments revealed significant bactericidal activity (i.e., 3-log reduction of the bacterial inoculum) of the dcm02 and dcm04 compounds only for the LVS strain. In conclusion, we have identified novel synthetic bis-indole compounds that are active against *F. tularensis* subsp. *holarctica*. They may be drug candidates for the development of new therapeutic alternatives for tularaemia treatment. Their further characterization is needed, especially identification of their bacterial targets.

Keywords: tularaemia, *Francisella tularensis*, bis-indolic compounds, antibacterial activity

INTRODUCTION

Francisella tularensis, the agent of the zoonosis tularaemia, may cause severe to fatal human infections. This intracellular, Gram-negative bacterium is highly infectious for humans and many animal species. No human-to-human transmission has been described so far and human infection may occur through direct contact with infected animals, ingestion of contaminated meat or water, arthropod bites, contact with contaminated environments and laboratory exposure to *F. tularensis* cultures (Dennis et al., 2001; Maurin et al., 2011). *F. tularensis* is a class A biological threat agent according to the CDC (Centers for Diseases Control and Prevention, Atlanta, Georgia, USA). The highly virulent *F. tularensis* subsp. *tularensis* strains (Jellison type A) are located in North America, whereas *F. tularensis* subsp. *holarctica* strains (Jellison type B) are found throughout the northern hemisphere. In Europe, tularaemia cases are often sporadic (Maurin et al., 2011), but outbreaks have recently been reported in many countries, including in Spain, Norway and Sweden (Pérez-Castrillón et al., 2001; Larssen et al., 2011; Rydén et al., 2012). The first-line therapy of tularaemia is based on a reduced number of antibiotics, including the aminoglycosides (gentamicin and streptomycin), the tetracyclines (e.g., doxycycline), and the fluoroquinolones (e.g., ciprofloxacin) (Johansson et al., 2002). Treatment duration is usually 7–10 days for gentamicin and ciprofloxacin, and 2–3 weeks for doxycycline. However, high rates of failure and relapse are observed in tularaemia patients, especially when treatment is delayed and/or lymph node suppuration occurs (Rotem et al., 2012).

The aminoglycosides such as gentamicin and streptomycin have a bactericidal activity against *F. tularensis in vitro*, and their use in tularaemia patients is associated with almost 100% cure rates (Kaya et al., 2011; Rotem et al., 2012). However, they are nephro- and ototoxic and can only be administrated parenterally (Tärnvik and Chu, 2007). Gentamicin is currently used in many countries where streptomycin is no longer available. However, treatment failures with this antibiotic have recently been reported in 11 paediatric patients with oropharyngeal tularaemia in Turkey, with successful recovery after switching to streptomycin (Kaya et al., 2011). Doxycycline can be administrated orally, with few side effects. However, the tetracyclines are contraindicated in

children under 8 years of age and in pregnant women because of the risk of permanent staining of the dental enamel and bone toxicity in the foetus (Tärnvik and Chu, 2007; Kaya et al., 2011). Treatment with this bacteriostatic antibiotic is associated with higher relapse rates as compared to aminoglycosides and fluoroquinolones, especially when treatment is delayed and/or of short duration (Dennis et al., 2001; Tärnvik and Chu, 2007). Doxycycline is administered for a minimum of 14 days (Dennis et al., 2001; Tärnvik and Chu, 2007). The fluoroquinolones (especially ciprofloxacin and levofloxacin) are preferred as first-line drugs for treatment of tularaemia cases of mild to moderate severity (Johansson et al., 2002). They are bactericidal against *F. tularensis in vitro*, orally administrable, and have few side effects. They can be administrated to young children but not to pregnant women (Johansson et al., 2000; Dennis et al., 2001; Tärnvik and Chu, 2007; Kaya et al., 2011). Ciprofloxacin is recommended as first-line drug in case *F. tularensis* is used as a biological weapon (Dennis et al., 2001; Rotem et al., 2012).

Other antibiotics such as the beta-lactams, the macrolides, cotrimoxazole, chloramphenicol, and rifampicin are not recommended for treatment of tularaemia. Beta-lactams are not effective both because they are inactivated by the class A beta-lactamase produced by *F. tularensis* (Antunes et al., 2012) and they are poorly effective against the intracellular form of this pathogen (Maurin et al., 2000). The macrolides are considered unreliable for treatment of tularaemia because most *F. tularensis* strains have natural high-level resistance to these antibiotics. Only azithromycin may be a possible alternative in pregnant women infected with type B biovar I strains (Dentan et al., 2013). Chloramphenicol and cotrimoxazole are poorly effective *in vitro* and potentially associated with severe side effects (Tärnvik and Chu, 2007). Rifampicin is active against *F. tularensis in vitro*, but its use as a monotherapy is usually associated with rapid selection of resistant mutants.

No natural strains of *F. tularensis* with acquired resistance to gentamicin, fluoroquinolones or doxycycline have been isolated so far. However, *in vitro* experiments have shown that mutants resistant to fluoroquinolones, rifampicin or macrolides can be selected easily (Tärnvik and Chu, 2007; Gestin et al., 2010; Sutera et al., 2014). At present, treatment failures and relapses are considered to be primarily related to delayed administration of appropriate antibiotic therapy rather than *in vivo* selection of antibiotic-resistant mutants (Dennis et al., 2001; Johansson et al., 2002; Kaya et al., 2011; Rotem et al., 2012). However, the bioengineering of genetically modified strains of *F. tularensis* resistant to first-line drugs for use as a biological warfare agent is a major concern. Thus, innovative antibiotics with original structures and bacterial targets, active against this highly virulent pathogen, would be beneficial not only to improve treatment efficacy in tularaemia patients, but also to reinforce our preparedness against the misuse of antibiotic-resistant *F. tularensis* strains.

We recently identified synthetic bis-indole derivatives as new antistaphylococcal compounds with preserved activity against multi-drug resistant strains of *Staphylococcus aureus*, including MRSA strains (Denis et al., 2013a,b). In this study, we evaluated the activity of four of the leading compounds against clinical isolates of *F. tularensis* subsp. *holarctica*.

MATERIALS AND METHODS

BIS-INDOLIC COMPOUNDS AND ANTIBIOTICS

The four bis-indolic compounds evaluated in this study (dcm01, dcm02, dcm03, and dcm04) were synthesized by the DCM (Département de Chimie Moléculaire, Université Joseph Fourier Grenoble-1, Grenoble, France) according to previously published protocols (Denis et al., 2013a,b). The structures of the tested bis-indole compounds are presented on **Figure 1**. We also used gentamicin (Panpharma, Fougères, France) and doxycycline (Sigma-Aldrich, Lyon, France) as controls. Stock solutions of the bis-indolic compounds were prepared at 12.8 g/L in 100% DMSO (Sigma-Aldrich, Lyon, France) and stock solutions of gentamicin and doxycycline were prepared in sterile distilled water. All were kept frozen at −80°C until used.

BACTERIAL STRAINS

All experiments were conducted in a biosafety level 3 laboratory. The use of *F. tularensis* strains was carried out under the approval of ANSM (Agence nationale de sécurité du médicament et des produits de santé). We tested 41 isolates (Ft1–Ft41) of *F. tularensis* subsp. *holarctica* identified to the subspecies level in our laboratory (French reference center for *Francisella*) by amplification and sequencing of the intergenic 16S-23S rRNA region (Maurin et al., 2011). Four isolates were obtained from dead hares and the 37 others from human samples (**Table 1**). The clinical strains corresponded to independent and sporadic tularaemia cases occurring throughout France between 2004 and 2013 (Maurin et al., 2011). We also tested reference strains including *F. tularensis* subsp. *holarctica* LVS NCTC10857, *F. tularensis* subsp. *novicida* CIP56.12 and *F. philomiragia* ATCC25015. *S. aureus* ATCC29213 was tested as a control strain susceptible to the tested bis-indole compounds (Denis et al., 2013a,b). The reference bacterial strains were purchased from the American Type Culture Collection (ATCC, Mannasas, VA, USA) or the Collection of the Pasteur Institute (CIP, Centre de Ressource Biologique de l'Institut Pasteur, Paris, France). All strains are kept frozen in cryotubes (MastDiagnostic, Amiens, France) at −80°C. When needed, they are grown on chocolate agar supplemented

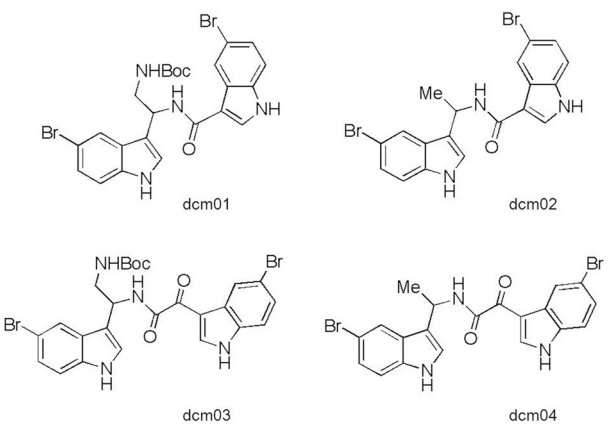

FIGURE 1 | Structures of the four bis-indole compounds.

Table 1 | Sources of the 41 isolates of *F. tularensis* subsp. *holarctica* (Ft1 to Ft41) used in this study.

Strain	Host	Year of isolation	Clinical sample
Ft1	Animal	UNK	Hare tissue
Ft2	Animal	UNK	Hare tissue
Ft3	Animal	UNK	Hare tissue
Ft4	Animal	UNK	Hare tissue
Ft5	Human	2004	Blood culture
Ft6	Human	2007	Blood culture
Ft7	Human	2006	Conjunctivitis
Ft8	Human	2007	Cutaneous ulcer
Ft9	Human	2007	Blood culture
Ft10	Human	2008	Mediastinal lymph node
Ft11	Human	2008	Pharynx
Ft12	Human	2008	Pharynx
Ft13	Human	2008	Pharynx
Ft14	Human	2008	Blood culture
Ft15	Human	2008	Cerebrospinal fluid
Ft16	Human	2008	UNK
Ft17	Human	2008	UNK
Ft18	Human	2008	Blood culture
Ft19	Human	2008	Blood culture
Ft20	Human	2008	Skin ulcer
Ft21	Human	2008	Conjunctivitis
Ft22	Human	2009	Whitlow
Ft23	Human	2009	Middle ear
Ft24	Human	2009	Lymph node
Ft25	Human	2010	Blood culture
Ft26	Human	2010	Blood culture
Ft27	Human	2010	Blood culture
Ft28	Human	2010	Lymph node
Ft29	Human	2010	Blood culture
Ft30	Human	2011	Lymph node
Ft31	Human	2011	UNK
Ft32	Human	2012	UNK
Ft33	Human	2011	Blood culture
Ft34	Human	2012	Blood culture
Ft35	Human	2012	Finger abscess
Ft36	Human	2012	UNK
Ft37	Human	2010	Blood culture
Ft38	Human	2012	Blood culture
Ft39	Human	2012	Whitlow
Ft40	Human	2012	Pleural fluid
Ft41	Human	2013	Pleural fluid

UNK, unknown.

with Polyvitex® (CHA-PVX medium, bioMérieux, Marcy l'Etoile, France) at 37°C in a 5% CO_2-enriched atmosphere.

DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATIONS

Minimum inhibitory concentrations (MICs) of the four bis-indolic compounds were determined against *S. aureus* ATCC29213, *F. philomiragia* ATCC25015 and all *F. tularensis* strains, using a broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI; M07-A8 Vol. 29, No. 2). Mueller-Hinton 2 broth supplemented with 2% PolyViteX® (MH2-PVX, bioMérieux, Marcy L'Etoile, France) was used as the antibiotic susceptibility testing medium for *F. tularensis* strains because of their fastidious nature. MH2 alone was used for other species. One row of a 96-well microtiter plate was filled with 75 µL of twofold serial dilutions of the tested bis-indolic compound in MH2-PVX medium, so as to obtain final bis-indolic concentrations ranging from 0.06 to 32 µg/mL in 0.5% DMSO. A bacterial inoculum (75 µL per well, 5×10^5 CFU/mL of final inoculum) was then added to each well. Antibiotic free cultures containing 0.5% DMSO were used as DMSO toxicity controls. MH2-PVX medium with 0.5% DMSO served as a negative control. Microplates were incubated at 37°C in a 5% CO_2 atmosphere. The MICs were read after 18 h culture incubation for *S. aureus* ATCC29213 and 48 h for *F. tularensis* strains. MICs corresponded to the minimum bis-indolic compound concentration that allowed complete inhibition of visual growth of bacteria. Experiments were conducted at least twice to confirm results. Following the same procedure but without DMSO, the MICs of gentamicin and doxycycline were determined against *F. tularensis* subsp. *holarctica* Ft6 and Ft24 strains and the control strain *S. aureus* ATCC29213.

DETERMINATION OF THE MINIMUM BACTERICIDAL CONCENTRATIONS

Minimum bactericidal concentrations (MBCs) were determined in triplicate experiments following CLSI recommendations (CLSI, M26-A, Vol. 19, No. 18), for the two most active compounds (dcm02 and dcm04) against three *F. tularensis* subsp. *holarctica* strains: the LVS strain and the two clinical strains Ft6 and Ft24. We used the same microdilution broth method described for MIC determination, but the primary bacterial inoculum was 10^6 CFU/mL. MBCs of gentamicin and doxycycline were determined in parallel as a bactericidal and a bacteriostatic control, respectively. After 48 h incubation at 37°C in a 5% CO_2atmosphere, MBCs were determined by plating 50 µL of tenfold serial dilutions of the bacterial suspensions of wells with no visible growth, and of the antibiotic-free control well, onto CHA-PVX medium. CFU counts were determined after 72 h incubation of the plates at 37°C, in a 5% CO_2 atmosphere. The detection limit was 20 CFU/mL. The MBC corresponded to the minimal antibiotic concentration which resulted in at least 99.9% reduction of the primary bacterial inoculum (i.e., 3 $\log_{10}$ reduction of bacterial titers).

TIME-KILL CURVES

Time-kill curves were determined for the LVS, Ft6, and Ft24 strains and the leading dcm04 compound. The primary inoculum calibrated at 10^6 CFU/mL was prepared in MH2-PVX medium and split into five 5-mL aliquots: one drug-free control; three others receiving 4, 8, and 16 times, respectively, the MIC of dcm04 for the tested strain, with 0.5% final concentration of DMSO in all three aliquots; and the last one receiving eight times the MIC of gentamicin for the tested strain, used as a positive control. Sterile MH2-PVX medium with 0.5% DMSO served as a negative control. Cultures were incubated 48 h at 37°C in 5% CO_2. At 0, 6, 12, 24, 36, and 48 h of incubation, a 50-µL aliquot was taken from

Table 2 | MICs (μg/mL) of *Francisella* strains for the four bis-indolic compounds: dcm01, dcm02, dcm03, and dcm04.

Bis-indole compound	*F. tularensis* subsp. *holarctica*				subsp. *novicida*	*F. philomiragia*
	FT1 to Ft41			LVS	CIP 56.12	ATCC25015
	MIC range	MIC_{50}	MIC_{90}	MIC	MIC	MIC
dcm01	2–>32	4	8	2	>32	>32
dcm02	4–8	4	8	2	32	16
dcm03	2–>32	4	8	2	>32	>32
dcm04	2–4	2	2	1	>32	4

each culture after shaking. Then 50 μL of ten-fold serial dilutions of each aliquot was plated on CHA-PVX medium. CFU counts were determined after 72 h incubation of the plates at 37°C in 5% CO_2. The detection limit was 20 CFU/mL. A 3-$\log_{10}$ or more reduction of the primary bacterial inoculum at any incubation time was considered a significant bactericidal effect. Experiments were conducted at least twice to confirm the results.

STATISTICAL ANALYSIS

A statistically significant decrease of viable bacterial counts in MBC assays was evaluated by one-tailed Student t-test using Statview® software. For each antibiotic concentration tested, we compared the bacterial count obtained after 48 h of incubation of cultures to the primary inoculum [i.e., log(N/N0)] and to a 3-log reduction cutoff. Significance was defined as a p-value < 0.05.

RESULTS

ALL TESTED SYNTHETIC BIS-INDOLIC COMPOUNDS ARE ACTIVE AGAINST *F. TULARENSIS* SUBSP. *HOLARCTICA* BUT NOT *F. TULARENSIS* SUBSP. *NOVICIDA*

MICs are represented in **Tables 2**, **3**. Almost all *F. tularensis* strains tested were susceptible to the four bis-indole derivatives. In contrast, the Ft5 strain was susceptible to dcm02 and dcm04 (MIC = 8 and 2 μg/mL, respectively), but resistant to dcm01 and dcm03 (MICs > 32 μg/mL). Dcm04 was the most active bis-indole compound with MICs ranging from 2 to 4 μg/mL and a MIC_{90} of 2 μg/mL. The MIC_{90} of the three other compounds was 8 mg/L. It should be noted that prolonged incubation of cultures only increased MICs by one dilution for some compounds. In comparison, the MIC of gentamicin against the LVS, Ft6 and Ft24 strains was 0.25 μg/mL and the MIC of doxycycline was 0.125 μg/mL against LVS and 0.25 μg/mL against the Ft6 and Ft24 strains. As for control strains, the *F. tularensis* subsp. *holartica* LVS strain was susceptible to the four bis-indole derivatives with a MIC of 1–2 μg/mL. In contrast, the reference *F. tularensis* subsp. *novicida* strain and the reference *F. philomiragia* strain were more resistant to these compounds. MICs of all bis-indole derivatives were ≥32 μg/mL for *F. tularensis* subsp. *novicida*, whereas dcm02 and dcm04 displayed lower MICs (16 and 4 μg/mL, respectively) against *F. philomiragia*.

MBC DETERMINATION AND TIME-KILL STUDIES REVEALED A BACTERICIDAL ACTIVITY AGAINST THE LVS STRAIN BUT ONLY BACTERIOSTATIC ACTIVITY AGAINST THE Ft6 AND Ft24 STRAINS

The bactericidal activities of the bis-indole compounds, gentamicin and doxycycline, were determined for the LVS, Ft6, and Ft24 strains (**Figures 2**, **3**). As expected, gentamicin displayed bactericidal activity against the three strains (> 3 $\log_{10}$ reduction of the primary bacterial inoculum, $p < 0.01$), with MBCs of 1 μg/mL for the LVS strain and 2 μg/mL for Ft6 and Ft24 strains. As for doxycycline, a significant reduction of the primary bacterial inoculum was observed (i.e., between 1 and 2 $\log_{10}$ at MIC × 64 for the LVS strain and MIC × 32 for the Ft6 and Ft24 strains; $p < 0.01$) but the 3-log reduction cutoff was not reached.

Table 3 | MICs (μg/mL) and MBCs (μg/mL) of the tree *F. tularensis* subsp. *holarctica* strains used for time-kill studies and MBC determination.

Bacterial strain	MIC (MBC)			
	dcm02	dcm04	Gentamicin	Doxycycline
F. tularensis subsp. *holarctica* Ft6	4	2	0.25 (2)	0.25
F. tularensis subsp. *holarctica* Ft24	4	2	0.25 (2)	0.25
F. tularensis subsp. *holarctica* LVS	2 (4)	1 (4)	0.25 (2)	0.125

MBCs of the dcm02 and dcm04 compounds (4 μg/mL for both compounds) were only two or four times their respective MICs for the LVS strain (**Table 3**), respectively. In contrast, MBCs could not be determined for the dcm02 and dcm04 compounds against the Ft6 and Ft24 strains. Here again reduction of the primary bacterial inoculum was significant (2-log reduction at concentrations up to MIC × 8 for dcm02 and MIC × 16 for dcm04, $p < 0.01$) but did not reached the 3-log cutoff. Higher concentrations of these compounds could not be tested because of their poor solubility.

Time-kill studies revealed a 3-$\log_{10}$ reduction of the primary inoculum of the LVS strain after 12 h incubation for gentamicin (MIC × 8) and 24 h for dcm04 (MIC × 4). As for dcm04, the same bactericidal kinetics were observed at 4, 8, and 16 times the MIC of this compound for the LVS strain. As for the Ft6 and Ft24 strains, we observed a progressive decrease of the bacterial load over the first 48 h of contact with dcm04 (**Figure 3**), but a 3-$\log_{10}$ reduction of the primary bacterial inoculum was never reached. Thus, the dcm02 and dcm04 compounds were only bacteriostatic against the clinical strains of *F. tularensis* subsp. *holarctica*.

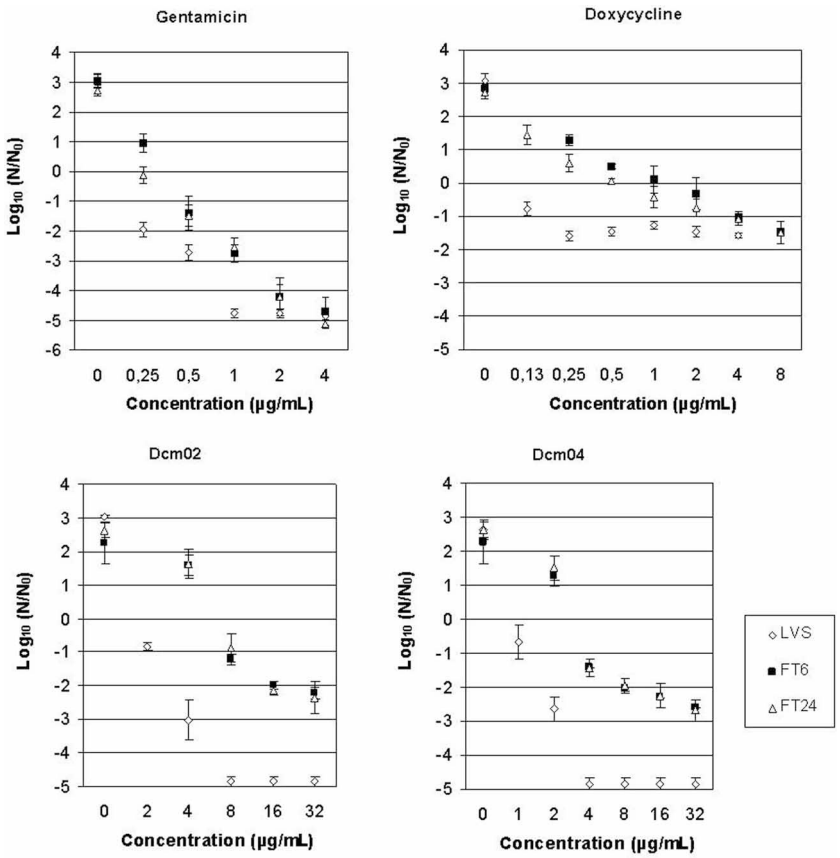

FIGURE 2 | MBC determination figures for dcm02, dcm04, gentamicin, and doxycycline against *F. tularensis* subsp. *holarctica* LVS, Ft6, and Ft24 strains, performed in triplicate experiments. Bacterial survival was monitored by measuring CFU/mL after 48 h of incubation. Error bars represent standard deviation.

DISCUSSION

Among the currently developed therapeutic alternatives for tularaemia, two promising original classes of compounds have recently been identified. First, substituted diphenyl ethers have demonstrated potent inhibition of ftuFabI Enoyl-acyl carrier protein reductase (England et al., 2009). This enzyme, absent in human cells, plays a key role in the type II fatty acid biosynthesis and has proved to be a useful target for growth inhibition of various pathogens such as *Mycobacterium tuberculosis*, *S. aureus* and *Plasmodium falciparum* (England et al., 2009; Lu et al., 2009; Hevener et al., 2012; Mehboob et al., 2012; Kingry et al., 2013). The leading compound SBPT04 has an MIC of 0.16 ± 0.06 µg/mL against *F. tularensis* LVS and Schu4 strains and also has a bactericidal activity with a MBC of 0.25 µg/mL. In a murine model of *F. tularensis* infection, this compound cleared bacteria by day 4 of treatment, without any relapse the following 30 days post-treatment (England et al., 2009). Secondly, screening of a library of more than 1000 2,5,6- and 2,5,7-trisubstituted benzimidazoles identified 21 leading derivatives exhibiting MICs between 0.35 and 48.6 µg/mL against the *F. tularensis* LVS strain. Their bacterial target remains uncharacterized, but these compounds may block polymerization of FtsZ, which is a homolog of tubulin/microtubule proteins found in eukaryotes, thus interfering with cell division processes (Kumar et al., 2013).

Here, we report that bis-indole derivatives in which the two indole groups are linked either with an amide (dcm01 and dcm02) or an α-keto-amide (dcm03 and dcm04) central linker exhibit antimicrobial activity against *F. tularensis* subsp. *holarctica*. These compounds were previously characterized as anti-staphylococcal drugs active against methicillin-resistant, vancomycin-intermediate, and fluoroquinolone-resistant *S. aureus* strains (Denis et al., 2013a,b). In this study, the 24 bis-indolic molecules evaluated were inactive against Gram-negative bacteria, including enterobacterial species (*Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, and *Enterobacter cloacae*), *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The MICs of two bis-indolic derivatives were lower (16 µg/mL) against *Haemophilus influenzae*. The cytotoxicity of the four bis-indole compounds we tested against *F. tularensis* strains was previously evaluated using three different cell lines: KB (human mouth carcinoma), MCR5 (human lung fibroblast) and HCT116 (human colon tumor) (Denis et al., 2013a,b). The IC_{50} determined using the HCT116 cell line were 1–5 times higher than the MICs found for *F. tularensis* strains.

We found a significant bacteriostatic activity of these tested bis-indole derivatives against 41 strains of *F. tularensis* subsp. *holarctica* isolated in France. Dcm04 appeared to be the most

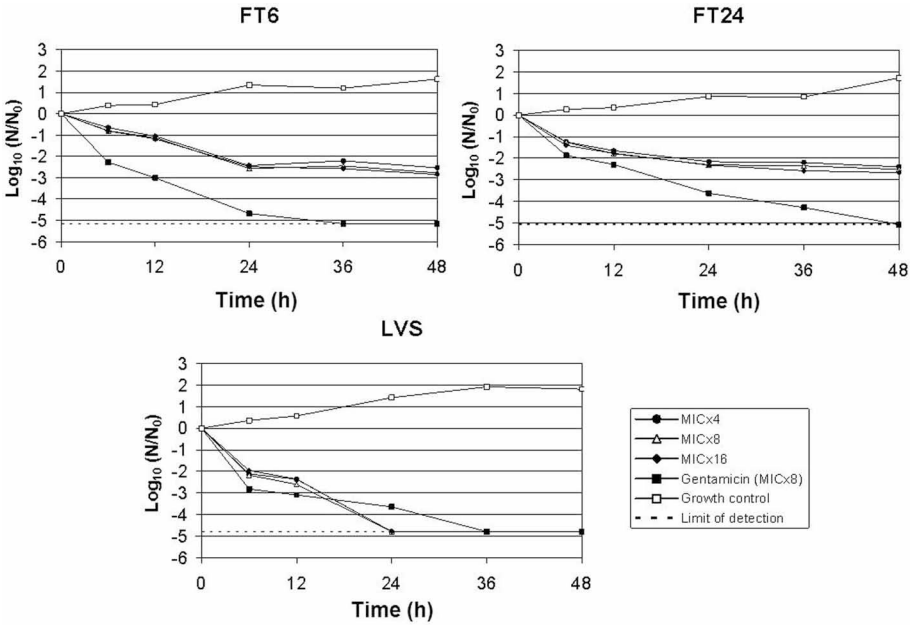

FIGURE 3 | Time-kill kinetics of dcm04 at 4, 8, and 16 times the MIC against *F. tularensis* subsp. *holarctica* LVS, Ft6, and Ft24 strains. Gentamicin at eight times the MIC was used as control of a bactericidal antibiotic. Two independent assays were performed but only one representative experiment is shown.

effective compound with a MIC_{90} of 2 μg/mL. The MICs ranged from 2 to 16 μg/mL when considering all four bis-indole compounds, except for a single strain that displayed higher MICs for the dcm01 and dcm03 compounds. The variations in antibiotic activities between the four compounds (especially between dcm01 and dcm03 vs. dcm02 and dcm04) may be related to differences in chemical structure, especially the presence of a large CH_2NHBoc chemical group in dcm01 and dcm03, whereas it is replaced by a methyl in dcm02 and dcm04. This large chemical group may limit access of dcm01 and dcm03 to their bacterial target or limit their penetration within bacteria. Surprisingly, the dcm02 and dcm04 compounds displayed a bactericidal activity against the virulence-attenuated LVS strain, but not the Ft6 and Ft24 clinical strains of *F. tularensis* subsp. *holarctica*. This was demonstrated both by MBC determinations and in killing curve experiments. The bactericidal activity of dcm04 against the LVS strain was not concentration-dependent but time-dependent. Hopefully, further structural optimization of these bis-indolic compounds and identification of their bacterial targets may enable us to obtain the same bactericidal activity for clinical strains of *F. tularensis* subsp. *holarctica*.

The activity of the bis-indole compounds also showed *Francisella* species and subspecies specificity, since these compounds were active against *F. tularensis* subsp. *holarctica*, only partially active (dcm02 and dcm04) against *F. philomiragia* and inactive against *F. tularensis* subsp. *novicida*. As a result, *F. tularensis* subsp. *novicida* cannot be used as an experimental model to identify the bacterial targets of these compounds, nor to evaluate the *in vivo* activity of the bis-indoles. Comparison of the complete genomes of *F. tularensis* subsp. *holarctica* LVS, OSU18, and FSC200, and that of *F. tularensis* subsp. *tularensis* Schu S4, previously identified a relatively limited number of specific genetic alterations (Petrosino et al., 2006; Rohmer et al., 2006) in the attenuated LVS strain. This might help in further investigations to search for an antibacterial target of these compounds in *F. tularensis*, or at least to explain the differences observed in susceptibility to the bis-indoles. Working hypotheses may include an increased affinity of the bis-indoles for their bacterial target in the LVS strain, a reduced penetration of the bis-indole in the clinical strains as compared to the LVS strain, partial inactivation of the bis-indoles in the clinical strains but not in the LVS strain, an escape pathway to the action of the bis-indoles in clinical strains but not in the LVS strain, and a lower efflux of these molecules in the LVS strain. Another hypothesis is that the bis-indole compounds are more active against the LVS strain because it belongs to the type B biovar II strains of *F. tularensis*, whereas the 41 clinical strains belong to biovar I of this sub-species. Biovar II strains naturally resistant to erythromycin are found in Central and Eastern Europe, and Asia (Kudelina and Olsufiev, 1980), whereas only the erythromycin-susceptible biovar I strains are found in France. We did not evaluate the activity of the bis-indoles against type A *F. tularensis* strains. Testing the activity of these compounds against a larger panel of *F. tularensis* strains will be needed to assess potential variability in susceptibility among different sub-species and biovars.

In conclusion, we have identified novel synthetic bis-indole compounds active against *F. tularensis* subsp. *holarctica* but not the closely related bacteria *F. tularensis* subsp. *novicida* and *F. philomiragia*. These compounds may be drug candidates for the development of new therapeutic alternatives for tularaemia treatment. Their bacterial targets remain to be characterized.

AUTHOR CONTRIBUTIONS

Research project design: Yvan Caspar, Max Maurin. Experiments: Yvan Caspar, Vivien Sutera, Sandrine Boisset. Writing: Yvan Caspar, Max Maurin.

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Uncovering the components of the *Francisella tularensis* virulence stealth strategy

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Over the last decade, studies on the virulence of the highly pathogenic intracellular bacterial pathogen *Francisella tularensis* have increased dramatically. The organism produces an inert LPS, a capsule, escapes the phagosome to grow in the cytosol (FPI genes mediate phagosomal escape) of a variety of host cell types that include epithelial, endothelial, dendritic, macrophage, and neutrophil. This review focuses on the work that has identified and characterized individual virulence factors of this organism and we hope to highlight how these factors collectively function to produce the pathogenic strategy of this pathogen. In addition, several recent studies have been published characterizing *F. tularensis* mutants that induce host immune responses not observed in wild type *F. tularensis* strains that can induce protection against challenge with virulent *F. tularensis*. As more detailed studies with attenuated strains are performed, it will be possible to see how host models develop acquired immunity to *Francisella*. Collectively, detailed insights into the mechanisms of virulence of this pathogen are emerging that will allow the design of anti-infective strategies.

Keywords: *Francisella tularensis*, FPI virulence genes, phagosome escape, unique LPS, stealth strategy, capsule mutant, virulent Schu S4

INTRODUCTION

Bacterial pathogens adopt a variety of different strategies to infect and grow within a chosen host. In general, opportunistic pathogens take advantage of a compromised host environment to employ their virulence factors to generate a productive infection. Overt pathogens, such as *Francisella tularensis*, have developed sophisticated strategies to alter and/or avoid recognition and antimicrobial killing mechanisms to reach or create a privileged growth site within a "normal" or healthy host, causing the disease tularemia. This organism causes disease by diverse routes, including oral, subcutaneous, and pneumonic; the respiratory route is of particular concern because infection with 50 or less organisms is associated with mortality rates of 30–60%, if untreated (Tarnvik and Berglund, 2003; McLendon et al., 2006). Regardless of the route of infection, the virulence strategy of *F. tularensis* involves entering host cells, escaping from the phagosome and growing within the cytosol of the infected host. Surprisingly, the host immune responses to these activities are delayed and significantly less than expected (Jones et al., 2012). Cells infected with *F. tularensis* quickly disseminate throughout the host to the liver and spleen. Uncontrolled growth in systemic organs causes host cell damage, hypersecretion of cytokines and death by "cytokine storm" (Sharma et al., 2011). Due to the extreme virulence and ease of aerosol dissemination of *F. tularensis*, the US Centers for Disease Control and Prevention have classified this organism as a Tier 1 select agent because of its potential development into a bioweapon (Dennis et al., 2001).

Here we review the literature describing the work that is characterizing the intracellular strategy of this organism as well as the interactions that occur between these organisms and a wide variety of different host cell types (Conlan and North, 1992; Forestal et al., 2003; Lindemann et al., 2007; Schulert et al., 2009; Horzempa et al., 2010). We will highlight studies that have used virulent *F. tularensis* strains since it is becoming clear that work with model organisms (i.e., *F. tularensis* subsp. *holarctica* LVS and *F. novicida*) have sometimes provided results that are different than those obtained with the virulent strains, perhaps not surprisingly since LVS is an attenuated vaccine strain. In addition, we discuss recent work in which mutagenesis studies have identified mutants with significantly altered virulence phenotypes that are yielding important insights into the immune evasion mechanisms of virulent *F. tularensis*. We will discuss the virulence roles of the unique *F. tularensis* LPS and capsule and we also report the presence of glycoproteins that carry O-antigen. In addition, we will discuss various aspects of *Francisella* immunology and how attenuated *F. tularensis* strains are providing opportunities to study adaptive immune in a murine model of infection. Finally, we will attempt to provide insights into how this accumulating body of work may direct efforts to develop strains that can provide protective immunity against *F. tularensis* infection.

GENETIC APPROACHES TO DISCOVER *Fcrancisella tularensis* VIRULENCE MECHANISMS

Efforts to understand the molecular details of the *F. tularensis* virulence strategy have progressed rapidly in the last decade. These research efforts have relied on genetic approaches to identify *Francisella* genes that are required for various aspects of pathogenesis. Qin and Mann (reference) performed transposon

mutagenesis in the Schu S4 strain and identified numerous genes required for growth in HepG2 cells, including metabolic genes (i.e., *car* and *pur* genes, *ggt*, etc.) and hypothetical genes that have been found in screens by numerous groups, including our own. Weiss et al. identified *F. novicida* genes required for systemic mouse disease by creating a library of random transposon insertions in the chromosome of *F. novicida* (Weiss et al., 2007). This work identified the genes on the *Francisella* Pathogenicity Island (FPI, required for phagosomal escape), LPS O-antigen biosynthetic genes and 44 previously unidentified genes as important for *F. novicida* virulence. Many of the genes found in these screens are important for modifying the intracellular growth niche of the organism, acquiring nutrients or in modifying the host immune response to the presence of the bacteria.

Our research group has performed a similar screen to identify genes in virulent *F. tularensis* Schu S4 required for growth in human macrophages (Lindemann et al., 2011). Using a transposon system similar to that of Weiss et al., pools of *F. tularensis* Schu S4 mutants were screened for strains that were significantly reduced in their ability to grow within human monocyte-derived macrophages (MDMs). That work identified *F. tularensis* Schu S4 mutants that were defective in production of capsule and O-antigen, defective in FPI gene expression, nutritional mutants, as well as many other mutants in genes with unknown functions. These mutants are yielding important insights into *Francisella* mechanisms of virulence and are providing avenues to study host immune responses to *Francisella* strains that have lost some of their virulence properties.

FACTORS IMPORTANT IN THE UPTAKE OF *Francisella tularensis* INTO PHAGOCYTIC CELLS

The virulence of *Francisella* is dependent upon the ability of the organisms to enter, persist and replicate within host cells as strains that have lost these abilities are avirulent (Brotcke and Monack, 2008; Charity et al., 2009). While it is not known which host cells provide growth niches that are essential to *Francisella* virulence, it is known that *Francisella* infects multiple cell types including phagocytes such as MDMs and polymorphonuclear leukocytes (PMN) as well as non-phagocytic epithelial and endothelial cells (Golovliov et al., 2003; Lauriano et al., 2004; Nano et al., 2004; McCaffrey and Allen, 2006; Qin and Mann, 2006; Schulert and Allen, 2006; Lindemann et al., 2007; Moreland et al., 2009). Upon contact with a phagocytic cell, *F. tularensis* can interact with different receptors, depending upon the opsonization state of the organism. In studies performed with non-opsonized *Francisella*, the mannose receptor plays an important role as non-opsonized Schu S4 had reduced uptake into macrophages acquired from mannose receptor knockout mice compared to macrophages from wild type mice (Geier and Celli, 2011). Consistent with this observation, blocking the mannose receptor with antibodies or soluble mannan significantly inhibits *Francisella* uptake (Balagopal et al., 2006; Schulert and Allen, 2006; Geier and Celli, 2011). However, deletion or blockade of the mannose receptor does not completely inhibit *Francisella* uptake, indicating that other receptors are involved in the uptake of the bacteria (Geier and Celli, 2011). While the reasons for the ability of this organism to use some many different receptors for internalization into phagocytic cells is not well understood, it does give the organism the capability to be internalized into a wide variety of different cell types. Entry into cells via different receptors doubtlessly activates different signaling cascades that elicit different cytokine responses from the host cells. Research efforts, by different groups, are aimed at understanding how the use of different receptors for internalization of *F. tularensis* impacts the infection dynamics between the pathogen and the host.

Studies of opsonized *Francisella* have demonstrated that the type of opsonization (serum vs. antibody) can alter the receptor employed for internalization of the organisms. In addition, independent of the method of opsonization, internalization of opsonized *Francisella* is significantly increased compared to non-opsonized organisms (Ben Nasr et al., 2006; Pierini, 2006; Geier and Celli, 2011). The targets of complement opsonization appear to be LPS and capsule, which protect the organism from the killing activity of complement while simultaneously stimulating the uptake of the bacteria into host cells (Clay et al., 2008). Several studies have implicated complement receptors in the uptake of *Francisella* strains (Schulert and Allen, 2006; Clay et al., 2008; Geier and Celli, 2011; Schwartz et al., 2012). Knockout of the CR3 receptor significantly reduces internalization of serum-opsonized Schu S4 in macrophages (Clay et al., 2008; Geier and Celli, 2011) whereas, serum-opsonized LVS utilizes CR4 to enter MDMs and dendritic cells (Ben Nasr et al., 2006; Schwartz et al., 2012). Both *F. tularensis* Schu S4 and LVS (serum opsonized) can use CR1 and CR3 for internalization into neutrophils (Schwartz et al., 2012). Additionally, both strains can utilize scavenger receptor A (SRA) for uptake into MDMs (Pierini, 2006). In contrast, IgG antibody-opsonized organisms have been shown to interact almost exclusively with Fc receptor (FcγR), since MDMs isolated from FcγR knockout mice are significantly reduced in their ability to internalize antibody opsonized Schu S4 (Geier and Celli, 2011). Antibody opsonization of Schu S4 has been associated with production of superoxide and decreased intracellular growth compared to non-opsonized bacteria (Geier and Celli, 2011). These data indicate that *Francisella* has evolved multiple mechanisms of internalization, which appear to be equally important, to take advantage of different host conditions and it is likely that the mechanism of uptake is important role in the subsequent interactions of the organism with its host.

Little is understood of the *Francisella* factors that mediate uptake into host cells. Recent data from our lab has demonstrated that the *Francisella* capsule and/or LPS are important in reducing uptake by phagocytes (Lindemann et al., 2011). Mutants in the *waaY* and *waaL* genes, which produce no capsule and lack the O-antigen side chain, displays ∼10 fold increase in uptake into MDMs compared to wild type Schu S4 (Lindemann et al., 2011; Rasmussen et al., 2014). One of these mutants also displays slightly increased uptake into epithelial cells (unpublished observation). The mechanism for this increased uptake is unknown but it is tempting to speculate that mutations that result in disruptions of capsule and LPS biosynthesis either change the charge of the bacterial surface or uncover other surface molecules that mediate uptake. While not much is known about the bacterial factors that facilitate entry of *Francisella* into various host

cells, strains that display increased uptake may be valuable in uncovering the mechanism of entry.

THE INTRACELLULAR INTERACTIONS OF *Francisella tularensis*

After entry, *F. tularensis* is located within a phagosome that begins to mature into a phagolysosome. Although maturation of the phagosome progresses significantly, *Francisella* has the ability to prevent fusion of the phagosome with the lysosome (Anthony et al., 1991; Checroun et al., 2006). During this process, the *Francisella*-containing phagosome (FCP) becomes decorated with both early and late endosomal markers: EEA-1, CD63, LAMP-1, LAMP-2, and Rab5 (Golovliov et al., 2003; Clemens et al., 2004; Checroun et al., 2006; Santic et al., 2006). Instead of fusing with the lysosome, the bacteria degrade the phagosomal membrane and egress into the host cytosol between 1 and 4 h post entry into the cell (Clemens et al., 2004, 2009; Chong et al., 2008). Interestingly, inhibition of phagosomal acidification delays *F. tularensis* escape and replication (Chong et al., 2008). The molecular mechanism that allows the bacteria to escape the phagosome is still uncharacterized but several studies have established that disruption of FPI genes renders the bacteria unable to escape the phagosome (Nano et al., 2004; Barker and Klose, 2007; de Bruin et al., 2007; Nano and Schmerk, 2007; Ludu et al., 2008; Buchan et al., 2009). In *F. tularensis* type A and type B strains both copies of the FPI must be mutated to inhibit phagosomal escape.

As *Francisella* reaches the cytosol the bacteria begin to replicate quickly, with data showing that virulent Schu S4 has an intracellular doubling time of ∼1 h (Chong et al., 2008). This intracellular growth appears to last up to 48 h *in vitro*. Depending upon the host cell type, the organisms can replicate 50–1500 fold (Lai et al., 2001; Qin and Mann, 2006; Bonquist et al., 2008; Schulert et al., 2009; Edwards et al., 2010). Eventually host cell resources are consumed and the cell dies, releasing bacteria into the extracellular environment (Lai and Sjostedt, 2003; Lai et al., 2004; Celli and Zahrt, 2013).

Several bacterial factors that are essential for cytosolic growth have been identified through genetic approaches. Many of these genes are involved in metabolic pathways such as purine biosynthesis (*purMCD*) or uracil biosynthesis (*pyrF*) (Pechous et al., 2006, 2008; Horzempa et al., 2010). Recent work using *F. novicida* demonstrated that the *FTN1586* open reading frame, an ortholog of *ansP*, encodes an asparagine transporter that is important for cytosolic growth (Gesbert et al., 2013). Similar roles in cytosolic growth have been identified for other transport proteins in the LVS strain, including, *FTL1645*, *FTL1586*, and *FTL0129* (Marohn et al., 2012). Other genes have been identified that are involved in cytosolic replication including *dipA*, *FTT0989*, *ripA*, and the γ-glutamyl transpeptidase, *ggt* (Brotcke et al., 2006; Fuller et al., 2008; Alkhuder et al., 2009; Wehrly et al., 2009; Chong et al., 2012).

Three genes, *migR*, *trmE*, and *cphA* have been identified from genetic screens and shown to be involved in modulating FPI expression and growth inside of MDMs (Buchan et al., 2009; Charity et al., 2009; Lindemann et al., 2011). It has recently been shown by our research group that each of these genes affects FPI expression by indirectly altering intracellular concentrations of the alarmone ppGpp (Faron et al., 2013). The ppGpp alarmone has been shown to strengthen the interaction of FevR and RNA polymerase to induce expression of FevR-dependent genes (Charity et al., 2009). Despite each of these mutants having reduced ppGpp concentrations, each mutant has a unique growth pattern in different *in vitro* infection models. The LVS Δ*trmE* strain did not grow inside of MDM cells, but replicated in both A549 and HEp-2 cells while the LVS Δ*migR* mutant only replicated in HEp-2 cells. The LVS Δ*cphA* strain was unable to grow in any of the three cell types (Faron et al., 2013). These data indicate that different host cells present different intracellular growth challenges to *Francisella*; the bacteria appear to respond to these challenges via different homeostatic feedback pathways that contribute to the intracellular ppGpp pools.

Evidence is accumulating that significant differences exist between virulent *F. tularensis* strains and LVS. For instance, mutation of *migR* decrease *F. tularensis* LVS virulence for mice but deletion of *migR* in Schu S4 has a very minor effect on mouse virulence (Buchan, 2009). It is possible that this virulence difference is due to steady-state FPI gene expression levels between Schu S4 and LVS, as *iglA-lacZ* reporter activity in Schu S4 is about 3-fold higher than that observed in the LVS strain (Faron et al., 2013).

We have recently identified another difference between *F. tularensis* Schu S4 and *F. tularensis* LVS. Using recently immortalized human AT-II cells (ABM), we have compared the ability of virulent Schu S4 and LVS to enter and replicate within these cells. Interestingly, Schu S4 grows ∼100-fold better in these airway epithelial cells than does LVS, suggesting that LVS attenuation may be partly due to an inability to effectively grow within epithelial lung cells (manuscript in preparation). To our knowledge, this is the first cell type in which such a stark difference in intracellular growth is observed between Schu S4 and LVS. These results indicate that it may be important to identify host cells that are key growth sites for the bacteria prior to efficient dissemination to distal organs. This knowledge may be vital in efforts to produce live attenuated vaccine strains.

THE ATYPICAL LIPOPOLYSACCHARIDE OF *Francisella tularensis*

An important contributing factor to the high virulence of this pathogen is that its early intracellular growth appears to be unchecked by significant host responses (Bosio, 2011). A factor that is important in *F. tularensis* immune evasion is the lipid A component of LPS. *Francisella* lipid A species are unusual in that they are asymmetrical, tetraacylated and have longer than normal fatty acids chains (16–18 carbons). This is in contrast to the lipid A from most Gram-negative bacteria that contain six acyl chains of 12–14 carbons in length and phosphate groups available for interactions with TLR-4 that stimulate strong proinflammatory responses (Raetz, 1990; Poltorak et al., 1998; Beutler and Poltorak, 2000). For *Francisella*, the non-prime phosphate on the di-glucosamine backbone can be shielded by a galactosamine while the phosphate on the prime side of the sugar backbone is often missing (Vinogradov et al., 2002; Phillips et al., 2004; Gunn and Ernst, 2007). Multiple species of lipid A with some or all of these differences may be present in the outer membrane of a single organism (reference). The differences in the *Francisella* lipid A

make the endotoxin of *F. tularensis* unable to bind to LBP and therefore unable to activate TLR-4 signaling pathways, rendering it inert compared to typical endotoxins (Hajjar et al., 2006; Gunn and Ernst, 2007). Several research groups are working to understand how acylation, length of fatty acid side chains and shielding the phosphates of the endotoxin contribute to the lack of bioactivity of the *F. tularensis* LPS (Kanistanon et al., 2012). These studies are providing important insights into how bacteria can modify their LPS to lessen the ability of the host to recognize their presence and activate key host cytokine pathways.

CHARACTERISTICS OF THE *Francisella tularensis* CAPSULE AND ITS ROLE IN BACTERIAL VIRULENCE

The role of the *Francisella* capsule in immune evasion and pathogenesis is an active area of investigation. Early work aimed at characterization of a crude preparation of the *Francisella* capsule revealed that it was composed of mannose, rhamnose, and dideoxy sugars (Hood, 1977). Sandstrom et al. created an acapsular *Francisella* strain and found that it was more sensitive to antibody-mediated killing (Sandstrom et al., 1988), although unfortunately this strain was not preserved for additional studies. Recent work using a monoclonal antibody directed against purified capsular material of *Francisella* has helped to characterize some aspects of this structure (Apicella et al., 2010). The capsule ranges in size from 100 to 250 kDa and it is present in all type A and B strains of *F. tularensis* that have been examined (Apicella et al., 2010). Immunization of mice with purified capsular material elicited circulating anti-capsule antibodies that protected from challenge with *F. tularensis* LVS but did not protect from *F. tularensis* Schu S4 challenge (Apicella et al., 2010).

Most bacterial capsules are large structures formed of repeating sugar subunits that are held together by glycosidic bonds and these structures associate with the bacterial outer membrane either directly or indirectly. There are four general classes of capsules for Gram-negative bacteria based upon biochemical, genetic, serological, and physical properties (Orskov et al., 1977; Daly et al., 1997). Of particular interest to this discussion of *Francisella* virulence factors are Group 4 capsules. Group 4 capsules are composed of similar (often identical) O-antigen sugars as those found in the LPS, contain acetimido sugars in their repeat unit structures, and are greater than 100 kDa in size (Whitfield, 2006). The capsule of *Francisella* has been shown to have a molecular weight of 100–250 kDa and contain the core sugar tetrasaccharide repeat of <2-acetamido-2,6-dideoxy-o-glucose (o-QuiNAc), 4,6-dideoxy-4-formamido-D-glucose (o-Qui4NFm), and 2-acetamido-2-deoxy-o-galacturnoamide (o-GalNAcAN), with the o-GalNAcAN unit present at twice the concentration of the other two sugars (Apicella et al., 2010; Wang et al., 2011). This is the exact composition of the sugars present in the *Francisella* repeating O-antigen subunits of the LPS (Vinogradov et al., 2002; Thomas et al., 2007). In a report characterizing the purified *Francisella* capsule, it was found that neither lipid A nor 2-keto-3-deoxyoctulsonic acid (KDO) were attached to the purified capsular structure, providing additional support that the LPS and capsule are distinct from each other (Apicella et al., 2010). These observations indicate that the *Francisella* capsule is similar to capsules produced by some strains of the Gram-negative bacteria *Vibrio cholera*, *Escherichia coli*, and *Salmonella enterica* which produce capsules that incorporate the LPS O-antigen (Jayaratne et al., 1993; Zhang et al., 2002; Peleg et al., 2005; Barak et al., 2007; Chen et al., 2007). Work to identify capsule biosynthetic genes has produced mutant strains that are disrupted for both capsule and LPS biosynthesis, indicating that the LPS and capsule biosynthetic pathways share many components for their assembly (unpublished observation). Efforts are still underway to identify the genes responsible for the unique biosynthetic steps in each pathway, such as the anchoring mechanism of capsule to the outer membrane, which is apparently distinct from the lipid A anchor of LPS (Bandara et al., 2011; Lindemann et al., 2011; Rasmussen et al., 2014).

One research group (Zarrella et al., 2011) has published data demonstrating that an alternate non-O-antigen capsule is present when *Francisella* is grown in brain heart infusion media (BHI). This high molecular weight alternate capsule species can be detected by PAGE in a *F. tularensis* LVS *wbtA* mutant that is unable to produce O-antigen. This indicates that this alternate capsule is distinct from the O-antigen capsule our research group has described. However, the composition of this structure was not reported. Another group also described the existence of a high molecular weight capsule-like material present in an *F. tularensis* LVS *wbtI* mutant strain, which is unable to produce O-antigen (Bandara et al., 2011). The authors reported that this capsule-like material was composed of glucose, galactose and mannose sugars. While it is not known if the structures reported by Zarella et al. and Bandara et al. are the same, both appear to be high molecular weight capsule-like molecules and both are present in strains with O-antigen defects.

F. tularensis PRODUCES PROTEINS THAT ARE GLYCOSYLATED WITH THE O-ANTIGEN

Efforts to understand the roles of bacterial glycoproteins have gained momentum from recent data indicating that some bacterial glycoproteins may play roles in host-pathogen interactions (Straskova et al., 2009; Egge-Jacobsen et al., 2011; Balonova et al., 2012). *Francisella* species have recently been shown to produce significant amounts of glycoproteins, many of which appear to be outer membrane proteins (Balonova et al., 2010). We have recently discovered that both virulent type A and B strains of *F. tularensis* produce O-antigen glycoproteins, detectable in whole cell lysates that bind to a monoclonal antibody specific for the O-antigen subunit (FB11, QED Biosciences). Interestingly, the *F. tularensis* LVS strain does not produce detectable quantities of these glycosylated proteins (**Figure 1A**). Furthermore, when the glycoprotein profiles of Schu S4 LPS O-antigen mutants [*FTT1236* (*waaY*) and *FTT1238c* (*waaB*)] were compared to the orthologous LVS mutants (*FTL0708* and *FTL0706*, respectively), we found that the LVS mutants lacked detectable O-antigen reactive protein bands that are present in the Schu S4 mutants (**Figure 1B**). Early work with these mutants obscured O-antigen protein reactivity due to proteinase K digestion during sample preparation (Lindemann et al., 2011). More recent work with non-proteinase K treated whole cell lysates has revealed that these strains do produce O-antigen reactive bands which can be detected by Western blots (**Figure 1C**).

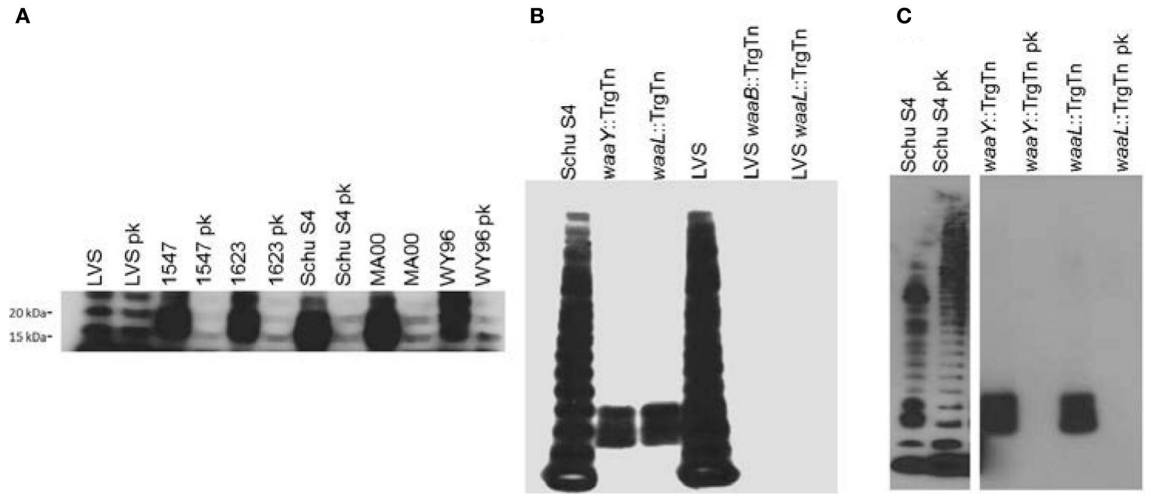

FIGURE 1 | *F. tularensis* strains produce O-antigen glycosylated proteins. One ml of *F. tularensis* broth cultures was centrifuged at 8000 × g for 2 min before resuspending the pellet in Buffer Part A, (6 mM Tris, 10 mM EDTA, and 2% [wt/vol] sodium dodecyl sulfate [pH 6.8]) and heated to 65°C to sterilize cultures. Bacterial lysates were incubated with, or without, proteinase K (New England Biolabs, Ipswitch, MA) at 37°C for 24 h before lyophilizing. Approximately 14 mg of bacterial material from each sample was mixed with NuPage (Life Technologies, Carlsbad, CA) sample reducing agent and buffer, boiled, and loaded into a 4–12% Bis-Tris NuPage gel and electrophoresed using NuPage MES SDS running buffer (Life Technologies, Carlsbad, CA). For immunoblots, samples were transferred to nitrocellulose and probed with FB11 primary antibody to detect O-antigen attached to LPS or to protein (QED Bioscience, San Diego, CA). Bands were visualized using goat anti-mouse IgG (H+L) conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) and SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL). **(A)** Western blot analysis of LPS preparations from *F. tularensis* LVS, virulent type B *F. tularensis* subsp.*holarctica* strains (1547 and 1623) and virulent type A *F. tularensis* subsp. tularensis strains (Schu S4, MA00, and WY96) using the O-antigen FB11 antibody for detection of bands containing O antigen, without or with proteinase K (pk) treatment. **(B)** Western blot of whole cell lysates of *F. tularensis* Schu S4, *F. tularensis* Schu S4 *waaY*::TrgTn and *F. tularensis Schu S4 waaL*::TrgTn, *F. holarctica* LVS, *F. holarctica* LVS *waaY*::TrgTn and *F. holarctica* LVS *waaL*::TrgTn probed with the anti-O antigen monoclonal antibody FB11 to detect the presence of LPS O antigen laddering in these strains. **(C)** Western blot analysis of *F. tularensis* Schu S4, *F. tularensis* Schu S4 *waaY*::TrgTn and *F. tularensis* Schu S4 *waaL*::TrgTn whole cell lysates without or with (pk) proteinase K treatmenst, using O-antigen FB11 antibody.

Another group has reported findings that are consistent with these observations. Balonova et al. (2012) reported the presence of a *Francisella* lipoprotein that is glycosylated with O-antigen sugars and that the glycosylated lipoprotein was absent in an *F. tularensis* subsp. *holarctica* FSC200 *wbtDEF* mutant (Balonova et al., 2012). We believe that protein O-antigen glycosylation, unique to virulent *F. tularensis* strains, may play a role in either the virulence of the organism or aid in evading the host immune response. Future work in the lab is focused on understanding the significance of O-antigen glycosylated proteins in *F. tularensis* pathogenesis.

DIFFERENCES IN ADAPTIVE IMMUNE RESPONSES TO VIRULENT *F. tularensis* AND *F. tularensis* LVS

One of the key features of the murine pneumonic tularemia model is the rapid time to death (∼5 days post infection), which precludes the development of a robust adaptive immune response. This aspect of *Francisella* virulence has made vaccine development a daunting challenge, and many labs have used *F. tularensis* LVS as a less virulent substitute to study aspects of B and T cell biology in sublethal infection and immunization. Evidence is accumulating that the host response to *Francisella* infection is strain-specific and responses that occur to *F. tularensis* LVS or *F. novicida* infection may be different than those observed for virulent *F. tularensis* (i.e., Schu S4) (Kurtz et al., 2013; Laws et al., 2013). This is logical; the LVS strain was purposefully selected as a vaccine strain based upon its attenuated virulence phenotype (Sandstrom, 1994; Elkins et al., 2003). A vaccine against fully virulent *Francisella* strains will almost certainly require stimulation of cellular immunity, though the mechanisms by which these processes can be directed against *Francisella* remain elusive (Ray et al., 2009; Conlan, 2011).

Protective antibodies were an early target of research into immunity to *Francisella*. In studies utilizing a Type A strain, Foshay (1946) showed that transfer of hyperimmune serum from horses or goats provided protection to ∼70–90% of rats infected subcutaneously, although the written details of these experiments are vague. More recent studies utilizing the mouse model have found contradictory results (Foshay, 1946; Kirimanjeswara et al., 2008). Kirimanjeswara et al. found that immune serum from LVS-immunized mice did not provide protection against Schu S4. *In vitro*, however, they observed that murine alveolar macrophages stimulated with interferon-γ were able to kill immune serum opsonized Schu S4 (Kirimanjeswara et al., 2008). While the latter result is intriguing, Crane et al. have shown that Schu S4 may be able to avoid this fate *in vivo* by interacting with the host serine protease plasmin. Schu S4 can bind to active plasmin, which can degrade *Francisella*-specific antibodies (Crane et al., 2009). The authors found that while antibody opsonization of both Schu S4 and LVS resulted in an increase in TNF-α, the presence of active plasmin on Schu S4, but not LVS, inhibited the induction of TNF-α in infected cells (Crane et al., 2009). These results

highlight a difference between the two strains that may have important implications for how virulent *Francisella* species avoid early detection and control. To that end, plasminogen knockout mice might be an interesting model to examine the role of antibody opsonization and plasmin during Schu S4 infection *in vivo* (Berri et al., 2013).

As with antibodies, the role of B cell activity in providing immunity to *Francisella* species is complex; however, it is clear that mice lacking B cells are less able to control or survive a *Francisella* infection (Crane et al., 2012). Data is emerging that a specific subset of B cells (B1a) can have almost opposite effects in the host response, depending on the infecting strain and the model of infection (Cole et al., 2009; Crane et al., 2013). Cole et al. demonstrated that immunization with lipopolysaccharide from LVS generated a B1a cell-dependent protective response against an intraperitoneal challenge with $\sim 10^3$ CFU of LVS (Cole et al., 2009). In contrast, Crane et al. utilized a short-term low dose antibiotic treatment after intranasal infection with Schu S4 (the convalescent model) and observed that mice largely deficient in B1a cells (XID mice) survived better than did wild type mice (Crane et al., 2013). The increased survival in these mice was found to be associated with a reduction in the anti-inflammatory cytokine IL-10, which is a potent inhibitor of IL-12. The latter cytokine stimulates interferon-γ production, and is necessary for the survival of tularemia (Crane et al., 2012). Curiously, Metzger et al. reported that IL-$10^{-/-}$ mice succumb to standard intranasal Schu S4 infection similar to wild type mice, though it should be noted that the time scales of infection are different between these studies and direct comparisons may not be appropriate (Metzger et al., 2013). The immune responses observed in the convalescent model would not have time to develop in a standard intranasal infection, although it would be of interest to see if IL-$10^{-/-}$ mice phenocopy XID mice in the convalescent model of Schu S4 infection.

One possible target of the anti-inflammatory activity of IL-10 may be at the interface of antigen presenting cells and T lymphocytes. Hunt et al. identified a factor released by *Francisella* infected cells that stimulated IL-10-dependent degradation of MHC class II molecules in macrophages (Hunt et al., 2012). Importantly, supernatants from Schu S4 infected macrophages have also been shown to stimulate the downregulation of MHC class II molecules (Wilson et al., 2009). These data suggest that antigen presentation to $CD4^+$ T cells may be reduced *in vivo*. This research group, as well as others, has also shown that *F. novicida* and LVS induce prostaglandin E2 (PGE2) production in infected macrophages (Woolard et al., 2007; Wilson et al., 2009). PGE2 has been shown to inhibit macrophage maturation, and might play a similar role in downregulating pathways important for intracellular killing of *Francisella*, as in *Burkholderia pseudomallei* infections (Zaslona et al., 2012; Asakrah et al., 2013). Interestingly, these groups showed that PGE2 induced by *Francisella* infection inhibited T cell proliferation and interferon-γ production *in vitro*. It has long been known that T cells are important for bacterial control and immunity to *Francisella*; for an excellent summary of this literature, see Cowley and Elkins' 2011 review (Cowley and Elkins, 2011; Crane et al., 2012; Eneslatt et al., 2012). Both $CD4^+$ T cells and $CD8^+$ T cells were required for survival of a primary Schu S4 infection in the convalescent model of tularemia, but only partial protection against a secondary challenge was observed for wild type mice (66% survival) (Crane et al., 2012). It is intriguing to speculate that the partial protection may be a function of a less than optimal T cell response mediated by PGE2, both in terms of antigen presentation and T cell proliferation. The use of knockout mice lacking various aspects of PGE2 production or signaling may be useful in testing this hypothesis. Our lab has preliminarily identified Schu S4 mutants that provide some protection against wild type Schu S4; it would be interesting to compare PGE2 levels during infection with our strains to those of other groups that have also shown protection (Conlan et al., 2010; Rockx-Brouwer et al., 2012). Additionally, we are developing vectors to express recombinant *Francisella* proteins fused to known $CD4^+$ and $CD8^+$ epitopes from lymphocytic choriomeningitis virus (LCMV) in an effort to quantify the magnitude of T cell proliferation and maturation state during both the immunization and challenge phases of protection studies.

SUMMARY

Significant advances have been made in understanding the virulence of *F. tularensis* over the last decade. The development of genetic tools has been critical in creating *F. tularensis* mutants that are defective in various aspects of virulence. The study of mutants has provided insights into the pathogenic mechanisms of *Francisella* and is pushing forward efforts to understand the mechanisms that this pathogen uses to evade host detection. The virulence strategy of *F. tularensis* has a two-pronged approach (**Figure 2**). A primary thrust of the *Francisella* virulence strategy is to reach the host cell cytosol as a privileged site for growth. The organism possesses a phagosomal escape mechanism, encoded on the FPI, which allows the organisms to disrupt the phagosomal membrane and enter the cytosol for growth. The FPI genes, which encode the capacity to lyse the phagosomal membrane, are central to the ability of the pathogens to reach the cytosol. Several labs are continuing work aimed at elucidating the molecular details of both the host and bacterial components of this process.

A second component of the *Francisella* virulence strategy employs the capsule and its unique LPS to avoid detection by host innate immunity mechanisms. The capsule and LPS provide protection from the host complement system by protecting the organism from complement opsonization and killing. Perhaps even more importantly, the unique *F. tularensis* LPS provides stealth to the overall virulence strategy as the lipid A does not activate the TLR4 cytokine response pathway.

We have also highlighted in this review some of the differences between laboratory strains of *F. tularensis* and virulent strains of *F. tularensis*. *F. tularensis* Schu S4 and LVS display significant differences in FPI gene expression levels with Schu S4 FPI expression being about 3-fold higher than LVS. Antibodies generated against *F. tularensis* LPS and capsule provide significant protection against murine challenge with *F. tularensis* LVS while little protection against Schu S4 challenge is observed. We also observe that virulent Schu S4 produces significant amounts of proteins glycosylated with O-antigen while LVS has almost undetectable levels of those glycosylated proteins, although the significance of these observations is still unknown. Finally, we have evidence

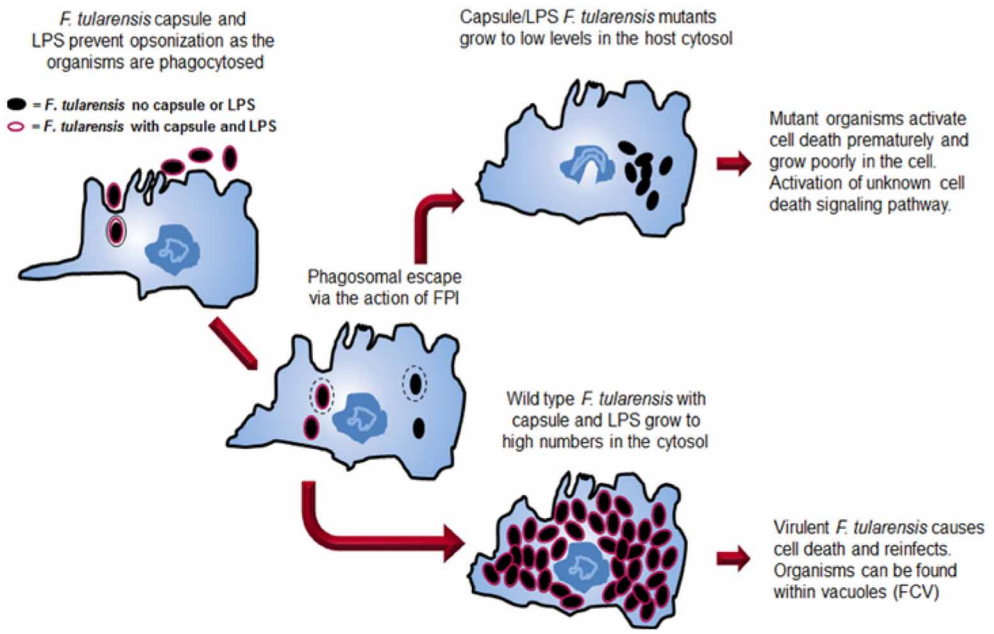

FIGURE 2 | Comparison of the intracellular growth strategies of wild type *F. tularensis* and a *F. tularensis* capsule/LPS mutant. Both wild type and mutant organisms are internalized into a host cell, although the absence of capsule and LPS allows increased internalization. Both strains can escape from the phagosome. The wild type strain grows to high numbers in the host cell cytosol before inducing death of the cell. At late stages, organisms can be found in *Francisella*-containing vacuoles (FCVs). In contrast, after mutant organisms escape the phagosome, they grow for a short time in the cytosol before the cell dies preventing additional growth of the organisms.

in our lab that LVS does not grow well in a human airway epithelial cell while Schu S4 grows to high levels (manuscript in preparation).

Importantly, there are many research groups actively trying to understand how virulent *F. tularensis* suppresses a variety of host immune responses including neutrophil and macrophage activation mechanisms. The molecular and cellular details of these virulence mechanisms continue to be pursued in detail with the goal of understanding the mechanisms which lead to the extreme virulence of this bacterial pathogen as well as to develop methods to create effective immune responses against *Francisella* infection.

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Targeting nutrient retrieval by *Francisella tularensis*

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Keywords: tularemia, legionella, autophagy, proteasome, legionnaires disease

Our continued progress in understanding microbial pathogenesis has been fueled, to a large extent, by the discovery of various translocation systems that inject a large cadre of eukaryotic-like and novel microbial effectors into the host cell. These effectors manipulate a myriad of host cell processes and subvert innate and adaptive immunity through novel and exciting mechanisms. The injected bacterial effectors and their host cell targets are potential candidates for anti-microbial therapy.

Nutrition and proliferation is fundamental to life, and all organisms have evolved to maximize their harvest of energy and biomass building blocks from available nutrients. This applies also to pathogens that colonize host tissues and cause disease. It is a simple logic that without proper nutritional resources for survival/proliferation in the host, bacterial pathogens do not cause disease. It can be challenging for microbial pathogens to obtain nutrients during infection, since part of the host innate defense is to restrict pathogen access to various essential nutrients (Abu Kwaik and Bumann, 2013). Successful pathogens have therefore evolved highly efficient nutrient retrieval strategies to counteract host nutritional limitation or deprivation of major sources of carbon and energy (Abu Kwaik and Bumann, 2013). Microbial acquisition of nutrients and metabolism *in vivo* is a major fundamental aspect of infectious diseases that impacts virulence, pathology, and efficacy of antibiotic treatment. Despite these simple facts, our knowledge of microbial nutrition *in vivo* still remains very limited, which is likely due to the experimental challenges in deciphering microbial nutrition and metabolism within complex microenvironments in the host. Numerous global genome-wide transcriptome and mutant screens have identified a large cadre of metabolic genes and pathways that are expressed *in vivo*, but their functional relevance remain largely unclear.

It has been widely presumed that the host cell cytosol is a rich haven of nutrients for microorganisms to proliferate in Abu Kwaik and Bumann (2013). However, recent evidence from studies on *Francisella tularensis* indicates that this presumption is short sighted (Alkhuder et al., 2009; Steele et al., 2013). After entry into mammalian and arthropod-derived cells, *F. tularensis* rapidly escapes from the acidified late endosome-like phagosome into the cytosol (Akimana and Kwaik, 2011; Asare and Abu Kwaik, 2011). While *F. tularensis* is auxotrophic for Cysteine, it is the least abundant and most limiting cellular amino acid in mammals (Price et al., in press). To counteract host limitation of Cys, *F. tularensis* exploits host glutathione (GSH) (Alkhuder et al., 2009; Meibom and Charbit, 2010), which is the most abundant source of Cys in the host cytosol (Franco et al., 2007). Glutathione is a non-ribosomal tri-peptide (L-γ-L-glutamyl-L-Cysteinyl-glycine) present in almost all eukaryotic cells and some prokaryotes, and its synthesis in eukaryotes is limited by the relatively low levels of cellular Cys (Franco et al., 2007). *F. tularensis* utilizes the enzyme γ-glutamyl transpeptidase (Ggt), which cleaves GSH to liberate Cys, raising the cellular levels of Cys needed to power intracellular proliferation in the host cell cytosol (Alkhuder et al., 2009; Meibom and Charbit, 2010). The severe intracellular growth defect of the *ggt* mutant of *F. tularensis* is rescued by supplementation of Cys (Alkhuder et al., 2009). In addition, requirement for high levels of Cys for *in vitro* growth of *F. tularensis* is bypassed by supplementation of GSH (Alkhuder et al., 2009). Therefore, Ggt should constitute a promising target for anti-microbial therapy to block access of *F. tularensis* to a rich source of an essential amino acid for intracellular proliferation.

Recent studies on the two intravacuolar pathogens, *Legionella pneumophila* (Price et al., 2011) and *Anaplasma phagocytophilum* (Niu et al., 2012), have indicated that the host cell cytosol does not have sufficient levels of amino acids needed to support the robust proliferation of these intra-vacuolar pathogens. To counteract this host limitation, the two pathogens target two major host degradation pathways to generate a surplus of amino acids to meet their demands for biomass buildup during their robust intracellular growth. While *L. pneumophila* promotes host proteasomal degradation (Price et al., 2011), *A. phagocytophilum* triggers the host autophagy degradation pathway (Niu et al., 2012). In either case, blocking the host degradation machinery blocks intracellular proliferation of the pathogen, and the block is relieved upon supplementation of excess amino acids (Price et al., 2011; Niu et al., 2012). In the case of *L. pneumophila*, supplementation of Cys, or Ser, pyruvate, or citrate also bypasses the requirement of host proteasomal degradation for intracellular proliferation (Price et al., 2011). This confirms that host proteasomal degradation provides carbon and energy sources to support the robust intra-vacuolar proliferation of the pathogen. It is possible that intra-vacuole pathogens may be inefficient in their retrieval mechanisms of host amino acids from the cytosol into their vacuoles. However, recent studies on *F. tularensis* supports the idea that the levels of host cell amino acids are simply not sufficient to support the high demands for carbon and energy to support the robust intracellular proliferation of bacterial pathogens that rely on amino acids as major sources of carbon and energy (Alkhuder et al., 2009; Steele et al., 2013).

To meet the demands for high levels of amino acids for its robust growth in the host cell cytosol, *F. tularensis* triggers the host macroautophagy degradation machinery, which is required for optimal intracellular bacterial growth (Steele et al., 2013). Similar to the approach used in *L. pneumophila* (Price et al., 2011), inhibition of the host degradation machinery blocks intracellular growth, but the block is relieved upon supplementation of excess amino acids or pyruvate (Steele et al., 2013). Interestingly, *F. tularensis* triggers a non-canonical ATG5-independent autophagy pathway that induces degradation of cellular proteins, which is predicted to generate a surplus of amino acids to support the robust intracellular bacterial replication (Steele et al., 2013). It would be interesting to identify the bacterial factor responsible for triggering host autophagy, as this would constitute a promising potential target for anti-microbial therapy to block intracellular proliferation of *F. tularensis* and manifestation of tularemia. It is also possible that the ATG5-independent autophagy pathway, which is triggered by *F. tularensis*, would constitute a potential target to prevent the infection and manifestation of the disease. One may also think that multi-targets of nutrient retrieval strategies that include Ggt and the bacterial autophagy inducer would constitute a strong approach for treatment of tularemia. As we continue to unravel mechanisms of nutrient retrieval by *F. tularensis* and other intracellular bacterial pathogens, we continue to expand our anti-microbial potential and promising targets to prevent tularemia and other infectious diseases for which no effective antibiotics are available any more.

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Neutrophils: potential therapeutic targets in tularemia?

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The central role of neutrophils in innate immunity and host defense has long been recognized, and the ability of these cells to efficiently engulf and kill invading bacteria has been extensively studied, as has the role of neutrophil apoptosis in resolution of the inflammatory response. In the past few years additional immunoregulatory properties of neutrophils were discovered, and it is now clear that these cells play a much greater role in control of the immune response than was previously appreciated. In this regard, it is noteworthy that *Francisella tularensis* is one of relatively few pathogens that can successfully parasitize neutrophils as well as macrophages, DC and epithelial cells. Herein we will review the mechanisms used by *F. tularensis* to evade elimination by neutrophils. We will also reprise effects of this pathogen on neutrophil migration and lifespan as compared with other infectious and inflammatory disease states. In addition, we will discuss the evidence which suggests that neutrophils contribute to disease progression rather than effective defense during tularemia, and consider whether manipulation of neutrophil migration or turnover may be suitable adjunctive therapeutic strategies.

Keywords: neutrophils, apoptosis, inflammation, *Francisella tularensis*, innate immunity

NEUTROPHILS IN INNATE HOST DEFENSE

Polymorphonuclear leukocytes (PMNs) are the most abundant leukocyte population in human blood and are rapidly mobilized to sites of infection (Kennedy and Deleo, 2009). In this locale they phagocytose microbes and utilize a combination of NADPH oxidase-derived reactive oxygen species (ROS), cytotoxic granule components and antimicrobial peptides to generate a highly lethal intraphagosomal environment (Nauseef, 2007; Kennedy and Deleo, 2009). In contrast to other leukocytes, neutrophils are short-lived and are preprogrammed to undergo constitutive (spontaneous) apoptosis 18–24 h after release into circulation, and under normal circumstances, PMN apoptosis is further accelerated by phagocytosis and oxidant production (Watson et al., 1996; Kobayashi et al., 2003c; Kobayashi and Deleo, 2009). Ground-breaking studies of DeLeo and colleagues revealed that both constitutive and infection-induced PMN death are controlled not only at the level of intracellular signaling, but also by global changes in gene expression (Kobayashi et al., 2002, 2003a,c), discoveries which necessitated revision of the long-standing notion that mature neutrophils were nearly transcriptionally inert (Jack and Fearon, 1988; Kobayashi and Deleo, 2009). Tight spatial and temporal control of PMN apoptosis is critical for elimination of infection and resolution of the inflammatory response, and during this process phagocytic and proinflammatory capacity are down-regulated, release of toxic cell components is prevented, and tissue damage is minimized (Kobayashi et al., 2003b,c; Fox et al., 2010). If this process is perturbed PMNs can develop a proinflammatory phenotype that promotes necrosis and granuloma formation and sustains infection (Kobayashi et al., 2003b, 2004). For this reason, defects in PMN turnover are indicative of an ineffective and dysregulated inflammatory response (Nathan, 2002). In keeping with this, recent studies revealed that neutrophils have immunoregulatory properties that directly influence the function of NK cells, DCs, macrophages and lymphocytes (Mantovani et al., 2011).

Abbreviations: ATII, alveolar type II cells; CDK, cyclin-dependent kinase; COPD, chronic obstructive pulmonary disease; DAMPs, danger-associated molecular patterns; LVS, live vaccine strain; MMP-9, matrix metalloproteinase-9; PICD, phagocytosis-induced cell death; PMN, Polymorphonuclear leukocyte; ROS, reactive oxygen species.

NEUTROPHILS AND TULAREMIA PATHOGENESIS

Francisella tularensis is a facultative intracellular pathogen that is distributed throughout the Northern hemisphere and two subspecies of this bacterium, *F. tularensis* subspecies *tularensis* (type A) and *F. tularensis* subspecies *holarctica* (type B) account for nearly all cases of human tularemia. Most studies of this organism have focused on macrophages as major vehicles for intracellular growth and bacterial dissemination from sites of infection to the liver and spleen (Chong and Celli, 2010). Nevertheless, *F. tularensis* is unusual in its ability to infect neutrophils and epithelial cells as well as mononuclear phagocytes, but relatively little is known about the shared and distinct contributions of these other cell types to disease (McLendon et al., 2006).

Aerosol infection of rhesus monkeys with virulent type B *F. tularensis* strains defined prominent features of pneumonic tularemia, and these studies were among the first to suggest a key role for neutrophils in tissue destruction and disease progression (Tulis et al., 1970; Schricker et al., 1972; Hall et al., 1973). These data demonstrate that large numbers of PMNs are present the lungs, and from day 2 onward alveoli and bronchioles become progressively clogged with neutrophils, bacteria and necrotic debris. Granulomas also begin to organize wherein live PMNs, bacteria and necrotic debris become enveloped by epithelial syncytia. A similar disease course has been described using rats, rabbits and mice (Dunaeva and Shlygina, 1975), and *ex*

vivo analyses indicate that PMNs contain viable bacteria. Rhesus monkeys, rabbits, mice (and many humans) do not survive acute infection with type A *F. tularensis* (Eigelsbach et al., 1962; Schricker et al., 1972). These organisms replicate much faster than type B isolates, and progression to moribund status is characterized by an accumulation of neutrophils and bacteria in the lung and extensive necrotic tissue damage (Conlan et al., 2003; Lamps et al., 2004; Bosio et al., 2007). In keeping with the histopathology data, flow cytometry analysis of mouse lung cells indicates that whereas alveolar macrophages account for ∼70% of *F. tularensis*-infected lung cells on the first day of infection, neutrophils are the major infected cell population by day 3, and the fraction of infected DCs and alveolar type II (ATII) cells is relatively low (Hall et al., 2008).

It has long been known that neutropenia or inherited defects in PMN function markedly increase susceptibility to infection. For this reason, neutrophil-depleting antibodies are often used to determine the role of this cell type in different diseases. Results of studies that used the first antibody developed for this purpose, RB6-8C5, suggested that PMNs are critical for host defense against *F. tularensis* (Sjostedt et al., 1994). However, subsequent studies revealed that RB6-8C5 causes depletion of both inflammatory monocytes and PMNs, necessitating a reinterpretation of prior results (Daley et al., 2008; Dunay et al., 2010). A subsequent study that used a lower dose of RB6-8C5 that is somewhat more selective for PMNs did not identify a role for these cells in control of *F. tularensis* (Kuolee et al., 2011). Using a different approach, Malik et al. discovered that inhibition of PMN migration into the lung allowed mice to survive what would otherwise be a lethal dose of type A *F. tularensis* or the live vaccine strain (LVS) (Malik et al., 2007). At the same time, studies of Elkins and colleagues suggest that tularemia severity is significantly increased under conditions that induce neutrophilia, and this correlates directly with enhanced hepatotoxicity (Bosio and Elkins, 2001; Mellilo et al., 2013). On balance, the data indicate that PMNs do not contribute to effective host defense during tularemia, and instead contribute to disease progression.

NEUTROPHIL CHEMOTAXIS

Mechanisms of neutrophil migration to sites of infection have been extensively studied (Craig et al., 2009; Balamayooran et al., 2010; Sadik et al., 2012; Fullerton et al., 2013). In the lung, inhaled bacteria typically interact with alveolar macrophages and ATII cells, and signaling downstream of pattern recognition receptors, including TLRs, triggers secretion of major neutrophil chemotactic agents, particularly IL-8 (CXCL8) in humans and KC in mice, as well as GRO-α, MIP-2 and MCP-1. Additional players include IL-1β, LIX/CXCL5, G-CSF, complement factor C5a and the eicosanoids LTB4 and PGE2, as well as Proline-Glycine-Proline (PGP), a peptide generated from collagen in the extracellular matrix by matrix metalloproteinases MMP-8 and MMP-9. As neutrophil function and phenotype are affected by the mechanism of recruitment, it is noteworthy that IL-8, KC, GRO-α, MIP-2, LIX, and PGP all bind CXCR1 and/or CXCR2 on PMNs. Neutrophils directly contribute to this process as well via secretion IL-8, IL-1β, LTB4, and MMP-9.

During tularemia a select subset of signals trigger PMN migration into the lung as the amount of IL-8 released by infected ATII cells, macrophages and endothelial cells is relatively low, and neither IL-8 nor MCP-1 appears to be essential (Gentry et al., 2007; Moreland et al., 2009). In marked contrast, *F. tularensis* specifically stimulates expression of MMP-9, and PGP is a major PMN chemoattractant in this system, as lung PMNs are markedly diminished in MMP-9 null mice and these animals are able to survive what would otherwise be a lethal dose of type A *F. tularensis* or the LVS (Malik et al., 2007), as noted above. In this regard, it is of interest that PGP is sufficient to recruit and maintain tissue neutrophils in in absence of other chemotactic agents and its stability and activity are enhanced by N-acetylation (Snelgrove et al., 2010), but whether this modification is induced during tularemia is unknown. PGE2 is produced by *F. tularensis*-infected macrophages and ATII cells (Woolard et al., 2007) and infected PMNs upregulate IL-1β (Schwartz et al., 2013), but the extent to which these agents contribute to PMN chemotaxis and phenotypic modulation during tularemia remains to be determined. A model that integrates these data is shown in **Figure 1**.

F. tularensis DISRUPTS NEUTROPHIL DEFENSE MECHANISMS

Francisella tularensis lipopolysaccharide (LPS) has an atypical structure that does not signal through toll like receptors 4 or 2. Nevertheless, LPS O-antigen and capsular polysaccharides act in concert to protect this organism from the lytic effects of serum complement (McLendon et al., 2006). This is significant as neutrophil uptake of *F. tularensis* is markedly enhanced by complement factors in normal human serum, and under these conditions phagocytosis is mediated by complement receptor 1 and complement receptor 3 (Schwartz et al., 2012b). Neutrophil receptors that confer inefficient phagocytosis of unopsonized *F. tularensis* remain obscure.

Under normal circumstances, phagocytosis is coupled to rapid activation of the Nox2 NADPH oxidase complex, such that toxic oxidants kill most bacteria within 60 min of infection (Deleo et al., 1999; Kobayashi et al., 2003a; Nauseef, 2007). In marked contrast, we and others demonstrated using several strains of type A and type B *F. tularensis* as well as LVS that this organism does not trigger a respiratory burst in human or monkey PMNs (Proctor et al., 1975; McCaffrey and Allen, 2006; McCaffrey et al., 2010). At the molecular level, forming *F. tularensis* phagosomes exclude flavocytochrome b558, which contains the catalytic core of the NADPH oxidase and also acts as a docking site in the membrane for the cytosolic subunits that are also essential for ROS production (McCaffrey and Allen, 2006; McCaffrey et al., 2010). Within minutes, the ability of infected PMNs to be activated by heterologous particulate and soluble stimuli is also profoundly impaired, and in this case *F. tularensis* acts at a later stage to inhibit the activity of enzyme complexes at the membrane (McCaffrey and Allen, 2006; McCaffrey et al., 2010). A similar, if not identical, mechanism of post-assembly NADPH oxidase inhibition also undermines the ability of specific IgG in anti-*F. tularensis* immune serum to enhance bacterial killing via neutrophil activation (McCaffrey and Allen, 2006; McCaffrey et al., 2010).

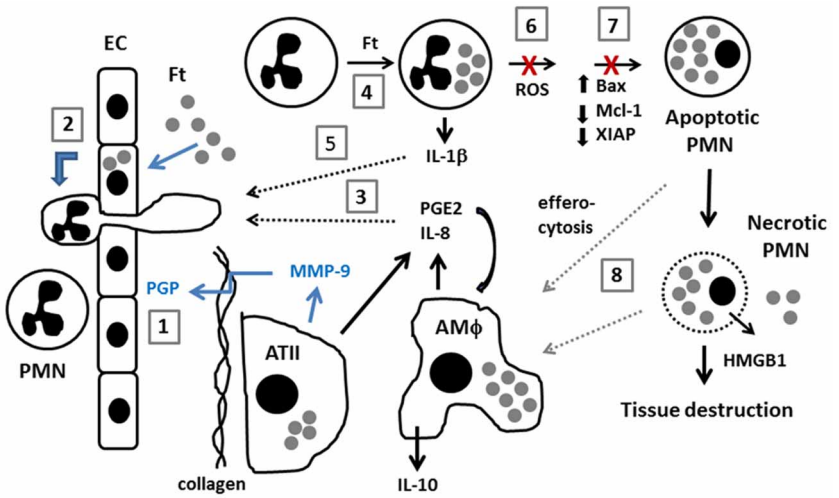

FIGURE 1 | Model of neutrophil dynamics in the *F. tularensis*-infected lung. Inhaled *F.* tularensis rapidly infects alveolar type II (ATII) cells and macrophages (AMΦ) (Gentry et al., 2007). (1) MMP-9, likely secreted by ATII cells, cleaves collagen, generating PGP which directly stimulates PMN recruitment from the bloodstream (Malik et al., 2007). (2) PMN migration is also stimulated by direct infection of pulmonary endothelial cells (EC) by an IL-8 and MCP-1-independent mechanism (Moreland et al., 2009). (3) *F. tularensis* stimulates release of PGE2 and IL-8 from AMΦ and ATII cells (Gentry et al., 2007; Woolard et al., 2007). PGE2 stimulates macrophage production of IL-10 (Hunt et al., 2012). (4,5) *F. tularensis* infects PMNs and upregulates IL-1β (Schwartz et al., 2013). The extent to which PGE2, IL-8 and IL-1β contribute to PMN chemotaxis and phenotypic modulation during tularemia remains to be determined (*dotted black arrows*). (6,7) *F. tularensis* inhibits PMN NADPH oxidase activity and prevents changes in gene expression that are critical for constitutive and phagocytosis-induced apoptosis (McCaffrey and Allen, 2006; McCaffrey et al., 2010; Schwartz et al., 2012a, 2013). (8) Efferocytosis of apoptotic PMNs is critical for control of infection and resolution of inflammation. Defects in apoptosis favor PMN necrosis, and subsequent release of cytotoxic cell components, and danger molecules such as HMGB1 exacerbate tissue destruction. Recent data suggest that efferocytosis and/or clearance of necrotic cell debris may be impaired (Mares et al., 2011) (*dotted gray arrows*).

Evasion of toxic oxidants at early stages of infection is followed by phagosome egress, and release of bacteria into neutrophil cytosol, and *F. tularensis* replicates to some extent in this locale, though but not to the same extent as in macrophages (McCaffrey and Allen, 2006; Schulert et al., 2009; Schwartz et al., 2012a; Long et al., 2013). FevR and MigR, major virulence regulators of *F. tularensis*, are essential for inhibition of neutrophil activation (Buchan et al., 2009; McCaffrey et al., 2010). The genes in the MigR/FevR regulon required for NADPH oxidase inhibition are as yet unknown, but are distinct from pathogenicity island genes required for phagosome escape and intracellular growth such as *iglI* and *iglJ* (McCaffrey et al., 2010; Long et al., 2013). Other genes such as *carA*, *carB*, and *pyrB* act indirectly via effects on pyrimidine biosynthesis (Schulert et al., 2009), and the role of the acid phosphatase AcpA in NADPH oxidase inhibition and virulence is controversial (Reilly et al., 1996; Child et al., 2010; McCaffrey et al., 2010; Mohapatra et al., 2010).

F. tularensis PROLONGS NEUTROPHIL LIFESPAN VIA EFFECTS ON SIGNALING AND GENE EXPRESSION

Neutrophils are short-lived and undergo constitutive apoptosis at a rate of 10^{11} cell per day in humans (Kennedy and Deleo, 2009). Although the specific events that initiate constitutive PMN apoptosis are unknown, it is clear that cell death is tightly regulated and that both intrinsic and extrinsic pathway caspases contribute to this process along with complex changes in gene expression that comprise and "apoptosis differentiation program," as noted above. Since the seminal studies of Watson (Watson et al., 1996) it has also been clear that phagocytosis and NADPH oxidase-derived ROS profoundly accelerate PMN apoptosis as compared with unstimulated controls (Kobayashi et al., 2003a, 2004). This "phagocytosis-induced cell death" (PICD) respone is also regulated at the level of gene expression, and is critical for effective host defense. Blockade of NADPH oxidase activity by *F. tularensis* suggested that this organism may not accelerate PMN death, and as such may not induce PICD. Indeed, our recent biochemical studies and analysis of PMN gene expression provide definitive evidence that *F. tularensis* not only fails to induce PICD, but also inhibits constitutive neutrophil apoptosis via effects on the intrinsic and extrinsic pathways, and in this manner profoundly prolongs cell lifespan (Schwartz et al., 2012a, 2013)

Specifically, the biochemical data indicate that the vast majority of infected PMNs do not progress to an apoptotic morphology within 48 h of infection with live *F. tularensis*, and in keeping with this PS externalization, processing and activation of caspases-8, −9, and −3, and DNA fragmentation are markedly impaired (Schwartz et al., 2012a). At the same time, *F. tularensis* significantly alters the expression of over 3400 human neutrophil genes between 3 and 24 h of infection, including 365 unique genes linked to apoptosis and cell survival (Schwartz et al., 2013). Of particular note are effects of *F. tularensis* on BAX, a proapoptotic member of the Bcl-2 family of proteins that plays a pivotal role in the intrinsic apoptotic pathway via disruption of the outer mitochondrial membrane. Upregulation of BAX is a hallmark of the PICD response (Kobayashi et al., 2002, 2003a, 2004). However, *BAX* mRNA and protein are

progressively downregulated by *F. tularensis* (Schwartz et al., 2013). It is therefore likely that blockade of the respiratory burst and downregulation of BAX synergize to prevent PICD during *F. tularensis* infection. At the same time, upregulation of several prosurvival factors and anti-apoptosis genes collaborate to diminish and delay the constitutive apoptosis program in PMN. Although not all of these data can be discussed here, sustained expression *BIRC4*, which encodes X-linked inhibitor of apoptosis protein (XIAP), and *CAST* which encodes calpastatin, likely account in large part for defective processing and activation of intrinsic pathway caspases (Schwartz et al., 2013). At the same time not all genes associated with enhanced PMN survival are modulated by this pathogen, as expression of IL-8 (CXCL8) is not induced and this cytokine is not secreted by PMNs infected with live *F. tularensis* (Schwartz et al., 2012a, 2013). In contrast, IL-8 appears critical for sustained survival of neutrophils infected with the obligate intracellular pathogen *Chlamydia pneumoniae* (van Zandbergen et al., 2004), and as such the data indicate that these two pathogens use distinct mechanisms to modulate PMN lifespan.

It is also important to note that apoptosis normally downregulates PMN functional capacity, and if apoptosis is inhibited these cells exhibit a sustained proinflammatory phenotype (Kobayashi et al., 2003b; Kennedy and Deleo, 2009). Consistent with this, *F. tularensis*-infected PMNs show enhanced expression of *VEGF, IL6, IL1B, CXCL1, OSM,* and *IL1RN* (Schwartz et al., 2013). Together, enhanced lifespan and proinflammatory capacity increase the probably of cell progression to secondary necrosis, which is characterized by spilling of DAMPs, alarmins, and other cytotoxic molecules that further amplify inflammation and cause extensive host tissue destruction (Kobayashi et al., 2003c; Fox et al., 2010; Silva, 2010).

TARGETING PMNs AS A CANDIDATE ADJUNCTIVE THERAPUTIC STRATEGY

Neutrophil accumulation and enhanced longevity, granuloma formation, and extensive tissue necrosis are benchmarks that define a defective inflammatory response (Nathan, 2002), and are also characteristic features of tissues infected with *F. tularensis* (Tulis et al., 1970; Schricker et al., 1972; Hall et al., 1973). The fact that blockade of PMN influx into the lung favors host survival without markedly altering tissue bacterial load (Malik et al., 2007) is consistent with an immunoregulatory role for PMNs in tularemia pathogenesis (Mantovani et al., 2011), as are the effects of *F. tularensis* on neutrophil activation state, lifespan and proinflammatory capacity that are noted above and summarized in **Figure 1**. Considered together, the data support a model in which neutrophils play a unique role in tularemia pathogenesis via dysregulation of the inflammatory response that is distinct from the role of macrophages as major vehicles for bacterial growth and dissemination. Thus, suitable points of therapeutic intervention may include PMN chemotaxis, apoptosis or activation state.

Inhibition of PMN apoptosis and aggressive neutrophilic inflammation also exacerbate the severity of pneumococcal meningitis (Koedel et al., 2009) and contribute significantly to lung destruction in human patients with chronic obstructive pulmonary disease (COPD) (Weathington et al., 2006). As in tularemia, PMN accumulation in the lungs of humans with COPD is driven by MMP-9-dependent production of PGP, and published data suggest that targeting this pathway with anti-PGP antibodies suppresses neutrophil responses and appears to have some therapeutic benefit in mouse COPD models (Weathington et al., 2006). Similarly, Arginine-Threonine-Arginine peptides block IL-8 and PGP signaling at the level of CXCR1 and CXCR2 to inhibit PMN migration and activation, and in this manner ameliorate lung destruction during emphysema (van Houwelingen et al., 2008). Other interventions that directly target this mechanism of PMN chemotaxis include small molecule inhibitors of MMPs such as GM6001, CP-471,474, and RS113,456 and enhancement of endogenous mechanisms of MMPs inhibition via intratracheal delivery of recombinant TIMPs (tissue inhibitors of metalloproteinases) (Djekic et al., 2009). As protein-based therapeutics are very expensive they are not optimal for treatment of chronic illnesses, yet could be of considerable benefit in the context of acute infectious diseases, including tularemia.

Other potential therapeutic targets include lipid mediators of the eicosanoid family, which are dysregulated in many critical illnesses. In particular, resolvin E1 is of interest as it is effective at concentrations as low as 1 nM, is beneficial for treatment of aspiration pneumonia, and is known to decrease lung PMNs via effects on ROS production, PICD and efferocytosis (Fullerton et al., 2013). Aspirin-triggered resolvins also appear to reduce mortality associated with systemic inflammatory response syndrome. On the other hand, excess or sustained production of PGE2 by macrophages and epithelial cells induces a state of "injurious resolution" that compromises Fc receptor function and NADPH oxidase activity and alters macrophage phenotype. This condition can occur in burn patients or infection with *Aspergillus*, and is reversed in by aspirin or cyclooxygenase inhibitors which reduce PGE2 levels by 95% (Fullerton et al., 2013). Although further analysis of eicosanoid profiles during tularemia is needed, these lipid mediators are of interest as PGE2 is induced by *F. tularensis* and dampens at least some aspects of the immune response (Woolard et al., 2007).

Other studies have begun to examine the potential therapeutic utility of PMN apoptosis induction. Cyclin-dependent kinases (CDKs) regulate growth of most cell types, yet are critical regulators of PMN viability and lifespan (Witko-Sarsat et al., 2011). Inhibition of CDKs with R-roscovitine and other related compounds can induce apoptosis and resolution of PMN-dominant inflammatory responses (Leitch et al., 2010), and in this manner accelerate recovery of mice with pneumococcal meningitis (Koedel et al., 2009). Moreover, our recent data indicate that CDK7 and CDK2 are induced in PMNs by *F. tularensis* (Schwartz et al., 2013). In summary, given the potentially short widow between the onset of symptoms, severe disease and death, future studies should consider whether agents that modulate PMN chemotaxis, directly target PMN apoptosis, or modulate the inflammatory response may be useful as adjunctive therapeutic agents when combined with antibiotics for treatment of tularemia.

RELEVANCE TO OTHER INFECTIOUS DISEASES

As in tularemia, PMNs are the most commonly infected cell type in the airway of persons infected with *Mycobacterium tuberculosis* (Lowe et al., 2012). PMN lifespan is prolonged, and protective CD4+ T cell-driven adaptive immune responses are curtailed at the level of DCs, as downstream responses are less efficient when these cells are directly infected than when antigens are acquired by efferocytosis of apoptotic, infected PMNs (Blomgran and Ernst, 2011). Thus, neutrophilia is associated with impaired control of infection and correlates directly with the severity of cavitary disease and tissue damage. Neutrophils may kill some *M. tuberculosis* early in infection, but PMN depletion at later stages appears to be beneficial (Lowe et al., 2012). Many factors drive PMN accumulation during tuberculosis including macrophage and DC-derived IL-8, G-CSF, and LTB4, as well as IL-1β, IL-8, and LTB4 from PMNs themselves (Lowe et al., 2012). A role for PGE2 has also been described, and MMP-9 may play a specific role in recruitment of PMNs to granulomas (Lowe et al., 2012; Hawn et al., 2013).

Brucella abortus replicates in an ER-derived vacuole in macrophages. Similar to *F. tularensis*, *B. abortus* LPS has low bioactivity, yet in contrast to *F. tularensis*, there are relatively few PMNs in the circulation or at sites of infection (Barquero-Calvo et al., 2007). At the same time, *B. abortus* is not efficiently killed by human PMNs *in vitro* (Kreutzer et al., 1979), and neutrophils do not appear to contribute directly to bacterial killing. Rather, control of infection is favored by PMN depletion during late stages of infection, which enhances lymphocyte activation and proinflammatory cytokine production ((Barquero-Calvo et al., 2007, 2013). Arthritis is a complication of *B. abortus* infection, and local secretion of MMP-2 and MMP-9 plays an important role in neutrophil recruitment and joint damage (Scian et al., 2011). In contrast, C5a, IL-1β, and LTB4 play dominant roles in neutrophil-mediated joint damage in rheumatoid arthritis (Sadik et al., 2012).

Finally, MMP-8 and MMP-9 also drive PMN recruitment to the lungs during cystic fibrosis, and this is perpetuated and amplified by MMP-9 released by PMN degranulation. PGP, particularly in its highly active N-acetylated form, is elevated in patient sputum (Xu et al., 2011). Thus, strategies that target MMPs may also be useful in this disease that is characterized by chronic neutrophilic inflammation. Considered together, the data summarized here demonstrate that PMNs play complex and important roles in infection, and therapeutic strategies that target this cell type are being developed and may have wide utility alone or in combination with antibiotics.

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Monocyte/macrophage inflammatory response pathways to combat *Francisella* infection: possible therapeutic targets?

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Francisella tularensis can bypass and suppress host immune responses, even to the point of manipulating immune cell phenotypes and intercellular inflammatory networks. Strengthening these responses such that immune cells more readily identify and destroy the bacteria is likely to become a viable (and perhaps necessary) strategy for combating infections with *Francisella*, especially given the likelihood of antibiotic resistance in the foreseeable future. Monocytes and macrophages offer a niche wherein *Francisella* can invade and replicate, resulting in substantially higher bacterial load that can overcome the host. As such, understanding their responses to *Francisella* may uncover potential avenues of therapy that could promote a lowering of bacterial burden and clearance of infection. These response pathways include Toll-like Receptor 2 (TLR2), the caspase-1 inflammasome, Interferons, NADPH oxidase, Phosphatidylinositide 3-kinase (PI3K), and the Ras pathway. In this review we summarize the literature pertaining to the roles of these pathways during *Francisella* infection, with an emphasis on monocyte/macrophage responses. The therapeutic targeting of one or more such pathways may ultimately become a valuable tool for the treatment of tularemia, and several possibilities are discussed.

Keywords: *Francisella*, monocytes, macrophages, signaling, host response

INTRODUCTION

Francisella tularensis is the Gram-negative causative agent of tularemia (Sjostedt, 2007). *F. tularensis* has been classified into distinct subspecies, including *F. tularensis* subsp. *tularensis* (*F. tularensis*; Type A), *F. tularensis* subsp. *holarctica* (*F. holarctica*; Type B), and *F. tularensis* subsp. *novicida (F. novicida)*, which may actually be a separate species (Johansson et al., 2010). *Francisella* is especially recognized for its low infectious dose and ability to cause severe illness and death, which justifies its categorization as a Category A select agent by the USA Centers for Disease Control and Prevention (CDC) (Sjostedt, 2007). Of note, the most life-threatening forms of tularemia are particularly associated with Type A infections regardless of host species (Mohapatra et al., 2013). Although known to infect a range of host organisms and cell types (Rick and Wu, 2007; Hall et al., 2008), *F. tularensis* has evolved to successfully infect human monocytes/macrophages where the bacteria escape the phagosome, replicate within the cytosol and then move on to other cells as the infected cells die (Clemens and Horwitz, 2007; Elkins et al., 2007; Jones et al., 2012; Celli and Zahrt, 2013). *In vivo*, macrophages appear to be the preferred host cell for *Francisella* (Sjostedt, 2003; Elkins et al., 2007).

One critical characteristic of *F. tularensis* is its ability to attenuate host inflammatory responses. Indeed, early studies in humans showed that *Franicsella*-infected individuals exhibited diminished cytokine responses to endotoxin (Greisman et al., 1963). In the murine system *F. tularensis* infection does not lead to a classic pro-inflammatory cytokine response, and this results in insufficient numbers of immune cells recruited to infection sites (Bosio et al., 2007). Further, murine studies have corroborated the findings of Greisman et al. (1963), who found that challenge with lipopolysaccharide (LPS) after infection did not lead to the production of pro-inflammatory cytokines such as Tumor Necrosis Factor α (TNFα) in mouse cell lines nor *in vivo* (Telepnev et al., 2003, 2005; Bosio, 2011). Similar findings have also been observed in *F. tularensis*-infected murine bone-marrow and alveolar macrophages following administration of the synthetic triacylated lipopeptide Pam_3CSK_4 (Crane et al., 2013).

Circulating monocytes constitute lower than 10% of blood immune cells, yet serve a critical role as primary responders to infection (Moser and Loetscher, 2001; Leavy, 2012). As mentioned above they are also prime targets of *Francisella* infection. Along with this, a higher percentage of monocytes are infected by *F. tularensis* than either *F. holarctica* or *F. novicida* during the course of infection (Hall et al., 2008). The focus of this brief review is on some of the critical intracellular response pathways involved with *Francisella* infection. The role of each pathway during *F. tularensis* infection is summarized, with an emphasis on monocyte/macrophage responses. Following this is a short discussion of potential non-antibiotic means of combating *Francisella* by modulating these response pathways.

Francisella AND TOLL-LIKE RECEPTORS

Host immune cells have evolved to contain an array of receptors which are vital for the detection of invading microbes and foreign materials. These surface- and endosomally-expressed sensors, termed pattern recognition receptors (PRR), can recognize highly conserved bacterial, viral, and fungal motifs (Brown et al., 2011). Toll-Like Receptors (TLR) are key PRR that are expressed by a variety of cells including monocytes and macrophages. *Francisella* directly interacts with the host cell through TLR2, a surface receptor that recognizes surface lipopeptides and peptidoglycan. In fact, TLR2$^{-/-}$ mice infected with *F. tularensis* Live Vaccine Strain (LVS) display markedly lower TNFα and Interleukin 6 (IL-6) production, decreased Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, and showed overall lower survival when compared to wild-type mice (Katz et al., 2006; Malik et al., 2006). Specific lipopeptides that can induce TLR2 signaling, particularly the triacylated 17-kDa membrane lipoprotein FTT0901/TUL4/LpnA (Sandstrom et al., 1987; Sjostedt et al., 1989, 1991) and the membrane lipoprotein FTT1103/FipB (*Francisella* infectivity potentiator protein B) (Qin and Mann, 2006; Qin et al., 2011), are present on *Francisella*'s surface (Thakran et al., 2008).

Modifications in TLR2 expression levels are associated with *Francisella* infections, and these can vary depending on the subspecies. For example, we have shown that *F. novicida* significantly increased TLR2 transcript after infection in primary monocytes while *F. tularensis Schu S4* decreased it (Butchar et al., 2008). In addition to altering receptor expression, both *F. tularensis* and *F. novicida* can downregulate the molecule Cluster of differentiation 14 (CD14) (Butchar et al., 2008). This is an important co-receptor for both TLR2 and TLR4. It is required by the host cell in order to generate a potent pro-inflammatory cytokine response to *F. tularensis* LVS, although it is not adequate for increasing survival *in vivo* (Chase and Bosio, 2010). In like manner, recent reports highlight the role of the downstream adapter Myeloid differentiation primary response 88 (MyD88), as mice lacking this molecule died rapidly when challenged with *F. tularensis* LVS (Collazo et al., 2006).

The importance of TLR4 in responding to LPS has long been recognized (Chow et al., 1999; Qureshi et al., 1999). *Francisella*, however, expresses an atypical LPS that does not strongly induce TLR4 (Duenas et al., 2006). This is attributed to the lack of two acetyl groups in its LPS, making it incapable of inducing a strong TLR4 response (Phillips et al., 2004). TLR signaling appears to be very effectively subverted by *Francisella*, and this may position TLR as well as downstream pathway members as prime candidates for targeted therapy (discussed in a later section).

INFLAMMASOME RESPONSES

Cystolic sensing mechanisms such as the multi-protein inflammasome play a prominent role in recognizing intracellular pathogens, including *Francisella*. This depends upon *Francisella's* escape from the phagolysosome (Mariathasan et al., 2005; Gavrilin et al., 2006). Inflammasomes regulate caspase activation through proteolytic cleavage, leading to Interleukins 1β and 18 (IL-1β and IL-18) processing. Cleavage of procaspase-1 to caspase-1 requires TLR2, as TLR2$^{-/-}$ mouse macrophages showed little caspase-1 24 h after infection with LVS (Dotson et al., 2013). Expectedly, caspases have been implicated in the regulation of *Francisella* infections. For example, mice lacking caspase-1 displayed higher bacterial numbers in organs following infection with *F. novicida* (Mariathasan et al., 2005; Jones et al., 2010).

Francisella's escape from the phagosome triggers Absent in Melanoma 2 (AIM2) inflammasome activation, as a subset of *F. novicida* have been shown to lyse within the cytosol and release AIM2-activating double-stranded deoxyribonucleic acid (dsDNA) (Fernandes-Alnemri et al., 2010; Jones et al., 2010; Rathinam et al., 2010). The importance of AIM2 in *Francisella* infection was clearly demonstrated by a study showing that AIM2$^{-/-}$ mice had increased organ bacterial burden and lower survival rates than wild-type following infection with *F. novicida* (Fernandes-Alnemri et al., 2010). In humans however, it has been shown that the NALP3 inflammasome was the primary driver of IL-1β production, with AIM2 contributing as well (Atianand et al., 2011).

The influence of *Francisella* on inflammasome activity appears to be subspecies-dependent. For example, recent data suggest that *F. novicida* does not inhibit inflammasome activation (Broz and Monack, 2011). In contrast, it has been shown that LVS delays inflammasome activation and cell death, an activity that requires the OmpA-like *Francisella* protein FTL_0325. *In vivo*, mice infected with LVS harboring a mutation in FTL_0325 showed significantly higher IL-1β by the first day after infection while mice infected with control LVS showed higher IL-1β at day 3. Importantly, mice infected with the LVS mutant that permitted earlier inflammasome activation showed a significantly lower bacterial load at day 3 (Dotson et al., 2013).

Virulent *Francisella* manipulates inflammasome responses by stimulating the activation of apoptosis-promoting caspase-3 rather than caspase-1 (Wickstrum et al., 2009; Bosio, 2011). Tissues from Type A *F. tularensis* infected mice expressed increased cleaved caspase-3, in contrast to the tissue responses of *F. tularensis* LVS- and *F. novicida*-infected mouse macrophages. In these cells, an increased caspase-1 dependent/caspase-3 independent inflammatory cytokine production was more evident (Wickstrum et al., 2009).

TYPE I AND II INTERFERONS

Interferons (IFNs) are host-produced proteins with an inherent role in pathogen clearance during infection. These Type I interferons induce signal transduction molecules, upregulate major histocompatibility complex (MHC) molecules and promote proliferation of T cells (Welsh et al., 2012). Importantly, they have been shown to be critical for inflammasome activation in response to *Francisella* (Henry et al., 2007). While usually associated with viral infections, interferons are also seen with *Francisella* infection. For example, *F. novicida* is able to induce a variety of Type I IFN-associated genes in mouse bone marrow-derived macrophages (BMM) (Henry et al., 2007), and Schu S4 upregulated IFNβ in human peripheral blood monocytes (Butchar et al., 2008). However, it has been shown that *Francisella* suppresses Type I interferon signaling. For example, the virulent *F. tularensis* strain Schu S4 inhibited the ability of dendritic cells to

produce IFNα and IFNβ (Chase et al., 2009). Infection of human monocytes with Schu S4 led to downregulation of IFNα receptors 1 and 2 (Butchar et al., 2008). As such, it appears as though the more virulent form of *Francisella* uses more than one means to combat Type I IFN signaling.

It has also been shown that infection of human primary monocytes with *F. tularensis* and *F. holarctica* not only leads to downregulation of Type I interferon pathway components but also Type II (Butchar et al., 2008; Cremer et al., 2011). IFNγ, a cytokine produced primarily by natural killer (NK) and T cells, regulates the immunological response to effectively clear pathogens. IFNγ can lower bacterial number following infection with LVS (Anthony et al., 1989; Polsinelli et al., 1994), and can reduce the intra-macrophage growth of LVS in a dose-dependent manner (Anthony et al., 1992). Later reports demonstrated that macrophages treated with IFNγ were more efficient in clearing *Francisella* via an increased ability to perform phagosome-lysosomal fusion (Santic et al., 2005). In human monocytes, both *F. tularensis* and *F. novicida* increased IFNγ ligand expression but decreased IFNγ Receptor 1 (Butchar et al., 2008). In conjunction, it has been shown in both human and murine monocytic cell lines that *F. novicida* induces Suppressor of Cytokine Signaling 3 (SOCS3) expression, suppresses Signal Transducer and Activator of Transcription 1 (STAT1) phosphorylation, and suppresses both Interferon gamma-induced protein 10 (IP-10) and Inducible Nitric Oxide Synthase (iNOS) production (Parsa et al., 2008a).

Because of the *Francisella*-mediated dampening of both Type I and Type II interferon signaling, there is a possibility that pharmaceutical delivery of interferons may help combat infection. Intron A (Spiegel, 1985), Rebif (Mantia et al., 2013), and Actimmune (Todd and Goa, 1992) are clinically approved drugs that deliver interferons alpha, beta, and gamma to the patient, respectively. They have been utilized for the management of Multiple Sclerosis, Chronic Granulomatous Disease (CGD), and Hepatitis B infection, but there is a possibility that one or more may aid against at least some forms of tularemia.

Another *Francisella* family member, *F. philomiragia*, is an opportunistic pathogen found with immunocompromised individuals. In particular, it is associated with the abovementioned CGD, which can lead to fatal septicemia (Seger et al., 1982; Mailman and Schmidt, 2005). Interestingly, both *F. tularensis* and *F. philomiragia* have been associated with chronic granulomas and necrotizing abscesses (Schmid et al., 1983; Nylund et al., 2006). Of particular importance, *F. philomiragia* has between a 70–85% homology to *F. tularensis* (Whipp et al., 2003), suggesting that both pathogens may to some degree respond to IFN treatment. However, Melillo et al. (2010) showed that IFNγ did not improve the ability of human macrophages to combat Schu S4. Further testing, perhaps with the use of monocytes, or using IFNγ plus other agents, may uncover a positive role of IFNγ against virulent *Francisella*.

THE ROLE OF NADPH

Target host cells of *Francisella* can respond to infection with the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Lindgren et al., 2005). Here we will focus on ROS, which are generated following the assembly of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) and are a crucial innate defense mechanism. Not surprisingly, however, *Francisella* has devised an array of techniques to inhibit ROS. These include techniques focused on hindering NADPH component assembly, obstructing ROS production from assembled NADPH oxidases and neutralizing generated ROS (Bosio, 2011; Jones et al., 2012). *Francisella*, including both virulent and less virulent strains, reduces ROS production in neutrophils and macrophages. The acid phosphatase AcpA has been shown to be important for inhibiting reactive oxygen species production in both macrophages and neutrophils (Mohapatra et al., 2010). Another report showed that in neutrophils, virulent *F. tularensis* both with and without an AcpA mutation, suppressed the production of superoxide anions from the NADPH oxidase complex (McCaffrey et al., 2010).

The live vaccine strain of *F. tularensis* was able to persist within neutrophils by avoiding acquisition of gp91/p22 plasma membrane and p47/p67 cytosolic NADPH subunits (McCaffrey and Allen, 2006). This supported the growth of *F. tularensis* LVS by hindering NADPH assembly. The importance of altering NADPH complexes is not unique to *Francisella*, as multiple bacteria including *Helicobacter pylori* and *Salmonella typhimuriu*m have been shown to alter NADPH oxidase assembly in cells (Gallois et al., 2001; Allen et al., 2005). If *Francisella* does encounter ROS, catalases and super oxide dismutases (SOD) enzymes are necessary for survival, as ΔSOD *F. tularensis* LVS have increased susceptibility to IFNγ-induced death (Melillo et al., 2009). Indeed, it has been shown that antioxidants produced by *Francisella* Schu S4 can dampen macrophage inflammatory responses (Melillo et al., 2010).

THE PI3K/Akt PATHWAY

Phosphatidylinositol 3′-kinase (PI3K) leads to activation of Akt, also known as protein kinase B (PKB/Akt) [please see (Hers et al., 2011; Hemmings and Restuccia, 2012) for brief reviews on the PI3K/Akt pathway, and (Cremer et al., 2011) for a short review within the context of *Francisella*]. The cellular processes mediated by PI3K include phagocytosis (Araki et al., 1996), autophagy (Petiot et al., 2000), cytokine production (Parsa et al., 2006), and oxidative burst (Chen et al., 2003; Hoyal et al., 2003). Hence, manipulation of PI3K may be advantageous for pathogens. For example, macrophages from PI3K-deficient mice show impaired nitric oxide production and increased predisposition to *Chlamydia pneumoniae* infection (Sakai et al., 2006). Stimulation of the PI3K/Akt pathway has downstream positive effects on NF-κB activation and host response (Rajaram et al., 2006). It has also been reported that PI3K and Akt are crucial in the production of RANTES ("regulated on activation, normal T cell expressed and secreted"), IL-6, and IL-12 following *F. novicida* infections (Parsa et al., 2006; Rajaram et al., 2006). *F. tularensis* Schu S4 but not *F. novicida* leads to downregulation of the regulatory p85 subunit of PI3K, as well as Akt itself (Butchar et al., 2008). Conversely, mice expressing constitutively active Akt (MyrAkt) did not succumb to *F. novicida* infections to the same extent as wild-type mice (Rajaram et al., 2006). Initiation of these pathways favors the host largely through the activation of NF-κB, which promotes survival, cytokine production, and phagosomal

maturation (Telepnev et al., 2005; Parsa et al., 2006). However, it has also been reported that wortmannin, by blocking Akt activation and mitogen-activated protein kinase phosphatase 1 (MKP1) upregulation, could enhance Mitogen-activated Protein Kinase 1 (MAPK1) and phosphorylation of the p38 MAPK, as well as cytokine release in murine BMM following infection with LVS (Medina et al., 2010). It was also shown that Complement component 3 (C3) opsonization of Schu S4 led to phosphorylation of Akt in human monocyte-derived macrophages (MDM) and that this led to an upregulation of the Erk inhibitor MKP-1 (Dai et al., 2013). Further experiments may be needed to tease out the role(s) of Akt during *Francisella* infection, but the differences seen are likely due to differences in complement, in bacterial subspecies and/or cell type. The results of Dai et al., however, point to the C3 pathway as a putative therapeutic target.

Downstream inhibitors of PI3K have also been shown to be involved with dampening responses following *Francisella* infection. Deletion of a key phosphatase, Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 (SHIP1) has been shown to permit greater cytokine production following infection of primary murine macrophages with *F. novicida* (Parsa et al., 2006). In addition, Phosphatase and tensin homolog (PTEN) is upregulated following *Francisella* Schu S4 infection of human MDM (Melillo et al., 2010). Both phosphatases serve to dampen PI3K activity, resulting in a lessening of responses such as cytokine production. SHIP1 has also been shown to attenuate Ras activity by binding Shc (Damen et al., 1996). MicroRNAs (miRs) are post-transcriptional regulators of gene expression and *Francisella* has developed methods to use microRNAs to its advantage (Cremer et al., 2009; Eulalio et al., 2012). Specifically, miR-155, which targets the 3′ UTR of SHIP1, is induced by *F. novicida* but not Schu S4, resulting in higher levels of SHIP1 with Schu S4 (Cremer et al., 2009).

THE RAS PATHWAY

Francisella also modulates the Ras-Raf-MAPK kinase-MAPK signaling pathway during infection (Al Khodor and Abu, 2010; Asare and Abu Kwaik, 2010). It has been shown that intracellular *F. novicida* triggers Ras activation within 15 min in human MDM. This occurs through Son of Sevenless 2 (SOS2)/Growth factor receptor-bound protein 2 (GRB2)/Protein kinase C α (PKCα) and Protein kinase C β1 (PKCβ1), which are essential for bacterial proliferation (Al Khodor and Abu, 2010). Along with proliferation, the Ras pathway has been linked to cell death associated with *Francisella* infection. Inhibition of MAPK1 phosphorylation prevented LVS-induced apoptosis in the J774.2 mouse macrophage cell line (Hrstka et al., 2005). Uptake of *F. novicida* also depends on MAPK1, via activation of Spleen tyrosine kinase (Syk) (Parsa et al., 2008b). Ras activation upon infection is not specific to *F. novicida*, as *Listeria monocytogenes* and *Helicobacter pylori* promote Ras activation during infection (Keates et al., 2001; Mansell et al., 2001).

In contrast to observations with *F. novicida*, it has been shown that C3-opsonized Schu S4 dampens activation of MAPK1, p38 MAPK, and NF-κB, along with cytokine production in human monocyte-derived macrophages (Dai et al., 2013). Cytokine responses to non-opsonized Schu S4 were stronger, although not as strong as the responses to *F. novicida*, and C3 opsonization did not alter the responses to *F. novicida* (Dai et al., 2013). These C3-mediated dampening effects appeared to be due to activation of the protein tyrosine kinase LYN (Dai et al., 2013). Hence, it appears as though there are differences in response to *F. novicida* and *F. tularensis* that suggest caution when making inferences from one to the other. Additional studies will be required in order to tease out the intricacies of virulent *Francisella* and Ras.

Effectively targeting the Ras pathway may provide a novel means of combating *F. tularensis*. Celecoxib, an FDA-approved cyclooxygenase 2 (COX-2) inhibitor normally administered as an anti-inflammatory agent, has recently been implicated in the upregulation of MAPK1 and/or p38 MAPK activity in head and neck squamous cell carcinoma cell lines, inhibiting their proliferation (Park et al., 2010). Importantly, a potent antimicrobial activity of celecoxib and a derivative has been reported, which appears specific against *Francisella*. Celecoxib and a pharmacologic derivative termed Compound 20, killed *F. novicida*, LVS, and Schu S4 in growth media. In addition, compound 20 inhibited the growth of *F. novicida* and Schu S4 in Raw 264.7 mouse macrophage cells (Chiu et al., 2009). Hence, celecoxib or related compounds may offer a dual effect against *Francisella*: promoting host cell responses and direct killing.

CAN WE FIGHT *Francisella* WITHOUT ANTIBIOTICS?

Research to date points to immunosuppression as a critical factor in the virulence of *Francisella*. This leads to the hypothesis that enhancing inflammatory responses would serve to combat infection. Although cytokines such as TNFα, IL-1β, and IFNγ are known to activate certain aspects of cellular immune responses and are known to be attenuated by *Francisella*, treatment with these or other such agents may not be sufficient to combat an antibiotic-resistant form of this pathogen. For example, even though IFNγ may promote phago-lysosomal fusion upon infection with the less virulent *F. novicida* (Santic et al., 2005), it does not appear to protect human MDM against Schu S4 (Melillo et al., 2010). Hence, although IFNγ may be important for combating *Francisella*, it in itself is not sufficient. Likewise, the specific therapeutic targeting of other pathways may not be sufficient to mount a successful immune response against *Francisella*. Another approach may be to stimulate the production and activation of monocytes through administration of a factor such as Granulocyte-macrophage colony-stimulating factor (GM-CSF). This factor acts on both monocytes and neutrophils, and has been tested extensively as an antitumor agent (Waller, 2007). Along with this, it can enhance the activity, including respiratory burst, of monocytes as has been shown *ex vivo* with septic patient monocytes (Williams et al., 1998). Although GM-CSF did not reduce intra-macrophage growth of LVS (Anthony et al., 1992), there is a possibility that it may show some efficacy *in vivo*.

Alternatively, it is likely that the simultaneous activation of multiple immune response pathways will be required. One potential non-antibiotic-based treatment that has already made its way into the cancer arena is the use of immunomodulatory agents. Indeed, this is being actively pursued as a potential treatment for sepsis as well as for several viral and bacterial infections (reviewed in Savva and Roger, 2013).

Immunomodulators have been studied and used for the treatment of cancer for well over 100 years. Tumors exert a strong immunosuppressive effect on host immune responses, even to the point where they co-opt immune cells for the production of factors that promote growth, survival, and angiogenesis (Becker et al., 2013; Kushner and Bautch, 2013). From this perspective, perhaps there are enough similarities between *Francisella* and tumor cells with regard to immunosuppression [e.g., both involve Transforming growth factor β (TGFβ) production (Bosio et al., 2007; Becker et al., 2013)] that these compounds would be effective in treating tularemia. In fact, it has recently been shown that administration of a TLR4 agonist conferred protection against *F. novicida* infection in mice (Lembo et al., 2008). It was later shown that a mix of DNA-liposome complexes plus *Francisella* membrane fractions could protect mice from *F. tularensis* infection (Ireland et al., 2010).

The first promising immunomodulator described in the literature was Coley's Toxin, produced by Coley (1891). This was a mix of bacteria that typically resulted in fever and malaise after injection but oftentimes led to the reduction or elimination of the patients' tumors. Since then, research has uncovered mechanisms both by which host immune cells respond to such "toxins" and by which tumor cells act to suppress immune responses (Becker et al., 2013; Broz and Monack, 2013). A later bacterially-based therapy was bacillus Calmette-Guerin (BCG), used as a tuberculosis vaccine and subsequently approved for treating bladder cancer (Vacchelli et al., 2013). Synthetic agents were also being developed such as imiquimod (Chen et al., 1988) and resiquimod (Tomai et al., 1995). Imiquimod (brand name Aldara) was approved in 1997 for the treatment of genital warts and certain skin cancers. Although such compounds are developed on an ongoing basis, the common theme is that as TLR agonists, they possess the ability to activate multiple immune-response-related pathways simultaneously (Brown et al., 2011). This fuller spectrum of activation, in contrast to single-pathway treatment such as with Interferons, carries the potential to more effectively combat *Francisella* infection.

Monocytes express most TLR, although with low levels of TLR9 and virtually no TLR3 (Hornung et al., 2002), so it is likely that most immunomodulators will lead to their activation. Perhaps as importantly, activation of monocytes with these compounds can also indirectly elicit responses from neighboring cells. For example, the TLR7/8 agonist resiquimod promoted the production of IFNγ from Natural Killer (NK) cells *in vitro*, but only through monocyte-derived IL-12 during co-culture (Hart et al., 2005). Direct or indirect effects on other cells have been well-documented as well. For example, resiquimod has been shown to promote dendritic cell maturation and antigen presentation (Ahonen et al., 1999), and treatment of PBMC with the TLR8-selective agonist VTX-2337 led to enhanced dendritic cell maturation as well as more effective priming of $CD8^+$ T cells (Stephenson et al., 2013). Hence, although monocytes respond strongly to such agonists, functional responses *in vivo* and in humans will result from the culmination of direct and indirect responses among multiple cell types. Although immunomodulators continue to be developed, it may be beneficial to begin testing the efficacy of agents that are currently approved for other uses in humans. For example, BCG and even perhaps the topically-applied Imiquimod could be tested in mouse models. There are numerous mouse models of infection that can be utilized (Conlan et al., 2011), so a plethora of possibilities exists.

Other than the well-characterized side effects associated with the use of immunomodulators such as fever, nausea and malaise (Witt et al., 1993; Goldstein et al., 1998; Pockros et al., 2007; Weigel et al., 2012), there are additional considerations. For example, as demonstrated by Ireland et al. with macrophages (Ireland et al., 2010), the timing of immunomodulator administration may be a critical factor in the efficacy of treatment. With *Francisella* in particular, the more virulent *F. tularensis* Schu S4 has been shown to alter the expression and function of immune response factors. For example, most TLR (as well as the MyD88 adaptor protein) in monocytes are down-regulated following infection with *F. tularensis* (Butchar et al., 2008). Similarly, it has been shown that *F. tularensis* can block NF-κB activation, PKB/Akt phosphorylation and cytokine production in macrophages (Melillo et al., 2010). Hence, within the context of tularemia it would be hoped that enough monocytes/macrophages (and other cells) would come into contact with the agonist before encountering *Francisella*, such that a more effective immune response could be attained. Along with timing, it is important to consider that repeated dosages of immunomodulators may not be fully effective. Endotoxin tolerance following an initial stimulus can lead to hyporesponsiveness to subsequent stimuli (Greisman and Hornick, 1975; West and Heagy, 2002; Morris and Li, 2012). It has been shown in a mouse model of tumor immunotherapy that systemic administration of the TLR7/8 agonist resiquimod led to such hyporesponsiveness, which was overcome by altering the timing of repeated injections (Bourquin et al., 2011). Based on this, it seems probable that immunomodulators by themselves will not be fully effective against *Francisella*.

The use of therapeutic antibodies within the cancer field has been ongoing since 1997 with the advent of Rituximab, and several others are in use or testing for a variety of cancers. Much of their efficacy has been attributed to antibody-dependent cellular cytotoxicity (ADCC) (Sliwkowski et al., 1999; Clynes et al., 2000), which monocytes are capable of performing (Shaw et al., 1978). There have also been efforts to engineer these antibodies for better binding, and/or for drug delivery to target cells (Vincent and Zurini, 2012).

Antibodies within the context of *Francisella* have also been examined preclinically, with promising results. (Stenmark et al., 2003; Stenmark and Sjostedt, 2004). Of particular note, human serum from a person infected with *F. tularensis holarctica* was able to confer protection against *F. tularensis holarctica* in mice (Stenmark et al., 2003). Stenmark and Sjostedt went on to show that immune serum led to increases in both TNFα and IL-12 (Stenmark and Sjostedt, 2004), which had been previously shown to be important within the context of *Francisella* infection (Stenmark et al., 1999). From a practical perspective, it may not be feasible to isolate anti-*Francisella* antibodies from people who survived infection and use them to treat currently-infected patients. In addition, there are currently few commercially-available antibodies against virulent *F. tularensis*.

However, DNA-based technology has made the production of monoclonal antibodies far less cumbersome so it is not unreasonable to predict that a battery of humanized antibodies could be available in the future. Furthermore, as with several antitumor antibodies, such new anti-*Francisella* antibodies may be engineered to enhance binding and/or immunogenicity. Due to the immunosuppressive nature of *F. tularensis*, however, it is possible that anti-*Francisella* antibodies alone will not be fully effective for all patients.

Perhaps a combination of immunomodulators and anti-*Francisella* antibodies should be explored, as it has been shown that the two together can lead to superadditive immune responses. For example, we found that treatment of human monocytes with the TLR7/8 agonist resiquimod led to synergistic increases in IgG-mediated TNFα production. Resiquimod also enhanced monocyte-mediated ADCC against a tumor cell line and synergistically improved the efficacy of antitumor antibody therapy *in vivo*. Interestingly, resiquimod modulated not only the function but also the expression of monocyte Fcγ receptors (FcγR), such that activating receptors were upregulated and the inhibitory FcγRIIb was downregulated (Butchar et al., 2010). Similarly, the TLR8-selective agonist VTX-2337 was shown to increase the effectiveness of NK cell-mediated ADCC (Lu et al., 2012). Although not all TLR agonists may modulate monocyte FcγR expression to equal extents, it is likely that at least one or more FDA-approved agents such as BCG could. Within the context of tularemia, this dual therapy might successfully combat the immunosuppressive effects of *F. tularensis* and direct the host immune cells specifically against this pathogen. It remains to be tested, however, whether such treatment can offset the *Francisella*-mediated suppression seen upon contact and phagocytosis. Lastly, from a treatment point of view, the synergistic effects of dual treatment might permit the use of lower dosages and thereby minimize untoward effects.

CONCLUSION

Francisella has evolved methods to escape and suppress host cell immune responses. This might be counteracted via the use of immunomodulatory agents or antibodies, and the combination of both may lead to the best results. Further research may lead to the successful development and testing of such agents.

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