

# Host–pathogen interactions and immune evasion strategies in *Francisella tularensis* pathogenicity

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**Abstract:** *Francisella tularensis* is an intracellular Gram-negative bacterium that causes life-threatening tularemia. Although the prevalence of natural infection is low, *F. tularensis* remains a tier I priority pathogen due to its extreme virulence and ease of aerosol dissemination. *F. tularensis* can infect a host through multiple routes, including the intradermal and respiratory routes. Respiratory infection can result from a very small inoculum (ten organisms or fewer) and is the most lethal form of infection. Following infection, *F. tularensis* employs strategies for immune evasion that delay the immune response, permitting systemic distribution and induction of sepsis. In this review we summarize the current knowledge of *F. tularensis* in an immunological context, with emphasis on the host response and bacterial evasion of that response.

**Keywords:** LVS, Schu S4, tularemia, host immunity, *Francisella tularensis*

## Introduction

The bacterium *Francisella tularensis* is the causative agent of the disease tularemia. Originally discovered in ground squirrels in Tulare County, CA, USA, *F. tularensis* has previously been termed *Bacterium tularense* and *Pasteurella tularensis*.<sup>1–3</sup> The first known infection of a human with *F. tularensis* was identified in 1913.<sup>2</sup> The bacterium has a coccobacillus shape, stains Gram-negative, and is nonmotile.<sup>4,5</sup>

*F. tularensis* exists as multiple subspecies, including the “type A” subspecies *F. tularensis tularensis* and the “type B” subspecies *F. tularensis holarctica* and *F. tularensis mediasiatica*. Additionally, there exists a related species, *Francisella novicida*.<sup>6–8</sup> Subspecies *F. tularensis tularensis*, *holarctica*, and *mediasiatica* can all cause infection in humans, although only subspecies *F. tularensis tularensis* is appreciably lethal. By contrast, *F. novicida* has been reported to cause infection only in immunocompromised individuals.<sup>7</sup>

Humans can contract infection with *F. tularensis* through several routes, including arthropod bites, contact with infected animals or animal carcasses, ingestion of contaminated materials, or inhalation.<sup>6,9</sup> Infection typically produces a febrile illness, although specific pathology and mortality rates are highly dependent upon the route of infection.<sup>1,2,6,10</sup> The most common presentation is glandular or ulceroglandular tularemia, in which infection occurs through the skin and causes localized inflammation and infection of local lymph nodes.<sup>6</sup> Ulcers produced by ulceroglandular tularemia are persistent, but the infection is rarely lethal and can often resolve without treatment.<sup>6</sup> *F. tularensis* can also cause typhoidal tularemia, which is characterized by systemic infection without lymphadenopathy or development of ulcers.<sup>6</sup> Typhoidal tularemia is more serious, with a mortality rate potentially as high as 60%.<sup>6</sup> Less commonly,

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infection can present as oropharyngeal or gastrointestinal tularemia resulting from ingestion of contaminated materials, or as oculoglandular tularemia as a result of infection of the conjunctiva of the eyes.<sup>6</sup> The most serious form of *Francisella* infection, pneumonic tularemia, is typically caused by inhalation of bacterial aerosols or airborne suspensions of infectious material. Type A *F. tularensis* is extraordinarily infectious and highly lethal when infection occurs through the respiratory route, with a case fatality rate in excess of 60% and a minimum infectious dose of fewer than 10 colony-forming units (CFU).<sup>10</sup>

The extreme respiratory infectivity of type A *F. tularensis* and its high survivability as an aerosol, combined with the high lethality of the pneumonic form of infection, have made the organism an attractive candidate for weaponization, and several governments produced large quantities of *F. tularensis* for that purpose during the 20th century.<sup>8,11–14</sup> Today, *F. tularensis* remains a potential agent of biological terrorism.<sup>14</sup> To date, there is no licensed vaccine against *F. tularensis*. An attenuated strain of type B *F. tularensis*, the live vaccine strain (LVS), was developed in the mid-20th century by serial passage of *F. tularensis holarctica* through mice.<sup>15,16</sup> LVS is attenuated in humans but retains virulence in mice. Salomonsson et al<sup>17</sup> found that complementation of LVS with the genes *pilA* and FTT0918 restored virulence to the level of virulent type B strains in subcutaneous infection. However, the mechanisms responsible for attenuation of LVS, particularly in models of respiratory infection, remain incompletely understood, leading to fears that LVS may revert to virulence or may cause disease in immunocompromised individuals.

## Virulence determinants

Several virulence determinants have been proposed for *F. tularensis*, many of which are expressed as components of the *Francisella* pathogenicity island.<sup>18</sup> These bacterial products, as well as others, augment virulence through several mechanisms, including suppression of the host immune response and facilitation of phagosomal escape and intracellular survival.

The *Francisella* pathogenicity island was first described in 2004.<sup>19</sup> It contains 17 open reading frames of varying size, several of which have been identified as essential for pathogenesis.<sup>19</sup> Intriguingly, the *Francisella* pathogenicity island is characterized by a lower percentage of guanine and cytosine nucleotides compared with the rest of the *Francisella* genome, which itself has a fairly low guanine and cytosine content.<sup>18,19</sup> In the study that identified the *Francisella*

pathogenicity island, the *pdpA* gene was identified as being essential for virulence, but its function and the functions of the other *pdp* genes are not known.<sup>19,20</sup> The *pdpD* gene was found to be present in *F. novicida* and in type A subspecies of *F. tularensis* but not in type B subspecies, and it has been implicated in intramacrophage survival.<sup>18,19</sup> Several genes within the *Francisella* pathogenicity island, including *vgrG*, *dotU*, and many of the *Igl* genes, share sequence homology as well as biochemical and structural characteristics with type VI secretion genes of other bacteria.<sup>21,22</sup> It is therefore not surprising that the products of *vgrG* and *IgII* have been found to be secreted by the bacterium during intracellular infection.<sup>23</sup> Several *Francisella* pathogenicity island proteins, including the IglC protein, have been shown to inhibit phagosome maturation.<sup>24,25</sup> These proteins also appear to be involved in escape from the phagosome and intracellular survival. IglC and *clpB* have also been implicated in evasion of immunity, as *F. novicida* mutants with defects in these genes fail to induce secretion of immunosuppressive prostaglandin E<sub>2</sub> (PGE<sub>2</sub>).<sup>26</sup> It is worth noting that most of the proteins secreted by the *Francisella* type VI secretion system, with the exception of *vgrG*, are unique to the *Francisella* genus.<sup>27</sup>

Control of reactive oxygen species (ROS) and nitrogen species is also an essential component of *Francisella* virulence. The enzyme KatG possesses both catalase and peroxidase properties, allowing it to detoxify hydrogen peroxide as well as reactive nitrogen species.<sup>28,29</sup> KatG plays a significant role in the virulence of *F. tularensis* LVS, as mutants lacking a functional *katG* gene failed to kill mice after intraperitoneal inoculation.<sup>29</sup> In contrast, *katG* mutants of Schu S4 were sensitive to hydrogen peroxide and reactive nitrogen species in vitro but retained lethality in mice, indicating that KatG is a sufficient but not necessary virulence factor.<sup>29</sup> Similarly, the superoxide dismutases SodB and SodC are essential for bacterial resistance to superoxide radicals, as *F. tularensis* LVS mutants of either enzyme are highly attenuated in mice challenged intranasally.<sup>30,31</sup>

## Host–pathogen interactions

### Bacterial survival and replication

*F. tularensis* has long been considered to be an intracellular pathogen. The first evidence that *F. tularensis* could replicate intracellularly came from studies of infected chick embryos and, later, HeLa cells.<sup>32,33</sup> Notably, these early investigations showed that bacteria were present in the cytoplasm of infected cells.

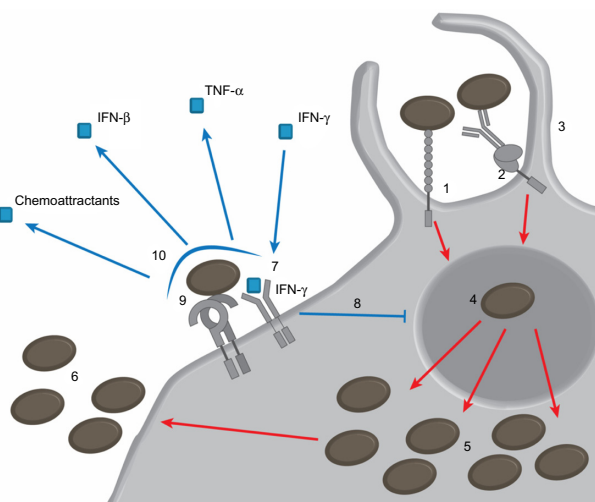
Later work demonstrated that *F. tularensis* is also capable of replicating within macrophages. In 1995, Fortier et al<sup>34</sup>

showed that *F. tularensis* LVS grew to high concentrations in the presence of peritoneal macrophages but not in the presence of lysed macrophages or in macrophage-conditioned media. *F. tularensis* is taken up by macrophages through a unique form of phagocytosis called “looping phagocytosis”, in which extensions of the cell membrane engulf a large volume of space surrounding the bacterium in an actin-dependent manner.<sup>35,36</sup> The large space within the loop does not result from the presence of a bacterial capsule, as the volume of the vacuole is reduced rapidly after phagocytosis.<sup>36</sup> Entry into the cell can involve a variety of surface receptors, including mannose receptors, Fc receptors, and complement receptors.<sup>35,37–40</sup> Both virulent and attenuated strains of *F. tularensis* survive phagocytosis by preventing acidification of the phagosome and arresting its maturation, and the organism ultimately escapes into the cytoplasm.<sup>41–43</sup> This process is summarized in Figure 1. There is evidence that following escape of *F. tularensis* LVS into the cytoplasm, a proportion of cytoplasmic bacteria re-enters the endocytic pathway through autophagy.<sup>44</sup> It is not certain whether this process is an adaptive strategy by the bacterium or a part of the cellular defense against infection; however, evidence suggests that autophagy provides intracellular bacteria with nutrients required for replication.<sup>45</sup> The replication process ends in the destruction of the host cell. Lai et al<sup>46</sup> have shown that infection of the macrophage-like J774 cell line with LVS

results in apoptosis. This apoptosis is not a self-sacrificing defense mechanism on the part of the cell, as apoptosis was not observed to reduce bacterial numbers.

*F. tularensis* may also be able to use nonmacrophage cells as hosts. LVS has been observed to replicate within alveolar type II (ATII) epithelial cells in vitro, and experiments using green fluorescent protein-expressing *F. tularensis* of both type A and type B strains have detected labeled bacteria within neutrophils after intranasal infection.<sup>47,48</sup> Experiments employing LVS and Schu S4  $\Delta$ *pyrF* mutants, which fail to replicate within primary macrophages, showed that such mutants were fully virulent in mice infected intranasally, suggesting that *F. tularensis* is not dependent upon replication within macrophages for survival and virulence.<sup>49</sup> Despite the proclivity of *F. tularensis* for intracellular replication, the majority of bloodborne bacteria of both type A and type B strains are extracellular.<sup>50,51</sup>

Iron is a key requirement for intracellular growth of *F. tularensis*.<sup>34,52,53</sup> Investigators have found that iron concentrations in growth media can affect expression of the *Francisella* pathogenicity island genes and can induce the organism to adopt a “host-adapted” phenotype.<sup>18,54</sup> There is evidence that *F. tularensis* actively modulates expression of transferrin receptors in order to promote delivery of iron to the phagosome during the early stages of intracellular growth.<sup>55</sup> However, iron uptake may paradoxically render bacteria more susceptible to intracellular killing by ROS.<sup>56</sup> The highly virulent type A strain Schu S4 has been shown to have a lower iron content than the attenuated type B strain LVS, which may be a factor in the latter’s reduced virulence.<sup>56</sup>



**Figure 1** *Francisella tularensis* (brown) binds to the cell surface using receptors such as the mannose receptor (1) or, in the case of opsonized bacteria, Fc receptors (2) or complement receptors. Bacteria enter the cell through looping phagocytosis (3) but survive by preventing maturation of the phagosome (4). *F. tularensis* escapes the phagosome to replicate in the cytoplasm (5), ultimately inducing apoptosis and escaping the cell (6). When macrophages are stimulated by exposure to IFN- $\gamma$  (7), they can restrict intracellular replication (8). Stimulation by IFN- $\gamma$  and stimulation of Toll-like receptor 2 (9) can also lead to secretion of inflammatory cytokines and chemoattractants (10).

**Abbreviations:** IFN, interferon; TNF, tumor necrosis factor.

## Pathogenesis

The earliest descriptions of tularemia included animals as varied as rodents, lagomorphs, and monkeys. These studies indicated that infection produced a febrile illness and generated foci of infection and necrotic inflammation in the lymph nodes as well as in dispersed organ sites.<sup>1,57</sup> Morphological changes to the liver and spleen, particularly splenomegaly, have also been observed.<sup>57,58</sup> Pneumonia is a frequent occurrence, even when the initial infection occurs through a route other than the respiratory route.<sup>10,59,60</sup> Following intravenous infection with *F. tularensis* LVS, infectious foci were detected in liver tissue after 16 hours, accompanied by infiltration of both neutrophils and monocytes.<sup>61</sup> Systemic dissemination also occurs following respiratory infection. Following aerosol infection with *F. tularensis* LVS, bacteria can be isolated from livers and spleens as early as 48 hours later.<sup>62</sup> In humans,

infection is known to produce fever, persistent malaise, and ulcerous lesions or buboes.<sup>2,10,59,60</sup>

A major factor in *F. tularensis* pathogenicity is its capacity for intracellular replication. It has long been recognized that *F. tularensis* can replicate within host cells,<sup>32,33</sup> and particularly macrophages.<sup>46,63,64</sup> This intracellular replication strategy contributes to pathogenesis as infected cells either apoptose or become lysed by uninfected immune cells.<sup>46,61</sup> Intracellular replication may also play a role in dissemination of the infection to distant sites. In 2008, Bar-Haim et al<sup>65</sup> showed that following intranasal *F. tularensis* LVS infection, dendritic cells trafficked to the mediastinal lymph nodes, which quickly became severely infected.

Death in *F. tularensis* infection appears to result from widespread sepsis and inflammation rather than bacterial pneumonia, even when infection occurs through the respiratory route.<sup>66-69</sup> In a study of pneumonic *F. tularensis* LVS infection, high levels of inflammatory cytokines and chemokines in the lungs and spleen, including interleukin (IL)-6, macrophage inflammatory protein 2, and chemokine ligand 2, were correlated with mortality.<sup>58</sup> Investigations involving respiratory infection with *F. tularensis* Schu S4 and *F. novicida* showed that lethal infection was associated with hypercytokinemia and biochemical markers for sepsis.<sup>66,69</sup> This sepsis may result from the fact that in *F. tularensis* infection, upregulation of cytokine production and inflammatory cell recruitment is delayed,<sup>66</sup> and the high degree of apoptotic cell debris associated with *F. tularensis* infection leads to alternative activation of macrophages, impeding bacterial clearance.<sup>70</sup> In lung infection, excessive levels of neutrophil recruitment may also contribute to pathology. Although neutrophil recruitment is delayed in *Francisella* infection,<sup>66</sup> high levels of neutrophil recruitment are associated with increased lung histopathology and, somewhat paradoxically, high bacterial burden.<sup>71</sup> These findings may be explained in part by observations that human neutrophils infected with *F. tularensis* LVS or Schu S4 exhibit a delay in time to apoptosis, preventing resolution of inflammation.<sup>72</sup>

## Innate immunity

The type 1-associated immune pathway is known to be an important factor in protection from *F. tularensis* infection. After cutaneous infection with *F. tularensis* LVS, immunized mice produced large quantities of IL-12 within 24 hours of infection.<sup>73</sup> Another study found that IL-12 p40-knockout mice failed to clear LVS administered intraperitoneally, even after immunization by sublethal intradermal infection.<sup>74</sup> These mice also had significantly lower levels of serum interferon

(IFN)- $\gamma$ , suggesting a connection between secretion of IL-12 and the IFN- $\gamma$  response. The p40 subunit of IL-12 was also shown to induce migration of dendritic cells from the lungs to the draining lymph nodes after LVS infection.<sup>75</sup>

After intradermal infection with LVS, IFN- $\gamma$  messenger ribonucleic acid expression can be detected within 48 hours in naïve mice and within 24 hours in immunized mice.<sup>73</sup> In *F. tularensis* infection, both natural killer (NK) cells and T-cells are important producers of IFN- $\gamma$ , although NK cells are the dominant source at early time points.<sup>76,77</sup> Following intranasal *F. tularensis* LVS infection, NK cells are among the first cells recruited to the lungs, and can be observed to secrete IFN- $\gamma$  within 72 hours of infection.<sup>76,78</sup> NK cells primed with bacterial deoxyribonucleic acid can reduce replication of *F. tularensis* LVS in macrophages, even in the absence of T- or B-cells, and this effect is dependent upon IFN- $\gamma$  and TNF- $\alpha$ .<sup>79</sup> IFN- $\gamma$  is essential to host survival following pneumonic infection, as IFN- $\gamma$  knockout mice challenged by the respiratory route exhibited greater rates of mortality and higher lung bacterial burden than wild-type (WT) mice.<sup>80,81</sup> Although exogenous treatment with IL-12 was shown to be protective against intranasal infection, this protection was not evident in IFN- $\gamma$  knockout mice, indicating that the protective effect of IL-12 is dependent upon IFN- $\gamma$  expression.<sup>81</sup>

Despite the capacity of *F. tularensis* to replicate intracellularly in vitro, it has been known for some time that IFN- $\gamma$ -stimulated macrophages play an important role in defense against this infection. In 1992, Fortier et al<sup>64</sup> showed that exposure of macrophages to IFN- $\gamma$  caused activation of these cells and allowed controlled replication of *F. tularensis* LVS in vitro. The authors attributed this to production of nitric oxide. A later paper by the same laboratory, however, showed that although IFN- $\gamma$ -stimulated alveolar macrophages produced nitric oxide, their antimicrobial activity against *F. tularensis* was independent of nitric oxide production.<sup>82</sup> In other macrophage populations, reactive nitrogen species appear to take on greater importance. A 2005 study indicated that IFN- $\gamma$ -stimulated peritoneal exudate cells secrete nitric oxide and control bacterial replication, but inducible nitric oxide synthase (iNOS) knockout mice or pharmacological inhibition of iNOS abrogated this bactericidal effect.<sup>83</sup> In contrast, knockout of p47, a component of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, which induces the respiratory burst, reduced but did not eliminate bactericidal activity.<sup>83</sup> IFN- $\gamma$ -treated alveolar macrophages produced large quantities of tumor necrosis factor (TNF)- $\alpha$ , but TNF- $\alpha$  is not essential for protection



in vitro when IFN- $\gamma$  is present. Alveolar macrophages treated with recombinant IFN- $\gamma$  produced reactive nitrogen species and controlled LVS burden equally well in the presence or absence of neutralizing monoclonal antibodies against TNF- $\alpha$ .<sup>82</sup> In vivo, both IFN- $\gamma$  and TNF- $\alpha$  were found to be essential for survival of primary intravenous infection in mice infected with *F. tularensis* LVS.<sup>84</sup> Neutralization of TNF- $\alpha$  or IFN- $\gamma$  in vivo rendered naïve mice unable to control bacterial replication after *F. tularensis* LVS infection. Immunized mice fared better at low infectious doses but proved unable to control infection against an intravenous dose exceeding 10<sup>6</sup> CFU.<sup>84</sup>

Macrophages can also respond to infection through means other than the production of nitric oxide or the respiratory burst. In 2007, it was found that infection of murine macrophages with *F. novicida* resulted in activation of the inflammasome in a manner dependent upon type I interferons.<sup>85</sup> Cole et al<sup>86</sup> found that macrophages detect *F. tularensis* LVS through both Toll-like receptor 2 (TLR2) and cytosolic signaling, resulting in secretion of IFN- $\beta$ . TLR2 knockout mice were found to be impaired in their ability to facilitate TNF secretion from macrophages, although this effect did not specifically require a complex of TLR2 with either TLR1 or TLR6.<sup>87</sup> Although IFN- $\gamma$  was found to restrict replication of the virulent type A strain Schu S4 within both murine bone marrow-derived macrophages and human blood monocyte-derived macrophages, this effect was found to be independent of either NADPH oxidase or iNOS.<sup>88</sup> The roles of IFN- $\gamma$  and TLR2 are summarized in Figure 1.

The role of neutrophils in *F. tularensis* infection is somewhat controversial and may be dependent upon the site of infection. Early research using the neutrophil-depleting monoclonal antibody RB6-8C5 indicated that neutrophils were essential for survival and control of bacterial replication when mice were infected intradermally or intravenously with *F. tularensis* LVS.<sup>89</sup> Intranasal infection resulted in recruitment of neutrophils to the lungs within 72 hours of infection.<sup>90</sup> However, the authors observed that cell depletion or increased recruitment of neutrophils to the lungs failed to affect either bacterial burden or time to death. Mice deficient in gp91<sup>phox</sup> were slightly more susceptible to low infectious doses, indicating a role for NADPH oxidase.<sup>90</sup> Some investigators have suggested that neutrophil recruitment results in harmful levels of inflammation after respiratory infection. Expression of matrix metalloproteinase 9 was associated with production of the neutrophil chemoattractant tripeptide proline-glycine-proline and with increased neutrophil recruitment and severe histopathology after *F. tularensis* infection.<sup>71</sup>

Knockout of IL-10 resulted in increased expression of IL-17, which led to increased neutrophil recruitment into the lungs after intranasal infection.<sup>91</sup> However, although IL-10 knockout mice were protected against cutaneous infection, they were more susceptible than WT mice to pneumonic infection.

Other cells present in the lung are also involved in *F. tularensis* infection. Following infection of mice with green fluorescent protein-expressing *F. tularensis* LVS and Schu S4, bacteria were detected by flow cytometry within ATII epithelial cells.<sup>47,48</sup> However, Gentry et al<sup>92</sup> showed that human ATII cells in an in vitro transwell system responded to both LVS and Schu S4 with NF- $\kappa$ B-dependent upregulation of IL-8. Mast cells have been found to inhibit replication of *F. tularensis* LVS within mouse macrophages through direct contact and via secretion of IL-4.<sup>93,94</sup>

## Cell-mediated immunity

As *F. tularensis* spends much of its existence within host cells, cell-mediated immunity likely plays a major role in control of tularemia. Early work indicated that splenocytes adoptively transferred from mice immunized with *F. tularensis* LVS could protect naïve mice from the virulent strain Schu S4.<sup>95</sup> However, this work failed to make a distinction between the potential roles of T-cells and B-cells. It was not until 1991 that experiments were conducted to determine the relative contributions of different splenocyte populations to adaptive immunity.<sup>96</sup> In those experiments, Fortier et al<sup>96</sup> inoculated splenocytes from LVS-immunized mice into naïve mice. Although immune splenocytes protected mice from an otherwise lethal intradermal LVS infection, depletion of T-cells removed the protective effect, implicating T-cells as a major component of the adaptive response to *F. tularensis*. In agreement with these results, vaccines designed to promote a cell-mediated adaptive immune response have had promising results. Mice vaccinated with *Salmonella typhimurium*, which was engineered to express the lipoprotein TUL4, had lower bacterial burdens after LVS challenge than mice vaccinated with WT *S. typhimurium*, even though the modified *S. typhimurium* produced a weaker adaptive response than LVS itself.<sup>97</sup> Although these mice generated antibody responses against TUL4, protection was abolished after cyclosporin A treatment to inhibit T-cell activation, suggesting that protection was predominantly cell mediated rather than humoral. Similarly, vaccination with immunostimulating complexes that were composed of micelles impregnated with TUL4 and the adjuvant Quil A, designed to promote T-cell responses, resulted in reduced bacterial burdens after LVS challenge.<sup>98</sup>

Another study found that in mice vaccinated with *F. tularensis* LVS and rechallenged 90 days later with Schu S4, survival was correlated with activation of T-cells in the spleen.<sup>99</sup> As bacterial burden up to day 4 of Schu S4 infection was found to be significantly different between LVS-immunized and nonimmunized mice in the spleen, but not in the lungs, the authors concluded that cell-mediated immunity conferred protection against systemic dissemination of *F. tularensis* rather than against replication in the lungs. However, another study found that airway administration of IL-17A, but not intraperitoneal administration, delayed time to death following respiratory *F. tularensis* LVS infection.<sup>100</sup> The T helper 1 (Th1)-associated transcription factor T-bet has been reported to play an important role in control of *F. tularensis* LVS infection. However, although T-bet knockout T-cells from lungs were unable to control intracellular bacterial replication in macrophages, T-bet knockout splenocytes were not deficient in this capacity, suggesting different roles for these two T-cell populations.<sup>101</sup>

Our understanding of the relative importance of different T-cell populations is still evolving. Cowley et al<sup>102</sup> showed that membrane-bound TNF- $\alpha$  expressed by CD8<sup>+</sup> and CD4<sup>+</sup> T-cells contributed to control of intracellular replication in macrophages but was essential only for protection mediated by CD8<sup>+</sup> T-cells. It was further found that IL-23-mediated stimulation of Th17 cells promoted secretion of IL-17A, which contributed to the Th1 and IL-12 responses against *F. tularensis* LVS.<sup>103</sup> This pathway was essential in controlling lung bacterial burden after intratracheal infection. Other investigators have found that airway administration of IL-23 and IL-17A delayed time to death (although these treatments did not increase survival), and neutralization of these cytokines by monoclonal antibody increased mortality.<sup>100</sup> Cowley et al<sup>104–106</sup> identified another population of T-cells that controls intracellular replication of *F. tularensis* LVS through IFN- $\gamma$ -dependent and -independent mechanisms, including production of IL-17A. These T-cells express CD3 and  $\alpha\beta$  T-cell receptors but do not express CD4, CD8, or NK cell markers.

Cell-mediated immunity can operate in synergy with humoral immunity to protect against *F. tularensis* infection. Although immune serum has been shown to protect against *F. tularensis* LVS infection, the protective effect of serum transfer is dependent upon both T-cells and IFN- $\gamma$ .<sup>96,107</sup> Mice vaccinated with *F. tularensis* lipopolysaccharide (LPS) can survive infection even in the absence of CD4<sup>+</sup> or CD8<sup>+</sup> T-cells, although depletion of CD8<sup>+</sup> T-cells results in delayed clearance.<sup>108</sup> However, serum transfer from mice immunized

with LPS and boosted with *F. tularensis* LVS failed to protect naïve mice depleted of either CD4<sup>+</sup> or CD8<sup>+</sup> T-cells.<sup>108</sup> A combination vaccine containing tetanus-toxin-conjugated O-polysaccharide (to generate an antibody response) and an LVS mutant lacking the O-polysaccharide (to generate a T-cell response) protected mice against intranasal and intradermal infection with the type A strain Schu S4 and the type B strain FSC 108 better than either vaccine component alone.<sup>109</sup> Humoral immunity may also enhance the T-cell response. It has been reported that immunoglobulin (Ig) A knockout mice recruited fewer IFN- $\gamma$ <sup>+</sup> T-cells to the lungs than WT mice 9 days after respiratory *F. tularensis* LVS infection.<sup>77</sup>

## Humoral immunity

The mouse antibody response to *F. tularensis* has been fairly well characterized. Mice infected with a sublethal infectious dose generate a robust antibody response characterized by specific IgG2 and IgM.<sup>107</sup> Rats generate a similar antibody profile against *F. tularensis* LVS.<sup>110</sup> Although antibodies are induced against a variety of bacterial antigens, a large proportion of the humoral response is directed against LPS in both mice and humans.<sup>111–113</sup>

Much of the early information pertaining to *F. tularensis* immunity was obtained from studies of the antibody response and from treatment of infected individuals with immune animal serum. Due perhaps to the variable virulence of different strains of *F. tularensis*, much of these early data were contradictory. In one of the earliest studies, Foshay<sup>60</sup> compared recovery times of tularemia patients who received only symptomatic treatment or streptomycin with those of patients who received immune serum or hyperimmune serum generated by inoculation of ungulates with formalin-fixed *F. tularensis* or highly virulent live cultures, respectively. It was found that serum treatment, especially hyperimmune serum, reduced the time necessary for recovery. Although use of animal serum in humans often led to “serum sickness”, this study was one of the first to indicate that humoral immunity could confer at least limited benefit against *F. tularensis*. Thorpe and Marcus,<sup>114</sup> on the other hand, found that passive serum transfer conferred only minor protection against virulent strains.

Other investigators have obtained evidence that antibodies contribute to survival of *F. tularensis* infection, especially against challenge with *F. tularensis* LVS, although they are not necessarily sufficient to ensure survival. Fortier et al<sup>96</sup> made the intriguing observation that, while transfer of immune serum to naïve animals could protect against an otherwise lethal challenge with *F. tularensis* LVS, transfer of T-cell-depleted splenocytes failed to confer protection,

suggesting a diminished role for B-cells. This work is partially contradicted by a report that showed higher bacterial burdens in the lungs, livers, and spleens of B-cell-knockout mice compared with WT mice after aerosol infection with *F. tularensis* LVS.<sup>80</sup> However, the authors also found no difference in mean time to death or liver pathology in B-cell-knockout mice versus WT mice after aerosol or intradermal infection.<sup>80</sup> Elkins et al<sup>115</sup> found that athymic nude mice challenged intradermally with a sublethal dose of *F. tularensis* LVS were able to survive subsequent intraperitoneal or intravenous infection with an inoculum of approximately 5,000 times the median lethal dose for nude mice. Priming with LVS did not protect against *S. typhimurium*, indicating that the protection was specific. As these mice were athymic, this protection was likely to be humoral in nature. Rhinehart-Jones et al<sup>107</sup> observed protection against *F. tularensis* LVS when serum from mice that had been infected intradermally with LVS was administered to naïve mice. These mice were also protected by transfer of LVS-specific IgG (but not IgM). Protection was found to be dependent upon host IFN- $\gamma$ , and nude mice were not protected by serum transfer, implicating a role for IFN- $\gamma$ -secreting T-cells in antibody-mediated clearance.<sup>107</sup> These results were corroborated by Sjöstedt et al,<sup>84</sup> who found that IFN- $\gamma$  and TNF- $\alpha$  were required for protection against secondary intravenous infection with *F. tularensis* LVS, although the requirement for these cytokines was reduced in immunized mice. When naïve and immunized mice were treated with neutralizing monoclonal antibodies against these cytokines, immunized mice were able to tolerate a 50-fold larger infectious dose than naïve mice. Kirimanjeswara et al<sup>116</sup> confirmed that immune serum could protect against *F. tularensis* LVS in an IFN- $\gamma$ -dependent manner, and further demonstrated that this protection was dependent upon Fc $\gamma$ R expression on phagocytes. Interestingly, Kirimanjeswara et al<sup>116</sup> were able to show protection even when serum was administered up to 48 hours after intranasal infection, validating early 20th century attempts to use immune sera therapeutically.<sup>60,116</sup> Transfer of immune serum or purified specific IgG has also been shown to protect rats against both *F. tularensis* LVS and Schu S4,<sup>110</sup> although rats have been reported to be more resistant to *F. tularensis* infection than mice.<sup>113</sup> Intriguingly, although targeting of *F. tularensis* Schu S4 to phagocyte Fc receptors by antibody opsonization enhanced phagocytosis, cellular entry using these receptors delayed bacterial replication and phagosomal escape and enhanced production of ROS.<sup>40</sup>

Several candidate antigens have been investigated as possible targets for protective humoral immunity. *F. tularensis*

LPS is poorly immunostimulatory in comparison with LPS from other Gram-negative bacteria;<sup>117</sup> however, much of the antibody response to *F. tularensis* is directed against LPS.<sup>112,113</sup> Fulop et al<sup>108</sup> have reported that mice immunized with LPS were protected against intraperitoneal *F. tularensis* LVS infection, and that serum from LPS-immunized mice was sufficient to protect naïve mice against LVS challenge. However, LPS immunization was insufficient to protect against challenge with the virulent strain Schu S4.<sup>108</sup> Twine et al<sup>118</sup> investigated the humoral response of BALB/c and CH3/HeN mice to *F. tularensis* LVS. Both mouse strains were protected against Schu S4 challenge by immunization with LVS, but C57BL/6 and DBA/2 mice were not protected. The authors found that the former strains of mice generated antibodies against numerous bacterial antigens, such as protein chain elongation factor thermo stable and peroxidase, which did not elicit antibody responses from unprotected strains.

Although IgG and IgM are highly upregulated following *F. tularensis* infection,<sup>107</sup> there is evidence to suggest that IgA is also a critical component of host defense. Baron et al<sup>78</sup> showed that WT C57BL/6 mice could be protected against lethal *F. tularensis* LVS infection by intranasal vaccination with inactivated *F. tularensis* and IL-12 as an adjuvant. However, IgA knockout mice could not be protected in this fashion. IgA knockout mice generate IgG and IgM responses to *F. tularensis* LVS that are comparable with responses of WT mice but exhibit reduced IFN- $\gamma$  responses, higher bacterial burden, and reduced survival following intranasal LVS infection.<sup>77</sup> The precise mechanisms responsible for IgA-mediated protection remain to be determined.

## Evasion of innate and adaptive immunity

*F. tularensis* is highly adept at evading recognition and destruction by the host immune system, as evidenced by the considerable delay between infection and the onset of the inflammatory response.<sup>66,119</sup> The pathogen employs multiple survival strategies, including intracellular replication, expression of an atypical LPS, and induction of alternative and aberrant activation of the immune response.

As has been described, *F. tularensis* is capable of replicating within host cells, including macrophages.<sup>32,33,64</sup> In the absence of opsonizing conditions (eg, when *F. tularensis* uses the mannose receptor to facilitate cell entry), *F. tularensis* rapidly leaves the phagosome and enters the cytoplasm, where replication occurs.<sup>40,41</sup> Although some bacteria remain extracellular throughout infection,<sup>50,51</sup> cytosolic replication may provide some protection against

detection by TLRs and other surface receptors, and from clearance by stimulated phagocytes. However, entry into the cytosol allows activation of other cellular defenses such as the inflammasome.<sup>85</sup>

Although the *F. tularensis* LPS has been used successfully to vaccinate mice against intraperitoneal infection with *F. tularensis* LVS,<sup>120,121</sup> it is well known that the LPS of *F. tularensis* is poorly immunogenic and, in particular, is a poor stimulator of TLR4.<sup>117,122</sup> This low level of TLR4 recognition has been ascribed to the unusual structure of the lipid A component.<sup>123</sup> Whereas the highly immunogenic lipid A of many Gram-negative species is hexa-acylated with short acyl chains, the lipid A of *F. tularensis* is tetra-acylated with long acyl chains.<sup>117,123,124</sup> *F. tularensis* LVS has also been reported to alter expression of its carbohydrate capsule, which incorporates polymers of LPS O-antigen, in order to evade recognition by host immunity.<sup>125</sup>

*F. tularensis* also employs several active measures to inhibit or redirect the host immune response. In 2003, Telepnev et al<sup>126</sup> found that infection of the mouse macrophage J774A.1 cell line with *F. tularensis* LVS resulted in the cells becoming unresponsive to *Escherichia coli* LPS. Infected cells failed to degrade the NF- $\kappa$ B inhibitor I $\kappa$ B and secreted less TNF- $\alpha$  and IL-1 $\beta$ . These findings were corroborated in 2005 when Bosio and Dow<sup>127</sup> reported that LVS-infected bone marrow-derived macrophages and dendritic cells, and dendritic cells isolated from the airways of *F. tularensis* LVS-infected mice, failed to secrete increased quantities of TNF- $\alpha$  and IL-6 but instead secreted increased quantities of the immunosuppressive cytokine TGF- $\beta$ . Similar observations were reported in mice infected via aerosol with type A strain Schu S4.<sup>128</sup> These immunosuppressive effects have been observed in human cells. Telepnev et al<sup>129</sup> have reported that the THP-1 human macrophage cell line and human peripheral blood monocytes infected with *F. tularensis* LVS displayed a brief period of stimulation, including NF- $\kappa$ B activation and TNF- $\alpha$  secretion, followed by inactivation of the NF- $\kappa$ B response and suppression of cytokine secretion within 5 hours. Interestingly, *F. tularensis* LVS mutated in the gene *iglC*, a *Francisella* pathogenicity island gene that encodes a 23 kDa protein produced during infection of macrophages, was unable to suppress immune activation.<sup>19,24,126,129,130</sup>

In addition to downregulating inflammatory cytokine secretion, *F. tularensis* also upregulates anti-inflammatory cytokines and promotes alternative activation of immune cells. Murine macrophages and dendritic cells infected

with *F. tularensis* LVS exhibit alternative activation, characterized by increased expression of arginase-1, IL-4, IL-13, and TGF- $\beta$ .<sup>127,131</sup> *F. tularensis* LVS also induces secretion of anti-inflammatory PGE<sub>2</sub> from bone marrow-derived mouse macrophages, although *F. novicida* mutants that do not induce PGE<sub>2</sub> secretion do not exhibit a defect in intracellular replication.<sup>26,132,133</sup> PGE<sub>2</sub> elicited by *Francisella* infection was found to downregulate major histocompatibility complex class II through an intermediary host factor dubbed FTM $\Phi$ SN (*F. tularensis* macrophage supernatant), which drives production of IL-10.<sup>134,135</sup> Predictably, Schu S4 has also been found to suppress secretion of inflammatory cytokines by infected dendritic cells; moreover, Schu S4 infection of dendritic cells also blunted the inflammatory capacity of nearby uninfected cells.<sup>136</sup> Interestingly, human dendritic cells infected with Schu S4 were found not to secrete TGF- $\beta$ , which was reported to be upregulated in murine cells infected with LVS.<sup>136</sup> It has been suggested that alternative activation of macrophages results in part from the abundance of cell debris found in *Francisella*-infected lungs. *F. novicida*-infected J774 cells exposed to large quantities of necrotic cell debris produced high levels of arginase-1 and showed reduced capacity for efferocytosis, which would lead to further accumulation of cell debris in vivo.<sup>70</sup> The reduced secretion of inflammatory cytokines and increased secretion of anti-inflammatory cytokines ultimately result in a delay in the recruitment of inflammatory cells to the lungs following pulmonary infection.<sup>48,66</sup>

*F. tularensis* is able to suppress the immune response in granulocytes as well as macrophages and dendritic cells. A 2006 report indicated that *F. tularensis* LVS phagocytized by neutrophils inhibits assembly of the NADPH oxidase complex and therefore the respiratory burst, even when the neutrophils receive additional stimulation in the form of phorbol 12-myristate 13-acetate.<sup>137</sup> Later studies showed that *F. tularensis* LVS, as well as virulent type A and type B strains, can inhibit NADPH oxidase function postassembly, and that inhibition is dependent upon *fevR*, a regulator of the *Francisella* pathogenicity island.<sup>138,139</sup>

The signaling mechanisms by which *F. tularensis* modulates the immune response remain poorly understood. It has been reported that *F. novicida* inhibits cellular responses to IFN- $\gamma$  by interfering with STAT-1 signaling.<sup>140</sup> Nallaparaju et al<sup>141</sup> have found that the *Francisella* outer membrane protein C reduces IFN- $\gamma$  signaling in infected macrophages, preventing production of nitric oxide. It has also been shown that lipid products of *F. tularensis* Schu S4 but not LVS can



inhibit IL-12 p40 secretion by inhibiting translocation of NF- $\kappa$ B, IRF1, and IRF8.<sup>142</sup>

Various factors involved in immunosuppression by, and virulence of, *F. tularensis* appear to be upregulated as a specific response to the host environment. The *Francisella* pathogenicity island gene *igIC*, which is necessary for immunosuppression, is upregulated during replication in macrophages but not during replication in Chamberlain medium.<sup>126,130</sup> Similarly, it has been observed that bacteria grown in Mueller–Hinton medium, which contains concentrations of amino acids that exceed those available to *F. tularensis* during infection, expressed increased levels of SodB and MglB and reduced levels of IglB, IglC, and KatG and longer, more capsule-like O-antigen than bacteria grown in macrophages or in the more physiologically similar Brain Heart Infusion medium.<sup>54,125</sup> These differences may explain why *F. tularensis* LVS grown on Thayer–Martin agar or in Mueller–Hinton medium is initially immunostimulatory before adopting an immunosuppressive phenotype, as the bacteria acclimate to the cellular environment.<sup>129,143</sup>

## Conclusion

*F. tularensis* has been known to science for over a century and has been the subject of an impressive body of research over that time – research that has intensified and accelerated as a result of recently increased interest in biodefense.<sup>1,14</sup> A considerable breadth of information is available on the interaction of this pathogen with mammalian hosts. In particular, it is well established that *F. tularensis* maintains a low immunological profile during early infection, evading surveillance and replicating within the relatively protective environment of the host–cell cytoplasm before accumulated cell debris and sheer bacterial load make an intense immune response unavoidable. However, despite decades of work, we have yet to develop a safe, effective, and well-characterized vaccine that protects against respiratory infection involving highly virulent type A strains. Key to the development of such a vaccine will be research on the host response to *F. tularensis* type A strains at mucosal surfaces – a field that is gaining momentum. Ultimately, defining the differences between host responses to virulent and attenuated strains of *F. tularensis* may shed important new insights into development of effective prophylactic and therapeutic treatments.

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## Disclosure

The authors report no conflicts of interest in this work.

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