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The subversion of the immune system by *Francisella tularensis*

Catharine M. Bosio*

Immunity to Pulmonary Pathogens Section, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Tom Kawula, University of North Carolina at Chapel Hill, USA
Stefanie Vogel, University of Maryland, USA

***Correspondence:**
Catharine M. Bosio, Immunity to Pulmonary Pathogens, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840, USA.
e-mail: bosioc@niaid.nih.gov

Francisella tularensis is a highly virulent bacterial pathogen and the causative agent of tularemia. Perhaps the most impressive feature of this bacterium is its ability to cause lethal disease following inoculation of as few as 15 organisms. This remarkable virulence is, in part, attributed to the ability of this microorganism to evade, disrupt, and modulate host immune responses. The objective of this review is to discuss the mechanisms utilized by *F. tularensis* to evade and inhibit innate and adaptive immune responses. The capability of *F. tularensis* to interfere with developing immunity in the host was appreciated decades ago. Early studies in humans were the first to demonstrate the ability of *F. tularensis* to suppress innate immunity. This work noted that humans suffering from tularemia failed to respond to a secondary challenge of endotoxin isolated from unrelated bacteria. Further, anecdotal observations of individuals becoming repeatedly infected with virulent strains of *F. tularensis* suggests that this bacterium also interferes with the generation of adequate adaptive immunity. Recent advances utilizing the mouse model for *in vivo* studies and human cells for *in vitro* work have identified specific bacterial and host compounds that play a role in mediating ubiquitous suppression of the host immune response. Compilation of this work will undoubtedly aid in enhancing our understanding of the myriad of mechanisms utilized by virulent *F. tularensis* for successful infection, colonization, and pathogenesis in the mammalian host.

Keywords: *Francisella tularensis*, antibody, complement, oxidative burst, macrophage, dendritic cell, inflammation, suppression

INTRODUCTION

Francisella tularensis is a small, non-motile, Gram negative bacterium, and the causative agent of tularemia. It is also a facultative intracellular pathogen. There are four primary subspecies of *F. tularensis*. *F. tularensis* subsp. *mediasiatica* and *novicida* are attenuated in humans. *F. tularensis* subsp. *holarctica* causes a mild disease in people. *F. tularensis* subsp. *tularensis* causes severe disease in humans and other mammals following exposure to small numbers (<15 bacteria) of bacteria. *F. tularensis* can be transmitted following exposure to aerosols, contaminated biological products, e.g., animal carcasses, ingestion of contaminated water, or from the bite of infected arthropod vector (as reviewed, Nigrovic and Wingerter, 2008). Once inside the host *F. tularensis* can invade multiple cell types. However, antigen presenting cells (APC) such as macrophages and dendritic cells appear to be the primary cell types targeted by the bacterium at the outset of infection (Bosio and Dow, 2005; Bosio et al., 2007; Hall et al., 2007, 2008; Bar-Haim et al., 2008).

As an intracellular pathogen, *F tularensis* must confront antimicrobial defenses present in the host at multiple steps during infection. Subversion of host immune responses begins at the site of infection. Depending on the route of entry, and prior to meeting a desirable host target cell, the bacterium must first evade killing by serum components designed to eliminate pathogens in the extracellular space. These serum components can include complement present in both naïve and immune hosts. In the vaccinated host, or those previously exposed to *F. tularensis*, serum antibodies may also participate in extracellular detection of the invading organism. Once in contact with a suitable host cell, the bacterium faces additional hurdles in place to control bacterial replication. Two of the most formidable host defense systems faced by *F. tularensis* are the reactive oxygen and reactive nitrogen systems, ROS and RNS respectively. ROS and RNS can be triggered by multiple mechanisms. Thus, the bacterium is forced to possess an arsenal of evasion strategies to either prevent triggering and/or, in some circumstances, dismantling the machinery of ROS and RNS in the host. In this review we will discuss specific strategies utilized by *F. tularensis* to successfully evade detection by the host in the extracellular space as well as disruption of the ROS and RNS in the intracellular compartment that facilitates replication, dissemination, and virulence of this bacterium.

INTERFERENCE WITH HOST RESPONSE IN THE EXTRACELLULAR SPACE

SERUM MEDIATED KILLING

Depending on the immune status of the host, serum, and/or plasma can mediate killing of bacteria via two often intertwined pathways. First, both naïve and immune animals possess the complement system. The complement system, as originally described by Jules Bordet, is comprised of heat-labile components present in plasma that enhance phagocytosis and killing of microorganisms. Today, we understand that complement can act independently, or

in conjunction with, antibodies to control pathogens. The complement system itself is made up of three pathways: the classical pathway, the mannose-binding lectin (MBL) pathway, and the alternative pathway. Each of these pathways can interact directly with pathogens, although the initial proteins and complexes that bind bacterial surfaces vary. Regardless of the pathway or proteins that initially target the microorganism, the pathways converge with the generation of C3 convertase, an enzyme that cleaves C3 to C3b. C3b is the primary effector of the complement system. This protein can act in two ways. First, C3b may directly opsonize pathogens to facilitate their phagocytosis and clearance from the host. Second, C3b plays a role in the generation of C5b. C5b forms the base of the membrane attack complex (MAC) which, when assembled, can induce direct lysis of the bacterium. These pathways are extremely well studied and there are many excellent reviews and textbook chapters that discuss them in detail (Janeway et al., 2005). Thus, only aspects of these systems that have been shown to be directly involved in control of *F. tularensis* will be discussed here.

During the early years of research on immunity to *Francisella*, it was noted that these microorganisms were relatively resistant to killing following exposure to human serum (Lofgren et al., 1983). This suggested that one mechanism of immune evasion by virulent *F. tularensis* was resistance to the assembly of the MAC on their outer membrane. The specific mechanism by which *F. tularensis* is resistant to the assembly of MAC is not completely clear. In one study, the presence of capsule contributed to the evasion of killing by serum components (Sandstrom et al., 1988). However, it has also been shown that *F. tularensis* binds another serum component, Factor H (Ben Nasr and Klimpel, 2008). In that study, Factor H served to cleave C3b to its inactive form iC3b. Generation of the iC3b under these conditions led to inefficient assembly of the MAC. Importantly, generation of iC3b served a second function for the bacterium. Inactive C3b serves as an opsonin for pathogens present in the host vascular system. Pathogens coated in iC3b are targeted for phagocytosis by various cell types which encode the receptors that are capable of interacting with this protein, including macrophages, dendritic cells, and neutrophils (Plow and Zhang, 1997). The two primary receptors noted to interact with iC3b are the complement receptor complex 3 (CR3) which consists of CD11b and CD18 and complement receptor complex 4 (CR4) that consists of CD11c and CD18. Indeed, *F. tularensis* is more efficiently phagocytosed by macrophages and dendritic cells following opsonization with iC3b and it has been shown to utilize both CR3 and CR4 in this process (Ben Nasr et al., 2006). Thus, the ability of *F. tularensis* to participate in the generation of iC3b serves at least two roles. First, it interferes with the deposition of the MAC on the surface of the bacterium and second it facilitates uptake by cells which are favored by the bacterium for replication.

In addition to complement proteins, the immune host may also possess *Francisella* specific antibodies. These antibodies can interfere with the ability of bacteria to infect host cells. Although the role for antibody in *Francisella* immunity has been controversial, classical studies by Foshay demonstrated that passive transfer of hyperimmune serum into humans infected with virulent *F. tularensis* greatly enhanced their recovery (Foshay et al., 1947). Further, this therapy was at least as effective as administration of streptomycin. Importantly, as described in Foshay's manuscript, passive transfer of immune serum was an effective treatment for patients regardless of the form, e.g., pneumonic versus ulceroglandular, of tularemia they had. More recent studies using the mouse model of pneumonic tularemia have demonstrated that passive transfer of both immune serum and specific monoclonal antibodies can protect against a lethal challenge of the Live Vaccine Strain (Kirimanjeswara et al., 2007; Savitt et al., 2009). However, passive transfer of antibodies failed to increase survival of animals challenged with fully virulent, Type A strains of *F. tularensis* although extended mean time to death was noted (Kirimanjeswara et al., 2008).

The failure of antibodies to contribute to survival of Type A infections in mice is not understood. One explanation may lie with the bacterium itself. Recently, our laboratory has demonstrated that antibody mediated opsonization of LVS and the Type A strain *F. tularensis* subsp. *tularensis* strain SchuS4 resulted in similar phagocytosis of each strain of bacterium by mouse macrophages. Importantly, we also demonstrated that phagocytosis of antibody opsonized bacteria provoked a pro-inflammatory response from infected macrophages (Crane et al., 2009). This was notable since it is widely accepted that one of the primary virulence mechanisms possessed by *F. tularensis* is its ability to infect host cells without eliciting pro-inflammatory cytokines such as TNF-α and IL-6 (Telepnev et al., 2003; Bosio and Dow, 2005; Chase et al., 2009). Production of these cytokines during infection is important because they could contribute to the activation of the ROS and RNS pathways that participate in control of *F. tularensis* infection (Fortier et al., 1992). Thus, the ability of both antibody opsonized LVS and SchuS4 to be phagocytosed by host cells and induce cytokine production from infected macrophages did not explain the failure of passively transferred antibody to protect against SchuS4 infections in mice.

To address this issue we turned to factors available in the host, but not routinely present among *in vitro* culture systems, which may influence the interaction of *F. tularensis* with antibody. One such factor is the host serine protease plasmin. Plasmin is generated following interaction of components of the host plasminogen system. The host plasminogen system is a key proteolytic system for dissolution of fibrin clots, migration of host cells through tissues, and the penetration of those cells through protein barriers (as reviewed, Plow et al., 1995). Many bacteria have been noted to bind plasminogen and plasmin and utilize these host proteins to enhance virulence (Lahteenmaki et al., 2001). Indeed, virulent *F. tularensis*, but not attenuated LVS, bound the active protease plasmin. Further, plasmin coated *F. tularensis* inhibited opsonization by *Francisella* specific antibody. The reduced ability of antibody to opsonize plasmin coated, virulent *F. tularensis* resulted in production of significantly less cytokines compared to opsonized, non-plasmin coated controls (Crane et al., 2009). These data suggested that an additional mechanism by which virulent *F. tularensis* subverts host immune responses is by utilizing host proteolytic machinery to degrade antibodies that may participate in protective responses.

INTERFERENCE WITH HOST RESPONSE AT THE CELLULAR LEVEL

INTERACTION WITH CELL SURFACE RECEPTORS

Once the bacterium has traversed the serum and contacted a target host cell, a series of new hurdles that may interfere with successful colonization of the host confront *F. tularensis*. Cellular defense often

begins with the interaction of the pathogen with receptors present on the host cell surface. The vast majority of receptors that interact with invading pathogens are termed pattern recognition receptors (PRR) due to their ability to recognize and bind conserved motifs present on multiple microorganisms. These PRR include, but are not limited to, scavenger receptors (SR), mannose receptors (MR), C-type lectins, and toll-like receptors (TLR). In some circumstances co-receptors and/or binding partners are required to optimally induce signaling though these receptors. For example, CD14 is a promiscuous co-receptor that acts to enhance signaling through TLR4 and TLR2 (Ulmer et al., 1999; Jiang et al., 2000).

Typically, engagement of many PRR results in the secretion of multiple cytokines and chemokines by the host cell. These soluble mediators then activate anti-microbial pathways and facilitate migration of effector cells to the site of infection. Thus, there are two strategies a pathogen may adopt to avoid engagement of these receptors. One is to possess ligands that fail to interact with the receptor or do so inefficiently. Another strategy is to engage receptors that fail to promote strong inflammatory responses. Both of these strategies are utilized by *F. tularensis*.

For example, as found in other Gram negative bacteria, *F. tularensis* possesses lipopolysaccharide (LPS) as part of its outer membrane. However, unlike LPS associated with *E. coli* or *Salmonella* species, the LPS associated with Type A subspecies of *Francisella* is an extremely weak TLR4 agonist (Phillips et al., 2004; Duenas et al., 2006) The poor stimulatory activity of LPS associated with this subspecies is attributed to the presence of only four acyl groups on their LPS (Phillips et al., 2004). Optimal signaling of LPS through TLR4 requires at least six acyl groups (Park et al., 2009). Thus, another mechanism by which *F. tularensis* evades detection by the host is modulation of ligands present on its surface to poorly interact with PRR that aid in altering the host cell to invading pathogens.

In addition to LPS, *Francisella* possess other TLR agonists. For example, Tul4 is lipoprotein that induces signaling responses via TLR2 (Thakran et al., 2008). Tul4 is present on the surface of the bacterium and thus represents a ligand that could alert the host cell to the presence of *F. tularensis* prior to phagocytosis of the bacterium. Yet, despite the presence of Tul4 as an available TLR2 agonist, strong inflammatory responses are not observed in host cells infected with virulent *F. tularensis*. One explanation for lack of detection of Tul4 on the surface of *F. tularensis* by specific host cells may lie in the absence of co-receptors present on select target cells. As stated above CD14 acts as a co-receptor to enhance interaction of microbial ligands for several PRRs including TLR2. At least two primary target cells of *F. tularensis*, alveolar macrophages and dendritic cells fail to express or, only express minimal concentrations of CD14 on their surface. Indeed, when dendritic cells were supplemented with soluble CD14 they secreted several pro-inflammatory cytokines following infection with *F. tularensis* (Chase and Bosio, 2010). *In vivo*, addition of CD14 at the time of intranasal infection induced production of TNF-α and IL-6 and was correlated with control of bacterial replication and dissemination (Chase and Bosio, 2010). However, it should be noted that despite early control of *F. tularensis* infection in mice receiving CD14, supplementation of this receptor *in vivo* did not result in increased survival (Chase and Bosio, 2010). Thus, while early detection of *F. tularensis* and induction of modest inflammatory response can impact replication the bacterium can ultimately overcome this response. The potential mechanisms by which *F. tularensis* overcomes the inflammatory response is discussed below.

A second strategy used by *F. tularensis* to evade detection at the level of the host cell surface is to engage receptors that fail to induce and/or suppress inflammatory responses. Utilization of the MR and CR3 are considered fairly innocuous routes of entry for intracellular pathogens since neither are associated with induction of signaling cascades that result in production of pro-inflammatory cytokines (Aderem and Underhill, 1999; Zhang et al., 2005). It has been demonstrated that, depending on the environment or conditions of infection, *F. tularensis* can utilize both MR and CR3 for entry into host cells. As discussed above, when opsonized with serum *F. tularensis* binds iC3b and gains entry into host cells via the CR3 receptor. Under non-opsonizing conditions, similar to that found in airways, *F. tularensis* utilizes the MR for entry in macrophages (Schulert and Allen, 2006). Therefore, *F. tularensis* evades detection at the point of entry in the host in three ways: (i) the bacterium has modified cell surface structures that enable it to avoid interaction with host receptors that are associated with induction of inflammation, e.g., TLR4; (ii) it targets cells that lack co-receptors which facilitate binding to receptors that could alert the host cell to invasion; and (iii) it utilizes receptors that fail to initiate production of pro-inflammatory cytokines.

THE INTRACELLULAR COMPARTMENT

Host defense by invading pathogens is not limited to detection of the microorganism at the surface of the cell. Mammals also possess an array of defense complexes, intracellular receptors, and signaling pathways that enable the host to control and eliminate the unprepared pathogen. Thus, as an intracellular pathogen, survival and replication of *F. tularensis* relies on its ability to interfere or modulate these intracellular defense mechanisms.

As observed in other bacterial infections, *F. tularensis* is susceptible to killing by reactive oxygen and reactive nitrogen species generated by the host (Fortier et al., 1992; Bosio and Elkins, 2001; Ireland et al., 2010). Thus, it is unsurprising that *F. tularensis* is capable of evading destruction by these toxic molecules. The evasion of products associated with an oxidative burst can be partially attributed to the neutralization of oxidative species by enzymes encoded by *F. tularensis*, e.g., catalase and superoxide dismutase (Lindgren et al., 2007). However, there is also evidence that *F. tularensis* is capable of directly interfering with the assembly of complexes in the host cell responsible for generating oxidative species. Allen and colleagues demonstrated that, following phagocytosis of opsonized *F. tularensis* by polymorphonuclear cells (PMN), the generation of superoxide anions via the NAPDH oxidase was actively inhibited. They then demonstrated that *F. tularensis* directly interfered with the phosphorylation of the p47 subunit of the NADPH oxidase. More recently, it was shown that interference with NADPH oxidase assembly occurred at two separate points. In addition to inhibiting activation of the p47 subunit, virulent *F. tularensis* also inhibited accumulation of the $gp91^{phox}$/$gp22^{phox}$ heterodimer (also known as flavocytochrome b_{558}) in neutrophils. Further, virulent *F. tularensis* also suppressed the ability of human neutrophils to assemble the NADPH oxidase following exposure to unrelated stimuli (McCaffrey et al., 2010).

In addition to directly interfering with assembly of the machinery responsible for focusing degradative enzymes at invading pathogens, *F. tularensis* has the capability of modulating this response in an indirect fashion. It has been suggested that attenuated strains of *F. tularensis* induce alternative activation of macrophages (Shirey et al., 2008). One property of alternatively activated macrophages is a dampened ability to activate reactive nitrogen species (Gordon, 2003). As described above RNS can contribute to the control of *F. tularensis* infections. Thus, provoking a state of alternative activation in host cells could give the invading bacterium an advantage for unrestricted replication in the host cell. However, it is not known if virulent strains of *F. tularensis* can provoke a similar response in resting macrophages. A more recent report provided evidence that *F. tularensis* inhibited oxidative burst via antioxidant scavenging systems associated with the bacterium (Melillo et al., 2010).

Pro-inflammatory cytokines such as TNF-α and IL-12 have been shown to contribute to the control of *F. tularensis* infections (Elkins et al., 1996, 2002). Some of these cytokines, e.g., TNF-α, may act directly on the APC to induce oxidative burst. Alternatively, other pro-inflammatory cytokines, e.g., IL-12, may act indirectly via the activation of effectors cells such T cells or NK cells to produce IFN-γ which then activates the APC. Thus, inhibition of the production and secretion of these pro-inflammatory cytokines by infected cells could also limit anti-microbial oxidative burst. The first evidence that *F. tularensis* was capable of modulating host cell production of cytokines was observed in humans infected with *F. tularensis* followed by exposure to endotoxin. Unlike uninfected controls, humans suffering from pneumonic tularemia failed to mount an inflammatory response following administration of endotoxin isolated from unrelated bacteria (Greisman et al., 1963). Later, *in vitro* studies using the murine macrophage cell line J774 demonstrated that *F. tularensis* actively suppressed the ability of host cells to produce TNF-α in response to *E. coli* LPS (Telepnev et al., 2003). In this report, a 23-kDa protein was found to be essential for suppression of TNF-α and IL-1β production in J774 cells, although the specific mechanism by which this protein interfered with host cell function was not described. Later studies conducted by our laboratory demonstrated that the *F. tularensis* mediated suppression of cytokine production also occurred following *in vivo* pulmonary infection (mirroring the original observations in humans) and, importantly, was extended to human dendritic cells (Bosio et al., 2007; Chase et al., 2009). The specific mechanism(s) by which virulent *F. tularensis* interferes with the ability of host cells to mount inflammatory responses has not been fully elucidated, although it is a subject of intense study by our lab and others. Recent work by Huang and colleagues demonstrated that LVS lacking a gene previously identified to be required for intracellular proliferation (RipA) provoked secretion of greater quantities of IL-1β and TNF-α in mouse macrophages and the human monocytic cell line, THP-1 cells (Fuller et al., 2008; Huang et al., 2010). It was not determined if RipA encoded a protein that directly interfered with induction of inflammatory responses in these cells or if the heightened inflammatory response observed in LVSΔripA mutants was a result of poorly replicating bacteria. Similarly, LVSΔmviN bacteria also elicited a stronger inflammatory response in mouse macrophages compared to wild type LVS (Ulland et al., 2010). However, unlike RipA, mviN was not required for intracellular replication of LVS. Thus, the effect mviN had on elicitation of inflammation was presumably not due to bacteria that were compromised for growth in the intracellular compartment. The role of RipA and mviN in infections mediated by virulent *F. tularensis* has not been explored. However, these data generated with LVS provided promising evidence that novel genes encoded by *F. tularensis* can contribute to the immunosuppression host cells.

CONCLUDING REMARKS

Francisella tularensis is a remarkable bacterial pathogen. In the early days of *F. tularensis* research, when scientists were first characterizing the bacterium, it was apparent that the "success" of this pathogen was tied to its ability to modulate and evade the immune system. This modulation was evident in two central observations. First, the ability of this microorganism to cause acute, lethal, disease in the mammalian host following exposure to relatively miniscule numbers of bacteria suggested it was capable of readily evading innate host defense mechanisms present at the outset and throughout the infection. Second, the lack of development of long lived immunity in laboratory workers who had survived a primary infection pointed to manipulation of adaptive immunity (Jellison, 1974). As discussed herein, it is clear that *F. tularensis* possesses a myriad of mechanisms by which to manipulate immunity. Further, current data suggests that this subversion begins in the extracellular compartment and continues throughout the intracellular life cycle of the bacterium. We have just begun to uncover the collection of immune evasion strategies embodied by this organism. Due to the multiple pathways *F. tularensis* influences as it traverses host environment, continued research into the specific mechanisms by which *F. tularensis* evades, modulates, and suppresses the host immune response will undoubtedly enhance our understanding of tularemia, infectious disease, and regulation of host immunity as a whole.

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