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A COMPLEX INTERACTION: COOPERATION BETWEEN MULTIPLE MACROPHAGE DEFENSE PATHWAYS IN RESPONSE TO *FRANCISELLA NOVICIDA* INFECTION

By

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B.S., Georgia State University, 2005

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An abstract of

A dissertation submitted to the Faculty of the

James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

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Program in Microbiology and Molecular Genetics

2012

Abstract

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Early detection of microorganisms by the innate immune system is provided by Toll-like receptors (TLRs), and activation of these receptors leads to the expression of proinflammatory cytokines. Some intracellular bacteria can subvert the TLR response by rapidly escaping the phagosome and replicating in the cytosol. However, these bacteria are recognized by the AIM2 inflammasome, a multi-protein complex comprised of the sensor protein AIM2, the scaffolding protein ASC and the cysteine protease caspase-1. Inflammasome activation leads to the release of proinflammatory cytokines IL-1β and IL-18 and death of the infected cell, an important host defense that eliminates the pathogen's replicative niche. While TLRs and inflammasomes are critical for controlling *Francisella novicida* infection, it was not known whether these host pathways cooperate to activate defenses against this intracellular pathogen. We showed that TLR2 contributes to rapid inflammasome activation since in its absence there was a delay in cell death, caspase-1 activation and IL-18 release in macrophages and *in vivo*. These data show that the host coordinates signals between two spatially separated host defense pathways to more rapidly respond to bacterial infection. In spite of this, *F. novicida* evades TLR2 activation by repressing the expression of bacterial lipoproteins (BLPs) using the protein FTN_0757. A Δ*FTN_0757* mutant of *F. novicida* induced robust TLR2-dependent cytokine production in macrophages, produced increased amounts of BLPs compared to wild-type bacteria, and was severely attenuated *in vivo*. One BLP, FTN_1103, was significantly upregulated in the Δ*FTN_0757* mutant, and deletion of *FTN_1103* led to a significant decrease in cytokine and BLP production and partially rescued the *in vivo* attenuation of the Δ*FTN_0757* mutant. Taken together, these data reveal a novel mechanism of immune evasion that is likely used by other intracellular bacterial pathogens to escape host defenses.

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Acknowledgements

It takes a village to raise a child is an old African proverb that I think accurately describes my journey to completing my graduate degree. With the continued support from my friends, family and mentors that I've picked up along the way, I can truly say that I have been fortunate to such a great group of people on my side.

I would like to thank Dave for giving me the opportunity to complete my thesis research in your laboratory. This was a learning process for both of us. I am grateful for having the opportunity to work side-by-side with you (except the days when we started at 7:30 am), which helped me master difficult techniques and learn the discipline it takes to be a successful scientist. I am thankful for the support that you have given me over the years and will miss having you there find the good in my "bad" data. Brooke and Tim, I will truly miss our lively and sometimes weird "coffee time" discussions. I really appreciate the support and input that I have received from both of you over the years.

I would like to thank my committee members (Jyothi Rengarajan, Rama Amara, Andrew Neish, Bill Shafer and Charles Moran) for their advice, support and guidance. To Bill, I chose not to let that moment define me 3.5 yrs ago and thanks for the words of encouragement. To Andrew, I would like to thank you for all of the advice and support that you have given me over the past year.

I would like to thank Helder, Rajesh, Oscar, David, Joanna and Benton for the lively lunchtime debates about science and politics in the basement cafeteria. Benton, since our days on BioBus, you always been a great friend and supporter, and I am truly thankful for that. I would like to thank my BioBus family, Michelle, Chandan, Dana, Bryan, and Dr. Baumstark, for their continued support over the years. Dr. B, you are personally and professionally an inspiration to me, and I thank you for setting the ball in motion by sparking my interest in teaching and research.

I would like to thank my friends Jasmine Rainey, Kim McCarthy, Kristi Porter, Brea Duval, Willie Johnson, Colleen Reynoso and Paul Musey for lending an ear and providing me with support throughout this challenging time, and thanks for understanding that you are appreciated even though sometimes you don't hear from me for a month or two.

To my family, I would like to thank all of the members of the Daniel family for seeing me through the good times and bad. I would like to thank my mom for allowing me to choose my own path in life and being there to support and guide me along the way. I know that I don't express this much, but you are truly an inspiration to me. There are no words that can express the amount of strength, determination and hard work it took for you to send all three of us to college by yourself. I strive to work as hard as you and treat others with unconditional kindness (even though it is hard for me sometimes) as you have done over the years. To Kanisha, thanks for the countless hours that you spent listening to me talk about science. I guess all of those talks have paid off now. I would like to dedicate this dissertation to the two people, Jeanette and Hosea Daniel, who greatly

shaped me into the person that I am today, but unfortunately are not around to see one of my greatest accomplishments. To Cocoa, thank you for spending countless nights being ignored and sometimes accidently left at daycare because my experiment ran late…a dog is truly a woman's best friend.

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Chapter 1: Introduction

Francisella tularensis

1.1. History, Epidemiology, and Taxonomy

The Gram negative, nonmotile, bacterium *Francisella tularensis* is the etiological agent of the disease tularemia. Reports of tularemia-like illnesses date back as far as 1653 in lemmings (1). In 1911, American scientist George McCoy identified this coccobacillus as the causative agent of a plague-like disease afflicting ground squirrels in Tulare County, California, and he named it *Bacterium tularense* and the disease tularemia (2). There have been occurrences of tularemia in invertebrates, such as ticks and mosquitos, and vertebrates including mice, squirrels, rabbits, muskrats and humans (3). In fact, the first human case of tularemia was reported in 1914 in a 21-year old male who worked as a meat cutter in Ohio (4). In 1925, Japanese physician Hachiro Ohara, who named the disease Yato-byo (wild rabbit disease), used his wife as a test subject to show that the infectious agent could be transmitted from infected rabbits to humans by direct contact with the skin (5). Around the same time as Ohara's discovery, American scientist Edward Francis identified *B. tularense* as the causative agent of deer-fly fever and linked it to the human infection previously reported by McCoy (6). In addition, he published several studies charactering the clinical pathology associated with human tularemia, describing disease transmission via insect bite or direct contact, and linking *B. tularense* as the causative agent of tularemia, Yato-byo and tularemia-like diseases reported in Europe (7). In 1947, taxonomists discovered that *B. tularense* did not share the same properties as other members of the *Bacterium* genus and reclassified it as *Francisella tularensis* to honor the significant contributions of Dr. Francis (6).

Francisella belongs to the family *Francisellaceae* and is currently the only genus in this family. *F. tularensis* has been divided into three subspecies: *tularensis*, *holarctica* and *mediasiatica*. Each subspecies varies in virulence and geographical prevalence (Table 1). For example, *F. tularensis* subspecies *tularensis* (Type A) is the most virulent subspecies and causes a fatal disease in humans and animals. It has only been isolated in North America and accounts for approximately 90% of human infections in this continent (8). *F. tularensis* subspecies *holarctica* (Type B) is less virulent than *F. tularensis* subspecies *tularensis*, causing a milder, slower-progressing disease in humans and animals. It has been isolated throughout the Northern Hemisphere and is mostly associated with disease in semiaquatic rodents such as muskrats, beavers and ground voles (6). *F. tularensis* subspecies *mediasiatica* usually has only been isolated from Central Asia, and its degree of virulence in rabbits is similar to *F. tularensis* subspecies *holarctica*. There have been very few studies on this organism and very little is known about its infection in humans.

F. novicida was originally isolated from a water sample taken from Ogden Bay in Utah and classified as a species of *Francisella* in 1959 (6). It has a vey low virulence level and primarily causes disease only in immunocompromised people. Due to its DNA relatedness, high frequency of transformation between *F. tularensis*, ability to cause disease in humans, and biochemical similarities, it was proposed that *F. novicida* be designated as a subspecies of *F. tularensis* in 1989 (9). We chose to use *F. novicida* as a model organism to study *F. tularensis* pathogenesis because of its extensive and almost exclusive use in studying inflammasome activation and high level of conservation of virulence genes between the two species. More importantly, there are similarities

between the intracellular lifestyle of *F. novicida* and other intracellular pathogens, including *Listeria monocytogenes*, which allows us to gain a better understanding of how these bacterial pathogens interact with host cells.

1.2. Tularemia

There are several different forms of tularemia and the clinical manifestations depend on the subspecies causing the infection as well as the route of entry. Tularemia symptoms (high fever, chills, malaise and headache) are nonspecific and usually appear after a 3 to 5 day incubation period (6). The most common form is ulceroglandular tularemia (approximately 75% of patients), which is caused by either insect bite (tick or mosquito vector) or direct contact of the skin or mucous membranes with contaminated animals (10). Ulceroglandular tularemia is characterized by the formation of a painful lesion at the site of infection that later ulcerates, usually within a week. The draining lymph node closest to the ulcer then becomes enlarged and tender. In the absence of antibiotic treatment, 30-40% of infected individuals experience continuous swelling of the lymph nodes and suppuration (pus formation) (6). All virulent *F. tularensis* subspecies are capable of causing this form of tularemia.

Pneumonic tularemia occurs in approximately 30% of patients who have ulceroglandular tularemia, but can also develop after inhalation of aerosolized bacteria (10). The outcome of this respiratory infection depends of the causative subspecies. For example, pneumonic tularemia caused by *F. tularensis* subspecies *holarctica* is not fatal (6), but inhalation of as few as 10 colony forming units of *F. tularensis* subspecies *tularensis* can cause a potentially lethal infection (11). The mortality rate for pneumonic

tularemia can be up to 50% in the absence of antibiotic treatment (10). However, antibiotic therapy can reduce the mortality rate to less than 2% (6). The current first-line treatment for tularemia is a 7-10 day course of aminoglycosides, such as streptomycin or gentamicin (10).

1.3. *F. tularensis* as a Bioweapon

F. tularensis has been considered a potential agent for use in biological warfare for some time. During World War II, the United States, Russia and Japan initiated research programs aimed at testing *F. tularensis* and other microorganisms for use as biological weapons, as well as developing tools for disseminating these agents. However, there is no direct evidence of *F. tularensis* actually being used as a bioweapon during the war. A former Russian biological weapons scientist, Kenneth Alibek, suggested that tularemia outbreaks that affected tens of thousands of Soviet and German soldiers on the Eastern European front during World War II may have been the result of intentional use (12), although his allegations have not been substantiated.

During the Cold War era, the United States military expanded its biological warfare program to include offensive programs that focused on developing vaccines, antisera and therapeutic agents to protect troops against potential biological attack (13). During this time, they also developed *F. tularensis* subspecies *tularensis* strains that were resistant to all available antibiotics and vaccines (10), and performed challenge studies (aerosol and intracutaneous) with military personnel and civilian volunteers to determine their susceptibility to *F. tularensis* infection and the effectiveness of a live-attenuated vaccine that had been developed (13). By the 1960s, *F. tularensis* was among a long list of

biological agents that had been weaponized and stockpiled by the United States military (13). The 1972 Convention on the Prohibition of the Development, Production, and Stockpiling of Bacteriological and Toxin Weapons and Their Destruction, or Biological Weapons Convention, was put into action. This treaty bans the development, possession, acquisition and stockpiling of biological and toxin weapons and equipment used to disperse these agents (14). By the early 1970s, the United States military had disarmed its program and destroyed its arsenal of biological weapons.

Despite the enforcement of the Biological Weapons Convention, the potential for a biological attack initiated by rogue countries still remains. It has been estimated that dispersal of 50 kg of virulent *F. tularensis* over a metropolitan area with a population size of 5 million individuals would result in 250,000 incapacitating casualties including 19,000 deaths, and illness would persist for several weeks and relapses would occur during the ensuing weeks or months (12). Due to its extremely low infectious dose, ease of transmission and high mortality rate, *F. tularensis* has been classified as a Category A Select Agent by the Centers for Disease Control and Prevention. To provide perspective, *Bacillus anthracis*, *Clostridium botulinum* toxin, *Variola major, Filoviruses* and *Arenaviruses* are also included on this list of biological agents that pose a considerable threat to national security. Taken together, the ability of *F. tularensis* to be easily weaponized and the massive casualties and panic that could ensue after an attack both exemplifies the importance of getting a better understanding of the pathogenesis of this highly virulent bacterium, and developing a safe and effective vaccine and other therapeutics to prevent and treat tularemia.

In remaining sections, *Francisella* will be used when referring to data that has been shown for more than one *Francisella* subspecies. However, a specific *Francisella* spp. will be named if the finding only relates to it.

2. Innate Immune Response to *Francisella* Infection

2.1. Macrophages

Macrophages have been considered the *in vivo* replicative niche of *Francisella,* and establishing an infection in these phagocytes is critical for bacterial survival *in vivo*. However, a comprehensive analysis of host cells infected by different *Francisella* species following intranasal inoculation revealed that all of the tested strains initially resided in alveolar macrophages, but after three days these strains were primarily associated with neutrophils (15). This finding shows that the interaction between *Francisella* and host immune cells may be more complex than first considered. Taken together, this study supports the notion that interactions between *Francisella* and macrophages may be necessary for the bacteria to survive and could potentially promote the expression of virulence factors necessary for infecting other host cells such as neutrophils. More studies are needed to truly understand the contribution of different host cells in the establishment of *Francisella* infection *in vivo*.

Francisella enters macrophages by inducing the formation of distinctive pseudopod loops around the bacterial cell (16) (Figure 1). Many bacterial pathogens enter macrophages via non-inflammatory receptors to avoid inducing an immune response (17). The host signaling pathways and bacterial factors that are important for *Francisella* uptake are not known, and this is currently an area of investigation. Following uptake, *F.*

tularensis is trafficked into a phagosome, which it subsequently escapes to replicate in the cytosol (18). Escaping the phagosome enables the bacteria to avoid destruction by antimicrobial peptides, nitric oxide and acid, which are induced in the phagolysosome.

Despite entering the macrophage through non-inflammatory receptors, *Francisella* is still recognized by pattern recognition receptors (PRRs) as it traffics through the macrophage. Activation of PRRs, such as Toll-like receptors, can result in the induction of proinflammatory cytokines that act to recruit other inflammatory cells to the site of infection and activate macrophage defenses (discussed in detail in section 2.2). Unlike less virulent *Francisella* strains, *F. tularensis* is able to suppress the production of proinflammatory cytokines, such as IL-6 and TNF-α, in infected macrophages (19). The virulence factors that contribute to this suppression are not known, but it is known that the induction of the immunosuppressive lipid prostaglandin E_2 (PGE_2) (20) and anti-inflammatory cytokines TGF-β and IL-10 (19, 20) likely contribute to the suppression of proinflammatory cytokine production.

Following cytosolic replication, *F. tularensis* can enter the autophagic pathway (21). Autophagy is a process carried out by eukaryotic cells in which cytoplasmic material is engulfed into double membrane-bound vacuoles called autophagosomes that subsequently fuse with lysosomes for degradation (22). This pathway was originally described as being important for maintaining cellular homeostasis, but it is now evident that it is also important for host defense. Some viruses and pathogenic bacteria hijack the autophagic pathway to exit host cells (23). It is not clear whether autophagy is a host-induced response to control infection or a *Francisella*-induced mechanism to promote virulence. *Francisella* autophagosomes contain MHC II (24), suggesting that the host

might induce autophagy to degrade bacteria and present their antigens to activate the immune system and control bacterial replication. However, *F. tularensis* downregulates the expression of *atg5*, *beclin 1* and several other autophagy-related genes (25) during macrophage infection, supporting the notion that *Francisella* may act to subvert this host defense system.

2.2. Toll-like Receptors

The macrophage, which acts as a sentinel of the innate immune system, is equipped with numerous membrane bound and cytosolic pattern recognition receptors (PRRs) that detect microbes by recognizing conserved microbial components known as pathogen-associated molecular patterns (PAMPs). Recognition of PAMPs by PRRs enables the macrophage to rapidly mount a proinflammatory response aimed at eliminating microorganisms. One of the best-characterized families of PRRs is the Toll-like receptors (TLRs). TLRs are type I integral membrane proteins that recognize bacterial PAMPs such as lipopolysaccharide (TLR4), bacterial lipoproteins (TLR2), flagellin (TLR5), and CpG DNA (TLR9) (26). Following ligand binding, all TLRs except TLR3 signal through the adaptor protein MyD88 to the activate the transcriptional regulator NF-κB, which promotes the expression of proteins involved in host defense including the proinflammatory cytokines IL-6, TNF-α, and IL-1β (26).

TLR4, which recognizes bacterial lipopolysaccharide (LPS), is one of the most well-characterized TLRs. TLR4, in a complex with MD-2 and CD14, binds to the lipid A portion of LPS at the surface of host cells (27, 28). Studies in human cells indicate that TLR4 is maximally activated by hexa-acylated lipid A with 12-14 carbon acyl side

chains, and altering the length of the side chains or changing the charge of lipid A can dampen the intensity of TLR4 activation (29). Bacterial pathogens have exploited this strict requirement for TLR4 recognition of LPS by altering the length of their acyl chains in their lipid A (30). The LPS of *Francisella* is tetra-acylated with 16-18 carbon acyl side chains, resulting in weak stimulation of TLR4 (31).

TLR2 predominantly recognizes bacterial lipoproteins (BLPs) but is capable of recognizing a diverse set of microbial ligands, including lipoteichoic acid and peptidoglycan from Gram-positive bacteria, lipoarabinomannan from mycobacteria and glycosylphosphatidylinositol anchored lipids from *Trypanosoma cruzi* (32, 33). The ability of TLR2 to respond to such a diverse set of PAMPs it attributed to its ability to form complexes with TLR1, TLR6 or CD36 (32). TLR2 recognizes diacylated and triacylated lipoproteins by forming a heterodimer with TLR6 or TLR1, respectively (34-36). Subsequent to ligand binding, the TLR2-ligand complex is internalized by macrophages into the phagosome, where TLR2 becomes enriched and initiates a MyD88-dependent signaling cascade that results in the activation of NF-κB (37). In addition to its canonical role in inducing proinflammatory cytokine and nitric oxide production, TLR2 also acts to promote rapid activation of cytosolic defenses (discussed in detail in Chapter 2).

TLR2 is the primary TLR involved in recognizing and responding to *F. tularensis* and this pathogen has either developed ways of subverting or is incapable of activation other TLRs. For example, and *Francisella* lacks genes encoding flagella (38), and therefore does not activate TLR5 (39, 40). Additionally, this bacterium has not been shown to activate TLR9 *in vitro*, and it is likely that bacteria escape the phagosome

before it can become activated. Furthermore, TLR9 plays a minor role, if any, in providing the host with protection against *F. tularensis* infection *in vivo* (41). In addition to TLR2 being the primary activator of the proinflammatory cytokine response in macrophages during *Francisella* infection, TLR2 and the adaptor protein MyD88 are critical for host defense against *Francisella* infection since mice lacking these proteins are more susceptible to infection than their wild-type counterparts (41-43).

2.3. Cytosolic Defenses

When *Francisella* reaches the cytosol, it has trafficked past TLRs and phagosomal defenses. It is nonetheless faced with a formidable challenge: replicate to high numbers without triggering an effective immune response. This is all the more challenging since the process of bacterial replication results in the release of PAMPs that can be recognized by cytosolic PRRs. Like the host cell surface and phagosome, the cytosol is equipped with numerous PRRs that recognize an array of bacterial products and elicit an immune response aimed at clearing the invaders. One large family of cytosolic PRRs is the NOD-like receptor (NLR) family whose 22 members respond to a diverse set of PAMPs including peptidoglycan (NOD1 and NOD2), flagellin (NLRC4, NAIP5 and NAIP6), components of bacterial type III secretion systems (NLRC4), as well as damage induced by pore-forming toxins (NLRP3) (44, 45). NOD1 and NOD2 are membrane-associated NLRs that detect muropeptide subunits of peptidoglycan and induce NF-□B activation and proinflammatory cytokine production (46). A role for NODs during *Francisella* infection has yet to be described, but these receptors may recognize and respond to peptidoglycan from this pathogen. Alternatively, *Francisella* may subvert NODs by

modifying its peptidoglycan (similar to *Listeria monocytogenes* which N-deacetylates its peptidoglycan) (47) or by suppressing the NOD signaling pathway.

F. novicida induces type I IFN (IFN) production through an unknown cytosolic PRR (48). The type I IFN family includes numerous IFN-α proteins, a single IFN-β protein, and other IFNs (49). These secreted cytokines have a well-established role in interfering with viral replication but can also be induced in response to bacterial infection (50). However, the adaptor protein STING (stimulator of interferon genes) is required for type I IFN production during *F. novicida* infection and is speculated to act downstream of the yet to be identified cytosolic PRR. STING is known to induce type I IFN production in response to cytosolic double-stranded DNA derived from transfected plasmids, viruses and some bacteria (51). Therefore, it is likely that *Francisella* DNA, which has been observed in the host cytosol by confocal microscopy (52, 53), is the ligand that activates this cytosolic defense system.

It is not clear how *Francisella* DNA reaches the cytosol to trigger type I IFN, but bacterial escape from the phagosome is required for induction of this host response (48, 53). Perhaps damage incurred by *Francisella* in phagosomes allows for the release of DNA from ruptured phagosomes following escape. Interestingly, an auxotrophic mutant of *Francisella* that escapes the phagosome but cannot replicate in the cytosol failed to trigger cytosolic defense pathways (54), suggesting that bacterial replication is required to increase the amount of DNA to a threshold level to which the host responds. In this context, mechanisms of maintaining structural integrity, such as LPS modifications and capsule formation, may prevent damage to the bacteria and the release of DNA in the

cytosol, and therefore would be hypothesized to promote subversion of these cytosolic defenses.

In addition to triggering the type I IFN pathway, cytosolic DNA released during *Francisella* infection can also be recognized by the PRR absent in melanoma 2 (AIM2) (52), whose expression is upregulated by IFN-β (53). AIM2 is a member of the PYHIN (Pyrin and HIN-200) family of proteins that binds double-stranded DNA through a HIN-200 domain (55-57). AIM2 contributes to host defense by initiating the formation of a multiprotein complex called the inflammasome that is comprised of a PRR (from the NLR or PYHIN families), the scaffolding protein ASC, and the cysteine protease caspase-1 (58). Inflammasome activation causes infected cells to undergo an inflammatory form of programmed cell death called pyroptosis (59). This cell death may release bacteria into the extracellular environment where they can no longer replicate and can easily be taken up by cells such as neutrophils that are not permissive for replication (60). Pyroptosis is accompanied by the release of the proinflammatory cytokines IL-1β and IL-18 from dying cells that act to recruit and activate other immune cells, further promoting bacterial clearance. AIM2 inflammasome activation is essential for controlling *F. novicida* infection since mice lacking components of this defense system succumb to infection much more rapidly than their wild-type counterparts (57, 61). In contrast, the NLRC4 and NLRP3 inflammasomes do not play an obvious role in combating *Francisella* infection in mice (61, 62), likely because *Francisella* does not encode known activators of these PRRs.

Since inflammasome activation plays such a critical role in controlling *Francisella* infection, it would seem likely that this stealth pathogen would evolve

mechanisms to try to modulate this host defense pathway. Unlike less virulent *Francisella* species, *F. tularensis* fails to efficiently activate the inflammasome (63). Macrophages and dendritic cells infected with *F. tularensis* secreted very low levels of the inflammasome-dependent cytokine IL-18 *in vitro*, and there was very little caspase-1 activation induced in the spleen and liver of infected mice (63). Additionally, microarray analysis of human monocytes infected with *F. tularensis* revealed that this pathogen downregulates the expression of several genes belonging to the TLR and type I IFN pathways (25, 64). TLR2 signaling is necessary for the expression of IL-1β and accelerates the rate of inflammasome activation during *F. novicida* infection (65), while type I IFN is essential for inflammasome activation (66). Therefore, hampering two major host defense pathways that contribute to inflammasome activation could lead to a lack of activation of this complex during *F. tularensis* infection. It is likely that highly virulent *F. tularensis* also has additional ways by which to limit inflammasome activation.

Several *Francisella* genes have been implicated in modulating inflammasome activation (66, 67). However, Peng *et al*. recently showed that these genes were not important for actively modulating the inflammasome (68). Instead, the increased induction of macrophage death triggered by a panel of mutants lacking genes encoding membrane-associated proteins was due to increased bacteriolysis in the cytosol that allowed for the leakage of DNA and increased inflammasome activation. This study suggests that maintenance of membrane integrity is critical for *Francisella* to prevent the release of PAMPs and induction of the inflammasome. *F. tularensis* may also directly suppress inflammasome activation although genes important for direct suppression of the

inflammasome complex by *F. tularensis* have not been identified. However, the presence of such genes in *F. tularensis* but not the less virulent *Francisella* species could explain their divergence in activation of this complex.

During the latter stages of *F. tularensis* infection, infected cells undergo caspase-3-dependent programmed cell death or apoptosis. Unlike pyroptosis, this form of cell death is non-inflammatory (69). Wickstrum *et al*. observed a significant increase in apoptosis in the livers and spleens of *F. tularensis*-infected mice between days 3 and 4 post-infection. This spike in apoptosis was preceded by an exponential increase in bacteria and antigen distribution in the infected organs (63). *F. tularensis* may direct host cells to undergo apoptosis instead of pyroptosis following cytosolic replication, facilitating dissemination to neighboring cells without triggering a strong inflammatory response. Additionally, phagocytosis of apoptotic bodies by activated macrophages impairs the ability of these cells to produce proinflammatory cytokines (70). In the context of an *F. tularensis* infection, these macrophages that have been rendered immunologically silent by taking up *Francisella*-containing apoptotic bodies could serve as reservoirs for further replication. Interestingly, it is not clear how *Francisella* egresses from infected cells and disseminates throughout the host. These impaired macrophages could also serve as Trojan horses trafficking the bacteria systemically.

Replicating in the cytosol of host cells without inducing an inflammatory response is one of the most challenging yet critical immune subversion tactics employed by the human pathogen *F. tularensis* during infection. Although many of the virulence determinants that aid in evasion of cytosolic defenses are not currently known, it is likely

that there are numerous genes necessary to subvert recognition by multiple PRR, activation of cell death pathways, and autophagy-mediated killing.

3. Adaptive Immune Response to *Francisella* Infection

Pulmonary infection caused by *F. tularensis* in humans is characterized by an initial delay in inflammatory responses during the first 72 hours (71). Although it is not entirely clear how *Francisella* induces this immunoquiescent state, modulating the activity of innate immune cells that contribute to early cell-mediated immunity likely plays a significant role. Antigen-presenting cells (APCs), such as macrophages and dendritic cells, play an important role in early innate defense against *Francisella* infection. The ability of these cells to present microbial antigens and activate T cells enables them to serve as a bridge between the innate and adaptive immune systems. During the earliest stages of infection, *Francisella* primarily resides in macrophages (15). Therefore, it is not surprising that this pathogen uses multiple subversion mechanisms (i.e., trying to facilitate cell entry via non-activating macrophage receptors, escaping phagosomal killing, modulating cytosolic defense pathways) to systematically disarm macrophage defenses. However, when activated by the proinflammatory cytokine IFN-γ, these phagocytes are capable of overcoming the modulatory effects exerted by *Francisella* and preventing its replication (72, 73). Primarily produced by NK cells and dendritic cells during infection, IFN-γ regulates over 200 genes, many of which are involved in host defense pathways important for enhancing nitric oxide production, inducing autophagy, and increasing major histocompatibility complex (MHC) class I and II antigen presentation (74, 75). Activation of these pathways could collectively enhance

Francisella clearance and promote host survival. Moreover, a lack of IFN-γ *in vivo*, either through genetic disruption (76) or anti-cytokine treatment (72), increased susceptibility of mice to *Francisella* infection, indicating that this cytokine is an important mediator of host defense *in vivo*.

Since IFN-γ has such a pleiotropic effect on macrophage activation, any mechanisms of suppressing the action of this cytokine would likely have broad effects on promoting *Francisella* pathogenesis. *Francisella* directly impairs the ability of macrophages to respond to IFN-γ stimulation during infection by downregulating the expression of the alpha subunit of the IFN-γ receptor (IFNGR), which is critical for signaling (77). In the event that signaling through the IFNGR does occur, *Francisella* blocks the phosphorylation and subsequent activation of Stat1 (through an unknown mechanism), a transcriptional regulator that is required for IFNGR signaling (78). To further ensure that the IFN-γ signaling pathway is decommissioned during infection, the bacteria induce an increase in the expression of SOCS3, a protein that negatively regulates this pathway (64). Activation of the TLR signaling pathway enhances IFN-γ-dependent cytokine production in macrophages (79). Therefore, suppression of TLR signaling by mechanisms could also dampen activation of the IFN-γ pathway.

In addition to suppressing IFN-γ signaling, *F. tularensis* induces macrophages to produce the immunomodulatory lipid prostaglandin E_2 (PGE_2) (Figure 2) (20). PGE_2 inhibits T cells from producing IFN-γ, favoring bacterial replication (80). PGE_2 also induces the expression of a >10 kD protease-resistant host factor that promotes ubiquitin-dependent lysosomal degradation of MHC II molecules, resulting in a nearly complete absence of MHC II on the surface of macrophages (81). Therefore, by inducing PGE_2,

Francisella inhibits bacterial killing mediated by IFN-γ and also dampens the induction of adaptive immune responses by limiting antigen presentation through MHC II.

Dendritic cells play a critical role in directing cell-mediated immunity by producing cytokines that control the way in which T cells are activated and the immune responses that they subsequently promote. In accordance with this pathogen's strategy to shutdown cell-mediated immune responses, *F. tularensis* suppresses proinflammatory cytokine production from these cells (19). Among these cytokines is IL-12 that is important for the development of IFN-γ-producing T cells (82). Surprisingly, inhibition of IL-12 by *F. tularensis* is mediated by IFN-β production (83). Type I IFNs contribute to inflammasome activation by *Francisella*. However, there was no correlation between IFN-β production and inflammasome activation during *F. tularensis* infection. Considering that *F. tularensis* dampens the expression of genes in the type I IFN pathway, it is possible that this pathogen suppresses the expression of type I IFNs to a level that is below the threshold for inflammasome activation, but adequate for IL-12 suppression.

Taking advantage of the immunomodulatory ability of dendritic cells and macrophages, *F. tularensis* redirects macrophages to become alternatively activated (84). Alternatively activated macrophages exhibit a skewed inflammatory profile that is less antimicrobial with decreased levels of nitric oxide production upon IFN-γ stimulation and an impaired ability to kill bacteria (85, 86). Additionally, *F. tularensis* directs dendritic cells to produce the anti-inflammatory cytokines TGF-β and IL-10 (19, 20). This greatly contributes to the absence of an inflammatory response early in infection because IL-10 inhibits macrophage proliferation and proinflammatory cytokine production (87). In

addition, it can lead to reduced MHC II expression, which would suppress adaptive immune responses (88).

Treating dendritic cells with IL-10 or TGF-β can direct them to become tolerogenic, inhibiting their ability to activate T cells that induce an inflammatory response (88). These cytokines also promote the development of regulatory T cells that can suppress the inflammatory activity of other T cells (89). In fact, pulmonary regulatory T cells develop in the lungs during *F. tularensis* infection, and this development correlates with an increase in bacterial burden (90). Though it is currently unclear how *F. tularensis* redirects APCs toward an anti-inflammatory response, inducing immune tolerance through the induction of IL-10 or TGF-β is a survival strategy used by pathogens such as *Yersinia pestis, Coxiella burnetii and Chlamydia pneumonia* (91). In the case of *Yersinia pestis*, IL-10 production is induced by the type III secretion system effector protein, LcrV (92, 93). Further characterization of *Francisella* secretion systems and effector proteins may shed light on similar mechanisms of immune modulation.

Cell-mediated immunity is generally accepted as being essential for controlling intracellular bacterial infection. In accordance with this dogma, antibodies have been shown to provide little protection against *Francisella* infection, but antibody-independent B cell responses are important for early protection (94). However, *Francisella*, like some other intracellular bacterial pathogens (95) is also capable of infecting and replicating inside of B cells (96), although it is not known whether this pathogen can modulate their activity in order to evade immune detection. *Salmonella typhimurium* has been shown to induce MyD88-dependent IL-10 production in splenic B cells during systemic infection, promoting its survival *in vivo* (97). As discussed earlier, IL-10 induces a tolerogenic state

in immune cells, and it is possible that *Francisella*-induced IL-10 production in APCs could also alter the function of B cells during infection.

Taken together, *F. tularensis* uses multiple strategies to modulate APCs in order to subvert subsequent activation of the adaptive immune system. This modulation contributes not only to the failure in immune activation during the initial stages of infection, but also helps provide a suitable environment for replication in subsequent phases of infection by dampening adaptive responses such as the activation of $CD8^+$ T cells which could kill infected host cells harboring *Francisella*.

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Table 1

	***F. tularensis* Schu4**	***F. holarctica* LVS**	***F. novicida* U112**
Genome Size (bp)	1,892,819	1,895,998	1,910, 031
G + C content (%)	32.26	32.15	32.47
Predicted ORF	1445	1380	1731
Sequence Similarity (%)	98.1 to U112 99.2 to LVS	97.8 to U112 99.2 to Schu4	97.8 to LVS 98.1 to Schu4
Biosafety Level	3	2	2
Intracellular Replication in Macrophages	Yes	Yes	Yes
LD_{50} in Humans (CFU)	<10	$<10^3$	$>10^3$
LD_{50} in Mice (CFU)	<10	<1	$<10^3$
Geographic Distribution	North America	Northern Hemisphere	Global

Adapted from Hall (15) and Schneeklet (98)

Figure 1

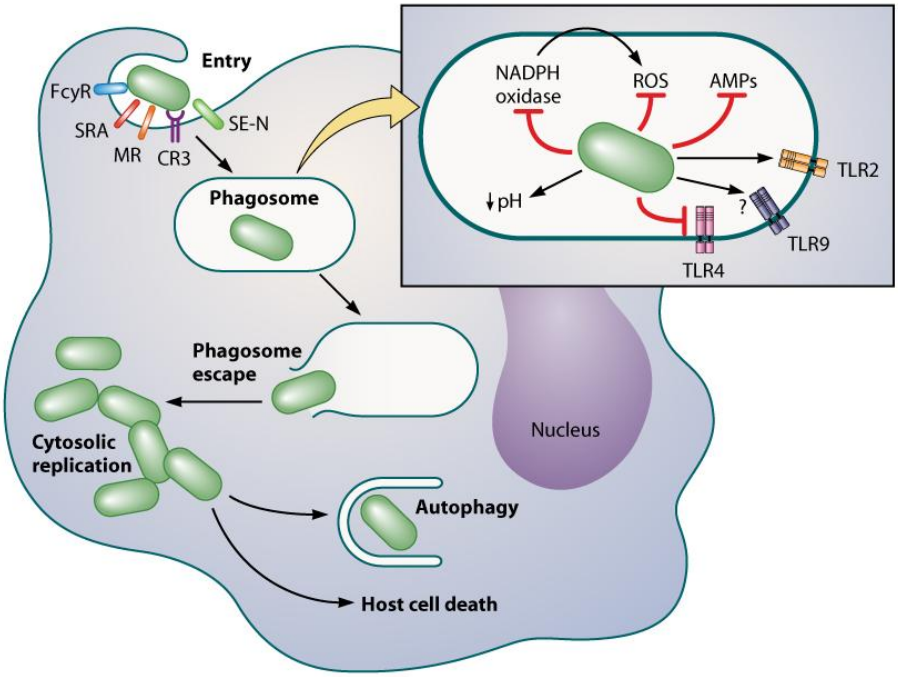

Figure 1. The Intracellular Life Cycle of *Francisella* in Macrophage. *Francisella* enters macrophages through noninflammatory receptors, such as Fc-gamma receptor (FcγR), scavenger receptor A (SRA), mannose receptor (MR) or complement receptor (CR3), and induces the formation of pseudopod loops. It is then trafficked through the macrophage inside of a phagosome, where it blocks NADPH oxidase and detoxifies reactive oxygen species (ROS) prior. Shortly after entering the macrophage, *Francisella* escapes the phagosome and replicates within the cytosol. Cytosolic bacteria can trigger the inflammasome, which leads to host cell death or associate with autophagosomes. This figure was conceptualized by Brooke Napier (Jones et al. 2012 *MMBR*, in press).

Figure 2

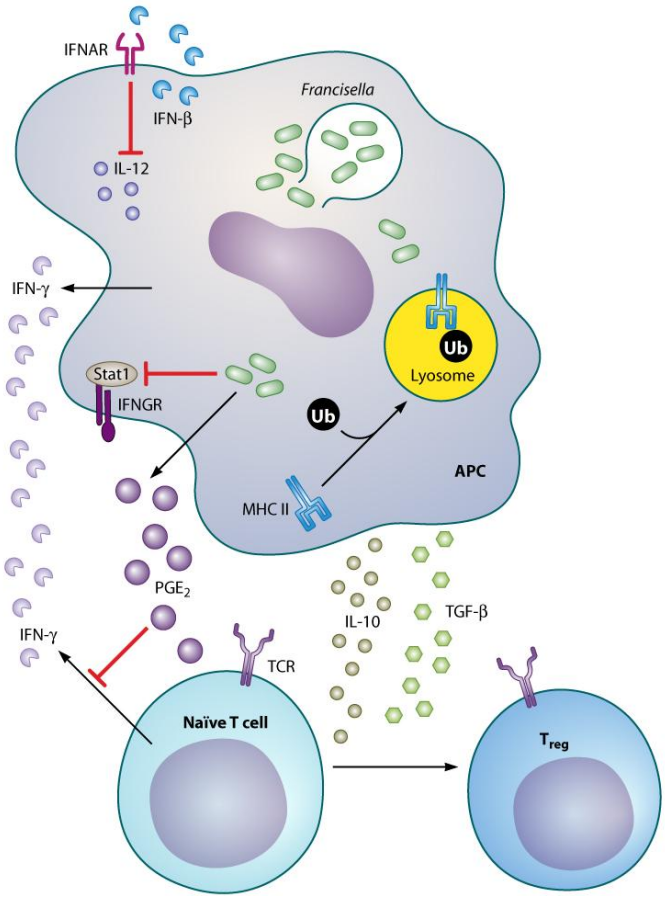

Figure 2. *Francisella* Interactions with the Adaptive Immune System. *Francisella* infects antigen-presenting cells (APCs) and dampens their ability to produce proinflammatory cytokines. IL-12 is blocked via the induction of beta interferon (IFN-β). IFN-γ is produced by dendritic cells and T cells during *Francisella* infection and it induces macrophages to kill the bacteria. *Francisella* blocks signal transduction through the IFN-γ receptor (IFNGR) by inhibiting Stat1 phosphorylation and through the induction of prostaglandin E2 (PGE_2) that inhibits IFN-γ production by T cells. To further hinder adaptive responses, *Francisella* hampers T cell activation by inducing the degradation of major histocompatibility complex class II (MHC II) (indicated by its

absence from the cell surface, tagging with ubiquitin (Ub), and trafficking towards the lysosome) and directing APCs to produce the anti-inflammatory cytokines TGF-β and IL-10. TGF-β promotes the development of regulatory T cells (T_{reg}) that are able to suppress inflammation and cell-mediated immune responses.

Chapter 2: TLR2 Signaling Contributes to Rapid Inflammasome Activation during *Francisella novicida* Infection

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Published in *PLoS ONE*, 2011

Volume 6, pg. e20609

All experiments were performed by Crystal Jones except for Figure 7, which was performed jointly with David Weiss. This manuscript was written by Crystal Jones and David Weiss.

Abstract

Early detection of microorganisms by the innate immune system is provided by surface-expressed and endosomal Toll-like receptors (TLRs), and activation of TLRs leads to the expression of proinflammatory cytokines such as IL-6 and IL-1β. Some intracellular bacteria subvert the TLR response by rapidly escaping the phagosome and entering the cytosol. However, these bacteria can be recognized by the inflammasome, a multi-protein complex comprised of a sensor protein, ASC and the cysteine protease caspase-1. Inflammasome activation leads to release of the proinflammatory cytokines IL-1β and IL-18 and death of the infected cell, an important host defense that eliminates the pathogen's replicative niche. While TLRs and inflammasomes are critical for controlling bacterial infections, it is unknown whether these distinct host pathways cooperate to activate defenses against intracellular bacteria. Using the intracellular bacterium *Francisella novicida* as a model, we show that TLR2$^{-/-}$ macrophages exhibited delayed inflammasome activation compared to wild-type macrophages as measured by inflammasome assembly, caspase-1 activation, cell death and IL-18 release. Components of the TLR2 signaling pathway, MyD88 and NF-κB, were required for rapid inflammasome activation. Furthermore, TLR2$^{-/-}$ mice exhibited lower levels of cell death, caspase-1 activation, and IL-18 production than wild-type mice upon *F. novicida* infection. These results show that TLR2 is required for rapid inflammasome activation in response to infection by cytosolic bacterial pathogens. In addition to further characterizing the role of TLR2 in host defense, these findings broaden our understanding of how the host integrates signals from spatiotemporally separated PRRs to coordinate an innate response against intracellular bacteria.

Introduction

The mammalian innate immune system defends against a variety of microbial pathogens. Early detection of invading microorganisms is provided by germline-encoded pattern recognition receptors (PRRs) that recognize conserved microbial components known as pathogen-associated molecular patterns (PAMPs). The macrophage, which acts as a sentinel of the innate immune system, is equipped with numerous membrane bound and cytosolic PRRs that can detect microbes. Recognition of PAMPs by PRRs enables the macrophage to rapidly mount a proinflammatory response aimed at eliminating microorganisms. One of the best-characterized families of PRRs is the Toll-like receptors (TLRs). TLRs are type I integral membrane proteins that recognize PAMPs such as lipopolysaccharide (TLR4), bacterial lipoproteins (TLR2), flagellin (TLR5), and CpG DNA (TLR9) (1). TLRs detect these foreign molecules at the plasma membrane and within phagosomes, resulting in the initiation of signaling cascades that lead to the activation of the transcriptional regulator NF-κB and the expression of proteins involved in host defense including the proinflammatory cytokines IL-6, TNF-α, and IL-1β (1).

Some bacterial pathogens evade TLRs by physically escaping the phagosome and reaching the cytosol where they replicate (2, 3). In order to detect and defend against these pathogens, the host uses cytosolic sensors such as the Nod-like receptor (NLR) and AIM2-like receptor (ALR) families. NLRs, such as NLRP3, respond to a diverse set of stimuli (4) while ALRs can bind to double-stranded DNA released from viruses and bacteria (5). Detection of PAMPs by either NLRs or AIM2 activates the inflammasome, a multiprotein complex composed of a sensor protein (NLR/AIM2), the adaptor protein

ASC, and the cysteine protease caspase-1 (6). Inflammasome activation leads to death of the infected macrophage, which is a host defense response that is thought to be protective since it eliminates the pathogen's replicative niche (7). In addition to inducing host cell death, inflammasome activation also leads to the caspase-1-mediated processing and maturation of the pro-forms of the proinflammatory cytokines IL-1β and IL-18 and their release from dying cells.

While both TLRs and the inflammasome can be activated during infection, if and how these host defense systems cooperate during infection remains unclear. However, these host defenses have been shown to work together under other circumstances. For example, the NLRP3 inflammasome is activated by a two-step process, which is initiated by priming macrophages with LPS or other TLR agonists to induce the expression of NLRP3 (8). This priming step is also required to induce the expression of pro-IL-1β, whereas expression of pro-IL-18 is constitutive in macrophages (9). Enhanced NLRP3 expression is required for responsiveness to the second stimulus, ATP, which signals through the $P2X_7$ receptor ($P2X_7R$) (10) and induces inflammasome activation, leading to cell death and the release of both IL-1β and IL-18. While this suggests that TLRs can act to help facilitate NLRP3 inflammasome activation under certain conditions, it is unclear if this type of cooperation occurs during the activation of inflammasomes in the context of a bacterial infection.

Francisella novicida is a Gram-negative intracellular bacterium that has been used as a model organism to study various aspects of inflammasome activation (11). Shortly after being internalized by a macrophage, this bacterium escapes the phagosome and begins to replicate in the cytosol where it is recognized by the AIM2 inflammasome

(12, 13), but not the NLRP3 (10) or NLRC4 inflammasomes (14). *F. novicida* is closely related to the highly virulent *F. tularensis*, which causes the disease tularemia in humans and has been categorized as a potential bioweapon by the Centers for Disease Control and Prevention (15). Additionally, *F. novicida* causes a tularemia-like disease in mice, allowing for the study of the role of the inflammasome in host defense during *in vivo* infection (16).

Although *Francisella* species are capable of invading an array of cells during infection (17), macrophages are considered to be the first cells that the bacteria encounter, and this interaction is critical for establishment of disease. TLR2, which signals in response to *Francisella* lipoproteins (18), is the primary TLR involved in recognizing and responding to this pathogen. *Francisella* LPS contains modifications that abrogate TLR4 recognition (19), the bacterium lacks genes that encode flagella (20) and therefore does not activate TLR5 (21, 22), and TLR9 plays a minor role, if any, in providing the host with protection against infection *in vivo* (23). Furthermore, TLR2 and the TLR adaptor protein MyD88 are critical for host defense against *Francisella* infection since mice lacking these proteins are more susceptible to infection than their wild-type counterparts (23-25). After *F. novicida* escapes the phagosome and replicates in the cytosol of macrophages, it is recognized by the AIM2 inflammasome complex which is essential for controlling *F. novicida* infection *in vivo* since mice lacking AIM2, ASC, or caspase-1 are highly susceptible to infection (12, 14).

Due to the sequential activation of TLR2 and the AIM2 inflammasome during *F. novicida* infection of macrophages and the critical role that both of these recognition systems play in host defense against this bacterium, we tested whether these systems

cooperate during infection. We found that TLR2 contributes to the rapid induction of inflammasome assembly, caspase-1 activation, host cell death and IL-18 release, since $TLR2^{-/-}$ macrophages have a significant delay in these inflammasome-dependent responses during *F. novicida* infection. Furthermore, TLR2 contributes to rapid inflammasome activation by signaling through MyD88 and NF-κB in infected macrophages. These findings were further validated with *in vivo* experiments showing that TLR2 contributes to inflammasome activation during *F. novicida* infection. Together these results highlight a novel way in which TLR2 contributes to innate immune signaling during bacterial infection. Furthermore, these findings demonstrate that innate recognition systems localized in different macrophage compartments that are activated at different times during infection, can cooperate to provide a multi-tiered defense response against intracellular bacterial pathogens.

Materials and Methods

Bacterial strains and growth conditions

Francisella novicida strain U112 and an isogenic *F. novicida mglA* mutant previously described (26) were obtained from Dr. Denise Monack (Stanford University, Stanford, CA). *E. coli* DH5α was obtained from Invitrogen (Carlsbad, CA). All bacterial cultures were grown overnight at 37°C with aeration in tryptic soy broth (BD Biosciences, Sparks, MD), and *F. novicida* cultures were supplemented with 0.2 % L-cysteine (BD Biosciences). Bacteria were killed by incubating in a water bath heated to 100°C for 30 minutes. Heat-killed bacteria were plated on tryptic soy agar (TSA) supplemented with 0.1 % L-cysteine to ensure that all bacteria were killed after treatment.

Mice

Wild-type and $P2X_7R^{-/-}$ C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). $TLR2^{-/-}$ and $MyD88^{-/-}$ C57BL/6 mice were generous gifts from Dr. Bali Pulendran (Emory Vaccine Center, Atlanta, GA). All animals were housed under specific pathogen-free conditions in filter-top cages at the Emory Vaccine Center vivarium and provided with sterile water and food *ad libitum*. All animal studies were reviewed and approved by the Emory University Institutional Animal Care and Use Committee (protocol #069-2008Y).

Macrophage experiments and infections

Murine bone marrow-derived macrophages (BMDM) were prepared from wild-type, $TLR2^{-/-}$, $MyD88^{-/-}$ and $P2X_7R^{-/-}$ C57BL/6 mice and cultured as described (11).

Macrophages were cultured in 96-well plates ($8x10^4$ cells/well) or 6-well plates ($1.8x10^6$ cells/well) in high glucose Dulbecco's modified Eagle's medium (DMEM) (Lonza, Walkersville, MD) supplemented with 10 % heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 10 % L929-conditioned media (conditioned DMEM) overnight. The media was removed and bacteria were added at the indicated multiplicities of infection (MOI) expressed as bacteria per macrophage. Plates were spun for 15 minutes at 2,000 rpm at room temperature to promote uptake of bacteria. Macrophages were incubated for 30 minutes at 37°C and washed two times before adding warm conditioned DMEM. To assess TLR signaling, macrophages were treated with Pam_3CSK_4 (100 μg/ml) (Invivogen, San Diego, CA), UltraPure LPS (100 μg/ml) (Invivogen), heat-killed *F. novicida* (100 killed bacteria/macrophage) and supernatants were collected after 6 h. The concentration of IL-6 in culture supernatants was quantified by ELISA (BD Biosciences). To inhibit NF-κB, macrophages were treated with 20 μM of caffeic acid phenethyl ester (CAPE) (EMD, Gibbstown, NJ) for 2 hours prior to infection (27, 28). For cell death assays, culture supernatants were collected at the specified timepoints after infection and cell death was quantified colorimetrically using the Cyto-Tox96 lactate dehydrogenase (LDH) release kit according to the manufacturer's instructions (Promega, Madison, WI). IL-18 (MBL International Corporation, Woburn, MA) and IL-1β (BD Biosciences) levels in the supernatants were quantified by ELISA.

Immunoblotting

Macrophages were infected with bacteria at a multiplicity of infection of 100:1 and lysed with Lysis Buffer (2 mM DTT and 10 % NP-40) supplemented with Complete Protease

Inhibitor Cocktail (Roche, Indianapolis, IN). Proteins were resolved by SDS-PAGE using 4-15 % polyacrylamide mini-gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. Blots were probed with rat anti-mouse caspase-1 p20 clone 4B4.2.1, rat anti-mouse ASC clone 8E4.1 (both generous gifts from Dr. Sanjeev Mariathasan, Genentech, South San Francisco, CA) and anti-mouse β-actin (Sigma-Aldrich, St. Louis, MO) antibodies. Proteins were visualized by SuperSignal ECL substrate (Thermo Scientific, Rockford, IL) and detected using the UVP Multispectral Imaging System (Upland, CA).

Macrophage staining and immunofluorescence

Macrophages were seeded onto glass cover slips in 24-well plates (3×10^5 cells/well) and infected as described above. Infected cells were washed and fixed with 4 % paraformaldehyde for 10 minutes at 37°C. Cells were incubated with primary antibodies against ASC (clone 8E4.1) and the p10 subunit of caspase-1 (Santa Cruz Biotechnology Inc, Santa Cruz, CA) for 30 minutes at 37°C. Following incubation, cells were washed three times with PBS and incubated with the appropriate Alexa Fluor–conjugated secondary antibodies and phalloidin stain (Invitrogen) for 30 minutes at 37°C. Cells were washed three times and cover slips were mounted over SlowFade Gold antifade reagent containing DAPI (Invitrogen). Images were obtained using a Zeiss Axioscope Z.1 microscope equipped with a Zeiss Imager 2.1 camera, and images were taken at 40x magnification. AxioVision software 4.6.3 was used for image acquisition. Speck formation was quantified by measuring the percentage of macrophages containing ASC or ASC-caspase-1 specks in a total of 10 fields for each sample. To measure phagosomal

escape, macrophages were pre-chilled at 4°C and infected with *F. novicida* at an MOI of 100:1. Cells were then rapidly warmed for 5 min in a 37°C water bath to allow for bacterial uptake. Macrophages were incubated for 10 min at 37°C, then washed three times with warm DMEM to remove extracellular bacteria, and incubated at 37°C until the indicated timepoints. At 30 and 60 minutes post-infection, cells were washed three times with PBS and fixed with 4 % paraformaldehyde for 10 minutes at 37°C. Following fixation, cells were incubated with primary antibodies against LAMP-1 (1D4B) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and *F. novicida* (generous gift from Dr. Denise Monack, Stanford University, Stanford, CA) for 30 minutes at 37°C. Following incubation, cells were washed three times with PBS and incubated with the appropriate Alexa Fluor–conjugated secondary antibodies and phalloidin stain (Invitrogen) for 30 minutes at 37°C. Cover slips were mounted over SlowFade Gold antifade reagent containing DAPI. Images were obtained using a Zeiss Axioscope Z.1 microscope equipped with a Zeiss Imager 2.1 camera, and images were taken at 63x magnification. To visualize and enumerate bacteria colocalizing with LAMP-1, Volocity software 5.5 (Improvision) was used to assemble z-stacks into 3D images.

Mouse infections

Groups of five wild-type and TLR2$^{-/-}$ mice were infected intraperitoneally with 2 x 10^6 CFU of *F. novicida* suspended in 500 μL of sterile PBS. At 4 hours post-infection, blood was collected from the mice and serum was prepared. The livers of infected mice were harvested and divided. One half of a lobe of the liver was homogenized and dilutions

were plated on Mueller-Hinton agar supplemented with 0.1 % L-cysteine for enumeration of bacterial levels in each organ. The other half was frozen in O.C.T. compound (TissueTek, Torrance, CA) and stored at -80°C until being sectioned.

Tissue staining and immunofluorescence

Frozen livers from infected mice were cut into 8 μm sections. To visualize cell death, liver sections were stained using a fluorescent In Situ Cell Death Detection Kit (Roche) according to the manufacturer's instructions. Bacteria were labeled using a chicken anti-*F. novicida* antibody (generous gift from Dr. Denise Monack, Stanford University, Stanford, CA). Coverslips were mounted over SlowFade Gold antifade reagent containing DAPI (Invitrogen). The number of TUNEL positive cells per field was calculated in a total of 10 fields for each sample. For detection of active caspase-1, liver sections were stained with goat anti-caspase-1 p20 antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) and donkey anti-goat IgG-FITC (Santa Cruz Biotechnology, Inc). Mature caspase-1 p20 was quantified using single channel densitometry to measure the total fluorescence emission of FITC-labeled liver sections. Images were obtained using a Zeiss Axioscope Z.1 microscope equipped with a Zeiss Imager 2.1 camera, and images were taken at 20x magnification. AxioVision software 4.6.3 was used for image acquisition and densitometry measurements.

Statistical analysis

The statistical significance of all data in this manuscript was analyzed using the Student's t-test or Mann-Whitney test with GraphPad software (GraphPad, La Jolla, CA).

Results

TLR2 contributes to rapid inflammasome activation during *F. novicida* infection

TLR signaling has been shown to act in synergy with cytosolic host defense systems to elicit a proinflammatory response against bacteria (8, 29-32). Considering these findings and the fact that *F. novicida* activates TLR2 and the cytosolic AIM2 inflammasome, we investigated whether TLR2 contributes to inflammasome activation during *F. novicida* infection. While LPS-prestimulated macrophages are often used to study inflammasome activation, we used unstimulated macrophages in our experiments for several reasons: 1) to examine the role of TLR2 during natural infection in the absence of exogenous stimuli, 2) to mimic the early events of natural infection, and 3) because *F. novicida* makes an LPS with very low stimulatory activity (19) and TLR4 is not normally activated during infection with *Francisella*.

The hallmarks of inflammasome activation are host cell death, proteolytic maturation of caspase-1, and the secretion of the proinflammatory cytokines IL-1β and IL-18 (6). We first assayed for host cell death by quantifying the level of lactate dehydrogenase (LDH) released into the supernatants of infected macrophages after infection. At 6 h post-infection, soon after *F. novicida* starts to replicate in the cytosol (33), 12 % of wild-type macrophages were dead while no death was detectable in TLR2$^{-/-}$ cells (Figure 1A). The delayed cell death phenotype was observed in TLR2$^{-/-}$ macrophages up to 10 h post-infection. This delay in cell death was observed at MOIs of 10:1 and 100:1 (Figure 1B). By 14 h post-infection, the levels of cell death in wild-type and TLR2$^{-/-}$ macrophages were equivalent (~ 96 %) (Figure 1A), demonstrating that TLR2 contributes to rapid inflammasome-dependent cell death but is not absolutely

required for this process. This is in contrast to macrophages from ASC$^{-/-}$ and caspase-1$^{-/-}$ mice, which did not undergo cell death between 6 and 14 h post-infection (data not shown), in agreement with previous studies (14). The rapid cell death response was dependent on escape of the bacteria into the cytosol since infection with an *F. novicida* strain unable to escape the phagosome (due to a point mutation in the *mglA* gene, which encodes a transcriptional regulator required for phagosomal escape) did not induce host cell death (Figure 1B). This is also in agreement with previous studies (14).

To ensure that the delayed induction of cell death exhibited by TLR2$^{-/-}$ macrophages was not due to an inability of *F. novicida* to reach and replicate in the cytosol of these cells, we measured phagosomal escape and intracellular replication. Between 30 and 60 minutes post-infection, there was a decrease in the number of LAMP-1 associated bacteria (Supporting Figure 1A, and in agreement with kinetics observed previously (11, 34)), which is indicative of bacteria escaping the phagosome. Since phagosomal escape is required for *Francisella* to replicate in macrophages (26, 35), we measured the intracellular replication of the bacteria and found that they were able to replicate with similar kinetics in both wild-type and TLR2$^{-/-}$ macrophages (Supporting Figure 1B), also consistent with previous studies (25, 36). Taken together, these results demonstrate that the role of TLR2 in promoting rapid inflammasome activation during *F. novicida* infection cannot be explained by differences in bacterial trafficking or replication.

To further test the role of TLR2 in inflammasome-dependent responses, we measured the levels of IL-1β and IL-18 in macrophage supernatants following infection with *F. novicida*. IL-1β secretion is often used as a marker for inflammasome activation,

but TLR2 regulates the expression of pro-IL-1β in response to *Francisella* infection, complicating the use of this cytokine as a marker for inflammasome activity (37). As expected, supernatants collected from TLR2$^{-/-}$ macrophages infected with *F. novicida* for 6.5 h at an MOI of 100:1 were devoid of IL-1β, whereas wild-type macrophages secreted IL-1β (Figure 1C). Since we cannot separate the role of TLR2 in transcriptionally controlling pro-IL-1β expression from an effect on inflammasome activation, we did not use IL-1β as a marker in this study. Instead we used IL-18 which is constitutively transcribed and expressed at the protein level independently of TLR2 (Supporting Figure 2A, B) (9). At 6.5 h post-infection, the supernatants of wild-type macrophages infected with *F. novicida* contained significantly increased levels of IL-18 compared to infected TLR2$^{-/-}$ macrophages (Figure 1D). Furthermore, IL-18 release was also dependent on bacterial escape into the cytosol since the *mglA* mutant did not induce this response. The role of TLR2 in promoting IL-18 secretion further demonstrates that TLR2 plays a role in the rapid induction of the inflammasome during *F. novicida* infection.

TLR2 contributes to rapid inflammasome assembly and caspase-1 processing in response to *F. novicida* infection

Since inflammasome activity is dependent upon assembly of this complex (38), we investigated whether the delay in host cell death and IL-18 release exhibited by TLR2$^{-/-}$ macrophages during *F. novicida* infection was due to a delay in complex formation. Inflammasome complexes are formed when ASC is recruited from a diffuse localization in the cytosol to a single cytosolic focus, to which procaspase-1 is recruited (39). Using fluorescence microscopy to visualize inflammasome complex formation, we found that

TLR2$^{-/-}$ macrophages exhibited delayed ASC foci formation compared with wild-type macrophages during *F. novicida* infection (Figure 2A). At 5.5 h post-infection, before any detectable cell death had occurred, 12 % of wild-type macrophages infected with *F. novicida* contained ASC foci while only 3 % of infected TLR2$^{-/-}$ macrophages contained ASC foci (Figure 2B). Interestingly, TLR2$^{-/-}$ macrophages also contained fewer ASC foci colocalizing with caspase-1, compared to wild-type macrophages (Figure 2C). This defect was still apparent after macrophages began to undergo cell death at 6 h post-infection (Figure 2B, C and Figure 1A). To ensure that the inflammasome complexes formed in response to *F. novicida* infection were dependent on ASC, we infected ASC$^{-/-}$ macrophages with *F. novicida* and found that these macrophages were unable to form ASC or caspase-1 foci (Figure 2A-C). These results demonstrate that TLR2 promotes rapid inflammasome assembly in response to *F. novicida* infection.

Proteolytic maturation of caspase-1 is dependent on inflammasome assembly and required for inflammasome activity (38, 40, 41). Therefore, we measured the levels of processed caspase-1 in wild-type and TLR2$^{-/-}$ macrophages following *F. novicida* infection. At 6 h post-infection, we detected the processed p20 subunit of caspase-1 in lysates from infected wild-type, but not TLR2$^{-/-}$macrophages (Figure 3). We could detect processed caspase-1 at 7.5 h post-infection in TLR2$^{-/-}$ macrophages, but the level was still less than that observed in wild-type macrophages (Supporting Figure 3). This difference in caspase-1 maturation was independent of any differences in procaspase-1 levels, which were equivalent in wild-type and TLR2$^{-/-}$ macrophages (Figure 3). Furthermore, the expression of the other known inflammasome components ASC (Figure 3) and AIM2 (Supporting Figure 4) were also equal in infected wild-type and TLR2$^{-/-}$ macrophages,

demonstrating that induction of the expression of inflammasome components does not explain the role of TLR2 in promoting inflammasome activity. In agreement with the data from Figure 1, bacterial escape into the cytosol was essential for the induction of caspase-1 processing since the *mglA* mutant did not induce this response (Figure 3). Taken together, these data demonstrate that TLR2 promotes rapid inflammasome assembly during *F. novicida* infection of macrophages, leading to more rapid caspase-1-processing and inflammasome activation.

Components of the TLR2 signaling pathway are required for rapid inflammasome activation

Since we have shown that TLR2 signaling contributes to rapid *F. novicida*-induced inflammasome activation, we examined whether TLR2 signaling alone was sufficient to induce this response. Macrophages treated with the TLR2 agonist Pam_3CSK_4 or heat-killed *F. novicida*, which contains bacterial lipoproteins (BLPs) that activate TLR2, did not induce any host cell death, suggesting that TLR2 signaling alone is not sufficient for this response (Supporting Figure 5). In addition, macrophages infected with the *mglA* mutant strain of *F. novicida*, as a control, were unable to induce host cell death. To demonstrate the TLR2-activating capacity of these stimuli, we measured the levels of IL-6 in the supernatants of stimulated wild-type and $TLR2^{-/-}$ macrophages. All of the stimuli induced IL-6, confirming that our preparations were active (Supporting Figure 5A). As expected, Pam_3CSK_4, heat-killed *F. novicida*, and the *mglA* mutant all induced IL-6 in a strictly TLR2-dependent manner. As a control for the specificity of the $TLR2^{-/-}$ macrophages, we demonstrated that LPS, a TLR4 agonist,

induced IL-6 secretion in both wild-type and TLR2$^{-/-}$ macrophages. Collectively, and in agreement with previous results (14, 42), these data show that TLR2 signaling alone is not sufficient to induce inflammasome activation.

Next, we sought to determine if known components of the TLR2 signaling pathway contribute to rapid inflammasome activation in response to *F. novicida* infection. The TLR2 adaptor protein MyD88 is absolutely required for *Francisella*-induced cytokine production in macrophages (36), and we therefore investigated if it also played a role in rapid inflammasome activation. MyD88$^{-/-}$ macrophages infected with wild-type *F. novicida* exhibited significantly less cell death (Figure 4A) and secreted less IL-18 (Figure 4B) than wild-type macrophages, showing that MyD88 is involved in rapid inflammasome activation. The rapid cell death response was also dependent on phagosomal escape since infection with the *mglA* mutant strain of *F. novicida* did not induce host cell death (Figure 4A). This difference was not due to an inability of *F. novicida* to replicate in MyD88$^{-/-}$ macrophages (Supporting Figure 1). To determine whether NF-κB-dependent signaling played a role in rapid activation of the inflammasome during *F. novicida* infection, we pre-treated macrophages with caffeic acid phenethyl ester (CAPE), which has previously been shown to block NF-κB translocation to the nucleus of macrophages infected with *F. novicida* (27, 28). CAPE-treated wild-type and TLR2$^{-/-}$ macrophages did not undergo cell death (Figure 5A) or secrete IL-18 (Figure 5B) after *F. novicida* infection. These results demonstrate that the TLR2 signaling components MyD88 and NF-κB play a role in inducing rapid inflammasome activation during *F. novicida* infection.

F. novicida-induced inflammasome activation is independent of the $P2X_7R$ pathway

TLR signaling has previously been shown to be involved in inflammasome activation when triggered in conjunction with the $P2X_7$ receptor ($P2X_7R$) pathway, which leads to activation of the NLRP3 inflammasome (10). We investigated whether the $P2X_7R$ pathway is involved in rapid inflammasome activation in response to *F. novicida* infection, which would potentially explain how TLR2 contributes to this process. We infected $P2X_7R^{-/-}$ macrophages with wild-type *F. novicida* and found that the levels of cell death (Figure 6) and IL-18 secretion (data not shown) were similar to the levels measured in infected wild-type macrophages at 7 h post-infection. In contrast, $TLR2^{-/-}$ macrophages displayed defective responses. These findings demonstrate that *F. novicida* induces rapid inflammasome activation through a $P2X_7R$-independent mechanism. This is in agreement with results from us and others demonstrating that *F. novicida*-induced inflammasome activation is independent of NLRP3 (10, 12, 43). Therefore, the previously identified TLR/$P2X_7R$/NLRP3 pathway cannot explain the role of TLR2 in rapid inflammasome activation by *F. novicida*.

TLR2 plays a role in inflammasome activation in response to *Listeria*

Since we have shown that TLR2 is required for rapid inflammasome activation in response to *F. novicida*, we tested whether TLR2 plays a similar role in inflammasome activation during infection with another cytosolic bacterial pathogen known to activate the AIM2 inflammasome, *Listeria monocytogenes* (5). We found that 15 % of wild-type macrophages infected with *L. monocytogenes* at an MOI of 20:1 underwent cell death at 1 h post-infection, while only 3 % of $TLR2^{-/-}$ macrophages died (Supporting Figure 6).

This difference in cell death was also exhibited in macrophages infected at an MOI of 1:1 at 5 h post-infection (data not shown). These results provide evidence demonstrating that TLR2 promotes inflammasome activation in response to multiple cytosolic bacterial pathogens.

TLR2 is required for rapid *F. novicida*-induced inflammasome activation *in vivo*

We have shown that TLR2 signaling is required for rapid inflammasome activation in response to *F. novicida* infection in macrophages. To determine whether TLR2 plays a similar role in inflammasome activation *in vivo*, we infected wild-type and TLR2$^{-/-}$ mice intraperitoneally with 2 x10^{6} CFU of wild-type *F. novicida* and assayed for inflammasome activation using several readouts. TUNEL staining was used to assess the amount of host cell death in the livers of infected mice. At 4 h post-infection, we observed increased TUNEL staining in the livers of wild-type mice in comparison to the livers of TLR2$^{-/-}$ mice (Figures 7A, B). This response was independent of any differences in bacterial loads in infected livers since wild-type and TLR2$^{-/-}$ mice harbored equivalent levels of bacteria at this early timepoint (Fig. 7C). This result is consistent with our finding that TLR2$^{-/-}$ macrophages exhibited significantly less cell death in response to *F. novicida* infection *in vitro* (Figure 1).

To determine whether TLR2 contributes to caspase-1 activation *in vivo*, fluorescence microscopy was used to visualize the mature 20 kD subunit of active caspase-1 in the livers of infected wild-type and TLR2$^{-/-}$ mice. We observed increased levels of active caspase-1 in the livers of wild-type mice compared to TLR2$^{-/-}$ mice 4 h post-infection (Figures 7D, E). Previous studies have shown that the inflammasome can

be visualized microscopically and that *F. novicida* colocalizes with this complex during macrophage infection (13, 44). We observed *F. novicida* colocalizing with mature caspase-1 in the livers of infected mice, which may represent the first time that the active inflammasome has been visualized during bacterial infection *in vivo*.

Active caspase-1 cleaves pro-IL-18 resulting in its release into the extracellular environment. Therefore, we measured the amount of IL-18 present in the serum of infected mice. The concentration of IL-18 measured in the serum of $TLR2^{-/-}$ mice was significantly reduced when compared to wild-type mice following infection (Figure 7F), and this was not due to differences in the expression of IL-18 mRNA (data not shown). Taken together, these results show that TLR2 is required for rapid inflammasome-dependent host cell death, caspase-1 activation and IL-18 production during *F. novicida* infection *in vivo*.

Discussion

Upon infection of macrophages, *F. novicida* is initially recognized by TLR2 at the plasma membrane and in the phagosome, leading to the induction of a proinflammatory response dependent upon MyD88 and NF-κB (21, 37). Subsequently, the bacteria escape the phagosome and reach the cytosol where they are recognized by the inflammasome, leading to death of the infected macrophage and the release of the proinflammatory cytokines IL-1β and IL-18 (14). Here, we show that TLR2, in addition to inducing proinflammatory cytokine production through the canonical MyD88/NF-κB pathway, contributes to rapid inflammasome activation in response to *F. novicida* infection.

TLR2$^{-/-}$ macrophages exhibited a delayed inflammasome response compared to wild-type macrophages, as indicated by delayed inflammasome assembly, levels of caspase-1 maturation, host cell death and IL-18 secretion (Figures 1-3). Interestingly, TLR2$^{-/-}$ macrophages did undergo inflammasome-dependent cell death after *F. novicida* infection, in contrast to ASC$^{-/-}$, caspase-1$^{-/-}$ and AIM2$^{-/-}$ macrophages, which do not die up to 14 h post-infection ((12, 14) and data not shown). This suggests that TLR2 is not absolutely required for inflammasome activation, but instead contributes to rapid induction of inflammasome activity. Consistent with these *in vitro* findings, we observed decreased levels of mature caspase-1 and host cell death in the livers of *F. novicida*-infected TLR2$^{-/-}$ mice compared to wild-type mice, as well as decreased levels of IL-18 in the serum (Figure 7). Recently, it was reported that highly virulent *F. tularensis* strains induced caspase-3-dependent cell death in mice (45). It would be interesting to test whether TLR2 contributes to cell death during infection with these strains as well. Taken

together, these findings clearly demonstrate that TLR2 plays an important role in promoting rapid inflammasome activation during *F. novicida* infection.

The role of TLR2 is not explained by differences in the expression levels of AIM2 inflammasome components since TLR2 signaling does not significantly alter the expression of AIM2 (Supporting Figure 4), ASC or caspase-1 (Figure 3). It is, however, possible that TLR2 signaling induces the expression of an as yet unidentified inflammasome component, explaining its effect on inflammasome activation. AIM2 expression is dependent on type I interferons (IFN) including IFN-β(46), which is also required for *F. novicida*-induced inflammasome activation (47). Therefore, the role of TLR2 in rapid inflammasome assembly could be explained if TLR2 controlled IFN-β expression. In fact, Cole *et al* found that TLR2 regulates IFN-β expression in macrophages during *F. tularensis* Live Vaccine Strain (LVS) infection (37, 48). However, we found equivalent levels of IFN-β mRNA in wild-type and $TLR2^{-/-}$ macrophages following *F. novicida* infection (data not shown), in agreement with previous reports demonstrating that TLR adaptor proteins do not play a significant role in IFN-β expression during *F. novicida* infection (47). In addition, our findings showed that AIM2 expression was not dependent on TLR2 signaling (Supporting Figure 4). Discrepancies between the present study and those of Cole *et al* could be due to differences in the kinetics of the induction of IFN-β in macrophages infected with *F. novicida*, which is more rapid than during *F. tularensis* LVS infection (37, 47). This rapid induction of IFN-β by *F. novicida* may mask the potential contribution of TLR2 to its expression.

AIM2 inflammasome activation has previously been shown to occur in response to bacterial and viral infections (5), but can also be induced by transfection of double-stranded DNA into host cells, bypassing the need for TLRs (44, 49). Our finding that TLR2 is not absolutely required for *F. novicida*-induced inflammasome activation is in agreement with the ability of the AIM2 inflammasome to be activated independently of TLRs. However, in the context of *F. novicida* infection, we find that TLR2 nonetheless promotes more rapid AIM2 inflammasome activation (Figure 1A). To the best of our knowledge, the contribution of TLR signaling in promoting rapid AIM2 inflammasome activation during infection with the intracellular bacterium *F. novicida* is novel.

TLR signaling has been linked to NLRP3 inflammasome activation when macrophages are costimulated with TLR ligands and extracellular ATP, which activates the $P2X_7R$ pathway (10). However, we find that the $P2X_7R$ pathway is not required for rapid *F. novicida*-induced inflammasome activation (Figure 6), and we and others have previously shown that this process occurs independently of NLRP3 (10, 12, 43). This demonstrates that the role of TLR2 in promoting rapid inflammasome activation cannot be explained by cooperation with the $P2X_7R$/NLRP3 pathway. However, TLR2 signaling through NF-κB induces the expression of a multitude of proteins involved in host defense, including antimicrobial peptides (50). It is possible that TLR2 indirectly promotes inflammasome activation by increasing the expression of NF-κB-dependent antimicrobial peptides, which damage the bacteria, leading to increased release of bacterial DNA, the activator of the AIM2 inflammasome. Whether antimicrobial peptides or other defenses contribute to release of bacterial DNA and inflammasome activation is an important future question to answer.

Additionally, we show that TLR2 contributes to inflammasome activation in response to infection by *L. monocytogenes*, a cytosolic bacterium that is also recognized by AIM2 (Supporting Figure 6). In a previous study by Özören *et al*, TLR2 was shown to play no role in inflammasome activation in thioglycollate-elicited peritoneal macrophages infected with *L. monocytogenes* at an MOI of 50:1 (51). The high multiplicity of infection used by the authors of that study, which was higher than those used in the present study, could have forced the infection to proceed more rapidly, thereby masking the role of TLR2 in rapid inflammasome activation. In fact, we found that bone marrow-derived macrophages infected with *L. monocytogenes* at MOIs of 50:1 and 100:1 did not require TLR2 for inflammasome activation (data not shown). It would be interesting to test whether TLRs play a role in promoting more rapid AIM2 inflammasome activation during infections with other pathogens that activate AIM2 such as vaccinia virus and murine cytomegalovirus (5).

Our results suggest that TLR2 and the AIM2 inflammasome provide an integrated, multi-tiered recognition and defense system against intracellular bacteria. TLR2 recognizes the presence of both pathogenic and nonpathogenic bacteria at the plasma membrane and in the phagosome of macrophages, whereas the AIM2 inflammasome recognizes pathogenic bacteria that escape the phagosome and reach the cytosol. The initial induction of the TLR2 signaling pathway promotes more rapid AIM2 inflammasome activation only in the event that bacteria reach the cytosol. This enables the macrophage to elicit an innate response based on the level of the threat imposed by invading bacteria. Inflammasome-mediated host cell death is a protective host response that removes the bacterium's replicative niche, but also comes with a significant cost

since it depletes macrophages as well. Therefore, this response is tightly regulated and only induced when the macrophage encounters a heightened level of danger that is triggered when bacteria reach the cytosol. Taken together, we propose a model in which macrophages integrate signals from the spatiotemporally separated TLR2 signaling pathway and AIM2 inflammasome complex during infection in order to mount an appropriate innate immune response against invading bacteria.

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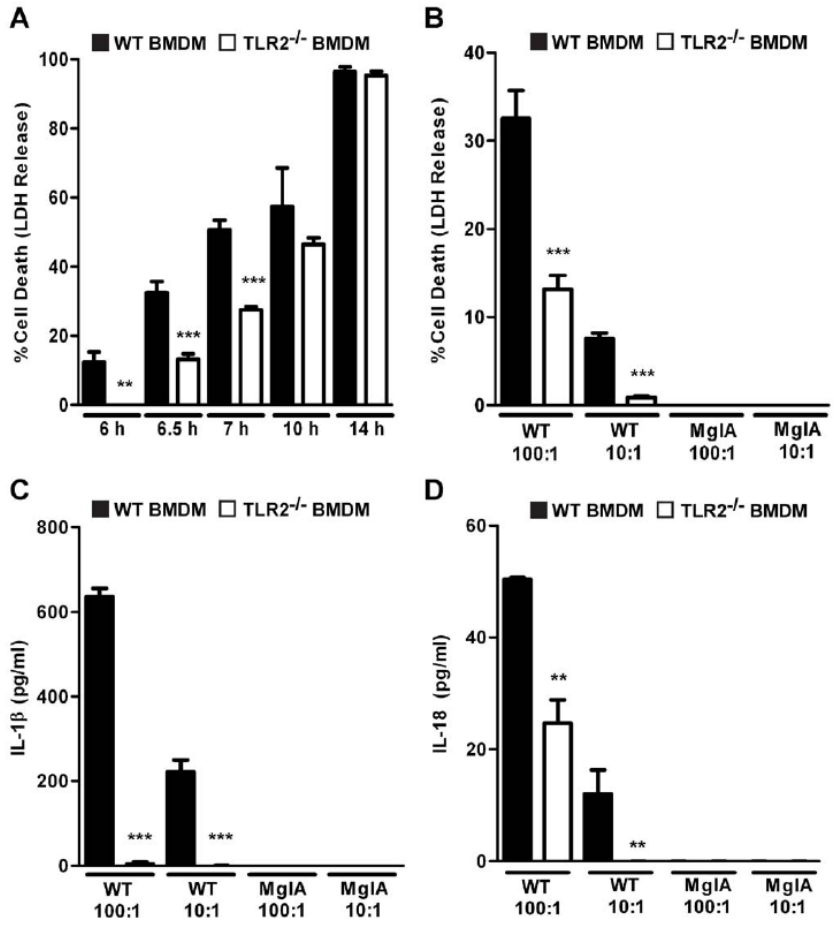

Figure 1. TLR2 is required for rapid inflammasome activation in response to *F. novicida* infection. (A) Bone marrow-derived macrophages (BMDM) from wild-type and TLR2$^{-/-}$ C57BL/6 mice were infected with wild-type *F. novicida* at an MOI of 100:1 and supernatants were collected at 6-14 h post-infection for quantification of macrophage death as measured by LDH release. (B) Macrophages were infected with wild-type (WT) and *mglA* mutant strains of *F. novicida* (MglA) at the indicated MOIs for 6.5 h and supernatants were collected for quantification of cell death, and levels of (C) IL-1β and (D) IL-18. Data are representative of five independent experiments. Error bars represent the standard deviation of triplicate samples. *** $p \leq 0.0007$, ** $p < 0.0085$.

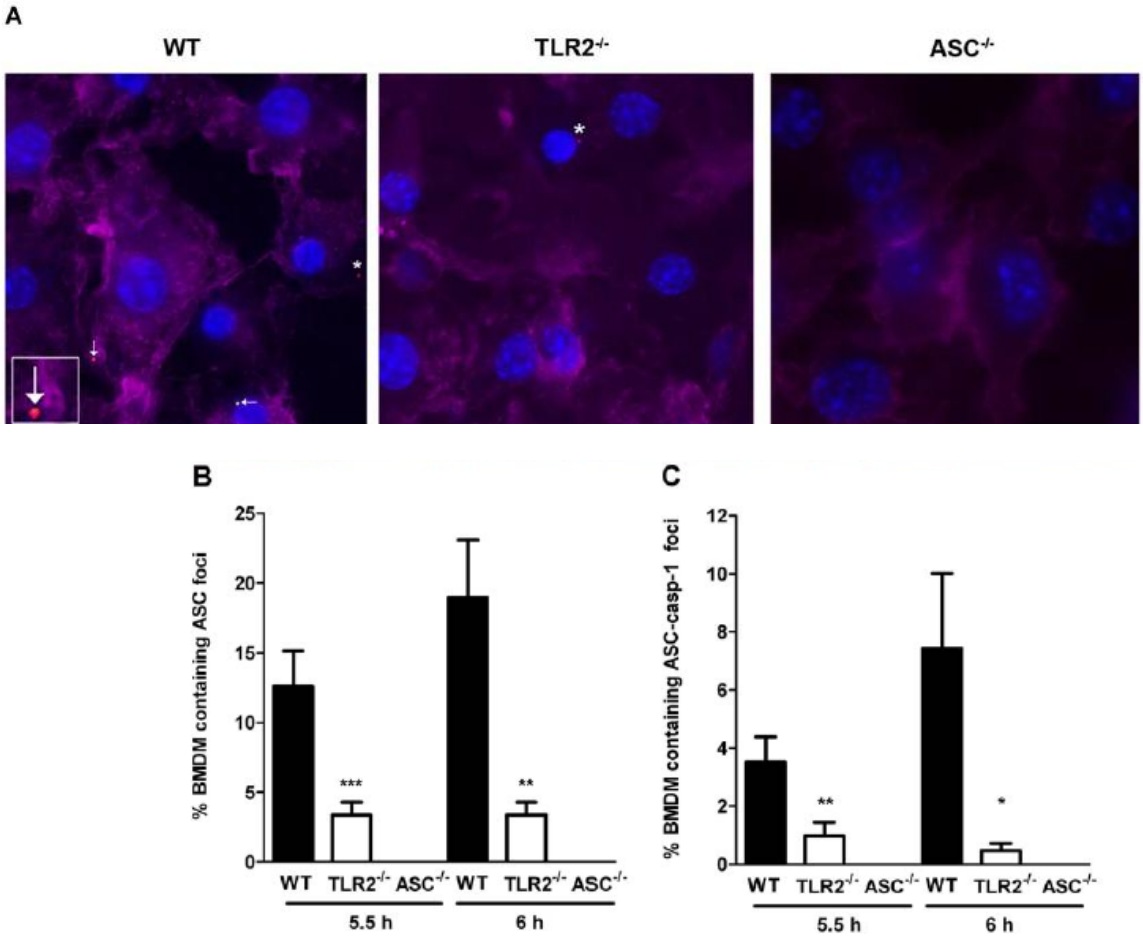

Figure 2. TLR2 contributes to rapid inflammasome assembly during infection with *F. novicida*. Wild-type and TLR2$^{-/-}$ BMDM were infected with *F. novicida* at an MOI of 100:1 for 6 h. (A) Macrophages were stained for the presence of caspase-1 (green), ASC (red), DNA (blue) and actin (purple) and visualized by fluorescence microscopy. Stars show ASC foci and arrows point to ASC and caspase-1 colocalized foci. Inflammasome assembly was quantified by counting the number of macrophages containing (B) ASC foci or (C) ASC-caspase-1 colocalized foci. Error bars represent the standard error of the mean (SEM). Data are representative of two independent experiments, in which at least 200 macrophages were counted for each sample. *** $p = 0.0008$, ** $p < 0.0040$, * $p = 0.0167$.

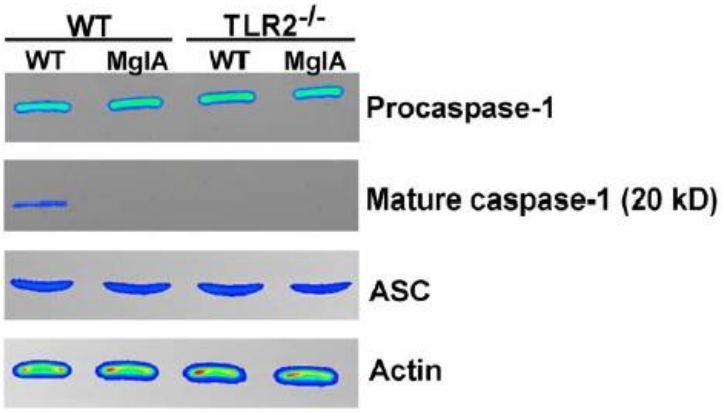

Figure 3. TLR2 is required for rapid activation of caspase-1 during *F. novicida* infection. BMDM were infected with wild-type (WT) and *mglA* mutant strains of *F. novicida* (MglA) at an MOI of 100:1 and lysed at 6 h post-infection. The levels of procaspase-1, mature caspase-1 (p20 subunit) and ASC in infected macrophages were measured by Western blot. β-actin was used as a loading control. Data are representative of three independent experiments.

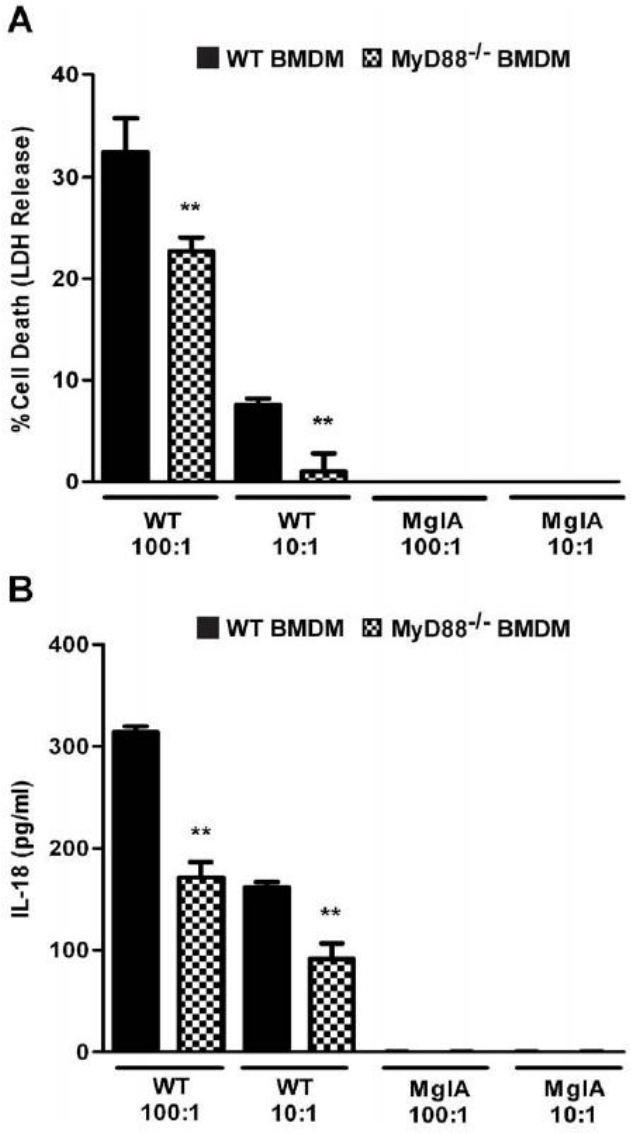

Figure 4. MyD88 is involved in rapid inflammasome activation in response to *F. novicida* infection. BMDM from wild-type and MyD88$^{-/-}$ C57BL/6 mice were infected with wild-type (WT) and *mglA* mutant strains of *F. novicida* (MglA) at the indicated MOIs. At 6.5 h post-infection, (A) death of the infected macrophages was measured by LDH release and (B) the concentration of IL-18 in the supernatants was quantified by ELISA. Data are representative of four independent experiments. Error bars represent the standard deviation of triplicate samples. ** $p < 0.0090$.

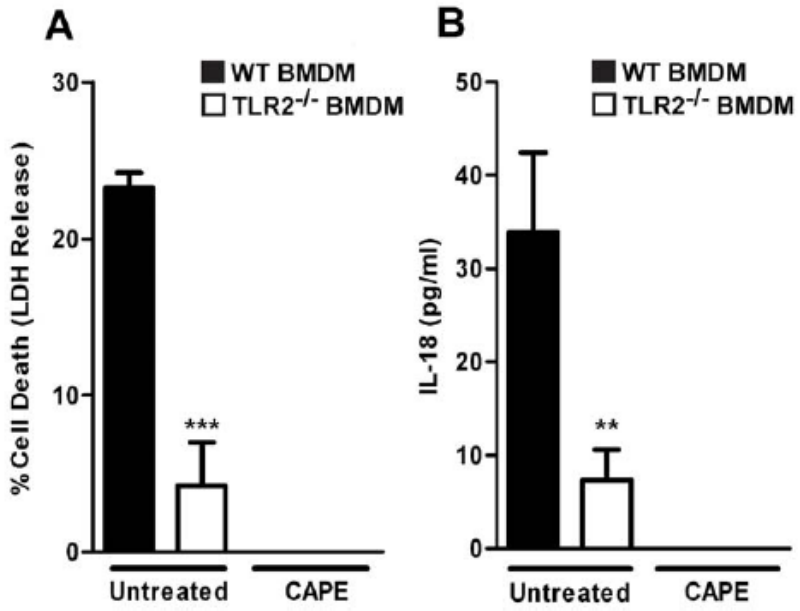

Figure 5. NF-κB activation contributes to rapid inflammasome activation during *F. novicida* infection. Macrophages were left untreated or treated with 20 μM of CAPE (NF-κB inhibitor) for 2h, then infected with *F. novicida* at an MOI of 100:1. Supernatants were collected for measurement of cell death by quantifying LDH release (A) and IL-18 secretion (B) at 6.5h post-infection. Data are representative of four independent experiments. Error bars represent the standard deviation of triplicate samples. *** $p \leq 0.0004$, ** $p = 0.0074$.

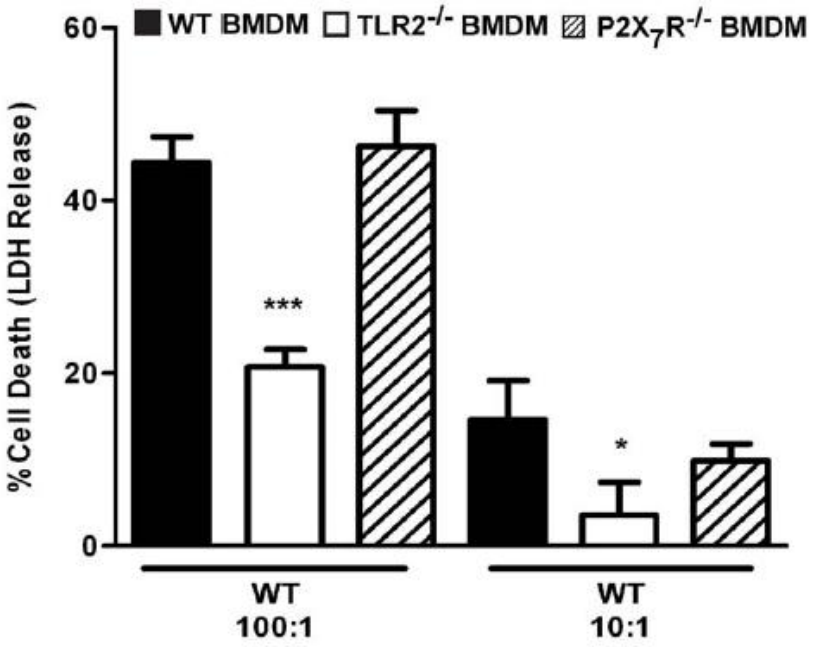

Figure 6. The $P2X_7$ receptor is not required for *F. novicida*-dependent inflammasome activation. BMDMs from wild-type, $TLR2^{-/-}$ and $P2X_7R^{-/-}$ mice were infected with wild-type *F. novicida* at the indicated MOIs. At 7h post-infection, host cell death was measured by quantifying the amount of LDH released into the supernatant. Data are representative of three independent experiments. Error bars represent the standard deviation of triplicate samples.

*** $p \leq 0.0003$, * $p = 0.0319$.

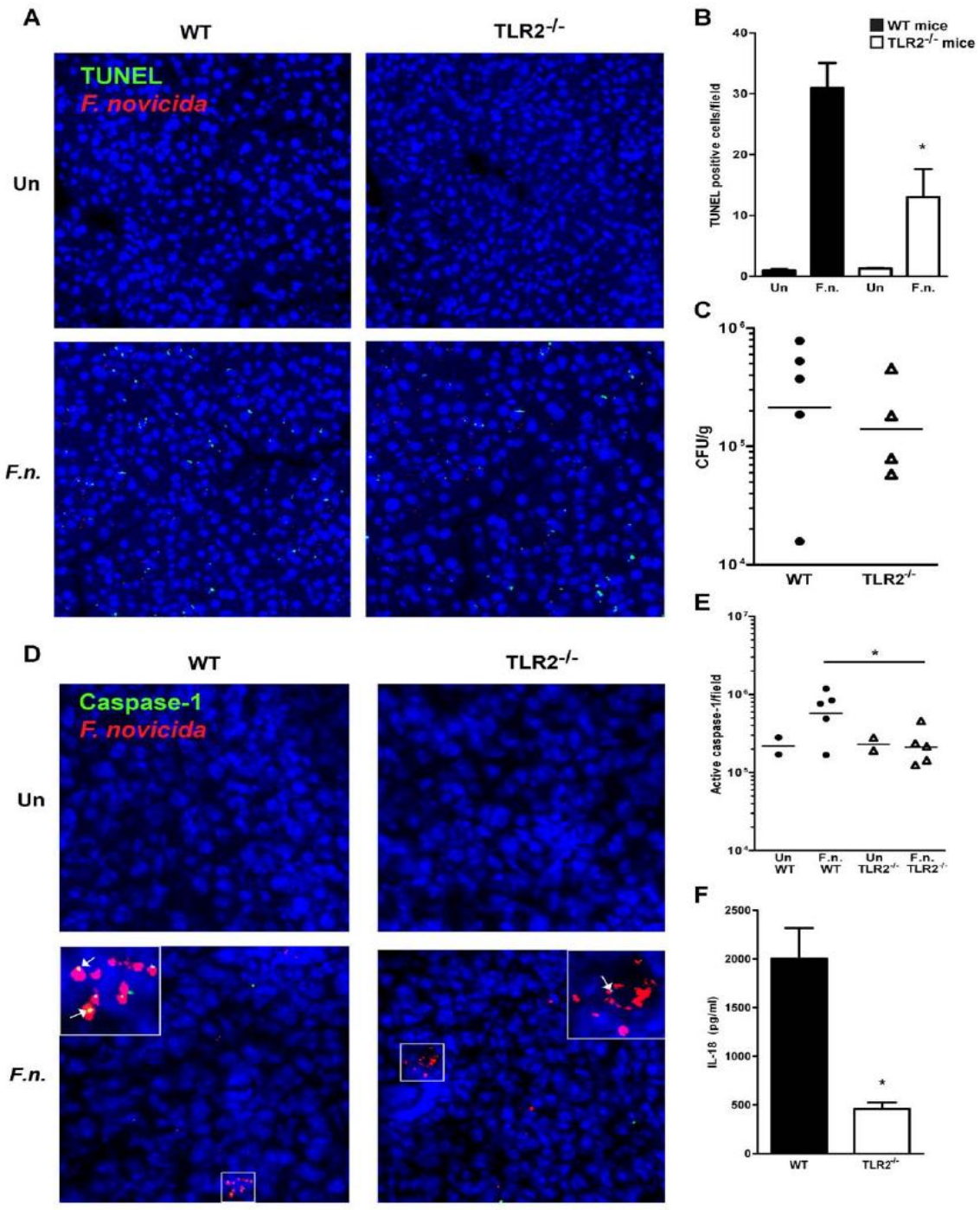

Figure 7. TLR2 plays a role in inflammasome activation *in vivo*. Wild-type and TLR2$^{-/-}$ mice were left uninfected (Un) or infected intraperitoneally with 2.4 x 10^6 CFU of wild-type *F. novicida* (*F.n.*) and livers were harvested at 4 h post-infection. (A) Cell death in the liver was visualized by TUNEL staining, and (B) the number of TUNEL

positive cells per field was quantified. (C) The bacterial burden in the liver of infected mice was determined by counting colony-forming units. (D) Sections were stained for presence of active caspase-1 (p20) in the livers of uninfected and *F. novicida*-infected mice, and (E) the level of caspase-1 activation was quantified by measuring the total fluorescence intensity emitted by the FITC-labeled p20 subunit of capase-1. (F) The amount of IL-18 present in the serum of *F. novicida*-infected mice was measured by ELISA. Data are representative of three independent experiments. Error bars represent the standard deviation of triplicate samples. ** $p = 0.0030$, * $p \leq 0.0370$.

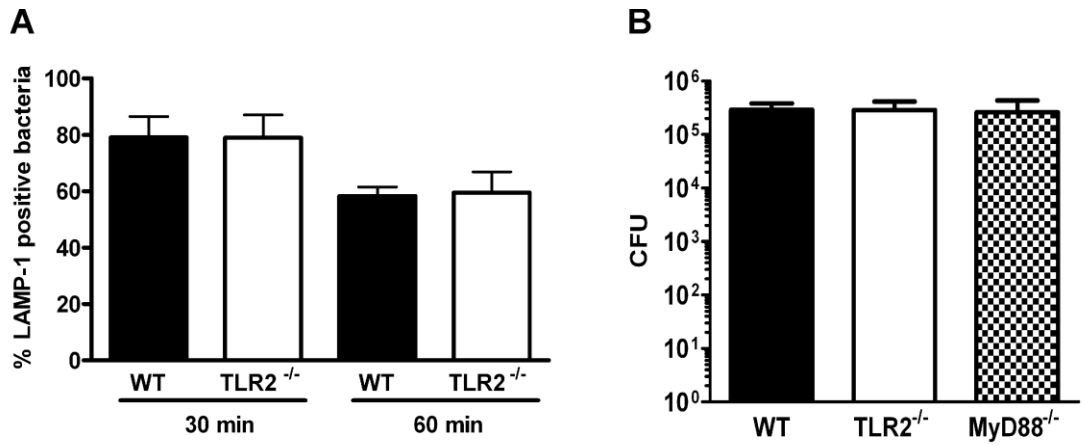

Figure S1. Phagosomal escape and intracellular replication of *F. novicida* in BMDM. (A) Macrophages were infected with *F. novicida* at an MOI of 100:1 and stained as described in the *Materials and Methods*. At 30 and 60 minutes postinfection, the number of bacteria colocalized with LAMP-1-containig vacuoles was enumerated. At least 100 bacteria were analyzed per sample. (B) Macrophages were infected with *F. novicida* at an MOI of 20:1. At 5 h post-infection, before any visible cell death occurred, macrophage lysates were plated and grown overnight. To determine the bacterial load, colony forming units (CFU) were counted.

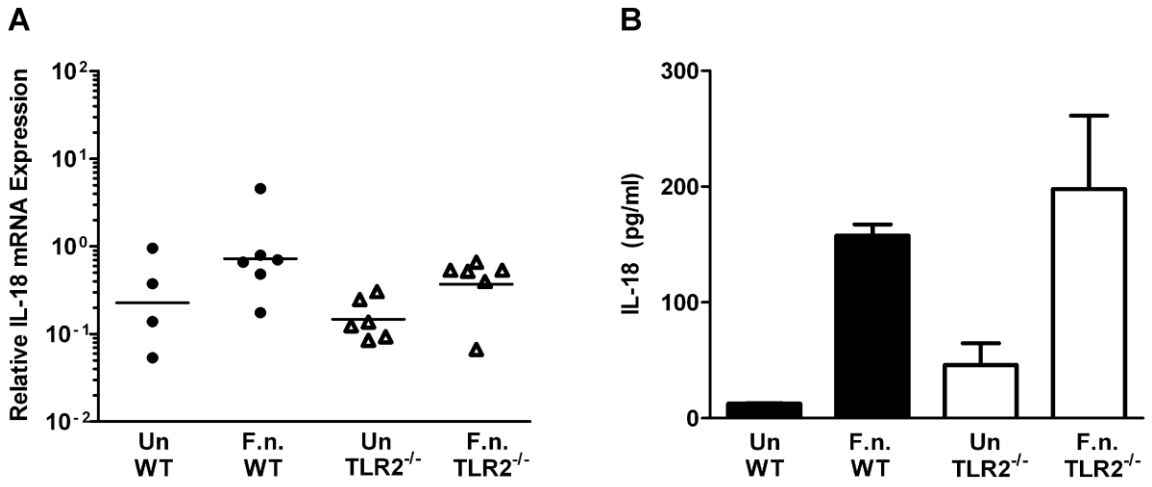

Figure S2. TLR2 is not required for IL-18 expression. BMDM from wild-type and TLR2$^{-/-}$mice were left uninfected (Un) or infected with *F. novicida* (F.n.) at an MOI of 100:1. At 5.5 h post-infection, macrophages were lysed. (A) RNA was harvested and quantitative real-time PCR was used to determine the level of IL-18 expression, which is represented relative to the expression of the housekeeping gene β-actin. (B) The total amount of IL-18 present in each lysate was measured by ELISA. Data are representative of three independent experiments.

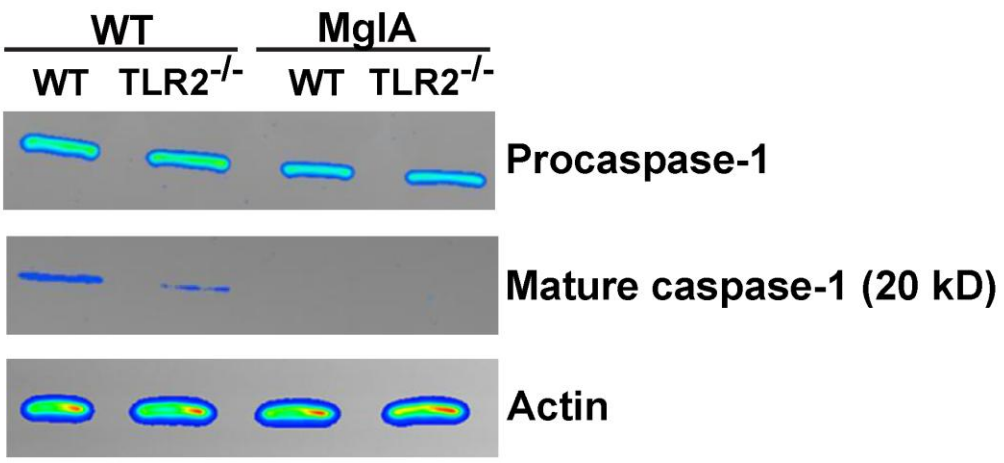

Figure S3. TLR2$^{-/-}$ macrophages exhibit delayed caspase-1 activation in response to *F. novicida* infection. WT and TLR2$^{-/-}$ BMDM were infected with wild-type and the *mglA* mutant strain of *F. novicida* at an MOI of 100:1 for 7.5 h. Macrophage lysates were collected, and the presence of procaspase-1, mature caspase-1 (p20 subunit) and β-actin was visualized by western blot.

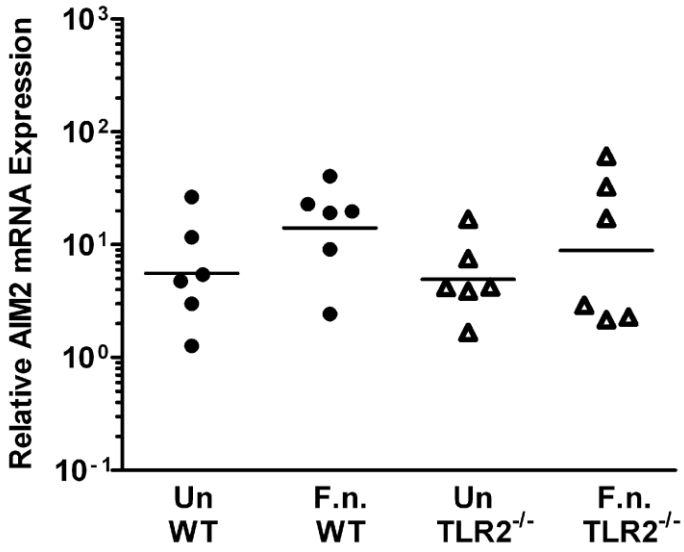

Figure S4. TLR2 is not required for AIM2 expression. BMDM from wild-type and TLR2$^{-/-}$ mice were left uninfected (Un) or infected with *F. novicida* (F.n.) at an MOI of 100:1. At 5.5 h post-infection, macrophages were lysed and the total RNA was harvested. Quantitative real-time PCR was used to determine the level of AIM2 expression, which is represented relative to the expression of the housekeeping gene β-actin. qRT-PCR analysis was used to quantify AIM2 expression because we were unable to detect the protein by western blot in macrophages that were not pre-treated with IFN-β. Data are representative of three independent experiments. Bars represent the geometric mean for each group.

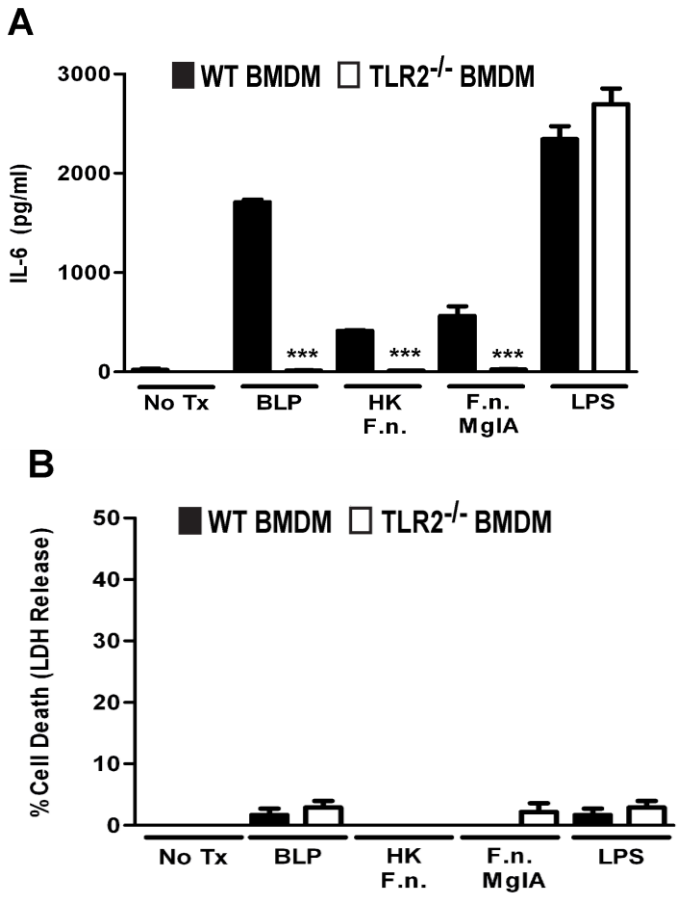

Figure S5. TLR2 signaling is not sufficient to induce cell death. BMDMs from wild-type and TLR2$^{-/-}$ mice were either left untreated (no Tx), stimulated with Pam_3CSK_4 (BLP), heat-killed wild-type *F. novicida* (HK F.n.), or Ultrapure *E. coli* LPS or infected with the *mglA* mutant strain of *F. novicida* (F.n. MglA) at an MOI of 100:1. At 7h, (A) the concentration of IL-6 in the supernatants was measured by ELISA, and (B) cell death was measured by quantifying the amount of LDH released into the supernatants. Data are representative of three independent experiments. Error bars represent the standard deviation of triplicate samples. ***p values < 0.0008

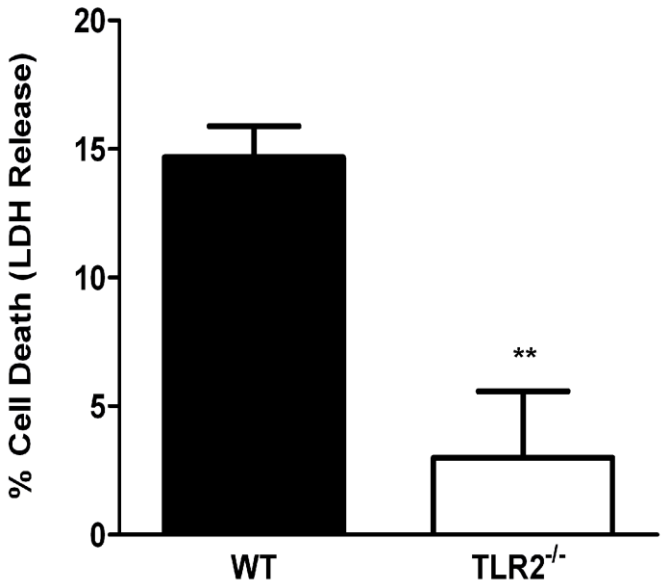

Figure S6. TLR2 plays a role in inflammasome activation during *Listeria* infection. Wild-type and TLR2$^{-/-}$ BMDM were infected with *L. monocytogenes* EGD-e at an MOI of 20:1 for 1 h. At the indicated timepoints, cell death was measured by quantifying the amount of LDH released into the supernatants. Data are representative of three independent experiments. **p = 0.0021

Chapter 3: Repression of Bacterial Lipoprotein Production by *Francisella novicida* Facilitates Evasion of Innate Immune Recognition

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*These authors contributed equally to the work in this manuscript. David Weiss performed all aspects of the microarray experiment and Helder Nakaya performed the bioinformatics analysis on the microarray data.

Abstract

Innate recognition systems, including the Toll-like receptors (TLRs), play a critical role in activating host defenses and proinflammatory pathways in response to infection. Pathogens have developed strategies to subvert TLRs in order to survive and replicate within the host. The model intracellular pathogen, *Francisella novicida*, modulates host defenses to promote survival and replication in macrophages. TLR2, which recognizes bacterial lipoproteins (BLPs), is critical for activating host defenses and proinflammatory cytokine production in response to *Francisella* infection. Here we show that the *F. novicida* protein FTN_0757 acts to repress BLP production, dampening TLR2 activation. The Δ*FTN_0757* mutant strain induced robust TLR2-dependent cytokine production in macrophages compared to wild-type bacteria, and produced increased amounts of BLPs. The deletion of one BLP (FTN_1103) from Δ*FTN_0757* decreased the total BLP concentration to near wild-type levels and correlated with a decrease in the induction of TLR2 signaling. The overproduction of BLPs also contributed to the *in vivo* attenuation of the Δ*FTN_0757* mutant, which was significantly rescued when *FTN_1103* was deleted. Taken together, these data reveal a novel mechanism of immune evasion by the downregulation of BLP expression to subvert TLR2 activation, which is likely also used by other intracellular bacterial pathogens.

Introduction

Early detection of microbial pathogens by pattern recognition receptors (PRRs) is an important component in the initiation of an effective immune response aimed at clearing infections (1). One group of PRRs, the Toll-like receptors (TLRs), are type I integral membrane proteins present on the surface of a diverse set of host cells, as well as in endosomes. TLRs are responsible for the recognition of a number of different microbial components, or pathogen-associated molecular patterns (PAMPs) (2). For example, lipid A from Gram-negative bacteria is recognized by TLR4, flagellin by TLR5, CpG DNA by TLR9, and bacterial lipoproteins (BLPs) by TLR2 (3-6). Upon recognition of their cognate PAMP, TLRs signal to activate transcription factors, including NF-κB, which lead to the production of proinflammatory cytokines, chemokines, and antimicrobial peptides (2). Production of these proteins stimulates an array of host defenses including the activation of macrophages and the recruitment of neutrophils, which help to fight infection (2, 7).

Pathogens have developed a variety of mechanisms to prevent TLR signaling. Some pathogens secrete effector proteins into host cells that block components of the TLR signaling pathways. For example, the enteropathogenic *Escherichia coli* effector NleE directly blocks NF-κB activation by preventing IκB degradation, and the *Brucella* spp. effector Btp1 binds the cytosolic TIR domain of TLR2 and TLR4, preventing the recruitment of downstream signaling proteins (8-10). Pathogens can also prevent TLR signaling by modifying PAMPs. For instance, alterations in the amino acid sequence of the flagellin monomer allow *Helicobacter* spp. to prevent recognition by TLR5 (11). Specific modifications to the structure of lipid A, such as the addition of acyl chains

(*Salmonella* spp.) or the removal of acyl chains (*Yersinia pestis*), facilitate evasion of TLR4 signaling (12, 13). Furthermore, recognition of pathogens by TLRs can be subverted by preventing the release of PAMPs. For example, the masking of flagella by a lipid membrane sheath is used by *Vibrio* spp. to prevent recognition of flagellin by TLR5 (14, 15).

The Gram-negative bacterium *Francisella novicida*, a model intracellular pathogen closely related to highly virulent *F. tularensis*, has evolved strategies to subvert host defense proteins including TLRs (16, 17). Similar to other *Francisella* spp., *F. novicida* can infect and replicate within host macrophages, which express numerous TLRs (18, 19). Extensive modification of its lipid A, including alterations in the length and number of acyl chains, results in a lack of signaling through TLR4 (20, 21). This is a critical component of *Francisella* pathogenesis since mutants that cannot modify lipid A are severely attenuated *in vivo* (20). Furthermore, *Francisella* does not encode flagellin and therefore does not activate TLR5 signaling (22, 23). The bacteria are, however, recognized by TLR2, which plays an important role in host defense as indicated by the increased susceptibility to infection of mice lacking TLR2 (22-25). Thus, molecular strategies used by *Francisella* spp. to subvert TLR2 signaling would likely promote pathogenesis.

The specific proteins used by *Francisella* spp. to suppress host defenses, and their mechanisms of action, are largely unknown. We and others previously used *in vivo* genetic screens to identify critical *Francisella* virulence determinants (26-30). One gene that we identified, *FTN_0757* (also termed *FTT_0584* before the *F. novicida* genome was sequenced), is necessary for *F. novicida* virulence in mice and has been shown to be

involved in the suppression of several pro-inflammatory cytokines (26, 30). However, its mechanism of action is unknown. Therefore, we set out to elucidate how *FTN_0757* contributes to the subversion of innate inflammatory responses and better define the breadth of its effect on the host response.

Here, we demonstrate that FTN_0757 action leads to the suppression of a large panel of NF-κB-dependent genes, as well as genes encoding other host defense proteins. We show that the increased production of cytokines and chemokines in response to infection by the *FTN_0757* mutant is due to hyperstimulation of TLR2. More specifically, we show that FTN_0757 functions to limit the expression and production of BLPs that induce proinflammatory mediators through TLR2. One BLP, FTN_1103, is highly overproduced in the *FTN_0757* mutant and accounts for the majority of the increased BLP content. Deletion of *FTN_1103* from the *FTN_0757* mutant significantly reduces the activation of TLR2 and rescues the virulence defect of the mutant *in vivo*. To our knowledge, this is the first demonstration that suppression of BLP content by an intracellular pathogen allows subversion of TLR2-dependent responses and promotes virulence. Furthermore, this work may provide insights into ways by which other pathogens escape recognition by TLR2.

Materials and Methods

Bacterial strains and growth conditions

Francisella novicida strain U112 was kindly provided by Dr. Denise Monack (Stanford University, Stanford, CA). All bacterial cultures were grown overnight at 37°C with aeration in tryptic soy broth (TSB) supplemented with 0.2% L-cysteine (BD Biosciences, Sparks, MD). When necessary, the media was supplemented with kanamycin (30 μg/ml) or tetracycline (20 μg/ml).

Bacterial mutagenesis

Mutant strains (Δ*FTN_0757* and Δ*FTN_1103*) were constructed by allelic replacement as described previously (31, 32) using primers in Table 1. To excise the Flippase Recognition Target (FRT)-flanked kanamycin resistance cassette and create unmarked strains, the kanamycin-resistant mutants were transformed with plasmid pLG42 encoding the Flp-recombinase, performed as previously described (33). The Δ*FTN_0757*/Δ*FTN_1103* deletion strain was generated by transforming an unmarked Δ*FTN_0757* strain with genomic DNA from the marked Δ*FTN_1103* strain, and selecting for kanamycin resistance. *FTN_1103* was complemented in *cis* into the Δ*FTN_0757*/Δ*FTN_1103* deletion strain through allelic replacement, as described previously.

Preparation of bacterial fractions

Overnight cultures of bacteria were subcultured 1:50 into 50ml of TSB with 0.2% cysteine and grown to an OD_{600} of 0.9 – 1.0. Cultures were centrifuged at 5,000 x *g* for

10 minutes to pellet the bacterial cells. For cell-free supernatants, the remaining supernatant was passed through a 0.22μm filter (Millipore, Billerica, MA), and stored at -20°C until use. For heat-killed bacteria, the bacterial pellet was resuspended in PBS (Lonza, Walkersville, MD), boiled at 100°C for 1 hour, and then stored at -20°C until use. For isolation of membrane fractions, resuspended cells were lysed via freeze-thawing for three cycles. The cell lysate was then centrifuged at 10,000 x *g* for 10 minutes to remove unlysed cells. The cleared supernatant was then centrifuged at 120,000 x *g* for 2 hours to pellet the total membrane fraction. Membrane pellets were resuspended in 1ml PBS and stored at -20°C until use. For enrichment of bacterial lipoproteins, pelleted membrane fractions were resuspended in 200μl PBS and 500μl *n*-butanol (Sigma-Aldrich, St. Louis, MO) and centrifuged at 27,000 x *g* for 90 minutes. The aqueous phase, containing an enrichment of bacterial lipoproteins, was collected and stored at -20°C until use (34). Protein fractions were normalized either by colony forming units (cfu) or by protein concentration, measured via the bicinchoninic acid assay (Thermo Scientific, Waltham, MA), as indicated. Twenty micrograms or 10^8 cfu-equivalents of each fraction were separated via 12-20% SDS-PAGE (Bio-Rad, Hercules, CA) and stained with Coomassie Blue G-250 (Teknova, Hollister, CA).

Microarray analysis

All RNA samples were checked for purity using a ND-1000 spectrophotometer (NanoDrop Technologies) and for integrity by electrophoresis on a 2100 BioAnalyzer (Agilent Technologies). The samples were amplified using the Nugen WT Pico Kit (NuGEN Technology) and the target reactions were run with 25 ng of total RNA. The

amplification products were processed through the EXON Module (NuGEN Technology), which creates sense-strand cDNA targets. The sense strand cDNA Targets were then fragmented and labeled using NuGEN's FL-Ovation™ cDNA Biotin Module V2 (NuGEN Technology). Labeled targets were hybridized to GeneChip® Mouse Gene 1.0ST arrays (Affymetrix, Inc.), following Standard Nugen Protocols for target hybridization to the Affymetrix Gene Arrays. The hybridizations were run for 16 hours, 45°C, 60 rpm in an Affymetrix Hybridization Oven 640. The Cartridge arrays were washed and stained using the Affyemtrix Fluidics Stations 450, following Affymetrix protocols. Scanning was performed on an Affymetrix GeneChip 3000 7G scanner, and Affymetrix GCOS software was used to perform image analysis and generate raw intensity data. Probe sets of all samples were normalized by RMA, which includes global background adjustment and quantile normalization. Using the gene annotation provided by Affymetrix, we discarded 11,537 probe sets that did not match to known genes. Student's t-test ($p < 0.02$) and a fold-change filter (mean fold-change > 25%) were used to identify genes differentially expressed in macrophages infected with Δ*FTN0757* strain compared to those infected with wild-type strain for 4 hours. The expression levels of NF-□B-regulated genes were visualized using Ingenuity Pathway Analysis (Ingenuity Systems) software.

Protein identification by mass spectrometry

After staining with Coomassie Blue G-250, the band of interest was excised and subjected to in-gel digestion (12.5 μg/ml trypsin). Extracted peptides were loaded onto a C_{18} column (75μm inner diameter, 15cm long, ~300nl/min flow rate, 1.9μm resin) (Dr.

Maisch GmbH, Ammerbuch-Entringen, Germany) and eluted using a 10-30% gradient (Buffer A: 0.1% formic acid, 1% ACN; Buffer B: 0.1% formic acid, 99.9% ACN). The eluted peptides were detected by Orbitrap (300-1600 *m*/*z*; 1,000,000 automatic gain control target; 500-ms maximum ion time; resolution, 30,000 full-width at half-maximum) followed by ten data-dependent MS/MS scans in the linear ion trap quadrupole (2 *m*/*z* isolation width, 35% collision energy, 5,000 automatic gain control target, 200-ms maximum ion time) on a hybrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The acquired tandem mass spectrometer (MS/MS) spectra were searched against and a decoy-concatenated *F. novicida* database (3,393 redundant protein targets) from the NCBI RefSeq protein database project (September 2011) using the Sorcerer-SEQUEST Algorithm version 3.11 r11 (Sage-N Research, San Jose, CA). Search results were filtered with a 1% FDR and summarized by in-house programs, as described by Gozal et al (35).

Macrophage experiments and infections

Murine bone marrow-derived macrophages (BMDM) were prepared from 6-8 week old wild-type and TLR2$^{-/-}$ C57BL/6 mice and cultured as described (26). Macrophages were cultured in 96-well plates (5-8x10^4 cells/well) in high glucose Dulbecco's modified Eagle's medium (DMEM) (Lonza, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 10% L929-conditioned media (conditioned DMEM) containing M-CSF (macrophage colony stimulating factor) overnight. The media was removed and bacteria were added at a multiplicity of infection (MOI) of 20 or 100 bacteria per macrophage. Plates were centrifuged for 15 minutes at

335 x *g* at room temperature to promote uptake of bacteria. Macrophages were incubated for 30 minutes at 37°C and washed two times before adding warm conditioned DMEM. The concentrations of IL-6, KC, and MIP-1β in culture supernatants at the indicated timepoints after infection were quantified by ELISA (BD Biosciences, Sparks, MD). For treatment with bacterial components, cells were washed gently and media containing heat-killed bacteria, membrane fractions, or bacterial lipoprotein fractions at the given concentrations was added. Macrophages were stimulated for the indicated duration of time, before the cell-culture supernatant was collected.

RNA extraction and quantitative real-time PCR

Overnight cultures of the indicated bacteria were subcultured 1:50 into 50mL TSB with 0.2% L-cysteine and grown to an OD_{600} of 0.9 to 1.0. RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) and purified using the RNeasy Mini Kit (Qiagen, Germantown, MD) according to the manufacturers' instructions. Quantitative real-time RT-PCR (qRT-PCR) was performed using the Power Sybr Green RNA to CT 1-Step Kit (Applied Biosystems, Carlsbad, CA) and gene-specific primers (Table 1) using an Applied Biosystems StepOne Cycler. Relative transcript levels were calculated by normalizing C_T values to DNA helicase II (*uvrD, FTN_1594*).

Mouse infections

For competition experiments, groups of five wild-type C57BL/6 mice were infected subcutaneously with a 1:1 ratio of wild-type and the indicated mutant strain of *F. novicida* (total of 1 x 10^5 cfu) in sterile PBS. At 48 hpi, the skin, spleen and liver of

infected mice were harvested and homogenized in PBS. Serial dilutions were plated on Mueller-Hinton agar supplemented with 0.1% L-cysteine with or without kanamycin for enumeration of bacterial burden in each organ. The competitive index (CI) was calculated using the formula, CI = (mutant cfu in output/wild-type cfu in output)/(mutant cfu in input/wild-type cfu in input). All experimental procedures were approved by the Emory University Institutional Animal Care and Use Committee (protocol #069-2008Y).

Statistics

All experiments were analyzed using the unpaired Student's *t* test except Figure 6A, which was analyzed by a Mann-Whitney test. The CI values from competition experiments were analyzed using the one-sample Student's *t* test and CI values of the *ΔFTN_0757* and *ΔFTN_0757/ ΔFTN 1103* strains were both significantly different from 1 (***$p < 0.0001$).

Results

FTN_0757 Suppresses TLR2-dependent Proinflammatory Responses in Macrophages

Previous studies by our laboratory and others showed that a Δ*FTN_0757* mutant of *F. novicida* induced increased macrophage secretion of several proinflammatory cytokines compared to wild-type bacteria (26, 30). However, the full scope of this hyperinflammatory response and the mechanism underlying this phenotype were unclear. As a first step towards defining the extent of this effect, we sought to measure the breadth of the inflammatory response induced by the Δ*FTN_0757* mutant during macrophage infection using microarray analysis. We harvested RNA from murine bone marrow-derived macrophages (BMDM) infected with wild-type *F. novicida* or the Δ*FTN_0757* mutant, as well as uninfected controls. Microarray analysis revealed that there was a broad and robust increase in the number and magnitude of macrophage genes expressed in response to infection by the Δ*FTN_0757* mutant compared to wild-type bacteria (Tables S1, S2 and Figure 1A). Furthermore, Ingenuity Pathway Analysis revealed that host defense pathways consisting of genes encoding PRRs that recognize bacteria and proteins important for proinflammatory cytokine signaling were induced to higher levels in Δ*FTN_0757*-infected macrophages than in macrophages infected with wild-type bacteria (Figure S1A). This analysis also identified 53 NF-κB-regulated genes as being induced in macrophages infected with the Δ*FTN_0757* mutant, compared to only 25 genes in wild-type-infected macrophages (Figures S1B, C).

To validate the microarray results, we first measured IL-6 production since it was the most differentially expressed gene in macrophages infected with the Δ*FTN_0757*

mutant compared to those infected with wild-type bacteria (Figure 1A). In agreement with our microarray data, macrophages infected with the Δ*FTN_0757* mutant secreted significantly higher levels of IL-6 than wild-type-infected macrophages (Figure 1B). This response was dependent on TLR2 since TLR2$^{-/-}$ cells did not produce detectable levels of IL-6 (Figure 1B). In addition to increased cytokine production, macrophages infected with the Δ*FTN_0757* mutant secreted significantly higher amounts of the chemokines KC and MIP-1β compared to those infected with wild-type bacteria, further validating our microarray results (Figures 1C, D). This response was also TLR2-dependent. To ensure that differences in cytokine and chemokine production were not due to differences in the ability of these strains to replicate within macrophages, we assessed the bacterial burden of infected macrophages. We found that both wild-type and Δ*FTN_0757* bacteria replicated with the same kinetics in wild-type and TLR2$^{-/-}$ macrophages (Figure S2). Taken together, these findings validate our microarray results and clearly demonstrate that TLR2 is required for the hyperinflammatory response elicited in macrophages infected with the Δ*FTN_0757* mutant.

FTN_0757 Represses Production of Bacterial Lipoproteins

Since we have shown that infection with Δ*FTN_0757* elicits a broad and robust increase in TLR2-dependent signaling compared to wild-type bacteria, we sought to explore the mechanism responsible for this phenotype. Bacteria can block TLR signaling through active processes such as secretion of effectors, or dampen host signaling by modulating PAMPs in numerous ways that lessen the immunostimulatory capacity of the bacteria (8-13). To test whether the FTN_0757-dependent subversion of TLR2 signaling

was an active process requiring live bacteria, we treated macrophages with heat-killed preparations of wild-type and Δ*FTN_0757* mutant bacteria. We used IL-6 as a marker for the proinflammatory response due to its robust induction during macrophage infection with Δ*FTN_0757* (Figures 1A, B). We found that heat-killed Δ*FTN_0757* induced a significant increase in IL-6 production compared to killed wild-type bacteria, and that this response was completely dependent on TLR2 (Figure 2A). This demonstrated that the suppression of cytokine production by FTN_0757 is not dependent on an active process, but is instead due to a difference in a heat-resistant component(s) of the bacteria. Since the immunostimulatory moiety of BLPs is heat-resistant, BLPs signal through TLR2, and *Francisella* is known to encode BLPs that activate TLR2 (22-25, 36, 37), we hypothesized that changes in BLPs were responsible for the hyperinflammatory phenotype of Δ*FTN_0757*.

BLPs are located in bacterial membranes, so we next isolated the total membrane protein fraction from the wild-type and Δ*FTN_0757* strains and tested them for their TLR2-stimulating activity. The membrane fraction from Δ*FTN_0757* induced increased IL-6 production compared to the wild-type fraction (Figure 2B). This response was TLR2-dependent, similar to what we observed with the heat-killed preparation (Figure 2A) and infection with live bacteria (Figure 1B). This is consistent with the hypothesis that differences in BLPs are responsible for the hyperinflammatory phenotype of Δ*FTN_0757*. To further explore this possibility, we fractionated and quantified BLPs from the total membrane protein fraction of each strain. Strikingly, we found that the Δ*FTN_0757* strain contained roughly twice as much total BLP as wild-type bacteria (Figure 2C). This data provides a potential explanation for the increased induction of

proinflammatory cytokines elicited by Δ*FTN_0757*, since higher levels of BLPs would likely lead to increased TLR2 activation. To rule out the possibility that BLPs from the Δ*FTN_0757* strain had increased activity on a molar basis compared to BLPs from the wild-type strain, we treated macrophages with equal concentrations of the BLP fraction from each strain. Both BLP fractions induced an equivalent amount of IL-6 production in a TLR2-dependent manner (Figure S3), demonstrating that the BLPs from each strain had the same intrinsic TLR2-stimulating activity. Taken together, these data suggest that the hyperinflammatory phenotype of Δ*FTN_0757* is due to its increased BLP content leading to more robust TLR2 activation, rather than differences in the ability of its BLPs to act as TLR2 ligands.

FTN_0757 Represses the Expression of the Bacterial Lipoprotein FTN_1103

The significant increase in BLP concentration in the Δ*FTN_0757* strain could be due to an increase in the levels of a small number of specific BLPs, or a more global increase in overall BLP production. In order to differentiate between these possibilities, we analyzed the respective protein composition of the BLP fraction from each strain via SDS-PAGE. While most protein bands were present at similar levels, a specific band of approximately 30 kD was highly enriched in the BLP fraction of the Δ*FTN_0757* strain compared to wild-type (Figure 3A). Utilizing an LC-MS/MS peptide mass fingerprinting approach, we identified the band to be FTN_1103. Although annotated in the NCBI database as a hypothetical protein, FTN_1103 contains the typical amino acid motifs associated with BLPs, including a positively charged N-terminal region, a hydrophobic H-region, and a conserved lipobox motif (Leu-Gly-Ser) adjacent to the invariant cysteine

at residue 29, which would serve as a lipidation site (Figure 3B). Also, consistent with its presence in the BLP fraction, FTN_1103 is predicted to be a BLP by the PRED-LIPO lipoprotein prediction server with a reliability score of 0.996 (38). Together, these data suggest that FTN_1103 is a BLP, which we showed is highly overproduced in Δ*FTN_0757*.

To further prove that the protein overproduced in Δ*FTN_0757* is indeed FTN_1103, we generated a Δ*FTN_0757*/Δ*FTN_1103* double deletion strain and analyzed its BLP content. The enriched protein that we previously identified as FTN_1103 was absent in the BLP fraction of the Δ*FTN_0757*/Δ*FTN_1103* mutant, confirming its identity as FTN_1103 (Figure 3A). We next measured the proportion of the increased BLP pool in Δ*FTN_0757* that was due to the increase in production of FTN_1103. Deletion of *FTN_1103* in the Δ*FTN_0757* strain led to a large reduction in the total BLP concentration, almost to the level present in the wild-type strain (Figure 4A). This data indicates that overproduction of one specific BLP, FTN_1103, is the major cause of the increased BLP content in Δ*FTN_0757*. This also suggests that the increased TLR2-stimulating activity of the Δ*FTN_0757* strain may in large part be due to the overproduction of FTN_1103.

FTN_0757 Represses FTN_1103 to Evade TLR2 Activation in Macrophages

To determine whether overproduction of FTN_1103 in Δ*FTN_0757* was the major basis for the increased TLR2 activation induced by this strain, we treated macrophages with either heat-killed preparations or total membrane protein fractions derived from wild-type, Δ*FTN_0757*, or Δ*FTN_0757*/Δ*FTN_1103*. Similar to our

previous observation, macrophages stimulated with preparations from Δ*FTN_0757* elicited a significantly increased TLR2-dependent IL-6 response as compared to those treated with preparations from the wild-type strain (Figures 4B, C). However, macrophages treated with equivalent fractions from the Δ*FTN_0757/*Δ*FTN_1103* mutant secreted significantly lower levels of IL-6 compared to those treated with Δ*FTN_0757* preparations (Figures 4B, C). Deletion of *FTN_1103* did not reduce the TLR2-stimulatory activity of Δ*FTN_0757* completely to wild-type levels. This is likely due to smaller increases in the production of other BLPs and correlates with the incomplete reduction of BLP levels in the Δ*FTN_0757/*Δ*FTN_1103* mutant (Figure 4A). These data demonstrate that the major cause of the increased TLR2-stimulating capacity of killed and membrane preparations of Δ*FTN_0757* is the overproduction of FTN_1103.

We next set out to determine whether FTN_1103 overproduction contributed to the increase in TLR2-dependent cytokine production elicited by Δ*FTN_0757* during infection of macrophages. As a control, we first tested whether Δ*FTN_0757* overexpressed *FTN_1103* during infection of macrophages. We isolated total RNA from macrophages infected with wild-type or Δ*FTN_0757* strains. We found that Δ*FTN_0757* maintained a significant increase in expression of the transcript for *FTN_1103* during macrophage infection compared to wild-type, similar to what we observed with bacteria grown in rich media (Figures 5A, B). However, it is important to note that we did not see a significant difference in gene expression of other predicted BLPs (such as *dsbA/ FTN_0771*) or genes within the *Francisella* Pathogenicity Island (FPI) between the wild-type and Δ*FTN_0757* strains (data not shown). Together these data show that FTN_0757

is required to repress expression of *FTN_1103* during growth in rich media as well as during intracellular infection.

We next infected macrophages with the Δ*FTN_0757*/Δ*FTN_1103* mutant and notably, observed the amount of IL-6 induced by this strain was much less than that induced by macrophages infected with Δ*FTN_0757* (Figure 5C). This is consistent with our results for stimulation with heat-killed preparations and membrane protein fractions (Figures 4B, C). As a further control, we genetically restored *FTN_1103* into the Δ*FTN_0757*/Δ*FTN_1103* strain. Following infection of macrophages with this strain, we observed a restoration of the hyperinflammatory defect of the Δ*FTN_0757* strain (Figure S4). Collectively, these data show that the TLR2-dependent cytokine response induced by Δ*FTN_0757* is due to the over-production of BLPs, and that FTN_1103 is the major BLP contributing to this phenotype.

FTN_0757 Repression of BLP Expression is Critical for *F. novicida* Virulence *in vivo*

The Δ*FTN_0757* mutant is severely attenuated in mice compared to wild-type bacteria (26). Since deletion of *FTN_1103* in the Δ*FTN_0757* mutant rescued the majority of its hyperinflammatory phenotype during macrophage infection, we tested whether it would also rescue its virulence attenuation *in vivo*. First, as a control, we tested whether *FTN_1103* expression was upregulated in the Δ*FTN_0757* mutant during *in vivo* infection. We measured the level of *FTN_1103* expression in the wild-type and Δ*FTN_0757* strains at 6 hours after subcutaneous infection of mice, an early timepoint when the loads of each strain were similar (data not shown). We found that the

FTN_1103 transcript was indeed present at higher levels in the Δ*FTN_0757* mutant compared to wild-type bacteria (Figure 6A). This demonstrates that the regulation of *FTN_1103* by FTN_0757 occurs during *in vivo* infection, similar to our findings with bacteria grown in rich media and during macrophage infection (Figures 5A, B).

To determine whether overexpression of FTN_1103 contributed to *in vivo* attenuation of the Δ*FTN_0757* mutant, we performed competition experiments. We infected mice subcutaneously with a 1:1 mixture of wild-type and either the Δ*FTN_0757* or Δ*FTN_0757/*Δ*FTN_1103* mutant and enumerated bacteria in the spleen at 48 hpi. The Δ*FTN_0757* mutant was >100,000-fold attenuated compared to wild-type bacteria (Figure 6B), in agreement with our previous work (26). In contrast, the Δ*FTN_0757/*Δ*FTN_1103* mutant was present at much higher levels than the Δ*FTN_0757* mutant, and was only ~100-fold attenuated compared to wild-type. This represents 1,000-fold complementation of the Δ*FTN_0757* mutant as a result of deleting *FTN_1103.* The lack of complete complementation correlates with the BLP content, macrophage stimulation and infection experiments using the Δ*FTN_0757/*Δ*FTN_1103* strain, and might be attributed to an increase in the expression of other BLPs in the Δ*FTN_0757* mutant (Figures 4, 5). As a control, we tested the phenotype of the Δ*FTN_1103* mutant and found that it was present at levels close to those of the wild-type strain, indicating that *FTN_1103* alone does not play an important role in virulence under these infection conditions. Taken together, these data indicate that increased FTN_1103 production in the Δ*FTN_0757* mutant significantly contributes to its attenuation *in vivo*.

Discussion

The mammalian innate immune system utilizes pattern recognition receptors, such as TLRs, to rapidly detect and respond to bacterial infection. Activation of TLRs results in the induction of numerous host defenses and a proinflammatory response aimed at clearing the invading bacteria. However, some bacterial pathogens have developed strategies to modulate TLR pathways. For example, *Salmonella* evade TLR5 activation by repressing flagellin production (39), and *Yersinia* secretes the effector protein YopJ that inhibits TLR-mediated NF-κB activation in host cells (40). Here we show that *F. novicida* downregulates the expression of BLPs during infection to evade TLR2 activation and promote its pathogenesis in the host.

We first set out to broadly characterize the hyperinflammatory phenotype of the Δ*FTN_0757* mutant by performing microarray analysis of macrophages infected with this strain or wild-type bacteria. We found that macrophages infected with the Δ*FTN_0757* mutant induced a robust increase in the expression of genes belonging to host defense pathways involved in the detection of bacteria by PRRs, NF-κB signaling, reactive oxygen and nitric oxide production, and cytokine and chemokine signaling, as compared to macrophages infected with the wild-type strain (Figure S1). Since the initial macrophage proinflammatory response to *Francisella* infection is primarily TLR2-dependent (41), we hypothesized that FTN_0757 was acting to subvert this pathway. Indeed, we found that the hyperinflammatory response to the Δ*FTN_0757* mutant in macrophages was completely TLR2-dependent (Figures 1B-D), demonstrating that FTN_0757 is important for modulating the activation of TLR2 by *F. novicida*.

Investigation into the mechanism underlying the robust TLR2-dependent inflammatory response induced by Δ*FTN_0757* revealed that this mutant produced 2-fold more BLP (TLR2 ligand) than wild-type bacteria, suggesting that overexpression of BLPs caused the increased activation of TLR2 (Figure 2C). LC-MS/MS analysis identified FTN_1103 as the most highly induced BLP in the membranes of the Δ*FTN_0757* mutant as compared to those of the wild-type strain (Figure 3). While FTN_1103 is annotated as a hypothetical protein, sequence analysis revealed that it contains the canonical BLP lipidation sequence. Furthermore, deletion of *FTN_1103* from the Δ*FTN_0757* mutant not only decreased the total membrane BLP concentration to almost wild-type levels (Figure 4A), but also significantly reduced the magnitude of the TLR2-dependent inflammatory response induced by *FTN_0757*-infected macrophages (Figure 5C). Together, these findings show that FTN_0757 acts to repress BLP expression, leading to evasion of TLR2 recognition.

In addition to its upregulation in the Δ*FTN_0757* mutant during growth in rich media (Figure 5A) and infection of macrophages (Figure 5B), we demonstrated that *FTN_1103* was also upregulated in this strain during *in vivo* infection (Figure 6A). Similar to the almost complete complementation of BLP levels and macrophage TLR2 activation, deleting *FTN_1103* from the Δ*FTN_0757* mutant resulted in a restoration of its ability to replicate *in vivo* to almost wild-type levels (Figure 6B). However, a Δ*FTN_1103* mutant did not exhibit a significant decrease in *in vivo* fitness (Figure 6B), leading us to conclude that FTN_1103 alone does not significantly contribute to *F. novicida* pathogenesis. These data demonstrate that the overexpression of FTN_1103 is the major cause of the attenuation of the Δ*FTN_0757* mutant during *in vivo* infection.

In addition to contributing to the evasion of innate defenses, suppression of BLP expression can also play an important role in evading adaptive immune responses. The spirochete *Borrelia burgdorferi* downregulates the expression of one of its most highly immunogenic BLP, OspC, at the onset of the humoral immune response in order to avoid detection by antibodies and subsequent killing (42). *B. burgdorferi* strains that are unable to downregulate OspC expression *in vivo* are rapidly cleared from mice in an antibody-dependent manner (43). Our data suggest that another consequence of OspC downregulation may be the evasion of TLR2 activation. As TLR2 signaling can contribute to antibody responses (44), this may represent a two-pronged approach to block this critical host defense: dampening the TLR2 activation signal as well as limiting the expression of a major antigen against which the antibody response is directed. By extension, the Δ*FTN_0757* mutant may induce a more potent antibody-mediated immune response than wild-type bacteria, and therefore downregulating FTN_1103 may also lead to evasion of antibody responses.

Our findings reveal a novel mechanism utilized by *F. novicida* to evade TLR2 activation. To our knowledge, this is the first demonstration of an intracellular bacterial pathogen that downregulates BLP expression to evade innate immune recognition. Since TLR2 is essential for the initial macrophage inflammatory response to *Francisella* infection, evading TLR2 activation may provide this bacterium precious time to reach its cytosolic replication niche without triggering a robust host response. This may represent a new paradigm used by other intracellular pathogens to evade TLR2 and possibly adaptive immune responses.

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Table 1. Primers used in this study

Primer Name	Sequence
FTN_0757 Deletion	
757-F1	tgaatcaaattcaagcacac
757-arm1-rev	tatcgataccgtcgacctcaaaatga
757-FRT-fwd	ccgagtaaaagaggtcattttgaggt
757-FRT-rev	gctaatttcataccaagcatgcatag
757-arm2-fwd	tatcgatcctgcagctatgcatgctt
757-R1	aactaacgacatctttccag
FTN_1103 Deletion	
1103-F1	ttagctgtaatggaggtatt
1103-arm1-rev	ttatcgataccgtcgacctcatttttc
1103-FRT-fwd	gttgtaggggattattgaaaaatgag
1103-FRT-rev	cttaagcaaaaaatctctgattagcat
1103-arm2-fwd	tatcgatcctgcagctatgctaatca
1103-R1	cgatagtgatagtagagctg
FTN_1103 Complementation	
1103comp-F1	tagtaatgtgatcggagtgt
1103comp-arm1-rev	atcgataccgtcgacctcgttaagtt
1103comp-FRT-fwd	tgggctgttggccaacttaacgaggt
qRT-PCR	
uvrD-RT-fwd	gggatgtcgccttttgattttc
uvrD-RT-rev	ctcttttgtcccttgtgcttgc
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1103-RT-rev	acccaactcaccatcgccaca

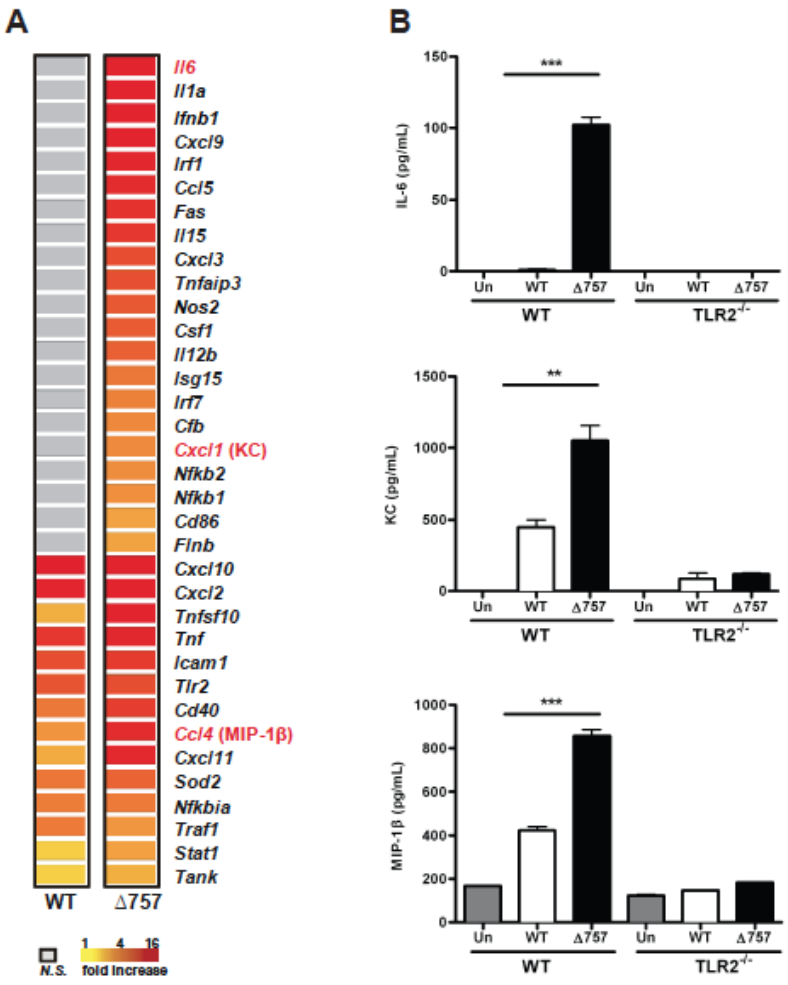

Figure 1. The Δ*FTN_0757* mutant induces robust TLR2-dependent macrophage activation. Wild-type and TLR2$^{-/-}$ bone marrow-derived macrophages (BMDM) were either left untreated (Un) or infected with wild-type (WT) or the Δ*FTN_0757* mutant (Δ757) of *F. novicida* for 4 hours at an MOI of 100:1. (A) A heat map of immune genes differentially expressed in infected macrophages compared to uninfected macrophages. The total amount of (B) IL-6, KC and MIP-1β secreted into the culture supernatant by was measured by ELISA. Data are representative of three independent experiments. Error bars represent the standard deviation of triplicate samples. **p = 0.0069, ***p < 0.0005.

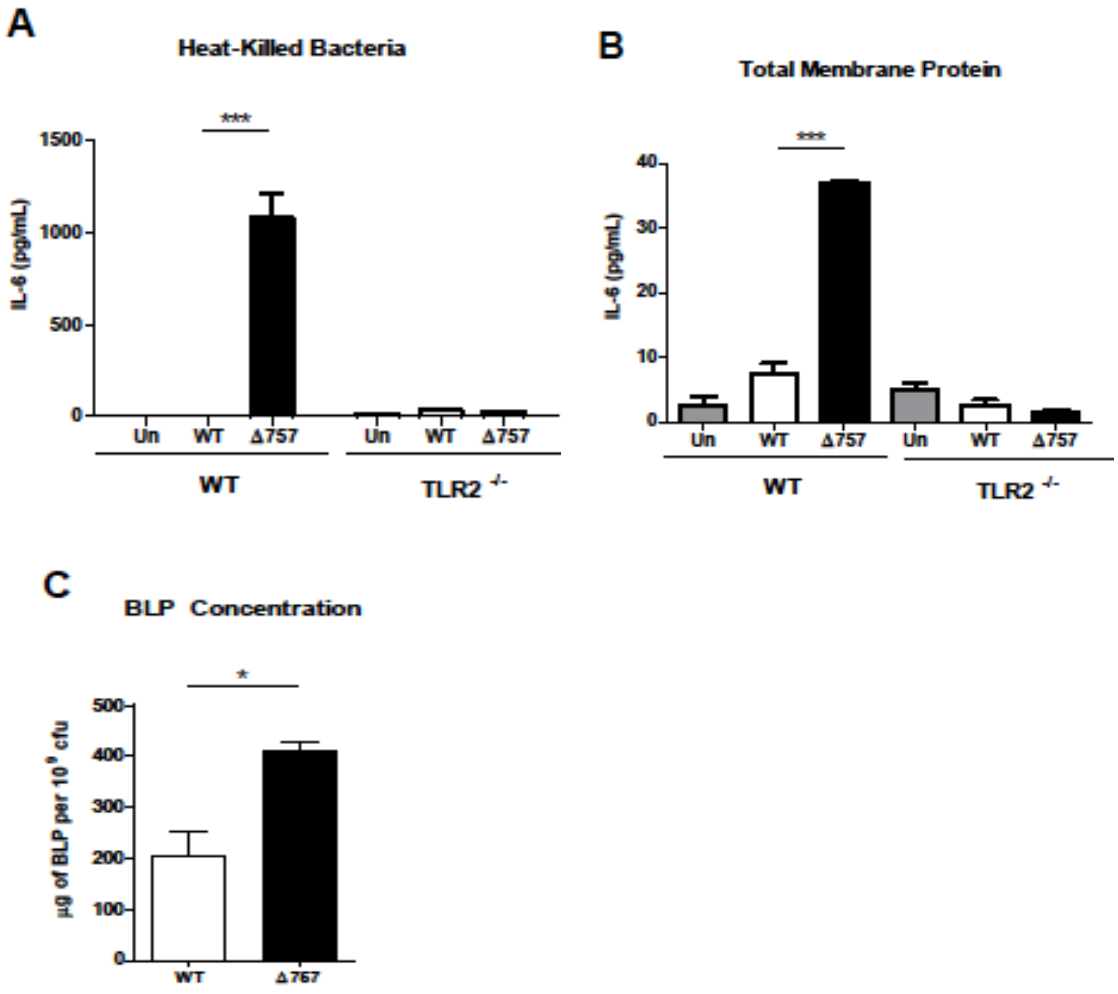

Figure 2. The Δ*FTN_0757* mutant has increased TLR2-stimulating activity and BLP levels. Wild-type or TLR2$^{-/-}$ macrophages were stimulated with (A) heat-killed wild-type or Δ*FTN_0757* (Δ757) at a ratio of 20:1 bacterial cell equivalents per macrophage or (B) total membrane protein fractions derived from the indicated strains at a 1:1 ratio. At 4 hours, supernatants were collected and IL-6 concentrations were quantified by ELISA. (C) BLPs were extracted from the total membrane protein fraction and their concentrations measured via the BCA assay and normalized to bacterial cfu. Bars represent the mean and standard deviation. Data are representative of at least 2 independent experiments. *$p \leq 0.05$, ***$p < 0.0001$.

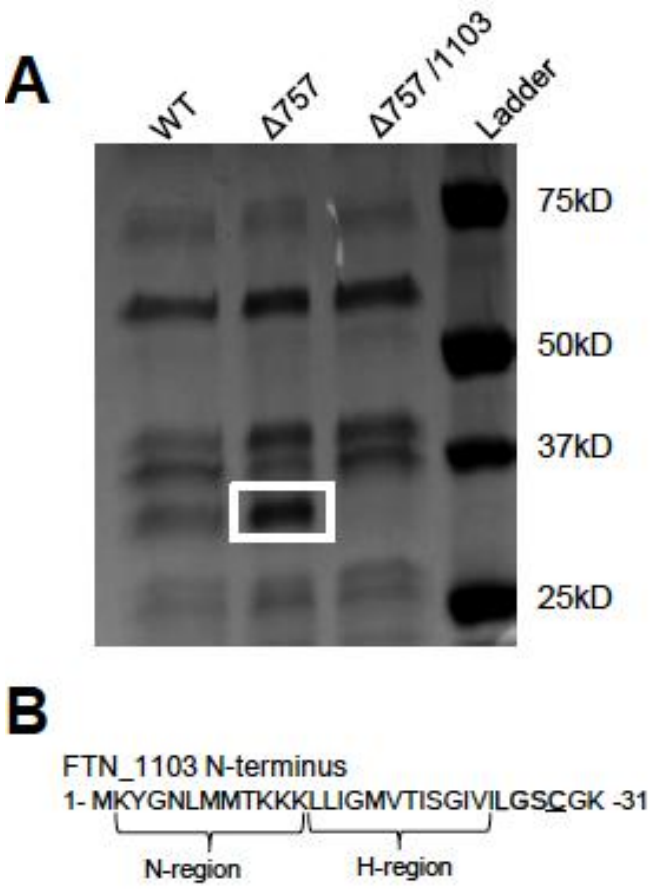

Figure 3. The Δ*FTN_0757* mutant produces increased amounts of the BLP FTN_1103. (A) 10^8 cfu equivalents of the BLP fraction from wild-type or the Δ*FTN_0757* mutant (Δ757) were separated by SDS-PAGE and stained with Coomassie G-250. The most enriched band in the Δ*FTN_0757* lane (white box) compared to the wild-type lane was subjected to LC-MS/MS analysis and identified as FTN_1103. Deletion of *FTN_1103* from the Δ*FTN_0757* mutant resulted in loss of the enriched band. (B) The N-terminus of FTN_1103 contains canonical BLP motifs including a positively charged N-region, hydrophobic H-region, a lipobox motif (bold), and conserved cysteine (underline).

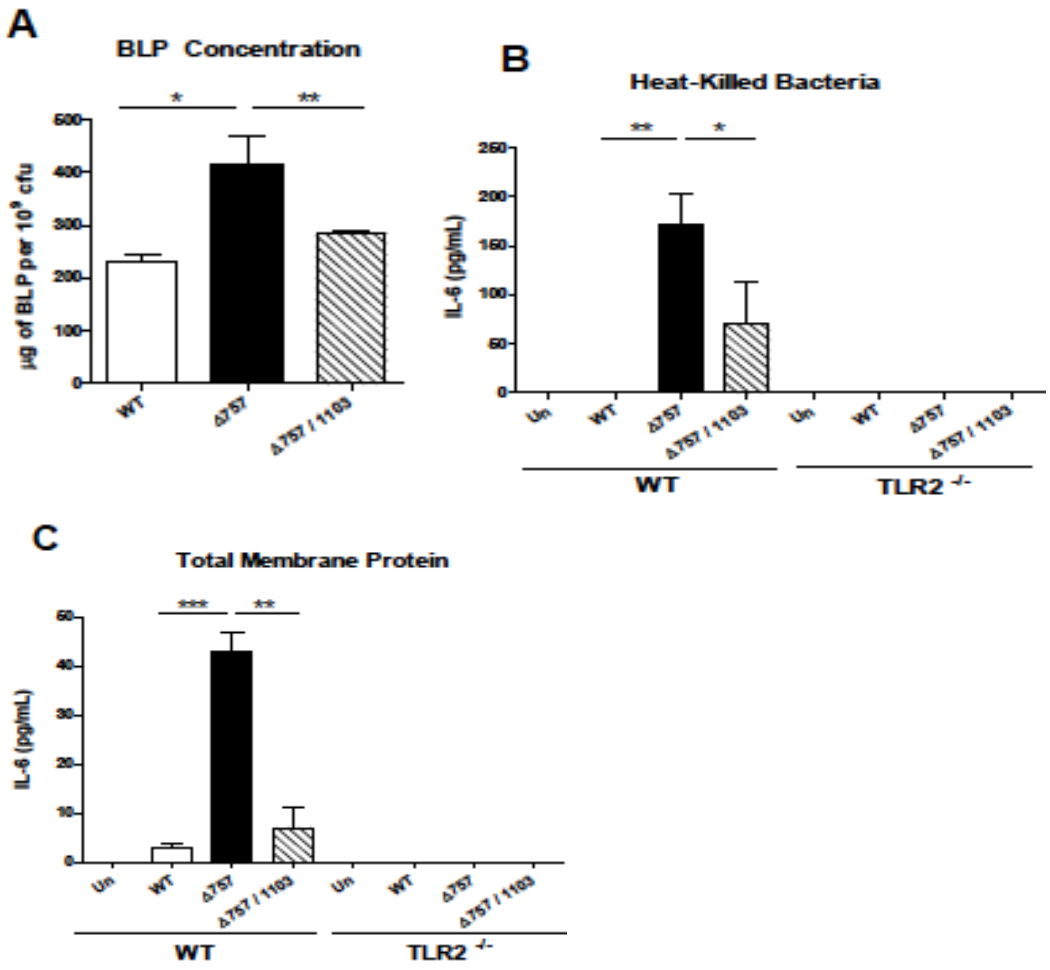

Figure 4. FTN_1103 is responsible for increased TLR2 signaling in the Δ*FTN_0757* mutant. (A) BLPs were extracted from the total membrane protein fraction of wild-type, the Δ*FTN_0757* mutant (Δ757), or the Δ*FTN_0757*/Δ*FTN_1103* (Δ757/1103) strains, and their concentrations measured via the BCA assay and normalized to bacterial cfu. Wild-type or TLR2$^{-/-}$ macrophages were stimulated with (B) heat-killed bacteria at a ratio of 20:1 bacterial cell equivalents per macrophage, or (C) total membrane protein fractions at a 1:1 ratio for 4 hours. Supernatants were collected and IL-6 concentrations were quantified by ELISA. Bars represent the mean and standard deviation. Data are representative of at least 2 independent experiments. *$p \leq 0.05$, **$p \leq 0.005$, ***$p < 0.0001$.

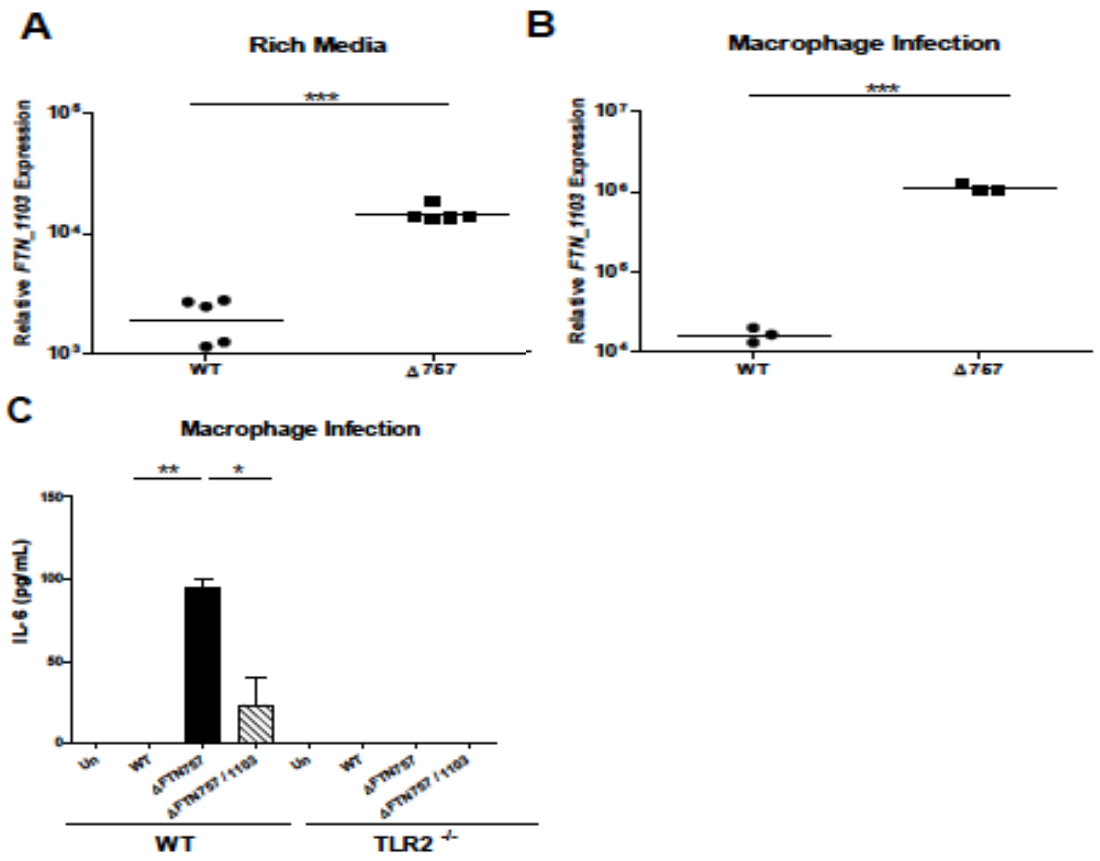

Figure 5. FTN_0757 represses expression of *FTN_1103* during macrophage infection to prevent TLR2 signaling. Following (A) log phase growth in rich media or (B) infection of macrophages with wild-type or the Δ*FTN_0757* (Δ757) strains at an MOI of 100:1 for 4 hours, RNA was harvested. qRT-PCR was performed for *FTN_1103* and $\Delta\Delta C_T$ values were normalized to those of the helicase, *uvrD* (*FTN_1594*). Points represent independent samples and bars represent the geometric mean. (C) Wild-type or TLR2$^{-/-}$ macrophages were infected with wild-type, Δ*FTN_0757* (Δ757), or Δ*FTN_0757*/Δ*FTN_1103* (Δ757/1103) strains at an MOI of 20:1. Supernatants were collected at 4 hpi and IL-6 concentrations were quantified by ELISA. Bars represent the mean and standard deviation. Data are representative of at least 3 independent experiments. *$p \leq 0.05$, **$p \leq 0.005$, ***$p < 0.0001$.

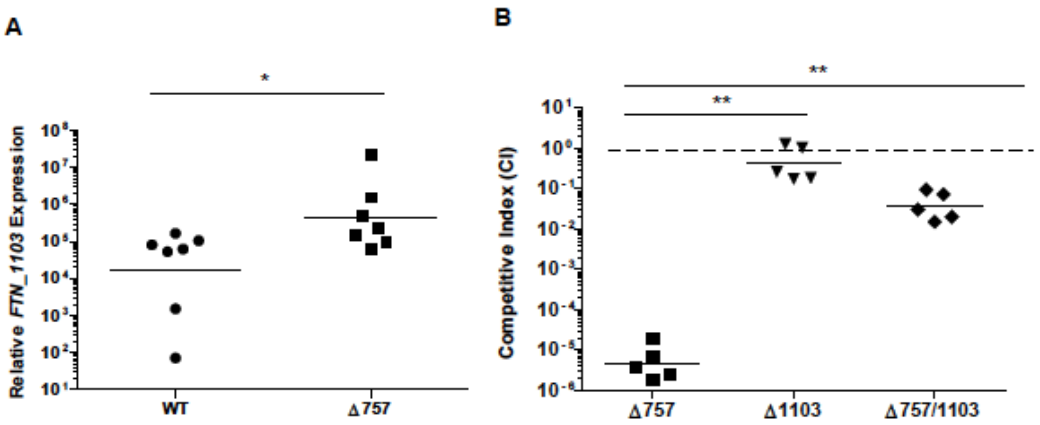

Figure 6. Deletion of *FTN_1103* significantly rescues the *in vivo* virulence defect of the Δ*FTN_0757* mutant. (A) Wild-type C57BL/6 mice were infected subcutaneously with 2x10^6 cfu of wild-type *F. novicida* or the Δ*FTN_0757* mutant. At 6 hpi, the skin was harvested and RNA was extracted. Quantitative real-time PCR was used to determine relative expression of *FTN_1103*, which was normalized to the constitutively expressed *uvrD*. (B) Mice were infected subcutaneously with a 1:1 mixture of wild-type *F. novicida* and either the Δ*FTN_0757* (Δ757), Δ*FTN_0757*/Δ*FTN_1103* (Δ757/1103), or Δ*FTN_1103* mutant (Δ1103). At 48 hpi, the spleen was harvested and the colony forming units (cfu) for each strain were enumerated after overnight growth. The competitive index (CI) = (mutant cfu_{output} / wild-type cfu_{output}) / (mutant cfu_{input} / wild-type cfu_{input}). Bars represent the geometric mean of CI values from each group of mice. Data shown are representative of three independent experiments. *$p \leq 0.05$, **$p \leq 0.005$.

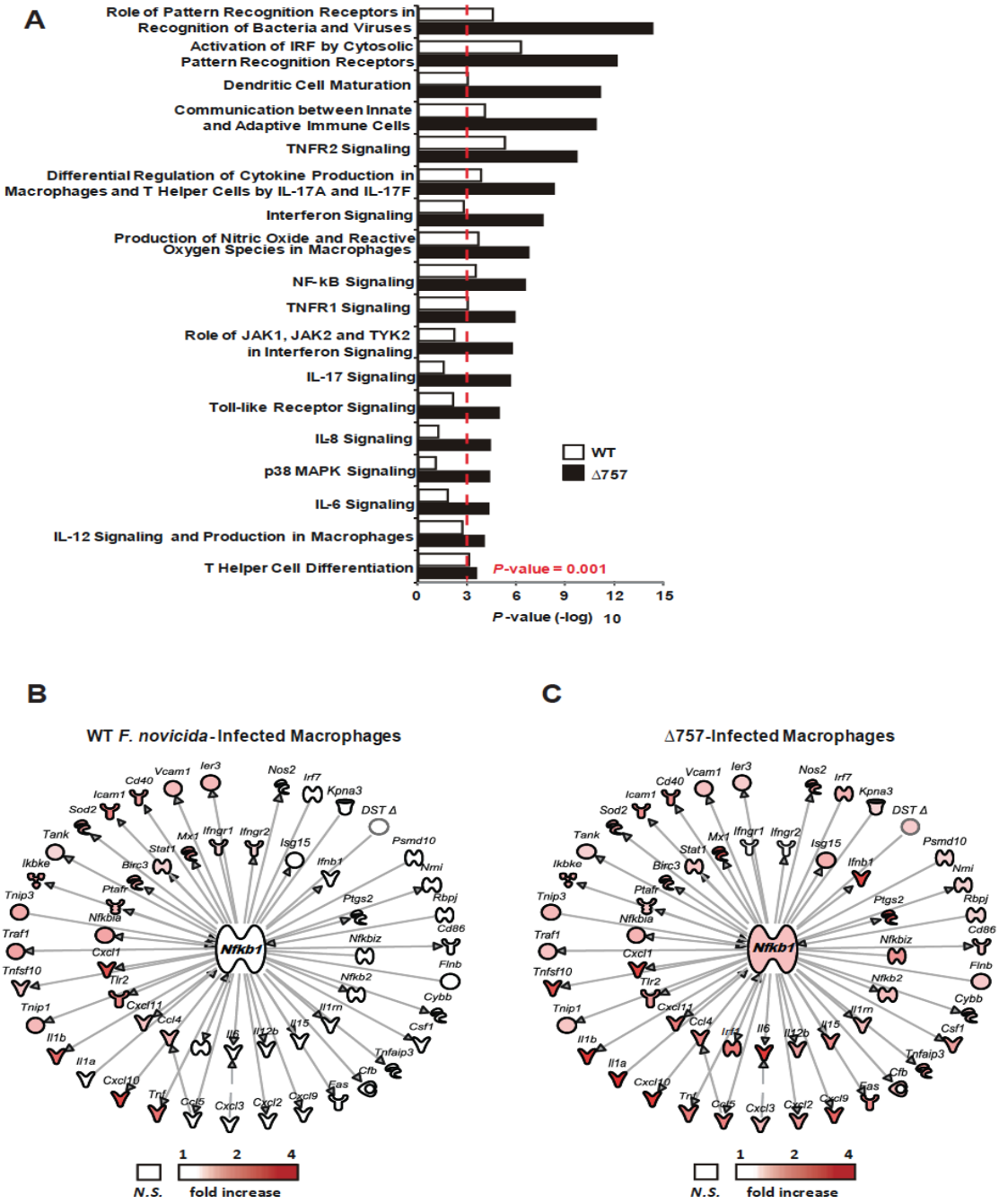

Figure S1. Global transcriptional response to infection with the Δ*FTN_0757* mutant in macrophages. (A) Ingenuity Pathway Analysis was used to identify innate immune pathways whose genes were differentially expressed in macrophages infected with wild-type *F. novicida* (WT) or the Δ*FTN_0757* mutant (Δ757) at 4 hpi. The red dashed line

represents a p-value (right-tailed Fisher Exact Test) cutoff of 0.001. (B, C) A network map of NF-κB-related genes differentially expressed in macrophages infected with (B) WT or (C) Δ757 at 4 hpi. Solid and dashed lines represent direct and indirect interactions reported for the genes, respectively. The colors represent the mean fold-change in gene expression at 4 hpi compared to control in two biological replicates.

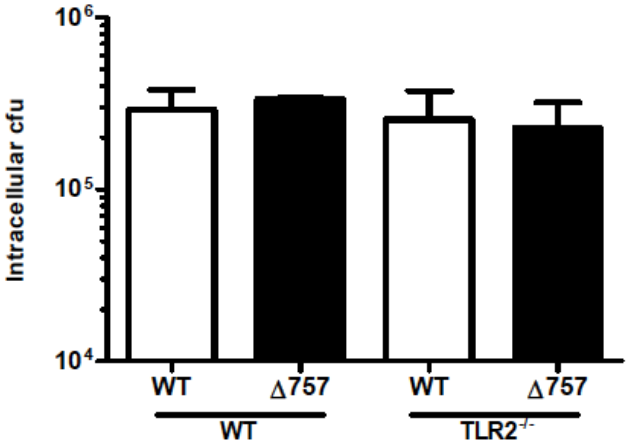

Figure S2. Intracellular replication of Δ*FTN_0757* in macrophages. (A) Wild-type or TLR2$^{-/-}$ macrophages were infected with wild-type (WT) or the Δ*FTN_0757* mutant (Δ757) bacteria at an MOI of 20:1. At 5 hpi, macrophage lysates were plated, grown overnight, and colony-forming units (cfu) were counted. Data are representative of three independent experiments. Error bars represent the standard deviation of triplicate samples.

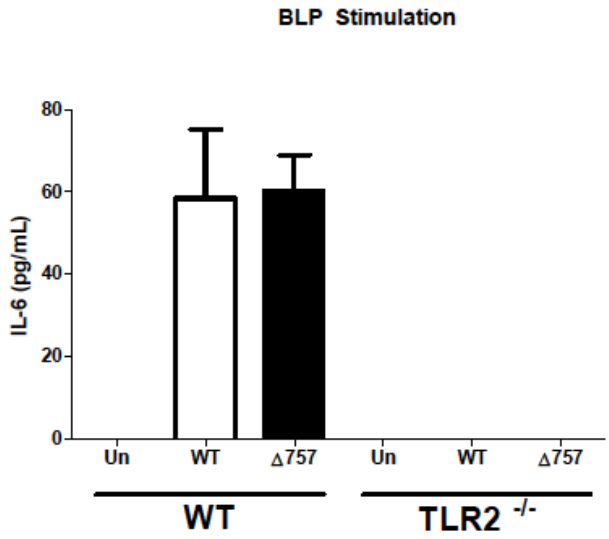

Figure S3. BLP from wild-type and Δ*FTN_0757* strains activate TLR2 with equal potency. Wild-type or TLR2$^{-/-}$ macrophages were unstimulated (Un) or stimulated with 1μg of total BLP preparations from wild-type (WT) or Δ*FTN_0757* (Δ757) strains. At 4 hours post-stimulation, supernatants were collected and IL-6 concentrations were quantified by ELISA. Bars represent the mean and standard deviation.

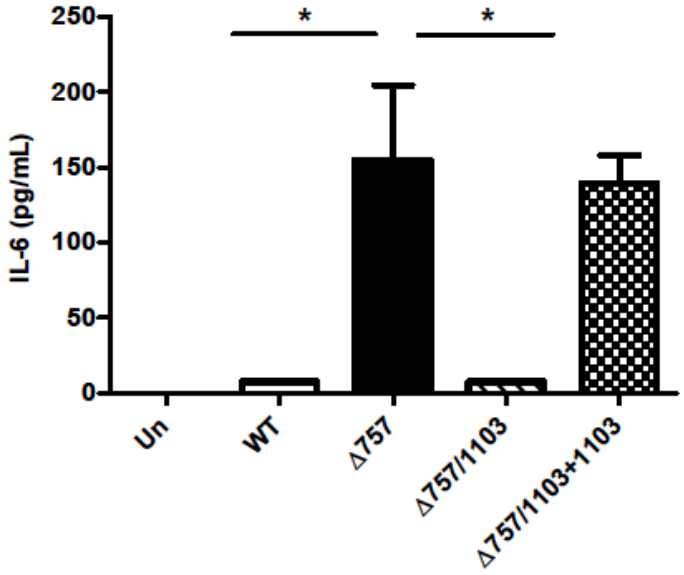

Figure S4. Complementation of the Δ*FTN_0757*/Δ*FTN_1103* strain with *FTN_1103* restores the induction of inflammatory signaling. Macrophages were untreated (Un) or infected with wild-type (WT) *F. novicida*, Δ*FTN_0757* (Δ757), Δ*FTN_0757*/Δ*FTN_1103* (Δ757/1103), or the Δ*FTN_0757*/Δ*FTN_1103* mutant complemented with *FTN_1103* (Δ757/1103 + 1103), at an MOI of 20:1. At 4 hpi, the concentration of IL-6 secreted into the culture supernatant was measured by ELISA. Data are representative of three independent experiments. Error bars represent the standard deviation of triplicate samples. *$p < 0.05$.

Chapter 4: Discussion

Discussion

The innate immune system detects the presence of bacterial pathogens using a number of pattern recognition receptors (PRRs), including TLRs, C-type lectin receptors and NLRs (1). Activation of these pathways can promote bacterial phagocytosis (2) and lead to the activation of transcription factors such as NF-κB, AP1 and IRFs that direct the expression of inflammatory cytokines and antimicrobial peptides (3). Using the Homeland Security Advisory System (4) as an analogy, macrophage antibacterial defenses can be color-coded and divided into three zones that are successively activated as bacteria migrate to the inside of the cell (Figure 1).

The extracellular or Code Yellow defenses are important for detecting bacterial invaders via PRRs, and intrinsically (by promoting phagocytosis and upregulation of antimicrobial peptide and nitric oxide production) and extrinsically (through cytokine and chemokine production) alerting the immune system to the presence of the enemy. Phagosomal or Code Orange defenses respond to bacteria that have entered the phagocyte, by acidifying the phagosome and attacking bacteria with antimicrobial peptides and other degradative agents in the phagolysosome. This pathway can be activated in conjunction with the Code Yellow defenses and is capable of clearing nonpathogenic bacteria. However, bacterial pathogens use a variety of immune evasion mechanisms to escape killing at this stage of the attack (5). Intracellular bacteria, including *Francisella* spp., evade the host attack by escaping the phagosome and replicating in the cytosol where they are nonetheless faced with the challenge of escaping cytosolic or Code Red defenses. These defenses include ubiquitination, autophagy-mediated degradation and inflammasome activation. This schematic (Figure 1)

exemplifies the range of outcomes of interactions between the macrophage and bacteria, but it does not take into account the toll that host cells pay when activating these defenses.

With activation of Code Yellow defenses, there is very little or no fitness cost to the host. In fact, simultaneous activation of these defenses can enhance the magnitude of its response to microorganisms. For example, murine macrophages simultaneously stimulated with poly I:C (a TLR3 ligand) and CpG DNA (a TLR9 ligand) produced increased amounts of nitric oxide, IL-12, TNF-α and IL-6 compared to macrophages that received a single treatment of either ligand (6). More importantly, this amplified inflammatory response can also promote host resistance to microbial infection. On the contrary, Code Orange defenses cause some loss of fitness since degradative enzymes in the lysosome can cause damage to the phagolysosomal compartment (7). This would allow for the leakage of phagolysosomal components, including PAMPs, which could activate cytosolic defenses. Code Red defenses, particularly inflammasomes, cause the most severe consequence to host cells since their induction leads to host cell death. Regulating the activation of defense pathways that have higher fitness costs is necessary to prevent macrophages from undergoing cell death each time they encounter a bacterium, to maintain the integrity of the innate immune system.

Cooperation can be defined as a type of interaction where two or more individual host defense pathways optimally induce the same effector mechanism when pathways are activated, often resulting in synergy (8). This is exemplified by the relationship between TLRs (Code Yellow-Orange defenses) and inflammasomes (a Code Red defense), two spatially separated and temporally activated host defense pathways that individually

activate distinct antimicrobial defenses and collectively rapidly respond to intracellular bacteria by inducing cell death and facilitating IL-1β and IL-18 release. The canonical pathway for inflammasome activation requires input from two signals: (1) TLR activation by a microbial ligand to induce the expression of the inflammasome substrate IL-1β, and (2) activation of a cytosolic inflammasome sensor (NLR or AIM2) by either a PAMP or host-associated danger signal (DAMP) (9). Having this two-step activation requirement is a critical regulatory mechanism that enables host cells to assess the level of threat imposed by an invading microorganism and to elicit an immune response that will optimally clear the microbe while imposing a minimal fitness cost to the host.

The findings in this dissertation provide an additional layer of defense imparted by TLRs that aid in inflammasome activation. In particular, TLR2 activation promotes the assembly and rapid activation of the AIM2 inflammasome during *F. novicida* infection (Chapter 2). In accordance with the previously stated paradigm, TLR2 activation is not absolutely required for AIM2 inflammasome activation (cell death, caspase-1 activation and IL-18 release), but it is necessary for speeding up the kinetics of inflammasome activation and for the expression of the IL-1β transcript. The timing in which the macrophage deploys its defenses and the bacterial pathogen employs its evasion strategy is an important factor in determining the outcome of the infection.

Therefore, coupling TLR2 activation, which occurs at the onset of *Francisella* infection, to inflammasome assembly could potentially give host cells an advantage over bacterial pathogens. It would be speculated that delays on the host side can turn an easily clearable infection into a devastating disease for the host, and delays in the bacterium's ability to evade the TLR2/inflammasome pathway could prevent the pathogen from

causing disease and allow the host to easily clear the infection. Indeed, TLR2$^{-/-}$ mice have increased bacterial loads >4 hours post-infection (unpublished data) and are more susceptible to *F. novicida* infection compared to their wild-type counterparts (10). It is possible that this phenotype in TLR2$^{-/-}$ mice is due to an abrogation of the proinflammatory cytokine response that is important for clearing *Francisella* infection. It is also very likely that a delay in *in vivo* inflammasome activation also contributes to a lack of bacterial clearance since cell death is a protective mechanism for the host that exposes bacteria to immune cells, such as neutrophils, that can more efficiently control *Francisella* infection (11, 12). By assigning a role for TLR2 in regulating IL-1β expression and inflammasome assembly kinetics, the host creates a fail-safe defense system. Activation of this host defense pathway allows for the expression of IL-1β and promotes rapid inflammasome assembly. However, in the event that bacterial pathogens are able to evade TLR2 activation, the inflammasome can still be activated and the potent proinflammatory cytokine IL-18, which is constitutively expressed independently of TLR2 (Chapter 3), can also be released.

The coupling of host defense pathways that respond to bacterial infection may be beneficial to the host since it adds an additional layer of protection against bacterial infection, but it can make it easier for a pathogen to develop a single mechanism to evade both host defense pathways. With TLR2 playing a role in proinflammatory cytokine production and inflammasome activation during *Francisella* infection, this pathogen is under selective pressure to develop a mechanism for evading this TLR, which plays a critical role in deciding its fate inside the host. Unlike *Yersinia* which secretes effector proteins into macrophages to block TLR signaling, *Francisella* does not encode a type III

secretion system that would allow for the delivery of effector proteins (13). Interestingly, *Francisella* does encode a type VI secretion system that could deliver effector proteins, but this secretion system and putative effector proteins have not been characterized (14). Another strategy used by pathogens to evade TLR activation is to modify their PAMPs. However, this approach cannot be employed by *Francisella* to evade TLR2 since this receptor recognizes the N-terminal signal sequence of bacterial lipoproteins (BLPs) called the lipobox, and modifying this region would likely interfere with attachment of lipoproteins to the bacterial membrane and potentially be detrimental to the bacteria.

The findings in this dissertation present a new paradigm for bacterial evasion of TLR2: repressing BLP expression. Through the action of FTN_0757, *F. novicida* repressed BLP expression to evade TLR activation (Chapter 3), and repressing BLP expression also provided a means for this pathogen to evade TLR2-dependent inflammasome activation (unpublished data), but it is important to note that macrophages are still capable of undergoing cell death. These studies show that maintaining a relatively low concentration of BLPs in the outer membrane is critical to bacterial virulence. The mechanism of FTN_0757 regulation of BLP expression is unclear. While its sequence does not contain any homology to proteins known to function in gene regulation, its position in the genome provides some clues about its possible function. FTN_0757 lies upstream of a gene encoding a small RNA (*ftrA*). Considering that this protein contains a putative endonuclease domain, it could be acting in conjunction with this small regulatory RNA to control the expression of BLPs in a manner similar to the DICER system in eukaryotes. More experimentation is necessary to uncover the

mechanism of FTN_0757 regulatory action, and this is currently an area of active investigation in our laboratory.

Taken together the findings presented in this dissertation provide a more in-depth understanding of how the host couples TLR2 signaling to inflammasome activation in order to reserve activation of this lethal defense pathway to occur only in response to an uncontrollable bacterial threat. Taking into account the ability of cytosolic bacteria to breach all exterior defenses and replicate in the cytosol, the macrophage optimizes its response to intracellular bacterial pathogens by integrating signals from defense pathways in one or more zones of defense. This layering of host defense pathways provides the host with protection against bacterial pathogens that have evolved several strategies for evading host defenses and minimizes the overall fitness cost incurred by responding to bacterial infection.

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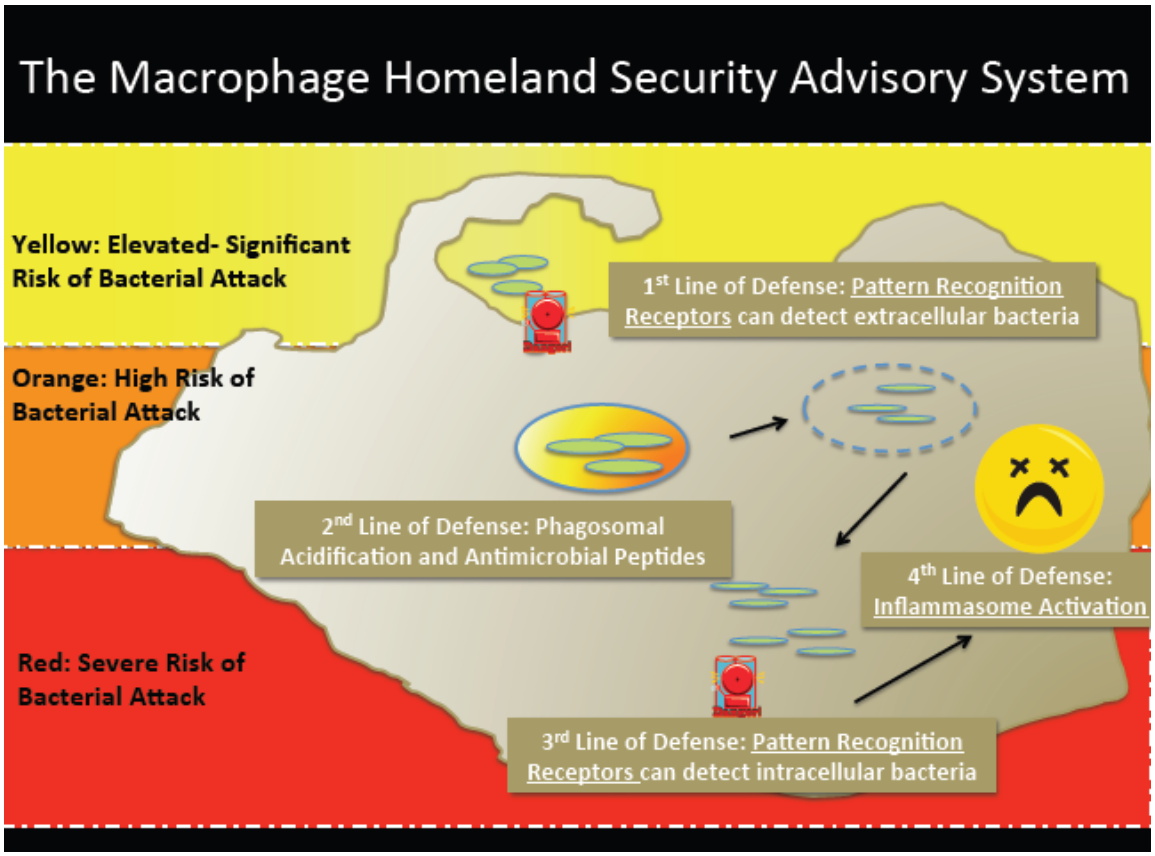

Figure 1. Macrophage defense against bacterial infection. Macrophage defenses against bacterial infection can be divided into three zones: extracellular defenses, phagosomal defenses and cytosolic defenses. These defenses are deployed by the macrophage based on the level of threat imposed by bacteria.