Interactions between inflammasomes and the stealth pathogen, *Coxiella burnetii*

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2017

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Program Authorized to Offer Degree:

Pathology

University of Washington

Abstract

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Coxiella burnetii, a zoonotic pathogen and agent of Q fever, is an obligate intracellular bacterium that hijacks host cell machinery to form a large, acidified replicative vacuole from which it injects proteins into the cytosol through a Type IV secretion system. Type IV secretion is required for *C. burnetii* intracellular growth and modulation of host cell responses, including apoptosis, a non-inflammatory, caspase-mediated programmed cell death, to establish a replicative niche and persistent infection. In contrast to apoptosis, pyroptosis is a pro-inflammatory, caspase-mediated cell death, and plays a key role in the innate immune response to microbial infections.

Pyroptosis is regulated by inflammasomes – macromolecular complexes that contain cytosolic sensors, for example NLRP3 and NLRC4, and the effector cysteine protease, caspase-1. Highly adapted pathogenic bacteria have evolved mechanisms to subvert inflammasomes by direct inhibition or avoiding detection. Hosts have counter-adapted strategies as inflammasomes are dispensable to immune responses in some *in*

vivo models. We found that *C. burnetii* primes but avoids cytosolic detection by NLRP3 inflammasomes and these interactions are independent of Type IV secretion.

We established an *in vivo* mouse model to determine the biological relevance of inflammasomes during immune response to *C. burnetii*. Following pulmonary infection, mice deficient in caspase-1 or NLRP3 had significantly higher bacterial burdens in the lung than wild-type mice. However, by the end of the 21-day infection, pulmonary bacterial burdens were similarly decreased amongst wild-type and knock-out mice. Furthermore, *Nlrp3*$^{-/-}$ and *Casp1/11*$^{-/-}$ mice had similar development and resolution of gross and histologic lesions and macrophage kinetics as for wild-type mice. Together, these data indicate that like other effective pathogens, *C. burnetii* has developed mechanisms to subvert host inflammasomes. Likewise, hosts utilize other pathways to combat infection further supporting the theory that inflammasomes are not required but are important for the enhancement of adaptive immune responses to pathogens. By identifying mechanisms of inflammasome evasion by *C. burnetii*, Q fever pathogenesis and pathophysiology is better understood, advancing diagnostic, prophylactic, and treatment strategies.

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LIST OF ABBREVIATIONS

ASC	Apoptosis-associated speck-like protein containing a CARD
BMDM	Bone marrow derived macrophages
CARD	Caspase activation and recruitment domain
Casp1	Caspase-1
Caspase	Cysteinyl aspartate-specific proteinase
Cb	*Coxiella burnetii*
LDH	Lactate Dehydrogenase
LPS	Lipopolysaccharide
NMI, NMII	Nine Mile Phase I, Nine Mile Phase II
NLR	Nucleotide-binding domain, LRR-containing receptor
NLRC4	NLR, CARD-containing 4
NLRP3	NLR, PYD-containing 3
MOI	Multiplicity of infection
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PYD	Pyrin domain
Stm	*Salmonella enterica* serovar Typhimurium
T3S, T3SS	Type III secretion, Type III secretion system
T4S, T4SS	Type IV secretion, Type IV secretion system
TLR	Toll-like receptor
WT	Wild-type
Yptb	*Yersinia pseudotuberculosis*

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ACKNOWLEDGMENTS

I would like to thank my thesis advisor, Dr. Charles Frevert, for his mentorship and support and Drs. Donald Knowles and Denny Liggitt for providing me the opportunity to pursue research training. I would especially like to thank our collaborators in the Cookson Lab and Histology Imaging Core and Dowon An for her technical expertise. Many thanks to Dr. Robert Heinzen and lab members for their generous support and providing bacteria, antibodies, and advice. I would like to express gratitude to my committee members Drs. Brad Cookson, Piper Treuting, William Mahoney, and Deborah Fuller. I give special thanks to Drs. William Mahoney and Piper Treuting for reading this dissertation. I thank my parents, Fran and Larry, and sister Meghan for their continued support and encouragement. Finally, I thank my husband, Devin for his unwavering love and inspiration and my daughters Phoebe and Fern, for keeping things in perspective.

Chapter 1. Introduction

Coxiella burnetii, the agent of Q fever

Query ("Q") fever is an overall poorly understood, globally reported, zoonotic disease caused by the obligate intracellular Gram-negative bacterium, *Coxiella burnetii*. *C. burnetii* is endemic worldwide with an extensive list of hosts, though the absolute prevalence in the animal kingdom is unknown.(1) Ruminant livestock species, particularly sheep, goats, and cattle are the most important reservoir hosts yet other domestic animals and arthropods have been rarely implicated in human exposures.(1, 2) Ruminant reservoir hosts are typically asymptomatic but intermittently shed bacteria.(3, 4) In naïve herds, *C. burnetii* infection of pregnant females results in placentitis and epizootic abortions, and subsequently, billions of bacteria are deposited into the environment.(3, 4) Once expelled, the small cell variant (SCV), which is the infectious form of *C. burnetii*, is resistant to numerous elements including ultraviolet radiation, disinfectants, and heat.(5) Thus, *C. burnetii* may persist in the environment for months to years to become aerosolized and inhaled by other hosts.(3) Humans are most commonly infected by inhalation of bacteria and those in proximity to reservoir hosts, either by occupation or geography, are at increased risk of infection.(1, 2) Primary pulmonary infection results following inhalation with as few as 1-2 organisms.(1) This low infectious dose, resistance to environmental elements, ability to aerosolize, and previous attempts of weaponization earn *C. burnetii* a Tier 2 Select Agent classification by the Center for Disease Control (CDC).(6) Ruminant Q fever places a significant economic

burden on livestock producers; however, these are relatively minor when compared to the economic impact on the human health care system.(7)

Once inhaled, SCVs are phagocytosed by alveolar macrophages, via interactions with αvβ3 integrin and/or complement receptor 3 (CR3) resulting in actin re-organization and uptake.(8, 9) Ligation with either host receptor depends on the LPS structure of *C. burnetii*.(10) Loss of the methylated sugars of the O-antigen core lead to a shift from phase I to phase II bacteria, referred to as phase variation.(11) This was first documented in *C. burnetii* during *in vitro* culture though antibodies to both phase variants are found in Q fever patients and so are utilized in serologic tests.(12) Phase variation is an evasive mechanism of Gram-negative bacteria and in *C. burnetii* Nine Mile (NM) strain it results in a permanent chromosomal deletion within the encoding region of LPS.(9, 13, 14) This renders Nine Mile Phase II (NMII) avirulent based on lack of clinical disease and normothermia in guinea pigs and immunocompetent mice.(12, 15) Avirulent NMII is exempt from Select Agent status and can be cultivated and studied in Biosafety Level 2 (BSL2) facilities.(6, 15) *In vitro* kinetics are identical between Nine Mile Phase I (NMI) and NMII strains and both induce similar cytokine responses in mouse and human cells *in vitro*.(9, 16) Thus NMII strain has been used increasingly in the study of host immune responses to this highly adapted pathogen.

C. burnetii is exquisitely adapted to intracellular life, not only surviving but thriving within host target cells of the monocyte-macrophage system.(6, 17, 18) Following internalization, the bacteria hijack host vesicle pathways for the development of a spacious, acidified, host-derived (parasitophorous) vacuole (PV) (Fig. 1).(17-19) After PV acidification, *C. burnetii* undergoes a morphological and metabolic switch to an

active, non-infectious large cell variant (LCV; >1μm) and commences exponential growth.(1, 5, 17)

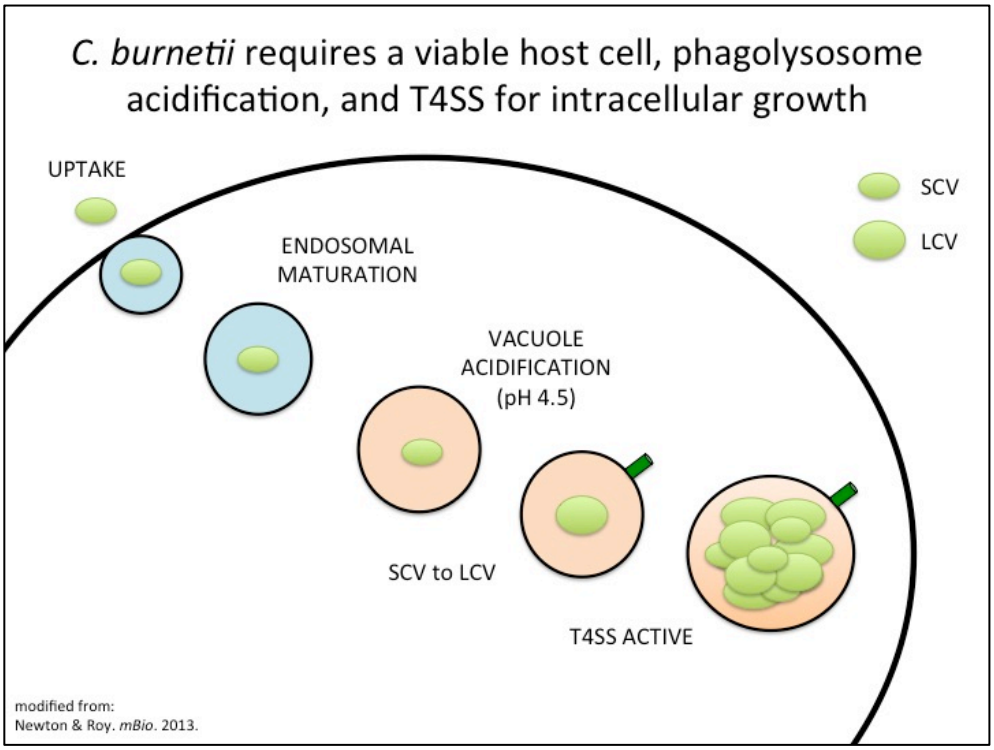


Figure 1. *Coxiella burnetii* depends on the host cell for uptake, endosomal maturation, and acidification of the vacuole through endosomal-lysosomal fusions. The infective form, the small cell variant (SCV) is internalized, then switches to the metabolically active form (large cell variant, LCV), and begins to replicate. At this time, the Type IV secretion system is activated and begins to secrete bacterial effector proteins into the host cytosol, modulating host machinery and regulating the formation and maintenance of the large parasitophorous vacuole. This figure is a modification of Figure 1 in the work of Newton & Roy et al. mBio, 2013.

Coxiella-containing vacuoles have sequential labeling of proteins signifying well-characterized interactions with host endosomal, autophagic, lysosomal, and phagocytic

pathways.(17, 18, 20, 21) This precise vesicular pathway entails active modulation of these and other host cell pathways that are similar to other obligate intracellular pathogens. (17, 19, 22-28) This modulation results in: interference with host kinase and phosphatases; alteration of macrophage phenotypes (polarization); down regulation of inflammatory mediators and attenuation of adaptive immunity; prevention of oxidative burst and nitrogen intermediates; and manipulation of host cell death pathways including apoptosis, autophagy, and pyroptosis.(17, 19, 22-28)

Many mechanisms of host modulation are dependent on secretion of bacterial proteins into host cytosol through a type IV secretion system (T4SS), which has been described for several intracellular bacteria.(20, 29-32) Using bioinformatics, fusion protein reporter and functional assays, T4S secretion of effector proteins was found to be critical to *C. burnetii* including PV formation and maintenance, intracellular replication, and inhibition of apoptosis.(29, 33-36) Mutant bacteria lacking the T4SS (Δ*dotA*) survive within cells but have defective PV formation, intracellular growth, and anti-apoptotic effects.(37, 38)

A subset of translocated T4SS effectors, the ankyrin-repeating domain (Ank) proteins, are of special interest in that they contain eukaryotic-like leucine-rich repeat domains.(23, 39) In eukaryotes, Ank proteins are ubiquitous and involved in myriad signaling pathways, regulating protein-protein interactions.(39) At least ten *C. burnetii* Ank proteins are translocated during infection, though only one, AnkG, has a confirmed function.(40-42) Following T4S into the host cytosol, AnkG binds p32 on the outer mitochondrial membrane, inhibiting cytochrome c release and intrinsic apoptosis of the host cell.(40)

Interestingly, different *C. burnetii* isolates have heterogeneity among all Ank proteins at the genome, proteome, and secretome level, which further supports their role in virulence in pathogenicity.(42) More recently, an exhaustive screen of encoded *C. burnetii* T4SS proteins, including all known Ank proteins, revealed only one protein (non-Ank protein), IcaA, that is capable of attenuating inflammasome-mediated cell death, known as pyroptosis.(27) This T4SS protein was shown to act upon caspase-11, the cysteine protease associated with non-canonical inflammasomes with no direct inhibition on caspase-1 and canonical inflammasomes.(27) To date, there are no known T4SS effectors secreted by *Coxiella burnetii* that interact with canonical inflammasomes.

Inflammasomes

Host immunity to microbial infections is governed by an initial robust innate response, which relies on an array of highly conserved receptors, known as pattern recognition receptors (PRRs).(43) Included in this group are Toll-like receptors (TLRs), C-type lectin receptors, RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs), and Nod-like receptors (NLRs).(43-45) Together, these receptors provide a broad and redundant repertoire of defenses against numerous infectious (i.e. pathogens) and non-infectious cellular stressors. NLRs are a large family of proteins that contain a conserved NOD motif and act as cytosolic sensors, able to detect various pathogen- and danger-associated molecular patterns (PAMPs and DAMPs, respectively).(43, 45, 46) Activation of NLRs and similar sensors results in recruitment and formation of macromolecular complexes known as inflammasomes. Inflammasome formation involves the recruitment and cleavage of the pro-form of the effector cysteine protease, caspase-1, which in turn

leads to cleavage, activation, and secretion of the pro-inflammatory cytokines, IL-1β and IL-18.(46)

The inflammasomes identified thus far are defined by their cystolic sensor: NLRP1/NALP1b; NLRC4/IPAF; NLRP3/NALP3; and AIM2 (absent in melanoma 2). Some inflammasomes have a growing list of ligands, while others have only few known stimuli.(43, 46) For example, NLRC4 inflammasomes are rapidly activated following detection of bacterial flagellin.(28, 46) Some pathogenic bacteria can stimulate more than one inflammasome, highlighting the importance and reduncancy of these pathways to innate immunity.(47-52) For example, *Legionella pneumophila, Yersinia pseudotuberculosis,* and *Salmonellla enterica* activate NLRC4/IPAF inflammasomes when expressing flagellin but can also be detected by NLRP3 when flagellin is absent or down-regulated.(51, 53, 54)

The NLRP3 inflammasome is the most widely studied of caspase-1-mediated (canonical) inflammasomes and is activated by numerous microbial products including bacterial toxins and viral RNAs, as well as non-microbial substances such as uric acid.(44, 45) In contrast to NLRC4 inflammasomes, NLRP3 inflammasome activation requires two signals; the first (signal 1) is referred to as "priming", which involves Toll-like receptor (TLR)- signaling and NFκB-mediated upregulation of NLRP3 and pro-forms of IL-1β and IL-18 (Fig. 2).(55) Signal 2 involves the NLRP3 sensing of cytosolic damage signals (from cellular or DNA injury), reactive oxygen species, or changes in ionic balances, which results in aggregation of NLRP3 proteins. NLRP3 aggregates then co-localize with ASC, a bipartite adaptor protein that bridges NLRP3 with pro-caspase-1, and aids in the recruitment of pro-caspase-1 to form the inflammasome complex.(56, 57)

Within the mature inflammasome, pro-caspase-1 is cleaved to its active form. Active caspase-1 subsequently cleaves pro-forms of IL-1β and IL-18 to their active forms, which are then secreted. Active caspase-1 also leads to pore formation (through cleavage of gasdermin D) and pyroptosis.(44, 45, 58)

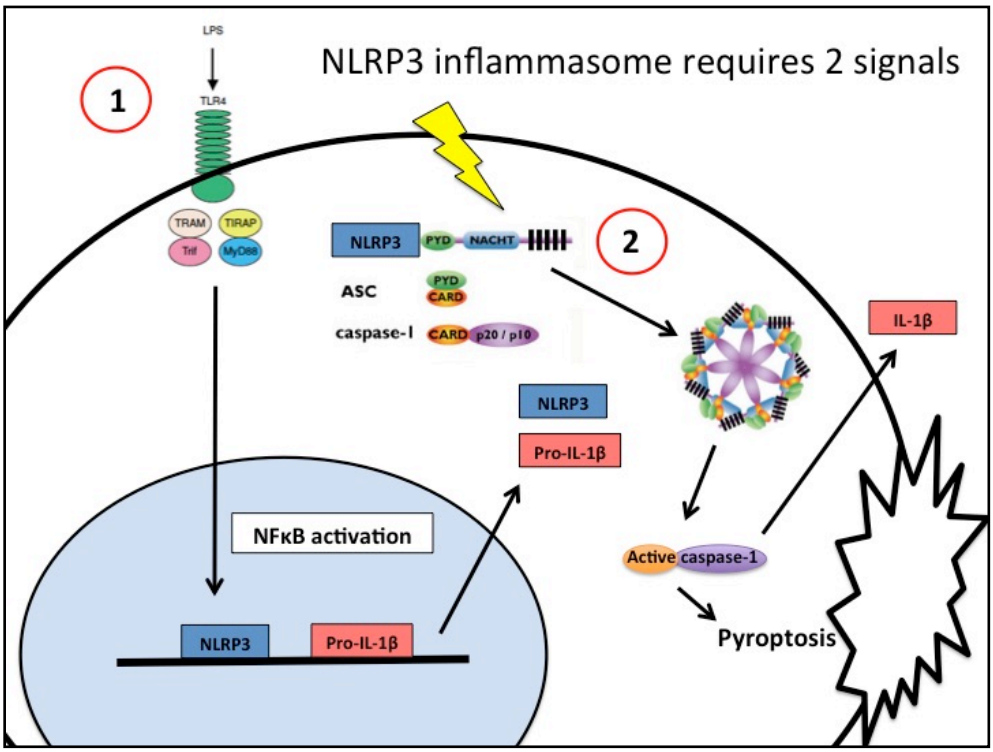


Figure 2. The NLRP3 inflammasome requires 2 signals for activation: 1) signal 1 or priming involves upregulation of NLRP3 and pro-IL-1β through TLR-signaling and NfκB activation. 2) signal 2 occurs following the detection of a damage- or pathogen-associated molecular pattern (DAMP and PAMP, respectively), which results in the aggregation of NLRP3, co-localization with the adaptor protein, ASC, and recruitment of pro-caspase-1. Following inflammasome formation, pro-caspase-1 is cleaved and activated. Active caspase-1 cleaves IL-1β resulting in secretion and through gasdermin-D, causes pore formation and pyroptosis.

In contrast to apoptosis, a caspase-mediated and non-inflammatory form of cell death, pyroptosis is an inflammatory process that results in plasma membrane pore formation, ion flux, and release of cytoplasmic contents into the extracellular space.(59) Upon cell lysis, intracellular pathogens are released and re-exposed to phagocytes and immune cells, enhancing the host response.(50, 60) Thus, inflammasomes are considered critical to the development of an inflammatory response sufficient to combat microbial infections and for the clearance of intracellular pathogens.(44, 50, 61, 62)

Recent studies have linked non-canonical inflammasomes, mediated by caspase-11 in mice and caspase-4 and 5 in humans, to canonical (caspase-1-mediated) NLRP3 inflammasomes during Gram-negative sepsis and when bacterial LPS accesses and is detected in the host cell cytosol.(63-66) Importantly, mice deficient in caspase-1 also have a dysfunctional allele for caspase-11 therefore studying the interplay of the canonical and non-canonical inflammasomes during infections *in vitro* or *in vivo* is complicated.(27) Regardless, the relationship between non-canonical and NLRP3 inflammasome activation exemplifies the evolution of redundant and complimentary pathways for microbial detection in the mammalian immune system.(50, 53)

It is not surprising that successful intracellular pathogens have evolved mechanisms to subvert inflammasomes through avoidance of detection or direct inhibition of inflammasome components.(53, 54, 67, 68) Examples include the down-regulation of flagellin by *Salmonella enterica* serovar Typhimurium to avoid detection by NLRC4, and the T3SS effector YopM of *Yersinia pseudotuberculosis,* which binds to pro-caspase-1 inhibiting its recruitment to the forming inflammasome.(53, 54, 67, 69) The importance of inflammasomes during natural infections with highly adapted

pathogens has been questioned as many *in vivo* studies demonstrate that inflammasomes can be dispensable to successful host immune responses and survival.(53, 70) This paradox likely exemplifies host counter-adaptations and the development of redundant immune responses to ensure the survival of the host.(47, 53, 71) Nevertheless, inflammasomes are still regarded as key components of the immune response through enhancement of an adaptive response, particularly cell-mediated immunity.

As an obligate intracellular pathogen, *C. burnetii* has evolved an arsenal to evade immune responses and inhibit cell death as it targets and resides within macrophages, the cells central to both innate and adaptive immunity. Modulation of programmed cell death is an important ability for intracellular pathogens - *C. burnetii* has a T4SS effector protein, AnkG, which inhibits apoptosis.(40) Although *C. burnetii* has a T4SS effector protein, IcaA, that by an unknown mechanism can attenuate capase-11, the interactions between *C. burnetii*, canonical inflammasomes, and caspase-1-mediated cell death (pyroptosis) remain largely unexplored.(27, 54)

Q fever pathophysiology

After initial infection with *C. burnetii*, clinical outcomes of Q fever are considered to be highly dependent on host immune responses, specifically macrophage activation states and robust cell-mediated immunity, and to a lesser extent, virulence of the bacterial strain.(10) Approximately 60% of acute Q fever patients are asymptomatic, fewer develop transient influenza-like symptoms with alveolitis and mild pneumonia; and rare patients require hospitalization due to fulminant pneumonia or hepatitis.(10) A small percentage of acute Q fever patients may progress to a chronic form of the disease

months to years later with development of splenic and hepatic granulomas.(1, 10, 72) Those at risk of developing chronic Q fever include: patients with valvulopathy, of which a large percentage develop frequently fatal chronic endocarditis; and various immunocompromised states, including pregnancy when hormonally-induced anti-inflammatory states increase a women's risk for infection.(10, 73, 74) Diagnosis of Q fever is difficult due to lack of or vague symptoms during the acute phase and inconsistent or contradictory diagnostic results in chronic disease.(75-78) Current diagnostic strategies include serologic testing (antibody), molecular detection of bacterial DNA (PCR), antigen detection using immunohistochemical techniques, and cytokine detection and profiling.(75, 77, 79, 80)

Recent advances in molecular analyses have allowed for more accurate characterization of host immune responses to bacterial infections, including *C. burnetii*.(81-83) Acute Q fever is characterized by a Th1-response, which is protective and dependent on IFN-γ, resulting in efficient antimicrobicidal activities and bacterial clearance by classicaly activated (M1) macrophages.(25, 84, 85) In contrast, chronic Q fever patients demonstrate an abnormal cytokine profile, defective microbicidal killing by professional phagocytes, and alternatively activated (M2) macrophages.(25, 86, 87) *C. burnetii* induces macrophages to produce cytokines or express surface receptors typical of both M1 and M2 programs: IL-6 and CCL8 (formerly MCP-2); and TGF-β1, IL-10, mannose receptor, and arginase (Arg-1), respectively.(25, 80, 85) Of note, reactive oxygen species and reactive nitrogen intermediates contribute to the control of *C. burnetii* infection in activated (M1) host macrophages and can be inhibited by *C. burnetii*.(25, 26, 86, 88) Host detection also plays an important role in the predisposition to the

development of chronic Q fever; polymorphisms in the pattern recognition receptors TLR1 and NOD and adaptor protein MyD88 were found to contribute to this risk.(89) The host target cell of *C. burnetii*, the alveolar macrophage, has surface markers and expression patterns associated with alternative activation (M2) compared to other resident macrophages, for instance the M1-activated hepatic Kupffer cells and splenic red pulp macrophages.(90-93) Not suprisingly, monocytes derived from chronic Q fever patients regain an effective microbicidal response following treatment with IFN-γ (shifting towards M1) and bacterial replication is enhanced with IL-10 treatment (shifting to M2).(87, 94) Indeed, changes in macrophage polarization (i.e. plasticity) have been demonstrated during microbial infections and are highly dependent on cytokine signaling and microenvironments with likely are influenced by the inciting pathogen.(25, 81, 95) The mechanisms determining host responses, specifically those linking innate and adaptive immunity, to *C. burnetii* are not fully realized despite decades of research

Models of Q fever

Though *C. burnetii* has been studied extensively since its discovery nearly a century ago, much remains unclear regarding the mechanisms underlying bacterial virulence, host immune response, clinical manifestations, and persistence (or clearance) of this exquisitely evolved pathogen. Various models of Q fever have been developed over the years and include traditional lab animal models and novel approaches including *ex vivo* tissue systems and *Drosophila* studies.(6, 96, 97)

Guinea pigs have been used extensively to study Q fever because they develop a febrile response with clinical signs useful for evaluation of vaccine and therapeutic

effects *in vivo*.(15) Characteristic Q fever lesions found in this model include splenomegaly, granulomatous pneumonitis and bronchointerstitial pneumonia and splenic and hepatic microgranulomas.(15) Furthermore, guinea pigs can develop chronic disease including lesions of the heart valves consistent with endocarditis.(98) Though some inbred mouse strains, like C57BL/6, are considered resistant to *C. burnetii* based on normothermia and healthy appearance, through our and others studies, it is clear that inbred mice can develop similar histologic lesions to human Q fever and guinea pig models.(99-102) Not surprisingly, there are inter-species differences in macrophages and several studies demonstrate that mouse cells are less permissive to *C. burnetii* vacuole development and replication than those from outbred mice; this finding is in part due to induction of higher levels of nitric oxide in mice.(103-105) Amongst inbred mice, there are documented differences in baseline macrophage polarization states (M1/Th1 vs. M2/Th2) thus varied susceptibilities to certain pathogens – specifically C57BL/6 mice are known to be Th1-dominant with robust cell-mediated immunity and IFN-γ production in contrast to the Th2-dominant BALB/c strain with the A/J mouse as an intermediate phenotype.(106, 107) Despite this, mouse (C57BL/6) alveolar macrophages are both susceptible to *C. burnetii* infection and permissive to bacterial growth.(108, 109)

Mouse models have been used to elucidate mechanisms of host detection and responses to *C. burnetii* by taking advantage of their clinical resistance to the avirulent NMII strain, in particular, that is increasingly utilized in Q fever research. Host TLR-signaling during *C. burnetii* infection is complicated as a result of bacterial LPS (phase) variation, with engagement of either TLR4 or TLR2 depending on LPS structure.(11, 102, 104, 110) As such, *in vitro* and *in vivo* studies have produced conflicting results

though, notably, there are differences in infection protocols including route of administration, bacterial phase variants, mouse strains, and cell types used.(25, 102, 104, 111, 112) Nevertheless, in human Q fever patients, polymorphisms in TLRs and adaptor molecules correlate to increased risk of bacterial persistence and chronic disease, which indicates these receptor pathways are important players in Q fever pathogenesis.(89)

In previous *in vivo* mouse models of Q fever, TLR2 and TLR4 were shown to be involved in but dispensable to limiting bacterial growth following intraperitoneal infections.(104) However, TLR2 was more important to limiting growth and dissemination following pulmonary infection.(112) TLR2, TLR4, and the adaptor molecule MyD88 were not required for inflammatory responses to intratracheal treatment with *C. burnetii*, yet infection-induced morbidity was attributed to MyD88 signaling.(113) *In vitro* studies using bone marrow derived macrophages and NMII *C. burnetii* found that TLR2-MYD88-mediated production of TNF was critical to the C57BL/6 mouse's ability to attenuate intracellular growth and clear infection.(16) *In vivo* models of pulmonary NMI *C. burnetii* infection corroborated these findings, further indicating the critical roles of the adaptor protein MyD88 and the pro-inflammatory cytokine TNF in the response to *C. burnetii.* Recent studies utilizing *Drosophila* have corroborated the critical role of TNF during *C. burnetii* infection.(96)

Type I interferon signaling, once thought to be important in the immune response to *C. burnetii*, appears to have a more complex role.(113) *Ifnar*$^{-/-}$ mice were reportedly protected from virulent NMI *C. burnetii*-induced weight loss and bacterial dissemination contrary to the hypothesis that this signaling pathway is important to a successful host response.(113) It was shown that Type 1 interferon actually promoted infection and

bacterial replication within the spleen of *Ifnar*$^{-/-}$ mice when administered peripherally but reduced bacterial burdens locally when instilled in the lung.(113) Type I interferon signaling is critical to immunity to intracellular pathogens and is linked to non-canonical inflammasomes through upregulation of caspase-11.(64) Notably, the only *C. burnetii* T4SS effector protein that engages inflammasomes thus far is IcaA, which attenuates caspase-11-mediated (non-canonical) pore formation by an unknown mechanism.(27)

A plethora of information regarding host immunity to *C. burnetii* has been gained with mouse models.(99, 100, 102, 114) By using clinically resistant C57BL/6 mice and the NMII strain to produce characteristic Q fever histologic lesions following a more natural route of exposure (pulmonary), we have further elucidated immune mechanisms important to Q fever pathogenesis.

Despite a century of research since its discovery, *C. burnetii* intracellular biology and Q fever pathogenesis in both human and animal hosts are not fully understood. As such, current diagnostic and preventative strategies are unsatisfactory and there is no vaccine approved by the Federal Drug Administration (FDA) for prophylaxis in humans or the reservoir ruminant species. Since a massive outbreak in The Netherlands during 2007-2010, Q fever has become a major public health concern in the United States given the lack of a Federal Department of Agriculture (FDA)-approved vaccine for animals and humans, increased incidence of urban farming of livestock host species, and potential bioterrorist attacks. By studying host-pathogen interactions using mouse models, the pathophysiology of Q fever and the evasive mechanisms of *C. burnetii* can be better understood leading to the development of preventative and therapeutic strategies to combat persistent infections during a potential future outbreak in the U.S. and abroad.

Chapter 2.

Experimental Procedures

In vitro Materials and Methods

Cell culture

Primary bone marrow derived macrophages (BMDM) from male and female wild-type (WT) C57BL/6-J (Jackson Laboratories) or knockout (*Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Tlr2/4*$^{-/-}$, *MyD88*$^{-/-}$, *Trif*$^{-/-}$, *Casp1/11*$^{-/-}$, *Nlrp3*$^{-/-}$, and *Nlrc4*$^{-/-}$) mice were cultured in DMEM (Invitrogen) supplemented with 10% FCS, 5 mM HEPES, 0.2 mg/ml L-glutamine, 0.05 mM 2-ME, 50 mg/ml gentamicin sulfate, and 10,000 U/ml penicillin and streptomycin with 30% L cell-conditioned medium. Wild-type mice were originally sourced from Jackson Laboratories. *Casp1/11*$^{-/-}$ mice were a gift from Dr. Craig Roy of Yale University (51) and *Nlrp3*$^{-/-}$ and *Nlrc4* mice were a gift from Dr. Ed Miao of University of North Carolina at Chapel Hill (115). *Tlr4*$^{-/-}$, *Tlr2/4*$^{-/-}$, *MyD88*$^{-/-}$; *Tlr2*$^{-/-}$; and *Trif*$^{-/-}$ mice were provided by the labs of W. Altemeier, A. Haijar, and M. Gale, respectively (U. Washington). Propagated macrophages were then harvested using cold PBS containing 1mM EDTA, suspended in phenol red-free antibiotic free DMEM supplemented with 5% or 10% fetal calf serum (FCS) depending on length of infection or treatment. When indicated, LPS stimulation was achieved by treating macrophages with *Salmonella minnesota* R595 lipopolysaccharide (LPS; 100 ng/ml; List Biologicals) for either 4 hours to approximately 18 hours prior to treatment with inflammasome activators (nigericin) or infections. Experiments measuring IL-1β release in the supernatants, caspase-1 activation or foci formation were performed in the presence of 5 mM glycine to reduce cell lysis.(116)

Bacteria

All *Coxiella* infections were achieved using Nine Mile Phase II (NMII; RSA clone 439) *C. burnetii* [wild-type, mCherry-expressing, or T4SS-deficient (Δ*dotA*)] at multiplicities of infection (MOI) of 20 unless otherwise indicated. Bacteria were diluted in cell media based on genome equivalents (GE) of frozen stocks, added to plate wells, then centrifuged onto cells for 3 minutes at 150 x gravity (g). *Salmonella enterica* serovar Typhimurium infections were performed at MOI 10 for 1 hour following growth in lysogeny broth (LB) overnight at 37°C, dilution of 1:15, and growth for 3 hours in LB containing 0.3 M NaCl. *Yersinia pseudotuberculosis* (*Yptb*; JOEHKM strain lacking flagellin and T3SS effectors)(59) infections were performed at MOI 20 for 90 minutes following growth overnight at 25°C in LB, dilution of 1:40 into LB with 20 mM MgCl2 and 20 mM Na2C2O4, and growth at 25°C for 1 hour and then 37°C for 2 hours. Both *Stm* and *Yptb* were then washed in PBS, quantified using a Beckman Coulter Multisizer 4, then added at pre-determined MOI, and spun onto macrophages for 3 minutes at 150 x g.

Reagents & Assays

To induce NAIP5/NLRC4 inflammasomes, cells were infected with *Stm*. For NLRP3-specific caspase-1 activation, 10 μM nigericin (Sigma-Aldrich) and *Yptb* were used. Cytotoxicity was assessed by measuring lactate dehydrogenase (LDH) release into cell supernatants (CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega). IL-1β-secretion was measured in cell supernatants using a sandwich ELISA assay (DuoSet®, R&D Systems®) following filtration by 0.22-mm filter (Corning).

Immunocytochemistry, Caspase-1 Detection, and Microscopy

To evaluate inflammasome foci *in vitro*, mouse macrophages were seeded onto 12mm coverslips within 24-well plates (Corning). Following infections and treatments, cells were washed with 0.1% Bovine Serum Albumin (BSA) in PBS and then fixed with 4% paraformaldehyde or washed, fixed, and permeabilized using the Cytofix/Cytoperm™ kit (BD Biosciences). Primary antibodies used were NLRP3 (Santa Cruz; sc-66846) and ASC (Millipore; 04-147) with species-targeted fluorescent secondary antibodies (AlexaFluor®). To evaluate the presence of active caspase-1 in cells, the FAM-YVAD-FMK caspase-1 detection assay (FLICA™, ImmunoChemistry Technologies, LLC) was used according to manufacturer's instructions. Nuclear visualization was achieved through Hoescht staining. All cells were similarly washed; coverslips mounted on slides, sealed, and then visualized using a Leica SL Confocal microscope or DeltaVision Elite Microscopy Imaging System (GE Healthcare). At least 10 separate high power (400X) fields were evaluated to determine presence of NLRP3, ASC, or active caspase-1 (FLICA) foci. Images of representative fields were captured at 400X or 600X magnification and analyzed using ImageJ.(1.47v, National Institutes of Health).

Immunoblot analysis

Macrophages were lysed with 1x SDS sample buffer (Invitrogen) and then eluted with boiling at 100°C for 10 minutes. Lysates were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane, and detected by the indicated antibodies, Immobilon chemiluminescence system (Millipore), and Odyssey (Li-Cor). Primary

antibodies used included NLRP3 (Cell Signaling; D4D8T), pro-IL-1β (R&D Systems; AF-401-NA), NLRC4 (Millipore; 06-1125), and actin (Abcam; ab6276). Target protein relative fluorescent units (RFU) were normalized to actin.

Statistical Analysis

GraphPad Prism was used to calculate statistical significance by one- or two-way ANOVA (*P <0.05, **P < 0.01, ***P < 0.001) with Bonferonni post-tests, unless otherwise indicated. Data from *in vitro* experiments are representative of at least three independent experiments.

In vivo **Materials and Methods**

Mice

Wild-type and knockout (*Casp1/11*$^{-/-}$, *Nlrp3*$^{-/-}$, *Tlr4*$^{-/-}$, *MyD88*$^{-/-}$) C57BL/6 mice were bred and housed at University of Washington School of Medicine. Wild-type mice were originally sourced from Jackson Laboratories. *Casp1/11*$^{-/-}$ mice were a gift from Dr. Craig Roy of Yale University (51) and *Nlrp3*$^{-/-}$ mice were a gift from Dr. Ed Miao of University of North Carolina at Chapel Hill.(115) *Tlr4*$^{-/-}$ and *MyD88*$^{-/-}$ mice were provided by the lab of Dr. William Altemeier. All experiments were performed in accordance with protocols approved by the UW Institutional Animal Care and Use Committee (IACUC). During infections, mice were housed in the UW South Lake Union Vivarium ABSL2 facility with the following room environmental parameters: room temperature was held at a range of 68 to 79°F with the goal of 72° F and an acceptable temperature variation of no more than a 4°F over a 24-hour period. The acceptable room

humidity range was 30 to 70%. Room lighting was programed for 14 hours of light and 10 hours of dark. Mice were housed in HEPA filtered ventilated shoe box cages and the ventilation in the animal housing room was at least 10 to 15 fresh air changes per hour per room. All mice were provided environmental enrichment, fed rodent diet *ad libitum* and had free access to fresh water at all times. Daily health checks were performed by the UW Husbandry Staff whom are overseen by board certified lab animal veterinarians.

Bacteria

All *Coxiella burnetii* pulmonary infections were achieved using avirulent Nine Mile Phase II (NMII; RSA clone 439) suspended in 50 μl PBS. Each mouse was infected with $1x10^7$ bacteria.

Pulmonary infections

Twelve to sixteen week old male and female mice (n = 4-8 per genotype per time point; approximately 50% male and 50% female) were infected with Nine Mile Phase II (NMII; RSA clone 439) *Coxiella burnetii* ($1x10^7$ bacteria) suspended in 50 μl phosphate buffered saline (PBS) via oropharyngeal instillation under isoflurane anesthesia. Control mice (n = 4-6 per genotype; approximately 50% male and 50% female) were instilled with PBS only. Mice were monitored for clinical signs such as lethargy, hunched posture, ruffled fur, or increased respiratory rate and effort, throughout the experiment. Mice were weighed at 3, 7, 14, and 21 days post infection and those at endpoint were sacrificed via isoflurane anesthesia and exsanguination via cardiocentesis. The left bronchus was tied

off using monofilament suture, and the left lung removed and frozen at -80°C. Spleens were weighed and compared to body weight; most of the spleen was frozen at -80°C.

Histopathology

The remaining right lung was instilled at 20 mmHg pressure and immersion fixed in 10% neutral buffered formalin (NBF). A portion of the spleen and liver were immersion fixed in 10% NBF. Following fixation of lung, hepatic, and splenic tissues, tissues were trimmed then routinely processed, embedded, and sectioned at 5 μm prior to hematoxylin and eosin staining. Slides were cover-slipped and evaluated for histologic changes by a ACVP board certified veterinary pathologist (M. A. Delaney).

Immunohistochemical staining and quantitative microscopy

Polyclonal antibodies to *C. burnetii* NMII (gift from Robert Heinzen) and F4/80 clone BM8 (Invitrogen, MFF48000) were applied to lung tissue sections at 1:1000 and 1:200, respectively, and detected using 3,3'-Diaminobenzidine (DAB) chromogen in a Leica Bond-Max Automated Immunostainer. Following counterstaining with hematoxylin and coverslipping, the slides were scanned in brightfield at 200X magnification using the Hamamatsu NanoZoomer Digital Pathology System. The digital images were then imported into Visiopharm® software for quantitative analysis. Using the Visiopharm® Image Analysis module, regions of interest (ROIs) were manually drawn around lung and spleen in each sample. The software converted the initial digital image into grayscale values using two features, RGB-B and HDAB - DAB. Visiopharm® software was then trained to label positive *C. burnetii* (NMII) or F4/80 staining and the hematoxylin

counterstain using a threshold of pixel values. All ROIs were processed in batch mode using this configuration to generate the desired outputs as a ratio of positive staining over total area (ROI) evaluated.

Statistical Analysis

GraphPad Prism was used to calculate statistical significance by one- or two-way ANOVA (*P <0.05, **P < 0.01,***P < 0.001) with Bonferonni post-tests, unless otherwise indicated. The PBS control group and each infection group were composed of at least 4 mice including 2 from each sex.

Chapter 3.

Investigating interactions between *C. burnetii* and inflammasomes *in vitro*

Results

Nine Mile Phase II (NMII) *C. burnetii* develops a permissive infection in C57BL/6 bone marrow derived macrophages up to 72 hours *in vitro*.

To establish our *in vitro* model, we first verified that *C. burnetii* is able to develop an infection in bone marrow derived macrophages (BMDM) from wild-type C57BL/6 mice. Macrophages were infected with avirulent Nine Mile Phase II (NMII) mCherry-expressing *C. burnetii* at multiplicities of infection (MOI) of 10, 20, and 50. After 24, 48, and 72 hours of infection, NMII *C. burnetii* were detected within BMDM by microscopy (Fig. 1.1). Infection at MOI 20 for 24 hours was identified as the infectious dose and time point for further studies as most macrophages contained mCherry-expressing bacteria and following 72 hours of infection; a large percentage of macrophages had aggregates of intracellular bacteria indicative of a successful infection and bacterial replication (Fig. 1.1).

Nine Mile Phase II (NMII) *C. burnetii* does not induce caspase-1 activation, IL-1β secretion, and pyroptosis *in vitro*.

To determine if *C. burnetii* activates caspase-1 in our *in vitro* model, BMDM from male and female wild-type C57BL/6 mice were infected with avirulent Nine Mile Phase II (hereafter, NMII) at a multiplicity of infection of 20 (MOI 20). Following 24, 48, and 72 hours of infection, caspase-1 activation was determined by the presence of

bright green fluorescent foci using FAM-YVAD-FMK caspase-1 detection assay (Fig. 1.2 A, B). For a positive control in all studies, robust caspase-1 activation was achieved by infecting LPS-primed (18h) BMDM with *Salmonella enterica* serovar Typhimurium (*Stm)* at a MOI 10 for 1 hour to stimulate the NLRC4 inflammasome or treating with nigericin (10 uM) for 30 minutes following 4 hours of LPS-priming to induce NLRP3 inflammasome activation (Fig. 1.2 A, B). For a negative control, LPS-primed BMDM were left untreated or uninfected. In contrast to *Stm*-infected and nigericin-treated macrophages, in which a large percentage contained discrete green fluorescent cytoplasmic (active capase-1) foci accompanied by nuclear condensation (Fig. 1.2 A), NMII-infected cells and uninfected macrophages had similar findings with no discrete cytoplasmic foci and normal nuclear detail with discernable heterochromatin and nucleoli throughout all time points indicating viable cells with lack of caspase-1 activation (Fig. 1.2 A, B).

Filtered cell supernatants were applied to a sandwich ELISA to quantify IL-1β secretion of uninfected, infected, and treated cells. *Stm*-infected and nigericin-treated macrophages were utilized as positive controls for intact NLRC4 and NLRP3 inflammasome pathways, respectively, within the propagated macrophages *in vitro*. As expected, high concentrations of IL-1β were detected in supernatants of the positive control macrophages with negligible levels found secreted from those infected with NMII (Fig. 1.2 C).

Cytotoxicity of *Stm*-infected (NLRC4), nigericin-treated (NLRP3), and NMII-infected macrophages was assessed by measuring lactate dehydrogenase (LDH) release into cell supernatants. As expected, *Stm*-infected and nigericin-treated macrophages

released large amounts of LDH into cell supernatants indicating a high percentage of cell death (Fig. 1.2 D). NMII-infected cells demonstrated minimal cell death at 24 hours post-infection (Fig. 1.2 D), which persisted throughout 72 hours of infection (data not shown).

Together these results demonstrate that NMII *C. burnetii* infection in mouse BMDM does not result in caspase-1 activation, IL-1β secretion, or cell death during the first 72 hours *in vitro.*

NMII *C. burnetii* infection does induce NLRP3-ASC foci formation *in vitro*.

To elucidate if NMII *C. burnetii* are detected by NLRP3 within the cytosol leading to formation of NLRP3 and ASC foci, BMDM from wild-type C57BL/6 mice were infected with NMII (MOI 20 for 24h) and then labeled with antibodies targeting NLRP3 and ASC. In addition, FLICA™ assay was used to detect active caspase-1. As positive and negative controls, BMDM were treated with nigericin as previously described or left uninfected, respectively. As expected, BMDM treated with nigericin had prominent ASC foci that co-localized with NLRP3 and active caspase-1 confirming cytosolic detection (i.e. signal 2), inflammasome formation, and caspase-1 activation (Fig. 1.3). Macrophages with inflammasome foci (NLRP3-ASC-caspase-1) often had condensed nuclei representing pyroptosis (Fig. 1.3). Like uninfected macrophages (Fig. 2A), NMII-infected macrophages had no evident NLRP3 or ASC foci indicating a lack of cytosolic detection (Fig. 1.3). Consistent with previous results, NMII-infected macrophages contained no active caspase-1 foci and had normal nuclei as for uninfected cells (Fig. 1.3).

These findings demonstrate that NMII does not induce NLRP3-ASC foci formation, signifying that neither these bacteria nor their products are detected by NLRP3 within the cytosol. By avoiding cytosolic detection by NLRP3, there is deficient signal 2 and no recruitment, cleavage, or activation caspase-1, and no subsequent pyroptosis or IL-1β secretion.

NMII *C. burnetii* is unable to inhibit and instead potentiates caspase-1-mediated cell death and IL-1β secretion in the presence of potent inflammasome agonists.

Previous studies show that the intracellular pathogen, *Yersinia pestis* produces and secretes (T3SS) a protein, YopM, that binds caspase-1 and blocks inflammasome formation (66). To test if NMII is able to inhibit or attenuate caspase-1 activation in the presence of potent inflammasome stimuli, naïve BMDM were pre-infected with NMII (MOI 20 for 24 hours) and then infected with *Stm* (MOI 10 for 1 hour) to induce NLRC4/IPAF inflammasomes or infected with *Yersinia pseudotuberculosis* (*Yptb;* MOI 20 for 1.5 hours) to induce NLRP3 inflammasomes. In addition, nigericin (10 μM for 30 minutes; a bacterial toxin and specific NLRP3 agonist) was used to induce NLRP3 activation following 4 hours of LPS treatment. For negative controls, BMDM were left uninfected or untreated. To confirm that *Stm*-, *Yptb*-, and nigericin-induced cell death and IL-1β secretion are caspase-1-mediated, BMDM form C57BL/6 mice deficient in caspase-1 (*Casp1/11*$^{-/-}$) were utilized in parallel experiments.

As expected, BMDM infected with *Stm* and *Yptb* and those treated with nigericin had large amounts of LDH released into supernatants (i.e. cytotoxicity) and high levels of IL-1β secretion when compared to uninfected and NMII-infected cells (Fig. 1.4)

Surprisingly, wild-type BMDM that were NMII-infected prior to *Stm* or *Yptb* infection or nigericin treatment had significantly higher cytotoxicity than from those macrophages infected with only *Yptb* or treated with nigericin alone (Fig. 1.4 A, C, E). As expected, all *Casp1/11*$^{-/-}$ BMDM had minimal or no measurable cytotoxicity (Fig. 1.4 A, C, E). Similarly, uninfected BMDM and those infected with only NMII had negligible or undetectable levels of IL-1β in the supernatants (Fig. 1.4 B, D, F). Pre-infection with NMII resulted in significantly higher IL-1β secretion from cells subsequently infected with *Stm* and *Yptb* or treated with nigericin (Fig. 1.4 B, D, F). *Casp1/11*$^{-/-}$ cells had minimal or no measurable IL-1β secretion (Fig. 1.4 B, D, F).

These data show that cell death (i.e. pyroptosis) and IL-1β secretion during *Stm* and *Yptb* infection and following nigericin-treatment are mediated by caspase-1 and that rather than attenuating inflammasome formation, pre-infection with NMII *C. burnetii* potentiates caspase-1-mediated (inflammasome) processes *in vitro*. This confirms that NMII *C. burnetii* is unable to inhibit or attenuate activation of either the NLRC4 or NLRP3 inflammasomes, thus caspase-1 in the presence of canonical stimuli. Importantly, NMII *C. burnetii* potentiated caspase-1 activation induced by three different inflammasome agonists, thus it appears NMII *C. burnetii* does not act directly upon caspase-1.

Potentiation by NMII *C. burnetii* of caspase-1-mediated cell death is not seen in LPS-primed macrophages.

To determine the mechanism for potentiation of inflammasomes by NMII, cytotoxicity was measured following standard infection protocols with the addition of

LPS treatment of BMDM for approximately 18 hours before infections with *Stm* and *Yptb*. In LPS-stimulated BMDM, the potentiation of pyroptosis was abolished as similar cytotoxicity was seen in BMDM left uninfected or pre-infected with NMII *C. burnetii* (Fig. 1.5 A, B). As expected, IL-1β secretion was significantly increased from cells that were stimulated with LPS prior to infection with *Stm* or *Yptb* when compared to naïve BMDM (Fig. 1.5 C, D). However, potentiation by NMII *C. burnetii* is still evident as there were significant increases in IL-1β secretion from BMDM pre-infected with NMII even after LPS stimulation (Fig. 1.5 C, D).

Together these data suggest NMII *C. burnetii,* as a Gram-negative bacterium, functions as a source of LPS to prime NLRP3 inflammasomes resulting in a potentiation of the downstream effects: pyroptosis and IL-1β secretion, following activation by potent inflammasome agonists. The potentiation of cytotoxicity is lost in LPS-stimulated BMDM. However in the case of IL-1β secretion, NMII *C. burnetii* maintains an effect with slightly, albeit significantly higher IL-1β levels in pre-infected BMDM. Interestingly, *C. burnetii* was also able to potentiate *Stm*-induced caspase-1 activation through NLRC4 inflammasomes. Though not required for rapid activation, LPS may enhance NLRC4-mediated effects. We wondered if NMII *C. burnetii* was able to upregulate both NLRP3 and pro-form of IL-1β and how it compared to treatment with LPS. Furthermore, we wanted to determine any effects of *C. burnetii* infection on NLRC4.

NMII *C. burnetii* primes NLRP3 inflammasomes.

In order to confirm that NMII *C. burnetii* primes NLRP3 inflammasomes, BMDM from wild-type mice were infected with NMII *C. burnetii* as previously described or left uninfected. During the last 4 hours of infection, BMDM were either treated with LPS (+) or left untreated (-). Cell lysates were separated by SDS-PAGE and probed for NLRP3, NLRC4, and pro-form of IL-1β and normalized to β-actin. At 24 hours of infection, NMII-infected BMDM lysates contained increased NLRP3 protein levels compared to uninfected cells with and without LPS treatment (Fig. 1.6 A, B). LPS treatment of BMDM prior to NMII *C. burnetii* infection also resulted in increased NLRP3 protein levels. Pro-IL-1β proteins were not detected in uninfected BMDM. With LPS treatment, there was a robust increase in pro-IL-1β in both uninfected and NMII-infected BMDM (Fig. 1.6 A, C). There appeared to be an additive effect with higher protein levels in BMDM infected with NMII and subsequently treated with LPS (Fig. 1.6 A, C). There was also an evident increase in pro-IL-1β following NMII infection alone (Fig. 1.6 A, C). Next, we probed for NLRC4 and found that *C. burnetii* and LPS induced mild increases in NLRC4 compared to uninfected, naïve BMDM. (Fig. 1.6 D, E). These data demonstrate that NMII *C. burnetii* are able to prime (i.e. provide signal 1 to) NLRP3 inflammasomes during early infection with robust upregulation of NLRP3 and less robust upregulation of pro-IL-1β in the absence of LPS.

Upregulation of NLRP3 by NMII *C. burnetii* is dependent on TLR2-MYD88 and independent of TLR4 and TRIF signaling.

To elucidate signaling mechanisms of NLRP3 inflammasome priming by *C. burnetii*, BMDM from mice deficient in TLR4, TLR2, TLR2 and 4, and the adaptor

molecules, MyD88 and TRIF were infected and NLRP3 levels were measured by immunoblotting and compared to wild-type (WT) cells as previously described. NLRP3 levels in NMII-infected *Tlr2*$^{-/-}$, *Tlr2/4*$^{-/-}$ and *MyD88*$^{-/-}$ BMDM were markedly reduced compared to WT macrophages indicating TLR2 and MyD88 are required for NLRP3 priming by NMII *C. burnetii* (Fig. 1.7 A, B). Furthermore, NLRP3 levels of infected macrophages from *Tlr4*$^{-/-}$ and *Trif*$^{-/-}$ mice were equivocal to those seen in WT macrophages, suggesting that these molecules are dispensable for NLRP3 priming by NMII *C. burnetii* (Fig. 1.7 A, B).

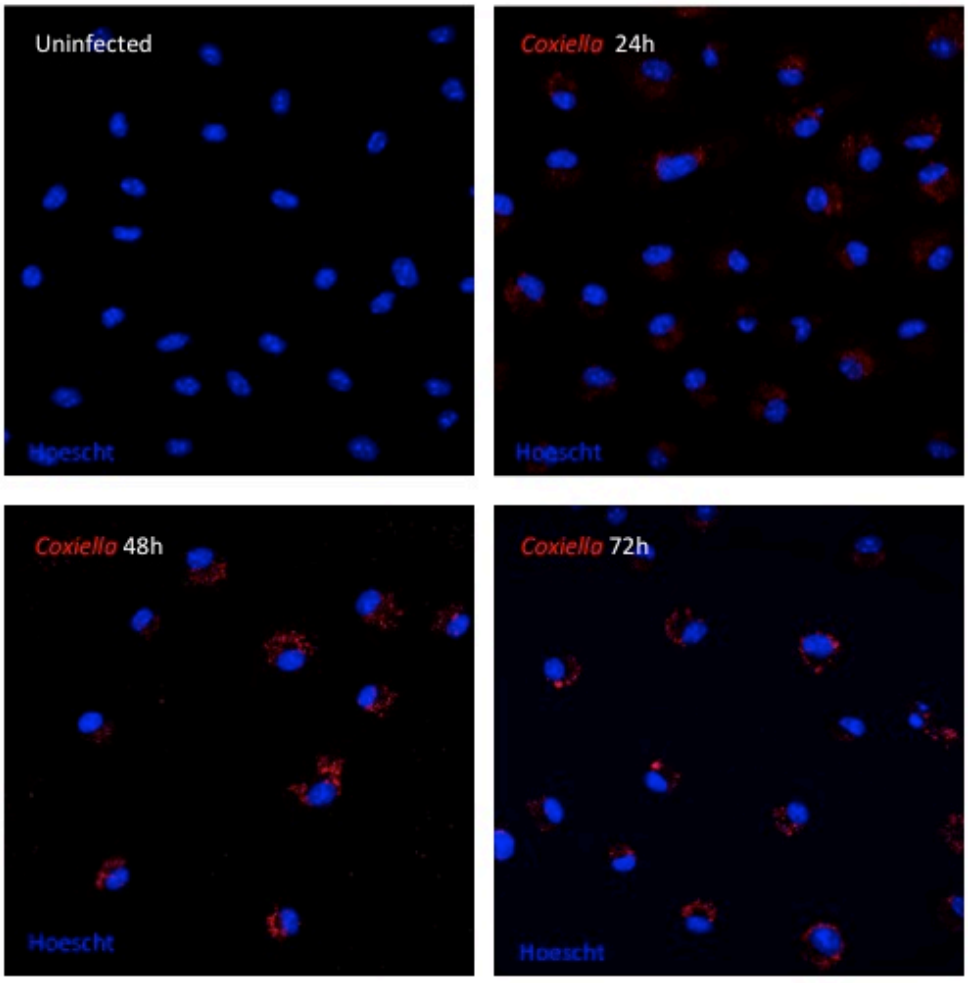
NMII *C. burnetii* mutants lacking the Type IV Secretion System (Δ*dotA*) do not cause cell death or IL-1β secretion but prime and potentiate NLRP3 inflammasomes.

In order to determine if the type IV secretion (T4SS) of *C. burnetii* affects priming of NLRP3 inflammasomes, caspase-1 activation or its downstream effects, BMDM from wild-type mice were infected with mutant NMII lacking the T4SS (Δ*dotA*) or wild-type NMII *C. burnetii* (*Cb*) or left uninfected (UI). For NLRP3 priming experiments, BMDM were either treated with LPS (+) or left untreated (-) for the last 4 hours. Cell lysates were separated by SDS-PAGE and probed for NLRP3 and pro-IL-1β. Compared to uninfected BMDM, those infected with wild-type NMII *C. burnetii (Cb)* and Δ*dotA C. burnetii* had similarly robust upregulation of NLRP3 regardless of LPS stimulation (Fig. 1.8 A, B). Pro-IL-1β had more modest increases induced by Δ*dotA* compared to NLRP3 as for wild-type NMII (Fig. 1.8 A, C). In addition, cytotoxicity and IL-1β secretion were assessed using nigericin (10 μM) treatment as a positive control and

as a NLRP3 agonist to determine if: a) Δ*dotA* NMII induces pyroptosis and IL-1β secretion; b) Δ*dotA* NMII potentiates these caspase-1-mediated effects as for wild-type NMII *C. burnetii*. BMDM infected with Δ*dotA* did not exhibit cytotoxicity or secrete IL-1β (Fig. 1.8 D, E). Furthermore, pre-infection with Δ*dotA* resulted in significantly higher pyroptosis and IL-1β secretion (Fig. 1.8 D, E); thus indicating that *C. burnetii's* T4SS is not required for priming of or avoidance of cytosolic detection by NLRP3 inflammasomes.

Figures

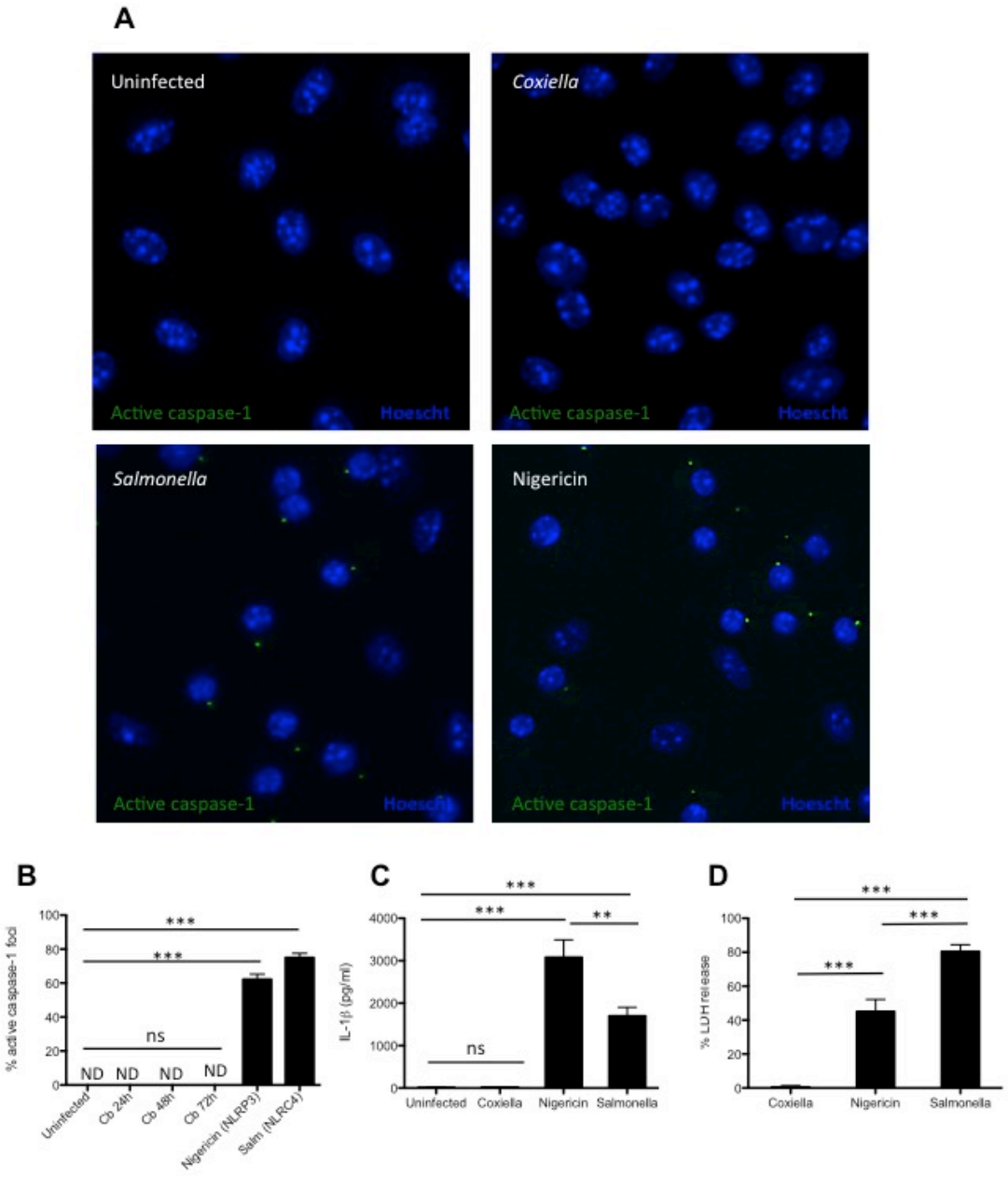
Figure 1.1.



NMII *C. burnetii* develops a permissive infection in mouse bone marrow derived macrophages up to 72 hr *in vitro.*

Bone marrow derived macrophages (BMDM) from wild-type C57BL/6 mice were infected with mCherry-expressing NMII *C. burnetii* at MOI 20 following 18 hr of LPS stimulation. At all time points, a large percentage of NMII-infected macrophages had evident intracellular bacteria (red) with increasing amounts noted at 48h and 72h post infection. Data is representative of at least 3 individual experiments.

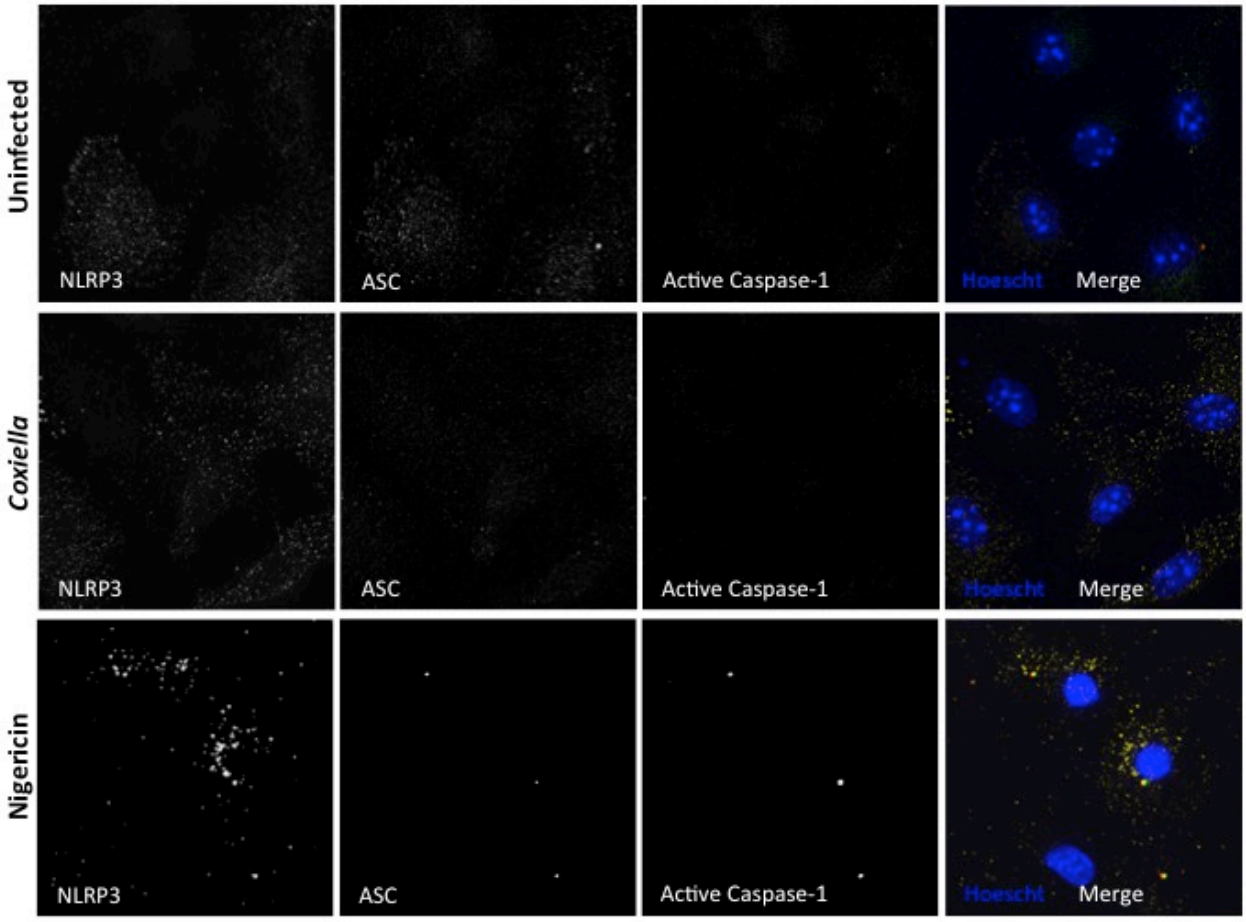
Figure 1.2.



NMII *C. burnetii* does not induce caspase-1 activation, IL-1β secretion, or cell death in mouse macrophages. Mouse BMDM were infected with NMII (*Coxiella*, MOI 20, 24 hr*)* or *Salmonella (Stm)*, treated with nigericin, or left uninfected. **(A)** Caspase-1 activation, specifically foci formation, was quantified using a caspase-1 detection assay.

Coxiella-infected and uninfected macrophages do not exhibit active caspase-1 foci within the cytoplasm in contrast to nigericin-treated and *Stm*-infected BMDM, many of which contain intense green foci within the cytoplasm, often adjacent to a condensed (pyroptotic) nucleus. **(B)** BMDM containing active caspase-1 foci were quantified and percentages calculated. Large percentages of nigericin-treated and *Stm*-infected BMDM contain active caspase-1 foci whereas uninfected and *Coxiella*-infected BMDM have no evident foci. **(C)** Uninfected and *Coxiella*-infected secreted negligible levels of IL-1β, in contrast, nigericin-treated and *Stm*-infected BMDM, which secreted large amounts of IL-1β. **(D)** Lactate dehydrogenase levels from BMDM were also measured to determine cytotoxicity. Compared to spontaneous cell death of uninfected macrophages, nigericin-treated and *Stm*-infected BMDM had increased cytotoxicity. NMII-infected BMDM had zero to minimal cytotoxicity. Data represents at least 3 individual experiments. One-way ANOVA with Bonferroni's Multiple Comparison Test, (***) $p < 0.0001$. ND = not detected.

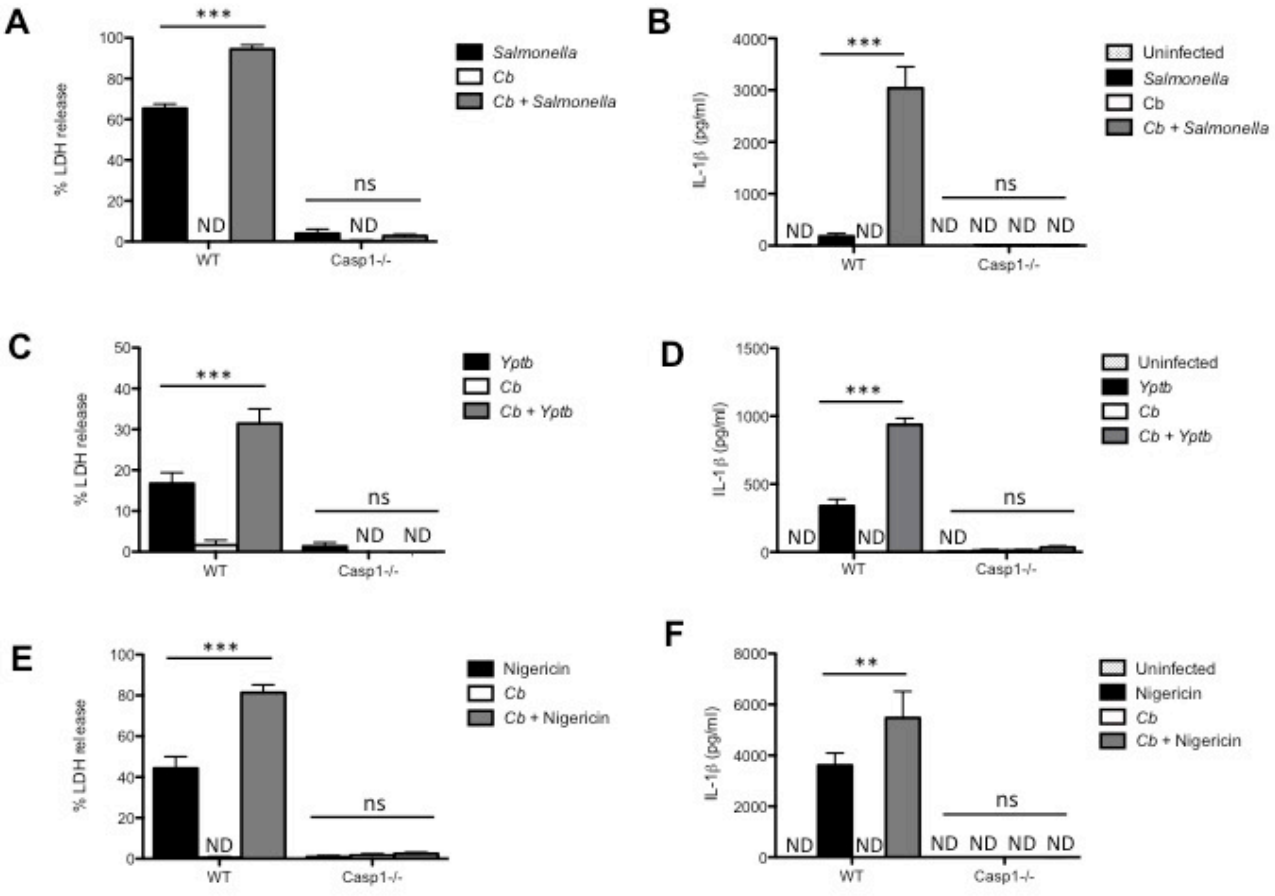
Figure 1.3.



NMII *C. burnetii* does not induce NLRP3:ASC foci formation or caspase-1 activation in contrast to macrophages treated with NLRP3 agonist nigericin.

Uninfected, *Coxiella*-infected, and nigericin-treated BMDM were labeled with antibodies targeting the cytosolic sensor NLRP3 and the adaptor protein ASC in addition to staining with FAM-YVAD-FMK (FLICA™, active caspase-1) and Hoescht (DNA, blue). Nigericin-treated macrophages have aggregates of NLRP3 with multifocal co-localization with both ASC and active caspase-1, with condensed (pyroptotic) nuclei, representing inflammasome formation. In contrast, uninfected and *Coxiella*-infected BMDM have no NLRP3, ASC, or active caspase-1 staining.

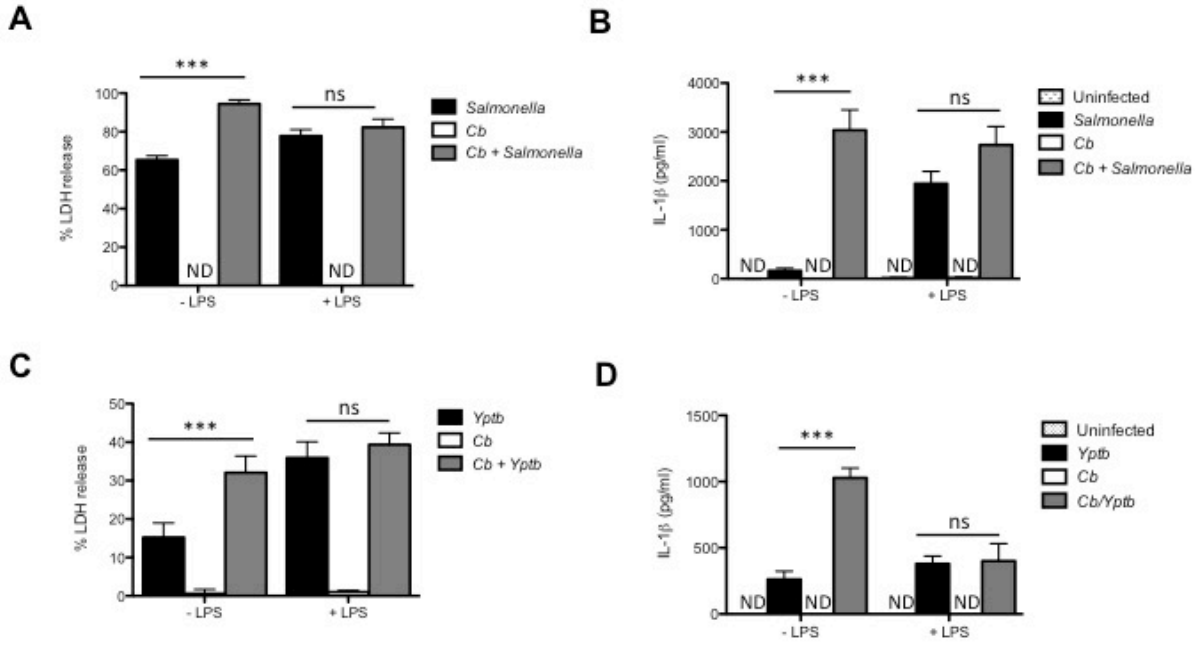
Figure 1.4.



NMII *C. burnetii* does not inhibit and instead potentiates caspase-1-mediated cell death and IL-1β secretion induced by potent inflammasome agonists. (A, C, E) Wild-type mouse BMDM were pre-infected with NMII (gray bars) and then infected with *Stm* or *Yptb* or treated with nigericin. NMII-pre-infected BMDM had increased cytotoxicity compared to BMDM infected with *Stm* or *Yptb* or treated with nigericin alone (black bars). No cytotoxicity is detected in NMII-infected BMDM. Minimal or no cytotoxicity is detected in *Stm*- or *Yptb*-infected or nigericin-treated BMDM from *Casp1/11*$^{-/-}$ mice. **(B, D, F)** IL-1β secretion is significantly elevated in *Stm-, Yptb*-infected, and nigericin-treated BMDM pre-infected with NMII (gray bars) compared to BMDM only infected

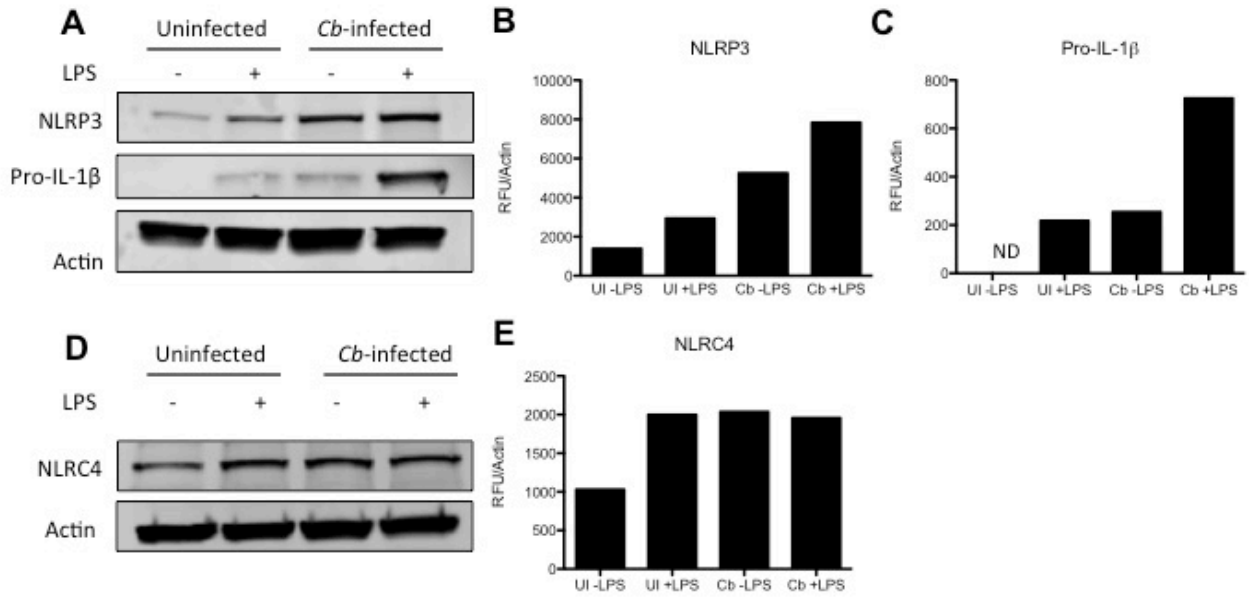
with *Stm* or *Yptb* or treated with nigericin (black bars). No IL-1β secretion is detected in uninfected and NMII-infected BMDM (white bars) or in BMDM from *Casp1/11*$^{-/-}$ mice. Data represents at least 3 individual experiments. Two-way ANOVA with Bonferroni's Multiple Comparison Test, (**) $p<0.001$, (***) $p<0.0001$, ns = not significant, ND = not detected.

Figure. 1.5.



Potentiation by NMII *C. burnetii* of caspase-1-mediated cell death is lost or reduced with LPS stimulation. Compared to naïve BMDM (- LPS) BMDM treated with LPS (+ LPS) during pre-infection with NMII (gray bars) have lost and reduced potentiation of cytotoxicity and IL-1β secretion, respectively. Data represents at least 3 individual experiments. Two-way ANOVA with Bonferroni's Multiple Comparison Test, (*) $p < 0.01$, (**) $p < 0.001$, (***) $p < 0.0001$. ND = not detected

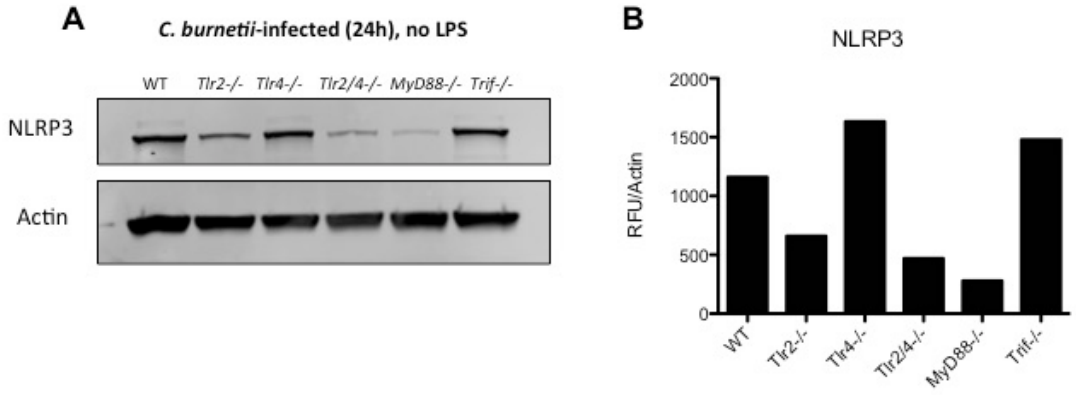
Figure 1.6.



NMII *C. burnetii* upregulates NLRP3 and pro-form of IL-1β in the absence of LPS. NLRP3 inflammasome priming was demonstrated by infecting wild-type mouse BMDM with NMII *C. burnetii* (MOI 20, 24 hr) and compared to uninfected (UI) BMDM. During the last 4 hr of infection, BMDM were treated with LPS (100ng/ml; +) or left untreated (-). Protein levels of NLRP3 and pro-IL-1β were evaluated by immunoblot, quantified by densitometry, and normalized to actin. **(A)** *Coxiella* (*Cb;* NMII)-infected BMDM lysates contain increased NLRP3 and pro-IL-1β levels in the absence of LPS stimulation compared to uninfected, naïve BMDM and uninfected, LPS-stimulated (UI, +) BMDM. **(B)** Actin normalization highlights the increased NLRP3 levels seen in *Cb*-infected BMDM subsequently treated with LPS. **(C)** *Cb*-infected BMDM lysates also have slightly increased levels of pro-IL-1β compared to uninfected BMDM. However, LPS-treated BMDM have much higher protein levels comparatively. **(D, E)** In contrast, NLRC4 levels were overall lower than NLRP3 and similar amongst LPS-treated and

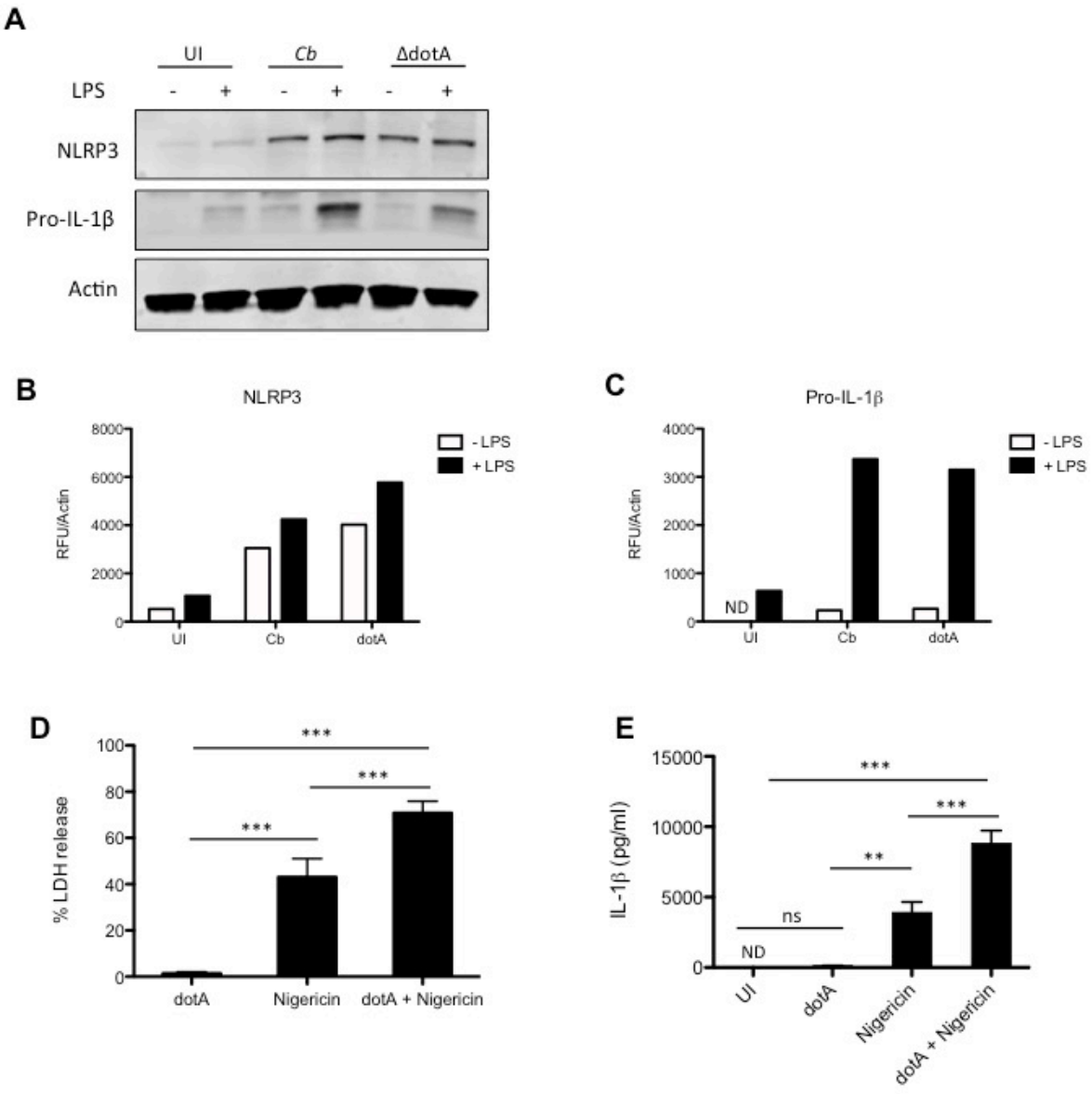
NMII-infected BMDM but higher than uninfected, naïve BMDM. ND = not detected. Immunoblots and graphs are representative of at least 2 individual experiments.

Figure 1.7.



NMII *C. burnetii* upregulation of NLRP3 requires TLR2 and MyD88 signaling. BMDM from wild-type (WT) and *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Tlr2/4*$^{-/-}$, *MyD88*$^{-/-}$, and *Trif*$^{/-}$ mice were left uninfected or infected with NMII *C. burnetii* (MOI 20, 24 hr). Lysates were separated by SDS-PAGE and probed for NLRP3 and actin. **(A)** In NMII-infected BMDM, there is a marked reduction in NLRP3 levels in *Tlr2*$^{-/-}$ and *MyD88*$^{-/-}$ BMDM when compared to WT BMDM. *Tlr4*$^{-/-}$ and *Trif*$^{/-}$ BMDM lysates are similar to WT BMDM levels. **(B)** Representative densitometry and actin normalization highlight these differences, indicating MyD88 and TLR2 are involved in NLRP3 priming by NMII *C. burnetii*. Immunoblots and graphs are representative of at least 2 individual experiments.

Figure 1.8.



T4SS-deficient NMII *C. burnetii* (ΔdotA) upregulates NLRP3 and potentiates downstream effects but does not itself induce cell death or IL-1β secretion.

Wild-type BMDM were left uninfected (UI) or infected with wild-type NMII *C. burnetii* (*Cb)* or with a NMII mutant lacking T4SS (Δ*dotA*) and then left untreated (-) or treated with LPS (+). Lysates were separated by SDS-PAGE and probed for NLRP3, pro-IL-1β,

and actin. **(A)** BMDM infected with Δ*dotA* have similar NLRP3 and pro-IL-1β levels compared to wild-type NMII. With the addition of LPS, NLRP3 levels are increased in uninfected, NMII-infected, and Δ*dotA*-infected BMDM. In contrast, BMDM infected with NMII or Δ*dotA* have slightly increased levels of pro-IL-1β compared to uninfected BMDM, but less than uninfected BMDM treated with LPS. **(B)** Actin normalization of NLRP3 levels demonstrates the increases seen upon NMII infection (white bars) and the additive effect of LPS treatment (black bars). **(C)** Actin normalization of pro-IL-1β levels highlights the marked increases in this protein following LPS treatment (black bars) and comparatively, the less robust regulation induced by NMII and Δ*dotA* infections alone (white bars). Blots and graphs are representative of at least 2 experiments. **(D, E)** Wild-type BMDM were left uninfected or infected with Δ*dotA* and subsequently treated with nigericin, then cytotoxicity and IL-1β secretion were measured as previously described. BMDM infected with Δ*dotA* have minimal cytotoxicity **(D)** and IL-1β secretion **(E)**. BMDM pre-infected with Δ*dotA* and then treated with nigericin have significantly increased cytotoxicity and IL-1β secretion compared to BMDM treated with nigericin alone. Data represents at least 3 individual experiments. Two-way ANOVA with Bonferroni's Multiple Comparison Test, (*) $p<0.01$, (**) $p<0.001$, (***) $p<0.0001$, ND = not detected.

Discussion

Recent epizootics in ruminant reservoirs and human populations have rejuvenated research efforts into the molecular mechanisms underlying the complicated and dynamic host-pathogen interactions that occur during Q fever.(2, 117) Here, we examined the interactions between a conserved innate defense mechanism, the inflammasome, and the highly adapted obligate intracellular bacterium, *Coxiella burnetii*.

We first established an *in vitro* model using the clinically resistant C57BL/6 mouse as a source for bone marrow derived macrophages (BMDM), a cell type considered less permissive to NMII *C. burnetii.* In our hands, we demonstrated productive infection up to 72 hours *in vitro*. During this time frame, we saw no evidence of cell death, caspase-1 activation, or substantial IL-1β secretion. We showed that NMII *C. burnetii* does not induce caspase-1 activation or IL-1β secretion in mouse BMDM similar to recent studies.(16, 27) Since *C. burnetii* lacks a gene encoding flagellin, but has a T4SS that secretes bacterial proteins and products into the cytosol, we turned our focus to the NLRP3 inflammasome. We hypothesized that *C. burnetii* avoids detection by the receptors involved in the NLRP3 inflammasome, which include those at the cell membrane (TLRs) and cytosolic sensors, specifically NLRP3, and adaptor protein, ASC.

For the first time, we demonstrated that *C. burnetii* is not detected in the host cytosol by NLRP3 due to lack of NLRP:ASC foci formation, indicating lack of signal 1. Without NLRP3 sensing and subsequent ASC foci formation, there is no recruitment and activation of caspase-1 or the downstream effects of pyroptosis and IL-1β secretion. Thus, the activation of NLRP3 by sensing of a PAMP or DAMP (signal 2) during *C. burnetii* infection of BMDM is either avoided or actively inhibited.

To determine if NMII *C. burnetii* can actively inhibit or attenuate inflammasomes, we used potent agonists of the NLRP3 and NLRC4 inflammasomes in BMDM pre-infected with NMII. Surprisingly, we found that NMII was unable to attenuate inflammasomes and instead NMII-infected BMDM had higher cytotoxicity and IL-1β secretion. This inability to attenuate inflammasomes corroborates results from previous studies utilizing different agonists including *Legionella pneumophila* for induction of NLRC4 inflammasomes.(16, 27) It has also been demonstrated that *C. burnetii* NMII is unable to inhibit the AIM2 inflammasome induced by dsDNA.(27) Collectively, these data indicate that *C. burnetii* does not inhibit inflammasomes at the level of the effector protease, caspase-1, or downstream from caspase-1 activation. By using different inflammasome stimuli and a lower infectious dose of *C. burnetii*, we were able to uncover host-pathogen interactions missed by previous studies. The novel finding of NMII *C. burnetii*'s potentiation of inflammasomes warranted investigation into possible mechanisms. Being a Gram-negative bacterium, we hypothesized *C. burnetii* provided LPS stimulation to BMDM resulting in NLRP3 priming (signal 1) and upregulation of pro-IL-1β and subsequent increases in cytotoxicity and IL-1β secretion.

Through immunoblotting, we found that NMII *C. burnetii* robustly upregulates NLRP3 and to a lesser extent pro-IL-1β in the absence of LPS (Fig. 4). Interestingly, NMII-infected BMDM had higher levels of NLRP3 following LPS treatment, which indicates an additive effect. Furthermore, LPS was shown to be an extremely effective agent for the induction of pro-IL-1β. This priming resulted in the potentiation of caspase-1-mediated effects when *C. burnetii*-infected BMDM were subsequently treated with potent NLRP3 agonists, nigericin and *Yptb*. What was also surprising was the

potentiation noted in *Stm*-infected BMDM pre-infected with NMII. It is well know that some pathogenic bacteria can stimulate more than one inflammasome, highlighting the importance of inflammasomes to the innate response during microbial infections (47-51, 71). Specifically, NLRC4 and NLRP3 inflammasomes may have redundant roles in response to *Salmonella enterica* serovar Typhimurium depending on bacterial flagellin up or down-regulation and length of infection (47, 48, 71). To elucidate the mechanism of *C. burnetii* inflammasome potentiation during co-infections with *Stm*, we looked at NLRC4 levels in NMII-infected BMDM and found that NLRC4 levels were mildly increased similar to uninfected LPS-treated BMDM. This suggests that though not required for activation or priming, LPS treatment does promote NLRC4 inflammasomes and this is likely the reason for the potentiation noted in *Stm*-infected BMDM pre-infected with NMII.

There has been increasing interest and study of the regulation of NLRP3 inflammasomes.(118) Our data suggest NLRP3 priming (signal 1) and subsequent activation (signal 2) are tightly controlled and pathogens likely influence regulation. Macrophage activation states are critical to immune responses overall but are also important to the individual cell's survival as this relates to caspase-mediated cell death.(119) Considering *C. burnetii* can persist in tissues for months and even years during chronic Q fever and that there is dysregulation of inflammatory cytokines in these patients, determining the effect of *Coxiella*-induced NLRP3 upregulation on macrophage polarization states, if any, could be enlightening.

By utilizing BMDM from genetically modified mice deficient in TLRs (TLR2, TLR4, TLR2/4) and adaptor molecules (MyD88, TRIF), we determined that NLRP3

priming by NMII *C. burnetii* is dependent on TLR2-MyD88 signaling and is independent of the TLR4-TRIF. There are conflicting theories as to the importance of TLR2 and TLR4 to the detection of *C. burnetii*. We have shown that though TLR2-mediated detection of NMII *C. burnetii* results in the upregulation of NLRP3, it does not result in inflammasome formation and subsequent caspase-1 activation. The lack of caspase-1 activation has been described previously in different cell types including mouse alveolar and bone marrow derived macrophages as were used in this study.(16, 27) Importantly, mutant *C. burnetii* lacking the T4SS (Δ*dotA*) do not induce caspase-1 activation, IL-1β secretion, or cell death despite robustly priming NLRP3 inflammasomes. This lack of inflammasome activation is similarly seen in C57BL/6 BMDM infected with mutant bacteria lacking the recently discovered IcaA (Inhibition of Caspase Activation) protein produced by *C. burnetii*.(27) IcaA attenuates the activation of caspase-1 that is triggered through the non-canonical caspase-11-mediated pathway by an unknown mechanism but does not act upon caspase-1 directly.(27) Thus, our data further suggest that the lack of caspase-1 activation by *C. burnetii* is not reliant on T4S or a specific T4S effector, and rather lack of cytosolic detection by NLRP3 of the bacteria and its products.

At least 130 effector proteins are translocated into the host cell cytosol through *C. burnetii*'s T4SS.(29, 33-36) Of these putative "effectors" one or several could potential function as inhibitors of caspase-1, NLRP3 and ASC foci formation, or other points of the NLRP3 inflammasome pathway. Recent advances in axenic culture and genetic manipulation have allowed for screening of some of these proteins. From the exhaustive screening by Cunha *et al.*, only one *C. burnetii* protein was found to inhibit cleavage of caspase-1, aptly named Inhibition of Caspase Activation, IcaA.(27) Even though the

probability of one of these foreign proteins being sensed seems considerable, collectively these data suggest *C. burnetii* effectively avoid cytosolic detection in a T4SS-independent manner negating the NLRP3 inflammasome as a player in the initial immune response.

Interestingly, B1a B cells derived from the peritoneal cavity of mice exhibited caspase-1-mediated cell death (pyroptosis) following infection with NMII *C. burnetii*.(120) This subset of B cells was previously found to be important to the immune response to *C. burnetii.* The authors deduced that NMII-induced caspase-1 dependent pyroptosis may require T4S, TLR2 signaling and NLRP3 inflammasomes.(120) Other cell types have been shown to behave differently following *C. burnetii* infection with divergent cell death responses depending on the cell's activation states and bacterial phase variation - cell types including human monocytes, monkey and mouse alveolar macrophages, and THP-1 cells.(23, 24, 108, 121) It is clear that host-pathogen interactions are specific to certain cells types and that there are overlapping mechanisms across immune cells to ensure a successful collective response to even the most highly adapted of pathogens. To better elucidate the the biological relevance of host inflammasomes during *C. burnetii* infection, we turned to an *in vivo* model, which encompasses entire organ systems, different immune cell types, and a longer course of infection following a natural route of infection.

Chapter 4.

Determining the biological relevance of inflammasomes in Q fever

Results

Pulmonary NMII *C. burnetii* infection causes transient splenomegaly, increased bacterial burdens and macrophage expansion in the lungs, and hepatic microgranulomas in wild-type C57BL/6 mice.

First, we established an *in vivo* model using NMII *C. burnetii* to infect wild-type C57BL/6(J) mice via a pulmonary exposure over a 3-week time interval. C57BL/6 mice were chosen as they are clinically resistant to NMII *C. burnetii* and there are numerous genetically modified mice deficient in receptors and adaptor molecules of interest on this background strain.(101, 104, 122) Furthermore, C57BL/6 mice are M1/Th1 skewed and therefore best represent the largest percentage of Q fever patients, those individuals who mount a successful M1/Th1 (cell-mediated) response and are able to clear the bacteria (Fig. 2.1). Wild-type C57BL/6 mice were infected with NMII *C. burnetii* via oropharyngeal instillation. Control mice were instilled with PBS only and were sacrificed at 3 or 7 days post instillation.

No mice showed clinical signs of disease or lost significant weight during the 3-week infections (Fig. 2.2). In NMII-infected mice, spleen size increased by 3 dpi, peaked at 7 dpi, and subsequently returned to the size of PBS-treated mice by 21 dpi (Fig. 2.3A-C, 2.4 A, B). At 3 dpi, NMII-infected mouse lungs had mild alveolitis and pyogranulomatous interstitial pneumonia with lymphoplasmacytic and histiocytic (i.e. macrophage) perivascular and peribronchiolar cuffing (Fig. 2.3F, G; Fig. 2.5A, E). *C.*

burnetii (NMII)-containing and F4/80-positive cells (macrophages) were most numerous in the lungs at 7 dpi and decreased significantly by 21 dpi (Fig. 2.5B, C). By 3 dpi, livers of NMII-infected mice had multifocal microgranulomas, characterized as variably sized nodular aggregates of F4/80-positive macrophages with fewer lymphocytes, plasma cells, and neutrophils centered on rare necrotic hepatocytes (Fig. 2.3I, J; Fig. 2.5D). Hepatic microgranulomas increased in number and size up to 7 dpi and then decreased by 21 dpi (Fig. 2.5D, G). Within the lung and liver of PBS-control mice, F4/80-labeled macrophages were limited to the perivascular and peribronchiolar regions and the reticuloendothelial framework, respectively (Fig. 2.5C-F). Low numbers of NMII *C. burnetii* were detected in the spleens and livers of infected mice as early as 3 dpi via immunohistochemistry, which confirms dissemination from the lungs in our *in vivo* model (Fig. 2.3E, H). Control (PBS-instilled) mice did not exhibit significant gross or histologic changes and NMII *C. burnetii* were not detected in any of the examined tissues (Fig. 2.3, Fig. 2.5). Within the lung and liver of PBS-control mice, F4/80-labeled macrophages were limited to the perivascular and peribronchiolar regions and the reticuloendothelial framework, respectively (Fig. 2.5C, D).

Together, these data demonstrate that C57BL/6 wild-type mice, though considered clinically resistant to NMII *C. burnetii*, in fact develop splenomegaly and consistent multi-organ histologic lesions following pulmonary infection. Furthermore, NMII *C. burnetii* are able to disseminate from the lungs, most likely via macrophages, as they are the predominant inflammatory cells within the pulmonary and hepatic lesions and a majority of bacteria were found in (F4/80 positive) macrophages.

NLRP3 and caspase-1 are dispensable to the resolution of splenomegaly, hepatic microgranulomas, and pulmonary bacterial burdens following pulmonary infection with *C. burnetii* NMII.

To determine the biological relevance of NLRP3 inflammasomes and the effector protease, caspase-1 to Q fever pathogenesis, we compared wild-type (WT) mice and mice deficient in NLRP3 and caspase-1 using our *in vivo* model. We evaluated WT, *Nlrp3*$^{-/-}$, and *Casp1/11*$^{-/-}$ mice (n = 4-8 per genotype; approximately 50% male, 50% female) over a 3-week period following oropharyngeal instillation of NMII *C. burnetii*. All mice were evaluated for clinical signs, body weight, splenomegaly, pulmonary bacterial burdens, and pulmonary and hepatic macrophage infiltrates.

None of the *Nlrp3*$^{-/-}$ and *Casp1/11*$^{-/-}$ mice had clinical signs or significant weight loss. Splenomegaly of *Nlrp3*$^{-/-}$ and *Casp1/11*$^{-/-}$ mice followed the same temporal trend as for WT mice (Fig. 2.6A). Furthermore, subgrossly and histologically, spleens from *Nlrp3*$^{-/-}$ and *Casp1/11*$^{-/-}$ mice were indistinguishable from WT mice at 3, 7, 14, and 21 dpi (see Fig. 2.3C,D; 2.4B). Lung and liver microscopic lesions of *Nlrp3*$^{-/-}$ and *Casp1/11*$^{-/-}$ mice were also similar to those in WT mice. Interestingly, at 7 dpi, NMII positive staining in the lungs was significantly higher in *Casp1/11*$^{-/-}$ ($p < 0.05$) and *Nlrp3*$^{-/-}$ ($p < 0.001$) mice; however by 21 dpi, there was no significant difference detected (Fig. 2.6B). F4/80 positive staining in the lungs was not significantly different between WT and knock-out mice at any time points (Fig. 2.6C). Macrophage abundance in the liver followed a virtually overlapping trend in the knock-out mice compared to WT mice (Fig. 2.6D). Together, these data suggest that NLRP3 and caspase-1 are dispensable to the development and resolution of splenomegaly, macrophage responses in the lung and

liver, and eventual decreases in pulmonary bacterial burdens following NMII *C. burnetii* pulmonary infection in C57BL/6 mice.

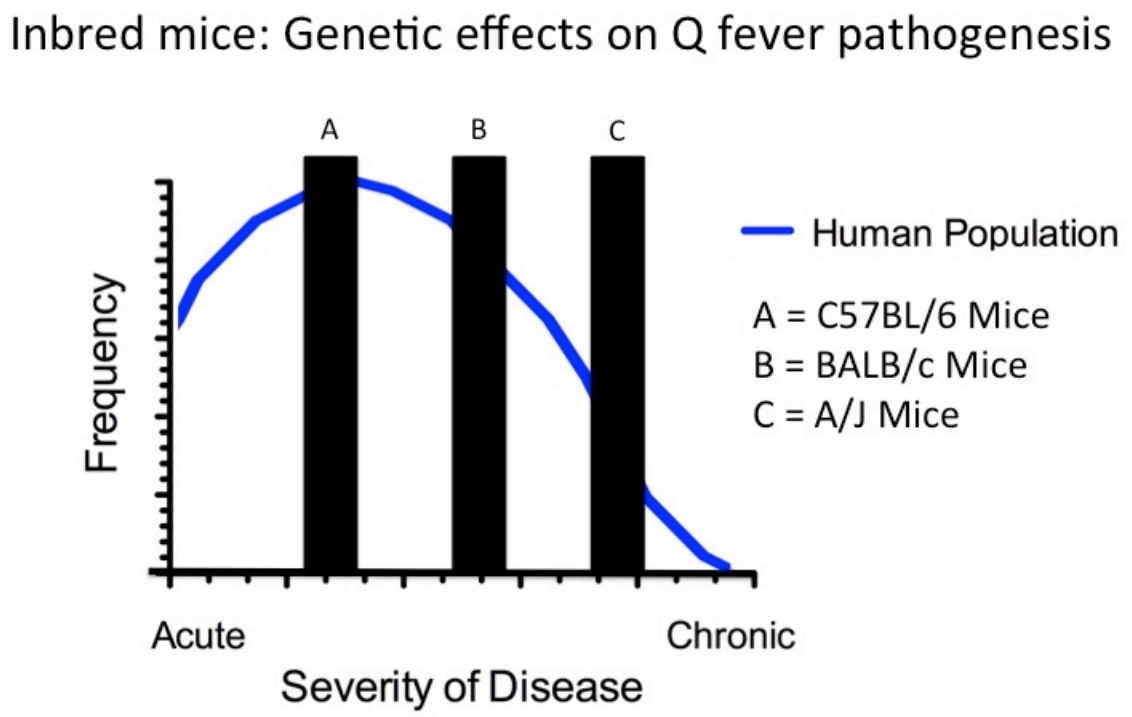
MyD88 is important for pulmonary clearance of NMII *C. burnetii* and macrophage responses in the lung and liver following airway exposure.

To determine the role of MyD88 and TLR4 in the resistance of C57BL/6 mice to NMII *C. burnetii*, we compared wild-type (WT) mice and *MyD88*$^{-/-}$ and *Tlr4*$^{-/-}$ mice. No mice exhibited significant weight loss, clinical signs, or mortality during the 14-day infection interval. The most striking finding was the significant increases in pulmonary bacterial burdens in mice deficient in MyD88 (Fig. 2.7B). At 14 days post infection, *MyD88*$^{-/-}$ mice had high levels of positive staining for NMII (Fig. 2.7B) despite decreasing levels of F4/80 positive macrophages in the lungs (Fig. 2.7C). At this time point (14 dpi), *Tlr4*$^{-/-}$ mice had similar pulmonary bacterial burdens as for WT mice but significantly increased macrophages compared to WT mice (Fig. 2.7B, C). Interestingly, *MyD88*$^{-/-}$ mice had persistent splenomegaly at day 14 post-infection when compared to WT and *Tlr4-*$^{-/-}$ mice (Fig. 2.5A). Splenomegaly was attributed to white pulp hyperplasia, and expansion of the red pulp by predominantly macrophage infiltrates as well as extramedullary hematopoiesis (data not shown; see Fig. 2.3C, D). In contrast, hepatic macrophages were significantly decreased in *MyD88*$^{-/-}$ mice at 7 days post infection compared to WT and *Tlr4*$^{-/-}$ mice (Fig. 2.5 D). Together these data suggest MyD88, but not TLR4, plays an important role in the recruitment, kinetics, and microbicidal activity of macrophages in the lungs and the clearance of NMII *C. burnetii* following pulmonary infection. Furthermore, MyD88 appears to be involved with the resolution of

splenomegaly and the macrophage response (microgranuloma formation) in the liver. Finally, TLR4 seems to be dispensable to the pulmonary clearance of NMII as well as splenic and hepatic macrophage kinetics following pulmonary infection, though TLR4 affects the activity of macrophages within the lungs with persistent macrophage infiltrates despite decreasing bacterial burdens.

Figures

Figure 2.1.

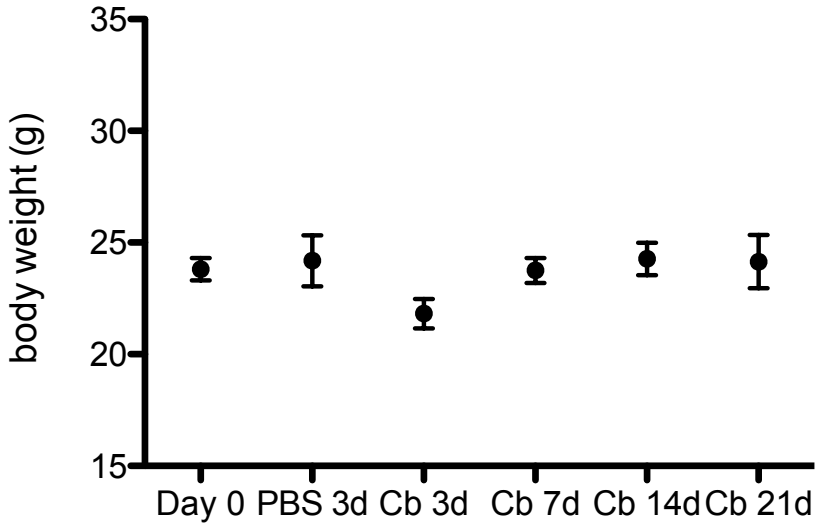


Modified from: Montagutelli X. *J Am Soc Nephrol* 2000.

Utilizing inbred mouse strains to model acute versus chronic Q fever in humans.

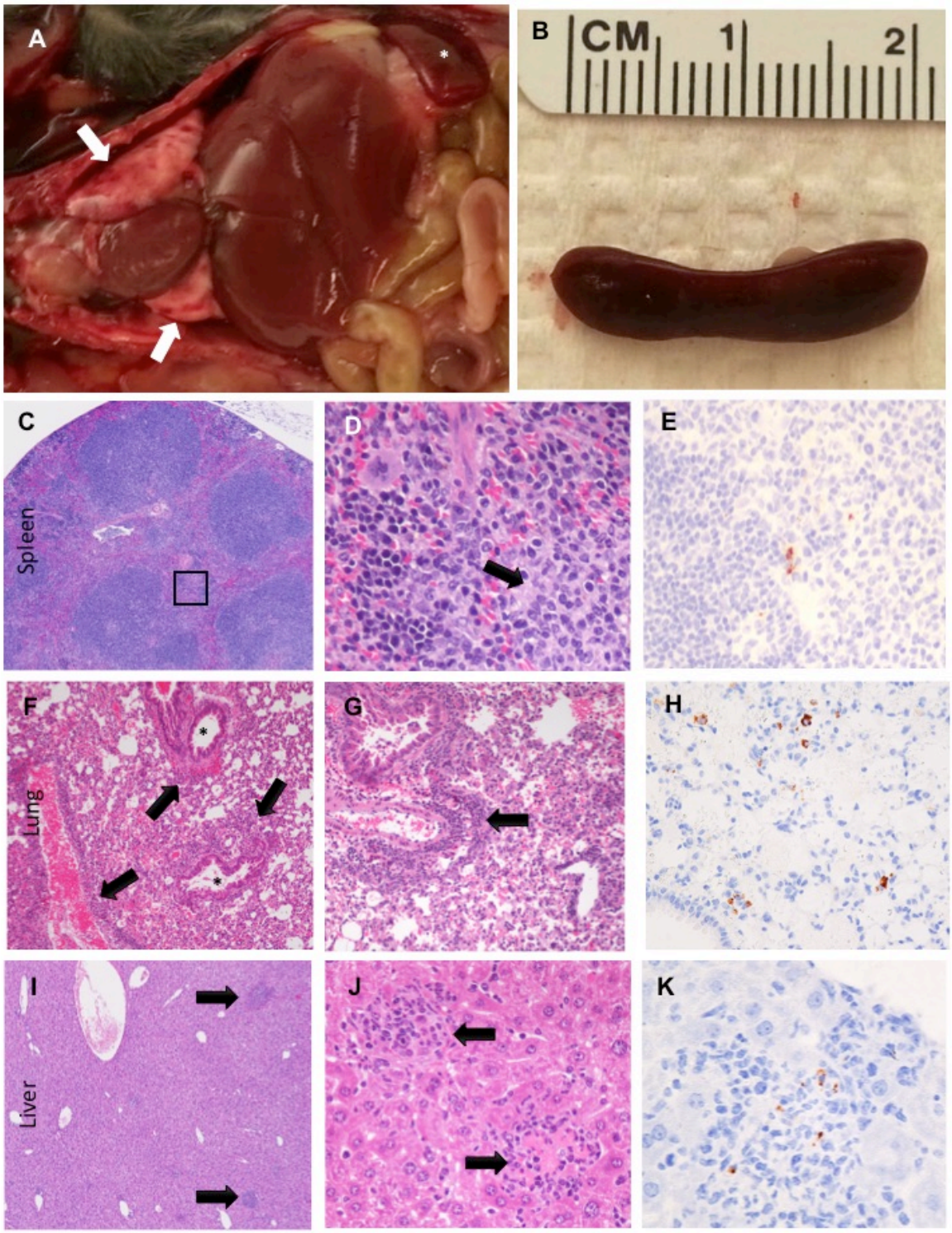
A majority of Q fever patients clear the infection due to a robust M1/Th1 response, as seen in C57BL/6 mice. In contrast, BALB/c mice are less able to clear the microbial infections due to a dominant humoral (M2/Th2) response. Historically, BALB/c and immuno-compromised or -modulated mouse genotypes (e.g. SCID, IL-10 over-expression) were used in attempts to model chronic Q fever in humans.

Figure 2.2



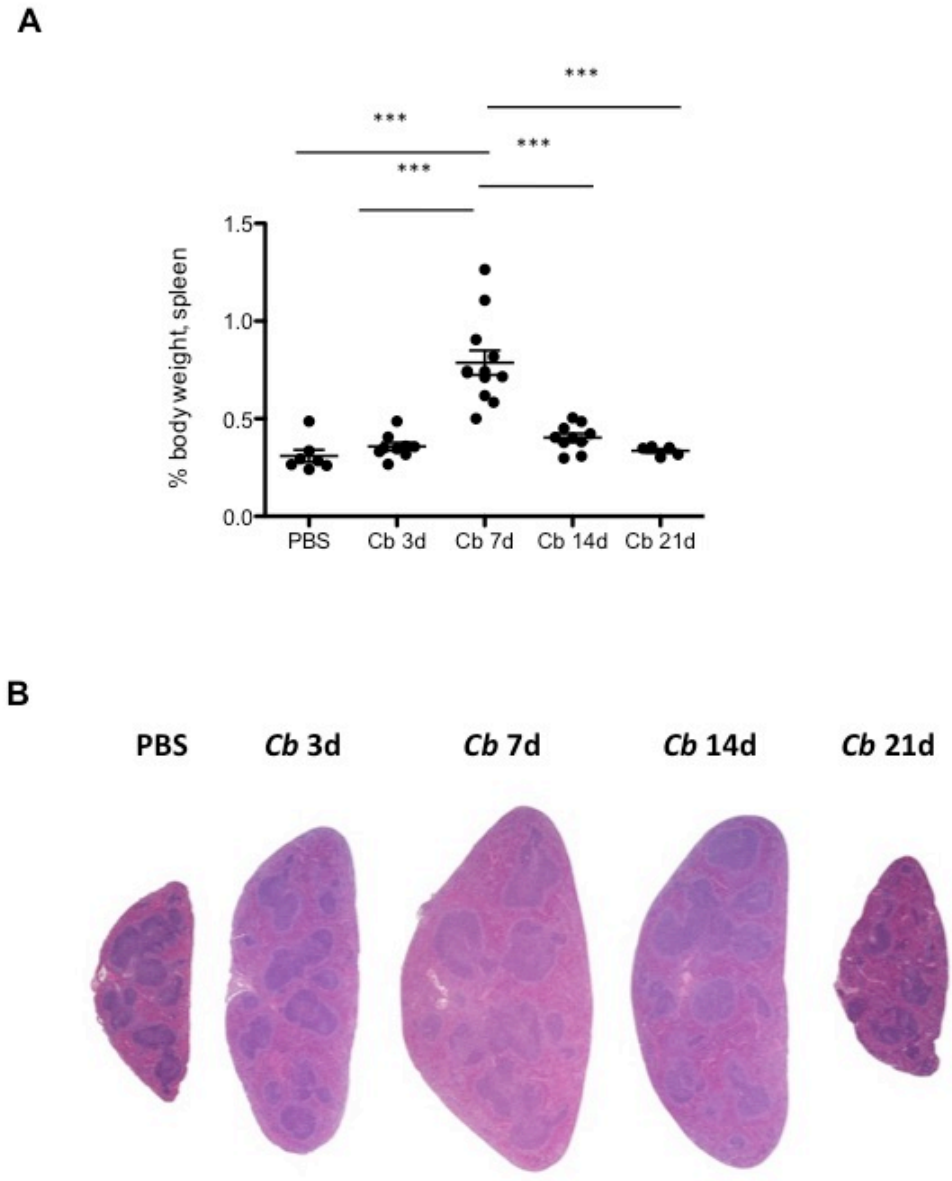
Wild-type C57BL/6 mice do not exhibit weight loss following pulmonary infection with NMII *C. burnetii*. At day 3 post infection (Cb 3d), there is a minimal decrease in average body weight compared to Day 0 and PBS-treated mice (PBS 3d) but by day 7 post infection (Cb 7d) and until the end of infection intervals (Cb 21d), body weights rebounded to Day 0.

Figure 2.3.



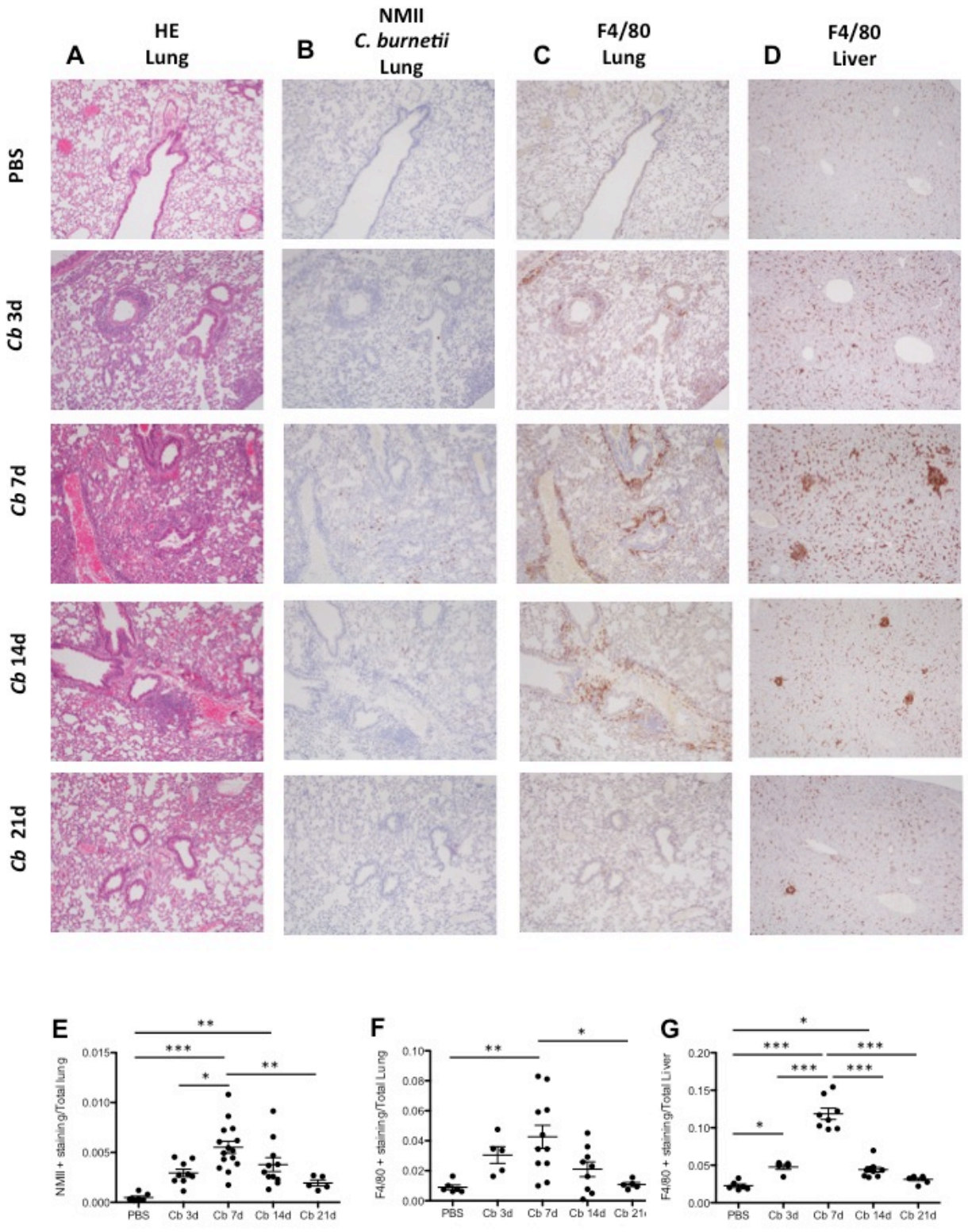
Wild-type C57BL/6 mice develop characteristic gross and histologic lesions of Q fever following pulmonary infection with NMII *C. burnetii* including splenomegaly, pneumonia, and hepatic microgranulomas with intralesional bacteria. (A) By 7 days post infection, mice have grossly evident pneumonia, characterized by mottled dark red and pink lungs that fail to collapse, and exude frothy fluid, indicative of edema (arrows). Splenomegaly is also noted grossly (*) **(B)**, and histologically corresponds to lymphoid hyperplasia and expansion of the red pulp **(C). (D)** The red pulp contains increased hematopoietic precursors (extramedullary hematopoiesis) and large round to polygonal cells with abundant eosinophilic cytoplasm, consistent with macrophages (arrow). **(F, G)** In the lungs, the vessels and bronchioles (*) are surrounded by dense cuffs of lymphocytes, plasma cells, and rare neutrophils (arrows). The alveolar spaces contain increased foamy macrophages. **(I, J)** The livers have multifocal, variably-sized nodular aggregates of macrophages with fewer lymphocytes and plasma cells often centered on few hypereosinophilic (degenerate) hepatocytes and cellular debris, characterized as microgranulomas (arrows). **(E, H, K)** Positive immunostaining for NMII *C. burnetii* show low numbers of intracellular bacteria (brown) within the splenic, pulmonary, and hepatic lesions. (**C, D, F, G):** Hematoxylin and eosin. (**E, H**): Immunohistochemistry, anti-NMII, DAB chromogen (brown) and hematoxylin counterstain (blue).

Figure 2.4



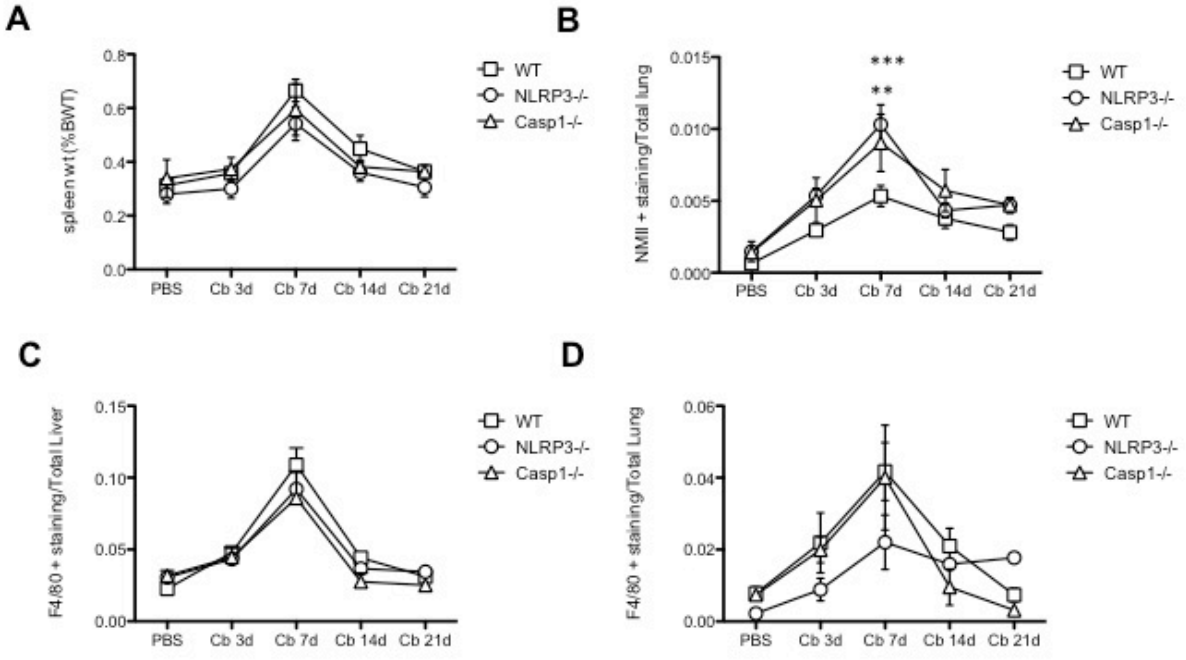
Splenomegaly develops and resolves following pulmonary infection with NMII *C. burnetii* in wild-type C57BL/6 mice. (**A)** Splenomegaly, as measured by percentage of body weight, is evident at 3 days post infection (3 dpi; *Cb* 3d), peaks at 7 days, and subsequently resolves by 21 dpi. **(B)** At the subgross level, spleens have lymphoid (white pulp) hyperplasia and increased macrophages and hematopoietic cells within the red pulp.

Figure 2.5


A
HE
Lung
B
NMII
C. burnetii
Lung
C
F4/80
Lung
D
F4/80
Liver
PBS
Cb 3d
Cb 7d
Cb 14d
Cb 21d
E
NMII + staining/Total lung
F
F4/80 + staining/Total Lung
G
F4/80 + staining/Total Liver
PBS
Cb 3d
Cb 7d
Cb 14d
Cb 21d

Pulmonary lesions and bacterial burdens, and pulmonary and hepatic macrophages are transiently increased during pulmonary infection with NMII *C. burnetii.* (A) Pulmonary lesions are evident by 3 dpi, characterized by peribronchiolar and perivascular mononuclear infiltrates, congestion, and increased alveolar macrophages. These lesions increase in severity by 7 dpi and begin to resolve by 14 dpi. **(D, G)** NMII *C. burnetii* are detected in the alveolar macrophages by 3 dpi, are increased at 7 dpi, and are reduced significantly by 21 dpi. **(E, H)** Pulmonary macrophages (F4/80 positive cells) show a similar trend as for bacteria with significant increases by 7 dpi. **(F, I)** Hepatic macrophages (F4/80 positive cells) are detected throughout the liver as part of the reticuloendothelial system, and in infected mice as multifocal nodular aggregates (i.e. microgranulomas). Hepatic macrophages are significantly increased by 7 dpi with marked reduction by 21 dpi. Each treatment group consists of at least 5 mice. Two-way ANOVA with Bonferroni's Multiple Comparison Test, (*) $p < 0.05$, (**) $p < 0.001$, (***) $p < 0.0001$.

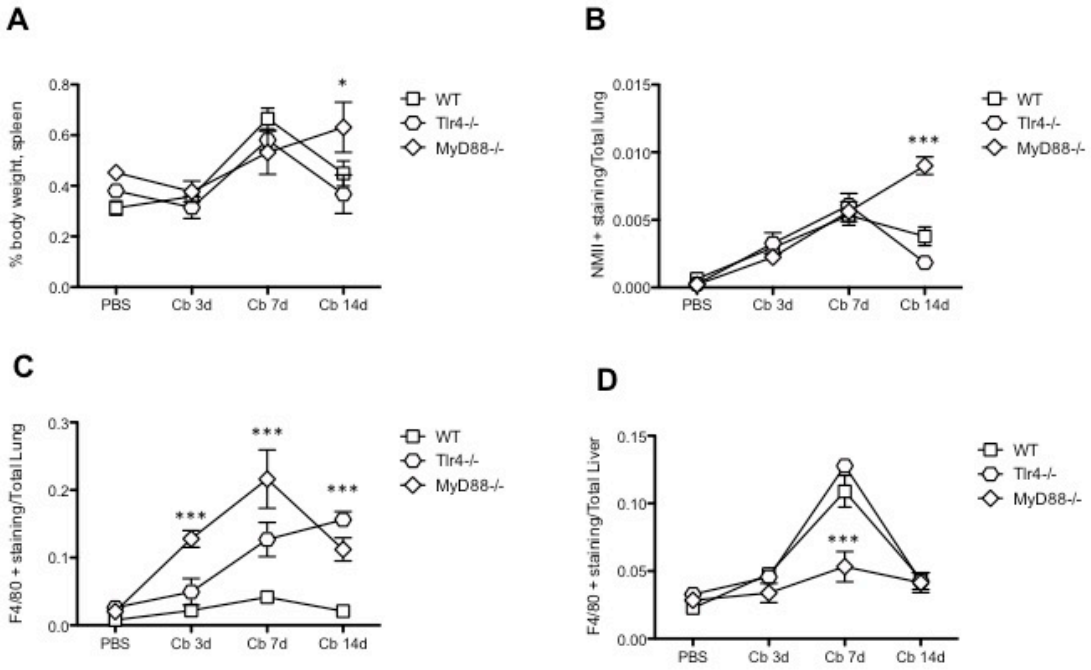
Figure 2.6.



NLRP3 and Caspase-1 are not required for resolution of splenomegaly and kinetics of macrophage infiltrates of the lung and liver but facilitate early pulmonary bacterial clearance in chronic NMII *C. burnetii* infection. (A) Development and resolution of splenomegaly as measured by percentage of body weight is similar in all mice over the 21-day infection interval. **(B)** At 7 days post infection (Cb 7d) pulmonary bacterial burdens are significantly higher in *Nlrp3*$^{-/-}$ and *Casp1/11*$^{-/-}$ than wild-type (WT) mice, however are not significantly different at any other time points. **(C)** Macrophages within the liver increased significantly by 7 dpi in all mice. There is no difference in hepatic macrophages between wild-type mice and mice deficient in NLRP3 or caspase-1 over the 21-day infection interval. **(D)** Pulmonary macrophages show similar trends as for pulmonary bacterial burdens, though there is no significant difference between WT mice and *Nlrp3*$^{-/-}$ and *Casp1/11*$^{-/-}$ mice. Each treatment group consists of at least 4 mice.

Two-way ANOVA with Bonferroni's Multiple Comparison Test, (**) $p < 0.05$, (***) $p < 0.001$.

Figure 2.7.



MyD88 is important for resolution of splenomegaly, pulmonary clearance of NMII *C. burnetii* and macrophage responses in the lung and liver following pulmonary infection. (A) Mice deficient in TLR4 were similar to WT with a reduction in splenic size at 14 dpi (Cb 14d); in contrast *MyD88*$^{-/-}$ mice had sustained splenomegaly at this time point. **(B)** NMII *C. burnetii* burdens in the lungs were similar between WT and *Tlr4*$^{-/-}$ mice whereas *MyD88*$^{-/-}$ mice had significantly increased pulmonary bacterial burdens. **(C)** Macrophage infiltrates in the lungs were significantly higher in both MyD88 and TLR4-deficient mice at 7 dpi. **(D)** Hepatic macrophages in *MyD88*$^{-/-}$ were significantly less than in WT and *Tlr4*$^{-/-}$ mice. Each treatment group consists of at least 4 mice. Two-way ANOVA with Bonferroni's Multiple Comparison Test, (**) $p < 0.05$, (***) $p < 0.001$.

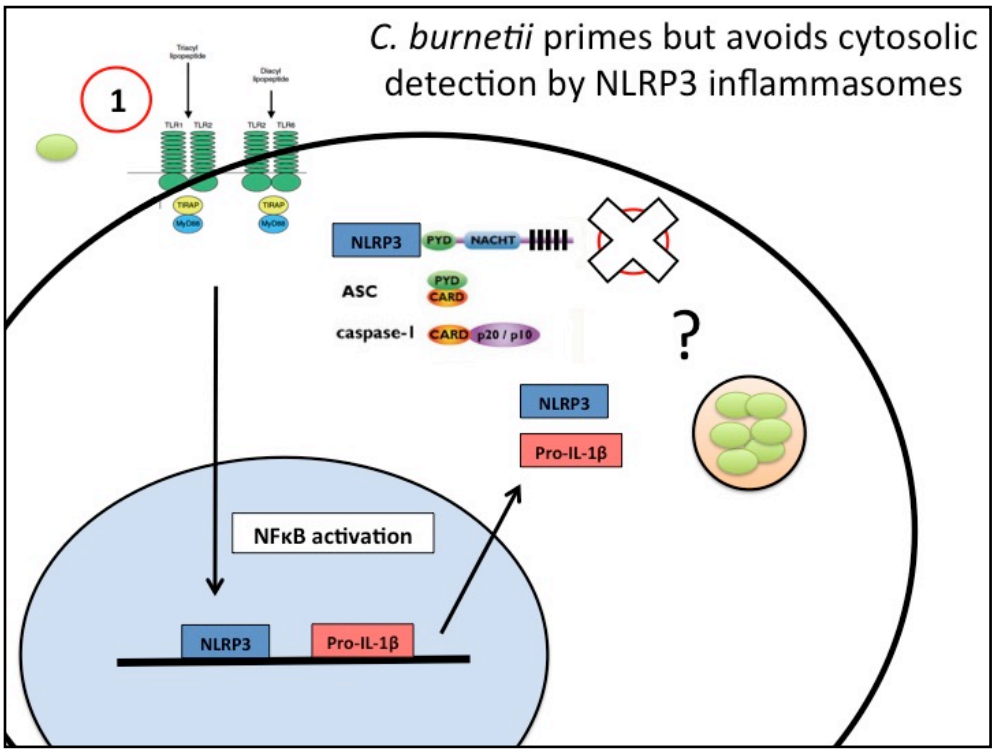
Discussion

Though C57BL/6 mice are thought to be resistant to NMII *C. burnetii,* through our and other studies it is clear that immunocompetent C57BL/6 mice can develop similar histologic lesions to human Q fever and the gold-standard animal model, guinea pigs following infection with NMII *C. burnetii* despite no weight loss (Fig. 2.2) or apparent clinical disease.(99-101) In a majority of human acute Q fever patients, infection may lead to transient pneumonitis and splenic inflammation.(1, 10) A small subset of human chronic Q fever patients may suffer from fulminant pneumonia, hepatitis, splenitis, and rarely endocarditis.(10) We found that the spleens of infected wild-type mice increased in size up to 7 days post infection (dpi) and resolved to normal size by 21 dpi (Fig. 2.4). Splenomegaly was also reported in previous *in vivo* studies following intraperitoneal infection of mice.(102, 104) Contributing to splenomegaly in our study were extensive macrophage infiltrates and increased extramedullary hematopoiesis, the latter of which is a common finding in mice following a systemic inflammatory response or antigenic stimulation (Fig. 2.3). Notably, bacteria were detected in the spleens and livers of infected mice by 7 dpi via immunohistochemical staining indicative of dissemination from the lungs (Fig. 2.3). We also determined that bacterial burdens and macrophage (F4/80 positive) populations increased following airway exposure, peaking at 7 dpi and then subsequently decreased, which correlated to resolution of pulmonary lesions (Fig. 2.5). Following a similar trajectory, hepatic microgranulomas increased up to 7 dpi and then decreased by 21 days. This *in vivo* model demonstrates that survival, weight loss, and clinical signs of disease are not sufficient to determine pathogenicity of an organism or resistance of a host. Furthermore,

by using C57BL/6 mice, our findings are more easily translated to those acute Q fever patients who successfully clear the infection during the initial infection period due to a robust cell-mediated (M1/Th1) response. A better understanding of the mechanisms required for the early clearance of *C. burnetii* may led to the development of immune therapies for chronic Q fever patients. Specifically, this model will be useful to study other important players in Q fever pathogenesis and extra-pulmonary dissemination, such as TLR-signaling pathways including adaptor molecules, cytokines and cytokine receptors, T helper cells and the adaptive immune response, virulence of *C. burnetii* strains, and potentially novel therapeutic, diagnostic, and preventative strategies.

Recent studies have shown that detection and restriction of *C. burnetii* NMII in C57BL/6 BMDM is dependent on TLR2, not TLR4, and subsequent cytokine responses, specifically TNF production, are critical to bacterial restriction and clearance.(16) Furthermore, cytosolic sensing was determined to be unimportant to resistance to *C. burnetii* growth in mouse macrophages.(16) Similarly, our *in vivo* findings suggest NLRP3 inflammasomes and caspase-1 are not necessary for the restrictive nature of C57BL/6 mice to *C. burnetii*, specifically during chronic infections and extra-pulmonary dissemination. Although TLR2-MyD88 signaling is important for NLRP3 inflammasome priming during *C. burnetii* infection, and the TLR2-MyD88 pathway appears to play a key role in bacterial restriction and clearance, NLRP3 inflammasomes and caspase-1 are not required for pulmonary bacterial clearance after the first week of infection. However, these components do appear to facilitate the clearance of *C. burnetii* during the early stages of infection (7 dpi) – this finding correlates to the theory that inflammasomes are

important for mitigating microbial infections while the host initiates an adaptive response.(53)



***Coxiella burnetii* – NLRP3 Inflammasome Interaction Model. *C. burnetii* is detected at the host cell membrane and induces the upregulation of NLRP3 and pro-IL-1β, thus providing signal 1 (priming) for the NLRP3 inflammasome. This priming corresponds to robust potentiation of inflammasome activation following stimulation by other potent agonists. However, *C. burnetii* or its bacterial products are not detected in host cytosol by NLRP3 and neither inflammasome formation nor activation occurs despite infection. Through unknown mechanisms, *C. burnetii* avoids cytosolic detection by inflammasomes.**

Our *in vitro* data (see Chapter 3) indicate NMII *C. burnetii* primes NLRP3 inflammasomes but avoids cytosolic detection. How do we reconcile the facilitation of pulmonary clearance of *C. burnetii* by both NLRP3 and caspase-1 at 7 days post infection

given the complete nullification of the NLRP3 inflammasome during *in vitro* infection? One possible mechanism for this dichotomy is the presence of NLRP3 agonists in the lungs during this peak inflammation and microbicidal activity in response to *C. burnetii* at 7 days post infection. It is likely that there is some level of cell death within the lungs and release of potential NLRP3 agonists (i.e. DAMPs and PAMPs) that provide Signal 2, inflammasome and caspase-1 activation. Examples of NLRP3 agonists include reactive oxygen species (ROS) or leaked lysosomal contents that may occur during the inflammatory response to *C. burnetii.* Based on our *in vitro* findings showing potentiation of inflammasomes by *C. burnetii*, it wouldn't be entirely surprising that there is some, albeit mild inflammasome activation during *in vivo* infections provided the degree of inflammation and microbicidal activity.

Another possibility to account for our *in vivo* findings at day 7 post infection, is the role of non-canonical inflammasomes, mediated by caspase-11 in mice, in the response to *C. burnetii* and its interplay with NLRP3 inflammasomes and caspase-1.(64) A recent study demonstrated that *C. burnetii* has a T4SS effector protein, IcaA, that can attenuate caspase-11 activation, thus mitigating NLRP3-mediated caspase-1 activation.(27) The caspase-1-deficient mice used in our studies are also deficient in caspase-11, so this effect could be a result of the lack of caspase-11 and not caspase-1.

Finally, the lungs, as an organ of the entire biological system of the mouse, are much more complex than a monoculture of bone marrow derived macrophages. Thus, additional factors and mechanisms such as cytokines and chemokines in the alveolar microenvironment, activation states of those macrophages newly recruited to the lungs, and trafficking of infected macrophages to other organs (e.g. spleen and liver) must be

considered when interpreting disparities between *in vivo* and *in vitro* findings. Importantly, NLRP3 and caspase-1 do not appear to be critical to the systemic responses to *C. burnetii* as evidenced by the nearly identical profiles of splenomegaly and hepatic macrophages.

Interestingly, our results from the 14-day infection pilot study using *Tlr4*$^{-/-}$ and *MyD88*$^{-/-}$ mice suggest MyD88 is a key player in the immune response to *C. burnetii*, especially for the pulmonary response and bacterial clearance. In addition, MyD88 appears to affect the systemic response to pulmonary *C. burnetii* infection following dissemination as macrophages fail to increase in the liver as for WT and *Tlr4*$^{-/-}$ mice and macrophages numbers dwindle in the lungs despite increasing bacterial burdens. Our data also suggest TLR4 contributes to the macrophage trafficking in the lungs as these cells increased up to day 14 post infection despite waning bacterial burdens. These findings indicating a requirement for MyD88 during a successful immune response to *C. burnetii* corroborate recent *in vitro* studies using NMII and C57BL/6 BMDM as well as *in vivo* studies using virulent NMI and warrants further investigation.(112) Notably, genetic studies of human Q fever patients found that polymorphisms in MyD88 are associated with increased risk of developing chronic disease.(89)

Chapter 5.

Future Directions

Here we have elucidated interactions between the stealth pathogen, *C. burnetii* and host inflammasomes. By developing both *in vitro* and *in vivo* models using C57BL/6 mice and NMII *C. burnetii*, we have dependable tools in order to study Q fever pathogenesis as it pertains to the human and ruminant reservoir hosts.

Future experiments may focus on the interactions of NMII *C. burnetii* and human monocyte/macrophages inflammasomes. Though inflammasomes are highly conserved immune platforms, there are known differences between mouse and human inflammasomes and IL-1β processing. Valuable insight will be gained by performing the above-mentioned experiments with human peripheral blood derived mononuclear cells (PBMC) or alveolar macrophages. Along these lines, similar studies can be carried out with cells (PBMC, alveolar macrophages) from ruminant (i.e. goat and sheep) to determine similarities and differences of their mononuclear/myeloid cells in regards inflammasomes and immune responses to *C. burnetii.*

Notably, we developed a method to determine distribution and quantities of NMII *C. burnetii* in lung tissue through immunohistochemistry and semi-quantitative digital analysis. To correlate these results with qPCR (i.e. genome equivalents) would be an important step to validate this methodology for future *C. burnetii* studies. Immunohistochemistry (IHC) was incredibly useful for detecting low numbers of *C. burnetii* in the spleen and liver. Future studies may better utilize IHC and flow cytometry as well as *in vivo* imaging platforms (IVIS® Spectrum) to determine recruitment of

immune cells into lungs, dissemination, clearance/persistence, and target cell types of *C. burnetii.*

Additional *in vivo* studies utilizing a M2/Th2-biased strain (i.e. BALB/c) to determine the effect of macrophage activation states would be beneficial. Also, *in vivo* studies focusing on MyD88 and TLRs (TLR2 and TLR4) and infections with NMII *C. burnetii* will be useful to compare to those previously conducted using NMI.(112, 123) Furthermore, our preliminary study had 14-day instead of 21-day infections, thus we do not know how MyD88 and TLR4 affect more chronic infections.

Besides NMII *C. burnetii*, all other variants and strains are Select Agents and require BSL3/ABSL3 facilities and practices. Nevertheless, it would be interesting to determine if NMI primes NLRP3 and potentiates inflammasomes in different cell types, including mouse BMDM, alveolar macrophages, and human and ruminant cells *in vitro*.

By determining if polymorphisms of TLRs and/or adaptor molecules exist in ruminant hosts that chronically shed *C. burnetii* similar to human patients with persistent infections (89, 124), valuable insight may be gained in order to better develop diagnostic, preventative, and treatment strategies. Specifically, by boosting the macrophage response in the lungs (through enhancement of M1 activity by TNF and IFNγ) and promoting inflammasome activation in the lungs, pulmonary clearance of *C. burnetii* may be enhanced and the dissemination of *C. burnetii* from the lungs to the spleen and liver may be encumbered resulting in a shorter disease interval and reduced morbidity.

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100. **Stein A, Louveau C, Lepidi H, Ricci F, Baylac P, Davoust B, Raoult D.** 2005. Q fever pneumonia: virulence of Coxiella burnetii pathovars in a murine model of aerosol infection. Infect Immun **73:**2469-2477.
101. **Zamboni DS.** 2004. Genetic Control of Natural Resistance of Mouse Macrophages to Coxiella burnetii Infection In Vitro: Macrophages from Restrictive Strains Control Parasitophorous Vacuole Maturation. Infection and Immunity **72:**2395-2399.
102. **Ochoa-Reparaz J, Sentissi J, Trunkle T, Riccardi C, Pascual DW.** 2007. Attenuated Coxiella burnetii phase II causes a febrile response in gamma interferon knockout and Toll-like receptor 2 knockout mice and protects against reinfection. Infect Immun **75:**5845-5858.
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104. **Zamboni DS, Campos MA, Torrecilhas AC, Kiss K, Samuel JE, Golenbock DT, Lauw FN, Roy CR, Almeida IC, Gazzinelli RT.** 2004. Stimulation of toll-like receptor 2 by Coxiella burnetii is required for macrophage production of pro-inflammatory cytokines and resistance to infection. J Biol Chem **279:**54405-54415.
105. **Zamboni DS, Rabinovitch M.** 2003. Nitric Oxide Partially Controls Coxiella burnetii Phase II Infection in Mouse Primary Macrophages. Infection and Immunity **71:**1225-1233.
106. **Watanabe H, Numata K, Ito T, Takagi K, Matsukawa A.** 2004. Innate immune response in Th1- and Th2-dominant mouse strains. Shock **22:**460-466.
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108. **Calverley M, Erickson S, Read AJ, Harmsen AG.** 2012. Resident alveolar macrophages are susceptible to and permissive of *Coxiella burnetii* infection. PLoS One **7:**e51941.
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111. **Ammerdorffer A, Schoffelen T, Gresnigt MS, Oosting M, den Brok MH, Abdollahi-Roodsaz S, Kanneganti TD, de Jong DJ, van Deuren M, Roest HJ, Rebel JM, Netea MG, Joosten LA, Sprong T.** 2015. Recognition of Coxiella burnetii by toll-like receptors and nucleotide-binding oligomerization domain-like receptors. J Infect Dis **211:**978-987.

112. **Ramstead AG, Robison, A., Blackwell, A., Jerome, M., Freedman, B., Lubick, K.J., Hedges, J.F., Jutila, M.A.** 2016. Roles of TLR2, TLR4, and MyD88 during pulmonary *Coxiella burnetii* infection. Infect Immun **84:**940-949.
113. **Hedges JF, Robison, A., Kimmel, E., Christensen, K., Lucas, E., Ramstead, A., Jutila, M.A.** 2016. Type I Interferon Counters or Promotes *Coxiella burnetii* Replication Dependent on Tissue. Infect Immun **84:**1815-1825.
114. **Meghari S, Bechah, Y., Capo, Christian, Lepidi, Hubert, Raoult, Didier, Murray, Peter, Mege, Jean-Louis.** 2008. Persistent *Coxiella burnetii* infection in mice overexpressing IL-10: An efficient model for chronic Q fever pathogenesis. PloS Pathoges **4:**e23.
115. **Miao EA, Alpuche-Aranda CM, Dors M, Clark AE, Bader MW, Miller MW, Aderem A.** 2006. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. Nature Immunol **7:**569-575.
116. **Fink SL, Cookson BT.** 2006. Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. Cell Microbiol **8:**1812-1825.
117. **Bjork A, Marsden-Haug N, Nett RJ, Kersh GJ, Nicholson W, Gibson D, Szymanski T, Emery M, Kohrs P, Woodhall D, Anderson AD.** 2014. First reported multistate human Q fever outbreak in the United States, 2011. Vector Borne Zoonotic Dis **14:**111-117.
118. **Gurung P, Li B, Subbarao Malireddi RK, Lamkanfi M, Geiger TL, Kanneganti TD.** 2015. Chronic TLR stimulation controls NLRP3 inflammasome activation through IL-10 mediated regulation of NLRP3 expression and caspase-8 activation. Sci Rep **5:**14488.
119. **Bergsbaken T, Cookson BT.** 2007. Macrophage activation redirects yersinia-infected host cell death from apoptosis to caspase-1-dependent pyroptosis. PLoS Pathog **3:**e161.
120. **Schoenlaub L, Cherla R, Zhang Y, Zhang G.** 2016. Coxiella burnetii Avirulent Nine Mile Phase II Induces Caspase-1-Dependent Pyroptosis in Murine Peritoneal B1a B Cells. Infect Immun **84:**3638-3654.
121. **Zhang Y, Zhang, G., Hendrix, L.R., Tesh, V.L., Samuel, J.E.** 2012. Coxiella burnetii induces apoptosis during early stage infection via a caspase-independent pathway in human monocytic THP-1 cells. PLoS One **7:**e30841.
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123. **Hawn TR, Berrington WR, Smith IA, Uematsu S, Akira S, Aderem A, Smith KD, Skerrett SJ.** 2007. Altered Inflammatory Responses in TLR5-Deficient Mice Infected with *Legionella pneumophila*. J Immunol **179:**6981-6987.
124. **Hawn TR, Verbon A, Lettinga KD, Zhao LP, Li SS, Laws RJ, Skerrett SJ, Beutler B, Schroeder L, Nachman A, Ozinsky A, Smith KD, Aderem A.** 2003. A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to Legionnaire's Disease. J Exp Med **198:**1563-1572.

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EDUCATION

University of Washington, School of Medicine
Doctor of Philosophy, Pathology, August 2017

University of Illinois College of Veterinary Medicine
Master of Science (non-thesis), January 2013

Tufts University Cummings School of Veterinary Medicine
Doctor of Veterinary Medicine, May 2008

University of Massachusetts at Amherst
Bachelor of Science *cum laude*, Animal Science, May 2002

POSTGRADUATE TRAINING

Resident, Zoological Pathology Program (2009 – 2012)
University of Illinois College of Veterinary Medicine

Intern, Agricultural Animal Medicine and Surgery (2008 – 09)
Washington State University College of Veterinary Medicine

BOARD CERTIFICATION

American College of Veterinary Pathologists, 2013

USDA ACCREDITATION

National Accreditation Number: 069284; Valid through 5/13/2019

LICENSURE

Washington Veterinary Board of Governors
License number: VT 60038456, Valid through 8/11/2018

BIBLIOGRAPHY
Peer reviewed

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Moshiri AS, Doumani R, Yelistratova L, Blom A, Lachance K, Shinohara MM, **Delaney M**, Chang O, McArdle S, Thomas H, Asgari MM, Huang ML, Schwartz SM, Nghiem P.

2017. Polyomavirus-Negative Merkel Cell Carcinoma: A More Aggressive Subtype Based on Analysis of 282 Cases Using Multimodal Tumor Virus Detection. *J Investigative Dermatology.* 137(4):819-827. doi: 10.1016/j.jid.2016.10.028. Epub 2016 Nov 1.

Delaney, MA, Ward, JM, Walsh, TF, Chinnadurai, SK, Kerns, K, Kinsel, MK, Treuting, PM. 2016. Initial Case Reports of Cancer in Naked Mole-rats (*Heterocephalus glaber*). *Veterinary Pathology*. 53(3):691-6, DOI: 10.1177/0300985816630796.

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Scoville, DK, White, CC, Botta, D, McConnachie, LA, Zadworny, ME, Schumch, SC, Hu, X, Gao, X, Dills, RL, Sheppard, L, **Delaney, MA**, Griffith, WC, Beyer, RC, Pounds, JG, Faustman, EM, Kavanagh, TJ. 2015. Susceptibility to quantum dot induced lung inflammation differs widely among the Collaborative Cross founder mouse strains. *Toxicol. Appl. Pharmacol*. 289(2):240-50, DOI:10.1016/j.taap.2015.09.019

Woodward-Davis, AS, Bergsbaken, T, **Delaney, MA,** Bevan, MJ. 2015: Dermal-Resident versus Recruited γδ T Cell Response to Cutaneous Vaccinia Virus Infection. *The Journal of Immunology*, 194(5), 2260-7, DOI:10.4049/jimmunol.1402438

Chang, M, **Delaney, M,** Hajjar, A, Wight, T, Frevert, C. Regulation of versican expression by bacterial infection is TLR4-dependent but MyD88-independent. *The FASEB Journal*, 28(1), Supp 1046.3.

Delaney, MA, Colegrove KM, Spraker, TA, Zuerner, RL, Galloway, RL, and Gulland, FMD. 2014: Isolation of *Leptospira* from a Phocid: Acute Renal Failure and Mortality from Leptospirosis in Rehabilitated Northern Elephant Seals (*Mirounga angustirostris)*, California, USA. *Journal of Wildlife Diseases,* 50(3), 621-7, DOI: 10.7589/2013-08-195

Nevitt, BN, Langan, JN, Adkesson, MJ, **Delaney, MA**, Rubin, DA, Muhlbauer, MC, and Colegrove, KM. 2013: Multifocal *Cryptococcus neoformans var. neoforms*: Infection, Treatment, and Monitoring Serial Computed Tomography in a Schmidt's Red-Tailed Guenon (*Cercopithecus ascanius schmidti*). *Journal of Zoo and Wildlife Medicine*, 44, 728-736, DOI: 10.1638/2013-0063.1

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Delaney, MA, Nagy, L, Kinsel, MJ, Treuting, PM. 2013: Spontaneous Histologic Lesions of the Adult Naked Mole-Rat (*Heterocephalus glaber*): a Retrospective Survey of Lesions in a Zoo Population. *Veterinary Pathology,* 50, 607-21, DOI: 10.1177/0300985812471543

Sasseville, V, Mansfield, K, Mankowski, J, Tremblay, C, Terio, K, Matz-Rensing, K, Gruber-Dujardin, E, **Delaney, MA**, et al. 2012: Meeting Report: Spontaneous Lesions and Diseases in Wild, Captive-Bred, and Zoo-Housed Nonhuman Primates and in Nonhuman Primate Species Used for Drug Safety Studies. *Veterinary Pathology*, 49, 1057-1069, DOI: 10.1177/0300985812461655

Delaney, MA, Singh, K, Murphy, CL, Solomon, A, Nel, S, and Boy, SC. 2012: Biochemical and Immunohistochemical Evidence of Ameloblastic Origin of Amyloid-Producing Odontogenic Tumors in Cats. *Veterinary Pathology*, 50, 238-242, DOI: 10.1177/0300985812452583

Delaney, MA, Terio, KA, Colegrove, KM, Briggs, MB, and Kinsel, MJ. 2012: Occlusive Fungal Tracheitis in Four Captive Bottlenose Dolphins (*Tursiops truncatus*). *Veterinary Pathology*, 50, 172-6, DOI: 10.1177/0300985812446153.

Allender, MC, Dreslik, M, Wylie, S, Phillips, C, Wylie, DB, Maddox, C, **Delaney, MA**, and Kinsel, MJ. 2011: *Chrysosporium* sp. infection in eastern massasauga rattlesnakes [letter]. *Emerging Infectious Diseases*, 17, 2383-4. DOI: 10.3201/eid1712.110240

Keating, J, **Delaney, M**, Meehan-Meola, D, Warren, W, Alcivar, A, Alcivar-Warren, A. 2007: Histological findings, cadmium bioaccumulation, and isolation of expressed sequence tags (ESTs) in cadmium-exposed, specific pathogen-free shrimp, *Liptopenaeus vannamei* postlarvae. *Journal of Shellfish Research*. 26, 1225-1237. DOI: 10.2983/0730-8000(2007)26[1225:HFCBAI]2.0CO;2

Alcivar-Warren, A, Meehan-Meola, D, Park, SW, Xu, Z, **Delaney, M**, Zuniga G. 2007: *SHRIMPMAP*: A Low-density, microsatellite-based linkage map of the Pacific whiteleg shrimp, *Liptopenaeus vannamei*: Identification of sex-linked markers in linkage group 4. *Journal of Shellfish Research*, 26, 1259-1277.

Miller-Sims, V, **Delaney, M**, Atema, J, Kingsford, M, and Gerlach, G, 2005: DNA Microsatellites in *Acanthochromis polyacanthus*. *Molecular Ecology Notes*, 5, 841-843.

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Delaney, MA, Follet, C, Ryan, N, Hanney, N, Lusk-Yablick, J. and Gerlach, G, 2002: Social Interaction and Distribution of Female Zebrafish (*Danio rerio*) in a Large Aquarium. *Biological Bulletin*, **203**, 240-241.

Abstracts

Delaney, MA, Ford JK, Tang K, Gaydos JK. 2016: Meosparasitic Copepod (*Pennella balaenopterae*) Infestation of a Stranded Offshore Killer Whale (*Orcinus orca*) in Southeast Alaska: Review of Significance as a Health Indicator in Cetaceans. International Association of Aquatic Animal Medicine, 47th Annual Conference Proceedings, Virginia Beach, VA.

Delaney, MA and Lahner L. 2016: Cardiomyopathy in a Captive North American River Otter (*Lontra canadensis*). C.L. Davis Marine Animal Pathology Workshop. International Association of Aquatic Animal Medicine, 47th Annual Conference Proceedings, Virigina Beach, VA.

Delaney, MA and Gaydos JK**.** 2015: Islet Cell Tumor in a North American River Otter (*Lontra canadensis*). C.L. Davis Marine Animal Pathology Workshop. International Association of Aquatic Animal Medicine, 46th Annual Conference Proceedings, Chicago, Illinois.

Anderson, E, Adkesson, MJ, **Delaney, MA**, Langan, JN, Sanchez, CR. 2012: Immune mediated hemolytic anemia secondary to disseminated B-cell lymphoma in a California sea lion (*Zalophus californianus)*. American Association of Zoo Veterinarians, 44th Annual Conference Proceedings. Oakland, California.

Delaney, MA, Adkesson, M, Terio KA, Queen, S, Mankowski J. 2011: Case 5, Disseminated giant cell disease in a Red-capped mangabey (*Cercocebus torquatus*). Primate Pathology Workshop. American College of Veterinary Pathologists, 62nd Annual Meeting Proceedings. Nashville, Tennessee.

Manire, C**, Delaney, MA,** and Landolfi, J**.** 2011: Lymphosarcoma in a Captive Bonnethead Shark (*Sphyrna tiburo*). International Association of Aquatic Animal Medicine. 42nd Annual Conference Proceedings. Las Vegas, Nevada.

Delaney, MA, and Singh, KS. 2010: Feline Amyloid-Producing Odontogenic Tumors (APOT) are Derived from Ameloblasts. American College of Veterinary Pathologists, 61st Annual Meeting Proceedings. Baltimore, Maryland.

Delaney, MA, and Pessier, AP. 2008: Microsporidiosis in Mountain Yellow Legged Frog (*Rana muscosa)* Tadpoles in Southern California. Association of Reptilian and Amphibian Veterinarians, 14th Annual Conference Proceedings, Los Angeles, California.

Delaney, MA, Meehan-Meola, D, Keating, JK, and Alcivar-Warren, A. 2007: Effects of Cadmium on Marine Shrimp Postlarvae (*Penaeus vannamei*): Histological Findings and Bioaccumulation of Cadmium-Exposed Shrimp and Isolation of Cadmium-Related Expressed Sequence Tags for Linkage Mapping. International Association of Aquatic Animal Medicine, 38th Annual Conference Proceedings, Lake Buena Vista, Florida.

Delaney, MA, Meehan-Meola, D, and Alcivar-Warren, A. 2006: Bioaccumulation of Cadmium in Marine Shrimp Postlarvae (*Penaeus vannamei)* and Isolation of Expressed Sequence Tags for Mapping and Microarray Studies. EcoHealth ONE Proceedings, Madison, Wisconsin.

TEXTBOOK CHAPTERS
First authorship

Urinary System, Chapter 16: Comparative Anatomy and Histology: A mouse and human atlas, 2nd edition. Elsevier. 2017.

Rodentia (Chapter 19); Lagamorpha (Chapter 20); Hyracoidea (Chapter 22): Pathology of Zoo and Wild Animals, 1st edition. Elsevier. 2017.

TEACHING

University of Washington Department of Comparative Medicine, *Seattle, WA*
CMED 512: Introduction to Anatomical Analytical of Animal Disease (pathology rotation)
CMED 514/518: Comparative Pathology Conference (general pathology of lab animals)

The Histochemical Society & Marine Biological Laboratory, *Woods Hole, MA*
Technical Faculty, Immunohistochemistry & Microscopy (intensive 5 day course)
2014, 2015, 2016

AWARDS, HONORS, AND STUDENT GRANTS

March 2013	Histochemical Society (HCS) Short Course Travel Award
September 2010	Phi Zeta Honor Society inductee, Mu Chapter, University of Illinois
May 2008	Dr. Alan Hart Clinical Proficiency Scholarship Award (IDEXX)
2004 - 2008	Dean's list (5 semesters), Tufts Univ. Cummings School of Vet. Med.
November 2007	AABP Externship Grant
August 2007	ACVP Externship Grant
May 2007	IAAAM Annual Meeting, Poster Session - Second Place Award
Summer 2006	National Institute of Health (NIH) Summer Research Grant

Summer 2005	National Institute of Health (NIH) Summer Research Grant
1998 - 2002	Dean's list (5 semesters), Univ. of Massachusetts at Amherst

PROFESSIONAL MEMBERSHIPS

American Association of Zoo Veterinarians (AAZV)
Ecohealth Alliance
Histochemical Society (HCS)
International Association of Aquatic Animal Medicine (IAAAM)
Society of Marine Mammalogy (SMM)
Wildlife Disease Association (WDA)

JOURNAL REVIEWER ASSIGNMENTS

Diseases of Aquatic Organisms
Environmental Science and Technology
Journal of the American Association for Laboratory Animal Science
Journal of Zoo and Wildlife Medicine
Journal of Herpetological Medicine and Surgery
Journal of Medical Microbiology
Journal of Morphology
Journal of Wildlife Diseases
PLoS One
Veterinary Pathology

ADDITIONAL COURSEWORK

March 2013	Immunohistochemistry & Microscopy The Histochemical Society & Marine Biological Laboratory	*Woods Hole, MA*
May 2007	AQUAVET II Marine Biological Laboratory & Cornell University An advanced two-week course in the comparative pathology of aquatic invertebrates and vertebrates used as laboratory animals.	*Woods Hole, MA*
August 2006	Forensic Science for Marine Biologists Shoals Marine Laboratory Practical techniques for the investigation of marine mammal strandings and the untimely death of marine vertebrates.	*Appledore Island, NH*
June 2006	MARVET Mote Marine Laboratory An introductory course in marine mammal medicine; marine mammal biology and medical applications in captivity.	*Sarasota, FL*
Spring 2006	Geographic Information Systems (GIS): Applications to Human and Animal Health Tufts University Cummings School of Veterinary Medicine A forty-hour elective course introducing the fundamental concepts of GIS using hands-on computer tutorials with ArcGIS software.	*North Grafton, MA*
Spring 2004	University of Massachusetts at Dartmouth Immunology (Graduate section)	*North Dartmouth, MA*

EXPERIENCE / EMPLOYMENT

March 17 – current	Anatomic Pathologist (part-time consulting) Sealife Response + Rehab + Research (SR3) Perform necropsies of regional marine wildlife species	*Seattle, WA*
Sept 15 – current	Anatomic Pathologist (part-time consulting) SpecialtyVETPATH Perform histologic evaluations of surgical biopsy specimens and necropsies of companion, exotic, aquatic, and zoo animals, and wildlife	*Seattle, WA*
Oct 13 – current	Senior Postdoctoral Fellow Univ. of Washington (UW), Dept of Comparative Medicine Eighty percent (80%) appointment for PhD research of *Coxiella burnetii* and 20% Senior Pathologist for DCM	*Seattle, WA*
Nov 12 – Sept 13	Visiting Scientist/Comparative Pathologist UW, Dept of Comparative Medicine Initiated research program of *Coxiella burnetii* and worked as Senior Pathologist for the Veterinary Diagnostic Lab, including the instruction of lab animal medicine residents.	*Seattle, WA*
Dec 04 - Mar 07	Student Technician - Clinical Pathology Lab Tufts University Veterinary Medical Center Performed CBC, serum chemistries, urinalyses, coagulation tests, and fluid analyses for small and large animal hospitals and wildlife clinic.	*North Grafton, MA*
Oct 03 - Aug 04	Research Assistant/Lab Technician Marine Biological Laboratory Maintained laboratory and carried out molecular techniques including DNA extractions, PCR, gel and capillary electrophoresis and microsatellite analysis for population genetic studies of various coral reef fish species. Performed behavioral experiments involving zebrafish and maintained the Zebrafish Facility.	*Woods Hole, MA*
Aug 02 - Aug 03	Research and Animal Care Assistant Marine Biological Laboratory Provided support to many areas of the Marine Resources Center including Animal Health Dept, Zebrafish Facility, and Rodent Facility. Prepared and maintained habitats for incoming and resident research species. Performed necropsies and administered treatments.	*Woods Hole, MA*

EXTERNSHIPS /PRECEPTORSHIPS

May - June 08	The Marine Mammal Center Three-week externship performing physical exams and necropsies of Northern Elephant seals, Pacific Harbor seals, and California sea lions. Assisted with wild capture, sampling, and tagging of Pacific Harbor seals with staff researchers.	*Sausalito, CA*
April - May 08	PAWS Wildlife Center Four-week externship working with native wildlife. Performed physical exams, venipuncture, anesthesia, surgeries, radiography, and necropsies.	*Lynnwood, WA*

Assisted in wild capture of Pacific Harbor seals with WA Marine Mammal Investigations Unit.

Mar - April 08 — Marine Mammal Care Center at Fort MacArthur — *San Pedro, CA*
Four-week externship performing daily physical exams, blood collections, treatment administration, husbandry, and necropsies of Northern Elephant seals, Pacific Harbor seals, Northern Fur seals, and California sea lions.

Feb - Mar 08 — Toronto Zoo — *Toronto, ON*
Four-week externship at the Animal Health Centre of the zoo. Participated in daily evaluations, diagnostics, treatments, and necropsies of various zoo animals.

January 2008 — Mill Creek Veterinary Services — *Visalia, CA*
Two-week externship at a 14-vet dairy practice that services large dairy farms in Tulare and neighboring counties. Performed rectal palpation on hundreds of cows for reproductive evaluation. Assisted in calvings and surgeries.

August 2007 — Zoological Society of San Diego — *San Diego, CA*
Wildlife Disease Laboratory at the San Diego Zoo
Four-week externship at the Pathology Department of the San Diego Zoo and Wild Animal Park.

VOLUNTEER WORK (select)

Sept 2015 — Kwa-Zulu-Natal Sharks Board — *Umhlanga, South Africa*
During a two-week trip, performed necropsies on over 20 by-caught cetaceans (bottlenose and humpback dolphins, false killer whale) as part of a long-term health and disease assessment headed by Drs. Stephanie Plon (Nelson Mandela Metropolitan University) and Emily Lane (National Zoological Gardens of South Africa)

May 03 - May 08 — Cape Cod Stranding Network — *Buzzards Bay, MA*
Performed necropsies and Level A data collection of stranded marine mammals including cetacean and pinniped species.

March 06 & 07 — Rural Area Veterinary Services — *Donalsonville, GA and Hoopa, CA*
Humane Society of the United States
Performed physical exams, anesthesia, and sterilization surgeries of dogs and cats in underserved regions.

Aug 02 - Aug 04 — Volunteer Harbor Seal Trainer — *Woods Hole, MA*
Woods Hole Science Aquarium, NOAA/NMFS
Performed daily feedings of resident harbor seals through public training sessions. Assisted in veterinary procedures involving restraint techniques.

Sept 02 - Aug 03 — Penguin Colony Associate Volunteer — *Boston, MA*
New England Aquarium
Prepared diet for daily feedings, fed the three species of penguins, and logged feedings and behavioral observations.

Sept – Oct 03 — CTD Watch stander — *Arctic West*
Shelf Basin Interaction Research Cruise
Woods Hole Oceanographic Institution

Performed conductivity, temperature, depth instrument (CTD) casts during five-week oceanography research cruise. Assisted SCRIPPS PhD student with passive acoustic monitoring of marine mammals.

June - Aug 02 Research Animal Care Intern *Woods Hole, MA*
Marine Biological Laboratory
Provided daily care to numerous types of marine and freshwater species; maintained aquatic habitat systems. Participated in research of the natural behavior of zebrafish.

CERTIFICATIONS

NAUI Advanced Scuba Certification (2003) *Key Largo, FL*

PERSONAL

U.S. Citizen, DOB 8/8/80, Married, 2 children, 1 dog, 5 chickens
Interests: traveling, SCUBA diving, hiking, swimming, gardening, beer brewing, cooking
Travel destinations: England, Ireland, Spain, France, Netherlands, Morocco, South Africa, Costa Rica, Italy, Slovenia, Thailand, Japan, Indonesia, Arctic West, Canada, US and British Virgin Islands.