The Dissertation Committee for Claire Alison Smalley Certifies that this is the approved version of the following dissertation:

The Activation of the Inflammasome during Rickettsial Infection

Committee:

David Walker, MD, Supervisor or Mentor

Rong Fang, MD PhD

Sanjeev Sahni, PhD

Nisha Garg, PhD

Edward Miao, MD PhD

Dean, Graduate School

The Activation of the Inflammasome during Rickettsial Infection

by

Claire Alison Smalley, B.S. M.S

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Dedication

This dissertation is dedicated to my parents, who instilled the value of education, and to Josh, who carried the weight of graduate school.

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The Activation of the Inflammasome during Rickettsial Infection

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Rickettsiae are Gram-negative, obligately intracellular bacteria that infect endothelial cells and macrophages. The molecular mechanisms involved in interaction of rickettsiae with macrophages, however, remain poorly understood. In this dissertation, I have investigated the canonical and non-canonical inflammasome activation by rickettsiae in mouse macrophages. The central hypothesis is that activation and priming by lipopolysaccharide (LPS) of caspase-11 and ASC-dependent inflammasomes in macrophages is essential for host clearance of rickettsia through mediating the secretion of IL-1β, IL-18, and induction of pyroptosis. Specific Aim 1 focuses on the role of caspase-11-dependent inflammasome activation in host response against rickettsial infection and the activation mechanisms involved. The hypothesis of this aim is that caspase-11 is likely not protective in a mouse model of rickettsial infection. In vitro, inflammasome activation in mouse BMM is ASC dependent and partially caspase-11 dependent. Specific Aim 2 has more clearly demonstrated the role of rickettsial lipopolysaccharide in priming and activating the inflammasome in mouse macrophages. Rickettsial LPS can act as the inflammasome-priming signal 1 component through TLR4, although other priming mechanisms do exist

for inflammasome activation in macrophages during rickettsial infection. Additionally, rickettsial LPS transfected to the cytosol of macrophages indicates that rickettsial LPS can be sensed intracellularly by caspase-11, though the availability and structure of this ligand may contribute to low levels of activation through the caspase-11-dependent inflammasome activation pathway during infection. Taken together, our data, for the first time, illustrate the activation of inflammasome by rickettsiae in macrophages and the mechanisms involved.

TABLE OF CONTENTS

List of Figures

List of Illustrations

List of Abbreviations

Ab	Antibody
ADP	Adenosine diphosphate
AG	Ancestral group
AIM	Absent in myeloma
ASC	Apoptotic speck like protein containing CARD domain
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid assay
Bcl-2	B-cell lymphoma-2
BMDC	Bone marrow derived dendritic cells
BMM	Bone marrow derived macrophage
BSL3	Biosafety level 3
CARD	Caspase activation and recruitment domain
NAIP	NLR family apoptosis inhibition protein
CD	Cluster of differentiation
CO2	Carbon dioxide
CoA	Acetyl coenzyme A
COX	Cyclooxygenase
CS	Citrate synthase
CTL	Cytotoxic T lymphocyte
CXCL	C-X-C motif chemokine ligand
DAMP	Danger associated molecular pattern

DC	Dendritic cell
DKO	Double knockout
DMEM	Dulbeccos modified eagle medium
DNA	Deoxyribonucleic acid
DUSP1	Dual specificity protein phosphatase 1
ELISA	Enzyme linked immunosorbent assay
ERK-2	Mitogen activated protein kinase
Fig.	Figure
FoxP3	Forkhead box P3
G6PD	Glucose 6 phosphate dehydrogenase
GM-CSF	Granulocyte macrophage colony stimulating factor
GSDMD	Gasdermin D
H&E	Hemotoxylin and esoin
HO-1	Heme- oxygenase
Hpi	Hour post infection
IACUC	Institutional animal care and use committee
IFN	Interferon
IFNAR	Interferon alpha receptor
IgM	Immunoglobulin M
IL	Interleukin
IPAF	Ice protease activating factor
IRF	Interferon regulatory factor
ISF	Israeli spotted fever

IV	Intravenous
LBP	Lipopolysaccharide binding protein
LD	Lethal dose
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
NFkB	Nuclear factor kappa B
MARCO	Macrophage receptor with collagenous structure
MD-2	Lymphocyte antigen 96
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MyD88	Myeloid differentiation primary response gene 88
NK	Natural killer
NLR	Nod like receptor
NOD	Nucleotide binding oligomerization domain like receptors
NOS	Nitric oxide
Omp	Outer membrane protein
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PFU	Plaque forming units
Pi	Post-infection
PMN	Polymorphonuclear Cells
POP2	Pyrin domain only protein

PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
RIP2	Receptor interacting serine/threonine protein kinase
RIPA	Radioimmunoprecipitation assay buffer
RLR	RIG-I like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Sca	Surface cell antigen
SDS-PAGE	Sodium dodecyl sulfate- gel electrophoresis
SFG	Spotted fever group
SKO	Single knockout
SPG	Succinic acid phosphate glycine buffer
STAT	Signal transducer and activator of transcription
T3SS	Type 3 secretion system
T4SS	Type 4 secretion system
TCA	Tricarboxylic acid cycle
TG	Typhus group
TH1/2	T-helper 1 or 2
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRAM	TIR containing adaptor molecule
Treg	T regulatory cell
TRG	Transitional group

TRIF	TIR containing adaptor inducing interferon β
WASP	Wiscott Alrich syndrome protein
WT	Wild-type

Chapter 1

INTRODUCTION

Rickettsia australis, the etiologic agent of potentially deadly Queensland tick typhus and a member of the spotted fever (SFG) group of *Rickettsia*, infects macrophages, dendritic cells, and its main target, endothelial cells (Dantas-Torres 2007, Sexton 1991, Walker 1989). SFG rickettsiae were responsible for over 1900 infections in the US alone in 2010, and mortality can approach 60% if untreated (Dantas-Torres 2007, Parola 2013).

Rickettsiae are transmitted through the bite of an infected tick and can cause significant human disease. Rickettsial infections are often hard to diagnosis, due to general symptoms, such as a headache, fever, and myalgia. Clinical features of rickettsial diseases correspond to damage done to target cells and organs. SFG rickettsia-infected individuals exhibit increased levels of IFN-γ, TNF-α, IL-6, and IL-10 during the acute phase of infection (de Sousa 2007). Rickettsial damage to endothelial cells results in a multifocal lymphohistiocytic immune response by the host. Endothelial cell damage, and following vascular injury results in significant increases in vascular permeability.

While late stage symptoms are quite severe, initial symptoms imitate other viral and bacterial illnesses. Generalized symptoms in early rickettsial disease lead to a delayed diagnosis. Rapid, sensitive tests are not available for commercial use, and sensitive testing, such as PCR, requires that samples are sent out to a specialized center that can take days for a confirmatory diagnosis (Portillo 2017). When diagnosed promptly, rickettsial diseases can be treated successfully with doxycycline. Some patients, however, develop severe

sequelae as a result of rickettsial infection and can include deafness, impaired vision, behavioral disturbances and other neurological defects.

Because of severe late stage disease, debilitating sequelae, lack of rapid and sensitive diagnostics and, coupled with high mortality in untreated patients and increasing incidence (Parola 2013), there is a need for an effective rickettsial vaccine. Our research will contribute to this goal by investigating inflammasome-dependent defense mechanisms in myeloid cells. Macrophages are important producers of cytokines, and as a cell type that is one of the first to respond to infection (Murray 2011), understanding how *early* cytokine production relates to disease will advance the knowledge of the immune response to rickettsial infection.

Vaccine development for rickettsial diseases has been a difficult undertaking because of the diverse spectrum of rickettsial groups. Strong CD8 and CD4 T-cell responses clear rickettsial infections, but no vaccine has been developed using cross-protective T-cell epitopes. Little is known about the first interactions rickettsiae have with the innate immune system, including the first interactions of rickettsiae with professional antigen presenting cells. Typically, the cytokines produced by macrophages upon infection with bacterial or viral pathogens lead to other immune cells production of pro-inflammatory cytokines, which polarizes the subsequent adaptive immune response. In the last ten years, the intracellular innate immune signaling platform in macrophages, known as the inflammasome, has been researched closely as a mechanism of early host immune defense. The inflammasome plays an important role in accelerating appropriate pro-inflammatory immune responses for the rapid clearance of intracellular pathogens. The gap in knowledge in our study is that it is largely unknown how the inflammasome

responds to rickettsial infection, and the mechanisms involved in proper inflammasome activation in response to rickettsiae.

REVIEW OF LITERATURE

Rickettsiae and Rickettsial Disease

Introduction

Rickettsiae are small, coccobacillary alpha-proteobacteria, and are obligately intracellular bacteria (Wolbach 1923). Pathogenic rickettsiae in the typhus and spotted fever groups are the most well-known due to their high virulence and mortality, especially in the pre-antibiotic era (Wolbach 1925, Wolbach 1950). There are four groups based on phylogenetic analysis: Ancestral group (AG), transitional group (TRG), typhus group (TG), and spotted fever group (SFG) (Gillespie 2008). Rickettsiae have been described in a variety of host genera, and have been found all over the world (Parola 2013). Rickettsiae are most commonly associated with arthropods, including ticks, mites, and fleas.

The rickettsial genome is quite small as a result of reductive evolution (Andersson 1998). *R. prowazekii*, for example, contains slightly over 1 million base pairs and encodes about 840 genes (Andersson 1998). Rickettsiae grow independently of a number of host cell processes, including protein synthesis, cell division, and synthesis of DNA and RNA (Weiss 1972, Weiss and Dressler 1952). Rickettsiae do not synthesize amino acids or nucleosides and rely on the host cell to provide them (Andersson 1998, Austin and Winkler 1987). Rickettsiae parasitize host ATP through a bacterial membrane-bound ATP/ADP translocase (Wolf 1999). Rickettsiae import host ATP for hydrolysis to generate membrane potential (Wolf 1999). The significant number of ATP/ADP translocase genes in some species of rickettsiae indicates that rickettsiae use a significant amount of ATP, or

compensate for low cellular ATP in the late stages of infection. Some species have full TCA cycles and respiratory complexes; however, they must also obtain metabolic intermediates from the host in order to run these cycles (Winkler 1986). Additionally, they contain no genes for anaerobic glycolysis (Bovarnick and Snyder, 1949).

Pathogenesis of Rickettsial Disease

Spotted fever group and typhus group rickettsiae mainly target endothelial cells, while macrophages and dendritic cells are considered secondary targets. Early in infection, rickettsiae target endothelia located in small and medium blood vessels (George 1993, Silverman 1984). As infection progresses, rickettsial dissemination through the bloodstream, and perhaps through immune cells such as macrophages, leads to disease of larger vessels (George 1993, Silverman 1984). For infection to occur, rickettsiae must adhere to and enter a metabolically viable host cell. Adherence is dependent upon surface cell antigen (Sca) proteins. At least 15 Sca genes have been identified in rickettsiae; however, the most conserved are *sca0*, *sca1*, *sca2*, and *sca5* (Blanc 2005). OmpA (encoded by *sca0*) and OmpB (encoded by *sca5*) proteins in particular play a critical role in attachment and invasion into the host cell. While OmpB has been shown to be sufficient for rickettsial binding and invasion (Chan 2009), there are likely other Sca proteins involved in this process.

Host cells take up the bacteria via induced phagocytosis in non-phagocytic cells by caveolin- and clathrin-dependent endocytosis. Rickettsiae bind to host cell Ku70 via OmpB

(Martinez 2005). Ku70, a DNA-dependent protein kinase, is present in lipid rafts at the surface of the host cell and has been suggested to have a function in cholesterol dependent invasion of the host cell (Martinez 2005, Ramm 1976). Binding of Ku70 by OmpB, which is associated with the host β_1 integrin, sets off a signaling cascade resulting in recruitment of Cdc42, phosphoinosotidyl 3-kinase, and src-family kinases. The signaling cascade causes phosphorylation of focal adhesion kinase, inducing Arp2/3 dependent phagocytosis of the bacteria (Martinez 2004).

SFG rickettsiae also utilize OmpA for adherence, which binds to host cell $\alpha_2\beta_1$ integrin (Monferran 2004). Other Sca proteins involved in invasion into host cells include Sca1 and Sca2. Sca1 is an autotransporter described in *R. conorii* (Riley 2010) and is necessary for *R. conorii* adherence to the host cell. Sca2 is sufficient for *R. conorii* adherence and invasion of host cells (Cardwell 2009). Interestingly, Cardwell et al. have suggested that Sca2-dependent adherence and invasion is independent of Ku70, indicating that additional mechanisms may be present for rickettsial entry into the host cell. Sca2 has also been implicated in mediating Arp2/3 independent mobility in SFG rickettsiae (Haglund 2010, Serio 2010).

Upon internalization, rickettsiae escape the phagosomal vacuole into the cytosol of the host cell where they live freely (Whitworth 2005). Once in the phagosomal vacuole, the microcapsular layer of rickettsiae interacts with the host vacuolar membrane. Here rickettsia produces hemolysin C, via the *tlyC* gene, and phospholipase D, via the *pldA* gene, for membrane disruption. Within thirty to fifty minutes, the membrane becomes osmophilic, and holes begin to appear, allowing rickettsia to escape into the cytosol. Therefore, rickettsiae presumably escape before phagolysosomal fusion.

Once in the cytosol, spotted fever group rickettsiae will induce polymerization of cellular actin by RickA, a protein mediating actin polymerization in SFG rickettsiae and recruitment of Arp2/3 complex. RickA contains a Wiscott-Aldrich syndrome protein (WASP) domain, enabling it to bind to actin monomers and the Arp2/3 complex (Gouin 2004, Jeng 2004). RickA is likely an important component of rickettsial virulence, as it is one of the proteins that enable SFG rickettsiae to move from cell-to-cell, generating a disseminated infection (Simser 2005). Typhus group rickettsiae, however, do not possess mechanisms of actin polymerization, or in the case of *R. typhi*, relatively little actin polymerization (Heinzen 2003). Typhus group rickettsiae grow and divide in the host cytosol until host cell lysis frees them into the extracellular space, allowing them to infect nearby cells (Schaechter 1957).

Components of a type 4 secretion system (T4SS) have been identified in the rickettsial genome (specifically *R. typhi*). However, expression of this system in host cells during rickettsial infection has not been reported (McLeod 2004). Rickettsial VirB/D T4SS has been annotated in the genome (Gillespie 2010), but given the high level of divergence of VirB between bacterial species, the structure of these proteins could vary significantly. Given the genetic intractability of rickettsiae and undefined expression levels of the VirB/D, the function and effectors translocated by this system are still unknown.

Endothelial Cell Responses to Rickettsial Infection

Endothelial cell damage from rickettsiae leads to endothelial cell dysfunction and endothelial cell activation (Rydkina 2010). This dysfunction results in acute phase

responses and alteration in coagulation. SFG rickettsial infection in endothelial cells *in vitro* results in increases in expression of tissue factor (Sporn 1994), E-selectin (Sporn 1993), and vascular adhesion molecules (Dignat-George 1997). There are also increases in plasminogen activator inhibitor (Drancourt 1990) and increase in secretion of von Willebrand factor (Teysseire 1992). Infection with TG rickettsiae increases levels of prostaglandins (Walker 1990). The vasoactive properties of this response are mediated by cyclooxygenase (COX), and the increase of this enzyme potentially increases vasculitis seen during rickettsial infection (Rydkina 2006). Increases in vascular permeability is a hallmark of rickettsial infection. Changes in the distribution of p120 and beta-catenin adherens junctions result in gaps between endothelial cells, likely due to nitric oxide independent mechanisms including increases in TNF-α and IL-1β (Woods 2008). Increases in endothelial cell activation and vasoactive mediators during infection result in vascular permeability and resulting edema.

Endothelial cells, upon infection with SFG rickettsiae *in vitro*, secrete IL-6, IL-8, and IL-1α (Rydkina 2006) through activation of MAPK and NF-kB dependent pathways. IL-6 is thought to play a role in mediating production of acute phase proteins, while IL-8 is thought to act as a chemoattractant for leukocytes (Kaplanski 2005). IL-1α, although fixed to the endothelial cell membrane, can induce secretion of IL-8 and IL-6 in nearby endothelial cells. Endothelial cells also increase expression of chemokine receptors such as CXC chemokine ligand (CXCL) 9 and 10 (Valbuena 2004). Inflammatory cytokines and chemokine production by endothelial cells occur at the peak of the CD8+ T-cell response, but it is still unclear whether these responses are protective or contribute to disease pathogenesis (Valbuena 2004). Additionally, pro-inflammatory cytokines IFN-γ, TNF-α,

and IL-1β, while important for induction of nitric oxide synthesis resulting in reducing bacterial burden, also leads to increasing oxidant activity that likely contributes to endothelial cell damage and dysregulation (Woods 2008).

Rickettsial infection damages endothelial cells by increases in oxidative stress mechanisms, including the production of superoxide anion, hydrogen peroxide, and hydroxyl radical (George 1993). These increases in oxidative stress coupled with upregulation in mRNA for antioxidants, such as hemeoxygenase-1 (HO-1) (Rydkina 2002), superoxide dismutase and glucose-6-phosphate dehydrogenase (G6PD), indicate that cell injury caused by oxidation and related mechanisms is an important pathology during rickettsial infection. Human studies show that patients that are deficient in G6PD are significantly more likely to die from rickettsial infection and experience more severe forms of the disease (Silpapojakul 1996). Activation of oxidative stress mechanisms including hydrogen peroxide and nitric oxide by stimulation of pro-inflammatory cytokines IFN-γ, TNF-α, and IL-1β have been shown to induce rickettsial killing (Feng 2000).

As obligately intracellular bacteria, rickettsiae induce a shift in endothelial cells toward increased NFκB activity for the prevention of programmed cell death mechanisms. The inhibition of NFκB in infected endothelial cells induces caspase-8/9 activation for pro-apoptotic pathways; therefore, activation of NFkB to maintain mitochondrial structure to protect against infection-induced apoptosis is essential for rickettsial survival (Joshi 2003). Infection with rickettsiae also results in inhibition mediated by NFκB for B-cell lymphoma proteins (Bcl-2) transcription, indicating that rickettsiae repress apoptosis in host endothelial cells for their intracellular survival and preservation of their replication niche (Joshi 2004).

Clinical Features of Rickettsial Infection

Typically, symptoms of rickettsial infections manifest between 2-14 days post-infection, with the most common time ~7 days post-infection. Symptoms of rickettsial infection are a severe headache, fever, nausea, and vomiting (Biggs 2016). Often patients will present with a maculopapular or petechial rash or an eschar at the site of a tick bite, which develops between three and 5 days after onset of fever (Walker 1987). Rocky Mountain spotted fever patients typically do not have eschars, but do develop a rash; Mediterranean spotted fever patients will usually present with eschars (tache noir) (Drage 1999, McGinley-Smith 2003, de Sousa 2005).

Disseminated and late stage infections can cause complications such as cerebral edema and pulmonary edema (Walker 1980, Marin-Garcia 1984). Patients may have necrosis of the extremities due to vascular dysfunction and destruction, resulting in amputation of digits and limbs (Archibald 1995). In patients that recover from severe disease, permanent sequelae may be present. Reported sequelae in patients include hearing loss, brain damage, peripheral neuropathy, motor dysfunction and speech disorders (Wei 1999, Archibald 1995). In fatal cases of rickettsioses, autopsies show interstitial pneumonitis and proteinaceous fluid in the lungs and alveolar spaces. Rickettsia is found in microvascular endothelium in brain tissues, with perivascular inflammation and infiltration of inflammatory cells (Roggli 1985). If not treated, Rocky Mountain spotted fever can have a case fatality rate of between 20-60%, while Mediterranean spotted fever has a case fatality rate of 4% (Parola 2013). Of the typhus group rickettsiae, murine typhus

has a case fatality rate of 1%, and epidemic typhus has a case fatality rate of 15%. Early treatment improves mortality rate, but if treated late, mortality rates remain relatively high (Biggs 2016).

Diagnosis of rickettsial disease is difficult, due to general symptoms and lack of diagnostic tests that are effective early in the disease course (McDade 1991). The strongest evidence of rickettsial infection is the comparison of acute and convalescent serum for a 4-fold or greater increase in antibody titer. PCR for rickettsial DNA is specific. However, this depends on the level of bacterial DNA in the serum. Too little DNA from serum samples taken in the windows before and after the peak of infection lead to false negative or delayed diagnoses, and is therefore not sufficiently sensitive.

Treatment of rickettsial infection, if diagnosed in time, is very responsive to tetracyclines, most notably doxycycline (Munoz-Espin 1986, Purvis 2000), which acts as a bacteriostatic agent.

Animal Models for Spotted Fever Rickettsioses

The best-known model for spotted fever rickettsioses is the *Rickettsia rickettsii* guinea pig model (Walker 1977). Guinea pigs are highly susceptible to *R. rickettsii* infection, and infection induces fever and disseminated vasculitis (Walker 1977), closely replicating human infection. Unfortunately, few immunological reagents and no gene-deficient animals are available for studying the immune responses in guinea pig models.

Other models of spotted fever group rickettsioses include the C3H/HeN mouse model for *Rickettsia conorii* (Walker 1997), which is to date the best mouse model for

rickettsioses. Upon infection with an inoculum slightly higher than LD50, infection of the endothelium is established after 24 hours. Disseminated vascular injury occurs over the course of infection, along with endothelial activation and hemostatic changes typical of rickettsial infection in humans. While functioning as an excellent model for investigating the pathology of rickettsial infection, the C3H/HeN background does not have gene deficient knockout mice, and cannot be used for mechanistic studies.

The model for TRG rickettsia *R. australis* currently provides the best model of rickettsioses utilizing the C57BL/6J background mice. *R. australis* causes invasive disease but maintains low toxicity in adult mice (Bell and Pickens 1953). The C57BL/6J mouse model also mimics the disseminated endothelial infection and pathology of SFG rickettsioses seen in human patients (Walker 2001, Feng 1993). Additionally, the C57BL/6J mouse background enables mechanistic studies involving gene knockout mice for host defense against rickettsial infection.

Arthropod Vectors and Transmission

Spotted fever group rickettsiae are best known by their arthropod vector, the tick. Ixodid ticks (hard ticks), especially *Dermacentor* ticks, are responsible for the majority of SFG rickettsioses, *R. rickettsii*, in the Americas (Burgdorfer 1975). SFG rickettsia is maintained by transstadial (passing infection from one life stage to the next, e.g. nymph to adult) and transovarial (female to egg to larva) transmission, and acquisition of rickettsia from an infected mammalian host (Burgdorfer 1962, Burgdorfer 1966). It has been hypothesized that mammals may serve as an amplifying host for transmission to larvae as

nymph ticks. Some studies have indicated interference and competition between rickettsial species for transmission and transovarial maintenance in their tick vector (Macaluso 2002), which may affect the distribution and number of ticks infected with highly pathogenic SFG rickettsia.

Tick saliva has been implicated in promoting rickettsial infection (Kovar 2004) by decreasing activity of responding immune cells such as NK cells and dendritic cells (Kubes 1994, Cavassani 2005) and reducing production of pro-inflammatory cytokines such as IL-12 and IFN-γ (Kotsyfakis 2006). Immunomodulatory mechanisms of tick saliva, mediated in part by sialostatin, have been shown to dampen innate immune responses to both tickbite as well as the invading rickettsiae (Kotsyfakis 2006).

Typhus group rickettsia, however, is spread by the flea, *Xenopsylla cheopsis*, or louse, *Pediculus humanus* (Wolbach 1925). Both fleas and body lice bite multiple hosts, depositing rickettsiae-infected feces that are scratched into the bite site. Fleas and body lice can therefore spread TG rickettsia to multiple individuals (Azad 1997). *R. prowazekii* infected lice succumb to infection with this rickettsia 1-2 weeks after infection. Epidemic typhus occasionally breaks out into human populations; however, the natural reservoir has not yet been confidently identified (Azad 1990). Patients with recrudescent *R. prowazekii* infection could be a source of new outbreaks of epidemic typhus (Azad 1997). *R. typhi*, the causative agent of murine typhus, spread by oriental rat fleas found on rats and cat fleas found on opossums, is not lethal to its arthropod host, and can thus be maintained transovarially (Azad 1985).

Innate Immune Response to Rickettsial Infection

After initial damage to and activation of endothelial cells, acute phase responses begin to recruit innate immune cells to sites of rickettsial infection. Macrophages and monocytes may contribute to establishment of rickettsial infection and the resulting pathogenesis (Curto 2016). Macrophages, natural killer cells, and dendritic cells produce T-cell activating cytokines and chemokines during rickettsial infection.

Rickettsia infects bone marrow derived dendritic cells (BMDC) *in vitro*. Once rickettsia infects DC, DC upregulate production of both receptors and cytokines (Fang 2007). Receptors such as CD40, CD80, and MHCII are increased, and cytokine secretion of IL-2, IL-12p40 and IL-23 also increases, which is important for TH1 polarization (Fang 2007). DC is able to present rickettsial antigens to both CD4+ T-cells and CD8+ T-cells, and stimulate the production of IFN-γ (Fang 2007). Studies have also shown that infected DC can both present to and activate CD8+ T-cells in the absence of CD4+ T-cells. Indeed, transfer of rickettsia-infected DC to lethally infected mice protects against death by limiting bacterial burden, whereas LPS stimulated control DC were partially protective (Jordan 2009). These results suggest a role for TLR4 in DC-mediated protection, which results in increased T-cell and NK cell activation, as well as increased IFN-γ.

Following on this study, Fang *et al* delved into the mechanisms by which DC mediate protection *in vivo*. Infecting DC from *R. conorii*-susceptible C3H/HeN mice and DC from resistant C57Bl/6J mice shows that B6 DC internalize and kill rickettsiae more effectively (Fang 2007). These DC express higher levels of MHCII, IL-12p40, and are more effective at stimulating IFN-γ priming for T cells. In contrast, C3H/HeN DC do not

differentiate CD4+ T-cells and instead result in expanding the population of FoxP3+CD4+ Treg cells (Fang 2009).

Further DC studies using *R. australis* in C57BL/6J mice show that MyD88-deficient mice have decreased expression of MHC-II and lower production of IL-12p40 (Bechelli 2016). Additionally, serum levels of IFN-γ were significantly decreased in MyD88-deficient mice, further emphasizing the role DCs have in bridging the innate and adaptive immune responses to rickettsiae. The role of MyD88 as an adaptor protein for TLR2, TLR4, and TLR9 suggests the importance of innate signaling mechanisms in downstream adaptive immunity (Bechelli 2016).

Data from previous studies have indicated that priming through TLR2, TLR4, and TLR9 may contribute to host immunity during rickettsial infection. TLR2 increases cell activation upon infection with *R. akari*, a TRG rickettsia (Quevedo-Diaz 2010). A TLR4 point mutation in C3H/HeJ mice causes increased susceptibility to *R. conorii* infection (Jordan 2008). Treatment before infection with a TLR9 ligand increases protection against *R. australis* infection (Xin 2012). Additionally, the role of NOD receptors in signaling is hypothesized for generating anti-rickettsial responses (Sahni 2013). NOD1 and NOD2 are intracytoplasmic sensors of components of peptidoglycan and potentially contribute to protection via inducing NFκB activity.

NK cells are an important IFN-γ producing cell type early in infection. Protection in rickettisal infection has been associated with NK production of IFN-γ (Billings 2001). Fang et al. have found that NK cells mediate the innate phase of host immune response during *R. conorii* infection through production of IFN-γ (Fang 2012). NK cells also

contribute to protection from endothelial cell damage during rickettsial infection, likely through perforin production (Fang 2012).

Though not well characterized in the context of rickettsial infection, dermal macrophages are thought to be some of the first cells rickettsiae infect upon entering the host (Curto 2016). Macrophages produce significant levels of pro-inflammatory cytokines (Murray 2011). Relatively few studies detail rickettsial killing mechanisms of macrophages; however, it has been reported macrophages reduce tryptophan availability by increasing expression of degradative enzymes to starve the bacteria (Feng 2000). While the innate immune responses produce cytokines and chemokines, robust cellular immunity is an important factor in host immunity during rickettsial infection.

Adaptive Immune Response to Rickettsial Infection

A strong adaptive immune response by CD8+ and CD4+ T-cells is required for clearance of rickettsiae. Walker et al. found that CD8+ depleted mice were persistently infected during an ordinarily sublethal challenge with *R. conorii*, and a high proportion of these animals died due to uncontrolled infection. While it is noted that CD4+ cells are an important source of IFN-γ during an infection, which helps endothelial cell rickettsicidal activity, CD4+ cell depletion did not alter the outcome of infection (Walker 2001). CD4+ T-cells are prolific producers of IFN-γ, further polarizing CD8+ T-cells to increase rickettsicidal activity.

CD8+ T-cells are essential for producing sterilizing immunity during rickettsial infection. Studies have shown that MHC I and not MHC II cells infected with *R. typhi* were

killed via T-cell mediated cytotoxicity (Rollwagen 1985). Additional studies have confirmed this report, demonstrating that *R. conorii*-infected endothelial cells and *R. australis*-infected splenocytes are also targeted by cytotoxic T-lymphocytes (CTLs) via MHC-I (Rollwagen 1986). Walker et al. have found that CD8+ T-cells are necessary and sufficient to eliminate rickettsiae through perforin and granzyme (Walker 2000). Mice deficient in MHC-I receptor are 50,000 times more susceptible to infection with *R. australis* than wildtype mice (Walker 2000). IFN-γ deficient mice, as well as perforin-deficient mice, are over 100 times more susceptible and over 1000 times more susceptible to rickettsial infection, respectively (Walker 2000). Together, these data suggest that cytotoxic activity of CD8 T-cells is more important in altering the outcome of infection than the IFN-γ that is produced. Additionally, these data indicate that other cytotoxic capacities of CTLs, such as Fas/FasL or granulolysin activities, could be responsible for clearing infected cells (Walker 2000).

Protection against re-infection is partially mediated by antibody production. Antibodies raised against OmpA and OmpB are protective against lethal rickettsial infection in a passive protection study (Feng 2004a), but antibodies against these proteins appear after convalescence and therefore do not contribute to clearing initial infection (Feng 2004a). Protection by these antibodies is attributed to increased opsonization and phagocytosis of extracellular bacteria (Feng 2004b). Studies suggest that antibodies against rickettsiae interfere with the ability of the bacteria to escape the phagosomal vacuole, and lead to increased intracellular killing through nitric oxide production or tryptophan degradation (Feng 2004b).

Role of IL-1 Family Cytokine in Adaptive Immunity

IL-1 has been implicated as an important cytokine for enhancing T-cell proliferation and cytotoxic responses. CD8+ T-cell expansion in lymph node and spleen is associated with increased expression of IL-1RI receptor (Ben-Sasson 2013). CD8+ T-cell expansion is correlated with IL-1-mediated suppression of apoptosis in this cell population (McAleer 2007, von Rossum 2011), which is also in CD4+ T cells. Entry or retention in the liver or lung tissue by antigen-educated CD8+ T-cells is dependent on IL-1 at the tissue level. IL-1, therefore, appears to be an important mediator in vascular endothelial cell permeability for effector cells (Ben-Sasson 2013). CD8+ T-cell dependence on IL-1 for regulating adhesion and transmigration into the tissue through chemokine and adhesion molecule expression has been well-described (Ding 2000, Pietschmann 1992, Wang 1993, Kanda 1995). Priming of CD8+ cells increases levels of granzyme B expression and cytotoxic T-cell activity; however, increased granzyme B as well as increased IFN-γ production by CD8+ cells requires contact with other cells that have increased IL-1RI receptor expression (Ben-Sasson 2013). These data suggest that other stimulating factors coordinate with IL-1 to enhance antigen-specific T-cell expansion (Thompson 2012).

The Inflammasome

Introduction

The inflammasome is a multi-protein intracellular signaling platform. Briefly, the inflammasome consists of a sensor protein (or Nod-like protein, NLR), an adaptor protein (ASC, or apoptotic speck-like containing protein), and the inactive zymogen, pro-caspase-1. Upon stimulation, the inflammasome will assemble and transduce signals, resulting in the autoproteolytic cleavage of pro-caspase-1 to activated caspase-1. Activated caspase-1 will continue to cleave the pro- forms of IL-1β and IL-18 into their active components, to be released from the cell.

Mechanism and Regulation of Canonical Inflammasome Activation

Inflammasomes function by two separate signals. Signal 1 is a priming signal, stimulated through TLR or RLR signaling. TLR signaling causes translocation of NF-kB to the nucleus and transcription of pro-inflammasome components, including pro-caspase-1 or pro-caspase-11, and pro- IL-1β and pro-IL-18. Signal 2 is an activating signal that is highly dependent on the type of cellular assault (Kofoed 2011), and the NLR transducing the signal can be stimulated by toxins, bacterial components such as flagellin or intracellular LPS, ROS production, and ion flux.

In classical inflammasome, NLRP3 is activated via a number of cellular perturbations, resulting in ion flux of potassium and sodium, disruption to the mitochondria, ROS, or phospholipid cardiolipin (Sutterwala 2006, Mariathasan 2006, Kanneganti 2007). NLRP3 is believed to sense the ion flux or cellular perturbations that change redox status. It is possible that stress to the endoplasmic reticulum (ER) results in changes in concentrations of Ca^{2+} ions, resulting in damage to the mitochondria, increasing

ROS, culminating in the activation of NLRP3. Regardless of the stress, NLRP3 activation recruits ASC and pro-caspase-1 (Sutterwala 2006) for the processing of pro- IL-1β and pro-IL-18.

IPAF is activated by components of bacterial secretion systems (Miao 2006). Components of T3SS and T4SS, and even flagellin activate IPAF, and *Salmonella*, *Shigella*, *Legionella*, and *Pseudomonas* all activate this NLR. The CARD domain of IPAF/NLRC4 interacts directly with the CARD domain of pro-caspase-1, eliminating the need for ASC; however, infections with some bacteria require ASC adaptor activity for the optimal production of inflammasome-related cytokines. IPAF can associate with other sensor proteins, such as NAIP, and downstream, cell death resulting from NLRC4 activation is ASC-independent (Mariathasan 2004). Direct interaction of NLRC4 with a ligand has not yet been studied, raising the possibility that NLCR4 functions similarly to NLRP3 as an intracellular sensor for a common pathway for PAMP signaling (Kofoed 2011).

NRLP1 is activated by toxins, particularly anthrax toxin. NLRP1 contains a CARD domain and therefore does not need to interact with ASC for signaling capabilities (Hsu 2008). The only known ligand for NLRP1 is anthrax lethal toxin. K+ efflux may play a role in activation of this NLR (Hsu 2008).

AIM2 is activated by dsDNA (Burckstummer 2009). AIM2 is not an NLR, but a HIN-200 protein. AIM2 directly binds dsDNA, and ASC adaptor function is essential for full inflammasome activation (Burckstummer 2009). AIM2 can bind viral, bacterial, or eukaryotic DNA (Hornung 2010).

Mechanism and Regulation of Non-canonical Inflammasome Activation

The non-canonical inflammasome is marked by recruitment and activation of caspase-11. Caspase-11 (caspase-4 and caspase-5 in humans) is an inflammatory caspase, similar to caspase-1 (Kayagaki, Aachoui 2013). The only known ligand for caspase-11 activation is hexacylated LPS from Gram-negative bacteria (Kayagaki 2011, Hagar 2011). Caspase-11 has been reported to facilitate other cellular functions, including the modulation of actin polymerization to promote the fusion of phagolysosomes containing pathogenic bacteria (Akhter 2012). This function of caspase-11 may be relevant in non-LPS containing infectious disease models, as studies have reported host protection in response to *Aspergillus fumigatus*, fungi which do not possess LPS. Host control of infection is dependent on caspase-1 and caspase-11 (Karki 2015, Man 2017). Others have reported that *Ehrlichia*, a close relative of rickettsiae, activate caspase-11 during infection (Chattoraj 2013). *Ehrlichia* also does not encode LPS (Chattoraj 2013), and it has been suggested that activation of caspase-11 might induce actin-mediated phagosomal activity to eliminate or restrict pathogens *in vivo* (Man 2017).

Non-canonical inflammasomes are primed via TLR, Type I, or Type II IFN. One of the best-described mechanisms for priming of caspase-11 occurs through IFN-alpha and IFN-β stimulation through the IFNAR, resulting in STAT1 activation and pro-caspase-11 transcription. IFN-γ may also prime pro-caspase-11 through STAT1 activation (Broz 2012, Rathinam 2012).

Pro-caspase-11 detects intracellular LPS via guanylate binding protein (GBP) assistance (Pilla 2014). GBPs are recently described proteins that are inducible by IFN signaling and

have been shown to be involved in lysing vacuoles containing bacteria to expose them to the cytosolic space, where they can be recognized by the inflammasome (Finethy 2015). After pathogen detection, pro-caspase-11 undergoes autoproteolytic cleavage, then cleaves the N-terminal of Gasdermin D, which goes on to form pores in the membrane of the host cell. Caspase-1 has also been shown to cleave Gasdermin D in certain circumstances (He 2015).

Gasdermin D is the relatively recently discovered executioner of pyroptosis, or inflammatory cell death (Shi 2015, Kayagaki 2015). Pyroptosis is marked by caspase-1 or caspase-11 activation, resulting in cellular contents spilling into the extracellular space (Liu 2016). The cellular characteristics of pyroptosis are similar in caspase-1 dependent and caspase-11 dependent versions: cell swelling, Annexin V positivity, and TUNEL positivity. Mitochondria start to lose membrane integrity, and chromatin condensation can be seen, while DNA laddering is markedly absent (Liu 2016). Pyroptosis is considered more inflammatory than necrosis due to the activated inflammatory caspases, and significantly more inflammatory than apoptosis, which is immunologically silent. Pyroptosis is considered to be an important mechanism of cellular defense against invading pathogens. Not only does pyroptosis result in spilling of the activated and inflammatory caspases for increased immune cell recruitment, pyroptosis exposes the invading pathogen to the extracellular space (Liu 2016). Neutrophils responding to this inflammatory release can take up pathogens and kill them via ROS (Jorgenson 2016). Another function of Gasdermin D is its proposed role in the secretion of IL-1β (He 2015). While there are many mechanisms of IL-1β secretion, it is possible that Gasdermin D facilitates the passive release of active IL-1β through the pores formed during pyroptosis.

Gasdermin D cleavage results in pyroptosis and canonical NLRP3 activation via ion flux (Yang 2015). Upon activation of the caspase-11 dependent canonical inflammasome, NLRP3 can then signal through ASC and caspase-1 to cleave pro- IL-1β and pro-IL-18 for secretion from the cell. Alternatively, caspase-11 can bypass NLRP3/ASC and induce pro-caspase-1 to undergo autoproteolytic cleavage, to then cleave pro-IL-1β and pro-IL-18 into their active forms (caspase-11 dependent noncanonical inflammasome). It is not yet understood how these different pathways are programmed.

LPS and Innate Immunity

LPS a component of the extracellular "slime layer" of Gram-negative bacteria. LPS is recognized by TLR4 on host cells. TLR4 is a conserved Toll-like protein and is present in a wide variety of genera, a testament to its importance in host defense in a variety of life forms. TLR4 works in concert with two adaptor proteins, MyD88 and TRIF. Signaling through one or both of these adaptor proteins leads to priming of inflammasome responses, as well as priming for other cellular defense mechanisms (Medzhitov 1998, Yamamoto 2003, Kawai 1999).

LPS consists of three domains: Lipid A, the core oligosaccharide, and the O-antigen (Alexander 2001). Lipid A is comprised of a disaccharide backbone with up to six acyl chains (hexacylated). Most enteric bacteria, including *Salmonella sp.* and *E. coli*, produce hexacylated LPS, though numbers of acylations vary widely amongst families of bacteria (Galanos 1993). The core oligosaccharide consists of multiple sugar residues, and the O-

antigen consists of repeating sugar residues (Raetz 2002). Both have antigenic properties, but the core oligosaccharide antigen is less potent than that of the O-antigen (Raetz 2002).

LPS is present in the membrane of Gram-negative bacteria, and cannot be sensed by the immune system unless it released from the bacterial membrane. TLR4 together with MD-2 bind free LPS from the extracellular space or serum (Miyake 2006). When bound in the serum, LPS sensing requires lipid A binding protein (LBP). Once bound to LPS, LBP can transfer LPS to CD-14 on host cells, which then transfers LPS to MD-2 (Wright 1990). MD-2 bound LPS holds five of the six acyl chains in a groove, leaving the sixth acyl chain free to interact with TLR4. The amount of acyl chains on LPS directly correlates with its ability to induce signaling from TLR4 (Raetz 2002); LPS with four (tetraacylated) or fewer acyl chains are not antagonistic to TLR4. Acylation is important for caspase-11 mediated recognition of LPS as well; tetraacylated LPS is not recognized by caspase-11 either (Hagar 2011).

Many Gram-negative organisms take advantage of this variability of recognition of LPS by TLR4 and caspase-11. *Yersinia pestis* and *Francisella tularensis* exemplify this behavior. At environmental temperature, *Y. pestis* produces hexacylated LPS, but at host body temperature, *Y. pestis* alters its lipid A structure to produce tetraacylated LPS which cannot be recognized by TLR4 or caspase-11. These mechanisms enable *Y. pestis* to evade immune detection (Knirel 2005). *Helicobacter pylori* and *Porphyromonas gingivalis* both alter their lipid A structure for increased virulence as well (Gaddy 2015, Curtis 2011).

Very little is known about rickettsial LPS compared to the LPS of enteric organisms that are genetically tractable and easily grown, such as *Salmonella sp.* and *E. coli*. Few structural studies have been done using rickettsial LPS, likely due to a combination of the

large quantity and high purity of LPS needed for structural studies and the relatively low abundance of LPS found on rickettsial organisms. The rickettsial intracellular lifestyle contributes to this problem, as rickettsiae can only be grown in eukaryotic cells, including Vero, L929, or chicken embryos.

One such structural study of rickettsial LPS revealed that typhus group lipid A contains tri-, tetra-, and hexacylated lipid A species in varying quantities (Fodorova 2005). A significant minority of LPS species was not hexacylated, which may account for various studies that have indicated that LPS is non-endotoxic (Jordan 2008) and do not induce a Schwartzman's reaction in rabbits.

The O-antigen of rickettsial LPS is unique in that it lacks a heptose, one that is normally present in the O-antigen of other enteric pathogens, including *Salmonella* (Fodorova 2015, Amano 1998). Because the O-antigen is critical for host innate immune sensing, the synthesis of O-antigen without a heptose may aid in rickettsial evasion of the immune system.

Inflammasome in Anti-microbial Immunity

Caspase-1 and caspase-11 have been reported to be involved in host defense against many different types of pathogens, including bacteria, viruses, and fungi. Gram-negative bacteria are commonly associated with caspase-1 and caspase-11 activation; however, Gram-positive bacteria, viruses, and fungi induce host defenses related to inflammasome activation. Caspase-1/11 double knockout mice have decreased survival when infected with Gram-positive *Staphylococcus aureus* and *Streptococcus agalactiae* (Hanamsagar

2014, Costa 2012), but while these mice have no increase in bacterial burden in a model of *Staphylococcus* infection, they exhibit higher bacterial load in kidneys and lungs in *Streptococcus* models of infection (Costa 2012). Inflammasome activation with Gram-positive bacteria likely occurs through some component of their cell wall (Albiger 2007), or virulent proteins produced, such as *Bacillus anthracis* lethal toxin (Moayeri 2010).

Some viral infection models do not show any differences in survival in caspase-1/11 deficient mice, such as encephalomyocarditis virus (Rajan 2011). There is a growing body of literature; however, that shows viruses such as West Nile virus and influenza A virus are restricted by caspase-1 and caspase-11 (Thomas 2009, Ramos 2012). Caspase-1/11 mice exhibit decreased survival when infected with influenza A or West Nile virus. Mice infected with influenza A virus also have decreased levels of inflammasome-associated cytokines IL-1β and IL-18 and decreased IFN-γ producing CD4 and CD8 T-cells (Thomas 2009). Viral stimulation of proteins such as RIG-I and MAVS have been associated with inflammasome activation, often working in concert with sensors such as AIM2 and NLRP3 (Poeck 2010, Franchi 2014).

Protists and fungi activate caspase-1 and caspase-11. In fungal infections, caspase-1/11 knockout mice are susceptible to *Aspergillus fumigatus* infection, exhibiting reduced survival and increased organ damage compared with wild-type mice (Man 2017, Karki 2015). *Candida albicans* and *Paracoccioides brasiliensis* infections in caspase-1/11 knockout mice also cause increased mortality compared to wild-type mice (Hise 2009, Ketelut-Carniero 2015). The contributions of caspase-1 and caspase-11 to host defense are not yet understood in fungal infections, though caspase-11 modulation of phagosomal fusion with lysosomes may be a contributing factor (Ahkter 2015). The inflammasome

response to protists is varied. Infecting caspase-1/11 double knockout mice with *Leishmania major* and infecting caspase-11 single knockout mice with *Toxoplasma gondii* result in increased survival, demonstrating that inflammatory caspases likely play a role in establishment of infection or pathogenesis of the organism (Gurung 2015, Gorfu 2014). Other protists, including *Leishmania amazonensis* and *Trypanosoma cruzi*, cause decreased survival in caspase-1/11 knockout mice, suggesting a role for inflammatory caspase-mediated protection in these models (Lima-Junior 2013, Silva 2013, Dey 2014). The life cycle phases and variety of antigens in these lifecycles further complicates studying the interactions of protists with the inflammasome.

Inflammasome Response to Gram-negative Bacteria

Gram-negative bacteria typically activate the inflammasome in a number of ways. A number of different secretion systems (T3SS and T4SS, sensed by NLRC4/NAIP5), LPS (sensed by caspase-11), toxins (sensed by NLRP3 and NLRP1a/b), and outer membrane proteins are detected by one or more NLRs or other sensor proteins.

Due to the volume of virulence mechanisms Gram-negative human pathogenic bacteria possess, they have evolved mechanisms of inflammasome evasion, which attests to the importance of the inflammasome in vivo. *Salmonella*, *Listeria*, *Yersinia,* and *Burkholderia* all possess mechanisms of evasion. *Yersinia* produce proteins that inhibit activation of caspase-1, *Listeria* produces reduced amounts of flagellin, and *Burkholderia* reduces killing by PMN, likely in response to the downstream consequences of inflammasome activation (exposing bacteria to the extracellular space). Inflammasomes

significantly delay infection by human-adapted pathogens and are protective against opportunistic infections (Maltez 2016).

One of the most well-studied pathogens for inflammasome activation is *Salmonella*, which is considered a stereotypical enteric Gram-negative organism. *Salmonella* possesses several classically strong stimulators of NLR immunity, including hexacylated LPS, T3SS (SP-1 and SP-2), and flagellin (Miao 2011). LPS primes pro-inflammasome components through TLR4, and T3SS and flagellin activate inflammasome signaling through NLRP3 and NLRC4. *Salmonella* evades inflammasome activation by reducing the amount of flagellin produced, as well as transitioning their SP-1 T3SS, which is sensed by NLRC4, to the SP-2 T3SS, which does not induce activation. *Salmonella* can induce pyroptosis through the activation of caspase-11 via LPS and the subsequent activation of Gasdermin D downstream of both caspase-1 and caspase-11.

Due to their clinical relevance, the interactions of enteric Gram-negative organisms such as *Salmonella sp.* and *E. coli* with cellular innate immunity are well-documented. Studies using these organisms have clarified significant portions of inflammasome-based pathways. Obligately intracellular bacteria with significantly different lifestyles than enteric bacteria are starting to be recognized for their utility in probing previously unknown pathways of inflammasome recognition and inflammasome activation.

Inflammasome Response to Rickettsiales

Relatively little is known about the inflammasome response to rickettsiae compared to enteric pathogens such as *Salmonella* and *Escherichia*. The minimalist nature of

obligately intracellular bacteria means that many of these organisms do not possess typical virulence factors of other Gram-negative bacteria, and therefore interact with the inflammasome in a different manner. A handful of studies has been published on the interactions of *Anaplasma*, *Ehrlichia*, and *Coxiella* with the inflammasome, highlighting the relatively unknown nature of inflammasome activation in these organisms.

Ehrlichia have been shown to activate NLR signaling, TLR2, and Nod2 (Chattoraj 2013), independent of any major NLR typically associated with inflammasome activation. *Ehrlichia* have also been reported to activate caspase-11 in an LPS-independent manner. However, this mechanism remains to be clarified.

Anaplasma activate the caspase-1 dependent NLRC4 inflammasome, even though they do not possess T3SS or flagellin (Wang 2016). The NLRC4/ASC/caspase-1 axis regulates IL-18 and IFN-γ during infection (Pedra 2010). ASC is required for cytokine processing and releases from the cell in this infectious model, and ASC deficiency in this model results in significantly higher bacterial burdens.

Coxiella, a member of Legionellales, does not activate the inflammasome and even inhibits the caspase-11 dependent non-canonical NLRP3 inflammasome through IcaA (Cunha 2015). *Coxiella in vitro* produces LPS that is "silent" to TLR4 and caspase-11, due to its tetraacylated structure (Shannon 2005).

Previously, Smalley *et al.* have reported that spotted fever group rickettsiae activate the inflammasome through an ASC-caspase-1/11 dependent axis by 24h post-infection in bone marrow-derived macrophages (BMM). NLRP3 is involved in a time-dependent, transient, and tissue-specific manner *in vivo* and *in vitro*. While this may indicate that there are one or more NLRs involved in sensing rickettsiae during infection, the relative

"lateness" of inflammasome activation (peak at 24 hpi vs. 3-5 hpi in other organisms) demonstrates that rickettsiae possess yet undiscovered ways of interacting with the inflammasome.

Group-specific differences in inflammasome activation between rickettsiae may exist due to differential structure in lipopolysaccharide components and surface proteins, such as OmpB. Group-specific differences have been noted before in rickettsial infection of macrophages (Radulovic 2002). Rickettsial LPS, in particular, shows differential structure as demonstrated by a lack of cross-reactivity with monoclonal antibodies raised against the LPS of typhus and spotted fever groups (Amano 1998). Typhus group LPS has been defined, and shows majority hexacylated LPS, though there is a significant minority of tetraacylated and triacylated LPS species (Fodorova 2005). The lack of a heptose in the O antigen of rickettsial LPS may be important in triggering host responses, as heptose portions of O-antigen are commonly recognized by host pattern recognition receptors (PRRs). Both TLR4 and caspase-11 do not recognize minute changes in the structure of LPS (Chilton 2012). Indeed, TLR4 has greater difficulty recognizing pathogens with mutated O-antigen segments (Raetz 2002). Different acylation and O-antigen structures may account for the fact that rickettsial LPS does not produce classical endotoxic shock, and does not produce a Schwartzmann's reaction upon re-challenge with LPS in rabbits (Jordan 2008).

TLR4, however, is an important component of anti-rickettsial immunity, due to the increased lethality of *Rickettsia conorii* in C3H/HeJ mice, which have a point mutation in TLR4, compared with TLR4 intact C3H/HeN mice (Jordan 2009). Ultimately, C3H/HeJ mice were unable to generate the appropriate CD8+ T-cell response for sterilizing

immunity, implying that TLR4 priming plays an important role in rickettsial immunity. As discussed previously, TLR4 stimulation in dendritic cells leads to enhanced NK cell activation (Jordan 2009). Bechelli *et al.* have bolstered this finding with studies that highlight the importance of MyD88 in mediating instructive signaling in dendritic cells (Bechelli 2016), therefore confirming the involvement of a TLR4-dependent protective mechanism against rickettsial infection.

SIGNIFICANCE OF STUDY AND RESEARCH OBJECTIVES

Significance of Study

Rickettsiae of both the spotted fever and typhus groups represent a significant cause of morbidity and mortality worldwide. With an increase in encounters between humans and arthropods, especially the ticks and fleas that carry rickettsiae, it is becoming increasingly important to generate a pan-rickettsial vaccine. Attempts to generate a rickettsial vaccine have so far been unsuccessful, hobbled by group-specific immunity or sub-par generation of sterilizing immunity. New generations of vaccine and adjuvant development increasingly focus on the role of early cytokines for educating what will eventually become a sterilizing memory response to the pathogen in question (XXX). Indeed, the ability of IL-1 to enhance the expansion, differentiation, and effector capacity of T-cells implies that IL-1 might be investigated as adjuvant. The need for a pan-rickettsial vaccine, the relative lack of information on rickettsial interaction with innate immunity, and the importance of inflammasome-related cytokines in polarizing the appropriate adaptive immune response highlight the necessity in defining the initial host inflammasome-mediated immune events.

Research Objectives

The goal of the following research is split into two separate but related aims. We are interested in investigating the contributions of inflammatory caspases, caspase-1 and

caspase-11, to the generation of early pro-inflammatory cytokines IL-1β and IL-18 in macrophages. The major gap in knowledge is how rickettsiae are recognized in the cytosol of the infected host macrophage, and the contribution of inflammatory caspases to recognition and release of IL-1β, IL-18, and the induction of pyroptosis.

Research Objective 1

The first specific aim of this project was to reveal the role of caspase-11-dependent inflammasome activation in host response against rickettsial infection and the activation mechanisms involved.

The first subaim of the first aim was to identify the *in vivo* role of caspase-11 in host defense. Hypothesis: Caspase-11 is not essential for host defense against rickettsial infection.

The second subaim of the first aim was to determine whether caspase-11 is essential for IL-1 family cytokine secretion. Hypothesis: Caspase-11 is indispensable only for induction of pyroptosis in murine macrophages.

Research Objective 2

The second specific aim of this project was to elucidate the role of rickettsial lipopolysaccharide in priming and activating the inflammasome in mouse macrophages.

The first subaim of the second aim was to identify the mechanisms by which LPS, through TLR4, provide the priming signal for activation of caspase-11- or ASC- dependent inflammasome. Hypothesis: LPS of rickettsiae primes the ASC-dependent canonical inflammasome, which upon activation, leads to secretion of IL-18 and IL-1β in mouse macrophages *in vitro*.

The second subaim for the second aim was to identify the activation and regulatory mechanisms of the caspase-11-dependent inflammasome by cytosolic LPS. Hypothesis: Pro-caspase-11 is primed by interferon-γ, and activated by rickettsial LPS in the cytosol, leading to caspase-11 activation and pyroptosis.

Chapter 2

Materials and Methods

Rickettsia cultivation and purification

Rickettsia australis (Cutlack strain) were cultivated in Vero cells and purified as previously described with modifications (Fang 2007, Sears 2012). Briefly, infected cells were collected and suspended in SPG buffer (218 mM sucrose, 3.76 mM KH_2PO_4, 7.1 mM K_2HPO_4, 4.9 mM potassium glutamate) after sonication. The rickettsiae were placed on the top of 32%, 26% and 20% OptiPrep Density Gradient medium (Sigma- Aldrich, St. Louis, MO) in 6 × SPG bed. After centrifugation, rickettsiae were washed and collected. These stocks were used to infect macrophages *in vitro*.

Alternatively, *Rickettia australis* (Cutlack strain) were cultivated in Vero cells and purified via a cushion purification, as previously described (Fang 2007, Sears 2012). Briefly, infected cells were collected and suspended in SPG buffer after sonication. The rickettsiae were then placed on top of a 20% Optiprep density gradient in 1x SPG. After centrifugation, rickettsiae were washed with 1x SPG and collected. These stocks were used to infect macrophages *in vitro*.

Rickettsia australis (Cutlack strain) used for animal inoculation were cultivated in specific pathogen free embryonated chicken eggs. Yolk sacs from infected eggs were homogenized in a Waring blender and diluted to a 10% suspension in SPG buffer. All of these rickettsial stocks were quantified by plaque assay before use in experiments, as previously described (Feng 1993). The rickettsial stock was stored at -80°C until use. All the experiments

described in this study were performed in a certified biosafety level 3 (BSL3) laboratory at UTMB.

Generation of human macrophages

THP-1 cells were purchased from ATCC and cultured as previously described without antibiotics (Melehani 2015). THP-1 cells were differentiated in 100 μg/μl PMA (Sigma-Aldrich), reconstituted in DMSO (Sigma-Aldrich) for 16 h, followed by 24 h recovery in fresh medium. Cells were plated at a density of 1×10^6 cells per well in a 6-well-plate, and infected with *R. australis* at an MOI of 5.

Human peripheral blood monocytes (PBMC) were isolated from buffy coats obtained from the UTMB blood bank. Cells were isolated as previously described (Johnston 2015). Cells were plated at a density of 1×10^6 cells in each well in a 12-well-plate, and infected with *R. australis* at an MOI of 2 or 5.

Lipopolysaccharide purification

Rickettsiae were purified as described previously using the cushion purification. Rickettsiae were killed by exposure to 90°C for two hours in a certified biosafety level 3 (BSL3) lab at UTMB. Rickettsiae were then treated with 100 μg Proteinase K for 16h at 37°C. The resulting lysate was centrifuged at 12000 x g for 30 min at 4°C. The pellet was processed using the Intron Biotechnology LPS purification kit, as per directions from the manufacturer. Resulting rickettsial LPS was visualized and quantified via silver stain using known concentrations of *E. coli* LPS as a standard for quantification.

Immunoblotting

For evaluation of the activation of the inflammasome, uninfected and infected cells were lysed with RIPA lysis buffer (EMD Millipore, MA, 20–188) supplemented with protease inhibitors (Roche, IN, 05892970001). The soluble part of cell lysates was isolated by centrifugation and used for immunoblotting. Cell culture supernatants were processed by centrifugal filter units (3K) (Amicon) as described by the manufacturer. Briefly, 2 ml of supernatant were loaded onto columns and centrifuged at 7000 × g for 60 min at 4°C. Protein concentration was determined by BCA Assay (Pierce Biotechnology). The cell lysates and concentrated supernatant were separated by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane and probed with an anti-caspase-1 p20 antibody (EMD Millipore, MA, 06-503-I) for lysates, and anti-caspase-1 p10 antibody (sc-514, Santa Cruz Biotechnology) for concentrated supernatants. Immunoreactive bands were visualized using an appropriate secondary antibody and electrochemiluminescence detection reagents (Thermo-Scientific, Pierce, IL, 32106). Equal protein loading of the gels was controlled by normalization with β-actin with mouse monoclonal Ab (Sigma, MO, A1978) in the cellular lysates. The detection of pro-caspase-1 (45 kDa) and activated caspase-1 (10 or 20 kDa) is indicative of activation of inflammasome as described previously (Schroder 2010).

ELISA

Supernatants of cell cultures were collected and filtered to be rickettsiae-free before removal from the BSL3 laboratory. Cytokine concentrations in the culture supernatant were measured by using Quantikine enzyme-linked immunosorbent assay (ELISA) kits. Detection of cytokines in murine samples was performed using the ELISA kit from

eBioscience (San Diego CA). The limits of detection of the cytokines were as follows: IL-1β, 16 pg/ml; IL-18, 25.6 pg/ml; and IL- 10, 62.5 pg/ml. Measurement of IL-1β and IL-18 in human samples was performed using ELISA kits from R&D Systems (IL-18, limit of detection: 12.5 pg/ml) and eBioscience (IL-1β, limit of detection: 1pg/ml).

qPCR

To determine the rickettsial load in infected tissues, mouse lung, liver, and spleen were isolated from infected animals. Rickettsial loads were measured using quantitative PCR following DNA extraction as described in our previous studies with modifications (Fang 2012, Xin 2012). Briefly, tissues were first placed in RNALater (Thermo Fisher Scientific, Waltham, MA). Total DNA was extracted using a DNeasy tissue kit (Qiagen, CA, 69506), and rickettsial burdens were determined using an iCycler IQ from Bio-Rad (Hercules, CA). The following primers (SigmaGenosys, St. Louis, MO) and probes (Biosearch Technologies, Novato, CA) targeting *Rickettsia*-specific citrate synthase (CS) gene (*gltA*) as described previously (Fang 2012, Xin 2012) (*gltA* forward, GAGAGAAAATTATATCCAAATGTTGAT; *gltA* reverse, AGGGTCTTCGTGCATTTCTT; *gltA* probe, CATTGTGCCATCCAGCCTACGGT). The results were normalized to the weight of the same sample and expressed as copy number of CS genes per ng of tissue. Tissues from uninfected mice served as a negative control. Uninfected and *R. australis*-infected WT BMMs were collected in RNALater at the indicated time points. Total RNA was prepared using Qiagen RNeasy Mini kit (Valencia, CA) following the manufacturer's recommendations. Reverse transcription (RT) was performed using isolated and DNase-treated RNA with Bio-Rad iScript cDNA

synthesis kit (Hercules, CA). The resulting cDNAs were used as template for quantitative reverse transcription–polymerase chain reaction (RT-PCR). Gene expression of NLRP3 was determined using SYBR Green PCR Master Mix on an iCycler IQ (Bio-Rad, Hercules, CA) using primers targeting NLRP3 (Forward 5'-CCT TCA GGC TGA TCC AAG AG-3', Reverse 5'-GCC AAA GAG GAA TCG GAC AAC-3') and GAPDH (Forward 5'-ATGGTGAAGGTCGGTGTGAA-3', Reverse 5'- CTCCT TGGAGGCCATGTA-3') as described previously (Tsuchiya 2010, Valbuena 2003). Quantitative results were expressed as the mRNA relative ratio (2-ΔΔCt) normalized to the housekeeping gene as previously described (Livak 2001).

Mice and generation of bone marrow macrophage

Wild-type (WT) B6 mice, caspase-1/11-double knockout mice and C57BL/6N (B6N) mice were purchased from Jackson Laboratories (Bar Harbor, Maine). ASC-/- and NLRP3-/- mice were gifts of Dr. Vishva Dixit at Genentech (California, USA). Caspase 11-/- and caspase-1/11-/- mouse legs were from Dr. Edward Miao at UNC-Chapel Hill. Gasdermin D-/- mouse legs were from Dr. Joao Pedra at University of Maryland.

All mice were maintained and manipulated in an animal biosafety level-2 or 3 (ABSL2 or ABSL3) facility at the University of Texas Medical Branch, Galveston, TX. This study was carried out in strict accordance with the recommendations in the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All experiments and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee of the University of Texas Medical Branch at Galveston.

For *in vivo* experiments, mice were inoculated intravenously (i.v.) through the tail vein with *R. australis* at the dose indicated. After infection, mice were monitored daily for signs of illness until day 20 post infection (p.i.). No animal death was observed. Some of the mice were euthanized on days 2 and 4 p.i.. Mice were first anesthetized with inhalational isoflurane (Isoflurane1 USP, Piramal Healthcare Limited, 502321 Andhra Pradesh, India) and then euthanized by CO_2 narcosis and asphyxia followed by cervical dislocation. After the death of animals, mouse tissues including lung, liver, and spleen were isolated for evaluation of the bacterial replication and pathology by quantitative real-time PCR and histopathological analysis. All necessary precautions were taken to minimize the discomfort and pain to animals used in the experiments.

Generation of primary bone marrow-derived macrophages (BMMs) from 6–8-week old female WT mice, ASC-/- mice and NLRP3-/- mice was performed as previously described (Zhang 2008). Briefly, after femurs and tibias were dissected, bone marrows were flushed, and cells were cultivated in low-endotoxin DMEM/F12 containing 10% (v/v) newborn calf serum (Thermoscientific, Gibco, CA, 16010159) supplemented with either 20% supernatant from L929 cell culture or CMG cell culture, or recombinant M-CSF (PeproTech, NJ, 315–02) at 37°C in 5% CO_2. On day 6 of culture, cells were harvested and characterized by flow cytometric analysis after staining with anti-F4/80 and anti-CD11b antibodies. Cells were used if they contained 85 to 90% F4/80 (+) and CD 11b (+) cells. These cells were plated in 24-well plates at a density of 1×10^6 cells/ well in DMEM/F12 containing 10% newborn calf serum and used for experiments within 24 hrs.

CMG supernatant production

CMG cells were obtained from the Hwang Laboratory (U of Chicago). Cells were grown as previously described. Briefly, cells were grown to confluence in triple flasks. Conditioned supernatant was collected and used to differentiate WT B6 bone marrow-derived macrophages. Macrophages were differentiated and collected as previously described. Cells were stained with F4/80 and CD11b for flow cytometry to determine the percentage differentiation of bone marrow progenitors into macrophages. Only supernatant resulting in greater than 85% differentiation was used for experiments.

LPS transfection

LPS transfections were performed as previously described (Hagar 2011), with modifications. Briefly, 100 ng of rickettsial LPS or *Salmonella* LPS (List Biologicals) was added to Opti-Mem (Gibco), and 50 ng of Lipofectamine 2000 (Invitrogen) or DOTAP (Thermo-Scientific) was added to Opti-Mem and allowed to equilibrate for 5 min at room temperature. The LPS and Lipofectamine/DOTAP mixtures were combined and allowed to form complexes at room temperature for 30 min. Cell culture medium of BMM were replaced with Opti-Mem, and the LPS-transfection reagent complexes were added. Cells were centrifuged at 300 x g for 5 min, and then incubated at 4 hours at 37°C. Cell medium was replaced with DMEM/F12 and 10% v/v fetal bovine serum for 20 hours. Cell lysates and medium were collected after a total of 24 hours post-transfection.

Statistical analysis

For comparison of multiple experimental groups, the one-way analysis of variance (ANOVA) with Bonferroni's and Tukey’s procedure was used. Two-group comparison was

conducted using either Student t-test or Welch's t-test depending on whether the variance between two groups was significantly different. When two factors were included in the comparison, two- way ANOVA with Bonferroni post-test was used. For testing the difference in survival between different mouse groups, data were analyzed by the product limit (Kaplan-Meier) method. All the statistical analyses were performed using GraphPad Prism Software version 5.01. P-values of 0.05 or less were the threshold for statistical significance.

Chapter 3

Inflammasome Activation during Rickettsial Infection

Introduction

To address the research questions of the role of caspase-11, we first investigated whether rickettsiae could activate the inflammasome in macrophages. Although endothelial cells are the primary target cells for rickettsial infection, pathogenic rickettsiae also invade macrophages as observed in established animal models and in the arthropod feeding inoculation site (Walker 1999, Cragun 2010). In response to IFN-γ and TNF-α, macrophages are activated and serve as crucial effector cells mediating clearance of intracellular pathogens. Upon infection, perivascular infiltration of macrophages, together with lymphocytes and other cells, is a component of rickettsial vasculitis (Eremeeva 1999). Therefore, understanding the interactions of rickettsiae with macrophages will greatly increase our knowledge regarding the pathogenesis of rickettsial infections and immunity against rickettsiae. In the present study, we focused on interactions of inflammasome with *R. australis* in mouse and human macrophages. Inflammasomes have recently been recognized as important first line defenses against invading pathogens. As obligately intracellular bacteria that live freely in the host cell cytosol, rickettsial interaction with the inflammasome represents an important aspect to anti-rickettsial immunity that has previously been unexplored. For these studies, we hypothesize that *R. australis* are

recognized by cytosolic sensors, ASC-dependent inflammasome involving NLRP3, in macrophages leading to secretion of IL-1β and IL-18.

Results

First, to investigate the activation of inflammasome by rickettsiae in human macrophages, we first examined whether *R. australis* infects PBMC-derived macrophages by confocal immunofluorescence microscopy. *R. australis* (red) was detected in the cytosol (nucleus as blue) of infected human PBMC-derived macrophages, suggesting that rickettsiae were effectively taken up by and established infection in these cells (Figure represented in Smalley 2016). Interestingly, human PBMC-derived macrophages secreted a significantly higher level of IL-1β as early as 3 h p.i. compared to uninfected controls (Fig. 1A). The levels of IL-1β secreted by these infected macrophages were greater at 24 h p.i. compared

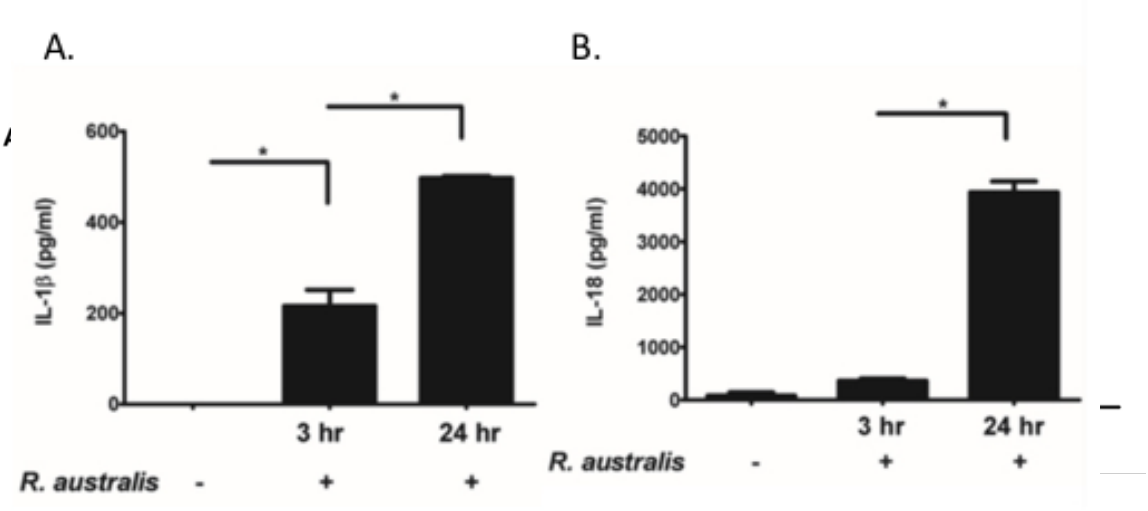

Figure 1. Infection of human PBMC-derived macrophages with *R. australis* and activation of inflammasome. Human PBMC-derived macrophages were prepared and infected with *R. australis*. Cells and culture supernatant were collected at 24 h p.i.. Secretion levels of IL-1β (A) and IL-18 (B) at 3 h and 24 h p.i. by human PBMC-derived macrophages infected with *R. australis* at an MOI of 2. Data represent two independent experiments with consistent results. Each experiment included at least 4 replicates. *, $p<0.05$

to 3 h p.i. (Fig 1B). At 24 h p.i., *R. australis* also induced a significantly increased level of IL-18 in human PBMC-derived macrophages (Fig. 1B). These results suggest that *R.*

australis infects primary human macrophages and promotes secretion of the inflammasome-derived cytokines including IL-1β and IL-18.

To further study the mechanisms involved in secretion of inflammasome-derived IL-1β in human macrophages, we examined the dependence of IL-1β secretion on caspase-1 in *R. australis*-infected THP-1 derived macrophages. Consistent with the results from human PBMC-derived macrophages (Fig. 1B), a significantly higher level of IL-1β was observed in *R. australis*-infected THP-1 derived macrophages compared to uninfected controls at 24 h p.i. (Fig. 2A). Interestingly, *Rickettsia*-infected THP-1 derived macrophages treated with an inhibitor of caspase-1 produced a significantly less fold change in IL-1β compared to untreated controls (Fig. 2B), suggesting that caspase-1 is critical for secretion of inflammasome-derived IL-1β in human macrophages upon rickettsial infection. Therefore, these results suggest that *R. australis* infects both human PBMC-derived macrophages and

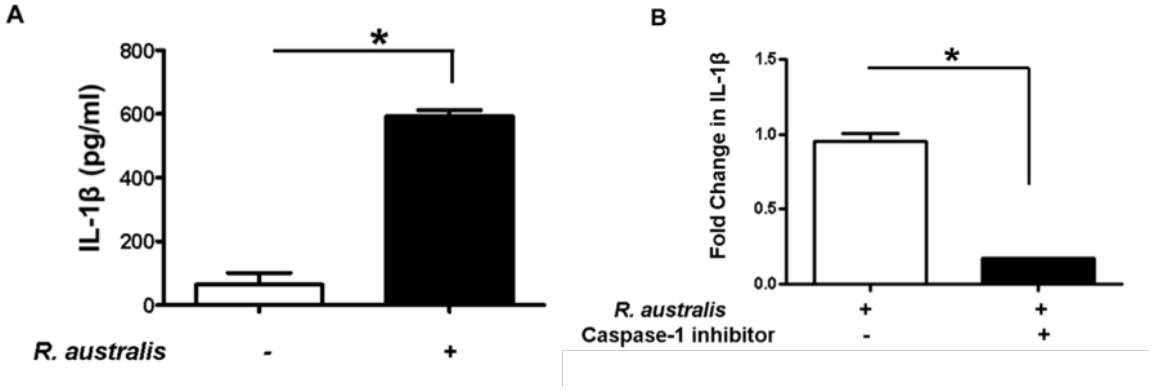

Figure 2. Activation of inflammasome by rickettsiae in THP-1 derived macrophages and caspase-1-dependent secretion of IL- 1β. Human THP-1 cells were differentiated to macrophages using PMA, and infected with *R. australis* at an MOI of 5. A, Infection with *R. australis* induced a significant increase in IL-1β production compared to uninfected cells at 24 h p.i.. B, Inhibition of caspase-1 significantly reduced the secretion levels of IL-1β by *R. autralis*-infected THP-1 derived macrophages. The fold change in IL-1β by treated cells vs. untreated controls was calculated and compared. Data represent two independent experiments with consistent results. Each experiment included at least 4 replicates. *, $p<0.05$.

THP-1 cells, and promotes caspase- 1-dependent secretion of IL-1β most likely via activating inflammasome pathway.

To investigate whether *R. australis* activates inflammasome in mouse macrophages, we first determined the secretion of inflammasome-derived IL-1 family cytokines including IL-1β and IL-18 by *R. australis*-infected mouse macrophages as well as investigating the kinetics- and dose-dependent mechanisms involved. As positive controls, WT BMMs stimulated with LPS plus ATP produced significantly higher levels of IL-1β and IL-18 compared to unstimulated controls. As shown in Fig. 3A, *R. australis* induced significant secretion of IL-1β at 8 h p.i. upon infection at a high dose (MOI of 6) and at 12 h p.i. at a low dose (MOI of 2). The levels of IL-1β induced by a high dose of *R. australis* were significantly higher than those with the low dose infection at 8 h, 12 h and 16 h p.i.. Interestingly, although the production levels of IL-1β increased over time, there was no difference between the levels at high and low doses of infection at 24 h p.i. (Fig. 3A). Very similar kinetics and dose-dependence of IL-18 production were observed in infected

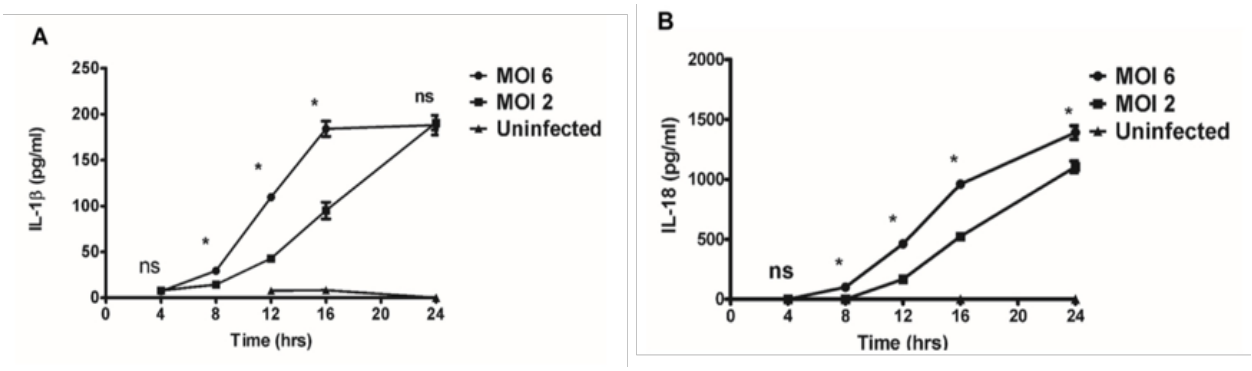

Figure 3. Kinetics and dose-dependent mechanisms of secretion of IL-1β and IL-18 by *Rickettsia*-infected BMMs. WT BMMs were isolated, cultivated, and infected with *R. australis* at MOI of 2 or 6. Cell culture supernatants were harvested at 4 hour intervals over 24 h p.i.. Secretion of IL- 1β (A) and IL-18 (B) was determined by ELISA. Data represent two independent experiments with consistent results. Each experiment included at least 4 replicates. *, $p<0.05$; ns, not significantly different.

BMMs (Fig. 3B) with the exception that a significant difference was observed between the levels at high and low doses after rickettsial infection at 24 h p.i..

Previous studies have demonstrated that rickettsiae induce negligible cytotoxicity in mouse peritoneal macrophages and the macrophage-like cell line, P388D1 (Radulovic 2002). In line with these previous studies, we did not find significantly reduced viability of mouse BMMs by trypan blue staining after infection with *R. australis* at 24 h p.i.. We also confirmed these results with LIVE/DEAD1 Fixable Dead Cell Stain by flow cytometric analysis (Supplementary Figure 1 represented in Smalley 2016). To further determine whether the secretion of IL-1β and IL-18 upon rickettsial infection is mediated by inflammasome, we investigated the activation of caspase-1 and the role of casp-1/ 11 in this process. The cell lysates of uninfected and infected BMMs showed expression of

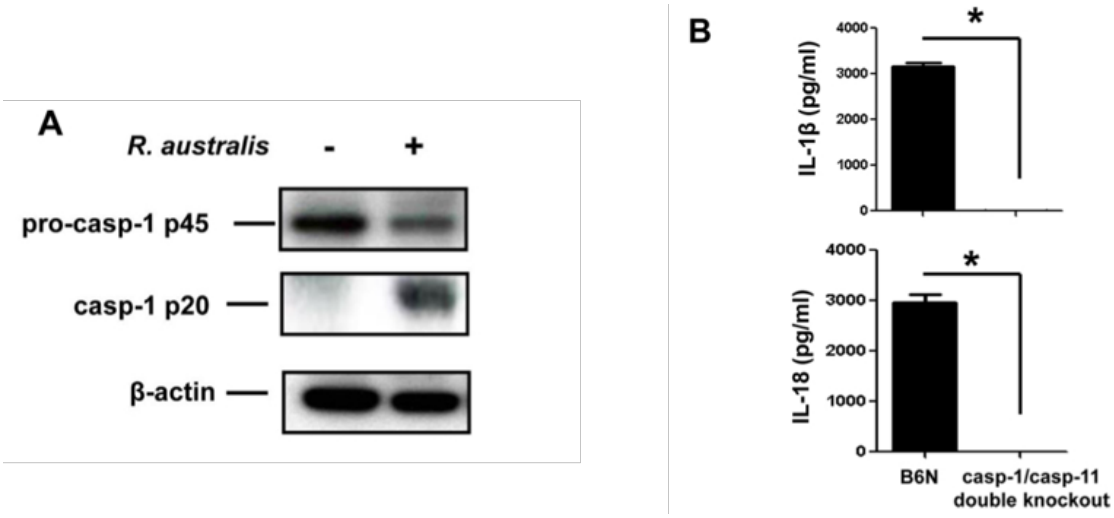

Figure 4. *R. australis* activated inflammasome in BMMs. A, WT BMMs were isolated, cultivated and infected with *R. australis* at an MOI of 10. At 24 h p.i., culture supernatant and cell lysates were collected. Cell lysates were processed for detection of activation of caspase-1. B, BMMs of B6N and caspase-1/11-double knockout mice were isolated and infected with *R. australis* as described above. The secretion levels of IL-1β and IL-18 were determined by ELISA. *, $p<0.05$

procaspase-1 (p45) while activated caspase-1 (p20) was detected only in infected samples (Fig. 4A). To confirm that the inflammasome pathway accounts for the production of

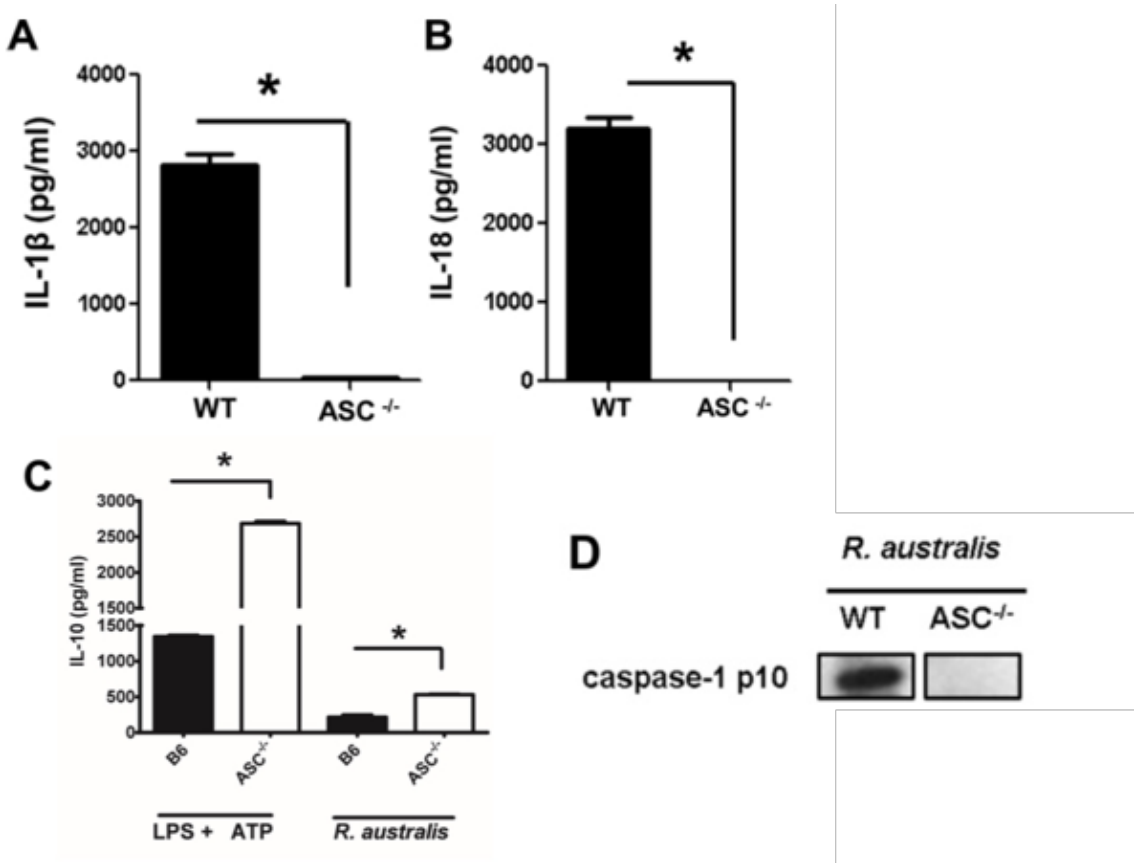

Figure 5. ASC-dependent inflammasome recognized cytosolic rickettsiae. BMMs of WT and ASC-/- mice were isolated, cultivated, and infected with *R. australis* at an MOI of 10 or treated with LPS plus ATP as described in Materials and Methods. At 24 h p.i., the secretion of IL-1β (A), IL-18 (B) and IL-10 (C) was assayed by ELISA. D, Activation of caspase-1 was determined by immunoblotting detection of the active unit p10 in the processed supernatant of infected WT and ASC-/- BMMs at 24 h p.i.. Data represent two independent experiments with consistent results. Each experiment included at least 4 replicates. *, $p<0.05$.

cytokines such as IL-1β and IL-18 in *R. australis* -infected macrophages, we employed caspase-1/11-double knockout mice and the corresponding controls, B6N mice. B6N mice have the same genetic background as the caspase-1/11-double knockout mice. In response to *R. australis* infection, BMMs from B6N mice produced significant levels of IL-1β and IL-18 (Fig. 4B). However, we did not detect any production of these cytokines in caspase-1/caspase-11-double knockout mice. These results confirmed that caspase-1 and/or caspase-11 are essential for production of IL-1β and IL-18, suggesting that *R. australis* activates inflammasome in mouse macrophages.

Next, we aimed to identify the NLRs involved in the recognition of *R. australis* in the host cell cytosol. Upon rickettsial infection, BMMs of ASC-/- mice failed to produce significant levels of IL-1β and IL-18 (Fig. 5A and 5B). To exclude the possibility of unresponsiveness of ASC-/- BMMs upon stimulation, we determined the production of IL-10, an inflammasome-independent cytokine. Interestingly, both *R. australis*-infected and LPS plus ATP-stimulated ASC-/- BMMs produced significantly higher levels of IL-10 than WT BMMs (Fig. 5C), suggesting that ASC-/- BMMs were responsive to rickettsial infection. These results also exclude the possibility that the incapability of ASC-/- BMMs to produce inflammasome-derived IL-1 family cytokines upon rickettsial infection was due to the failure of taking up *R. australis*. To confirm that the abolished secretion of IL-1β and IL-18 in ASC-/- BMM is not due to mechanisms other than failure of activation of caspase-1, we determined the cleavage of caspase-1 in the supernatant of infected WT and ASC-/- BMM by immunoblotting. As shown in Fig. 5D, at 24 h p.i., activated caspase-1 p10 was only detected in infected WT but not ASC-/- BMMs. Therefore, ASC, or ASC-dependent inflammasomes, were essential for the recognition of *R. australis* in the cytosol of mouse macrophages. Our results also suggest that ASC may suppress the production of IL-10 in response to infectious stimuli including rickettsial antigen

To further investigate the NLR inflammasome(s) responsible for recognition of rickettsiae in the cytosol, we first examined the transcriptional expression levels of NLRP3 in WT BMMs upon rickettsial infection. As early as 4 h p.i., infection with *R. australis* at an MOI of 6 significantly increased NLRP3 transcripts in WT macrophages (Fig. 6A). As infection progressed, the quantity of NLRP3 mRNA progressively decreased at 12 h p.i. compared to 4 h p.i.. The transcriptional levels of NLRP3 were not significantly increased at 12 h p.i.

compared to uninfected controls (Fig. 6A). To further investigate whether NLRP3 is responsible for recognition of rickettsiae in the cytosol, we infected NLRP3-/- and WT BMMs with *R. australis* at both high (MOI of 6) and low (MOI of 2) doses. At 12 h p.i., the secretion levels of IL-1β by NLRP3-/- BMMs were significantly decreased compared to WT counterparts in response to both low and high doses of *R. australis* (Fig. 6B). To further confirm that NLRP3 is involved in inflammasome activation by *R. australis*, we determined the cleavage of caspase-1 in the processed supernatant of WT and NLRP3-/-

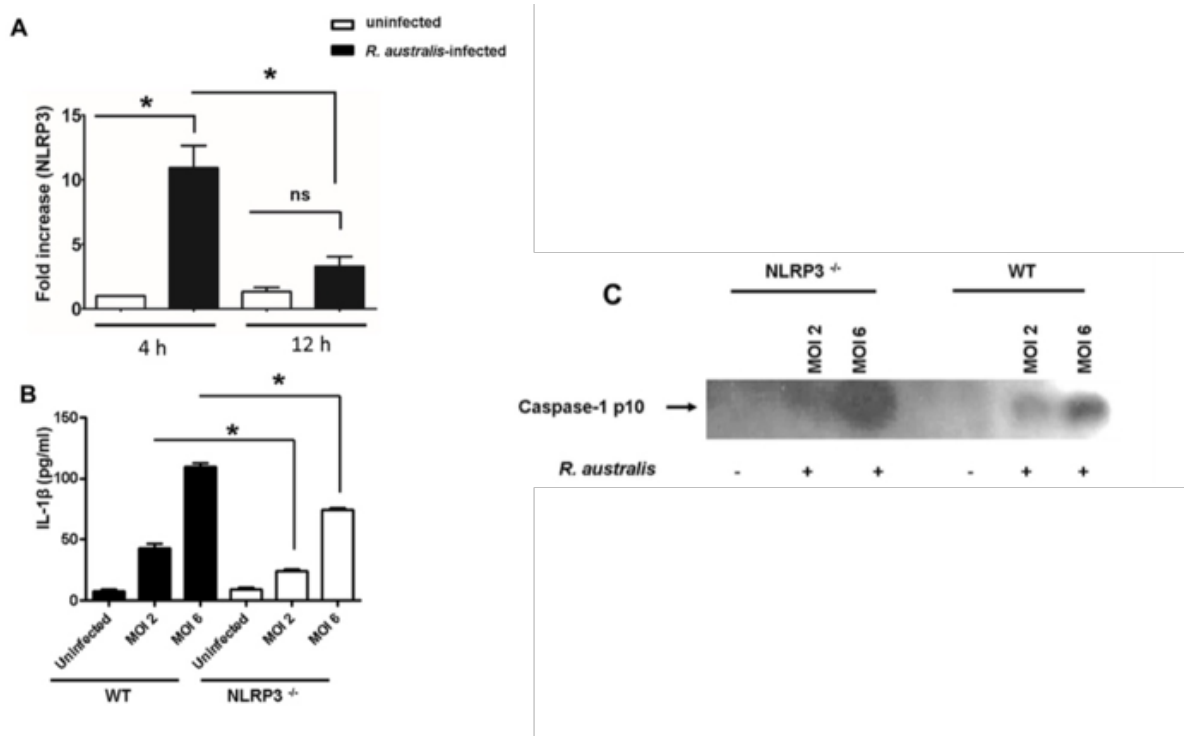

Figure 6. NLRP3 inflammasome was involved in recognition of rickettsiae at the early stage of infection in BMMs. WT and NLRP3-/- BMMs were isolated, cultivated, and infected with *R. australis* at MOIs of 6 and 2. The transcriptional levels of NLRP3 in WT BMMs at different time intervals of infection (at MOI of 6) were determined by RT-PCR as described in Materials and Methods (A). At 12 h (B) p.i., the secretion levels of IL-1β by WT and NLRP3-/- BMMs were determined by ELISA. The cleavage of caspase-1 was determined by detection of the active unit p10 in the processed supernatant at 12 h p.i. Data represent mean ± SD for at least 3 replicates each group. *, $p<0.05$ for a significant difference between WT and NLRP3-/- mice; ns, not significantly different.

BMMs by immunoblotting. As shown in Fig. 6C, at 12 h p.i., activated caspase-1 p10 was detected in the supernatant of infected WT BMM. The density of caspase-1 p10 was

correlated with the dose of rickettsial infection, suggesting a dose-dependent inflammasome activation mechanism. In line with Fig. 6B, the cleaved caspase-1 was detected in NLRP3 -/- BMMs infected with *R. australis* at a high dose infection (Fig. 6C). These results suggest that NLRP3 mediates the secretion of IL-1β by inflammasome pathway upon rickettsial infection and that there is an alternative NLRP3-independent pathway for inflammasome activation. These data also suggest that NLRP3 inflammasome contributes to recognition of *R. australis* in mouse macrophages. Thus, our results demonstrate that NLRP3 is activated by *R. australis* in BMMs. To further explore the role of NLRP3 inflammasome in host defense against rickettsial infections in vivo, we measured rickettsial loads in infected tissues and survival of infected NLRP3-/- and WT mice. Interestingly, the concentrations of *R. australis* in the spleen of NLRP3-/- mice were significantly higher than those in WT mice on day 4 p.i., but not at day 2 p.i., suggesting that NLRP3 contributes to rickettsial elimination in spleen (Fig. S2 represented in Smalley 2016). On days 2 and 4 p.i., rickettsial loads in liver and lung of NLRP3-/- mice were not significantly different from those in infected WT mice (Fig. S2 represented in Smalley 2016). Furthermore, compared to day 2 p.i., bacterial loads in tissues of NLRP3-/- mice on day 4 p.i. were greater, particularly in spleen, suggesting that rickettsial infection progresses in NLRP3-/- mice (Fig. S2 represented in Smalley 2016). We did not find any significant difference in the survival of NLRP3-/- and WT mice upon infection with *R. australis* at a dose of 2.8 × 105 plaque forming units (PFUs) (Fig. S3 represented in Smalley 2016). Furthermore, histopathological analysis did not show any significant difference in inflammatory infiltrations in infected lung, liver and spleen of infected NLRP3-/- and WT mice on either day 2 or day 4 p.i. (Fig. S4 represented in Smalley 2016).

These data suggest that the contribution of NLRP3 inflammasome to host control of *R. australis* in vivo is tissue- or cell type-specific. Taken together, these data suggest that NLRP3 inflammasome is not crucial to control rickettsial infection *in vivo* and only contributes to host control of rickettsiae in a tissue- or cell-specific mechanism.

Discussion

Here it has been demonstrated that cytosolic-replicating *R. australis* infects human primary and THP-1-derived macrophages, and induces the secretion of caspase-1-dependent cytokines, most likely through inflammasome pathway, which had never been reported previously. *R. australis* activated inflammasome in mouse macrophages via time- and dose-dependent mechanisms. ASC-dependent inflammasomes were responsible for recognition of *R. australis* in host cytosol while NLRP3 inflammasome significantly contributed to this process. The in vivo role of NLRP3 inflammasome in host immune responses to *R. australis* was tissue specific as evidenced by significantly increased bacterial loads in spleen, but not liver and lung, of NLRP3-/- mice compared to WT mice. More importantly, for the first time, we demonstrated that *R. australis* activated inflammasome in human macrophages with kinetics that differed from mouse macrophages. Our findings have provided novel knowledge of the mechanisms by which the host immune surveillance system interacts with *Rickettsia* via macrophages. *Rickettsia australis* activated ASC-dependent inflammasome in murine BMMs as indicated by the following evidence: 1) Secretion of IL-1β and IL-18 upon infection was completely abrogated in cells deficient in ASC and caspase-1/caspase-11-double knockout cells (Figs. 4 and 5); 2) *Rickettsia australis* infection induced cleavage of caspase-1 in the cell lysates and supernatant (Figs. 4A, 5D and 6C). Although neutrophil-dependent, inflammasome-independent processing of IL-1β has been described recently (Netea 2015), our current data excluded the possibility of inflammasome-independent processing and secretion of IL-1β

and IL-18 and demonstrated inflammasome activation by *R. australis* in macrophages. ASC serves as the essential adaptor molecule for several NLRs including NLRP3 and absent in melanoma 2 (AIM2) (Rathinam 2010, Rathinam 2012). Our results showed that NLRP3 contributed significantly to the activation of inflammasome by *R. australis* in BMMs. Future investigations are required to reveal the upstream signals mediating the activation of NLRP3 inflammasome by *R. australis*, such as potassium efflux (Matsuo 2015, Petrilli 2007), lysosomal degradation (Homung 2010), and ROS production (Tschopp 2010). Interestingly, we also found that a significant level of inflammasome-derived IL-1β secretion was NLRP3-independent (Fig. 6B), particularly at a high dose of infection, which suggests that ASC-dependent NLR inflammasomes other than NLRP3, potentially NLRP1 and/or AIM2, coordinate with NLRP3 or also play a significant role in the recognition of cytosolic *R. australis*. Furthermore, we did not find significant difference in IL-1β secretion by infected BMMs of NLRP3-/- mice compared to WT mice at 24 h p.i., a time at which the levels of IL-1β secretion reached a peak in macrophages of WT mice (Fig 3A). The differential contributions of NLRP3 to inflammasome activation by *R. australis* at early versus late time points are likely explained by two possibilities: 1) Inflammasomes other than NLRP3 play a major role in recognizing *R. australis* and the related danger signals at the late stage of infection; 2) NLRP3 inflammasome is down-regulated by other immune mechanisms such as caspase-11, autophagy, or cytokines specifically suppressive for NLRP3. These in vitro findings may account for the dispensable role of NLRP3 inflammasome in host control of *R. australis in vivo*. Considering the different proportions of cell types in spleen compared to liver and lung, *R. australis* may mainly activate NLRP3 inflammasome in leukocytes such as macrophages.

Interestingly, we found significantly enhanced host susceptibilities and increased rickettsial loads in tissues of mice deficient in ASC during rickettsial infection compared to WT mice in our preliminary in vivo studies. Our previous studies have clearly demonstrated that the *in vivo* production of IL-10 in murine models of fatal rickettsioses is associated with the severity of disease (Fang 2009). As shown in Fig. 5C, ASC significantly suppressed the secretion of IL-10 by *R. australis*-infected macrophages, implying that ASC may contribute to host resistance against rickettsiae. Although further investigations are required to completely understand how inflammasome contributes to host immunity *in vivo* against these intracellular bacteria, our data suggest that ASC/NLRP3 inflammasome plays a role in host defense. Among the different types of microbes, cytosolic bacteria are uniquely useful for investigating inflammasome activation mechanisms due to their biological characteristics and the site where inflammasomes initiate their recognition of microbes or microbial products. Inflammasome activation by rickettsial infection in macrophages was both time-and dose-dependent. Distinct from other facultatively cytosolic bacteria including *Listeria, Shigella, Burkholderia* and *Francisella,* rickettsiae are obligately cytosolic bacteria which quickly escape phagosomal vacuoles and replicate within the cytosol of host cells including macrophages. Mouse macrophages secrete IL-1β and IL-18 in response to *Listeria* at 5 h p.i. (Wu 2010), *Shigella* at 6 h p.i. (Cai 2012), *Burkholderia* at 4 h p.i.(Ceballos-Olvera 2011), and *Francisella* at 5 h p.i.(Jones 2010). Our data suggest that the kinetics and possibly the mechanisms of inflammasome activation by *R. australis* are distinct from other cytosolic bacteria. In response to *R. australis*, mouse macrophages secrete IL-1β and IL- 18 as late as at 8 h p.i. after a high dose of infection and at 12 h after a low dose of infection. The levels of these inflammasome-derived

cytokines increased progressively as the infection progressed and reached a peak at 24 h p.i. regardless of the dose. The delayed activation of inflammasome by *R. australis* in mouse macrophages compared with several facultatively cytosolic bacteria mentioned above suggests that this intracellular bacterium may initiate an evasion mechanism to escape inflammasome assembly at the early stage of infection. The dose-independent secretion of IL-1β at 24 h p.i. by infected mouse macrophages suggests that inflammasomes responsible for recognizing these intracellular bacteria at the late stage of infection are very sensitive to the activation of ligand(s) generated during rickettsial infection, and could be an ideal candidate for vaccine development targeting inflammasome activation in the future. While we have shown that *R. australis* activated caspase-1-dependent inflammasome in both murine and human macrophages, it remains unclear whether canonical or noncanonical inflammasomes are activated by rickettsiae. Kayagaki et al. demonstrated that the non-canonical inflammasome pathway engages caspase-11 to activate caspase-1 and the subsequent release of IL-1β and IL-18 (Kayagaki 2011). *Ehrlichia*, another obligately intracellular bacterial species closely related to rickettsiae, triggers cleavage of caspase-1 and IL-18 secretion in BMMs (Yang 2014). Interestingly, type I interferon signaling promotes host susceptibility to fatal ehrlichiosis potentially via activation of non-canonical inflammasomes (Yang 2014). Thus, it is an attractive hypothesis that caspase-11 mediates caspase-1 activation, which further processes IL-1β secretion in *R. australis*-infected murine macrophages. Recently several intracellular bacterial pathogens, including *Legionella pneumophila*, *Yersinia pseudotuberculosis*, and *Salmonella enterica serovar Typhimurium* (*S. Typhimurium*), were reported to activate both canonical caspase-1-dependent and non-canonical caspase-1-independent

inflammasomes in primary human macrophages (Casson 2015). Considering the fact that we have not examined caspase-11-dependent inflammatory cell death, pyroptosis, in infected human macrophages, our data suggest that *R. australis* at least activates canonical inflammasome in human macrophages. Furthermore, NLRP3 inflammasome has been described to be involved in both canonical and non-canonical inflammasome pathways (Kayagaki 2011 ,Ruhl 2015). In agreement with our data in murine macrophages, *R. australis* not only infects both human primary and THP-1 derived macrophages, but also activates the inflammasome in human macrophages. It is worth noting that: 1) The kinetics of secretion of IL-1β by *R. australis* -infected macrophages may differ from IL-18. We observed a significant difference in secretion levels of IL-18, but not IL-1β, in mouse macrophages at high and low doses after rickettsial infection at 24 h p.i. (Fig. 3). Furthermore, *R. australis* initiated significant secretion of IL-1β, but not IL-18, as early as 3 h p.i. in human primary macrophages (Fig. 1); 2) Induction of IL-1β by *R. australis* in human primary macrophages (3 h p.i.) occurred much earlier than in mouse macrophages (8 h p.i.) (Figs. 1B and 3A). These findings suggest that: 1) The inflammasome pathways mediating secretion of IL-1β are likely different compared to IL-18 during rickettsial infection; 2) Activation mechanisms of inflammasome by *R. australis* in human macrophages are potentially different from the mouse counterparts. In conclusion, *Rickettsia australis* activated ASC-dependent inflammasomes in which NLRP3 contributed significantly to recognition of the bacteria. Our data suggest inflammasomes other than NLRP3 might play a critical role in the cytosolic surveillance system at the late stage of rickettsial infection.

Chapter 4

Contribution of LPS to inflammasome activation

Introduction

Before the tightly controlled inflammasome signaling cascade is activated in any infectious disease model, a priming signal in the form of a pathogen associated molecular pattern (PAMP) or damage associated molecular pattern (DAMP) results in the upregulation of pro-IL-1 cytokines. One of the classical PAMPs is LPS from Gram-negative bacteria (Raetz 2010). LPS binding to TLR4 provides the first signal that leads to activation of NF-kB and the transcriptional up-regulation of pro- IL-1β and pro-IL-18. LPS is shed from Gram-negative organisms during active infection *in vivo*. Many cellular defense mechanisms are linked to LPS detection, underscoring the importance of this PAMP in pathogen recognition.

Since LPS is one of the most well-characterized PAMPs in other Gram-negative infectious disease models, we wished to determine the contribution of rickettsial LPS to priming and activating the inflammasome. Rickettsial LPS, as discussed previously, is understudied. Typically, members of the family of Rickettsiales have an altered form of LPS or no LPS, which may contribute to pathogenesis. Indeed, both *Orientia* and *Ehrlichia* lack LPS completely (Chattoraj 2013, Yang 2014).

The few studies that have been conducted on rickettsial LPS are conflicting, most of which have been done in typhus group rickettsiae. While studies indicate that typhus group LPS is endotoxic (Fumarola 1979) and can induce Schwartzmann's reaction (Schramek 1977), they agree that the level of endotoxicity is variable and reduced compared to control LPS from enteric pathogens. One study even questions the difference of rickettsial LPS from enteric LPS (Miragliotta 1981).

Many studies have indicated that typhus group rickettsiae and spotted fever group rickettsiae differ in cell surface antigens (Eisemann 1976, Osterman 1978). Some data suggest that typhus group LPS and spotted fever group LPS generate non-cross-reactive antibodies to their respective group LPS (Amano 1998).

Regardless of differences between enteric bacteria and group-specific differences, several studies have indicated the importance of TLR4 signaling in innate immunity during rickettsial infection. Jordan *et al* have demonstrated involvement of TLR4- stimulated dendritic cells in protective immunity against rickettsial infection (Jordan 2009). TLR4 competence in mice is associated with increased levels of IFN-γ and increased expansion of NK cells compared to TLR4-deficient mice. Additionally, NK cells from TLR4-competent mice demonstrated increased cytotoxicity and greater IFN-γ production, and NK cells from TLR4-deficient mice were activated by DC cells from TLR4 competent mice exposed to rickettsiae (Jordan 2009). TLR4 signaling during rickettsial infection has never before been investigated in macrophages. As pro-inflammatory cytokine producers and secondary targets of rickettsial infection, TLR4 signaling in macrophages potentially plays an important role in polarizing surrounding DC and NK cells to stimulate production of protective pro-inflammatory cytokines, aiding T-cell activation and differentiation.

Previously, we have demonstrated that rickettsiae activate inflammasome in both human and mouse macrophages. Here, we sought to investigate how rickettsiae prime and activate the inflammasome during infection. Our hypothesis is that rickettsial LPS acts as a priming signal for inflammasome activation through TLR4 in macrophages.

Results

It has been established that TLR4 plays a role in host innate and adaptive immunity during rickettsial infection (Jordan 2008, Jordan 2009); however, it is yet unknown how TLR4 contributes to cellular innate immunity, especially inflammasome-dependent immunity. To investigate TLR4 involvement in inflammasome activation, we infected TLR4$^{-/-}$ BMM with *R. australis* at an MOI of 6. Rickettsial infection in TLR4$^{-/-}$ BMM failed to produce any significant amount of IL-1β upon infection (Fig. 7A). Levels of IL-1β in *Salmonella*

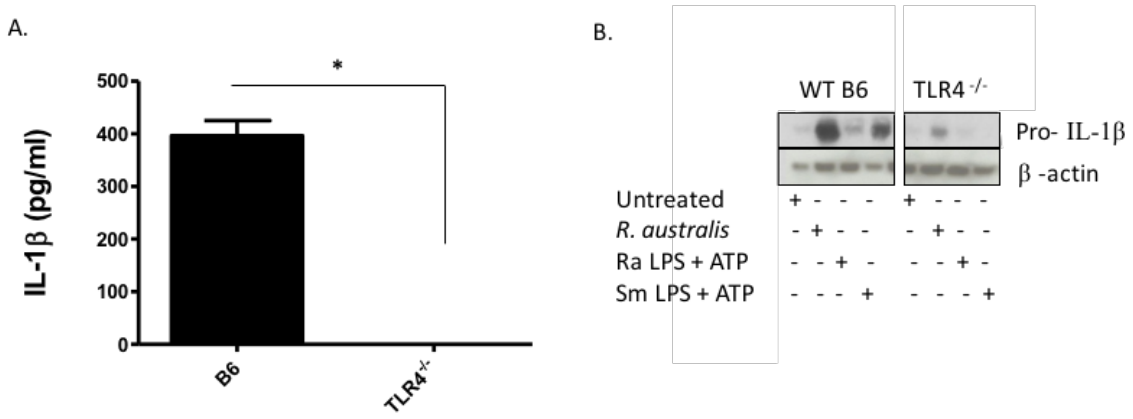

Figure 7 A and B. IL-1β secretion is ablated in TLR4-/- BMM. BMM were infected with MOI of 6 *R. australis* for 24h. A.)Supernatants were collected and tested by ELISA (R&D Systems) B.) Lysates were collected and probed with pro- IL-1β antibody (1:1000) from Cell Signaling. *, $p<0.05$

LPS and ATP stimulated cells in TLR4-/- BMM are significantly reduced, indicating that without TLR4, there is no priming of pro-IL-1 cytokines. To determine whether ablation of IL-1β secretion in BMM was due to a lack of priming or a lack of secretion, immunoblotting against pro- IL-1β in BMM lysate was performed (Fig. 7B). Interestingly, rickettsial infection in TLR4$^{-/-}$ BMM resulted in an increase in pro- IL-1β, indicating that there are other mechanisms of pro- IL-1β priming during infection. As expected, priming

with *Salmonella* LPS and ATP, as well as priming with rickettsial LPS and ATP, caused no increase in pro- IL-1β production in TLR4$^{-/-}$ BMM (Fig. 7B).

We next sought to confirm that rickettsial LPS is indeed the ligand for TLR4. Studies have indicated that rickettsial lipid A structure varies in the amount of acylations, and the rickettsial O-antigen has some slight structural differences from enteric bacteria. Some Rickettsiales, such as *Ehrlichia* and *Orientia*, do not have LPS (Yang 2014). Other Rickettsiales, such as *Coxiella*, possess LPS that is not recognized by TLR4 (Barry 2012). Therefore, we sought to confirm that rickettsial LPS could be identified by TLR4 to serve as the first signal for inflammasome activation. First, we isolated LPS from *R. australis*

Figure 7C. Purified rickettsial LPS. LPS was extracted from *R. australis* whole cells using Intron Biotechnology LPS extraction kit, and compared to *E. coli* LPS standards from List Biologicals.

(Fig. 7C). LPS was roughly quantified by densitometry via silver stain using *E. coli* LPS

standards. Our next step was to confirm that rickettsial LPS signals through TLR4. TLR4-/- BMM and WT B6 BMM were stimulated with 50 ng of *R. australis* LPS or *Salmonella Minnesota* LPS for 24 hr, and stimulated with 5 mM ATP for 1 hr (Fig. 7D). $TLR4^{-/-}$ BMM produced significantly less IL-1β than WT B6 BMM in response to rickettsial LPS

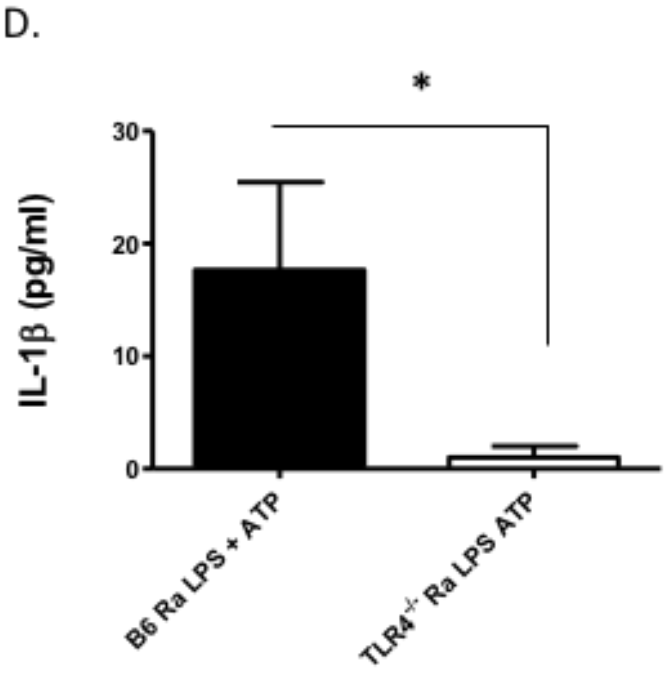

Figure 7D. IL-1β secretion in LPS-treated TLR4-/- BMM. Rickettsial LPS (Ra) was added to WT and TLR4-/- BMM at 50 ng/ml for 24h, and stimulated with 5 mM ATP for 1h prior to collection. Supernatant was collected and tested by IL-1β ELISA (R&D Systems). *, $p<0.05$

stimulation, indicating that rickettsial LPS signals through TLR4 providing the priming signal for inflammasome activation.

Rickettsial LPS and *Salmonella* LPS were used to treat WT B6 BMM in approximately the same quantity and stimulated with ATP as signal 1 and 2 for inflammasome activation, respectively (Fig. 8A). Rickettsial LPS and ATP stimulation led to significant amount of IL-1β secretion in WT B6 BMM, however, to a lesser extent than *Salmonella* LPS and ATP. The same is shown in cytotoxicity, measured by LDH release (Fig. 8B).

Immunoblotting in WT B6 revealed IL-1β production upon stimulation with rickettsial LPS and ATP (Fig. 8C). These results suggest that rickettsial LPS stimulation lead to the transcriptional upregulation and subsequent translation of pro- IL-1β, and that rickettsial LPS acts as signal 1 for inflammasome activation.

Since rickettsial LPS can act as signal 1 in WT B6 BMM, we next wondered what the role of TLR4 is in host immunity. TLR4 is considered to be a significant component of innate immunity during infection with many different Gram-negative bacteria. TLR4 is considered a significant component of host immunity in the C3H/HeN model of *R. conorii* (Jordan 2008). Here, we infected TLR4$^{-/-}$ and WT B6 BMM with *R. australis* at an MOI of 2 for 48 h (Fig. 9). TLR4$^{-/-}$ macrophages had significantly higher bacterial burden compared to WT BMM**.** Interestingly, bacterial burden in TLR4$^{-/-}$ BMM pre-treated with 40 ng/ml IFN-γ displayed significantly higher bacterial burden than untreated TLR4-/- (Fig. 10).

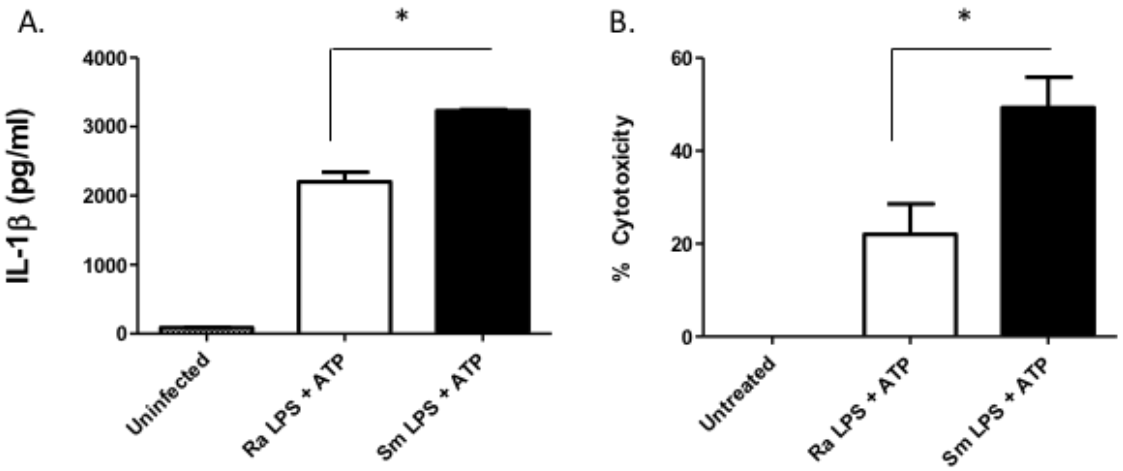

Figure 8A and 8B. Rickettsial LPS Primes the Inflammasome. WT B6 BMM were primed with 100 ng rickettsial (Ra) or *Salmonella* (Sm) LPS, and activated with 5 mM ATP. Samples were collected 24 hours post treatment. A) Active IL-1β secretion into the supernatant of MOI 6-infected WT B6 BMM B) Percentage cytotoxicity as measured by LDH assay in supernatant of MOI 6-infected WT B6 BMM. *, $p<0.05$

Discussion

The contribution of rickettsial LPS to the pathogenesis of rickettsial infections, especially in relation to cellular innate immunity, has been a matter of speculation for many years. Though multiple studies have touched on the structure of LPS, or the contribution of TLR4 to host defense during rickettsial infection, the interaction of TLR4 with rickettsial LPS, particularly spotted fever group LPS, has never been reported.

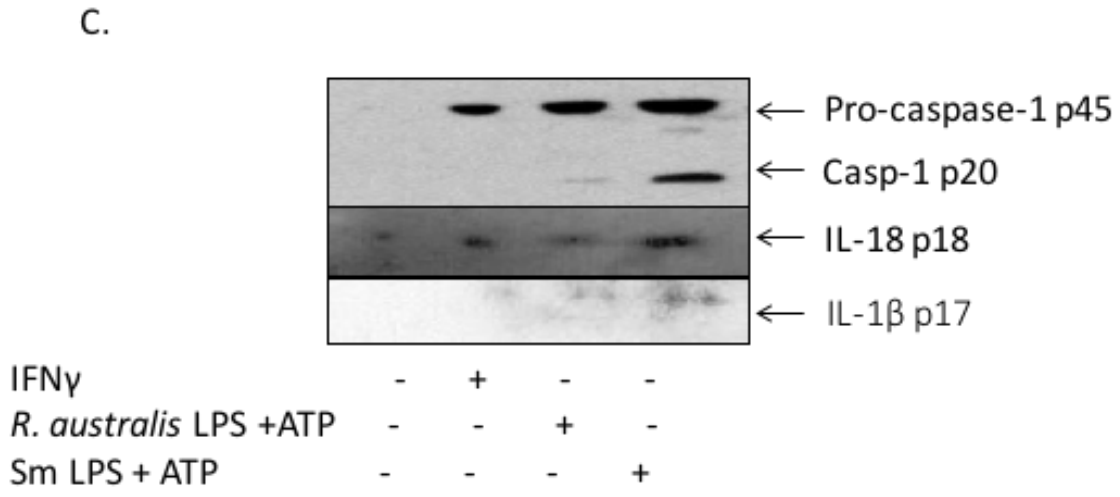

Figure 8C. Activation of Inflammasome Components in WT B6 BMM upon Treatment with LPS. WT B6 BMM were externally treated with ~100 ng rickettsial or *Salmonella* LPS for 24 h, and treated with 5 mM ATP for 1 hr. Supernatants were collected and concentrated. Adipogen anti-caspase-1 (1:500), Santa Cruz anti-IL-18 (1:1000), and Cell Signaling anti-IL-1β (1:1000) were used for detection

While rickettsial LPS here was not isolated via the standard phenol-chloroform extraction method, the extracted spotted fever group LPS shows differences in structure from *Salmonella minnesota* LPS via silver stain (Fig. 7C). These differences might be due to spacing between structural components of LPS, including Lipid A acylations and O-antigen (Fomsgaard 1990). These data also strongly agree with a study from Amano *et al.* (Amano 1993). Interestingly, the data shown in the manuscript of Amano *et al.,* Figure 1A,

lane 3 silver stain, shows SFG rickettsial LPS from *R. honei* TT-118 strain that agrees with *R. australis* LPS silver stain, especially the lowest two bands in the stain. Amano et al speculate that these lowest two bands ("fastest migrating" bands) are molecules containing the core oligosaccharide and lipid A portions. Furthermore, Amano *et al* speculate that these portions are not recognized by human IgM convalescent phase serum (Amano 1993). While this confirms structural differences between spotted fever group rickettsial and enterobacterial LPS, it lacks more specific data normally generated by mass spectrometry.

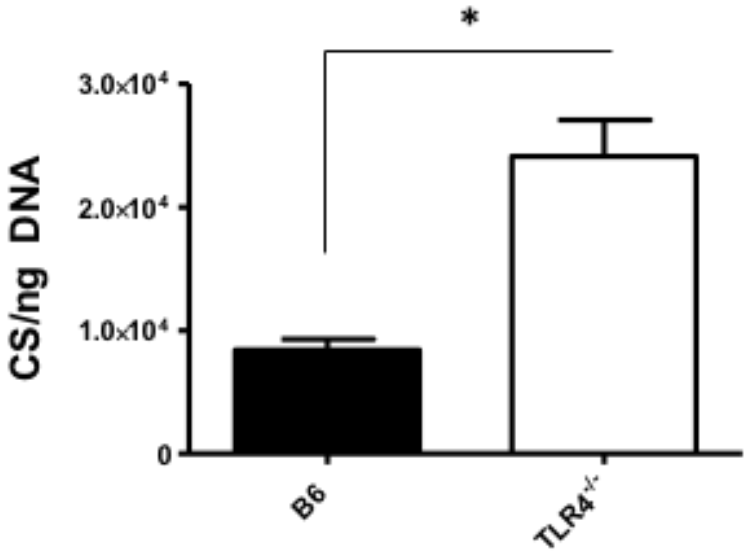

Figure 9. TLR4 contributes to host defense in BMM. Bacterial burden at 48h post-infection in BMM determined by qPCR. BMM were infected with MOI 2 *R. australis*. Cells were washed with PBS, and DNA was extracted using DNeasy Kit (Qiagen). qPCR was performed using probe for *R. australis* CS gene. *, $p<0.05$

Additionally, side-by-side silver stain imaging of typhus group and spotted fever group LPS has not yet been done, so structural differences between these two groups have not been studied in our system. Relatively lower levels of LPS in rickettsiae, 1-2% rickettsial biomass (Fodorova 2005), makes isolating and characterizing LPS difficult, which may account for the lack of structural data and studies. Nevertheless, these data confirm slight

differences in LPS structure between rickettsia and enterobacterial pathogens, which may have implications in pathogenesis and inflammasome signaling.

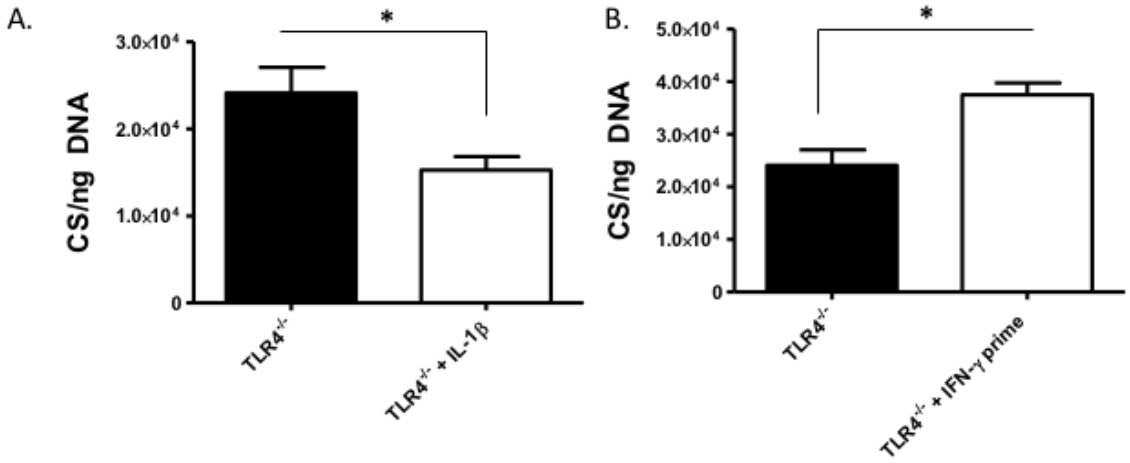

Figure 10. Bacterial burden in TLR4-/- BMM treated with cytokines. Bacterial burden at 48h post-infection in BMM determined by qPCR. BMM were infected with MOI 2 of *R. australis*. A.) r IL-1β (10ng/ml-Peprotech) was added upon infection, and medium was changed every 24h, or B.) IFN-g (40 ng/ml- R&D Systems) was used to prime 16h prior to infection. Cells were washed with PBS, and DNA was extracted using DNeasy Kit (Qiagen). qPCR was performed using probe for *R. australis* CS gene. *, $p<0.05$

Previously, Jordan *et al.* have published that *R. conorii* stimulate DC and *E. coli* LPS-stimulated DC protect from lethal *R. conorii* infection when adoptively transferred into infected mice (Jordan 2007). Jordan *et al*. suggested that TLR4 may play a role in DC-mediated protection, because *E. coli* LPS-stimulated DC were partially protective *in vivo* during *R. conorii* infection. Furthermore, both rickettsia and LPS stimulated DC produced IL-2 with similar kinetics, implying that this could be occurring through the same TLR (Jordan 2007). A study from Bechelli *et al* using MyD88-deficient DC shows the importance of TLR signaling in innate immunity. While these studies focus on DC, unpublished data show that MyD88 also plays an essential role in IL-1β secretion in BMM (unpublished data, Walker lab). MyD88 is an adaptor for multiple TLRs, including TLR4,

and we demonstrate here that TLR4 deficiency leads to complete ablation of inflammasome activation as measured by active, secreted IL-1β (Fig. 7A). This situation appears to be due to the lack of pro- IL-1β produced in TLR4-/- BMM in rickettsial infection (Fig. 7B). These results suggest that LPS-TLR4-MyD88 provide the priming signal for activation of the inflammasome by rickettsiae in mouse macrophages. It also excludes the possible involvement of the TLR4-TRIF signal in inflammasome activation.

We do find upregulation of pro- IL-1β in *R. australis*-infected TLR4$^{-/-}$ BMM lysate, suggesting that there are other TLRs involved in pro- IL-1β priming. Interestingly, we observed significantly more pro- IL-1β in *R. australis*-infected BMM, indicating the possibility that *R. australis* stimulates pro- IL-1β production via another pathway. It is likely that other TLRs are involved in sensing other rickettsial ligands, resulting in upregulation of pro- IL-1β, though it is still unclear which other rickettsial ligands are involved. Several studies have indicated that rickettsiae possess TLR2 and TLR9 ligands (Quevedo-Diaz 2010, Xin 2012), and since MyD88 is an adaptor for both TLR2 and TLR9, this increases the likelihood that one or both of these ligands are involved in inflammasome priming.

Since we see abrogation of inflammasome activation in TLR4$^{-/-}$ BMM (Fig. 7A) as well as abrogation of inflammasome activation in ASC$^{-/-}$ BMM (Fig. 5A) when infected with *R. australis*, inflammasome activation by rickettsiae is TLR4-dependent and ASC-dependent. This inflammasome pathway is not surprising, and is well described in models of many Gram-negative pathogens (Storek 2015). TLR4, and to some extent other TLRs, are required for inflammasome priming during rickettsial infection.

In terms of the contribution of TLR4 to immunity and bacterial burden, another publication from Jordan *et al* demonstrated that C3H/HeJ mice are more susceptible to rickettsial infection than C3H/HeN mice, due to the point mutation in the TLR4 receptor (Jordan 2008). C3H/HeJ mice produce significantly less IFN-γ, likely leading to a lack of TH1 polarization and proliferation. The mechanism of TLR4 deficiency leading to susceptibility to rickettsiae was speculated to be due to alteration in protective pro-inflammatory cytokines. In our studies, $TLR4^{-/-}$ BMMs exhibit higher bacterial burden compared to wild type BMMs (Fig. 9), consistent with the previous data showing that mice with a TLR4 mutation (C3H/HeJ) are significantly more susceptible to rickettsial infection than their C3H/HeN counterparts. Interestingly, however, when TLR4 BMM are primed with IFN-γ prior to infection, there is a higher bacterial burden. While there are reports of temporal priming requirements and pathway coordination between innate immune signaling pathways (Ward 2010, Kim 2014), including those of Type I/Type II IFN and TLRs, further studies are needed for clarification of results.

In conclusion, TLR4 is an important component of anti-rickettsial immunity, and TLR4 deficiency has noticeable affects on both innate and adaptive immunity. One of the mechanisms for the downstream effects of TLR4 deficiency could be an ablation of inflammasome activation, which effects other responding innate cells such as DC and NK cells. Without the proper polarizing cytokine signals, DC and NK cells have altered pro-inflammatory cytokines secretion, thereby affecting TH1 and CTL responses. These results clearly demonstrate that rickettsial LPS/TLR4 contributes to host protective immunity through inducing inflammasome activation in macrophages, which has never been reported.

Furthermore, rickettsial LPS is an important component of pathogenesis, though given the relatively low level of LPS produced and the altered structure of rickettsial LPS, rickettsiae do not seem to depend on their LPS as a virulence mechanism as heavily as their enteric gram-negative counterparts. It is possible that a dose-dependent mechanism exists during rickettsial infection. More structural data and mechanistic studies may help advance the field of knowledge of the contribution of rickettsial LPS to immune system evasion.

Chapter 5

The role of ASC-dependent inflammasome

Introduction

As discussed in previous chapters, rickettsiae induce inflammasome activation via upstream TLR4-dependent signals, and downstream, via the ASC-caspase-1/11-dependent pathway. We were next interested in the mechanisms by which ASC is able to regulate inflammasome activation by rickettsia. Volumes of literature establish ASC as the main adaptor protein and link between NLR and pro-caspase-1 (Schroder 2010, Rathinam 2012). A few studies have introduced the possibility that ASC may also have other roles inside the cell, including NFkB regulation. ASC plays a role in regulating MAPK/ERK2 pathways together with protein DUSP1 (Taxman 2011), and a study by Abdelaziz *et al* showed that a depletion of ASC resulted in greater NFκB activation, and greater bacterial survival (Abdelaziz 2011). Other studies have noted that ASC is a negative regulator of RIP2 protein (Sarkar 2006) and POP2 (Bedoya 2007), both of which act upon NFκB. Primarily, however, as the adaptor molecule for caspase-1, ASC regulation of IL-18 and IL-1β is tightly controlled so as not to lead to erroneous activation and downstream inflammation from these cytokines (Rathinam 2012).

While little data exist on IL-18 serum levels in rickettsial infection, IL-1β has been shown to be present early in rickettsial infection in human patients and mouse models (Parola

2013). Parola *et al* state that early phase infection with *R. conorii* ISF strain in human patients is associated with detectable levels of IL-1β in the serum. In mouse models, IL-1β is elevated at the peak of rickettsial infection, 4 days post-infection.

IL-1β is one of the most potent IL-1 family cytokines. Protection provided by IL-1β in infectious disease models is mainly associated with the ability of IL-1β to induce rapid neutrophil response and induction of a host of chemokines and cytokines (Sahoo 2011). Perhaps one of its most interesting functions, especially in regard to endothelial-targeting rickettsial infections, is the ability of IL-1β to activate endothelial cell adhesion molecules (Woods 2008). Amongst its pro-inflammatory abilities, several studies have explored the role of IL-1β in inducing ROS and NOS production, potentially making it an important cytokine for eradicating intracellular bacteria, like rickettsiae. Another function of IL-1β likely to be important for rickettsial infection is its ability to influence T-cell activation and differentiation, which is a critically important component of rickettsial clearance.

IL-18 secretion is classically associated with promoting IFN-γ production, primarily from NK cells, during infection (Dinarello 2003). The protective effects of IL-18 in infectious disease models mainly results from the ability of IL-18 to stimulate secretion of IFN-γ from both NK and T-cells. IL-18 has also been shown to have an effect in increasing levels of IL-8 and TNF-α, which are essential protective cytokines during rickettsial infection. Additionally, it amplifies cytotoxicity of CD8+ T-cells and NK cells (Sahoo 2011). This pathway is well-described in multiple infectious disease models, and has been linked to IFN-γ production by NK cells in models of other Rickettsiales such as anaplasma (Pedra 2007).

IFN-γ is an important cytokine in rickettsial infection, and is a necessary component of education and proliferation of cytotoxic T-cells (Tewari 2007). IFN-γ also enhances lysosome activity and induces iNOS production (Arunachalem 2000), increases expression of both MHC I and MHC II molecules (Zhou 2009, Steimle 1994), and suppresses TH2 polarization in CD4+ T-cells (Schulz 2009). All of these functions are potentially involved in rickettsial infection. The potential effect of ASC-dependent inflammasome activation on Type II IFN production and regulation makes investigating the innate-adapative immune response connection in rickettsial infection more imperative.

Given these important downstream effects of inflammasome-associated cytokines, we wondered if there was a role that the ASC-dependent inflammasome plays in both inducing innate rickettsicidal mechanisms as well as regulating adaptive immune responses to rickettsial infection through IL-1 family cytokine production. Our hypothesis for this set of experiments was that the ASC- dependent inflammasomes amplify or enhance IFN-γ production *in vivo* and ASC-dependent inflammasome activation contributes to bacterial clearance in BMM by mediating rickettsicidal IL-1β secretion.

Results

Previous data indicate that the ASC/caspase-1/11 axis is essential for inflammasome activation *in vitro* (Chapter 3, Smalley 2016). We hypothesized that ASC was protective *in vivo* in response to rickettsial infection. In a challenge model of *R. australis* using 0.5 LD_{50}, $ASC^{-/-}$ mice exhibited decreased survival compared to WT B6 mice (Fig. 11A); 90% of $ASC^{-/-}$ mice succumbed to infection by Day 7, while only 25% of WT B6 mice died by Day 9. These results are in line with the previous unpublished data from our lab that showed

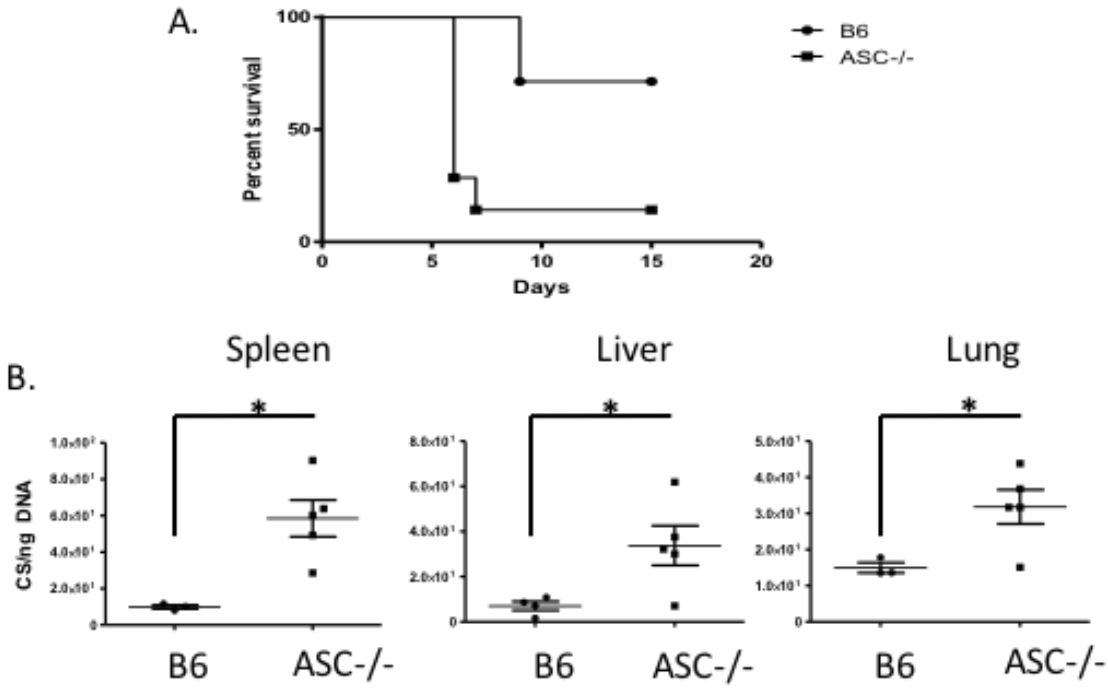

Figure 11. Survival and Organ Bacterial Burden in ASC-/- Mice. A.) Survival of 10-12 week old C57BL/6J and $ASC^{-/-}$ mice infected I.V. with 0.5 LD_{50} of *R. australis* from egg culture. B.) Bacterial burden at day 4 post-infection was measured by qPCR using CS gene in WT B6 and ASC-/- organs. *, $p<0.05$

100% of ASC-/- mice succumb to infection by Day 7.

To further investigate whether ASC mediates host protection through clearance of bacteria *in vivo*, infected tissues from these $ASC^{-/-}$ and WT mice were harvested on day 4 post-

infection. Bacterial burdens from lung, liver, and spleen showed a significantly higher level of bacteria in ASC$^{-/-}$ mice than in WT B6 mice (Fig. 11B).

Histopathology of these organs showed fewer, but larger, liver lesions in ASC$^{-/-}$ mice at

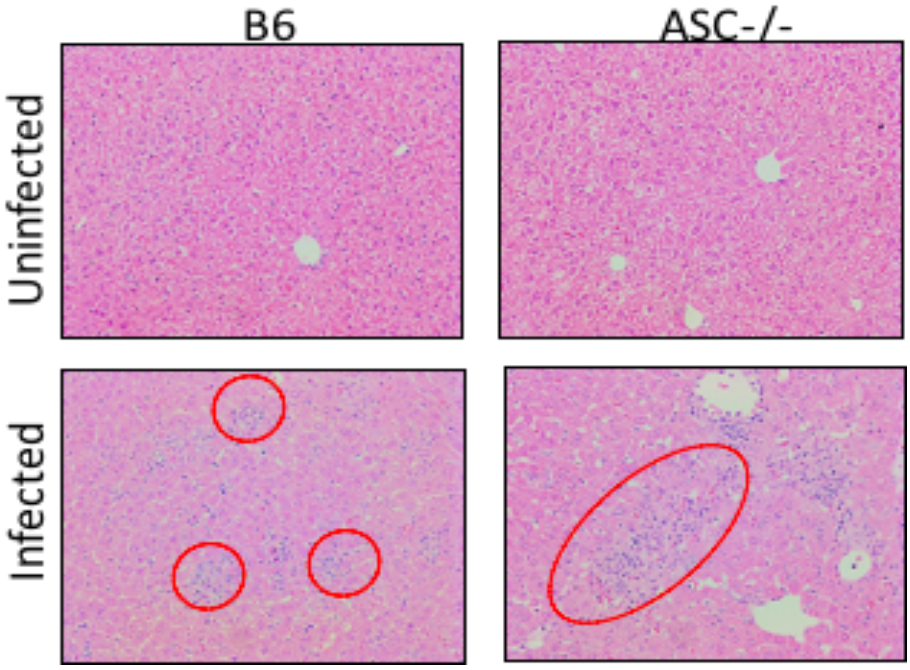

Figure 12A. Liver Histology in WT B6 and ASC-/- Mice. Liver samples were taken 4 days post-infection, embedded in paraffin, and stained with H&E for histological analysis.

day 4 post infection (Fig. 12A). Larger and more diffuse liver lesions were associated with poor control of infection, which correlates with both survival data as well as bacterial burden data. Lung histology showed no significant differences (Fig. 12B), and spleen histology showed an increase in white pulp at day 4 post-infection in both ASC$^{-/-}$ mice and WT B6 mice (Fig. 12C). While this appears to be important, future studies are required to explain how the expansion in white pulp contributes to host defense.

To further investigate the mechanisms by which ASC mediates protective immunity, we determined the *in vivo* production of pro-inflammatory cytokines including IFN-γ and IL-1β in the serum on day 4 p.i.. Indeed, ASC$^{-/-}$ mice had significantly decreased levels of

IFN-γ, IL-18, and IL-1β in the serum as compared to WT B6 mice (Fig. 13A-C). ASC$^{-/-}$ mice did not have significantly different levels of IL-10 in the serum; however, the concentration of IL-10 was greater than that in WT B6 mice (Fig. 13 D).

In finding that ASC$^{-/-}$ mice had increased mortality and decreased serum levels of

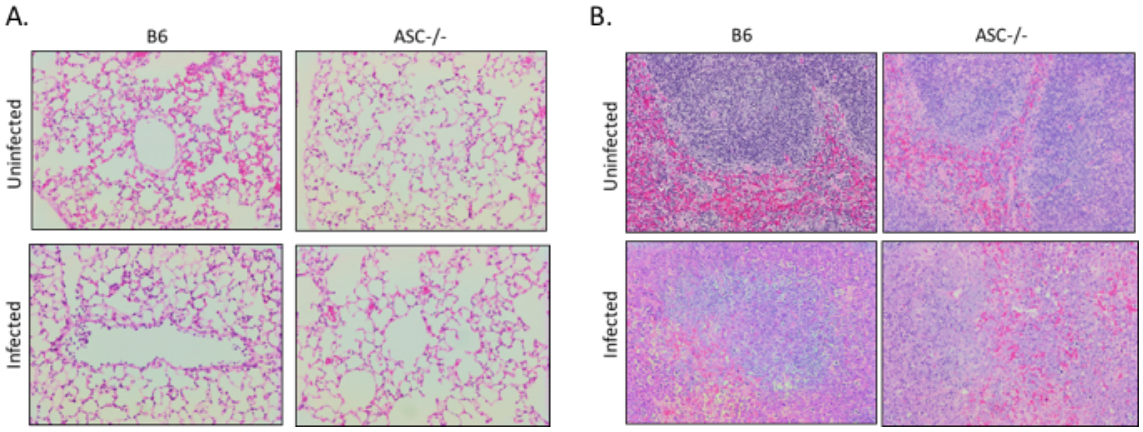

Figure 12 B and C. Histology of Lung and Spleen in WT B6 and ASC-/- Mice. Organ samples were taken 4 days post-infection, embedded in paraffin, and stained with H&E for histological analysis. A.) Lung samples for WT B6 and ASC-/- mice B.) Spleen samples for WT B6 and ASC-/- mice.

inflammasome-related cytokines, we examined previous data from Walker lab in IL-18R$^{-/-}$ mice. When infected with a sublethal dose of *R. australis*, IL-18R$^{-/-}$ mice are more susceptible to infection with *R. australis* than WT B6, and exhibit 40% mortality (unpublished data, Walker Lab). These data suggest that IL-18 signaling is protective *in vivo* and one of the inflammasome-mediated cytokines which contribute to host defense against rickettsial infection.

As shown in Chapter 3, ASC$^{-/-}$ bone marrow derived macrophages had complete ablation of both IL-18 and IL-1β production *in vitro* when infected with *R. australis* (Fig. 5A and 5B*)*. ASC$^{-/-}$ BMM also had a lack of caspase-1 activation as shown by immunoblot (Fig 5D). ASC$^{-/-}$ BMM produced increased pro- IL-1β upon infection with *R. australis*. Thus,

lack of active IL-1β secretion is not due to the reduced or abrogated levels of pro- IL-1β (Fig 14).

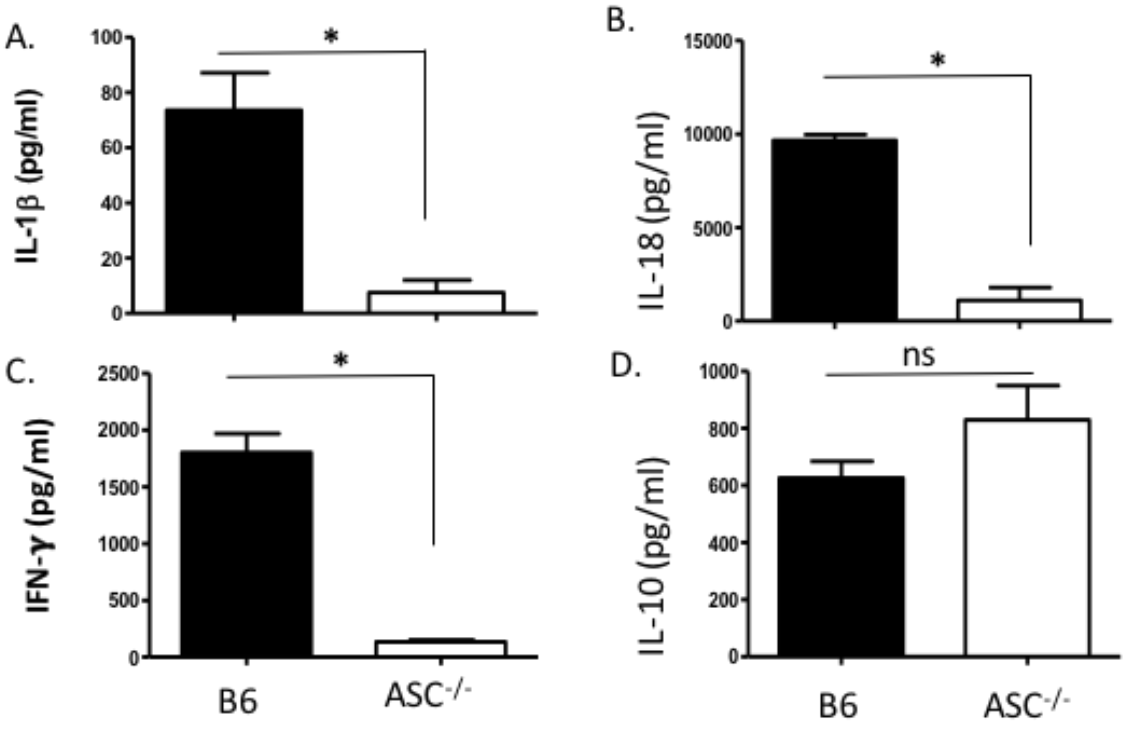

Figure 12. Serum Cytokine Levels in WT B6 and ASC-/- Mice. Serum was tested for cytokines by ELISA 4 days post-infection A.) IL-1β levels B.) IL-18 levels C.) IFN-γ levels D.) IL-10 levels. *, $p<0.05$

Since the secretion of inflammasome-related cytokines are deficient both in $ASC^{-/-}$ BMMs and in the sera of infected $ASC^{-/-}$ mice compared to WT controls, we next determined whether BMMs had a significantly higher bacterial burden as shown in the *in vivo* model. $ASC^{-/-}$ BMM were infected with *R. australis* with an MOI of 2, and cells were collected at 48 hpi. $ASC^{-/-}$ BMMs show significantly higher bacterial burdens than WT B6 BMM at 48 hpi (Fig. 15A). These data are also consistent with the *in vivo* model showing significantly increased bacterial burden in all of the collected infected tissues. Because $ASC^{-/-}$ BMM do not secrete IL-1β or IL-18, we wished to determine whether the higher bacterial burden

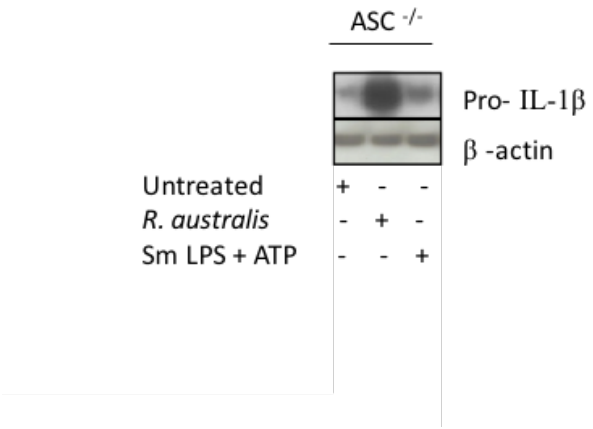

Figure 14. Upregulation of pro- IL-1β. ASC-/- BMM lysates from cells that were untreated, infected with MOI 6 of *R. australis*, or treated with 100 ng Sm LPS and 5 mM ATP for 24 h.

might be due to the lack of autocrine signaling from inflammasome-associated cytokines. Treatment of ASC$^{-/-}$ BMM with rIL-1β did not result in a lower bacterial (Fig. 15B). Since treating primary ASC$^{-/-}$ BMM with rIL-1beta had no effect, perhaps due to lack of IL-1R, we next infected RAW macrophages with *R. australis* and treated them with rIL-1β. RAW macrophages lack the ASC protein, and do not secrete active IL-1β when infected with *R. australis* (unpublished data, Walker lab). When RAW macrophages are infected with *R. australis* and rIL-1β is added at a concentration of 10 ng/ml, the bacterial burden decreases significantly (Fig. 16), indicating that rIL-1β plays a role in decreasing the bacterial burden *in vitro*.

To investigate if priming with inflammasome-independent cytokines might have an effect on bacterial burden in ASC$^{-/-}$ BMM, cells were primed overnight with IFN-γ, as previously described (Hagar 2011). ASC$^{-/-}$ BMM had significantly decreased bacterial burden at 48 hpi when primed overnight with 40 ng/ml IFN-γ compared to unprimed ASC$^{-/-}$ BMM (Fig.

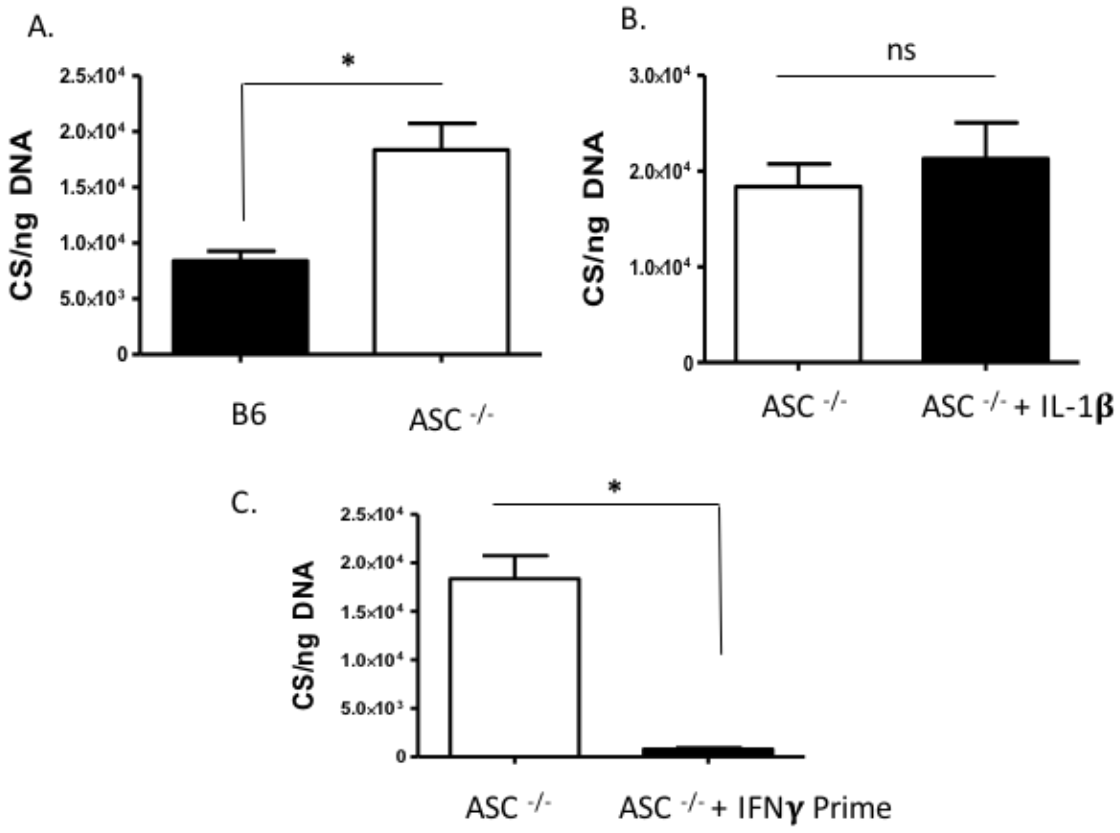

Figure 15. Bacterial Burden in WT B6 and ASC-/- BMM 48 hpi. Bacterial burden at 48h post-infection in BMM determined by qPCR. A.) BMM were infected with MOI 2 of *R. australis*. B.) Cells were untreated, or treated with 10 ng r IL-1β upon infection C.) Cells were untreated, or primed with 40 ng IFN-γ for 16h prior to infection. All cells were washed with PBS, and DNA was extracted using DNeasy Kit (Qiagen). qPCR was done using probe for *R. australis* CS gene. *, $p<0.05$; ns, not significantly different

15C). Both WT B6 and ASC$^{-/-}$ BMM primed with IFN-γ had significantly decreased bacterial burdens compared to their unprimed counterparts; however, primed WT B6 and primed ASC BMM bacterial burdens were not significantly different from each other, suggesting that rickettsial clearance by IFN-γ is an ASC-independent mechanism (Fig. 17). Additional studies in WT B6 BMMs using IFN-γ priming revealed increases in cytotoxicity at early stages on infection (Fig. 18). When primed with IFN-γ, cytotoxicity increased significantly compared to unprimed cells or cells primed with Type II Interferon (IFN-β). IFN-γ has been shown to upregulate pro-caspase-11 (Aachoui 2015) as well as upregulate

other cell defense mechanisms including ROS production, pro-IL-1β and pro-IL-18 (Rakshit 2014, Hayes 1995, Sodhi 1992). In this rickettsial infection model, IFN-γ likely promotes synthesis of pro-caspase-11 and other pro-inflammasome components, and upon infection with rickettsiae, the cell is able to mount an immune response faster and more efficiently. These data underscore the importance of IFN-γ during infection, and raises the possibility that macrophages need IFN-γ priming to reach their full inflammatory potential during early stages of infection.

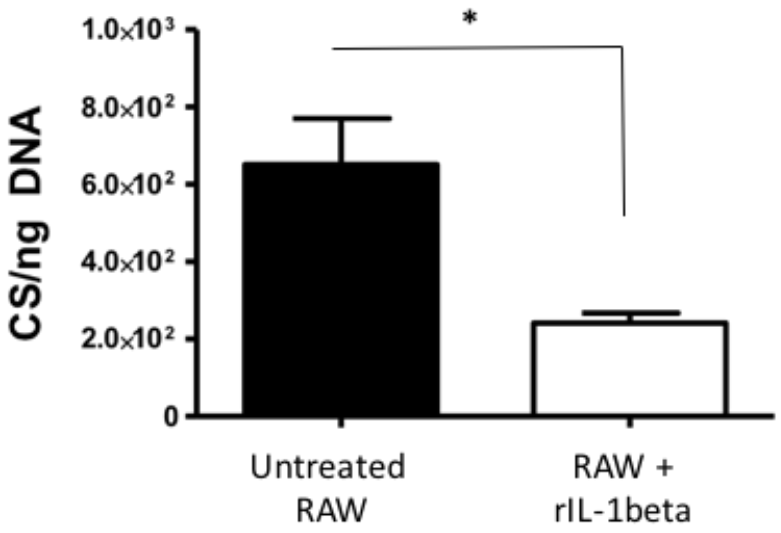

Figure 16. rIL-1β reduces Bacterial Burden in RAW Macrophages. Bacterial burden at 48h post-infection in BMM determined by qPCR. BMM were infected with MOI 2 of *R. australis*. rIL-1**β** (10ng/ml-R&D Systems) was added upon infection, and medium was changed every 24h. Cells were washed with PBS, and DNA was extracted using DNeasy Kit (Qiagen). qPCR was done using probe for *R. australis* CS gene. *, $p<0.05$

Discussion

ASC is protective *in vivo* during rickettsial infection, indicating that the inflammasome or cytokines secreted by inflammasome activation play a role in developing a sterilizing immune response. Because $ASC^{-/-}$ mice die by day 7 post-infection (Fig. 11A), it is likely

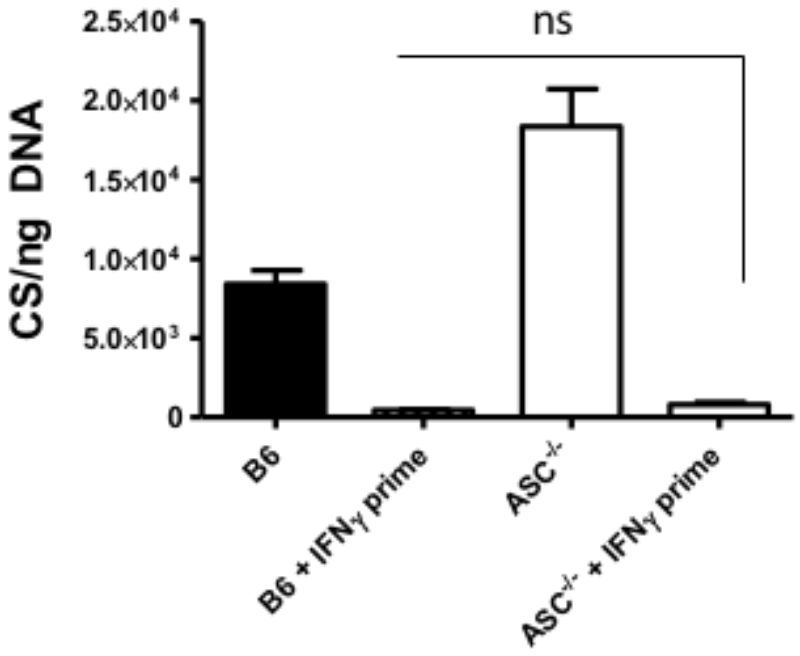

Figure 17. ASC contributes to bacterial clearance in BMM. Bacterial burden at 48h post-infection in BMM determined by qPCR. IFN-γ (40 ng/ml- R&D Systems) was used to prime 16h prior to infection. BMM were infected with MOI 2 of *R. australis*. Cells were washed with PBS, and DNA was extracted using DNeasy Kit (Qiagen). qPCR was done using probe for *R. australis* CS gene. ns, not significantly different

that during the adaptive immune response, CD4+ and CD8 + T-cells are not activated/primed at the optimal level, leading to defective development of effector T- cells. Several studies have reported the importance of ASC in sterilizing immunity (Pedra 2007, Abdelziz 2010).

At day 4 post infection, $ASC^{-/-}$ mice have significantly lower serum IFN-gamma (Fig. 13C), and, as expected, lower IL-18 and IL-1β in their serum than WT B6 mice (Fig. 13A

and 13B. ASC$^{-/-}$ mice also display slightly, though not significantly, elevated IL-10 levels (Fig. 13D). IL-10, a potent anti-inflammatory cytokine, has been shown by a number of researchers to be an important cytokine in rickettsial infection (Walker 2000, Vitale 2001, deSousa 2007). IL-10 is present in large quantities in a mouse model of rickettsioses after the immune control of the infection, but before convalescence (Walker 2000). In human infections, there are high levels of IL-10 in sera of patients with Mediterranean spotted fever (MSF) (Milano 2000).

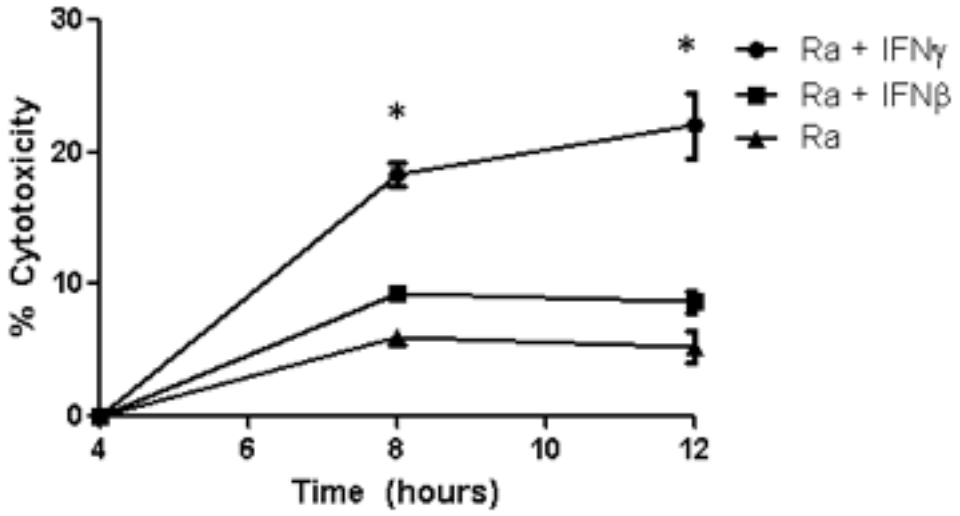

Figure 18. Kinetic IFN-priming in WT B6 BMM. WT B6 BMM were primed with either IFN-γ (40ng/ml- R&D Systems) or IFN-β (200 u/ml- PBL) for 16 hr prior to rickettsial infection with MOI 6 of *R. australis*. *, $p<0.05$

ASC$^{-/-}$ mice have higher bacterial burden in all organs (lung, liver, and spleen), and have significantly larger lesions in liver than B6 mice (Fig. 11B). These results suggest that ASC$^{-/-}$ mice have a stunted innate immune response at a crucial time point in rickettsial infection: the point when CD4 and CD8 T-cell responses are initiated.

At this time point, day 4 post-infection, NK cells are the most significant producers of IFN-γ (Fang 2012). Since ASC$^{-/-}$ mice have very low IFN-γ at day 4 post-infection, it is likely

that the lack of IL-18 and IL-1β result in subpar activation of NK cells, leading to lower levels of IFN-γ. Macrophage production of these two cytokines has been linked to effective NK activation and sustained NK cell responses (Mattiola 2015). Although NK cells have been reported to express low levels of IL-1RI compared to ILCs, there is evidence to suggest that NK cells upregulate detectable levels of IL-1RI, and that IL-1β has a biological effect on NK cells, especially when combined with IL-12, IL-15, and IL-23 (Hughes 2010, Cooper 2001, van de Wetering 2009). IL-18, however, is more commonly associated with NK cell activation. These phenomena have been described in multiple models of bacterial infection, including anaplasmosis and *Burkholderia pseudomallei* infection (Pedra 2007, Weirsinga 2007). IL-18 has been shown to be a prominent activator of IFN-γ secretion in NK cells via a positive feedback loop. Pedra *et al* have described, in a model of anaplasmosis, that IL-18$^{-/-}$ mice show the same level of mortality as ASC$^{-/-}$ mice, due to the lack of activation of NK cells and the subsequent lack of IFN-γ (Pedra 2007).

While ASC$^{-/-}$ mice in our model have significantly decreased IL-18 production (Fig. 13B), previous unpublished data have shown that rickettsia-infected IL-18R$^{-/-}$ mice have 25% mortality, compared with mice with ASC deficiency infected with the same dose of rickettsia, which is 90-100% lethal. Therefore, IFN-γ deficiency from the IL-18 positive feedback loop in NK cells is not entirely responsible for the level of mortality seen in ASC$^{-/-}$ mice. Given the low level of IL-1RI receptors on NK cells and the relatively smaller role of IL-1β priming in NK cells, it is unlikely that IL-1β deficiency alone results in decreased IFN-γ production. Thus, we looked to the role of IL-1β as potentially having some bactericidal activity *in vitro* using BMM.

ASC is clearly essential for IL-1β production in BMM (Fig. 5A). Previous data also indicate a lack of caspase-1 activation in the absence of ASC, indicating that inflammasome activation that is dependent on the ASC-caspase-1 axis. These data exclude the possibility that caspase-11 directly activates caspase-1, as ASC is necessary for caspase-1 activation. The possibility remains that activation of caspase-11 destabilizes the cellular membrane, leading to inflammasome activation through a yet unknown NLR-ASC-caspase-1 pathway. It is also possible that caspase-11 is responsible for pyroptosis. The role of caspase-11 in rickettsial infection will be more fully explored in Chapter 6.

In RAW macrophages, which naturally lack ASC protein, there is also a deficiency in IL-1β secretion (Fig. 16). When infected with rickettsia, RAW macrophages do not secrete IL-1β into the supernatant (Unpublished data, Walker Lab). When RAW macrophages were infected with rickettsia and then supplemented with recombinant IL-1β for 48 hours (Fig. 16), there was a significant decrease in bacterial burden compared to untreated RAW macrophages. This indicates that IL-1beta stimulates a rickettsicidal response in macrophages. The role of IL-1β as a defense mechanism against invading pathogens has been noted previously in the literature, especially in models of *M. tuberculosis* (Mtb). Jayaraman *et al* (Jayaraman 2013) have shown that IL-1β kills Mtb through the recruitment of ROS and NOS, through TNF receptor upregulation and caspase-3 activation.

While the $ASC^{-/-}$ BMM did not show reduced bacterial burden in response to rIL-1β added to the medium, it remains possible that this effect is explained by the previous report that primary derived BMM do not upregulate IL-1R. It is likely that the effect noticed by adding rIL-1β in RAW macrophages is the true effect of IL-1β on rickettsial infection in macrophages, as RAW macrophages remain IL-1R competent. The mechanisms that lead

to rickettsial clearance mediated by IL-1β have yet to be uncovered. It is possible that ROS and NOS account for the rickettsicidal mechanisms. Feng et al have demonstrated that NOS is a potent rickettsial killing mechanism in endothelial cells (Feng 2000), and likely in macrophages. Mechanistic studies with ROS and NOS inhibitors would clarify these links between IL-1β and rickettsial killing mechanisms.

While rIL-1β only showed significant effects in RAW macrophages, priming with IFN-γ led to dramatic effects on bacterial burden. IFN-γ, as mentioned previously, is a potent effector of macrophage activation and innate cellular pathogen killing pathways. Both WT B6 and $ASC^{-/-}$ BMM had significant decreases in bacterial burden at 48 hpi after IFN-γ priming (Fig. 15C). This mechanism of killing is ASC independent, and probably represents the ability of IFN-γ to prime caspase-11 and other pro-inflammasome components. Other data (Fig. 18) show that priming with IFN-γ before infecting with rickettsiae significantly increases LDH activity in the first 12 hours following rickettsial infection, even more so than priming with IFN-β. Increases in LDH activity suggest the involvement of caspase-11 and its ability to induce pyroptosis. By 12 hpi, cytotoxicity had increased to ~20% in IFN-γ primed cells compared with ~5% in unprimed cells. These data correlate with bacterial burden data at 48 hpi. Part of the decrease in bacterial burden at 48 hpi could due to increased pyroptosis. IFN-induced pyroptosis has never before been shown to be protective in rickettsial infection. Other possibilities in regard to decreased bacterial burden are increases in ROS and NOS production in IFN-γ primed macrophages. Previous studies have revealed increases in iNOS activity in cells exposed to IFN-γ, and previous studies have established NOS as a mechanism of rickettsial killing in endothelial cells and macrophages. It is likely that the increased levels of reactive oxygen species

resulting from IFN-γ stimulation are partially responsible for the decreased bacterial burden in BMM in our *in vitro* model. Altogether, these data underscore the importance of the inflammasome, particularly ASC, in rickettsial infection.

ASC has been broadly implicated as an important link between the innate and adaptive arms of the host response to pathogens (Schroder 2010). ASC appears to have the same function during rickettsial infection. A lack of ASC *in vivo* leads to decreased inflammasome associated cytokines, IL-1β and IL-18. The lack of IL-18 likely reduces the expansion and proliferation of NK cells in response to the lack of IFN-γ, which limits the expansion and proliferation of IFN-γ dependent TH1 CD4+ T-cells. Decreased IFN-γ levels likely impair expansion, proliferation, and cytotoxicity of CD8+ T-cells. This, together with slightly, though not significantly, elevated IL-10, further dampens any pro-inflammatory immune responses. The lack of IL-1β contributes to the dampened CD8+ T-cell response.

Additionally, ASC deficiency, leading to decreased IFN-γ, likely inhibits any cellular innate immune processes that are also dependent on IFN-γ, such as the priming of macrophages and MHC I/II expression in dendritic cells. Decreased macrophage priming could result in lower levels of ROS and NOS and a lack of pro-inflammasome components, leading to higher bacterial burdens.

Given the downstream importance of ASC in host defense, more studies are justified in fully investigating the repercussions of this inflammasome adaptor protein.

Chapter 6

Role of caspase-11 in inflammasome activation

Introduction

Since we have elucidated the role of ASC in rickettsial infection, we sought to investigate the downstream signaling driven by ASC in mediating the activation of caspase-1/caspase-11 axis. Caspase-1 and caspase-11 are closely related inflammatory caspases. They reside very close to each other in the genome (Kayagaki 2011), so much so that attempts at creating a caspase-1 single knockout animal model has been elusive. Currently, the only available caspase-1 single knockout mouse is a caspase-1/11 double knockout with a caspase-11 transgene added into the genome, utilized in a private lab and not commercially available. This makes studying caspase-1 difficult, and due to these constraints, we chose to investigate the respective roles of caspase-1 and caspase-11 using caspase-1/11 double knockout mice as well as caspase-11 single knockout mice. The caspase-11 knockout mouse on the C57Bl/6 background was generated by backcrossing C57Bl/6J wild-type mice with 129x1SvJ mice, which naturally lack caspase-11. These animals, while useful as a screen, have increased bactericidal capabilities in terms of enhanced NK cell activation (Sellers 2012, Man 2017) and are not an ideal model for studying the role of caspase-11 during infection.

Caspase-11 has relatively recently been clarified as an intracellular sensor of LPS (Kayagaki 2011, Hagar 2011). One of the ways caspase-11 is upregulated is upon binding

of TLR4 by LPS. TLR4 recruits the adaptor molecules TRIF and TRAM, which activate IRF3 for the production of Type I IFNα and IFN-β. Autocrine signaling from secreted Type I IFN engages the IFNAR, stimulating STAT1 and IRF9 pathways leading to caspase-11 upregulation. MyD88 has been shown, in some cases, to contribute once TLR4 is engaged by LPS (Broz 2012). Alternatively, caspase-11 can be upregulated by binding of Type II IFN, IFN-γ, by IFNR binding (Aachoui 2015). Because of the involvement of MyD88 in inflammasome activation in both DC and macrophages during rickettsial infection, we considered this as a possible mechanism for caspase-11 upregulation during rickettsial infection. Given the importance of IFN-γ in rickettsial infection, the possibility remains that IFN-γ plays a strong role in priming caspase-11 prior to inflammasome activation.

Following priming, caspase-11 binds intracellular LPS directly with the help of guanylate binding proteins, GBPs. GBPs have been implicated in lysing the vacuoles that some intracellular bacteria use as a replicative niche (Finethy 2015). Because rickettsiae escape the phagosome almost immediately upon entry into the host cell and replicate freely in the cytosol, we did not consider GBP involvement in this model. Caspase-11 is then activated by LPS of the invading Gram-negative organisms. As described in previous chapters, rickettsiae produce an atypical LPS in very small quantities. The ability of rickettsiae to grow and replicate in the intracellular space undetected for relatively long periods of time, as discussed in Chapter 3, is remarkable given the constant presence of caspase-11.

Caspase-11 can induce pyroptosis through activation and cleavage of Gasdermin D in order to expose the pathogen to the extracellular space. Pyroptosis, as an important mechanism of cell death in response to invading pathogens, is an intriguing idea for rickettsial

infection. In models of endothelial cell rickettsial infection, endothelial cells show little to no *inflammatory* cell death in response to infection as measured by LDH release and corresponding caspase-1/11 activation (unpublished data, Walker lab). While rickettsiae target mainly endothelial cells and it is possible that they have evolved specifically for the endothelial cell niche, we considered the possibility that rickettsiae do not induce pyroptosis through caspase-11 or even inhibit pyroptosis in order to maintain their intracellular niche.

In other circumstances, caspase-11 will destabilize the cellular membrane, resulting in ion flux and canonical inflammasome activation through NLRP3 (Yang 2015) and subsequent caspase-1 activation, IL-1β secretion, and IL-18 secretion. Given the involvement of caspase-11 in multiple pathways of inflammasome activation, we sought to understand the role of caspase-11 in activating the pathways leading to IL-1β and IL-18 secretion during rickettsial infection. Caspase-11 directly detects LPS in the cytosol of the cell, resulting in pyroptosis and inflammasome activation.

In the present studies, we sought to identify the contribution of rickettsial LPS to priming or activation of caspase-11 in regard to inflammasome activation. The hypothesis is that caspase-11 is partially required for IL-1β secretion and pyroptosis in BMM, and rickettsial LPS can directly activate caspase-11 when transfected to the host cytosol.

Results

To determine if caspase-11 deficiency would cause a dramatic increase in susceptibility to rickettsial infection, we infected 129X1SvJ mice with 0.5 LD_{50} *R. australis* egg stock. After 15 days infection, all mice had survived (Fig. 19A). Some mice became hunched and ruffled at day 4 post-infection; however, they recovered quickly and remained healthy until the end of the experiment. These results suggest that caspase-11 deficiency in rickettsial infection does not induce a significant and dramatic phenotype. In a caspase-11 single knockout strain, rickettsial infection may result in some mortality, but likely would not induce a dramatic phenotype similar to $ASC^{-/-}$.

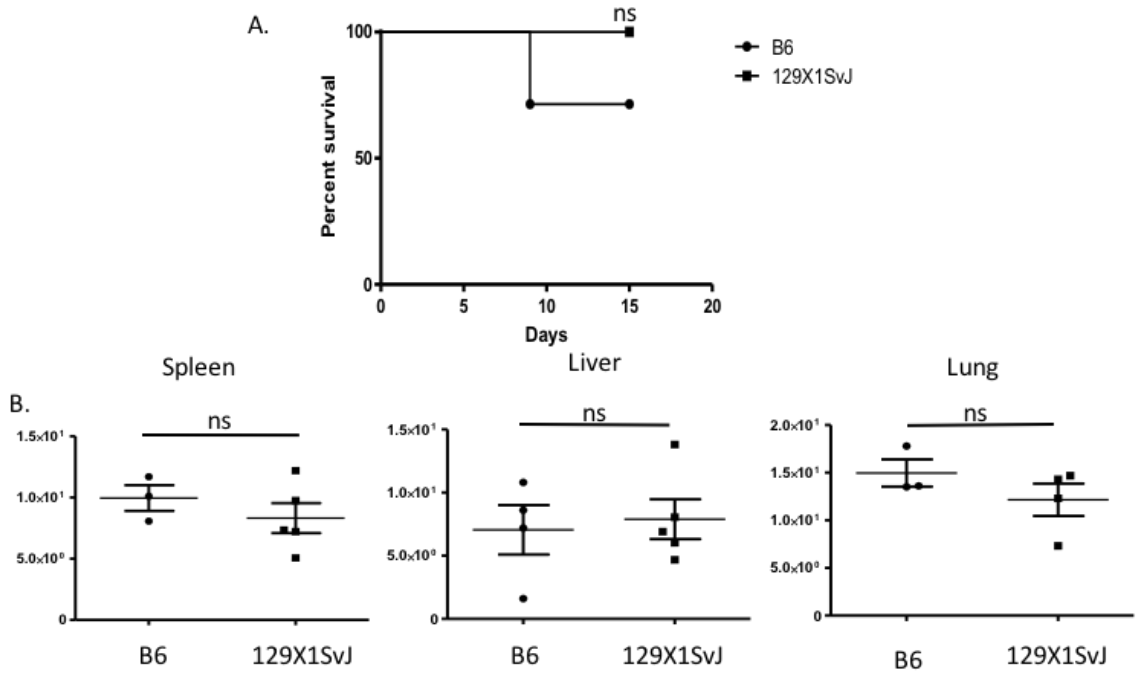

Figure 19. Survival and Organ Bacterial Burden in 129x1SvJ Mice. A.)Survival curve of 10-12 week old C57BL/6J and 129x1SvJ mice infected I.V. with 0.5 LD_{50} *R. australis* from egg culture. B.) Bacterial burden at day 4 post-infection was measured by qPCR using CS gene in WT B6 and 129x1SvJ organs. ns, not significantly different

To investigate the bacterial burden in 129X1SvJ mice, samples of lung, liver, and spleen were harvested at day 4 post-infection (Fig. 19B). All specimens were assayed by qPCR,

and bacterial burdens in liver, lung and spleen were not significantly different from those in WT B6 mice. These data are consistent with the survival data.

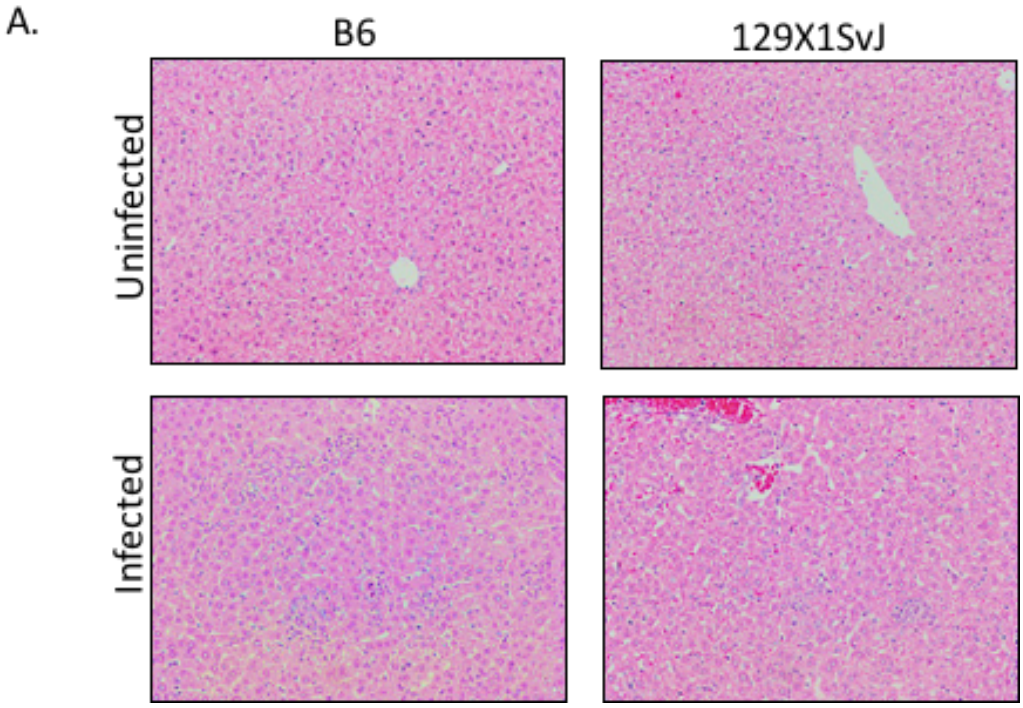

Figure 20A. Liver Histology in WT B6 and 129x1SvJ Mice. A.) Liver samples were taken 4 days post-infection, embedded in paraffin, and stained with H&E for histological analysis.

Liver, lung, and spleen samples were also collected on day 4 for histology. Liver histology showed significantly fewer lesions in the liver than in B6 mice (Fig. 20A). There were no significant differences between lung histology samples (Fig. 20B), and spleen histology showed expansion of the white pulp at day 4 post-infection (Fig. 20C); however, this does not seem to be clinically relevant as the levels of bacterial burden remained low at the same time point. Taken together, these data all matched survival and bacterial burden data. The data showed that 129x1SvJ mice have remarkable control over rickettsial infection, and therefore do not exhibit the same pathology that susceptible WT B6 mice show during infection. Serum cytokines IL-1β, IFN-γ, and IL-10 are all significantly decreased in

129x1SvJ mice compared to WT B6 mice at day 4 post-infection, indicating resistance to rickettsial infection (Fig. 21 A-C).

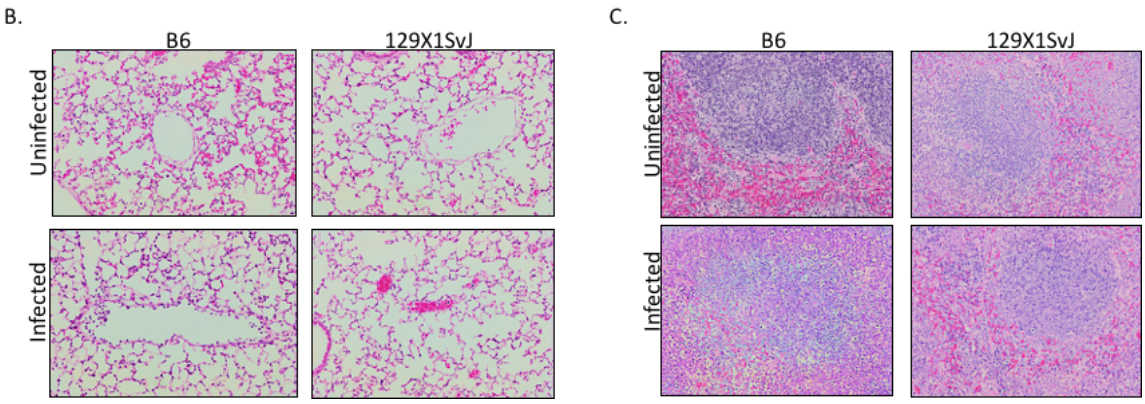

Figure 20 B and C. Histology of Lung and Spleen in WT B6 and 129x1SvJ Mice. Organ samples were taken 4 days post-infection, embedded in paraffin, and stained with H&E for histological analysis. A.) Lung samples from WT B6 and 129x1SvJ mice B.) Spleen samples from WT B6 and 129x1SvJ mice.

To investigate the role of caspase-11 in inflammasome activation *in vitro*, caspase-11 single knockout BMM were infected with MOI 6 of *R. australis* for 24 h (Fig. 22). Caspase11$^{-/-}$ BMM produced significantly lower levels of IL-1β upon infection compared to WT B6 BMM (Fig. 22A). Levels of TNF-α as a preliminary surrogate marker for cell-death in both genotypes are similar (Fig. 22B).

To determine whether IL-1β secretion is caspase-1 dependent, we also infected caspase 1/11-double KO BMM and compared them with caspase-11 single knockout BMM. In caspase-1/11- double KO BMM, inflammasome activation as measured by IL-1β secretion was completely ablated (Fig 23A). These data have been described previously (Chapter 3), but the experiment had not been done in the context of separating the respective contributions of caspase-1 and caspase-11. In this experiment, stimulation with LPS and ATP also resulted in no production of IL-1β, as expected. Levels of TNF-α in cell

supernatant were not significantly different from WT B6, showing that cell function is normal (Fig.23B).

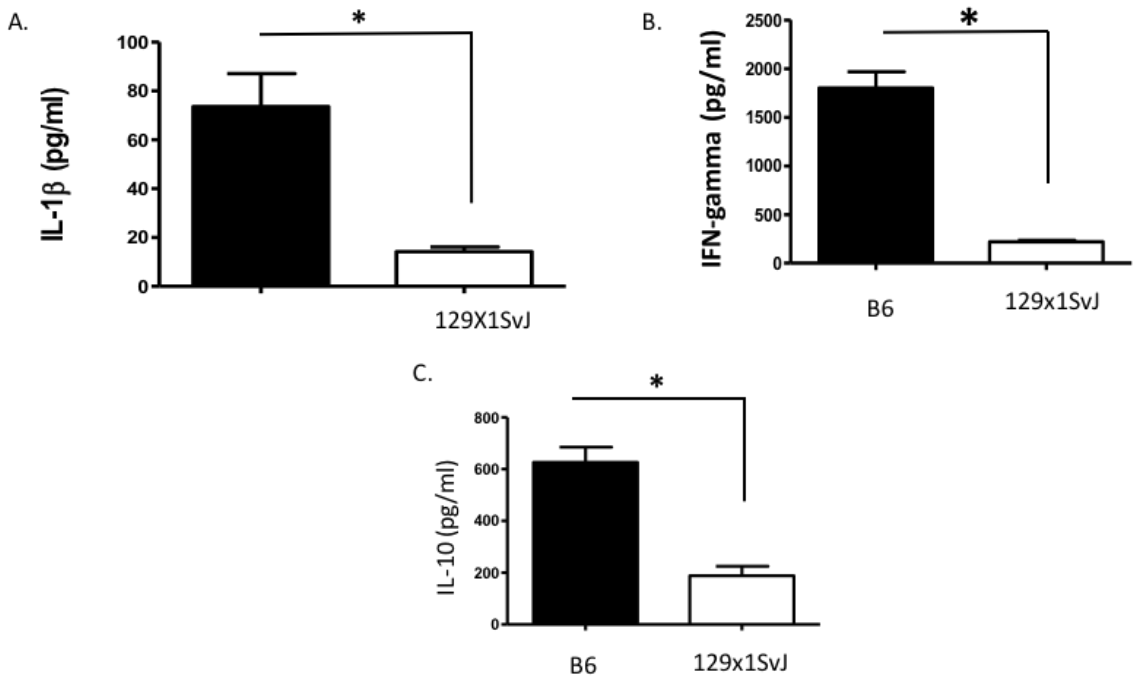

Figure 21. Serum Cytokine Levels in WT B6 and 129x1SvJ Mice. Serum was tested for cytokines by ELISA 4 days post-infection A.) IL-1β levels B.) IFN-γ levels C.) IL-10 levels. *, $p<0.05$

Next, we sought to investigate the roles that caspase-11 and caspase-1 play in host immunity by measuring the bacterial burdens in BMM. Caspase-11- single KO BMM had significantly higher bacterial burden than WT B6 BMM (Fig 24), and caspase-1/11- double KO BMM did not have significantly different bacterial burden compared with WT B6 BMM (Fig. 25). Because caspase-11- single KO BMM have high bacterial burdens, and caspase-1/11- double KO BMM are also caspase-11 deficient, these results show an inconsistency between these two genotypes, and could be due to decreased internalization of rickettsiae in caspase-1/11-double KO BMM.

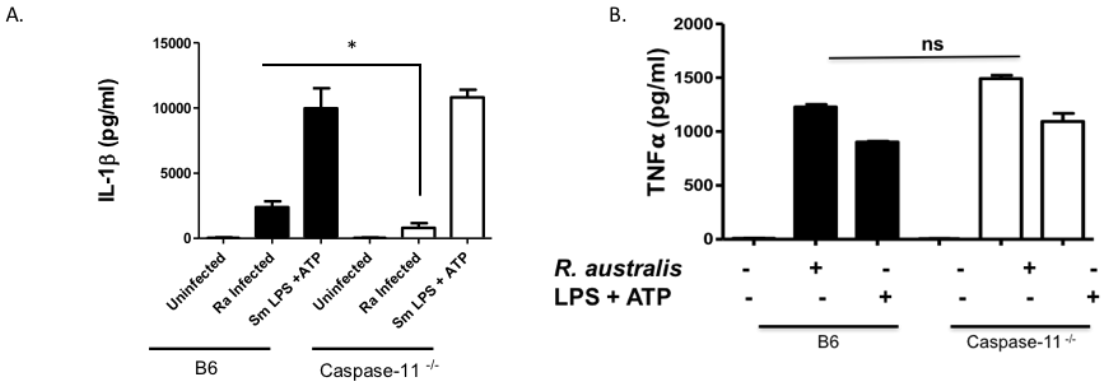

Figure 22. Caspase-11 is partially involved in IL-1β secretion. WT B6 BMM and caspase-11-/- BMM were infected MOI 6 of *R. australis*. Supernatants were collected and tested for A.) IL-1β secretion B.) TNF-α secretion. *, $p<0.05$; ns, not significantly different

To investigate whether rickettsial LPS could directly activate caspase-11 when transfected to the cytosol of BMM, rickettsial LPS was first isolated and quantified as discussed in Chapter 4. Rickettsial and *Salmonella* LPS were then transfected to primary B6 BMM using Lipofectamine. Cytotoxity assays (Fig. 26A) and caspase-1 immunoblots (Fig. 26B) showed that caspase-11 was activated by rickettsial LPS, with prior IFN-γ priming. The lack of *in vivo* activation of caspase-11, therefore, is likely explained by rickettsial evasion tactics, either by limiting the level of LPS freed in the cytosol, or by altering the structure of LPS. It is possible that rickettsiae alter the structure of LPS in a temperature-dependent manner, similar to other arthropod-borne gram-negative bacteria such as *Yersinia*. Further tests are necessary to determine if LPS structure is temperature and cell-type dependent.

Gasdermin D (GSDMD) is the downstream substrate of caspase-11. To see if GSDMD deficiency affected host defense in response to rickettsial infection, BMM were infected for 48h with MOI of 2 of *R. australis* (Fig. 27). GSDMD-deficient BMM have significantly higher bacterial burden than WT B6 BMM, suggesting that GSDMD plays a role in host defense against rickettsiae.

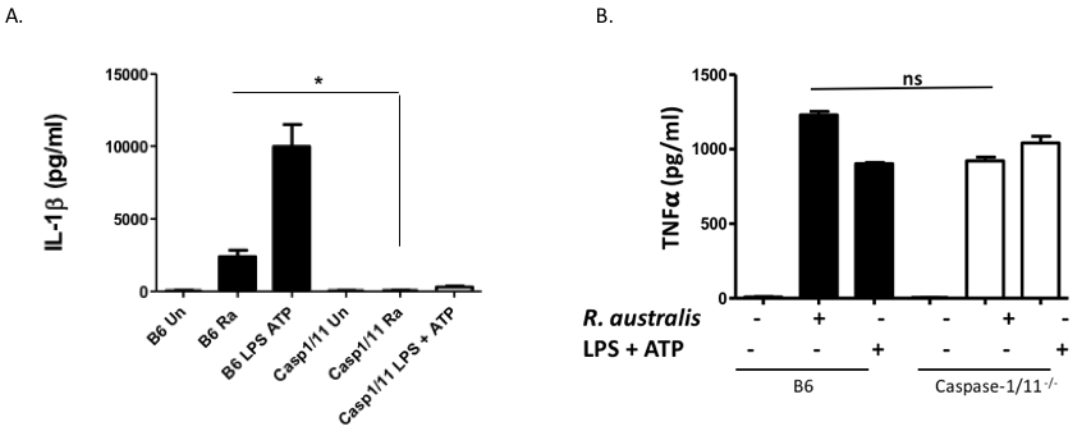

Figure 23. Caspase-1/11 is required for IL-1β secretion. WT B6 BMM and caspase-1/11-/- BMM were infected MOI 6 of *R. australis*. Supernatants were collected and tested for A.) IL-1β secretion B.) TNF-α secretion. *, $p<0.05$; ns, not significantly different

To determine if rickettsiae inhibit inflammasome activation, THP-1 derived macrophages were infected with MOI of 2 of *R. australis* for 24 hours after priming with multiple stimuli (Fig. 28). THP-1 derived macrophages were either infected with MOI of 2 *R. australis*, primed with *Salmonella* LPS for 19 hours then infected with MOI 2 of *R. australis* and stimulated with ATP 1 hr before collection, infected with MOI 2 of *R. australis* and stimulated with *Salmonella* LPS for 24 hrs and 1hr ATP stimulation, *Salmonella* LPS for 24 hours and ATP for 1 hr, or infected with MOI 2 of *R. australis* and stimulated with ATP for 1 hour. Infection with *R. australis* alone shows low cytoxicity (~10%) and moderate IL-1β production (~200 pg/ml). Classical inflammasome stimulation of *Salmonella* LPS and ATP show higher cytoxicity (~45%) and high IL-1β production (~600 pg/ml). With classical inflammasome stimulation of *Salmonella* LPS and ATP *plus* rickettsial infection, however, there is a significant decrease in both cytotoxicity and IL-1β production. These data suggest that rickettsiae have the ability to suppress or interfere with inflammasome activation. Also of note is that *R. australis* infection plus ATP stimulation do not result in

an increase in cytotoxicity or IL-1β production, again suggesting that rickettsia interfere with or suppress inflammasome activation.

Discussion

Taken together, these results indicate that although caspase-11 is partially involved in inflammasome activation, caspase-11 deficiency did not increase host susceptibility *in vivo*. This could be due to the background of the 129x1SvJ strain: they are markedly more resistant to infection than C57BL/6J mice (Fig. 19A). A dramatic phenotype in 129x1SvJ, such as extremely high mortality, might indicate that caspase-11 is heavily involved in inflammasome signaling and the downstream signaling of inflammasome related cytokines. The lack of a dramatic phenotype, however, suggests a subtle phenotype in

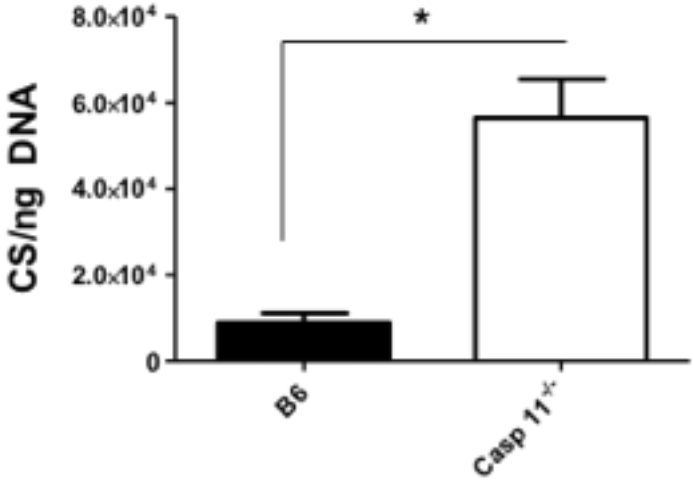

Figure 24. Caspase-11 contributes to host defense in BMM. WT B6 and caspase-11-/- BMM were infected with MOI 2 of *R. australis* for 48 hrs. Bacterial burden was determined by qPCR using CS gene as a standard. *, $p<0.05$

caspase-11 deficiency. Either caspase-11 is not involved in protection during rickettsial infection, or on the appropriate C57BL/6J background, caspase-11 deficiency might cause a slight increase in mortality. Since caspase-11 does play a role in IL-1β secretion, it is likely that caspase-11 does play a role, albeit not as dramatic a role as $ASC^{-/-}$, in inflammasome activation.

All data related to 129x1SvJ *in vivo* work correlates well with the survival data. Bacterial burden in lung, liver, and spleen in 129x1SvJ show no significant differences from WT B6 mice (Fig. 19B). Interestingly, serum cytokine levels of IL-1β and IFN-γ of 129x1SvJ were significantly lower than in WT B6 mice, at levels similar to $ASC^{-/-}$ mice (Fig. 21). This indicates that 129x1SvJ did not mount a large pro-inflammatory response against rickettsiae, confirming that they are resistant to rickettsial infection. A number of possibilities exist as to why these mice are more resistant than WT B6. Some studies show that 129x1SvJ mice have differential NK cell responses against pathogens (Sellers 2012), and other studies that show differences in phagocytosis (Sun 2011) between 129 and B6

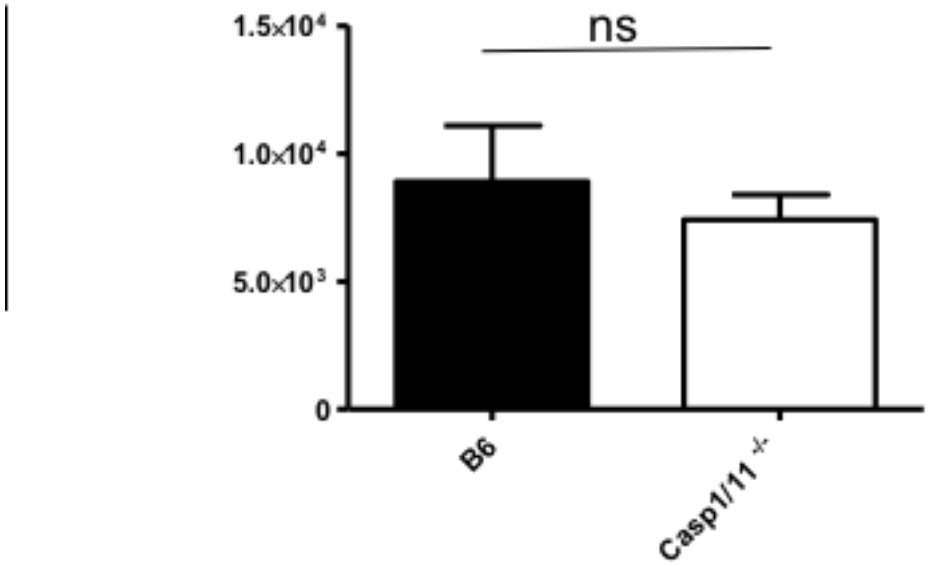

Figure 25. Bacterial burden in Caspase-1/11-/- BMM. WT B6 and caspase-1/11-/- BMM were infected with MOI 2 of *R. australis* for 48 hrs. Bacterial burden was determined by qPCR using CS gene as a standard. ns, not significantly different

genotypes. These differences may play a role in the ability of rickettsiae to enter and infect host cells. Sun *et al* demonstrated that differences in internalization between 129Sv alveolar macrophages and B6 alveolar macrophages were due to 129Sv macrophages expressing high levels of class A scavenger receptor MARCO, which is responsible for the

in vivo recognition of unopsonized bacteria and differences in internalization of bacteria (Sun 2011). This study, which was performed in a *Streptococcus* model, may have relevance in our model because of the reported involvement of MARCO with TLR4 (Chen 2010). TLR4 engagement with LPS leads to upregulation of MARCO in a MyD88-dependent and independent manner (Chen 2010). The upregulation of this receptor led to increased microbial capture and clearance before the bacteria can enter host cells *in vivo*; therefore, increased expression of MARCO may result in decreased levels of bacteria being internalized into the macrophage.

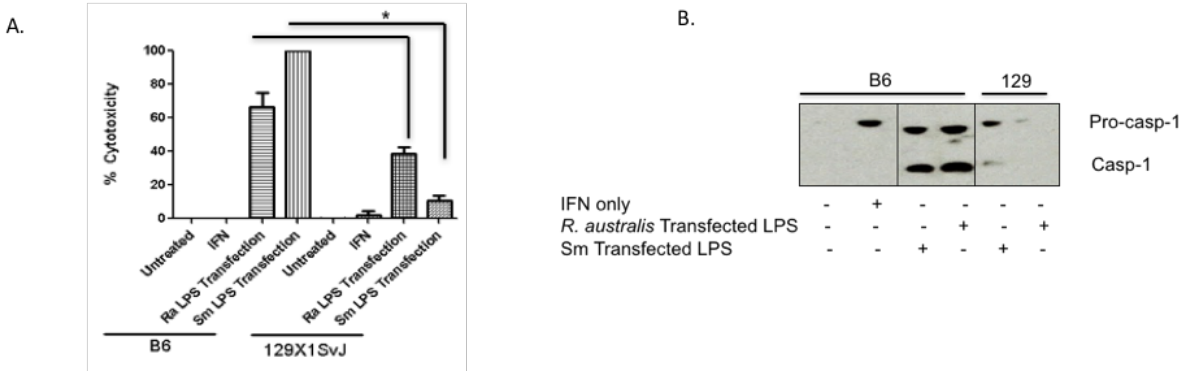

Figure 26. Rickettsial LPS transfection to the cytosol activates caspase-11. WT B6 BMM and 129x1SvJ BMM were primed for 16 hours with 40 ng of IFN-γ, then transfected with 100 ng of *R. australis* LPS or *Salmonella* LPS for 24 hours using Lipofectamine. Supernatant was collected after 24 hours A) Percent cytotoxicity was determined by LDH assay. B) Cell supernatant was concentrated and added to lysates. Immunoblot using anti-caspase-1 antibody from Adipogen (Casper-1). *, $p<0.05$

These differences in infectivity in our model, however, were not only seen *in vivo*; *in vitro* experiments using 129x1SvJ BMM show significant decreases in bacterial burden compared to WT B6 BMM (unpublished data). This result may also be due to differential internalization of rickettsiae in these cells compared to WT B6 BMM. Lower infectivity could account for a lack of inflammasome activation in 129x1SvJ BMM, as measured by secreted IL-1β and caspase-1 activation (unpublished data). Further studies are warranted

to investigate the role of autophagosomes in 129x1SvJ BMM, which may contribute to the low levels of inflammasome activation and bacterial burden *in vivo* and *in vitro*.

Our data in caspase-11- single KO BMM suggest that caspase-11 does play a partial role in inflammasome activation as measured by IL-1β secretion during rickettsial infection (Fig. 22A). When infected for 24 hours with MOI of 6, there was a significant difference in the levels of IL-1β between caspase-11- single KO BMM and WT B6 BMM. While significant, this difference is relatively small, indicating that caspase-11 does play a partial role in IL-1β secretion. These data indicate that the involvement of caspase-11 in IL-1β secretion is mediated via an unknown NLR with some NLRP3 involvement-ASC-caspase-1 pathway. Caspase-11 probably contributes to this pathway by inducing ion flux leading

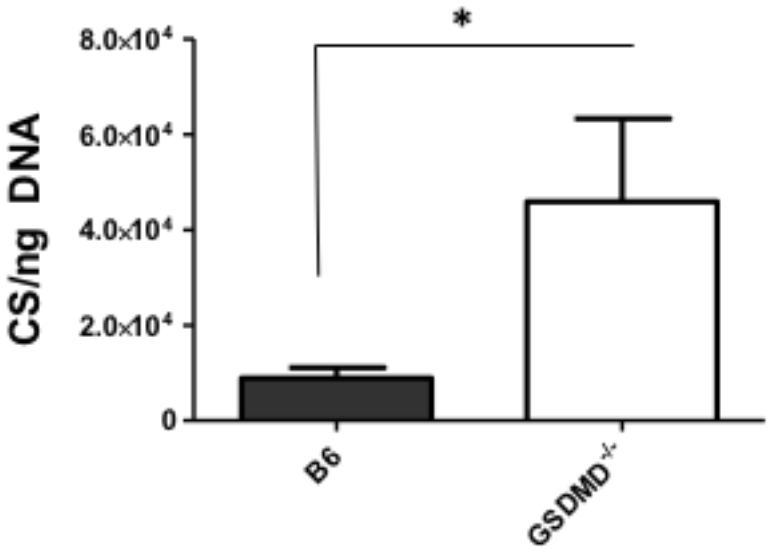

Figure 27. Gasdermin D is Involved in Host Defense in BMM. WT B6 and GSDMD$^{-/-}$ BMM were infected with MOI 2 of *R. australis* for 48 hrs. Bacterial burden was determined by qPCR using CS gene as a standard. *, $p<0.05$

to activation of an unknown NLR, in concert with NLRP3, at the early stages of infection. Another reason for partial involvement could be the relatively low abundance of rickettsial LPS. Chapter 4 briefly discussed the condition that rickettsial LPS accounts for a low

percentage of biomass in purified rickettsial pellets. The possibility remains that rickettsia shed such little LPS that caspase-11 isn't activated in sufficient levels to fully activate inflammasome-mediated secretion of IL-1β and IL-18. Another point of interest is that, as discussed in Chapter 3, IL-1β and IL-18 production in rickettsia-infected BMM begin to rise between 8-12 hpi, and peak at 24 hpi. Rickettsiae complete replication around 10-12 hours post-infection, and inflammasome activation could coincide with LPS shedding during replication.

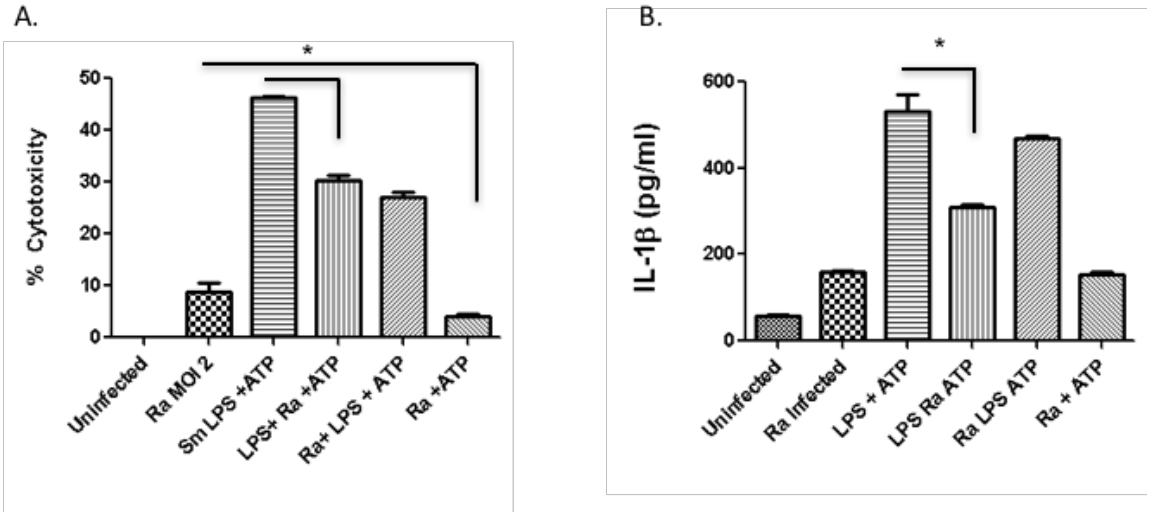

Figure 29. Rickettsial infection reduces cytoxicity and IL-1β production. THP-1 monocytes were differentiated into macrophages by PMA. THP-1 macrophages were infected with *R. australis* (MOI 2) for 24 hours. Cells were either treated with 100 ng Sm (*Salmonella minnesota*) LPS for 24h and 5 mM ATP for 1 hr (Sm LPS + ATP or rickettsial LPS +ATP), 100 ng Sm LPS for 24 h, *R. australis* MOI 2 infection for 5 hr, and 5 mM ATP for 1 hr (LPS+Ra+ATP), *R. australis* MOI 2 infection for 24 hr, 500 ng LPS for 5 hr, 5 mM ATP for 1 hr (Ra+LPS+ATP), or *R. australis* MOI 2 for 24 hr and 5 mM ATP for 1 hr (Ra+ATP). *, $p<0.05$

Partial involvement of caspase-11 could also stem from rickettsia's atypical LPS. Previous studies report that both TLR4 and caspase-11 recognize similar epitopes on lipid A: hexacylated lipid A species. Rickettsial LPS is recognized by TLR4 as measured by IL-1β secretion and increases in cytotoxicity, but this recognition of rickettsial LPS is always

lower than recognition of *Salmonella* or *E. coli* LPS (Fig 8). The variation in rickettsial LPS acylation, hexacylated with a significant minority of lipid A tri- and tetra-acylated, suggests that levels of recognition by both TLR4 and caspase-11 could be due to the relatively lower amount of rickettsial LPS that is hexacylated.

Regardless of lipid A acylations, rickettsial LPS seems to be recognized by caspase-11 upon transfection to the cytosol of BMM (Fig 26). Recognition of ~100 ng of rickettsial LPS by cytosolic caspase-11 implies that 1) rickettsial LPS is present in too low a quantity during infection to appropriately activate caspase-11 and 2) rickettsiae may alter LPS during infection to better evade host detection. The evidence for the former (Fig. 28) shows that rickettsial infection, together with classical inflammasome stimuli, results in a reduction of cytotoxicity and inflammasome activation as measured by IL-1β secretion. These data are interesting, and perhaps unsurprising. Rickettsiae reside solely in the host cytosol-recognition by the host via caspase-11 detection of LPS would certainly result in pyroptosis and the elimination of the intracellular replicative niche. Evasion of host detection mechanisms is paramount and favors survival of the organism. Although still unknown and its existence still needs to be tested, it is likely that rickettsiae encode some mechanism for inflammasome evasion, either through evading LPS recognition, production of an NLR analog, or a protein that inhibits inflammasome signaling. Many intracellular bacterial pathogens encode some form of virulence factor that contributes to evasion of host innate cellular immune mechanisms such as the inflammasome.

Caspase-11, while contributing partially to IL-1β secretion, also plays a role in rickettsial clearance in macrophages at 48 hpi (Fig 24). The bacterial burden in BMMs from caspase-11-single KO is significantly higher than in WT B6 BMM. Gasdermin D also plays a role

in limiting the bacterial burden, as bacterial burden in GSDMD-/- BMM is higher at 48 hpi as well (Fig. 27). It is likely that both caspase-11 and GSDMD restrict bacterial growth, presumably through their respective roles in inflammasome activation, or in mediating pyroptosis at later stages of infection (beyond 48 hpi). Interestingly, caspase-11 is known to have other roles besides that of noncanonical inflammasome activation, particularly that of modulating actin polymerization (Li 2007) and lysosome trafficking (Ahkter 2012). There may be unknown functions of caspase-11 during rickettsial infection that deserve greater study using actin polymerization-deficient *R. parkeri* strains.

Altogether, these data demonstrate a partial role for caspase-11 during rickettsial infection. Full activation of caspase-11 may be restricted by availability of rickettsial LPS, or by the structure of rickettsial LPS. Other roles of caspase-11 action, such as its function in actin polymerization independent of its inflammatory enzymatic activity, would be interesting subjects to pursue in the future.

Chapter 7

Discussion

Altogether, these data show the importance of the inflammasome for host immunity against rickettsiae. Rickettsiae activate inflammasome production of IL-1β and IL-18 beginning 8-12 hpi and peaking at 24 hpi. TLR4 is essential for inflammasome priming and subsequent activation; previous studies confirm that TLR4 is a critical component of host immunity (Jordan 2008). NLRP3 was not protective *in vivo*, but was found to be involved in time- and tissue-specific manner for inflammasome activation, suggesting that there are multiple NLRs that may cooperate in a time-dependent manner. ASC is a critical component of host innate immunity, discovered to be essential for both *in vivo* and *in vitro* protection against rickettsial infection. Caspase-1 is crucial for inflammasome activation *in vitro*, and caspase-11 is partially involved in inflammasome activation *in vitro*. Rickettsial LPS is likely the ligand for caspase-11 activation; however, the amount of ligand and the structure of the ligand may contribute to the minor involvement of caspase-11 in this model.

Overall, there are important differences in this model from other Gram-negative organisms, and different from other Rickettsiales. The TLR4-caspase-11-ASC-caspase-1 axis has been described in other infectious disease models; however, NLRP3 is generally considered a part of this pathway. The involvement of more than one NLR in caspase-1 dependent non-canonical inflammasome activation would be unique. Additionally, caspase-11 only plays a partial role in activation of this axis, further complicating the

caspase-1 dependent non-canonical pathway. Further studies are necessary to confirm the additional NLR(s) involved in these pathways.

The strong phenotype of ASC *in vivo* and *in vitro* indicates that ASC is protective during rickettsial infection. ASC protection is likely due to inflammasome-associated cytokines IL-1β and IL-18 protective downstream effects. IL-1β is potently pro-inflammatory, recruiting nearby immune cells to the site of infection and polarizing CD8+T-cells. IL-18 has a well described role in promoting production of IFN-γ through NK and TH1 CD4+ T-cells, which polarize CD8+ T-cells and promote cytoxicity. Caspase-1 is required for IL-1β and IL-18 processing, and is likely a component of this pathway as well. Further experiments utilizing ASC-deficient mice would answer several ongoing questions in rickettsiology, including the role of the inflammasome in dendritic cell mediated immunity, as well as the respective contributions of IL-1β and IL-18 to host defense during rickettsial infection.

Caspase-11 does play a partial role in inflammasome mediated secretion of IL-1β and IL-18, though the low abundance of rickettsial LPS and the atypical structure of rickettsial LPS make the contribution of caspase-11 less clear. Further studies investigating the involvement of caspase-11 in actin polymerization during rickettsial infection would be an interesting avenue to explore. One strain of rickettsia, *R. parkeri* RickA mutant, cannot polymerize actin for cell-to-cell spread, making it a perfect candidate to investigate this question.

Overall, this work begins to bridge a gap in rickettsiology between the role of the innate immune system and the role of the adaptive immune system. Here, we have laid the foundation for understanding how cellular innate immunology, the inflammasome,

contributes to downstream host protection during rickettsial infection. Though this work is instructive, many questions remain to be answered.

One of the most interesting questions, perhaps, is how rickettsiae are able to evade inflammasome activation for a relatively extended period of time. Many other Gram-negative organisms are identified and activate the inflammasome within hours post-infection. The finding that the peak of inflammasome activation in BMM is 24 hpi suggests that rickettsiae possess mechanisms for inhibiting inflammasome activation. Additionally, rickettsiae reside and replicate directly in the cytosol, without the "protection" afforded by residing in vacuoles like some other Rickettsiales (Moumene 2016). These are common

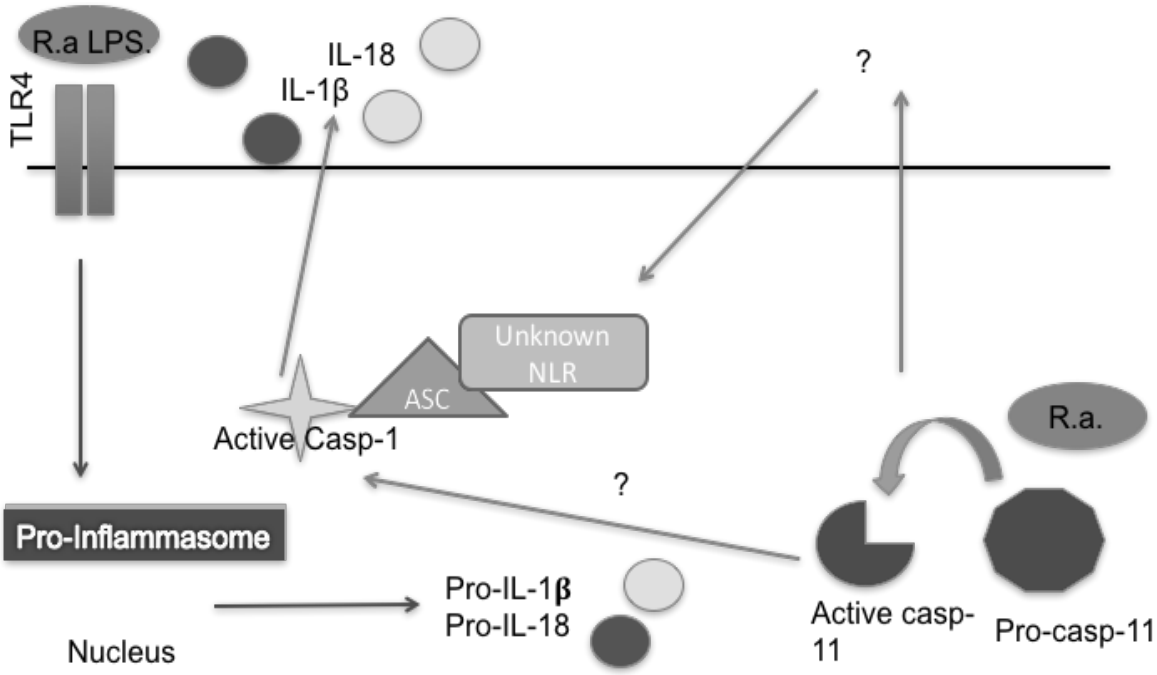

Illustration 1. Rickettsial Activation of the Inflammasome. Rickettsia prime inflammasome components through TLR4 interaction. Upon entering the cell, rickettsia activate the inflammasome via a partial NLRP3-ASC-Caspase-1-partial caspase-1-dependent pathway.

mechanisms amongst other pathogens: viruses, fungi, and bacteria have been shown to encode protein inhibitors that directly block inflammasome activation until the pathogen burden becomes too high (Brodsky 2010, Gregory 2011, Dotson 2013). Rickettsiae are

genetically intractable, making the identification and confirmation of a ligand for inflammasome inhibition difficult. The increasing availability and decreasing cost of CRISPR/Cas9 technology is a promising avenue for studying individual rickettsial proteins (McClure 2017). Nevertheless, rickettsiae are unique organisms that are useful for examining the basics of inflammasome biology.

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Vita

Claire Smalley was born June 1, 1988, in Redding, California to April Smalley and Lawrence Smalley. She attended Shasta High School, where she graduated in the top 5% of her class. She attended University of California at Davis, where she received a Bachelors of Science in Microbiology. She next attended University of Nevada at Reno, where she earned her Masters Degree of Science in Cellular and Molecular Biology. Claire was named on a patent for a novel method of identifying circulating antigen in human samples for her work at UNR. She next attended University of Texas, Medical Branch in Galveston. She is named as first or co-author on five manuscripts.

Permanent address: 15636 Ranchland Drive, Redding CA 96001

This dissertation was typed by Claire Smalley