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Investigating Inflammasome Activation in Response to Legionella Pneumophila and its Application to Other Bacterial Pathogens

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Abstract
The mucosal surfaces of metazoan organisms provide niches for colonization by commensal microbes. However, these barrier surfaces also encounter pathogens. Therefore, sentinel immune cells must be capable of distinguishing between pathogenic and non-pathogenic organisms to tailor appropriate immune responses. Virulent microorganisms often uniquely possess mechanisms for accessing the host cell cytosol. Therefore, to detect pathogens, innate immune cells encode cytosolic receptors, which recognize conserved, pathogen-associated molecular patterns. Many mammalian cytosolic receptors activate the inflammasome, a multi-protein complex that activates the host enzyme caspase-1. Caspase-1 mediates IL-1 family cytokine release and a pro-inflammatory form of cell death, which are important for host defense. The canonical inflammasome activates caspase-1, but recent studies have shown that a related enzyme, caspase-11, contributes to inflammasome activation. However, it remains unclear if caspase-11 mediates inflammasome responses against bacteria that use virulence-associated secretion systems to deliver bacterial products into the host cytosol. Additionally, humans encode two orthologs of caspase-11, caspase-4 and caspase-5, and it is unclear if either enzyme contributes to inflammasome activation in primary macrophages. Furthermore, the bacterial ligands that trigger inflammasome activation in human cells are poorly defined. Legionella pneumophila, which causes pneumonia, uses a specialized secretion system to access the host cytosol to establish a replicative niche in both murine and human cells. Therefore, we investigated the host and bacterial requirements for inflammasome activation in response to L. pneumophila, and we interrogated if these requirements are conserved for the response against other Gram-negative bacterial pathogens. Our studies demonstrate that caspase-11 contributes to IL-1 release and cell death in response to bacterial pathogens in murine macrophages, and we find that inflammasome activation requires the presence of virulence-associated secretion systems. Using neutralizing antibodies, we show that IL-1α and IL-1β have distinct roles in pulmonary defense against L. pneumophila in vivo. Through siRNA knockdown studies, we demonstrate that human caspase-4 has a conserved role in inflammasome activation in response to multiple Gram-negative bacterial pathogens. Finally, using bacterial mutants, we show that flagellin is a trigger for inflammasome activation in human macrophages. Overall, our studies help define the mechanism by which host cells initiate defense against bacterial pathogens.

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INVESTIGATING INFLAMMASOME ACTIVATION IN RESPONSE TO *LEGIONELLA PNEUMOPHILA* AND ITS APPLICATION TO OTHER BACTERIAL PATHOGENS

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ABSTRACT

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Cierra N. Casson

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The mucosal surfaces of metazoan organisms provide niches for colonization by commensal microbes. However, these barrier surfaces also encounter pathogens. Therefore, sentinel immune cells must be capable of distinguishing between pathogenic and non-pathogenic organisms to tailor appropriate immune responses. Virulent microorganisms often uniquely possess mechanisms for accessing the host cell cytosol. Therefore, to detect pathogens, innate immune cells encode cytosolic receptors, which recognize conserved, pathogen-associated molecular patterns. Many mammalian cytosolic receptors activate the inflammasome, a multi-protein complex that activates the host enzyme caspase-1. Caspase-1 mediates IL-1 family cytokine release and a pro-inflammatory form of cell death, which are important for host defense. The canonical inflammasome activates caspase-1, but recent studies have shown that a related enzyme, caspase-11, contributes to inflammasome activation. However, it remains unclear if caspase-11 mediates inflammasome responses against bacteria that use virulence-associated secretion systems to deliver bacterial products into the host cytosol. Additionally, humans encode two orthologs of caspase-11, caspase-4 and caspase-5, and it is unclear if either enzyme contributes to inflammasome activation in primary macrophages. Furthermore, the bacterial ligands that trigger inflammasome activation in human cells are poorly defined. *Legionella pneumophila*, which causes pneumonia, uses a specialized secretion system to access the host cytosol to establish a replicative niche in both murine and human cells. Therefore, we investigated the host and bacterial requirements for inflammasome activation in response to *L. pneumophila*, and we interrogated if these requirements are conserved for the response against other Gram-negative bacterial pathogens. Our studies demonstrate that caspase-11 contributes to IL-1 release and cell death in response to bacterial pathogens in murine macrophages, and we find that inflammasome activation requires the presence of virulence-associated secretion systems. Using neutralizing antibodies, we show that IL-1α and IL-1β have distinct roles in pulmonary defense against *L. pneumophila in vivo*. Through siRNA knockdown studies, we demonstrate that human caspase-4 has a conserved role in inflammasome activation in response to multiple Gram-negative bacterial pathogens. Finally, using bacterial mutants, we show that flagellin is a trigger for inflammasome activation in human macrophages. Overall, our studies help define the mechanism by which host cells initiate defense against bacterial pathogens.

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LIST OF ABBREVIATIONS

AIM2:	Absent in melanoma 2
ASC:	Apoptosis-associated speck-like protein containing a carboxy-terminal caspase recruitment domain
ATG5:	Autophagy protein 5
ATP:	Adenosine triphosphate
B6:	C57BL/6J mice
BALF:	Bronchoalveolar lavage fluid
BIRC:	Baculoviral inhibitor of apoptosis repeat-containing
BMDM:	Bone marrow-derived macrophage
CARD:	Caspase recruitment domain
CARDIAK:	Caspase recruitment domain-containing interleukin-1β converting enzyme associated kinase
CARDIF:	Caspase recruitment domain adaptor inducing IFN-β
cGAMP:	Cyclic guanosine monophosphate–adenosine monophosphate
cGAS:	Cyclic guanosine monophosphate–adenosine monophosphate synthase
cPOP:	Cellular pyrin domain-only protein
CLR:	C-type lectin receptor
CpG:	Cytidine-phosphate-guanosine
CRISPR:	Clustered regularly interspaced short palindromic repeats
CXCL1:	C-X-C-motif ligand 1
DMSO:	Dimethyl sulfoxide
ER:	Endoplasmic reticulum
GBP:	Guanylate-binding protein
HIN:	Hematopoietic expression, interferon-inducible nature and nuclear localization
IAP:	Inhibitor of apoptosis
ICE:	Interleukin-1β converting enzyme
IFI16:	Interferon-inducible protein 16
IFN:	Interferon
IFNAR:	Type I interferon receptor
IGIF:	Interferon-γ-inducing factor
IL:	Interleukin
IPAF:	Interleukin-1β converting enzyme-protease activating factor
IPS-1:	Interferon-β promoter stimulator 1
IRAK:	Interleukin-1 receptor-associated kinase
KC:	Keratinocyte-derived chemokine
LAMP-1:	Lysosomal-associated membrane protein 1
LCV:	*Legionella*-containing vacuole
LPS:	Lipopolysaccharide
LRR:	Leucine-rich repeat
MAVS:	Mitochondrial antiviral signaling
MDA5:	Melanoma differentiation associated gene 5
MDM:	Monocyte-derived macrophage
MOI:	Multiplicity of infection
MSU:	Monosodium urate
NAIP:	Neuronal apoptosis inhibitory protein

NF-κB:	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR:	Nucleotide-binding domain, leucine rich repeat containing protein
NLRC4:	NLR family, caspase recruitment domain containing 4
NLRP3:	NLR family, pyrin domain-containing 3
NLS:	Nuclear localization signal
NOD:	Nucleotide-binding oligomerization domain
PAMP:	Pathogen-associated molecular pattern
PBMC:	Peripheral blood mononuclear cell
Poly(I:C):	Polyinosinic-polycytidylic acid
PRR:	Pattern recognition receptor
PYHIN:	Pyrin and hematopoietic expression, interferon-inducible nature and nuclear localization domain-containing protein
RIG-I:	Retinoic acid-inducible gene I
RIP2:	Receptor-interacting protein kinase 2
RLR:	Retinoic acid-inducible gene I-like receptor
SPI-1:	*Salmonella* pathogenicity island I
ROS:	Reactive oxygen species
STING:	Stimulator of interferon genes
T3SS:	Type III secretion system
T4SS:	Type IV secretion system
T_H1:	T-helper type 1
TICAM1:	Toll-interleukin-1 receptor-domain-containing molecule 1
TIR:	Toll-interleukin-1 receptor
TLR:	Toll-like receptor
TNF:	Tumor necrosis factor
TRIF:	Toll-interleukin-1 receptor domain-containing adaptor protein inducing interferon-β
TRIM56:	Interferon-inducible tripartite-motif 56
UPR:	Unfolded protein response
UVB:	Ultraviolet B
WT:	Wild-type

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CHAPTER 1

INTRODUCTION

Sections of this chapter have been adapted from a published peer-reviewed mini-review titled “Inflammasome-mediated cell death in response to bacterial pathogens that access the host cell cytosol: lessons from *Legionella pneumophila*” by **Cierra N. Casson** and Sunny Shin. Frontiers in Cellular and Infection Microbiology, 2013.

The mucosal surfaces of metazoan organisms, such as the intestinal epithelium and upper respiratory tract, provide diverse niches for microbial colonization. However, in addition to commensal microbes, these barrier surfaces have the potential to encounter pathogenic organisms as well. When cells of the innate immune system encounter a microorganism, they must have the capacity to accurately determine if the microorganism is pathogenic or not to mount an appropriate immune response while minimizing damage to the host (Janeway and Medzhitov, 2002). If the immune response is unnecessarily over-exuberant, immune cells can cause damage to host tissue or induce autoimmunity (Dube et al., 2001; Kurashima et al., 2013; Meng et al., 2009). Conversely, if the immune response is not effective and the organism is virulent, the pathogen can replicate and cause damage to or even kill the host (Flynn et al., 1995; Franchi et al., 2012; Reddick and Alto, 2014). Therefore, the ability of the innate immune system to distinguish pathogenic organisms from non-pathogenic or commensal organisms is key for the maintenance of homeostatic barrier function and survival of the host (Belkaid and Artis, 2013; Belkaid and Hand, 2014).

A. Extracellular sensing of microorganisms

To detect pathogens, cells of the innate immune system encode pattern recognition receptors (PRRs), which detect conserved microbial products. These conserved microbial products are known as pathogen-associated molecular patterns (PAMPs), and they include components of the bacterial cell wall, such as lipopolysaccharide (LPS) and peptidoglycan, bacterial flagellin, and viral and bacterial nucleic acids (Janeway and Medzhitov, 2002). Germline-encoded PRRs are critical initiators of host defense against invading microorganisms (Medzhitov, 2007).

The C-type lectin receptors (CLRs) are a subset of PRRs that bind to carbohydrates and lipids in the extracellular space (Dambuza and Brown, 2015). The CLR dectin-1 responds to β-glucans from fungi (Brown and Gordon, 2001), while dectin-2 senses α-manins during fungal infection (Saijo et al., 2010). CLRs signal through Syk kinase to generate cytokines important for anti-fungal defense (Robinson et al., 2009; Rogers et al., 2005). Recently, however, it has been appreciated that CLRs can detect PAMPs from bacterial species as well, including mycobacterial components (Dambuza and Brown, 2015; Yonekawa et al., 2014).

The Toll-like receptors (TLRs) are a subset of PRRs that are surface and endosomally-associated trans-membrane proteins that detect PAMPs found in the extracellular space (Janeway and Medzhitov, 2002) through leucine-rich repeat (LRR) domains (Bell et al., 2003). TLR1,2,4,5, and 6 are found on the cell surface, while TLR3,7,8, and 9 are expressed in intracellular vesicles (Kawai and Akira, 2010). TLR2 detects bacterial lipoproteins (Aliprantis et al., 1999; Brightbill et al., 1999). TLR2 works in concert with TLR1 or TLR6 to form heterodimers to detect peptidoglycan and other lipopeptide components of Gram-positive bacterial cells walls and zymosan, a yeast cell wall component (Ozinsky et al., 2000; Takeuchi et al., 2001; 2002; Underhill et al., 1999). TLR4 detects LPS from Gram-negative bacteria (Medzhitov et al., 1997; Poltorak et al., 1998), and TLR5 detects bacterial flagellin (Hayashi et al., 2001). Some TLRs form complexes with accessory proteins to detect their ligands. For example, TLR4 requires CD14 (Haziot et al., 1996) and MD-2 (Shimazu et al., 1999) to efficiently detect LPS. Interestingly, the detection of PAMPs through any given TLR does not necessarily

distinguish between bacterial and viral organisms, as TLR4 recognizes a respiratory syncytial virus protein in addition to LPS (Kurt-Jones et al., 2000).

The endosomal TLRs (TLR3,7,8, and 9) respond to viral and bacterial nucleic acids. TLR3 detects double-stranded RNA, including the synthetic analog polyinosinic-polycytidylic acid (poly(I:C)) (Alexopoulou et al., 2001). TLR7 and TLR8 were originally shown to respond to small antiviral compounds, such as imidazoquinolines (Hemmi et al., 2002; Jurk et al., 2002), but they also sense single-stranded RNA (Diebold et al., 2004; Heil et al., 2004). TLR7 is particularly important for sensing single-stranded RNA during viral infection (Lund et al., 2004). TLR9 recognizes unmethylated bacterial 2'-deoxyribo cytidine-phosphate-guanosine (CpG) dinucleotides (Hemmi et al., 2000) and also detects CpG motifs during infection with DNA viruses, such as Herpes simplex virus-2 (Lund et al., 2003).

TLRs signal through the recruitment of Toll-interleukin-1 (IL-1) receptor (TIR) domain containing proteins to generate cytokine responses. TLRs 1,2,5,6,7, and 8 signal through the adaptor myeloid differentiation primary-response protein 88 (MyD88), while TLR3 signals through TIR-domain-containing adaptor protein inducing interferon-β (IFN-β) (TRIF)/TIR-domain-containing molecule 1 (TICAM1) (Hoebe et al., 2003; Medzhitov et al., 1998; Yamamoto et al., 2003). TLR4 can signal through both MyD88 and TRIF (Hoebe et al., 2003; Yamamoto et al., 2003), and there is recent evidence that TLR2 can signal through TRIF in response to certain lipopeptides (Nilsen et al., 2015). MyD88 signaling generally leads to pro-inflammatory cytokine production, while TRIF signaling leads to type I IFN production, and both pathways modulate signaling through the

transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to produce inflammatory mediators that provide defense against microbial organisms (Kawai and Akira, 2010). Therefore, TLRs are critical initiators of many immune responses. However, detection through TLRs alone is often not sufficient to distinguish between pathogenic and non-pathogenic organisms, as non-pathogens encode many of the same PAMPS as pathogens. For example, both commensal and pathogenic bacteria may express LPS if they are Gram-negative organisms, and both may express flagellin if they are motile organisms. Therefore, innate immune cells must possess additional PRRs to help distinguish between pathogenic and non-pathogenic microbes.

B. Intracellular sensing of pathogens

As TLRs cannot always distinguish between pathogens and non-pathogens, innate immune cells encode an additional set of PRRs to more specifically detect pathogenic microbes. Though PAMPs associated with commensal organisms may be sensed via surface or endosomal TLRs in the extracellular space, many pathogenic organisms uniquely have mechanisms for accessing the host cytosol, a cellular compartment where microorganisms are typically not found. Thus, to detect pathogenic activities, many cells encode cytosolic PRRs, such as retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Yoneyama et al., 2015) and nucleotide-binding domain, leucine rich repeat containing proteins (NLRs) (Harton et al., 2002), many of which respond to PAMPs that are present within the host cell cytosol.

RLRs detect cytosolic RNA, particularly during viral infection. The founding member of the RLR family, RIG-I, senses double-stranded RNA to initiate type I IFN production

(Yoneyama et al., 2004). Melanoma differentiation associated gene 5 (MDA5) is another cytosolic RLR that induces type I IFN through IRF-3 (Yoneyama et al., 2005). Though both RIG-I and MDA-5 detect double-stranded RNA, they are not completely functionally redundant, as they are each required for type I IFN production in response to distinct viruses (Kato et al., 2006). Both RIG-I and MDA5 signal through the adaptor protein interferon-β promoter stimulator 1 (IPS-1)/mitochondrial antiviral signaling (MAVS)/caspase recruitment domain (CARD) adaptor inducing IFN-β (CARDIF) to initiate antiviral defense (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005).

In addition to sensing RNA, mammalian cells have mechanisms for sensing cytosolic DNA independently of TLRs and inducing type I IFN as well (Stetson and Medzhitov, 2006; Stetson et al., 2008). For example, stimulator of interferon genes (STING) responds to non-CpG containing DNA during viral infection to trigger anti-viral defense (Ishikawa and Barber, 2008; Ishikawa et al., 2009). Interferon-inducible protein 16 (IFI16) (Unterholzner et al., 2010) and interferon-inducible tripartite-motif 56 (TRIM56) (Tsuchida et al., 2010) both interact with STING to mediate the response against cytosolic DNA. Recently, the cytosolic double-stranded DNA sensor cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) synthase (cGAS) was identified (Ablasser et al., 2013; Sun et al., 2013). cGAS generates the cyclic di-nucleotide second messenger cGAMP, which in turn binds to and activates STING to generate type I IFN (Wu et al., 2013).

Nucleic acids are not the only cytosolic PAMPs detected by mammalian PRRs. The nucleotide-binding oligomerization domain (NOD) proteins NOD1 (Bertin et al., 1999;

Inohara et al., 1999) and NOD2 (Ogura et al., 2001) are the founding members of the NLR family, and they respond to intracellular peptidoglycan (Chamaillard et al., 2003; Girardin et al., 2003a; 2003b; Inohara et al., 2003). Both NOD1 and 2 signal through the adaptor molecule receptor-interacting protein kinase 2 (RIP2)/CARD-containing IL-1β converting enzyme (ICE) associated kinase (CARDIAK) (Chin et al., 2002; Kobayashi et al., 2002). Signaling through NOD1 and 2 induces expression of pro-inflammatory cytokines, anti-microbial peptides, and chemokines important for inflammatory cell recruitment *in vivo* (Kobayashi et al., 2005; Masumoto et al., 2006).

Other members of the NLR family signal independently of RIP2 and activate multi-meric complexes known as inflammasomes. These NLRs respond to 'patterns of pathogenesis' and activities that are often associated with virulent organisms (Lamkanfi and Dixit, 2009; Vance et al., 2009), such as membrane disruption, delivery of bacterial molecules into the host cytosol via specialized secretion systems, or the use of pore-forming toxins (Davis et al., 2011; Franchi et al., 2012; Fritz et al., 2006; Vance et al., 2009). Inflammasome activation via NLRs initiates a cascade of events to produce key pro-inflammatory cytokines and activate a pro-inflammatory form of cell death that can eliminate the replicative niche for many pathogens (Rathinam et al., 2012a).

C. Caspase-1-dependent inflammasomes

The canonical inflammasome is a multi-protein complex that assembles in the cytosol to activate the host enzyme caspase-1, also known as ICE (Martinon et al., 2002). Caspase-1 is a cysteine protease with a catalytic cysteine at residue 285, and its active tetramer form is produced from two heterodimers of a 10kDa (p10) and 20kDa (p20)

subunit generated by auto-processing of the 45 kDa (p45) pro-enzyme (Cerretti et al., 1992; Thornberry et al., 1992; Walker et al., 1994; Wilson et al., 1994). Caspase-1 regulates secretion of IL-1 family cytokines and a pro-inflammatory form of cell death termed pyroptosis (Cookson and Brennan, 2001; Kuida et al., 1995; Li et al., 1995; Rathinam et al., 2012a). Caspase-1 processes IL-1β and IL-18 into their mature forms and aids in their secretion (Ghayur et al., 1997; Gu et al., 1997; Howard et al., 1991; Thornberry et al., 1992), and caspase-1-dependent cleavage of cytokines occurs within the cytosol of host cells (Singer et al., 1995). Caspase-1 does not cleave IL-1α, though it can aid in IL-1α secretion as well (Howard et al., 1991; Keller et al., 2008). IL-1 family cytokines act *in vivo* to enhance immune responses against invading microorganisms (Bohn et al., 1998; Dinarello, 2009; Labow et al., 1997). Additionally, caspase-1-mediated pyroptosis enhances clearance of bacterial pathogens *in vivo* (Miao et al., 2010a).

Each NLR responds to a distinct stimulus (or range of stimuli) when activating the inflammasome (Figure 1-1). Few NLRs have been shown to bind directly to their implicated activators, and some are triggered by a wide variety of stimuli. For example, NLR family, pyrin domain-containing 3 (NLRP3) responds to stimuli ranging from bacterial RNA (Kanneganti et al., 2006) to extracellular adenosine triphosphate (ATP) (Mariathasan et al., 2006) and uric acid crystals (Martinon et al., 2006). NLRP1 activates the inflammasome in response to both anthrax lethal toxin (Boyden and Dietrich, 2006) and infection by the intracellular parasite *Toxoplasma gondii* (Cavailles et al., 2014; Cirelli et al., 2014; Ewald et al., 2014; Gorfu et al., 2014). In murine cells, ICE-protease activating factor (IPAF)/NLR family, CARD domain containing 4 (NLRC4) mediates

inflammasome activation in response to three distinct stimuli—flagellin, the conserved inner rod component of the bacterial type III secretion system (T3SS) (PrgJ), and the T3SS needle protein (Franchi et al., 2006; Lightfield et al., 2011; Miao et al., 2006; 2010b; Yang et al., 2013). Biochemical studies have shown that the NLRs neuronal apoptosis inhibitory protein 5 (NAIP5) and NAIP6 co-immunoprecipitate with flagellin, while NAIP2 interacts specifically with PrgJ (Kofoed and Vance, 2011; Zhao et al., 2011). Additionally, NAIP1 specifically interacts with the needle protein of the T3SS (Yang et al., 2013) (Figure 1-2). NLRC4 appears to be an important adaptor for the NAIP receptors, acting as a scaffolding or signaling molecule downstream of NAIP1,2,5, and 6 to mediate inflammasome activation in response to all three stimuli.

The pyrin and hematopoietic expression, interferon-inducible nature and nuclear localization (HIN) domain-containing protein (PYHIN) protein absent in melanoma 2 (AIM2), though not an NLR, recognizes cytosolic double-stranded DNA to activate a caspase-1-dependent inflammasome (Hornung et al., 2009; Roberts et al., 2009). Unlike NLRP3, AIM2 has been crystalized with its ligand, showing a direct interaction between double-stranded DNA and the AIM2 sensor (Jin et al., 2012). The adaptor protein apoptosis-associated speck-like protein containing a carboxy-terminal caspase recruitment domain (ASC) often bridges the interaction between the NLRs or AIM2 and caspase-1, allowing for oligomerization and auto-processing of caspase-1 for activation (Mariathasan et al., 2004; Srinivasula et al., 2002). Caspase-1 auto-processing is required for cytokine cleavage and secretion, though cell death can occur independently of caspase-1 proteolysis (Broz et al., 2010).

D. IL-1 family cytokines

The IL-1 family of cytokines consists of seven agonistic cytokines (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ) and one anti-inflammatory member (IL-37) (Garlanda et al., 2013). The first identified members of the IL-1 family were IL-1α and IL-1β. IL-1α and IL-1β are pro-inflammatory cytokines that were fist called “lymphocyte-activating factors” in the 1970’s because of their potent ability to activate T-cells (Gery and Waksman, 1972; Gery et al., 1972). In addition to lymphocyte activation, IL-1α and IL-1β are important for many other immune processes, such as fever and inflammation, and were also called “leukocyte pyrogens” in the 1980’s because of their ability to mediate fever (Rosenwasser and Dinarello, 1981). However, it was acknowledged that “lymphocyte-activating factor” and “leukocyte pyrogen” were the same molecules, and they were re-classified as IL-1 (Dinarello et al., 1983). It also became apparent that IL-1 was actually two distinct molecules, IL-1α and IL-1β (March et al., 1985).

IL-1 family cytokines are uniquely regulated in that they do not require canonical endoplasmic reticulum (ER)/Golgi trafficking for secretion, unlike other pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and IL-6 (Monteleone et al., 2015). Both IL-1α and IL-1β lack signal sequences for canonical ER/Golgi trafficking and exit host cells via a poorly-characterized mechanism (Auron et al., 1984; Monteleone et al., 2015). IL-1β protein localizes mostly to the cytosol of LPS-stimulated monocytes (Singer et al., 1988), and IL-1β is not immediately released after stimulation. A ‘two-step’ model for secretion of IL-1β was first proposed when the observation was made that low doses of LPS induced intracellular production of IL-1β but did not result in extracellular release of IL-1β activity (Chin and Kostura, 1993; Newton, 1986). Therefore, release of IL-1α and

IL-1β is tightly regulated, with caspase-1 activation via the inflammasome as 'signal 2' for secretion of these potent inflammatory mediators (Keller et al., 2008). Caspase-1 cleaves the pro-form of IL-1β for maturation (Howard et al., 1991). IL-1α is not processed by caspase-1 but can be cleaved by calcium-dependent proteases known as calpains (Carruth et al., 1991; Howard et al., 1991). However, unlike IL-1β, full-length IL-1α is biologically active (Mosley et al., 1987), and the release of the full-length form is also tightly regulated (Howard et al., 1991; Keller et al., 2008). Though the mechanism leading to IL-1β secretion during inflammasome activation has been well-studied, the cellular factors that contribute to IL-1α release are less clear.

IL-1α and IL-1β both bind to and signal through the same receptor, the IL-1 receptor (IL-1R), *in vivo* (Dower et al., 1986). However, IL-1α has a higher affinity for the IL-1R than IL-1β on certain cell types, such as T-cells (Dower et al., 1986). Though IL-1α and IL-1β both bind the same receptor *in vivo*, there is evidence that suggests that they may have different intracellular regulations. For example, in human monocytes, IL-1α has a longer half-life, and its secretion is delayed compared to that of IL-1β (Hazuda et al., 1988). Unlike IL-1β, the pro-form of IL-1α contains a nuclear localization signal (NLS) and can translocate into the nucleus to promote production of other pro-inflammatory cytokines (Cheng et al., 2008; Wessendorf et al., 1993). Additionally, IL-1α and IL-1β have newly appreciated non-redundant roles *in vivo* as well, as recent data suggest that IL-1α is required for protection during disease and for mounting an inflammatory response against endogenous danger signals. For example, in a mouse model of *Mycobacterium tuberculosis* infection, wild-type mice do not lose weight or die from inhalation of the bacterium, but IL-1α-deficient mice succumb to infection, suggesting that IL-1β cannot

always compensate for the loss of IL-1α during disease (Mayer-Barber et al., 2011). Likewise, in a model of monosodium urate (MSU)-induced peritonitis, IL-1α is required for neutrophil recruitment to the peritoneal cavity (Gross et al., 2012; Martinon et al., 2006).

After the identification and characterization of IL-1α and IL-1β, several other members of the IL-1 cytokine family were identified. IL-18 was first named IFN-γ-inducing factor (IGIF) because of its ability to induce IFNγ from T-cells (Okamura et al., 1995). IL-18 is also processed by caspase-1 for maturation and activation during inflammasome activation and is important for T-helper type 1 (T_H1)-associated immunity (Ghayur et al., 1997; Gu et al., 1997; Kohno et al., 1997). IL-33 is an alarmin released from dying cells, and it induces T-helper type 2 (T_H2)-associated cytokines (Schmitz et al., 2005). Unlike IL-1β and IL-18, IL-33 is inactivated by caspase-1 cleavage because it is processed into a form that does not signal through its receptor, ST2 (Cayrol and Girard, 2009). IL-36α, IL-36β, and IL-36γ can amplify pro-inflammatory cytokine production by dendritic cells (Vigne et al., 2011), but they are not known to be cleaved by caspase-1 (Garlanda et al., 2013). IL-37 is processed by caspase-1 but dampens IFNγ production by blocking the ability of IL-18 to signal (Bufler et al., 2002; Kumar et al., 2002).

E. Cell death as an innate immune effector mechanism

In addition to release of IL-1 family cytokines, inflammasome activation leads to a pro-inflammatory form of cell death termed pyroptosis (Cookson and Brennan, 2001). Cell death is an important innate immune effector mechanism to aid in clearance of intracellular pathogens, as it can eliminate a pathogen's replicative niche. Additionally,

pro-inflammatory cell death can be critical for alerting neighboring cells to the presence of invading pathogens (Bergsbaken et al., 2009; Kono and Rock, 2008). Pyroptosis is critical both for clearance of bacterial pathogens and for release of important pro-inflammatory cytokines (Fink and Cookson, 2005; Miao et al., 2010a). Therefore, it has been difficult to separate the role of cell death from the release of inflammatory mediators *in vivo*. However, cell death itself can contribute to pathogen clearance independently of cytokine production during some bacterial infections. For example, caspase-1-deficient mice are more susceptible to infection with the Gram-negative bacterial pathogens *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), *Legionella pneumophila*, and *Burkholderia thailandensis,* and the defect is independent of the cytokines IL-1β and IL-18, implying that a cytokine-independent effector mechanism of caspase-1 controls bacterial infection (Miao et al., 2010a). However, it is still possible that an additional uncharacterized alarmin released during caspase-1 activation controls bacterial infection *in vivo*, as caspase-1 is known to regulate the unconventional secretion of many proteins outside of the IL-1 family cytokine members (Keller et al., 2008). Therefore, the biological role of host cell death independent of cytokine or alarmin release is still unclear.

F. The non-canonical inflammasome

Experiments examining inflammasome activation were first performed with macrophages from mice that lack caspase-1, and it was concluded that caspase-1 is solely responsible for inflammasome activation (Kuida et al., 1995; Li et al., 1995). However, the strain of mice used to generate the original caspase-1 knockout has a polymorphism in caspase-11 that eliminates caspase-11 protein expression. Thus, the original mice actually lack

both caspase-1 and caspase-11 (Kayagaki et al., 2011). Though it was reported that caspase-11 mediates septic shock *in vivo*, the cell-intrinsic role of caspase-11 in response to bacterial pathogens remained unclear (Wang et al., 1996; 1998). Recently, however, a non-canonical caspase-11-dependent inflammasome has been described that contributes to IL-1α, IL-1β, and IL-18 secretion and cell death in response to many Gram-negative bacteria. Caspase-11 is activated with delayed kinetics, taking 16-24 hours *in vitro*, in response to bacteria that do not typically access the host cell cytosol, such as non-pathogenic *Escherichia coli* (Kayagaki et al., 2011). For many Gram-negative bacteria, non-canonical inflammasome activation requires TRIF and type I IFN signaling downstream of TLR4 to upregulate caspase-11 (Broz et al., 2012; Gurung et al., 2012; Rathinam et al., 2012b). Additionally, caspase-11 is robustly activated by Gram-negative bacteria that escape the vacuole and replicate in the host cell cytosol, such as *B. thailandensis* (Aachoui et al., 2013). However, it is unclear if pathogens that naturally reside withinin vacuoles but use virulence-associated secretion systems to deliver bacterial products into the host cell cytosol activate caspase-11. These Gram-negative bacterial pathogens, such as *S.* Typhimurium and *L. pneumophila*, have been used to study caspase-1 activation, and they robustly activate the inflammasome in a manner requiring the T3SS (Chen et al., 1996; Hersh et al., 1999) or type IV secretion system (T4SS) (Sadosky et al., 1993; Zamboni et al., 2006).

G. *Legionella pneumophila* as a model organism to study inflammasome activation

L. pneumophila is a Gram-negative bacterium that causes the severe pneumonia Legionnaires' disease (Fraser et al., 1977; McDade et al., 1977). *L. pneumophila* was

first identified as the causative agent of pneumonia in 1977 (McDade et al., 1977) following a massive outbreak of disease that occurred at the American Legion convention in Philadelphia, Pennsylvania in the summer of 1976 (Fraser et al., 1977). After isolation of *L. pneumophila* as the etiological agent, it was retrospectively deduced, using preserved serum samples and serum-reactive tests, that *L. pneumophila* was the causative organism behind previous outbreaks of pneumonia with unidentified pathological agents, such as Pontiac Fever (McDade et al., 1977), ranging back to as early as 1947 (McDade et al., 1979; Thacker et al., 1978). Original epidemiologic evidence suggested that it was not person-to-person contact or foodborne illness that caused Legionnaires' disease (Fraser et al., 1977), and it was eventually determined that aerosolization of the bacterium likely occurred in the air conditioning system of the hotel where the American Legion convention occurred (Fraser and McDade, 1979). The natural host for *L. pneumophila* is free-living, fresh water and soil-dwelling amoebae (Rowbotham, 1980), and these infected amoebae can reside within industrial sources of fresh water, such as air-conditioning systems. It is now well-established that modern technologies that can aerosolize contaminated water droplets, such as heat, ventilation, and air conditioning systems and cooling towers, are often associated with outbreaks of respiratory disease caused by *L. pneumophila* (Fields, 1996). Once aerosolized, *L. pneumophila* can be inhaled into the mammalian lung to cause disease by first infecting alveolar macrophages (Nash et al., 1984).

L. pneumophila resides and replicates within a vacuole inside of human monocytic cells, and the bacterium recruits host ribosomes to the *Legionella*-containing vacuole (LCV) (Horwitz and Silverstein, 1980). *L. pneumophila* uses its *dot*/*icm*-encoded T4SS to

translocate effector proteins into the host cytosol to establish an ER-derived vacuole that supports bacterial replication (Berger and Isberg, 1993; Isaac and Isberg, 2014; Marra et al., 1992; Sadosky et al., 1993). It was first observed in the 1980's that live bacteria were needed to establish the LCV and recruit host-derived organelles (Horwitz, 1983). Within minutes of being phagocytosed by a host cell, *L. pneumophila* uses its T4SS to inhibit phago-lysosomal fusion (Berger et al., 1994). Bacterial mutants that lack the protein DotA, one components of the T4SS, fail to evade endocytic trafficking and localize to a lysosomal-associated membrane protein 1 (LAMP-1)-positive host compartment within 5 minutes of uptake (Roy et al., 1998). The locus encoding the T4SS was first characterized by identifying genes that, when mutated, resulted in a defect in intracellular multiplication (*icm*) (Marra et al., 1992) or a defect in organelle trafficking (*dot*) (Berger and Isberg, 1993). Evolutionarily, the T4SS is related to bacterial conjugative systems used to transfer DNA (Segal et al., 2005), and the T4SS is capable of conjugative transfer (Segal et al., 1998; Vogel et al., 1998).

The T4SS is a multi-subunit structure that is thought to form a channel from the inner membrane of Gram-negative bacterial species, through the outer membrane of the bacterium, and ending with a pore into the host cell membrane (Christie et al., 2014). Therefore, the T4SS physically translocates effector proteins and bacterial products from the bacterial cytosol, across the phagosomal membrane (Luo and Isberg, 2004), and directly into the host cell cytosol. There are two major classifications of the T4SS, the type IVA and type IVB secretion systems (Juhas et al., 2008). The most well-characterized T4SS is the type IVA VirB/virD4 secretion system from *Agrobacterium tumefaciens*, an oncogenic pathogen of plant cells. The *L. pneumophila* T4SS, a type

IVB secretion system, is encoded by 25 genes located across two different genetic loci that assemble to form the translocation apparatus (Segal et al., 2005). The T4SS is capable of creating a pore of ~3 nM in diameter in the host cell membrane (Kirby et al., 1998), though unlike the needle-like structure for the T3SS, which is ~20 Å in diameter, (Galán et al., 2014; Kubori et al., 1998), the T4SS pore has never been crystalized.

Currently, approximately 300 effector proteins have been identified that are translocated by the T4SS, and many effectors modulate host cell processes to establish a replicative niche for the bacterium (Isaac and Isberg, 2014; Nagai et al., 2002). Many of the effectors are functionally redundant in mammalian cells, as deletion of regions of the *L. pneumophila* genome containing ~100 effectors does not affect the ability of the bacterium to replicate within murine macrophages (O'Connor et al., 2011). It remains unclear precisely how the bacterial effector proteins interact with the T4SS to be secreted, though the C-terminal region of the effectors are critical for translocation to occur (Nagai et al., 2005).

L. pneumophila uses its T4SS to establish its replicative niche in both amoebae and mammalian macrophages (Rowbotham, 1980). However, the natural host for *L. pneumophila* is amoebae in aquatic reservoirs (Fliermans et al., 1981), and it is a recent, opportunistic human pathogen (Phin et al., 2014). Therefore, while the bacterium has evolved to evade amoebic host defenses, it is not thought to have evolved to evade mammalian-specific immune responses. Thus, as a consequence of accessing the host cytosol in mammalian cells, *L. pneumophila* triggers multiple pathways that elicit cell-intrinsic immune responses and induce cell death (Shin and Roy, 2008). These robust

immune responses make the bacterium valuable for studying host defense against intracellular pathogens, including studying inflammasome activation.

H. *Legionella pneumophila* and caspase-1-dependent inflammasome activation

It is well-understood that *L. pneumophila* triggers inflammasome activation and pyroptosis as a consequence of flagellin expression and T4SS activity (Figure 1-3). In murine macrophages, detection of flagellin by baculoviral inhibitor of apoptosis (IAP) repeat–containing 1e (BIRC1e)/NAIP5 mediates pyroptosis and contributes to restriction of *L. pneumophila* replication both *in vitro* and *in vivo* (Derré and Isberg, 2004; Growney and Dietrich, 2000; Kofoed and Vance, 2011; Wright et al., 2003; Zamboni et al., 2006; Zhao et al., 2011). Flagellin-deficient *L. pneumophila* (Δ*flaA* Lp) evade NAIP5-mediated restriction and replicate in NAIP5-sufficient macrophages from C57BL/6J (B6) mice, in part because they do not induce as much caspase-1-dependent cell death as wild-type (WT) Lp (Molofsky et al., 2006; Ren et al., 2006). NLRC4 also acts upstream of caspase-1 to induce flagellin-mediated restriction of replication, pore formation in the host membrane, and IL-1β release (Silveira and Zamboni, 2010; Zamboni et al., 2006). NLRC4 co-immunoprecipitates with NAIP5, consistent with the model that NLRC4 is an adaptor for NAIP5 (Kofoed and Vance, 2011; Zamboni et al., 2006; Zhao et al., 2011) (Figure 1-1). The NAIP5/NLRC4-dependent cell death induced in B6 macrophages requires cytosolic access, as T4SS-deficient mutants of *L. pneumophila* (Δ*dotA* Lp) do not activate the inflammasome. These data suggest that flagellin is translocated through the T4SS into the host cytosol during infection, though translocation of flagellin has not been shown experimentally.

A/J mice express a hypomorphic allele of NAIP5 (Diez et al., 2000), and A/J macrophages still activate caspase-1 in response to WT Lp under certain infection conditions (Lamkanfi et al., 2007). However, using *Naip5*$^{-/-}$ macrophages, it was shown that NAIP5 is required for caspase-1 activation in response to WT Lp (Lightfield et al., 2008). Interestingly, NAIP6 also interacts with *L. pneumophila* flagellin (Kofoed and Vance, 2011; Zhao et al., 2011). However, NAIP6 is insufficient for the restriction of *L. pneumophila*, as *Naip5*$^{-/-}$ macrophages and mice are permissive for infection (Lightfield et al., 2008), which may be due to lower expression levels of NAIP6 relative to NAIP5 in primary macrophages (Wright et al., 2003). NAIP5 and NLRC4 also contribute to the control of *L. pneumophila* replication by enhancing fusion of the *Legionella*-containing vacuole (LCV) with lysosomes during infections performed at a low multiplicity of infection (MOI) (Amer et al., 2006; Fortier et al., 2007). In addition, flagellin-dependent NLRC4 signaling leads to caspase-7-mediated restriction of *L. pneumophila* via enhanced lysosomal degradation of the bacterium (Akhter et al., 2009). NLRC4-mediated restriction *in vivo* is also partially caspase-1-independent through an unknown mechanism (Pereira et al., 2011). However, caspase-1 activation downstream of NLRC4 clearly induces pyroptosis and leads to IL-18 secretion both *in vitro* and *in vivo*, contributing to IFN-γ production and the subsequent resolution of pulmonary infection (Archer et al., 2009; Brieland et al., 2000; Case et al., 2009; Spörri et al., 2006). Thus, the NAIP5/NLRC4 inflammasome may control *L. pneumophila* replication through multiple mechanisms.

Not surprisingly, infection conditions, including MOI, can affect the detection of caspase-1 activation in response to *L. pneumophila*, as higher MOIs likely enhance the number of

macrophages that harbor bacteria. At higher MOIs, infection of B6 macrophages induces both NLRC4-dependent and NLRC4-independent inflammasome activation. NLRC4-independent caspase-1 activation and IL-1β and IL-18 secretion require ASC, although the identity of the *L. pneumophila*-derived signal sensed via ASC and the NLR upstream of ASC remain unknown (Case et al., 2009). Caspase-1 cleavage in the absence of ASC can be detected in either the supernatant or the cytosol, depending on the MOI (Abdelaziz et al., 2011a; Case et al., 2009). ASC also drives formation of a punctate structure containing caspase-1 and NLRC4 in *L. pneumophila*-infected macrophages (Case and Roy, 2011). At early timepoints, pore formation is not observed in the absence of NLRC4, though cell death still occurs in the absence of ASC. Recruitment of NLRC4 into the ASC complex appears to dampen NLRC4 activity because pyroptosis occurs at a higher rate in the absence of ASC (Case and Roy, 2011). However, further studies are needed to elucidate the temporal and spatial coordination of the ASC- and NLRC4-dependent inflammasomes and how they are triggered by *L. pneumophila*.

I. Inflammasome activation and autophagy

In murine macrophages, autophagy is induced shortly after phagocytosis of *L. pneumophila*, as components of the autophagy pathway co-localize with the LCV (Amer and Swanson, 2005). LCVs in A/J macrophages show delayed autophagosome maturation compared to LCVs in B6 macrophages, potentially contributing to increased replication of the bacterium. When expression of the autophagy component autophagy protein 5 (ATG5) is silenced, *L. pneumophila* replication in A/J macrophages increases. Additionally, replication of *L. pneumophila* decreases slightly when autophagy is induced exogenously, suggesting that autophagy contributes to restriction of *L. pneumophila*

replication (Matsuda et al., 2009). Under low MOI infection conditions where there is minimal induction of pyroptosis, it was revealed that the induction of autophagy dampens pyroptosis in response to *L. pneumophila*, and turnover of autophagosomes requires NAIP5, NLRC4, and caspase-1 (Byrne et al., 2013). Collectively, these data suggest that NAIP5 inflammasome activation contributes to the restriction of *L. pneumophila* replication by inducing autophagy and/or pyroptosis, depending on the MOI and amount of flagellin present. How competing host and bacterial factors influence the outcome of inflammasome activation and autophagy during infection remains unclear and may be clarified by studies examining the temporal regulation of inflammasome activation and autophagy at a single-cell level.

J. *Legionella pneumophila* and non-canonical inflammasome activation

Caspase-11 responds to *L. pneumophila* that escape from the vacuole and aberrantly enter the cytosol (Aachoui et al., 2013), a process that is not thought to occur physiologically during infection with WT Lp (Figure 1-3). The T4SS-translocated effector SdhA is critical for bacterial growth in primary murine macrophages (Laguna et al., 2006; Liu et al., 2008). Macrophages infected with Δ*sdhA* Lp undergo cell death because SdhA is required to maintain LCV membrane integrity (Creasey and Isberg, 2012). Therefore, Δ*sdhA* Lp aberrantly enter the host cytosol where they become degraded, induce type I IFN, and activate caspase-1 via AIM2 (Creasey and Isberg, 2012; Ge et al., 2012; Monroe et al., 2009). In addition, Δ*sdhA* Lp induce rapid caspase-11-dependent cell death independently of bacterial flagellin (Aachoui et al., 2013). It appears that AIM2 responds to cytosolic *L. pneumophila* by producing IL-1β, whereas caspase-11 mediates cell death. However, *L. pneumophila* does not normally enter the cytosol, so the

upstream mediators of caspase-11 activation may be different for Δ*sdhA*Δ*flaA* bacteria that enter the cytosol and Δ*flaA* bacteria that remain within the vacuole. Therefore, it remains unclear if caspase-11 is activated by *L. pneumophila* that remain within their natural vacuole during infection.

Though Δ*flaA* Lp avoid NAIP5/NLRC4-mediated pyroptosis and can replicate in B6 macrophages, Δ*flaA* Lp trigger an additional form of cell death (Case et al., 2009), and it is unclear if this cell death is mediated by caspase-11. However, caspase-11 has been shown to have a cell-intrinsic role in restricting growth of WT *L. pneumophila*. Caspase-11 contributes to NAIP5/NLRC4-mediated inflammasome activation and restricts WT Lp by enhancing phago-lysosomal fusion (Akhter et al., 2012). In its non-lytic role, caspase-11 modulates actin polymerization and phosphorylation of cofilin to promote lysosomal trafficking of pathogenic, but not non-pathogenic, bacteria. Additionally, caspase-11 contributes to control of WT Lp replication *in vivo* (Akhter et al., 2012). However, it is not known if a separate, non-canonical caspase-11-dependent inflammasome is activated by Δ*flaA* Lp independently of NAIP5/NLRC4. Non-canonical inflammasome activation is a recently described phenomenon, so there are many questions that remain unanswered. As *L. pneumophila* rapidly and robustly activates caspase-1 while residing within the LCV, it is likely that caspase-11 is activated during infection as well.

K. Inflammasome activation in human cells

Unlike macrophages from most inbred mouse strains, human cells are permissive for *L. pneumophila* replication. The mechanisms underlying inflammasome-mediated control of *L. pneumophila* replication in human cells are unclear. Humans express only one

homolog of the numerous murine NAIP paralogs (Scharf et al., 1996) (Figure 1-2). The homolog, human NAIP (hNAIP), restricts growth of WT Lp (Vinzing et al., 2008). Additionally, the human NLRC4 homolog, human IPAF (hIPAF), also restricts *L. pneumophila* replication. Overexpression of full-length hNAIP in HEK293T cells increases cell death in response to *L. pneumophila* (Boniotto et al., 2012), and overexpression of hNAIP in the murine macrophage RAW264.7 cell line mediates flagellin-induced pyroptosis and IL-1β secretion (Katagiri et al., 2012), suggesting that it may function similarly to NAIP5. However, unlike NAIP5, hNAIP does not co-immunoprecipitate with flagellin and instead interacts with the T3SS needle protein (Yang et al., 2013; Zhao et al., 2011) (Figure 1-2). Thus, it is unclear whether hNAIP senses flagellin or another *L. pneumophila*-derived ligand, how hNAIP restricts *L. pneumophila* replication, and if hNAIP contributes to cell death or IL-1β secretion in primary human cells.

The implication that the IPAF/NAIP/caspase-1-dependent inflammasome contributes to restriction of *L. pneumophila* is pervasive, though caspase-1 activation in response to *L. pneumophila* has not been explicitly shown in primary cells from humans, a naturally susceptible host. Immortalized human alveolar epithelial cells activate caspase-1 in response to *L. pneumophila*, though primary human monocytes and monocyte-derived macrophages (MDMs) do not produce detectable levels of processed or active caspase-1 (Abdelaziz et al., 2011b; Furugen et al., 2008; Santic et al., 2007). Additionally, the expression of ASC is moderately down-regulated in infected monocytes, potentially contributing to evasion of inflammasome activation in human cells by *L. pneumophila* (Abdelaziz et al., 2011b). Future studies in primary MDMs and human alveolar

macrophages are needed to clarify the role of the inflammasome in restricting *L. pneumophila* replication in human cells (Figure 1-4).

Another striking difference between mice and humans is that humans do not actually encode caspase-11 (Figure 1-5). Instead, humans encode two putative functional orthologs—caspase-4 and caspase-5 (Kamada et al., 1997; Kamens et al., 1995; Munday et al., 1995). In immortalized epithelial cells, caspase-4 mediates IL-18 release in response to infection with *S.* Typhimurium (Knodler et al., 2014). Additionally, overexpression of either caspase-4 or caspase-5 in THP-1 cells, an immortalized human monocytic cell line, restricts growth of WT *L. pneumophila* (Akhter et al., 2012). However, it is unclear if caspase-4 or caspase-5 is a functional homolog of caspase-11 in primary human macrophages, and a role for caspase-4 or caspase-5 in activating the inflammasome in response to *L. pneumophila* has not been interrogated.

L. Dissertation Aims

As *L. pneumophila* robustly activates innate immune responses in macrophages, it is a valuable tool for understanding how host cells are capable of mounting an immune response against intracellular pathogens. Additionally, *L. pneumophila* causes a severe form of pneumonia in humans, so understanding how the bacterium interacts with innate immune cells could provide novel insights into potential therapeutic targets during disease. Though caspase-1 is activated during *L. pneumophila* infection, murine macrophages still undergo cell death in a poorly characterized, caspase-1-independent manner, and the mechanism by which IL-1α is released during infection is unclear. Additionally, it is unclear how the inflammasome is activated in response to *L.*

pneumophila in primary human macrophages, as there are many important genetic differences between mice and humans. Therefore, the aims of the work presented in this dissertation were as follows:

1) To interrogate a role for caspase-11 in inflammasome activation in response to *L. pneumophila* and to determine if similar responses are mounted against other bacterial pathogens that use virulence-associated secretion systems.

Previous studies suggest that caspase-11 is activated in response to pathogens that escape the vacuole and replicate within the macrophage cytosol. However, many intracellular pathogens remain within vacuoles during infection of host cells, and it is unclear if caspase-11 is activated to mount an immune response against these vacuolar pathogens. In CHAPTER 3, we use caspase-1-deficient and caspase-11-deficient macrophages to determine the roles of caspase-1 and caspase-11 individually during infection with *L. pneumophila*. We find that caspase-11 controls IL-1α release and cell death and contributes to caspase-1-dependent IL-1β secretion. Caspase-11 activation requires the presence of the bacterial T4SS. Unlike during infection with other Gram-negative bacterial pathogens, we find that caspase-11 activation against *L. pneumophila* does not require IFNAR or TRIF. Finally, we show that caspase-11 is also activated in response to another Gram-negative bacterial pathogen, *Yersinia pseudotuberculosis*, and activation of caspase-11 requires the presence of the evolutionarily distinct T3SS. Therefore, caspase-11 robustly responds to the activities of virulence-associated secretion systems and controls IL-1α release during infection.

2) To investigate if IL-1α and IL-1β have non-redundant roles *in vivo* during a pulmonary model of *L. pneumophila* infection.

Previous studies suggest that though IL-1α and IL-1β bind the same receptor *in vivo*, they can play non-redundant roles in bacterial clearance and neutrophil recruitment. As IL-1α and IL-1β release are controlled by different intracellular mechanisms, it is possible that they play distinct roles during infection. In CHAPTER 3, we use neutralizing antibodies to deplete IL-1α and IL-1β during pulmonary *L. pneumophila* infection. We find that loss of IL-1α decreases neutrophil recruitment to the airway space during infection, whereas loss of IL-1β alone does not affect neutrophil recruitment. However, loss of both cytokines in combination has a dramatic effect on neutrophil recruitment and control of bacterial burdens. Therefore, IL-1α and IL-1β appear to have both distinct and overlapping roles in controlling pulmonary *L. pneumophila* infection.

3) To determine host factors that contribute to inflammasome activation in human macrophages in response to *L. pneumophila* and to determine if similar responses are mounted against other bacterial pathogens that use virulence-associated secretion systems.

There are many striking genetic differences between mice and humans. For inflammasome activation, humans do not encode caspase-11 but encode two putative orthologs—caspase-4 and caspase-5. It is unclear if caspase-4 or caspase-5 is a functional homolog of caspase-11 during infection of human macrophages. In CHAPTER 4, we use siRNA to target caspase-4 in primary human macrophages and examine

inflammasome activation. We find that *L. pneumophila* robustly triggers inflammasome activation in human macrophages in a manner that requires the T4SS, and this inflammasome activation is dependent on caspase-4. However, in contrast to murine cells, we find that caspase-4 controls IL-1α release and cell death but has no effect on IL-1β secretion. Finally, we find that caspase-4 controls IL-1α release for *Y. pseudotuberculosis* and *S.* Typhimurium, two distantly related Gram-negative bacterial pathogens that use a T3SS to access the host cell cytosol during infection. Therefore, caspase-4 has a conserved role in human macrophages in inflammasome activation against Gram-negative bacterial pathogens.

Another difference between mice and humans is that humans only encode one single NAIP protein, while mice encode many NAIP paralogs. Human NAIP is not thought to detect flagellin. Therefore, intracellular flagellin is not thought to be recognized by human cells during bacterial infection. However, in CHAPTER 5, we use bacterial mutants lacking flagellin to investigate a role for flagellin in activating the inflammasome in primary human macrophages. For both *L. pneumophila* and *S.* Typhimurium, we find that both IL-1α and IL-1β release are dependent on the presence of bacterial flagellin, suggesting that flagellin is actually sensed by human cells to activate the inflammasome during infection.

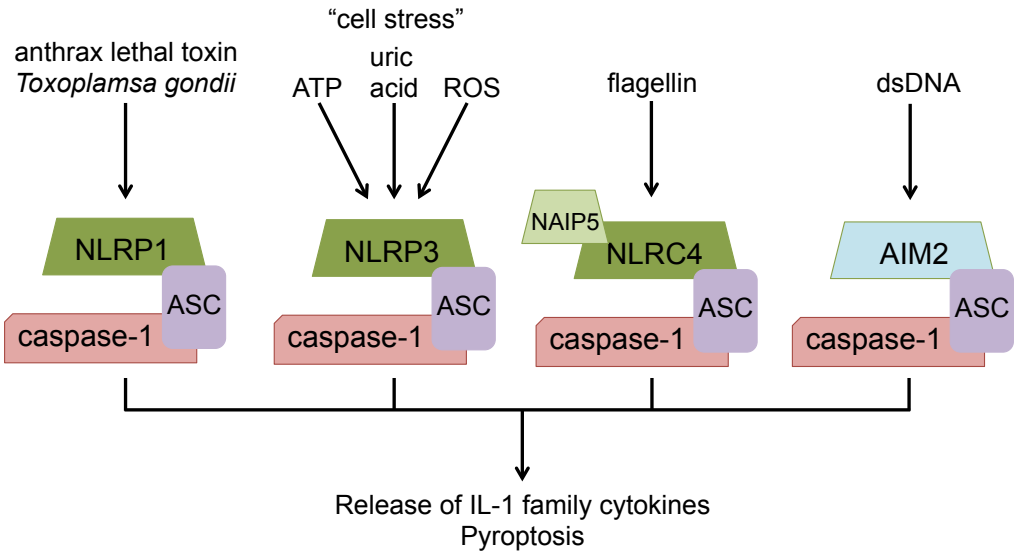

Figure 1-1. Canonical caspase-1-dependent inflammasomes are activated by distinct NLRs. Canonical inflammasomes activate caspase-1 to induce release of IL-1 family cytokines and a pro-inflammatory form of cell death known as pyroptosis. Each NLR upstream of caspase-1 responds to distinct stimuli to activate the inflammasome. NLRP1 senses anthrax lethal toxin and also responds to *Toxoplasma gondii* infection. NLRP3 responds to a wide variety of stimuli, including extracellular ATP, uric acid crystals, and reactive oxygen species (ROS). NLRP3 is thought to respond to general endogenous 'cell stress' signals. NLRC4 is an important adaptor for NAIP5-mediated recognition of cytosolic flagellin. AIM2, and PYHIN protein, detects cytosolic double-stranded DNA. The adaptor protein ASC bridges the interaction between the NLR or PYHIN protein and caspase-1 and allows for oligomerization and caspase-1 auto-processing for activation.

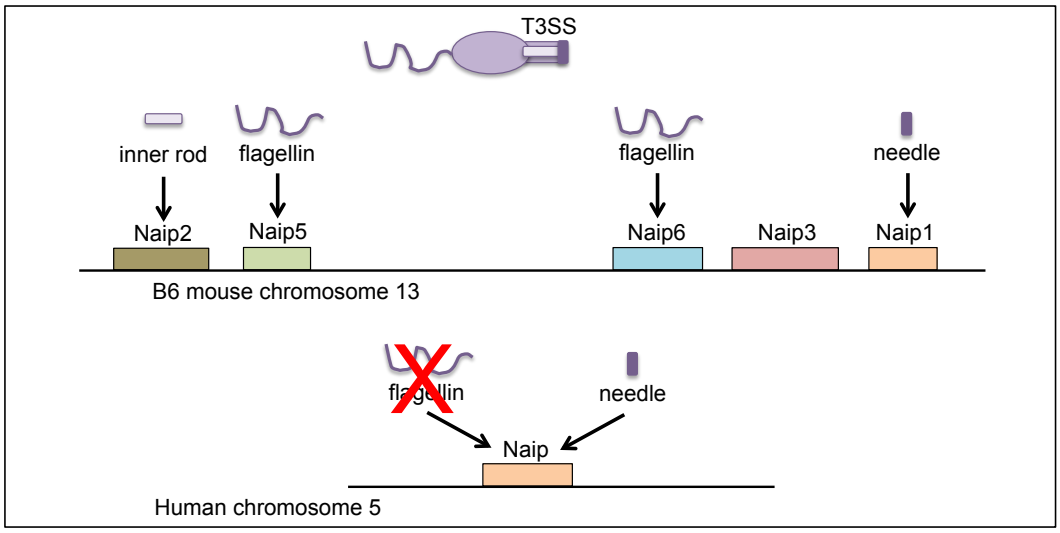

Figure 1-2. Murine NAIP paralogs recognize distinct stimuli and humans only encode a single NAIP protein. In B6 mice, murine NAIP5 and NAIP6 detect bacterial flagellin. NAIP2 detects the inner rod component of the T3SS, and NAIP1 responds specifically to the needle protein of the T3SS. In contrast to mice, humans encode a single NAIP protein. Human NAIP does not bind to flagellin and specifically responds to the needle protein of the T3SS.

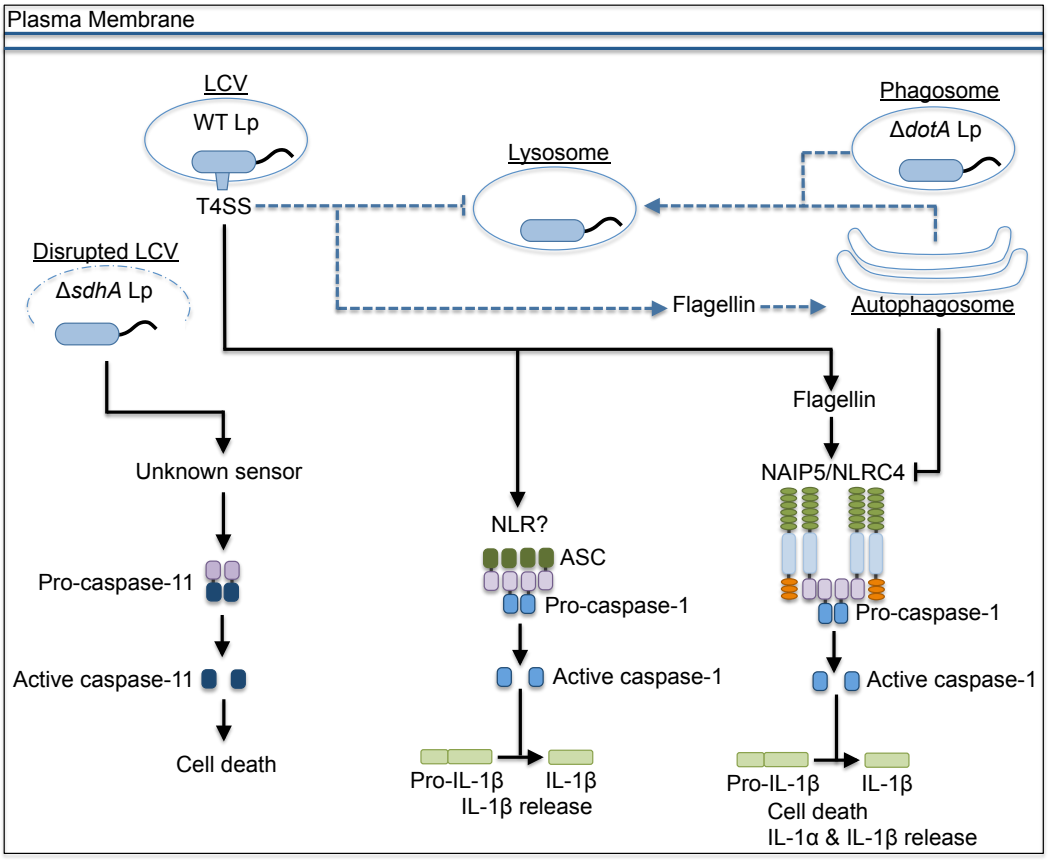

Figure 1-3. The inflammasome-mediated response to *L. pneumophila* in murine macrophages. *L. pneumophila* that do not express a functional T4SS (Δ*dotA* Lp) traffic to the lysosome, but wild-type *L. pneumophila* (WT Lp) use the T4SS to translocate effectors into the host cytosol to establish a replicative niche, the *Legionella*-containing vacuole (LCV), and block fusion with lysosomes. WT Lp triggers canonical caspase-1-dependent inflammasome activation through detection of translocated flagellin by NAIP5/NLRC4. ASC contributes to IL-1β secretion in response to WT Lp. Caspase-11 responds to bacteria that aberrantly enter the cytosol (Δ*sdhA* Lp) due to loss of LCV membrane integrity. Translocated flagellin triggers trafficking of WT Lp to the autophagosome, and induction of autophagy negatively regulates pyroptosis if there are low levels of flagellin in the host cell cytosol. Dashed lines represent vesicular trafficking patterns. Solid lines represent pathways for activation of the host response. Arrows at the end of lines represent induction, while flat bars at the end of lines represent inhibition.

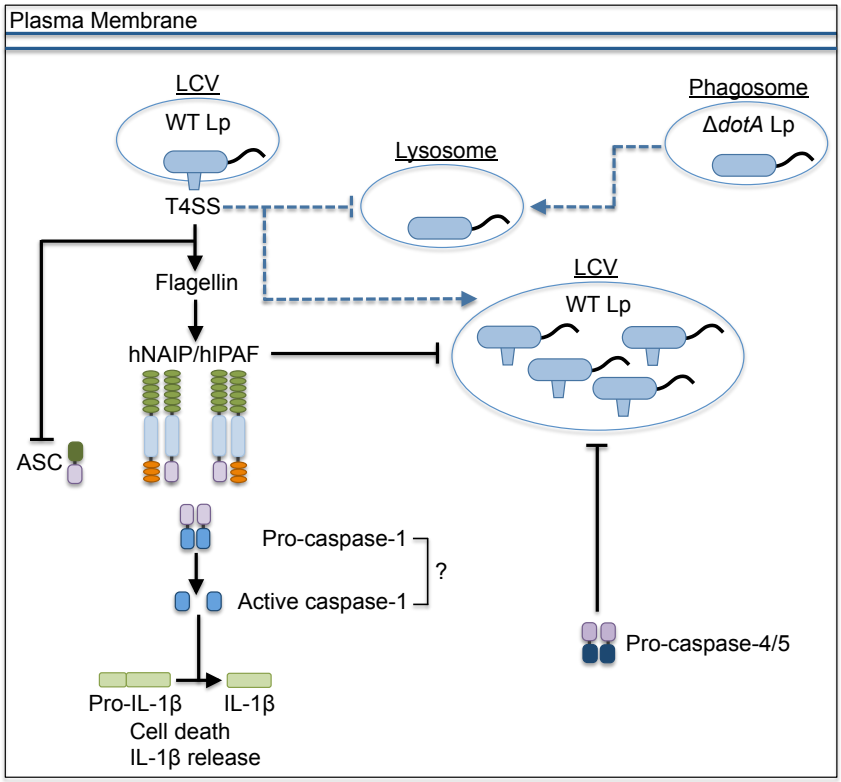

Figure 1-4. The inflammasome-mediated response to *L. pneumophila* in human cells. *L. pneumophila* that do not express a functional T4SS (Δ*dotA* Lp) traffic to the lysosome, but wild-type *L. pneumophila* (WT Lp) use the T4SS to translocate effectors into the host cytosol to establish a replicative niche, the *Legionella*-containing vacuole (LCV), and block fusion with lysosomes. The presence of flagellin triggers signaling through hNAIP/hIPAF that blocks replication of WT Lp, though it is unclear if caspase-1 is involved in restriction. T4SS activity down-regulates the expression of ASC. Overexpression of caspase-4 or caspase-5 blocks replication of WT Lp. Dashed lines represent vesicular trafficking patterns. Solid lines represent pathways for activation of the host response. Arrows at the end of lines represent induction, while flat bars at the end of lines represent inhibition.

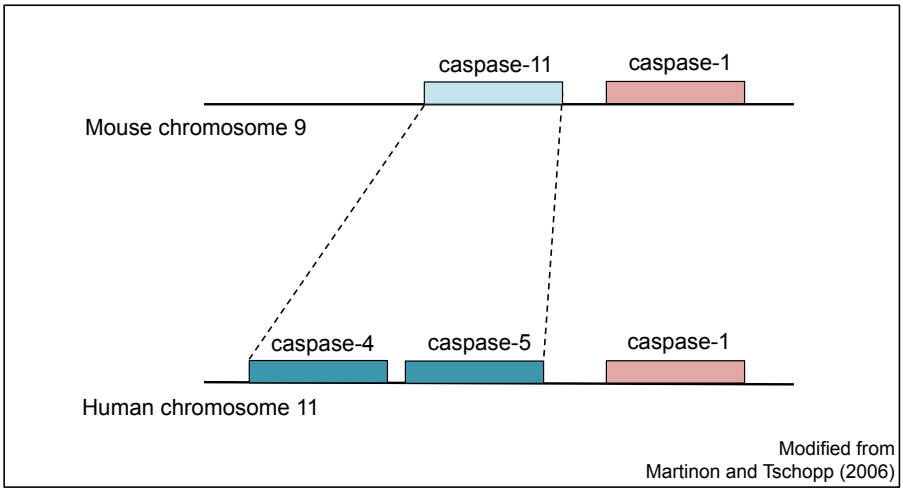

Figure 1-5. Humans do not encode caspase-11. Unlike mice, humans do not encode caspase-11. Instead, as the result of a duplication event, humans encode two putative functional orthologs—caspase-4 and caspase-5.

CHAPTER 2

MATERIALS AND METHODS

Ethics statement

These studies were carried out in strict accordance as defined in the federal regulations set forth in the Animal Welfare Act (AWA), the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and the guidelines of the University of Pennsylvania Institutional Animal Use and Care Committee. The protocols were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania (protocols #803465 and #803459).

Primary human samples

All studies on human peripheral blood mononuclear cells (PBMCs) were performed in compliance with the requirements of the US Department of Health and Human Services and the principles expressed in the Declaration of Helsinki. Samples obtained from the University of Pennsylvania Human Immunology Core are considered to be a secondary use of de-identified human specimens and are exempt via Title 55 Part 46, Subpart A of 46.101 (b) of the Code of Federal Regulations.

Bacterial strains

All experiments using *L. pneumophila* were performed with *Legionella pneumophila* serogroup 1 strains. Macrophages were infected with Lp02 (*thyA*), a thymidine auxotroph derived from strain Lp01 (Marra et al., 1992) or the Δ*dotA* (T4SS-) (Berger et al., 1994) and Δ*flaA* (FlaA-) (Ren et al., 2006) isogenic mutant strains. For *in vivo* studies, mice were infected with the Lp02 Δ*flaA* or the JR32 (Sadosky et al., 1993) Δ*flaA* isogenic mutant strain where indicated. For *in vitro* and *in vivo* studies, 48 hours prior to infection, *L. pneumophila* strains were grown in a stationary patch on charcoal yeast

extract (CYE) agar plates at 37°C. *Escherichia coli* BL21 strains were cultured in LB broth for 16 hours at 37˚C prior to infection. All experiments using *S.* Typhimurium were performed with *Salmonella enterica* serovar Typhimurium SL1344 strains. The strain SL1344 and the Δ*sipB* (T3SS-) and Δ*fliCfljB* (FliCFljB-) isogenic mutant strains were used. *S.* Typhimurium strains were grown overnight in LB broth with aeration at 37°C. Three hours prior to infection, *S.* Typhimurium strains were diluted into fresh LB with 300mM NaCl and then grown for 3 hours standing at 37°C to induce SPI-1 expression. All experiments using *Y. pseudotuberculosis* were performed with *Yersinia pseudotuberculosis* IP2666 strains. The strain IP2666 Δ*yopHOJMEK* (Δ6 Yp) (Lilo et al., 2008) and the Δ*yopB* (T3SS-) isogenic mutant (Palmer et al., 1998) strain were used. *Y. pseudotuberculosis* strains were grown overnight in 2xYT broth with aeration at 26°C. Three hours prior to infection, *Y. pseudotuberculosis* strains were diluted into fresh 2xYT with 20mM sodium oxalate and 20mM $MgCl_2$ and then grown for 1 hour with aeration at 26°C followed by 2 hours with aeration at 37°C.

Mice

C57BL/6J (B6) mice were purchased from Jackson Laboratories. *Casp1*$^{-/-}$*Casp11*$^{-/-}$ (Kuida et al., 1995), *Casp1*$^{-/-}$ (unpublished data, T.S. and R.A.F.), *Casp11*$^{-/-}$ (Wang et al., 1998), *Asc*$^{-/-}$ (Sutterwala et al., 2006), *Nlrc4*$^{-/-}$ (Lara-Tejero et al., 2006), *Asc*$^{-/-}$*Nlrc4*$^{-/-}$ (Case et al., 2009), *Ifnar*$^{-/-}$ (Müller et al., 1994), *Trif*$^{-/-}$ (Yamamoto et al., 2003), *Il1r1*$^{-/-}$ (Glaccum et al., 1997), and *Nlrp3*$^{-/-}$ (Martinon et al., 2006) mice are all on the B6 background. *Asc*$^{-/-}$, *Nlrc4*$^{-/-}$, and *Nlrp3*$^{-/-}$ mice were originally generated by Millenium Pharmaceuticals and were a kind gift of Richard Flavell. Animals were maintained in

accordance with the guidelines of the University of Pennsylvania Institutional Animal Use and Care Committee.

In vivo infection studies

8-12 week-old mice were anesthetized by intraperitoneal injection of a ketamine/xylazine/PBS solution at a dose of 100mg/kg ketamine and 10mg/kg xylazine. Mice were infected intranasally with 40µl of a bacterial suspension containing $1x10^6$ CFU *L. pneumophila* or PBS vehicle control. For antibody neutralization experiments, mice were injected intraperitoneally with 100µg anti-IL-1α antibody (clone ALF-161), 100µg anti-IL-1β antibody (clone B122), 100µg of each anti-IL-1α and anti-IL-1β antibody, or 100µg Armenian hamster IgG_1 isotype control antibody (eBioscience) 16 hours prior to intranasal infection. At the indicated timepoints after infection, mice were sacrificed, and the bronchoalveolar lavage fluid (BALF) and lungs were harvested. To determine bacterial load, the lungs were mechanically homogenized in sterile distilled H_2O and a portion of the lysate was spread onto CYE plates. Animal experiments were performed in accordance with approved University of Pennsylvania Institutional Animal Care and Use Committee protocols and procedures.

Murine macrophage experiments

Bone marrow was collected from the femurs and tibiae of mice by manually disrupting the bones with a mortar and pestle in RPMI and filtering the cells through a 70µM strainer. Bone marrow cells were differentiated into macrophages by culturing the cells in RPMI containing 30% L929 cell supernatant, 20% FBS, 100 IU/mL penicillin, and 100µg/mL streptomycin at 37˚C in a humidified incubator. The macrophages were

replated one day prior to infection in RPMI containing 15% L929 cell supernatant and 10% FBS. For experiments involving LPS-primed macrophages, macrophages in 48-well plates ($2.0x10^5$ cells/well) were pretreated with 0.5μg/mL LPS for 2.5 hours and either mock-infected with PBS, infected with *L. pneumophila* at an MOI=10 for 4 hours, or treated with 2.5mM ATP (Sigma-Aldrich) for 1 or 4 hours where indicated. For experiments performed in the absence of LPS priming, macrophages in 48-well plates ($2.0x10^5$ cells/well) were either mock-infected with PBS, infected with *L. pneumophila* at an MOI=10 for 16 or 20 hours, or infected with *E. coli* at an MOI=25 for 1 hour followed by gentamycin treatment (100μg/mL) for 15 hours. To assess the involvement of caspase-1 catalytic activity, macrophages were treated with 20μM or 40μM of the caspase-1 inhibitor YVAD-cmk (Bachem) or an equivalent volume of dimethyl sulfoxide (DMSO) (vehicle control) 0.5 hours prior to infection. For *L. pneumophila* and *E. coli* infections, bacteria were centrifuged down onto the macrophages at 1200 RPM for ten minutes prior to incubation. For *Y. pseudotuberculosis* infection, bacteria were washed three times with pre-warmed DMEM, added to the cells at an MOI=20, and centrifuged down onto the macrophages at 1000 rpm for 5 min. Cells were incubated at 37°C for 1 hour post-infection followed by addition of 100 μg/mL gentamicin. Supernatants were harvested 4 hours post infection for ELISA and LDH analysis.

Human macrophage experiments

THP-1 cells (ATCC TIB-202) were maintained in RPMI supplemented with 10% heat-inactivated FBS, 0.05mM β-mercaptoethanol, 100 IU/mL penicillin, and 100μg/mL streptomycin at 37°C in a humidified incubator. One day prior to infection, THP-1 cells were replated in media without antibiotics at a concentration of $2.0x10^5$ cells/well of a 48

well plate and incubated overnight with 200nM phorbol 12-myristate 13-acetate (PMA) to induce differentiation into macrophages. The media was replaced with warm media without antibiotics on the day of infection.

Primary human peripheral blood mononuclear cells (PBMCs) from de-identified healthy human donors were obtained from the University of Pennsylvania Human Immunology Core. PBMCs were pelleted at 200xg for 12 minutes and washed two times with PBS containing 0.5% BSA and 2mM EDTA. Monocytes were negatively selected using the Pan Monocyte Isolation Kit, Human (Miltenyi), which enriches for both CD14 and CD16 expressing human monocytes. After selection, monocytes were cultured in RPMI supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 100 IU/mL penicillin, 100µg/mL streptomycin, and 50ng/mL recombinant human M-CSF (Gemini Bio Products). Cells were cultured for 4 days in 10mL of media in 10cm dishes at $0.5x10^6$ cells/mL and then fresh media with 50ng/mL M-CSF was added and cells were cultured for an additional 2 days to complete differentiation into macrophages. One day prior to infection, monocyte-derived macrophages (MDMs) were gently detached and replated in media without antibiotics and with 25ng/mL M-CSF at a concentration of $1.25x10^5$ cells/well of a 48 well plate.

In experiments where macrophages were primed with LPS, cells were pre-treated with 0.5µg/mL LPS (Sigma-Aldrich) for 3 hours prior to infection. In experiments where macrophages were treated with YVAD, cells were pre-treated with 40µM Ac-YVAD-cmk (Bachem) or DMSO vehicle control for 0.5 hours prior to infection, and inhibitors were left on the cells for the remainder of the experiments. For infection with *L. pneumophila*,

bacteria were resuspended in PBS and added to the cells at an MOI=10 by spinning the bacteria onto the plate at 1200rpm for 10 minutes. Cells were infected for 4 hours when primed with LPS and 20 hours when unprimed. For infection with either *S.* Typhimurium or *Y. pseudotuberculosis*, bacteria were pelleted at 8000rpm for 3 minutes after induction and washed with PBS. Bacteria were then resuspended in PBS and added to the cells at an MOI=20 by spinning the bacteria onto the plate at 1200rpm for 10 minutes. Cells were infected for 1 hour before gentamycin was added to the media at 100μg/mL and then infection continued for an additional 3 hours. For all experiments, mock-infected cells were treated with PBS.

In experiments where macrophages were treated with LPS or IFNβ, cells were either left untreated (unstim.) or treated with 0.05 or 0.5μg/mL of *E. coli* LPS (055:B5 from Sigma) or 100 or 1000 U (Units) of IFNβ (recombinant human IFNβ purified in mammalian cells from R&D Systems) for the indicated amounts of time. In experiments where nigericin was used, cells were primed with LPS for 5 hours and then 10μM nigericin was added for an additional 2 hours.

THP-1 cells deficient for ASC (ASC-def) (Invivogen) and control empty vector containing THP-1 cells (Null) were maintained in RPMI supplemented with 15% heat-inactivated FBS, 100 IU/mL penicillin, 100μg/mL streptomycin, 100μg/mL Normocin (Invivogen), and 200μg/mL Hygromycin (for selection maintenance) at 37°C in a humidified incubator. One day prior to infection, ASC-def and Null THP-1 cells were replated in media without antibiotics at a concentration of $2.0x10^5$ cells/well of a 48 well plate and incubated overnight with 200nM phorbol 12-myristate 13-acetate (PMA) to induce differentiation

into macrophages. The media was replaced with warm media without antibiotics on the day of infection.

siRNA knockdown experiments

All Silencer Select siRNA oligos were purchased from Ambion via Life Technologies. For caspase-1, the siRNAs used were siRNA ID s2407, s2408, and s2409. For caspase-4, the siRNAs used were siRNA ID s2413, s2414, and s2415. The two available Silencer Select negative control siRNAs were purchased (Silencer Select Negative Control No.1 siRNA and Silencer Select Negative Control No.2 siRNA). Two days prior to infection, either THP-1 cells or primary human MDMs were replated in media without antibiotics in 48-well plates as described above. One day prior to infection, 10-30nM total pooled siRNA was transfected into the macrophages using Hiperfect (Qiagen), following the manufacturer's protocol for "Transfection of Differentiated Macrophage Cell Lines, Including THP-1." After 24 hours, the media was replaced with fresh warm media without antibiotics, and infections were performed as described above.

Transfection of intracellular LPS

Primary human MDMs replated at a concentration of $1.25x10^5$ cells/well of a 48 well plate were primed with 0.4μg/mL Pam3CSK4 (Invivogen) for 4 hours. The media was then replaced with fresh replating media, and cells were either treated with LPS alone (2 or 5μg/mL), mock transfected with Fugene HD (Promega) alone, or treated with a mixture of 0.75μL Fugene HD (0.25% v/v) plus LPS (2 or 5μg/mL) per well in 300μL media per well. Plates were then centrifuged at 2000rpm for 2 minutes before culturing at 37°C for 20 hours.

Cytotoxicity assays

Cells were infected or treated as described above, and harvested supernatants were assayed for loss of cellular membrane integrity via lactate dehydrogenase (LDH) activity. LDH release was quantified using the LDH Cytotoxicity Detection Kit (Clontech), following the manufacturer's protocol.

Immunoblot analysis

Cells were lysed directly in 1X SDS-PAGE sample buffer, and low volume (100 or 120μL/well of a 48 well plate) supernatants were mixed 1:1 with 2X SDS-PAGE sample buffer containing Complete Mini EDTA-free Protease Inhibitor Cocktail (Roche). Samples were boiled for 5 minutes, and proteins were separated by SDS-PAGE on a 12% gel and transferred to Immobilon P PVDF membranes (Millipore). Primary antibodies against mouse caspase-1 p10 (Santa Cruz Biotechnology), mouse caspase-11 (Sigma, clone 17D9), mouse IL-1β (R&D systems), human IL-1β (clone 3A6, Cell Signaling Technology), mature human IL-1β (clone 8516, R&D Systems), human caspase-1 p10 (polyclonal, Santa Cruz), cleaved human caspase-5 (polyclonal, Cell Signaling Technology), full-length human caspase-5 (clone EP876Y, GeneTex), human caspase-4 (polyclonal, Cell Signaling Technology), human ASC (polyclonal, Enzo Life Sciences), and β-actin (Sigma) were used. Anti-rabbit IgG (Cell Signaling Technology), anti-mouse IgG (Cell Signaling Technology), and anti-rat IgG (Santa Cruz Biotechnology or Jackson Immuno) HRP-conjugated secondary antibodies were used. For detection, SuperSignal West Dura or SuperSignal West Femto (Pierce Thermo Scientific) was used as the HRP substrate.

ELISA

Harvested supernatants from infected murine macrophages or the BALF from infected mice were assayed using capture and detection antibodies specific for IL-18 (MBL), IL-1α, IL-1β, IL-12p40 (BD Biosciences), and TNF (Biolegend). For human macrophages, cells were infected as described above, and the levels of cytokines were quantified in the harvested supernatants using paired antibody kits for human IL-1α (R&D Systems), IL-1β (BD Biosciences), and TNF (Biolegend).

qRT-PCR analysis

Cells were lysed directly in 350μL buffer RLT Plus with β-mecaptoethanol as per instructions in the RNeasy Plus Kit (Qiagen). Suspensions were vortexed and subsequently disrupted in a QIAshredder spin column (Qiagen) following the manufacturer's protocol. RNA was isolated following the manufacturer's protocol in the RNeasy Plus handbook (Qiagen). RNA was reverse transcribed into cDNA with Superscript II (Invitrogen) following the manufacturer's protocol. Quantitative PCR was performed with the CFX96 Real-time system from Bio-Rad using the SooFast EvaGreen Supermix with Low ROX kit (Bio-rad). The following primers were used (from PrimerBank): *CASP4* forward- CAAGAGAAGCAACGTATGGCA, *CASP4* reverse- AGGCAGATGGTCAAACTCTGTA, *CASP5* forward- TTCAACACCACATAACGTGTCC, *CASP5* reverse- GTCAAGGTTGCTCGTTCTATGG, *HPRT1* forward- CCTGGCGTCGTGATTAGTGAT, and *HPRT1* reverse- AGACGTTCAGTCCTGTCCATAA. For analysis, caspase-4 or caspase-5 mRNA levels were normalized to HPRT mRNA levels for each sample, and samples were normalized to untreated cells using the $2^{-\Delta\Delta CT}$ (cycle threshold) method to calculate fold induction.

Flow cytometry

To determine neutrophil recruitment to the airway, BALF cells were stained with Live/Dead Fixable Dead Cell Stain (Invitrogen), and antibodies specific for CD45, Gr-1 (eBioscience), and Ly6G (Biolegend). Data were collected with an LSRII flow cytometer (BD Biosciences), and post-collection data was analyzed using FlowJo (Treestar). Cells were pre-gated on singlets and live cells. Neutrophils were identified as being $CD45^+$, $Gr\text{-}1^+$, and $Ly6G^+$.

Statistical analysis

Graphpad Prism software was used for the graphing of data and all statistical analysis. For murine data, statistical significance was determined by the unpaired two-tailed Student's t test, one-way ANOVA with Tukey post-test, or two-way ANOVA with Bonferroni post-test. For human data, statistical significance was determined using the unpaired two-way Student's t-test for experiments in human cell lines and the paired two-way t-test for experiments comparing different treatments of primary human cells. For human data, all data are graphed so that each data point represents the mean of triplicate infected wells from independent experiments, and all statistical analyses were performed comparing the means of each experiment. Each individual experiment was performed a minimum of three separate times for human cell lines and at least 4 times using cells from different donors for primary human macrophages. Differences were considered statistically significant if the *P* value was <0.05.

CHAPTER 3

CASPASE-11 ACTIVATION IN RESPONSE TO BACTERIAL SECRETION SYSTEMS THAT ACCESS THE HOST CYTOSOL

This chapter appeared as a published peer-reviewed article titled “Caspase-11 activation in response to bacterial secretion systems that access the host cytosol” by **Cierra N. Casson**, Alan M. Copenhaver, Erin E. Zwack, Hieu T. Nguyen, Till Strowig, Bahar Javdan, William P. Bradley, Thomas C. Fung, Richard A. Flavell, Igor E. Brodsky, and Sunny Shin. PLoS Pathogens, 2013.

A. Abstract

Inflammasome activation is important for antimicrobial defense because it induces cell death and regulates the secretion of IL-1 family cytokines, which play a critical role in inflammatory responses. The inflammasome activates caspase-1 to process and secrete IL-1β. However, the mechanisms governing IL-1α release are less clear. Recently, a non-canonical inflammasome was described that activates caspase-11 and mediates pyroptosis and release of IL-1α and IL-1β. Caspase-11 activation in response to Gram-negative bacteria requires TLR4 and TRIF-dependent IFN production. Whether additional bacterial signals trigger caspase-11 activation is unknown. Many bacterial pathogens use specialized secretion systems to translocate effector proteins into the cytosol of host cells. These secretion systems can also deliver flagellin into the cytosol, which triggers caspase-1 activation and pyroptosis. However, even in the absence of flagellin, these secretion systems induce inflammasome activation and the release of IL-1α and IL-1β, but the inflammasome pathways that mediate this response are unclear. We observe rapid IL-1α and IL-1β release and cell death in response to the T4SS or T3SS of *Legionella pneumophila* and *Yersinia pseudotuberculosis*. Unlike IL-1β, IL-1α secretion does not require caspase-1. Instead, caspase-11 activation is required for both IL-1α secretion and cell death in response to the activity of these secretion systems. Interestingly, whereas caspase-11 promotes IL-1β release in response to the T4SS through the NLRP3/ASC inflammasome, caspase-11-dependent release of IL-1α is independent of both the NAIP5/NLRC4 and NLRP3/ASC inflammasomes as well as TRIF and type I IFN signaling. Furthermore, we find both overlapping and non-redundant roles for IL-1α and IL-1β in mediating neutrophil recruitment and bacterial clearance in response to pulmonary infection by *L. pneumophila*. Our findings demonstrate that

virulent, but not avirulent, bacteria trigger a rapid caspase-11-dependent innate immune response important for host defense.

B. Author summary

The inflammasome, a multiprotein complex, is critical for host defense against bacterial infection. The inflammasome activates the host protease caspase-1 to process and secrete IL-1β. Another caspase, caspase-11, can cause cell death and IL-1α release. The bacterial signals that trigger caspase-11 activation are poorly understood. A common feature of many bacterial pathogens is the ability to inject virulence factors and other bacterial molecules into the host cell cytosol by means of a variety of virulence-associated secretion systems. These secretion systems can introduce bacterial flagellin into the host cytosol, which leads to caspase-1 activation and cell death. However, many bacteria lack or down-regulate flagellin yet still activate the inflammasome. Here, we show that the T4SS of *Legionella pneumophila* and the T3SS of *Yersinia pseudotuberculosis* rapidly trigger caspase-11 activation in a flagellin-independent manner. Caspase-11 activation mediates two separate inflammasome pathways: one leading to IL-1β processing and secretion, and one leading to cell death and IL-1α release. Furthermore, we find these caspase-11-regulated cytokines are critical for neutrophil recruitment to the site of infection and clearance of non-flagellated *Legionella in vivo*. Overall, our findings show that virulent bacteria activate a rapid caspase-11-dependent immune response that plays a critical role in host defense.

C. Introduction

Antibacterial defense is initiated by germline-encoded PRRs, which detect conserved PAMPs (Janeway, 1989; Janeway and Medzhitov, 2002; Medzhitov, 2007). Plasma membrane-bound PRRs, such as the TLRs, detect PAMPs present in the extracellular space and endosomal compartments, whereas cytosolic PRRs, such as the NLRs, survey the host cytosol for the presence of invasive pathogens (Fritz et al., 2006; Harton et al., 2002; Inohara et al., 2005; Janeway and Medzhitov, 2002; Ting et al., 2006). Invasive microorganisms or other cellular stresses induce assembly of cytosolic protein complexes known as inflammasomes, which play a critical role in host defense (Davis et al., 2011; Martinon et al., 2002; Miao et al., 2010a; Rathinam et al., 2012a). Inflammasomes respond to a wide variety of activators, including bacterial pore-forming toxins and bacterial PAMPS, such as flagellin or RNA (Boyden and Dietrich, 2006; Franchi et al., 2006; Kanneganti et al., 2006; Mariathasan et al., 2006; Miao et al., 2006; Molofsky et al., 2006; Ren et al., 2006). Particular NLRs respond to their cognate stimuli and recruit the adapter protein ASC and pro-caspase-1 through homotypic protein-protein interactions between pyrin and CARD domains, leading to auto-processing and activation of caspase-1 (Dostert et al., 2008; Hornung et al., 2009; Kofoed and Vance, 2011; Srinivasula et al., 2002; Sutterwala et al., 2006). Caspase-1 is responsible for processing and secreting IL-1 family cytokines and mediates a pro-inflammatory cell death termed pyroptosis (Cookson and Brennan, 2001; Fink and Cookson, 2005; Martinon et al., 2002; Rathinam et al., 2012a).

Caspase-11 participates in the activation of a non-canonical inflammasome that induces cell death and the secretion of IL-1α and IL-1β in response to Gram-negative pathogens,

such as *Escherichia coli* and *Vibrio cholerae,* and to particular toxins, such as the cholera toxin B subunit (Broz et al., 2012; Gurung et al., 2012; Kayagaki et al., 2011; Rathinam et al., 2012b). This non-canonical, caspase-11-dependent response to Gram-negative bacteria is independent of virulence-associated secretion systems that deliver bacterial molecules into the host cytosol and requires LPS-induced TLR4 signaling through the adaptor TRIF and TRIF-dependent type I IFN production. Type I IFN signaling through the type I IFN receptor (IFNAR) is required for caspase-11 upregulation and activation, but how type I IFN mediates activation of caspase-11 is not well-defined (Broz et al., 2012; Gurung et al., 2012; Rathinam et al., 2012b). Caspase-11 contributes to NLRP3-dependent activation of caspase-1 and subsequent caspase-1-dependent IL-1β secretion and cell death. Caspase-11 also facilitates an NLRP3- and caspase-1-independent pathway that results in cell death and release of IL-1α (Broz et al., 2012; Gurung et al., 2012; Kayagaki et al., 2011; Rathinam et al., 2012b). This caspase-11-dependent, caspase-1-independent pathway is responsible for LPS-induced septic shock *in vivo* (Kayagaki et al., 2011; Wang et al., 1998). Although caspase-11 is activated in response to signals from Gram-negative pathogens and certain pore-forming toxins, whether caspase-11 contributes to inflammasome activation in response to virulence-associated secretion systems that deliver bacterial ligands into host cytosol is unknown.

Bacterial pathogens use evolutionarily conserved secretion systems, such as the T3SS or T4SS, to translocate effector proteins into the cytosol of host cells (Cornelis, 2006; Juhas et al., 2008). In addition to bona fide virulence factors, these secretion systems also translocate bacterial molecules such as flagellin or structural components of the

secretion machinery itself, which results in inflammasome activation (Brodsky and Monack, 2009; Miao et al., 2006; 2010b; Molofsky et al., 2006; Ren et al., 2006; Sun et al., 2007). *Legionella pneumophila*, an opportunistic pathogen that causes a severe pneumonia known as Legionnaires' disease (Fraser et al., 1977; McDade et al., 1977), uses its *dot/icm*-encoded T4SS as a virulence factor to translocate bacterial effector proteins into the host cell cytosol and establish a replicative vacuole (Berger and Isberg, 1993; Ensminger and Isberg, 2009; Hubber and Roy, 2010; Marra et al., 1992; Nagai et al., 2002; Roy et al., 1998; Segal et al., 1998; Vogel et al., 1998). *L. pneumophila* induces T4SS-dependent inflammasome activation through two genetically distinct pathways (Case et al., 2009). T4SS-mediated translocation of flagellin into the cytosol triggers caspase-1 activation and pyroptosis through the NLR NAIP5 in conjunction with another NLR, NLRC4 (Amer et al., 2006; Case et al., 2009; Lightfield et al., 2008; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). Caspase-1 activation is also triggered independently of the NLRC4/flagellin pathway through the adaptor protein ASC, but the bacterial factor that is recognized and the upstream proteins that regulate this pathway remain unknown (Case and Roy, 2011; Case et al., 2009). However, although ASC is necessary for robust secretion of IL-1β in response to *L. pneumophila* as well as a number of pathogens, such as *Salmonella* or *Yersinia* species, ASC is dispensable for induction of pyroptosis that is rapidly triggered in response to these infections. We therefore considered the possibility that in addition to its role in delayed inflammasome activation in response to Gram-negative bacteria, caspase-11 might participate in rapid cell death and release of IL-1α in response to the presence of bacterial pathogens that access the host cell cytosol by means of type IV and type III secretion systems.

Here, we demonstrate that IL-1α and IL-1β are rapidly released in response to bacterial T4SS activity independently of bacterial flagellin. In this system, we find that IL-1β secretion requires caspase-1, but caspase-1 is dispensable for cell death and IL-1α release in response to a functional *L. pneumophila* T4SS. Instead, caspase-11 is required for both IL-1α release and cell death in response to *L. pneumophila* T4SS activity. Consistent with recent findings, caspase-11 contributes to optimal NLRP3-mediated caspase-1 activation and IL-1β secretion in response to *L. pneumophila*. However, caspase-11-dependent IL-1α release and cell death in *L. pneumophila*-infected cells are independent of the NAIP5/NLRC4 and NLRP3/ASC inflammasomes. In contrast to the role of TRIF and IFNAR in the response against other Gram-negative bacteria, caspase-11 activation and cytokine release in response to the T4SS of *L. pneumophila* are independent of both TRIF and IFNAR signaling. We further demonstrate that T3SS activity of the unrelated pathogen *Yersinia pseudotuberculosis* induces a similarly rapid caspase-11-dependent response that also leads to cell death and release of IL-1α and IL-1β. Finally, we find that both IL-1α and IL-1β are critical *in vivo* for neutrophil recruitment and bacterial clearance. Overall, our data show that caspase-11 is poised to respond robustly to a conserved feature of pathogenic bacteria, bacterial access to the host cytosol through specialized secretion systems. This establishes caspase-11 as a critical regulator of immune system-mediated discrimination of pathogenic and nonpathogenic bacteria.

D. Results

LPS priming induces rapid IL-1α and IL-1β secretion in response to *L. pneumophila* T4SS activity

L. pneumophila infection induces IL-1α and IL-1β secretion that requires T4SS activity (Case et al., 2009; Shin et al., 2008). IL-1β secretion is regulated by a flagellin-dependent NAIP5/NLRC4 inflammasome and a poorly defined ASC inflammasome that both activate caspase-1 (Case and Roy, 2011; Case et al., 2009). The mechanisms underlying IL-1α secretion are less clear, but IL-1α secretion is still robustly induced by flagellin-deficient *L. pneumophila,* which do not activate the NAIP5/NLRC4 inflammasome (Shin et al., 2008). Recent studies have described a non-canonical inflammasome triggered in response to Gram-negative bacteria. This non-canonical inflammasome requires LPS for the upregulation and activation of caspase-11 and subsequent IL-1α and IL-1β release (Broz et al., 2012; Gurung et al., 2012; Kayagaki et al., 2011; Rathinam et al., 2012b). Whether caspase-11 is also activated in response to bacteria that use specialized secretion systems to translocate bacterial molecules into the host cytosol is unknown. We thus hypothesized that LPS priming would upregulate caspase-11, pro-IL-1α, and pro-IL-1β and allow for more robust and rapid IL-1α and IL-1β secretion in response to T4SS activity. To test this hypothesis, we first compared IL-1α and IL-1β release in unprimed and LPS-primed bone marrow-derived macrophages (BMDMs). As shown previously (Shin et al., 2008; Zamboni et al., 2006), unprimed BMDMs secrete robust levels of IL-1α and IL-1β by 20 hours post-infection with wild-type *L. pneumophila* (WT Lp) (Figure 3-1A). Slightly attenuated levels of secreted IL-1α and IL-1β are observed with flagellin-deficient *L. pneumophila* (Δ*flaA* Lp), which do not activate the NAIP5/NLRC4 inflammasome (Broz et al., 2012; Gurung et al., 2012;

Kayagaki et al., 2011; Molofsky et al., 2006; Rathinam et al., 2012b; Ren et al., 2006). Secretion of both cytokines is significantly diminished during infection with *L. pneumophila* lacking DotA, an essential component of the T4SS (Δ*dotA* Lp), and is significantly diminished in caspase-1/caspase-11-deficient (*Casp1*$^{-/-}$*Casp11*$^{-/-}$) macrophages as well (Figure 3-1A). The diminished IL-1 secretion induced by Δ*dotA* Lp is not due to a lack of pro-IL-1 production, as Δ*dotA* Lp and WT Lp induce robust levels of pro-IL-1β (Figure 3-2A). At 4 hours post-infection, unprimed macrophages do not secrete IL-1 (Figure 3-1B). However, LPS-primed cells rapidly secrete IL-1α and IL-1β, and this secretion is abrogated in *Casp1*$^{-/-}$*Casp11*$^{-/-}$ macrophages (Figure 3-1B). Secretion of IL-18, another IL-1 family cytokine, also requires T4SS activity and is eliminated in *Casp1*$^{-/-}$*Casp11*$^{-/-}$ cells (Figure 3-2B). Comparable levels of the caspase-1/caspase-11-independent cytokines IL-12 and TNF-α are secreted in the absence and presence of LPS priming (Figure 3-2C and 3-2D). These data suggest that LPS priming upregulates a factor required for rapid IL-1α and IL-1β release in response to *L. pneumophila* T4SS activity.

Caspase-1 catalytic activity is required for IL-1β but not IL-1α secretion

Secretion of IL-1β in response to both canonical and non-canonical inflammasome activation requires caspase-1 (Broz et al., 2012; Kayagaki et al., 2011; Thornberry et al., 1992). In contrast, IL-1α release downstream of the non-canonical inflammasome depends on caspase-11, and does not require caspase-1 (Kayagaki et al., 2011). To test if the catalytic activity of caspase-1 is required for IL-1α secretion in response to *L. pneumophila*, we inhibited caspase-1 catalytic activity with the pharmacological inhibitor YVAD-cmk (YVAD). Consistent with previous studies (Broz et al., 2010), IL-1β secretion

in response to *L. pneumophila* is substantially inhibited by YVAD. However, YVAD has no effect on IL-1α secretion, indicating that IL-1α release in response to *L. pneumophila* does not require caspase-1 catalytic activity (Figure 3-1C), as has been shown for other inflammasome activators (Gross et al., 2012). Given that IL-1α secretion occurs more rapidly upon LPS priming, is abrogated in *Casp1*$^{-/-}$*Casp11*$^{-/-}$ macrophages, and does not require caspase-1 catalytic activity, we considered the possibility that caspase-11 might participate in inflammasome activation during *L. pneumophila* infection.

Caspase-11 contributes to inflammasome activation in response to flagellin-deficient *L. pneumophila*

To test the genetic requirement for caspase-11 in the inflammasome response to *L. pneumophila*, we infected BMDMs from either caspase-1-deficient (*Casp1*$^{-/-}$) or caspase-11-deficient (*Casp11*$^{-/-}$) mice. In the absence of flagellin, caspase-11 is required for IL-1α secretion, whereas it is not essential for IL-1β secretion but contributes to maximal secretion (Figure 3-3A). These data suggest that caspase-11 is activated in response to *L. pneumophila* infection independently of flagellin. Indeed, there is robust processing and secretion of caspase-11 in response to WT and Δ*flaA* Lp (Figure 3-4). In accordance with previous findings (Broz et al., 2010; Kayagaki et al., 2011), caspase-1 is absolutely required for IL-1β secretion. In contrast, we observe robust IL-1α release even in the absence of caspase-1. Both IL-1α and IL-1β release in response to Δ*flaA* Lp are caspase-11-dependent in both primed and unprimed macrophages (Figure 3-3, 3-5A, and 3-5B), making *L. pneumophila* distinct from other Gram-negative bacteria that require priming to induce robust caspase-11 upregulation and activation (Rathinam et al., 2012b). Thus, while caspase-11 contributes to maximal caspase-1-dependent IL-1β

secretion, it is both necessary and sufficient for IL-1α release in response to flagellin-deficient *L. pneumophila*.

Cell death in B6 BMDMs is partially flagellin-dependent but is flagellin-independent in *Casp1*$^{-/-}$ BMDMs (Figure 3-3B). Importantly, cell death in response to flagellin-deficient *L. pneumophila* requires caspase-11, thus correlating caspase-11-dependent cell death with IL-1α release from host cells. In contrast, and consistent with previous findings (Kayagaki et al., 2011), LPS+ATP induces canonical caspase-1-dependent pyroptosis and secretion of IL-1α and IL-1β that is independent of caspase-11. Because caspase-1 must be processed to mediate IL-1β secretion (Broz et al., 2010), we examined whether caspase-1 processing is decreased in the absence of caspase-11, which could account for the decreased IL-1β secretion in response to Δ*flaA* Lp. Caspase-1 processing is slightly attenuated but not abrogated in response to Δ*flaA* Lp in *Casp11*$^{-/-}$ macrophages, consistent with the slight decrease in IL-1β secretion (Figure 3-3C and 3-5C). Thus, flagellin-deficient *L. pneumophila* trigger a canonical caspase-1-dependent inflammasome as well as a non-canonical caspase-11-dependent inflammasome.

Caspase-11 activation is independent of ASC and NLRC4

The ASC and NAIP5/NLRC4 inflammasomes are required for caspase-1 activation and IL-1β secretion in response to *L. pneumophila* (Case et al., 2009). To determine if these inflammasomes are also required for caspase-11 activation and IL-1α release, we infected ASC/NLRC4-deficient (*Asc*$^{-/-}$*Nlrc4*$^{-/-}$) BMDMs with *L. pneumophila*. *Asc*$^{-/-}$*Nlrc4*$^{-/-}$ BMDMs do not secrete IL-1β in response to either WT Lp, Δ*flaA* Lp, or LPS+ATP. However, *Asc*$^{-/-}$*Nlrc4*$^{-/-}$ BMDMs still release IL-1α in response to Δ*flaA* Lp in primed and

unprimed macrophages (Figure 3-6A and 3-7). Thus, unlike IL-1β, IL-1α is released independently of flagellin, ASC, and NLRC4. Accordingly, despite an absence of processed caspase-1 p10, robust levels of processed caspase-11 p26 are detected in the supernatants of *Asc*$^{-/-}$*Nlrc4*$^{-/-}$ cells infected with either WT or Δ*flaA* Lp but not in response to LPS+ATP (Figure 3-6B).

We next sought to determine whether IL-1α is also released independently of ASC and NLRC4 during *in vivo* infection. Because flagellin-deficient *L. pneumophila* do not activate the NLRC4 inflammasome (Case et al., 2009; Molofsky et al., 2006; Ren et al., 2006), infecting *Asc*$^{-/-}$ mice with Δ*flaA* Lp eliminates both the ASC and NLRC4 inflammasome pathways. Importantly, the level of IL-1β in the bronchoalveolar lavage fluid (BALF) 24 hours post-infection is significantly attenuated in *Asc*$^{-/-}$ mice infected with Δ*flaA* Lp (Figure 3-6C). In contrast, the level of IL-1α in the BALF is unaffected even in the absence of both the ASC and NLRC4 inflammasomes. Both IL-1α and IL-1β release are significantly diminished in caspase-1/caspase-11-deficient mice (Figure 3-8). Collectively, our data indicate that *L. pneumophila* triggers caspase-11 activation and IL-1α release independently of the ASC and NLRC4 inflammasomes during both *in vitro* and *in vivo* infection.

Caspase-11 mediates both NLRP3-dependent and NLRP3-independent inflammasome responses

L. pneumophila induces caspase-1 activation and IL-1β and IL-18 secretion through two genetically distinct pathways, one involving ASC and one involving NLRC4 (Figure 3-9A, 3-10A, and 3-10B) (Case et al., 2009). The upstream host and bacterial components of

the ASC-dependent response to *L. pneumophila* are still unknown, but are independent of the flagellin/NAIP5/NLRC4 pathway (Figure 3-9A and 3-10B) (Case et al., 2009). Because caspase-11 contributes to maximal IL-1β secretion in response to Δ*flaA* Lp, we further investigated the ASC-dependent mechanism of inflammasome activation. NLRP3, an NLR involved in inflammasome-dependent responses to a wide variety of pathogens, requires ASC to mediate caspase-1 processing during both canonical and non-canonical inflammasome activation (Kanneganti et al., 2006; Kayagaki et al., 2011; Mariathasan et al., 2004; 2006; Martinon et al., 2002). We therefore investigated the role of NLRP3 in the response to Δ*flaA* Lp. Notably, IL-1β and IL-18 secretion are abrogated during infection of NLRP3-deficient (*Nlrp3*$^{-/-}$) BMDMs with Δ*flaA* Lp in both primed and unprimed macrophages (Figure 3-9B and 3-11A, B, and C). Consistently, we do not detect processed caspase-1 p10 in the supernatants of *Nlrp3*$^{-/-}$ macrophages infected with Δ*flaA* Lp (Figure 3-9C). Thus, NLRP3 functions together with ASC, caspase-1, and caspase-11 to control IL-1β secretion in response to flagellin-deficient *L. pneumophila*. However, IL-1α release and cell death following infection with flagellin-deficient *L. pneumophila* are independent of NLRP3 (Figure 3-9B and 3-11A), indicating that caspase-11 also mediates an NLRP3-independent response towards flagellin-deficient *L. pneumophila*. Accordingly, NLRP3-dependent IL-1β secretion in response to flagellin-deficient *L. pneumophila* was inhibited by extracellular potassium, whereas NLRP3-independent caspase-11-dependent IL-1α secretion and cell death were not affected (Figure 3-11D and 3-11E).

Non-canonical inflammasome responses to *L. pneumophila* occur independently of TRIF and IFNAR

Recent data demonstrate that caspase-11 activation in response to a wide variety of Gram-negative bacteria requires TLR4 signaling through its adaptor TRIF and subsequent type I IFN production (Broz et al., 2012; Gurung et al., 2012; Rathinam et al., 2012b). To determine if *L. pneumophila* engages a similar TRIF and IFNAR-dependent pathway for caspase-11 activation, we infected TRIF-deficient (*Trif*$^{-/-}$) and IFNAR-deficient (*Ifnar*$^{-/-}$) BMDMs. Unlike the response to *E. coli*, *L. pneumophila* infection of unprimed macrophages triggered robust cell death and secretion of IL-1α and IL-1β that was independent of IFNAR and TRIF (Figure 3-12A and 3-12B). Consistently, priming with the TLR1/2 agonist Pam3CSK4, which results in TRIF- and IFNAR-dependent cytokine secretion and cell death in response to *E. coli* (Rathinam et al., 2012b), still induced cell death and cytokine secretion in TRIF- and IFNAR-deficient cells in response to *L. pneumophila* (Figure 3-13A and 3-13B). These data suggest that during *L. pneumophila* infection, caspase-11 is upregulated and activated independently of TRIF and IFNAR signaling. Indeed, caspase-11 is still robustly processed and secreted independently of IFNAR and TRIF (Figure 3-12C and 3-14). Notably, substantially upregulated levels of pro-caspase-11 are not observed in the lysates of cells infected with WT or Δ*flaA* Lp because both the pro and cleaved forms of caspase-11 are rapidly secreted into the cell supernatant upon infection (Figures 3-12C and 3-14). Accordingly, lysates from IFNAR- and TRIF-deficient macrophages infected with *L. pneumophila* express comparable levels of pro-caspase-11 to wild-type macrophages, whereas TRIF and IFNAR do contribute to upregulation of pro-caspase-11 in response to *E. coli* (Figure 3-15A, B, and C). When the macrophages are primed with LPS prior to

infection, there is a moderate contribution of TRIF and IFNAR signaling to inflammasome activation, consistent with the observation that LPS stimulates the TLR4-TRIF-IFNAR axis involved in caspase-11 upregulation (Figure 3-13C and 3-13D). Because the caspase-11-dependent response to *L. pneumophila* is TRIF-independent, we investigated whether the TLR signaling adaptor MyD88 contributes to caspase-11 upregulation. When immortalized macrophages deficient for both MyD88 and Trif (i*Myd88*$^{-/-}$*Trif*$^{-/-}$) were infected, caspase-11 upregulation was diminished in response to both WT and Δ*flaA* Lp (Figure 3-16A and 3-16B), and we were unable to detect caspase-11 activation. Thus, although TRIF is not required for caspase-11 activation, a TLR-dependent signal is likely required as the loss of both MyD88 and TRIF eliminates caspase-11 upregulation and activation.

Caspase-11 mediates inflammasome activation in response to *Yersinia pseudotuberculosis* type III secretion system activity

Because caspase-11 activation in response to *L. pneumophila* expressing a functional T4SS is so rapid and robust, we sought to test whether this robust caspase-11-dependent inflammasome activation might be a general response to the activity of specialized secretion systems that allow for bacterial access to the host cytosol. The *Yersinia pseudotuberculosis* T3SS induces inflammasome activation independently of bacterial flagellin and the known secreted effector proteins, and this inflammasome activation is important for bacterial clearance (Brodsky et al., 2010). As wild-type *Yersinia* induces cell death that is independent of both caspase-1 and -11 and requires the secreted effector YopJ (Brodsky et al., 2010; Lilo et al., 2008), we instead infected *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Casp1*$^{-/-}$, and *Casp11*$^{-/-}$ BMDMs with a strain of *Y. pseudotuberculosis*

that expresses a T3SS but lacks the six known secreted effectors (Δ6 Yp). Similarly to *L. pneumophila* infection, both IL-1α and IL-1β release in response to Δ6 Yp are caspase-11-dependent (Figure 3-17A). Again, caspase-1 is absolutely required for IL-1β secretion, whereas IL-1α is released independently of caspase-1. Secretion of IL-12, an inflammasome-independent cytokine, is unaffected (Figure 3-18). Cell death in response to Δ6 Yp is both caspase-1 and caspase-11-dependent, with a more dramatic reduction in death in *Casp11*$^{-/-}$ BMDMs (Figure 3-17B). Furthermore, *Y. pseudotuberculosis*-induced release of both IL-1α and IL-1β requires the presence of a functional T3SS, as *Y. pseudotuberculosis* unable to form a functional T3SS pore in the host cell plasma membrane (Δ*yopB* Yp) do not induce secretion of either cytokine. These data indicate a general role for caspase-11 in the induction of rapid cell death and robust release of IL-1α and IL-1β in response to bacterial secretion systems that are capable of accessing the host cell cytosol, but may be independent of the activities of specific bacterial effector proteins.

IL-1α and IL-1β control bacterial burden and neutrophil recruitment *in vivo*

As caspase-11 contributes to flagellin-independent IL-1α and IL-1β release from infected macrophages *in vitro* and IL-1α and IL-1β secretion is flagellin-independent *in vivo*, we wanted to determine the contribution of IL-1α and IL-1β to host defense against *L. pneumophila in vivo*. IL-1α and IL-1β both bind the IL-1R, which signals through the MyD88 adaptor protein (Adachi et al., 1998; Burns et al., 1998; Dower et al., 1986). As MyD88 is critical for control of *L. pneumophila* replication during *in vivo* infection but deletion of an individual MyD88-dependent TLR or a combination of TLRs does not recapitulate MyD88 deficiency, it is likely that other MyD88-dependent receptors,

including the IL-1R, may play a role (Archer and Roy, 2006; Archer et al., 2009; Hawn et al., 2007; 2006; Spörri et al., 2006). IL-1R signaling contributes to chemokine production by non-hematopoietic cells during infection with wild-type, flagellin-expressing *L. pneumophila* (LeibundGut-Landmann et al., 2011). However, the role of IL-1R signaling during infection with flagellin-deficient *L. pneumophila,* which do not activate the NAIP5/NLRC4 inflammasome, has not been investigated. We therefore infected B6 and IL-1R-deficient (*Il1r1*$^{-/-}$) mice intranasally with Δ*flaA* Lp and measured bacterial burden in the lung over the course of seven days. Though both B6 and *Il1r1*$^{-/-}$ mice received similar initial bacterial burdens, *Il1r1*$^{-/-}$ mice show a defect in bacterial clearance as early as 24 hours post-infection (Figure 3-19A). Bacterial burden remains elevated in the absence of IL-1R signaling, with the *Il1r1*$^{-/-}$ mice still exhibiting a log-increase in bacterial load at 120 hours post-infection. As IL-1R signaling is important for neutrophil recruitment (Miller et al., 2006), we examined whether *Il1r1*$^{-/-}$ mice have a defect in neutrophil recruitment to the pulmonary airway during *L. pneumophila* infection. Indeed, *Il1r1*$^{-/-}$ mice exhibit a significant decrease in neutrophil recruitment to the airway 24 hours post-infection, possibly contributing to their inability to efficiently clear the pathogen (Figure 3-19B and 3-19C).

The IL-1R signals in response to both IL-1α and IL-1β; however, these cytokines can play non-redundant roles in anti-bacterial defense (Mayer-Barber et al., 2011). To determine the relative contributions of IL-1α and IL-1β to neutrophil recruitment and bacterial clearance during *L. pneumophila* infection, we utilized neutralizing antibodies to selectively block either IL-1α or IL-1β prior to infection. Specific cytokine neutralization in the BALF could be observed 24 hours post-infection (Figure 3-20). Critically, IL-1α

neutralization alone significantly diminishes the percentage of neutrophils recruited to the BALF at 24 hours post-infection and results in a half-log increase in bacterial CFUs, in marked contrast to isotype control antibody or neutralization of IL-1β, which on its own did not have a significant effect (Figure 3-19D, E, and F). However, neutralization of both IL-1α and IL-1β fully recapitulates the magnitude of neutrophil reduction and defect in bacterial clearance observed in the *Il1r1*$^{-/-}$ mice. Collectively, these data indicate that although there are some overlapping roles for these cytokines during *L. pneumophila* infection, IL-1α plays a distinct role from IL-1β in driving neutrophil recruitment to the airway and mediating bacterial clearance.

E. Discussion

Inflammasomes respond robustly to conserved features of pathogenic microbes, such as pore-forming toxins or specialized secretion systems that access the host cytosol. Inflammasomes therefore play a central role in enabling the immune system to discriminate between virulent and avirulent bacteria (Vance et al., 2009). Recent reports show a role for caspase-11 in regulating the activation of a non-canonical inflammasome that promotes cell death as well as IL-1α and IL-1β secretion. This non-canonical inflammasome responds to both pathogenic and non-pathogenic Gram-negative bacteria independently of specialized secretion systems that translocate bacterial molecules into the host cytosol (Broz et al., 2012; Gurung et al., 2012; Kayagaki et al., 2011; Rathinam et al., 2012b). This pathway involves the TRIF- and IFNAR-dependent upregulation and activation of caspase-11 and occurs with relatively delayed kinetics in comparison to the response to pathogenic bacteria. Intriguingly, we find that the activity of the *L. pneumophila* Dot/Icm T4SS leads to rapid and robust caspase-11 activation

independently of the TRIF-IFNAR axis, and this activation triggers rapid cell death and release of both IL-1α and IL-1β (Figure 3-21). We extend these results to show that the evolutionarily distinct T3SS of another pathogen, *Y. pseudotuberculosis,* also rapidly triggers caspase-11-dependent responses. Collectively, our findings demonstrate that caspase-11 is critical for inflammasome activation in response to the secretion systems of virulent bacteria that enable bacterial molecules to access the host cell cytosol and demonstrate that IL-1α and IL-1β together play a crucial protective role during acute infection *in vivo*.

We demonstrate that in response to the activity of bacterial secretion systems that enable cytosolic access, caspase-11 contributes to NLRP3-mediated inflammasome activation and caspase-1-dependent IL-1β secretion and to a second ASC and NLRC4-independent pathway that does not require caspase-1 and leads to cell death as well as robust IL-1α release. These *L. pneumophila*-induced pathways are similar to recent findings with a number of Gram-negative bacterial pathogens, including *C. rodentium*, *E. coli*, and *S.* Typhimurium (Broz et al., 2012; Gurung et al., 2012; Kayagaki et al., 2011; Rathinam et al., 2012b). However, we observe rapid and robust T4SS-dependent activation of these two caspase-11-mediated pathways by *L. pneumophila*, whereas the response to Gram-negative bacteria lacking specialized secretion systems occurs less robustly and with much slower kinetics. Intriguingly, we observe a similarly rapid caspase-11-dependent induction of cell death and IL-1 release in response to the structurally and evolutionarily unrelated T3SS of *Y. pseudotuberculosis*. Importantly, this pathway is independent of host sensing of flagellin, as it is triggered by flagellin-deficient *L. pneumophila*, and *Y. pseudotuberculosis* downregulates flagellin expression when the

T3SS is expressed (Minnich and Rohde, 2007). Thus, our data suggest that the caspase-11 inflammasome is poised to respond robustly and rapidly to the activity of bacterial secretion systems that are capable of delivering microbial products into the host cell cytosol and may enable the host to respond to pathogens that evade flagellin-dependent responses. This could have significance for understanding the role of caspase-11 activation at mucosal sites colonized by large numbers of commensal bacteria. At mucosal barriers, it would be expected that the non-canonical inflammasome pathway would not be robustly activated by commensal bacteria but could respond rapidly to the presence of bacterial secretion systems that enable pathogen access to the host cytosol.

Our findings are consistent with recent observations that the *L. pneumophila* Dot/Icm T4SS triggers the caspase-11-dependent non-canonical inflammasome (Case et al., 2013), as well as the finding that bacteria that enter the cytosol either due to failure to maintain integrity of their replicative vacuoles or natural entry into the cytoplasm also trigger rapid caspase-11 activation (Aachoui et al., 2013). Thus, the pathway that leads to caspase-11 activation appears to be particularly sensitive to pathogens that 'violate the sanctity of the cytosol' (Lamkanfi and Dixit, 2009), either through the activity of specialized secretion systems that translocate bacterial molecules into the cytosol or through their direct entry into the host cell cytosol. Whether other pathogens that replicate within the cytosol, such as *Listeria* or *Shigella*, or cytosolic viruses possess mechanisms to evade this pathway remains to be determined.

L. pneumophila T4SS-mediated activation of caspase-11 differs from the other pathways of non-canonical inflammasome activation in several ways. First, *L. pneumophila*-mediated activation of caspase-11 does not require TRIF or IFNAR signaling. We observe a moderate dependence on TRIF and IFNAR signaling when macrophages are primed with LPS prior to infection, consistent with LPS-dependent upregulation of caspase-11 expression through the TLR4-TRIF-IFNAR axis (Broz et al., 2012; Gurung et al., 2012; Rathinam et al., 2012b). However, in the absence of LPS priming, TRIF and IFNAR signaling are dispensable for *L. pneumophila*-dependent caspase-11 activation. In this context, it is likely that MyD88 compensates for the absence of TRIF, as cells deficient for both MyD88 and TRIF failed to activate caspase-11 in response to *L. pneumophila*. Thus, although the TLR4-TRIF-IFNAR axis is required for caspase-11 activation in response to Gram-negative bacteria, a MyD88-dependent signal is sufficient for caspase-11 activation in response to pathogens that utilize virulence-associated secretion systems to translocate bacterial molecules into the host cytosol. It is possible that different signals are capable of activating caspase-11 through distinct pathways, but these pathways occur with distinct kinetics because they may indicate distinct levels of pathogenicity. Thus, while caspase-11 is robustly upregulated by LPS priming, this upregulation alone is insufficient for rapid activation in response to bacteria that lack specialized secretion systems, as Δ*dotA* or Δ*yopB* bacteria do not induce rapid cell death, even in primed cells. Collectively, these data indicate a two-signal model for rapid caspase-11 activation during infection with virulent bacteria, where bacterial PAMPs induce caspase-11 upregulation, but rapid caspase-11 activation requires a second, secretion system-dependent signal (Figure 3-21).

The specific secretion system-dependent signals responsible for caspase-11 activation are currently unknown. While rapid activation of caspase-11 requires the presence of a functional type III or type IV secretion system or cytosolic access of the bacteria, whether the signal is an as-yet-undefined translocated bacterial molecule or a cellular response to the pore forming activity of these systems remains to be determined. The delayed NLRP3- and caspase-11-dependent response to Gram-negative bacteria suggests that in addition to LPS-induced upregulation of inflammasome components, bacterial mRNA provides an additional signal for activating the NLRP3 inflammasome (Kanneganti et al., 2006; Sander et al., 2011), although the role of caspase-11 in this response has not been formally demonstrated. Activity of the type III or IV secretion systems may bypass the need for bacterial mRNA. Alternatively, these secretion systems may translocate bacterial RNA (Auerbuch et al., 2009; Monroe et al., 2009; Vance et al., 2009), and the rapid caspase-11-dependent response they induce could be due to more rapid delivery of bacterial mRNA into the host cell cytosol.

Furthermore, the host factors required for activation of the NLRP3-independent caspase-11-dependent inflammasome also remain to be identified. As this pathway is independent of flagellin sensing, NLRP3, ASC, and NLRC4, an unknown upstream sensor and/or adaptor may be involved in caspase-11 activation in response to a translocated bacterial substrate or an endogenous signal induced by infection. This sensor may also be upregulated by type I IFN signaling itself (Broz et al., 2012; Gurung et al., 2012; Rathinam et al., 2012b).

Our data show that IL-1α release during *L. pneumophila* infection is controlled by two independent pathways, one involving the flagellin-dependent NAIP5/NLRC4 and caspase-1-dependent inflammasome and a second pathway involving the NLRP3-independent caspase-11-dependent inflammasome (Figure 3-21). Though we demonstrate that IL-1α release has an important biological consequence *in vivo* for neutrophil recruitment and bacterial clearance, it is unclear if IL-1α release is regulated by unconventional secretion, as is the case for IL-1β (Keller et al., 2008). As both pathways that control IL-1α release also lead to cell death, our data are consistent with a model in which IL-1α is an endogenous alarmin that is released during cell death (Bianchi, 2007).

Interestingly, caspase-11 also contributes to control of flagellin-expressing *L. pneumophila* by serving as a component of an NLRC4-dependent inflammasome that promotes trafficking of the *L. pneumophila*-containing vacuole to lysosomes (Akhter et al., 2012). Thus, caspase-11 may function in multiple ways to control *L. pneumophila* infection. Importantly, we find that IL-1α, IL-1β, and IL-1R signaling play an important role in the control of *L. pneumophila* infection through efficient neutrophil recruitment to the airway. IL-1α and IL-1β play both distinct and overlapping roles in mediating neutrophil recruitment and controlling bacterial replication, as depletion of IL-1α alone showed a more pronounced defect in neutrophil recruitment and bacterial clearance than depletion of IL-1β alone, but loss of both cytokines resulted in a further reduction of neutrophil recruitment and an increased defect in bacterial clearance. Further analysis is required to define the relative contributions of the various caspase-11-mediated effector functions to the control of *L. pneumophila* replication *in vivo*.

In conclusion, these studies demonstrate that T3SS and T4SS activities trigger rapid and robust activation of caspase-11. This activation contributes to maximal NLRP3-dependent IL-1β secretion as well as to NLRP3-independent IL-1α release and host cell death. The downstream effector functions of these pathways are important for host defense against *L. pneumophila in vivo,* as IL-1α and IL-1β promote neutrophil recruitment to *L. pneumophila*-infected lungs and control pulmonary bacterial replication. Our results highlight the contribution of caspase-11 to rapid inflammasome activation and discrimination between pathogenic and nonpathogenic bacteria.

F. Acknowledgements

We are grateful to Junying Yuan (Harvard University) for permission to utilize *Casp11*$^{-/-}$ mice, Tiffany Horng (Harvard University School of Public Health) for providing *Casp11*$^{-/-}$ bone marrow, and Susan Weiss (University of Pennsylvania Perelman School of Medicine) for providing *Ifnar*$^{-/-}$ bone marrow. We also thank the Brodsky lab for valuable feedback on experimental design. Alan M. Copenhaver was instrumental in the design and execution of all *in vivo* experiments and was critical for performing flow cytometry. Erin E. Zwack performed the experiments involving *Y. pseudotuberculosis*. Hieu T. Nguyen aided in all *in vivo* experiments and performed ELISA. Till Strowig and Richard Flavell provided critical reagents. Bahar Javdan assisted in experiments involving YVAD. William P. Bradley performed immunoblot analysis. Thomas C. Fung aided in all *in vivo* experiments. Igor E. Brodsky and Sunny Shin assisted in the design of experiments, interpretation of data, and writing of the manuscript.

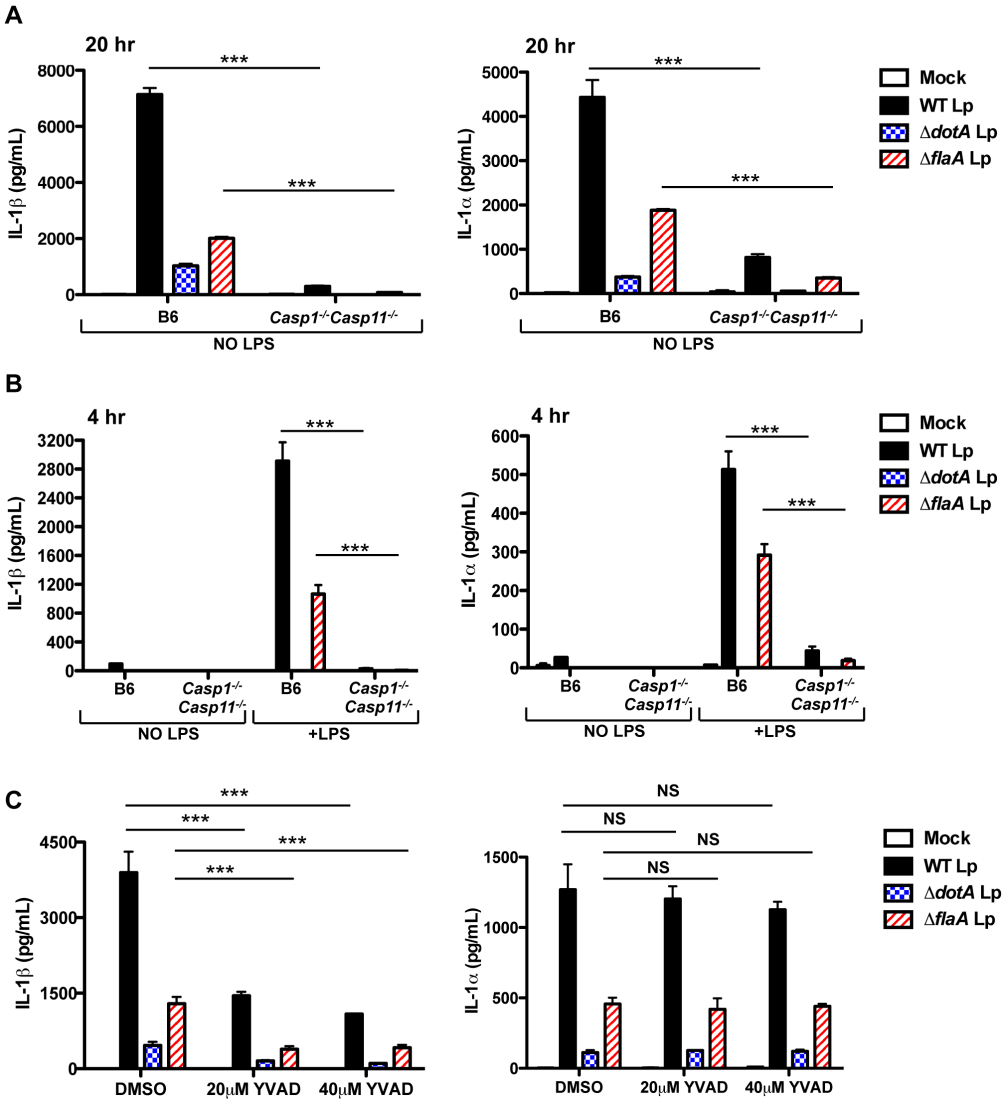

Figure 3-1: LPS priming induces rapid IL-1α and IL-1β secretion in response to *L. pneumophila* T4SS activity. (A) Unprimed B6 or *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs were infected with WT *L. pneumophila* (WT Lp), Δ*dot* Lp, Δ*flaA* Lp, or PBS (mock infection) for 20 hours. (B) B6 or *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs were either unprimed or primed with 0.5μg/mL LPS for 2.5 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS for 4 hours. (C) B6 BMDMs were pretreated with either 20μM or 40μM of the caspase-1 inhibitor YVAD-cmk or DMSO vehicle control for 0.5 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS for 20 hours. Levels of IL-1α and IL-1β in the supernatants were measured by ELISA. Graphs show the mean ± SEM of triplicate wells. Data are representative of three or four independent experiments. *** is $p<0.001$ by 2-way ANOVA with Bonferroni post-test. NS is not significant.

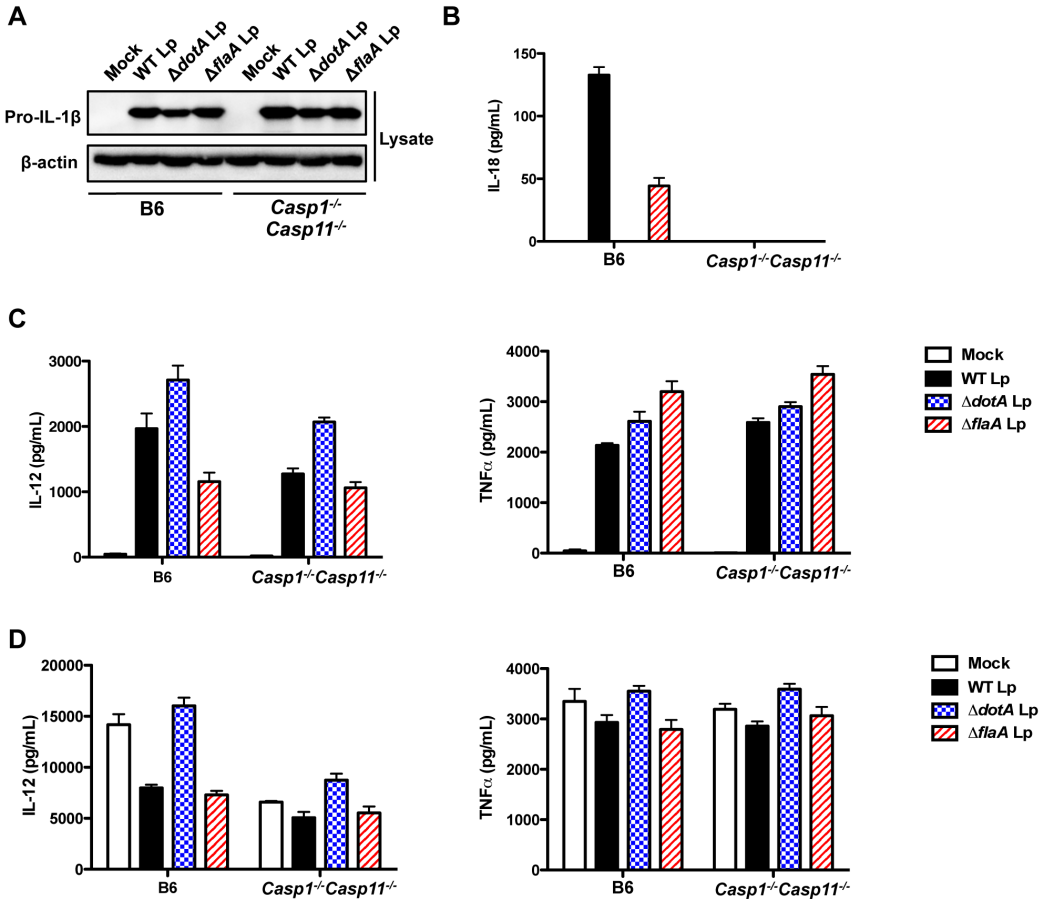

Figure 3-2. Caspase-1/caspase-11-deficient cells do not have a gross defect in cytokine secretion. (A) Unprimed B6 or *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs were infected with WT *L. pneumophila* (Lp), Δ*dotA* Lp, Δ*flaA* Lp, or PBS (mock infection) for 20 hours. Levels of full-length IL-1β (pro-IL-1β) and β-actin (loading control) in the cell lysates were determined by immunoblot analysis. (B) B6 or *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS for 4 hours. The level of IL-18 in the supernatants was measured by ELISA. (C) Unprimed B6 or *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs were infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS for 20 hours. Levels of IL-12 p40 and TNF-α in the supernatants were measured by ELISA. (D) B6 or *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS for 4 hours. Levels of IL-12 p40 and TNF-α in the supernatants were measured by ELISA. Graphs show the mean ± SEM of triplicate wells. Data are representative of two (B) or three (A, C, and D) independent experiments.

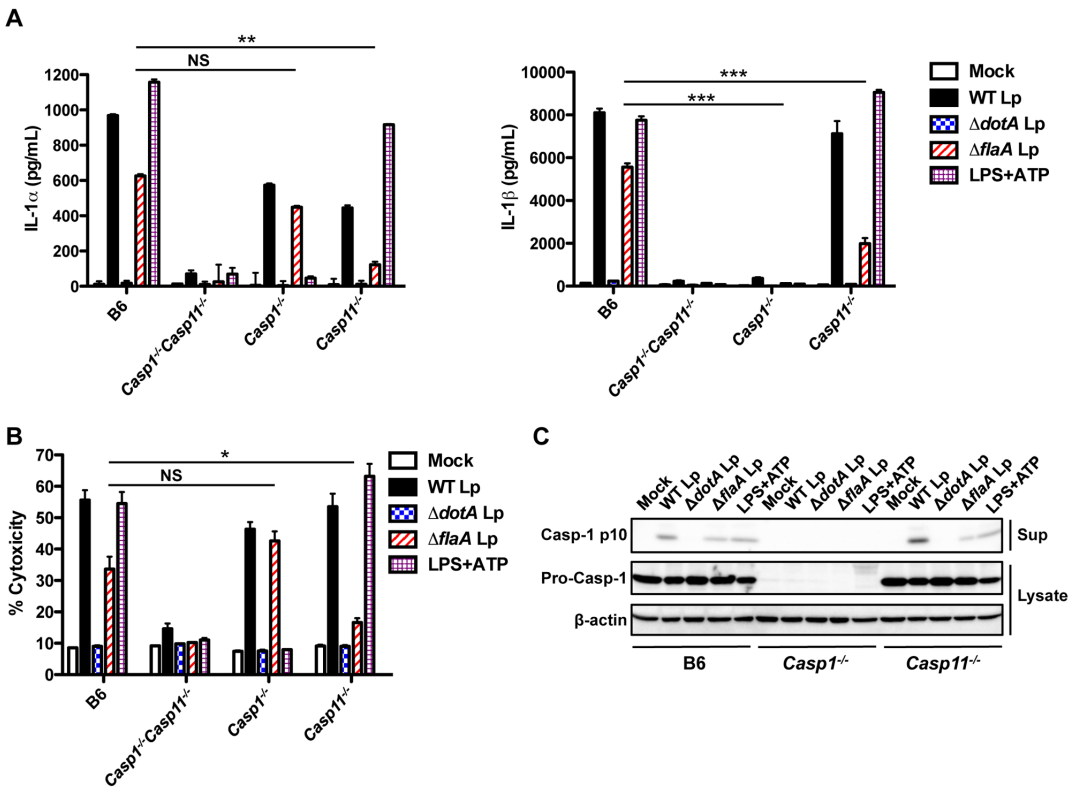

Figure 3-3. Caspase-11 controls the release of IL-1α and IL-1β and pyroptosis in response to flagellin-deficient *L. pneumophila.* B6, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Casp1*$^{-/-}$, or *Casp11*$^{-/-}$ BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT *L. pneumophila* (WT Lp), Δ*dotA* Lp, Δ*flaA* Lp, or PBS (mock infection) or treated with 2.5mm ATP for 1 (C) or 4 (A,B) hours. (A) Levels of IL-1α and IL-1β in the supernatants were measured by ELISA. Graphs show the mean ± SEM of triplicate wells. (B) Cell death (% cytotoxicity) was measured by LDH release into the supernatants relative to Triton X-100-lysed cells. Graphs show the mean ± SEM of triplicate wells. (C) Levels of processed caspase-1 (casp-1 p10) in the supernatants and full-length caspase-1 (pro-casp-1) and β-actin in the cell lysates were determined by immunoblot analysis. Data are representative of three independent experiments. *** is $p<0.001$ by two-way ANOVA with Bonferroni post-test, ** is $p<0.01$ by two-way ANOVA with Bonferroni post-test, and * is $p<0.05$ by unpaired t-test. NS is not significant.

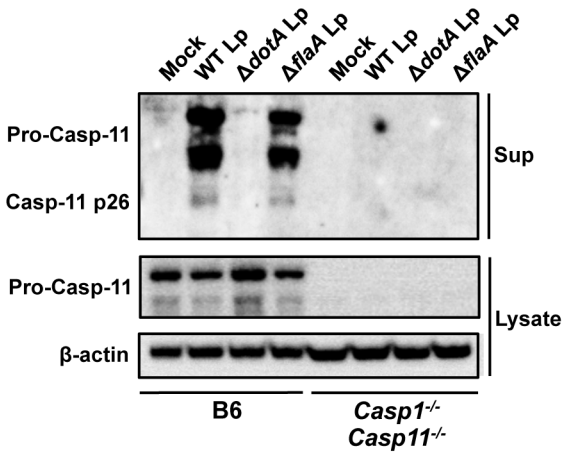

Figure 3-4. Caspase-11 is rapidly upregulated and secreted in response to *L. pneumophila*. B6 or *Casp1*$^{-/-}$*Casp11*$^{-/-}$ BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT *L. pneumophila* (Lp), Δ*dotA* Lp, Δ*flaA* Lp, or PBS (mock infection) for 4 hours. Levels of full-length caspase-11 (pro-casp-11) and active caspase-11 (casp11 p26) in the supernatants, and pro-casp-11 and β-actin (loading control) in the cell lysates were determined by immunoblot analysis.

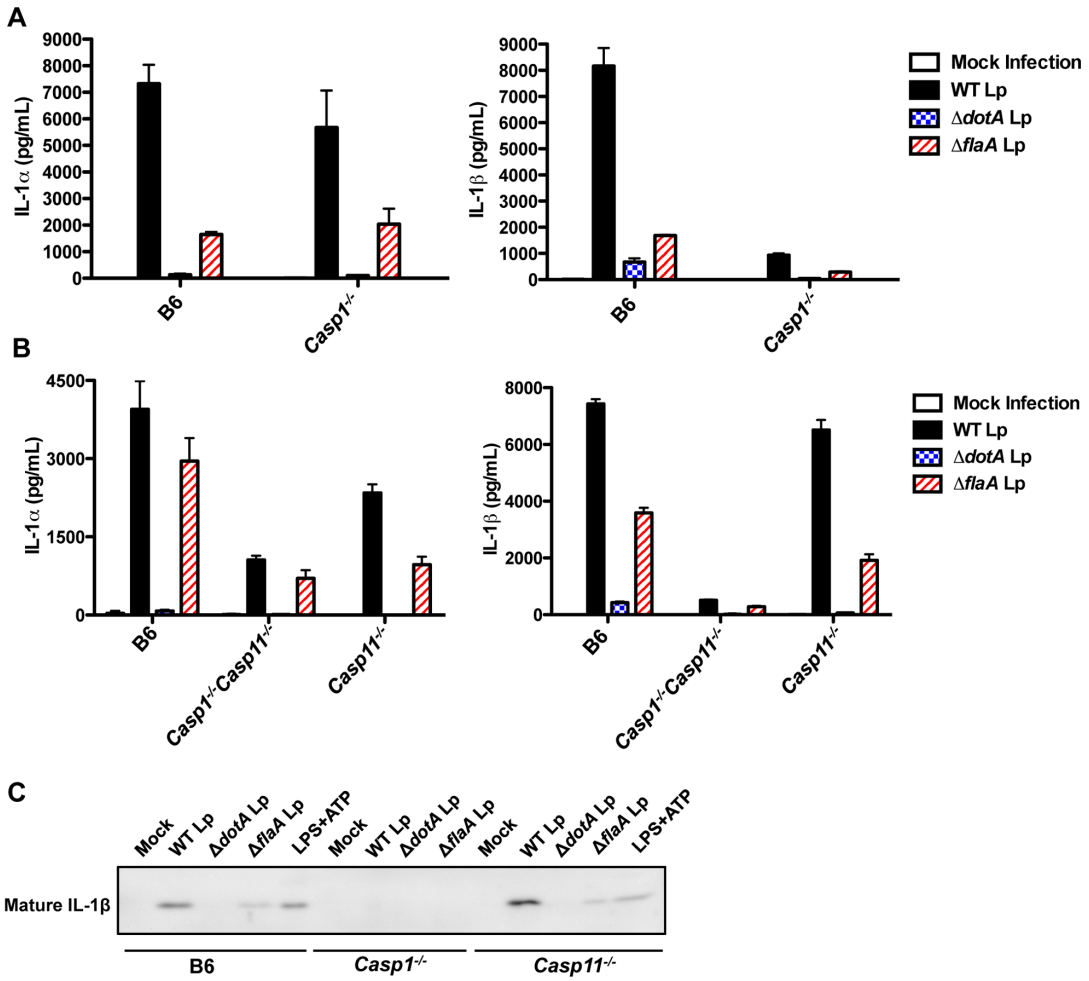

Figure 3-5. Caspase-11 is activated in response to *L. pneumophila* independently of macrophage priming. (A) Unprimed B6 and *Casp1*$^{-/-}$ BMDMs or (B) B6, *Casp1*$^{-/-}$ *Casp11*$^{-/-}$, and *Casp11*$^{-/-}$ BMDMs were infected with WT *L. pneumophila* (Lp), Δ*dotA* Lp, Δ*flaA* Lp, or PBS (mock infection) for 20 hours. Levels of IL-1α and IL-1β in the supernatants were measured by ELISA. Graphs show the mean ± SEM of triplicate wells. (C) B6, *Casp1*$^{-/-}$, or *Casp11*$^{-/-}$ BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS for 4 hours or treated with LPS+2.5mm ATP for 1 hour. Levels of mature IL-1β in the supernatant were determined by immunoblot analysis. Data are representative of two independent experiments.

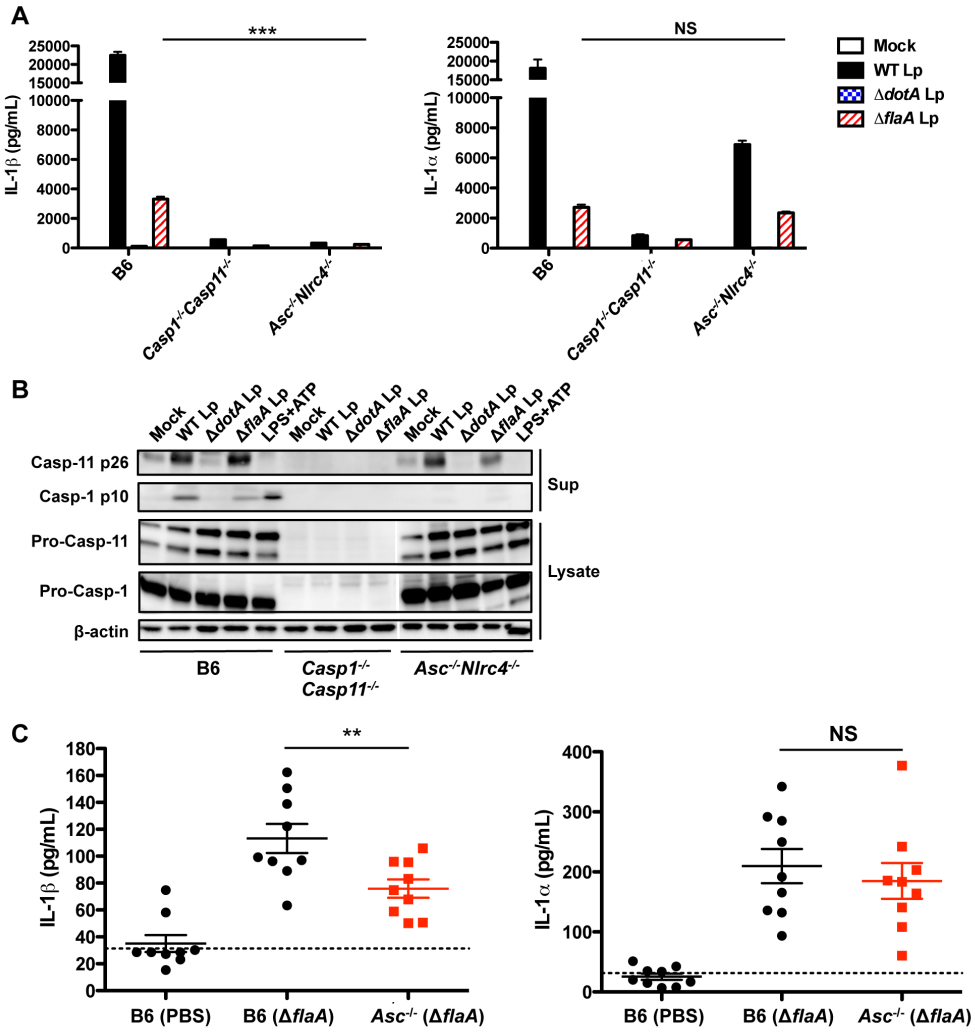

Figure 3-6. Caspase-11 activation is independent of ASC and NLRC4. (A) Unprimed B6, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, or *Asc*$^{-/-}$*Nlrc4*$^{-/-}$ BMDMs were infected with WT *L. pneumophila* (WT Lp), Δ*dotA* Lp, Δ*flaA* Lp, or PBS (mock infection) for 20 hours, and levels of IL-1α and IL-1β in the supernatants were measured by ELISA. Graphs show the mean ± SEM of triplicate wells. (B) Unprimed B6, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, or *Asc*$^{-/-}$*Nlrc4*$^{-/-}$ BMDMs were infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS (mock infection) for 20 hours or treated with LPS+ATP for 1 hour. Levels of processed caspase-1 (casp-1 p10) and caspase-11 (casp-11 p26) in the supernatants, and pro-caspase-1, pro-caspase-11, and β-actin (loading control) in the cell lysates were determined by immunoblot analysis. (C) 8-12 week old B6 and *Asc*$^{-/-}$ mice were infected intranasally with either 1x10^6 Δ*flaA* Lp or PBS. Bronchoalveolar lavage fluid (BALF) was collected 24 hours post-infection, and levels of IL-1α and IL-1β were measured by ELISA. Graphs show the mean ± SEM of 9 mice per group. Dashed line represents the limit of detection. Data are representative of three independent experiments (A,B) or are displayed as the pooled results of two independent experiments (C). *** is $p<0.001$ by two-way ANOVA with Bonferroni post-test. ** is $p<0.01$ by unpaired t-test. NS is not significant.

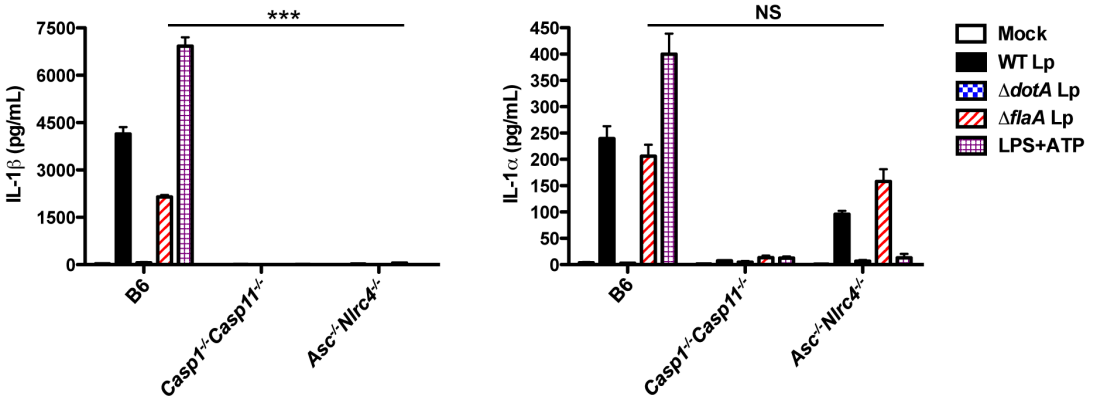

Figure 3-7. IL-1α release is ASC/NLRC4-independent. B6, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, or *Asc*$^{-/-}$*Nlrc4*$^{-/-}$ BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT *L. pneumophila* (Lp), Δ*dotA* Lp, Δ*flaA* Lp, or PBS (mock infection) or treated with 2.5mm ATP for 4 hours. Levels of IL-1α and IL-1β in the supernatants were measured by ELISA. Graphs show the mean ± SEM of triplicate wells. Data are representative of three independent experiments.

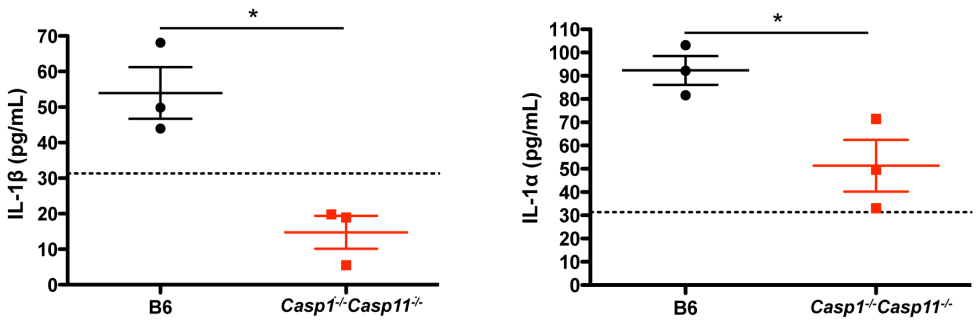

Figure 3-8. Both IL-1α and IL-1β secretion are caspase-1/caspase-11-dependent *in vivo.* 8-12 week old B6 or *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice were infected intranasally with 1x10^{6} Δ*flaA* Lp. BALF was collected 24 hours post-infection, and levels of IL-1α and IL-1β were measured by ELISA. Graphs show the mean ± SEM of three mice per group. Dashed line represents the limit of detection. * is $p<0.05$ by unpaired t-test.

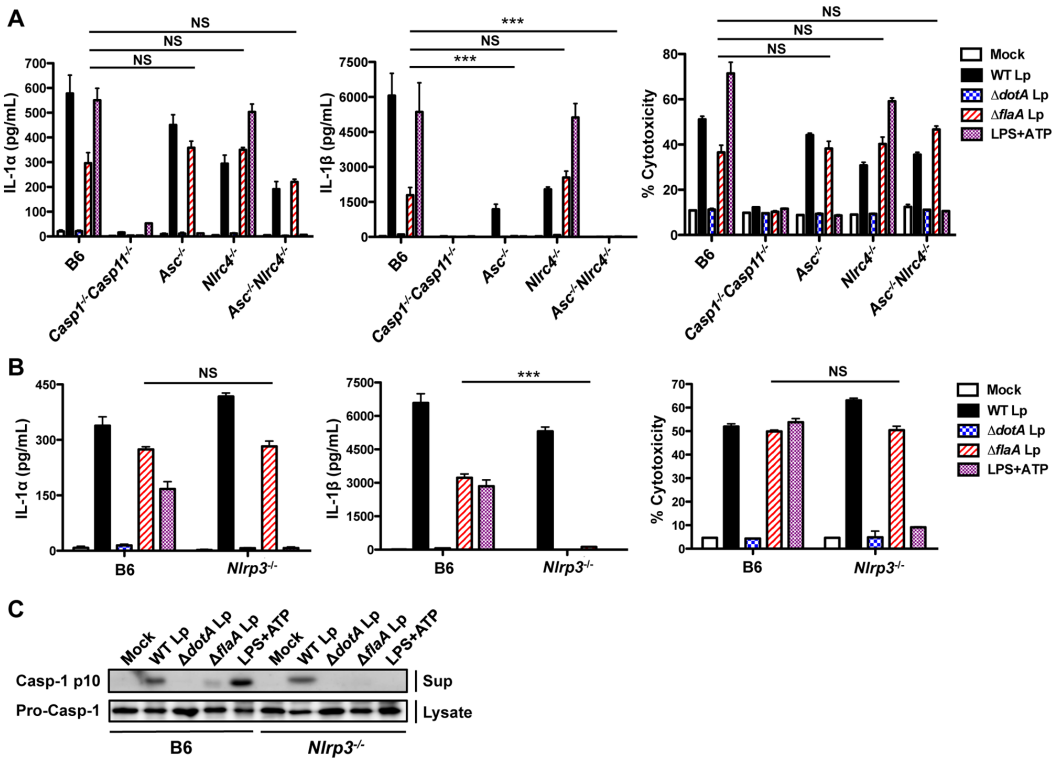

Figure 3-9. Caspase-11 mediates both NLRP3-dependent and NLRP3-independent immune responses. (A) B6, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Asc*$^{-/-}$, *Nlrc4*$^{-/-}$, or *Asc*$^{-/-}$*Nlrc4*$^{-/-}$ BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT *L. pneumophila* (WT Lp), Δ*dotA* Lp, Δ*flaA* Lp, or PBS (mock infection) or treated with 2.5mm ATP for 4 hours. Levels of IL-1α and IL-1β in the supernatants were measured by ELISA and cell death (% cytotoxicity) was measured by LDH release into the supernatants relative to Triton X-100-lysed cells. Graphs show the mean ± SEM of triplicate wells. (B and C) B6 or *Nlrp3*$^{-/-}$ BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS (mock infected) or treated with 2.5mm ATP for 1 hour (C) or 4 hours (B). (B) Levels of IL-1α and IL-1β in the supernatants were measured by ELISA and cell death (% cytotoxicity) was measured by LDH release into the supernatants relative to Triton X-100-lysed cells. Graphs show the mean ± SEM of triplicate wells. (C) Levels of processed caspase-1 (casp-1 p10) in the supernatants and pro-caspase-1 in the cell lysates were determined by immunoblot analysis. Data are representative of two (A,C) or three (B) independent experiments. *** is $p<0.001$ by one-way ANOVA with Tukey post-test. NS is not significant.

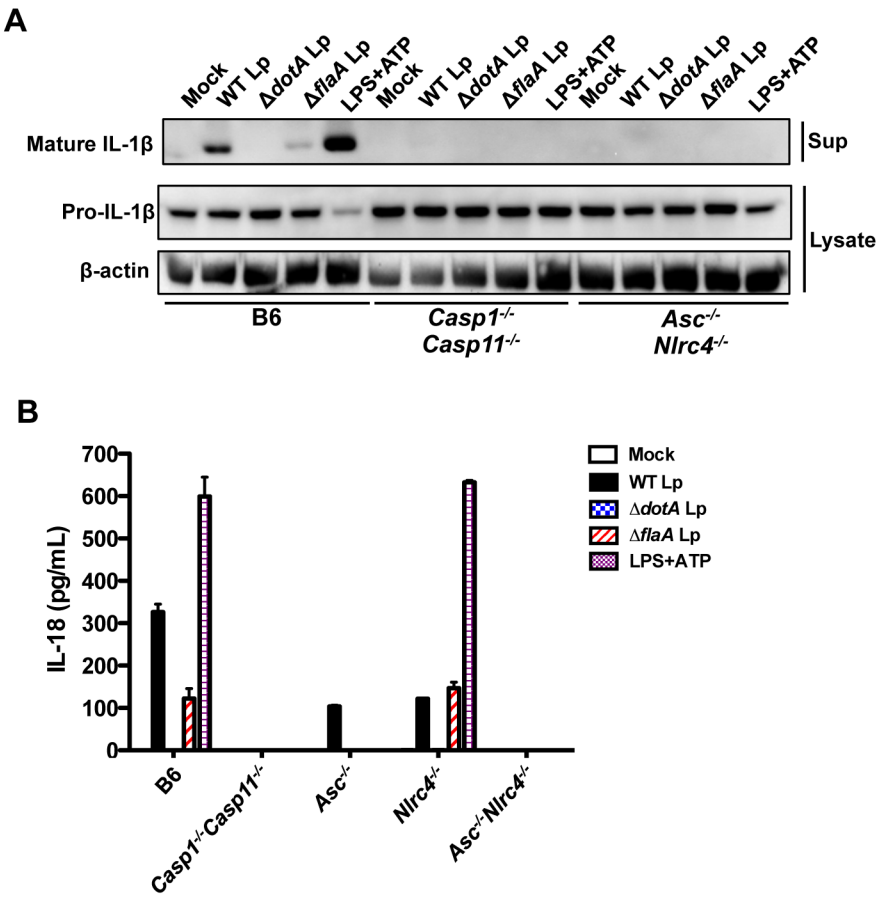

Figure 3-10. Mature IL-1β secretion is not always concordant with cell death. B6, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Asc*$^{-/-}$, *Nlrc4*$^{-/-}$, or *Asc*$^{-/-}$*Nlrc4*$^{-/-}$ BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT *L. pneumophila* (Lp), Δ*dotA* Lp, Δ*flaA* Lp or PBS (mock infection) for 4 hours or treated with 2.5mm ATP for 1 hour. (A) Levels of mature IL-1β in the supernatants, and full-length IL-1β (pro-IL-1β) and β-actin (loading control) in the cell lysates were determined by immunoblot analysis. Data are representative of two independent experiments. (B) The level of IL-18 in the supernatants was measured by ELISA. Graphs show the mean ± SEM of triplicate wells.

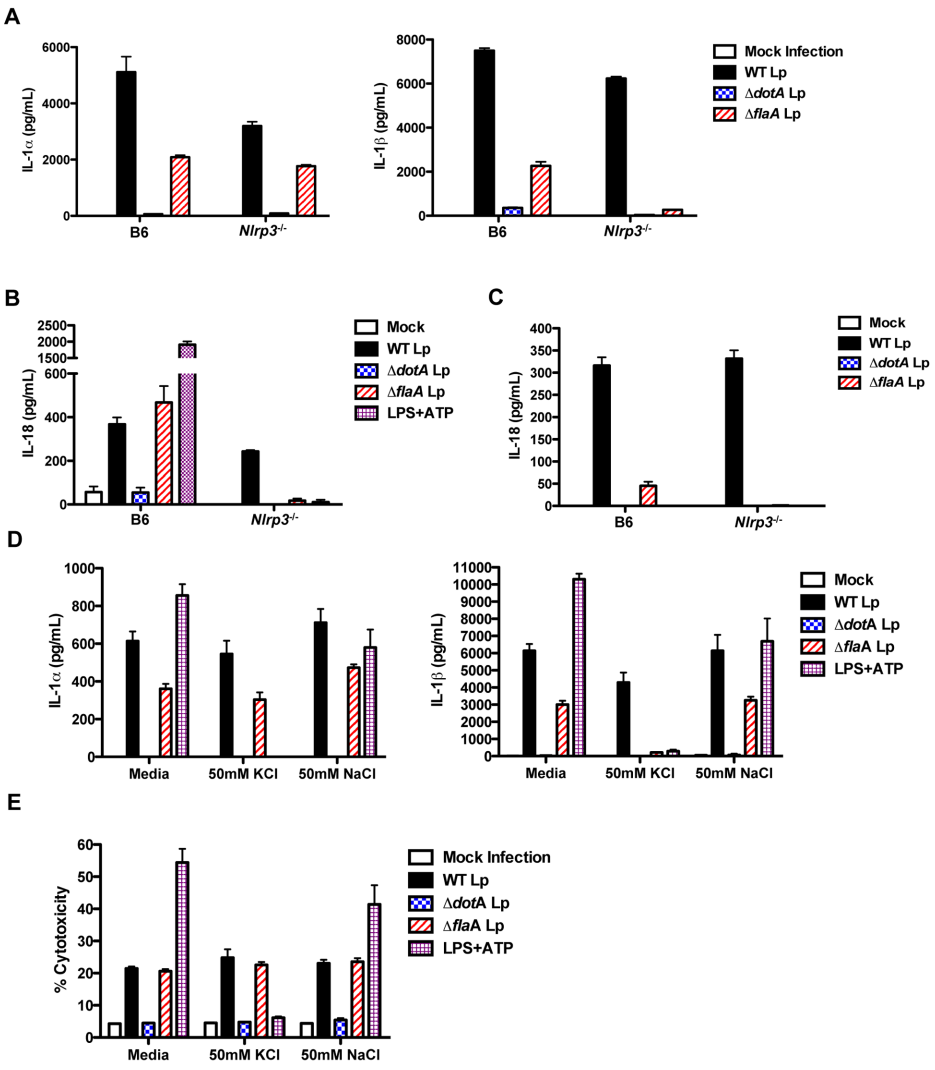

Figure 3-11. Flagellin-independent, NLRP3-dependent IL-1β secretion occurs independently of macrophage priming. (A) Unprimed B6 or *Nlrp3*$^{-/-}$ BMDMs were infected with WT *L. pneumophila* (Lp), Δ*dotA* Lp, Δ*flaA* Lp, or PBS (mock infection) for 16 hours. Levels of IL-1α and IL-1β in the supernatants were measured by ELISA. (B) B6 or *Nlrp3*$^{-/-}$ BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp or PBS (mock infection) or treated with 2.5mM ATP for 4 hours. The level of IL-18 in the supernatants was measured by ELISA. (C) B6 or *Nlrp3*$^{-/-}$ BMDMs were infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS (mock infection) for 16 hours. The level of IL-18 in the supernatants was measured by ELISA. (D and E) B6 BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp or PBS (mock infection) or treated with 2.5mM ATP for 4 hours. Where indicated, media alone, 50mM KCl, or 50mM NaCl were added prior to infection. (D) Levels of IL-1α and IL-1β in the supernatants were measured by ELISA. (E) Cell death was measured by LDH release. Graphs show the mean ± SEM of triplicate wells. Data are representative of two independent experiments (A-C).

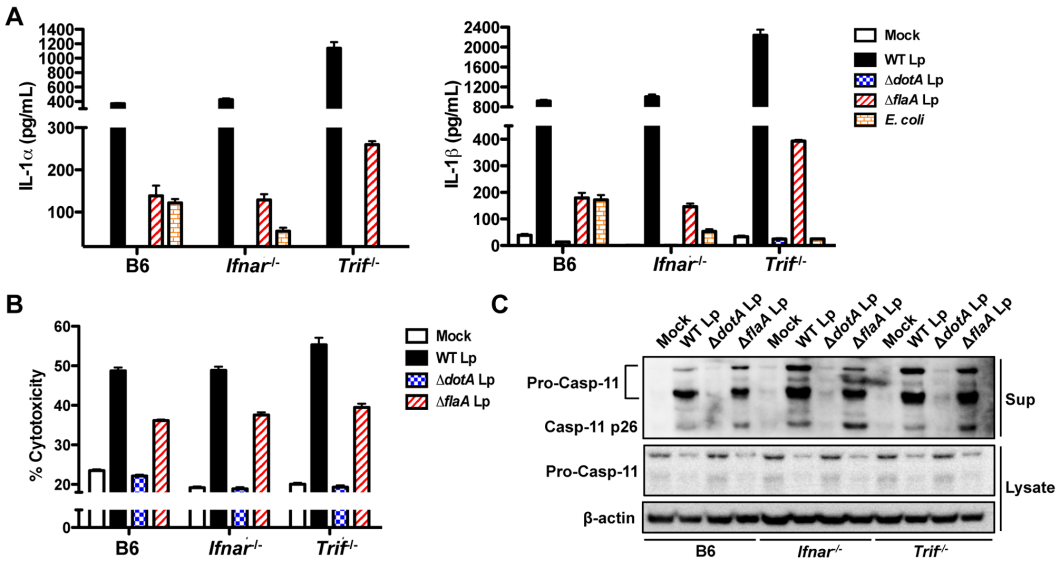

Figure 3-12. Non-canonical inflammasome responses to *L. pneumophila* occur independently of TRIF and IFNAR. (A) Unprimed B6, *Ifnar*$^{-/-}$, or *Trif*$^{-/-}$ BMDMs were infected with WT *L. pneumophila* (WT Lp), Δ*dotA* Lp, Δ*flaA* Lp, *E. coli*, or PBS (mock infection) for 16 hours. Levels of IL-1α and IL-1β in the supernatants were measured by ELISA. (B) Unprimed B6, *Ifnar*$^{-/-}$, or *Trif*$^{-/-}$ BMDMs were infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS (mock infection) for 16 hours. Cell death (% cytotoxicity) was measured by LDH release into the supernatants relative to Triton X-100-lysed cells. Graphs show the mean ± SEM of triplicate wells. (C) B6, *Ifnar*$^{-/-}$, or *Trif*$^{-/-}$ BMDMs were primed with 0.4μg/mL Pam3CSK4 for 4 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS for 16 hours. Levels of full-length caspase-11 (pro-casp-11) and processed caspase-11 (casp11 p26) in the supernatants and pro-casp-11 and β-actin (loading control) in the cell lysates were determined by immunoblot analysis. Data are representative of two independent experiments.

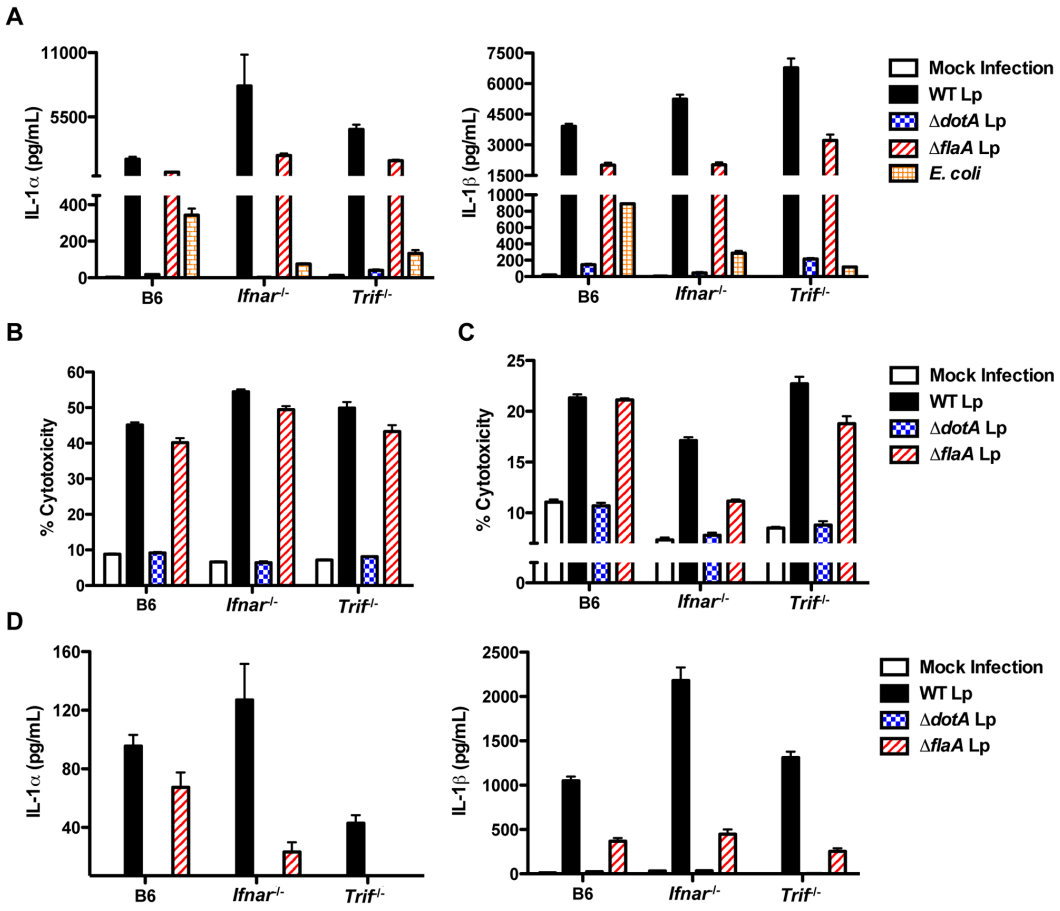

Figure 3-13. TRIF/IFNAR-independent IL-1 release occurs with Pam3CSK4-primed macrophages. (A) B6, *Ifnar*$^{-/-}$, or *Trif*$^{-/-}$ BMDMs were primed with 0.4μg/mL Pam3CSK4 for 4 hours and infected with WT *L. pneumophila* (Lp), Δ*dotA* Lp, Δ*flaA* Lp, *E. coli*, or PBS (mock infection) for 16 hours. The levels of IL-1α and IL-1β in the supernatants were measured by ELISA. (B) B6, *Ifnar*$^{-/-}$, or *Trif*$^{-/-}$ BMDMs were primed with 0.4μg/mL Pam3CSK4 for 4 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS for 16 hours. Cell death (% cytotoxicity) was measured by LDH release. (C) B6, *Ifnar*$^{-/-}$, or *Trif*$^{-/-}$ BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS for 4 hours. Cell death was measured by LDH release. (D) B6, *Ifnar*$^{-/-}$, or *Trif*$^{-/-}$ BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS for 4 hours. Levels of IL-1α and IL-1β in the supernatants were measured by ELISA. Graphs show the mean ± SEM of triplicate wells. Data are representative of two independent experiments.

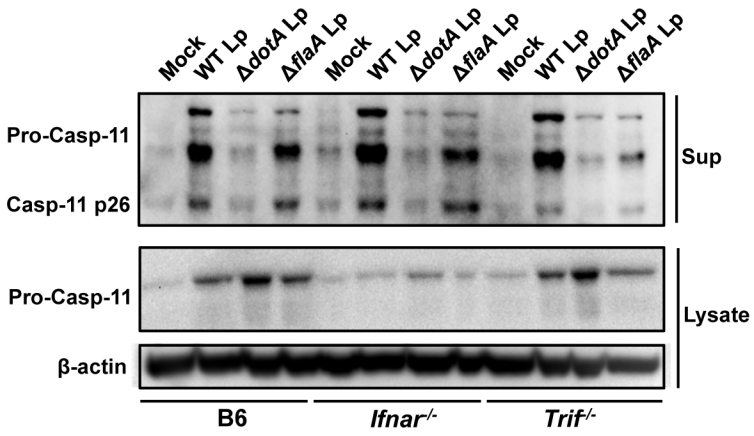

Figure 3-14. Caspase-11 is upregulated and secreted in an IFNAR- and TRIF-independent manner. Unprimed B6, *Ifnar*$^{-/-}$, or *Trif*$^{-/-}$ BMDMs were infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS for 16 hours. Levels of full-length caspase-11 (pro-casp-11) and active caspase-11 (casp11 p26) in the supernatants, and pro-casp-11 and β-actin (loading control) in the cell lysates were determined by immunoblot analysis.

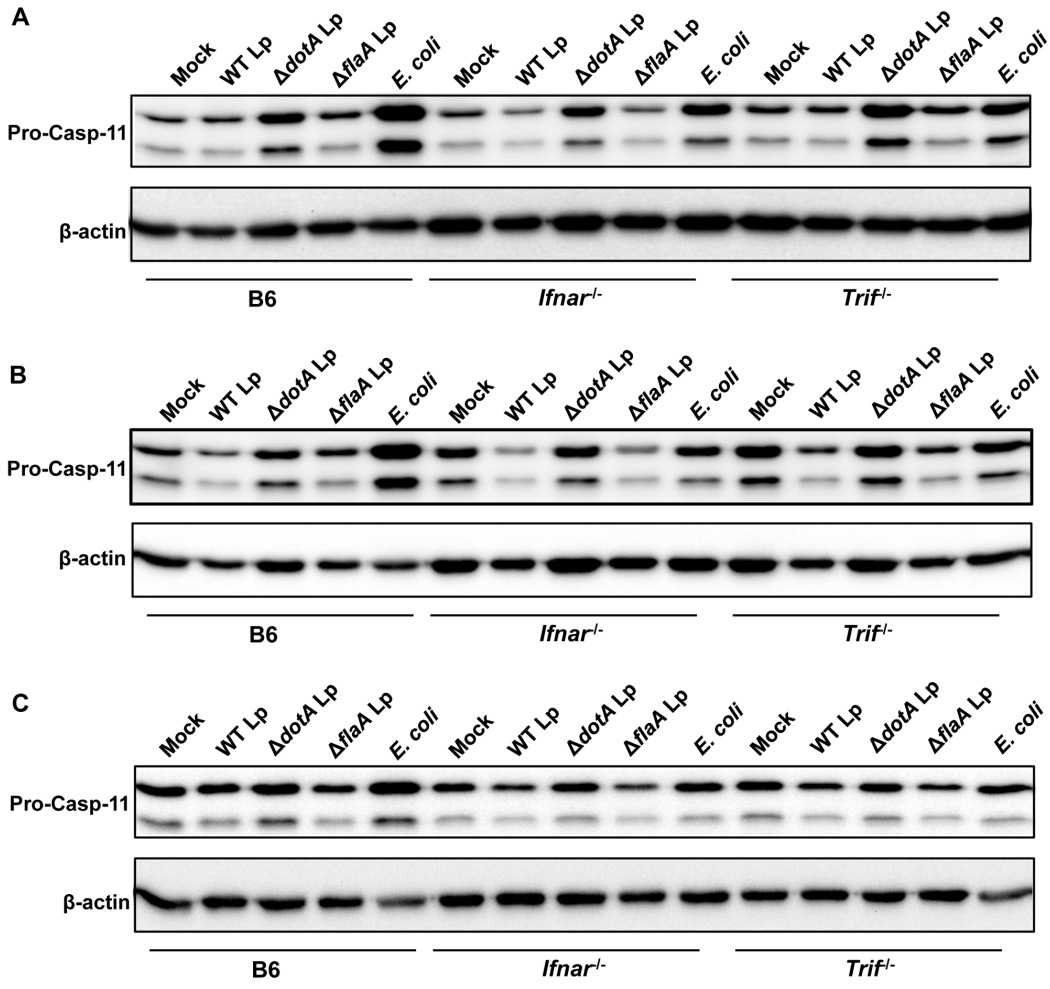

Figure 3-15. Detection of caspase-11 protein upregulation in cell lysates is moderate in response to *L. pneumophila.* (A) Unprimed B6, *Ifnar*$^{-/-}$, or *Trif*$^{-/-}$ BMDMs were infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, *E. coli*, or PBS (mock infection) for 16 hours. (B) B6, *Ifnar*$^{-/-}$, or *Trif*$^{-/-}$ BMDMs were primed with 0.4μg/mL Pam3CSK4 for 4 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, *E. coli*, or PBS for 16 hours. (C) B6, *Ifnar*$^{-/-}$, or *Trif*$^{-/-}$ BMDMs were primed with 0.5μg/mL LPS for 2.5 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, *E. coli*, or PBS for 4 hours. Levels of full-length caspase-11 (pro-casp-11) and β-actin (loading control) in the cell lysates were determined by immunoblot analysis.

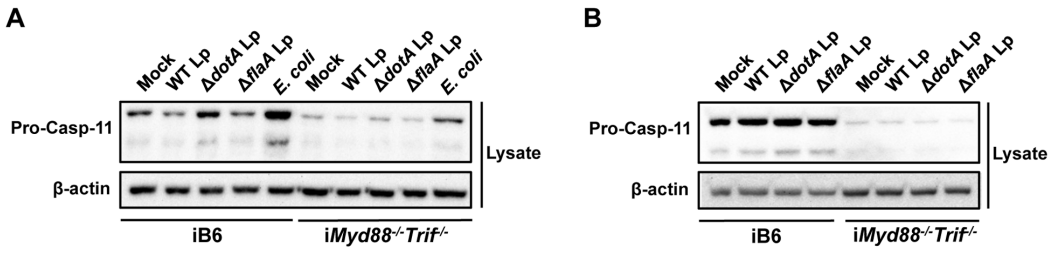

Figure 3-16. Caspase-11 is not upregulated in the absence of both MyD88 and TRIF. (A) Immortalized B6 (iB6) or MyD88/Trif-deficient (i*Myd88*$^{-/-}$*Trif*$^{-/-}$) BMDMs were primed with 0.4μg/mL Pam3CSK4 for 4 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, *E. coli*, or PBS (mock infection) for 16 hours. (B) iB6 or i*Myd88*$^{-/-}$*Trif*$^{-/-}$ macrophages were primed with 0.5μg/mL LPS for 4 hours and infected with WT Lp, Δ*dotA* Lp, Δ*flaA* Lp, or PBS (mock infection) for 4 hours. Levels of full-length caspase-11 (pro-casp-11) and β-actin (loading control) were determined by immunoblot analysis.

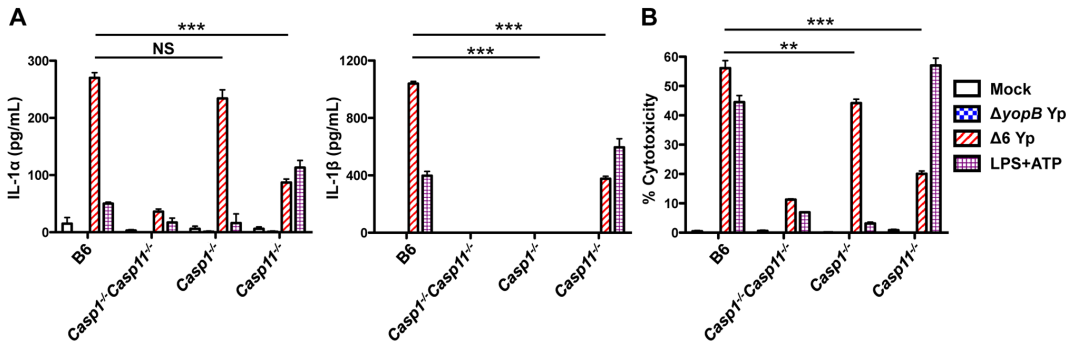

Figure 3-17. Caspase-11 mediates inflammasome activation in response to a functional *Yersinia* type III secretion system. BMDMs from B6, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Casp1*$^{-/-}$, or *Casp11*$^{-/-}$ mice were primed with 0.05μg/mL LPS for 4 hours and infected with type III secretion system-deficient *Y. pseudotuberculosis* (Δ*yopB* Yp), effectorless *Y. pseudotuberculosis* ΔHOJMEK (Δ6 Yp), or PBS (mock infection) or treated with 2.5mm ATP for 4 hours. (A) Levels of IL-1α and IL-1β in the supernatants were measured by ELISA. (B) Cell death (% cytotoxicity) was measured by lactate dehydrogenase (LDH) release relative to Triton X-100-lysed cells. Graphs show the mean ± SEM of triplicate wells. Data are representative of two independent experiments. *** is $p<0.001$ and ** is $p<0.01$ by two-way ANOVA with Bonferroni post-test. NS is not significant. This experiment was performed by Erin E. Zwack in Igor Brodsky's laboratory.

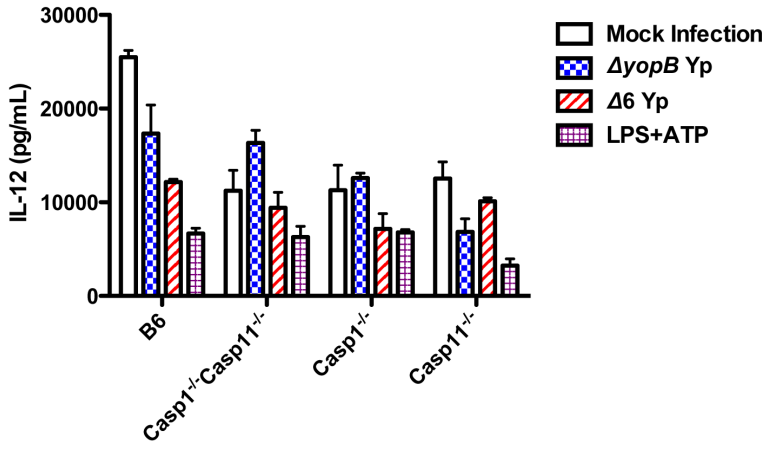

Figure 3-18. Caspase-11-deficient cells secrete comparable amounts of IL-12 in response to *Y. pseudotuberculosis*. B6, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Casp1*$^{-/-}$, or *Casp11*$^{-/-}$ mice were primed with 0.05μg/mL LPS for 2.5 hours and infected with type III secretion system-deficient *Y. pseudotuberculosis* (Δ*yopB* Yp), effectorless *Y. pseudotuberculosis* ΔHOJMEK (Δ6 Yp), or PBS (mock infection) or treated with 2.5mm ATP for 4 hours. The level of IL-12 p40 in the supernatants was measured by ELISA. Graphs show the mean ± SEM of triplicate wells. Data are representative of two independent experiments. This experiment was performed by Erin E. Zwack in Igor Brodsky's laboratory.

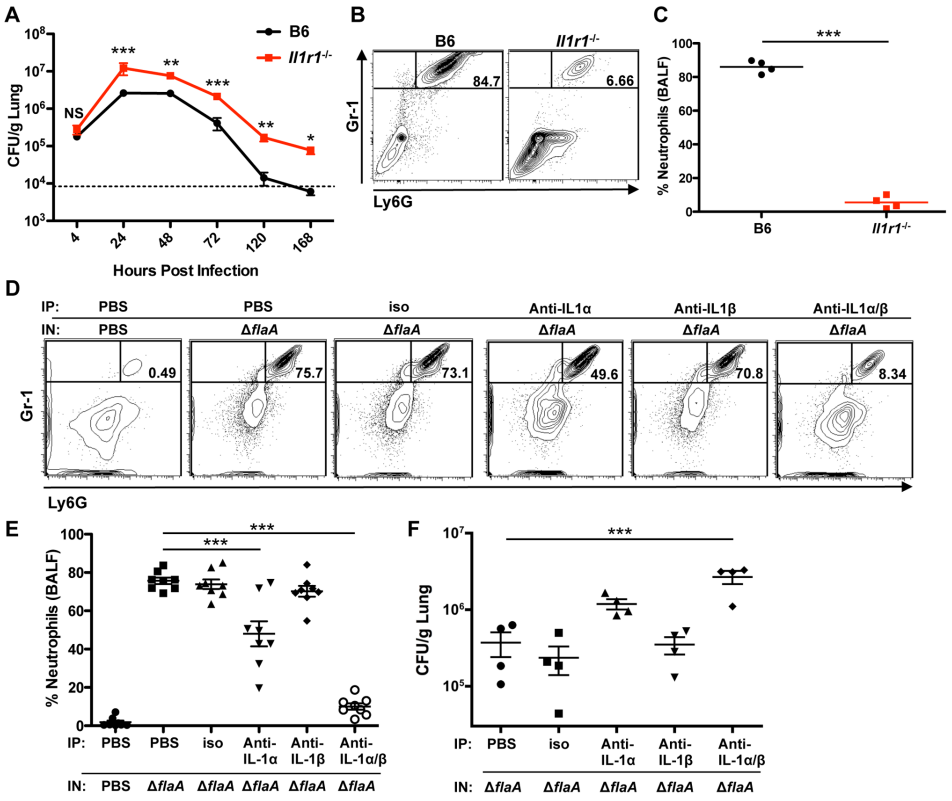

Figure 3-19. IL-1α and IL-1β control bacterial burden and neutrophil recruitment *in vivo*. (A) 8-12 week old B6 or *Il1r1*$^{-/-}$ mice were infected with 1x10^6 Δ*flaA L. pneumophila* intranasally (IN). Lungs were plated to quantify CFU per gram. Graph shows the mean ± SEM of three or four infected mice per group. Dashed line represents the limit of detection. (B and C) B6 or *Il1r1*$^{-/-}$ mice were infected with 1x10^6 Δ*flaA* Lp IN. 24 hours post-infection, bronchoalveolar lavage fluid (BALF) was collected and the percentage of neutrophils in the BALF was quantified by flow cytometry. Percentages are reported as the frequency of live cells in the BALF. (B) Representative flow cytometry plots showing the percentage of Gr-1$^+$Ly6G$^+$ neutrophils. (C) Graph showing the percentage of neutrophils. Each point represents an individual mouse and lines indicate the mean of 4 mice per group. (D, E, and F) B6 mice were injected intraperitoneally (IP) with either PBS, 100μg isotype control antibody (iso), 100μg anti-IL-1α antibody, 100μg anti-IL-1β antibody, or 100 μg each of anti-IL-1α and anti-IL-1β (anti-IL-1α/β) 16 hours before infection. The mice were then intranasally infected with either 1x10^6 Δ*flaA* Lp or mock infected with PBS. (D and E) 24 hours post-infection, BALF was collected and flow cytometry was performed to quantify the percentage of neutrophils. (D) Representative flow cytometry plots showing the percentage of Gr-1$^+$Ly6G$^+$ neutrophils. (E) Graph showing the percentage of neutrophils. Each point represents an individual mouse, lines indicate the mean of 8 mice per group, and error bars represent SEM. Shown are the pooled results of two independent experiments. (F) 72 hours post-infection, the lungs were plated to quantify CFU per gram. Each point represents an individual mouse. Line indicates the mean of 4 infected mice per group with error bars representing SEM. *** is $p<0.001$ by one-way ANOVA with Tukey post-test or unpaired t-test (C). **is $p<0.01$ and *is $p<0.05$ by unpaired t-test. NS is not significant.

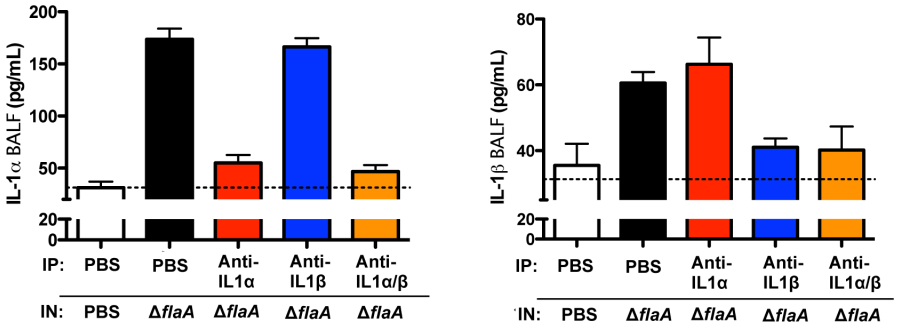

Figure 3-20. Intraperitoneally injected antibodies neutralize cytokine in the BALF. 8-12 week old B6 mice were injected intraperitoneally (IP) with either PBS, 100μg anti-IL-1α antibody, 100μg anti-IL-1β antibody, or 100μg each of anti-IL-1α and anti-IL-1β (anti-IL-1α/β) 16 hours before infection. The mice were then infected with either $1x10^6$ Δ*flaA* Lp or mock infected with PBS intranasally (IN). 24 hours post-infection, bronchoalveolar lavage fluid (BALF) was collected and the levels of IL-1α and IL-1β were measured by ELISA. Labels indicate what was received intraperitoneally (IP) and what was received intranasally (IN). Graphs show the mean ± SEM of 8 mice per group and represent the pooled results of two independent experiments.

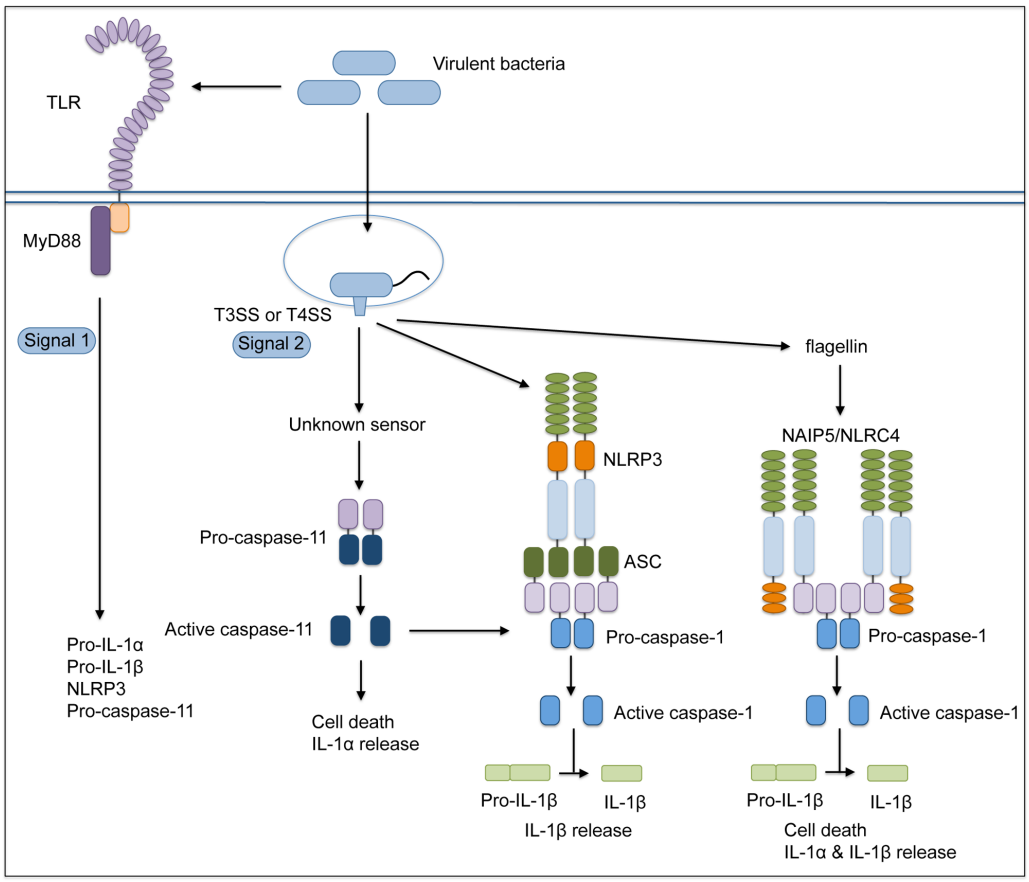

Figure 3-21. Caspase-11 controls multiple pathways of inflammasome activation in response to bacterial secretion systems that access the host cytosol. Three distinct inflammasome pathways are induced upon interaction of virulent bacteria with host cells. Translocation of flagellin into the host cytosol by specialized secretion systems triggers a NAIP5/NLRC4/caspase-1 inflammasome that leads to cell death, IL-1α, and IL-1β release. Virulent bacteria induce two separate pathways of caspase-11-dependent inflammasome activation through a two-signal model. First, TLR stimulation by PAMPs (signal one) leads to upregulation of pro-IL-1α, pro-IL-1β, NLRP3, and pro-caspase-11. Next, cytosolic detection of virulence activity, namely type III or type IV secretion (signal two), leads to caspase-11 processing and activation. Active caspase-11 contributes to NLRP3-mediated inflammasome activation and caspase-1-dependent IL-1β secretion. Caspase-11 also mediates caspase-1-independent cell death and IL-1α release through a pathway that is independent of the NLRP3/ASC and NAIP5/NLRC4 inflammasomes and involves an unknown host sensor.

CHAPTER 4

HUMAN CASPASE-4 MEDIATES NON-CANONICAL INFLAMMASOME ACTIVATION AGAINST GRAM-NEGATIVE BACTERIAL PATHOGENS

This chapter appeared as a published peer-reviewed article titled “Human caspase-4 mediates non-canonical inflammasome activation against Gram-negative bacterial pathogens” by **Cierra N. Casson**, Janet Yu, Valeria M. Reyes, Frances O. Taschuk, Anjana Yadav, Alan M. Copenhaver, Hieu T. Nguyen, Ronald G. Collman, and Sunny Shin. Proceedings of the National Academy of Sciences of the United States of America, 2015.

A. Abstract

Inflammasomes are critical for host defense against bacterial pathogens. In murine macrophages infected by Gram-negative bacteria, the canonical inflammasome activates caspase-1 to mediate pyroptotic cell death and release of IL-1 family cytokines. Additionally, a non-canonical inflammasome controlled by caspase-11 induces cell death and IL-1 release. However, humans do not encode caspase-11. Instead, humans encode two putative orthologs—caspase-4 and caspase-5. Whether either ortholog functions similarly to caspase-11 is poorly defined. Therefore, we sought to define the inflammatory caspases in primary human macrophages that regulate inflammasome responses to Gram-negative bacteria. We find that human macrophages activate inflammasomes specifically in response to diverse Gram-negative bacterial pathogens that introduce bacterial products into the host cytosol using specialized secretion systems. In primary human macrophages, IL-1β secretion requires the caspase-1 inflammasome, while IL-1α release and cell death are caspase-1-independent. Instead, caspase-4 mediates IL-1α release and cell death. Our findings implicate human caspase-4 as a critical regulator of non-canonical inflammasome activation that initiates defense against bacterial pathogens in primary human macrophages.

B. Significance statement

The innate immune system provides a first line of defense against invading pathogens. The inflammasome is an innate immune complex that activates inflammatory caspases upon infection, causing cell death and IL-1 cytokine release, which initiate defense against Gram-negative bacterial pathogens but also mediate septic shock. Many inflammasome studies have been performed using cells from mice, but mice and

humans differ in their complement of inflammatory caspases. Instead of caspase-11, humans encode the putative orthologs caspase-4 and caspase-5. Here, we show that caspase-4 plays a conserved role in inflammasome activation in response to virulent Gram-negative pathogens in primary human macrophages. Our findings provide important insight into how inflammasomes are regulated in human cells.

C. Introduction

PRRs of the innate immune system are critical for promoting defense against bacterial pathogens (Janeway and Medzhitov, 2002). Cytosolic PRRs are key for discriminating between pathogenic and non-pathogenic bacteria, as many pathogens access the host cytosol, a compartment where microbial products are typically not found (Harton et al., 2002). Cytosolic PRRs respond to patterns of pathogenesis that are often associated with virulent bacteria, such as the use of pore-forming toxins or injection of effector molecules through specialized secretion systems (Vance et al., 2009). A subset of cytosolic PRRs induce the formation of multi-protein complexes known as inflammasomes (Martinon et al., 2002). In mice, the canonical inflammasome activates caspase-1, an inflammatory caspase that mediates cell death and IL-1 family cytokine secretion (Kuida et al., 1995; Li et al., 1995). Additionally, the non-canonical inflammasome activates caspase-11 in response to many Gram-negative bacteria (Aachoui et al., 2013; Broz et al., 2012; Case et al., 2013; Casson et al., 2013; Gurung et al., 2012; Kayagaki et al., 2011; Lamkanfi and Dixit, 2014; Rathinam et al., 2012b). The canonical and non-canonical inflammasomes differentially regulate release of IL-1α and IL-1β (Kayagaki et al., 2011). Caspase-11 mediates LPS-induced septic shock in

mice (Kayagaki et al., 2011; Wang et al., 1998), and caspase-11 responds to cytoplasmic LPS independently of TLR4 (Hagar et al., 2013; Kayagaki et al., 2013).

In addition to its pathologic role in septic shock, the non-canonical inflammasome is critical for host defense in mice (Aachoui et al., 2013; Akhter et al., 2012). However, in humans, it is unclear whether an analogous non-canonical inflammasome exists. While mice encode caspase-11, humans encode two putative functional orthologs—caspase-4 and caspase-5 (Kamada et al., 1997; Kamens et al., 1995; Munday et al., 1995). All three inflammatory caspases bind directly to LPS *in vitro* (Shi et al., 2014). In murine macrophages, caspase-1 and -11 have both distinct and overlapping roles in the release of IL-1α and IL-1β and the induction of cell death (Kayagaki et al., 2011). However, the precise role of the human inflammatory caspases in the context of infection by bacterial pathogens remains unclear.

To elucidate how human inflammasome activation is regulated, we investigated the contribution of inflammatory caspases to the response against Gram-negative bacterial pathogens in human macrophages. Here, we show that both canonical caspase-1-dependent and non-canonical caspase-1-independent inflammasomes are activated in primary human macrophages and that caspase-4 mediates caspase-1-independent inflammasome responses against several bacterial pathogens, including *Legionella pneumophila*, *Yersinia pseudotuberculosis*, and *Salmonella* Typhimurium. Importantly, non-canonical inflammasome activation in human macrophages is specific for virulent strains of these bacteria that translocate bacterial products into the host cytosol via virulence-associated type III or type IV secretion systems. Thus, caspase-4 is critical for

non-canonical inflammasome responses against virulent Gram-negative bacteria in human macrophages.

D. Results

L. pneumophila induces both IL-1α and IL-1β release from human macrophages.

In murine macrophages, a canonical inflammasome leads to caspase-1 activation and IL-1β secretion, while a non-canonical inflammasome results in caspase-11 activation, cell death, and IL-1α and IL-1β release (Kayagaki et al., 2011). In human macrophages, it is unclear whether both canonical (caspase-1-dependent) and non-canonical (caspase-1-independent) inflammasomes are activated during bacterial infection (Lamkanfi and Dixit, 2014). To determine if both canonical and non-canonical inflammasomes are activated, we first used *Legionella pneumophila*, a pathogen that triggers robust inflammasome activation in murine macrophages and causes a severe form of pneumonia, Legionnaires' disease, in humans (McDade et al., 1977). To replicate within macrophages, *L. pneumophila* uses a T4SS to inject effector proteins into the host cell cytosol (Berger and Isberg, 1993; Isberg et al., 2009; Marra et al., 1992). Because *L. pneumophila* activates both the canonical and non-canonical inflammasomes in murine macrophages (Case et al., 2013; Casson et al., 2013), we examined whether the bacterium induces IL-1α and IL-1β release from human macrophages as well. First, we differentiated and infected the THP-1 monocytic cell line. Upon infection, THP-1 cells underwent death and released IL-1α and IL-1β in a manner requiring the presence of the bacterial T4SS (Figure 4-1A). THP-1 cells infected with bacterial mutants lacking a functional T4SS (T4SS- Lp) did not activate the

inflammasome but still upregulated pro-IL-1β, suggesting that the cells are capable of sensing T4SS- Lp (Figure 4-1B).

Inflammasome activation has been extensively analyzed in murine macrophages and in murine and human monocytic and epithelial cell lines. In human epithelial cell lines, non-canonical inflammasome activation leads to IL-18 release and cell death during *Salmonella* Typhimurium infection (Knodler et al., 2014). Additionally, non-canonical inflammasome activation occurs in a number of transformed cell lines in response to intracellular LPS (Knodler et al., 2014; Shi et al., 2014). However, we have limited understanding of inflammasome biology in primary human innate immune cells, particularly with respect to pathways that regulate IL-1α and IL-1β release. We therefore infected primary human monocyte-derived macrophages (MDMs) from healthy human donors with *L. pneumophila*. Importantly, cell death and IL-1 release in primary human macrophages required the presence of the bacterial T4SS (Figure 4-1C). Although T4SS-deficient bacteria did not elicit IL-1β secretion, pro-IL-1β was upregulated in all infected donor cells (Figure 4-1D). Mature IL-1β was detected in the supernatants of cells infected with wild-type *L. pneumophila* (WT Lp) but not with T4SS- Lp (Figure 4-2). The absence of inflammasome activation in T4SS- Lp-infected cells was not due to a lack of priming, as MDMs that were first directly primed with LPS and then infected with T4SS- Lp also did not activate the inflammasome (Figure 4-1E and 4-1F). Thus, *L. pneumophila* triggers robust T4SS-dependent inflammasome responses in primary human macrophages.

Caspase-1-dependent and -independent inflammasome pathways are activated during infection of human macrophages.

To determine whether both canonical (caspase-1-dependent) and non-canonical (caspase-1-independent) inflammasomes are triggered in human macrophages, we first examined the contribution of caspase-1 to inflammasome responses. We infected primary human MDMs and found that *L. pneumophila* infection induced caspase-1 cleavage into a 10kDa (p10) subunit that was released into the supernatant (Figure 4-3A). Caspase-1 was processed in response to WT Lp and not T4SS- Lp, suggesting that cytosolic sensing of T4SS activity controls caspase-1 cleavage into its active form. We next asked whether caspase-1 catalytic activity is required for inflammasome activation in human macrophages. We pre-treated primary human MDMs with Ac-YVAD-cmk (YVAD), a chemical inhibitor of caspase-1 activity, and examined inflammasome responses. In primary MDMs, caspase-1 activity played a major role in controlling IL-1β secretion in response to WT Lp (Figure 4-3B). Inhibition of caspase-1 catalytic activity had no significant effect on cell death or IL-1α release. This implies that as in murine cells, caspase-1-independent pathways contribute to IL-1α release in human cells, and these results were consistent with what was observed in THP-1 cells (Figure 4-3C). Importantly, the caspase-1-independent cytokine TNF was unaffected by inhibitor treatment. To further strengthen the link between caspase-1 and IL-1β secretion in human macrophages, we also knocked down caspase-1 in THP-1 cells (Figure 4-3D). Caspase-1 knockdown did not affect expression of caspase-4 or pro-IL-1β during infection with WT Lp but significantly reduced IL-1β secretion (Figure 4-3E). Thus, caspase-1 mediates IL-1β release but not IL-1α release, suggesting that another

inflammatory caspase may mediate IL-1α release during bacterial infection of human macrophages.

Caspase-4 contributes to non-canonical inflammasome activation in *L. pneumophila*-infected primary human macrophages.

As IL-1α release and cell death were independent of caspase-1, we considered the possibility that other inflammatory caspases mediate this response via non-canonical inflammasome activation. In murine macrophages, caspase-11 is robustly activated and cleaved in response to *L. pneumophila* (Casson et al., 2013), but whether caspase-4 or -5 is activated during infection of primary human cells is not known. Notably, we observed that caspase-4 was processed into a 32kDa (p32) subunit in primary human MDMs infected with WT Lp but not T4SS- Lp, again implying that primary human macrophages respond to the activity of the virulence-associated T4SS (Figure 4-4A). In contrast, we did not observe caspase-5 processing in response to WT Lp (Figure 4-5).

In murine macrophages, caspase-11 is upregulated in response to LPS, type I IFN, and IFNγ (Aachoui et al., 2013; Rathinam et al., 2012b). In immortalized epithelial cells, caspase-4 and -5 are both transcriptionally induced by IFNγ, and caspase-5 is upregulated by LPS in THP-1 cells (Lin et al., 2000; Ossina et al., 1997). However, the effect of type I IFN and LPS on caspase-4 and -5 expression in primary MDMs has not been examined. In MDMs, we observed that though both caspase-4 and -5 were transcriptionally induced by LPS and IFNβ (Figure 4-6A), only caspase-4 was translationally upregulated in response to both stimuli, while caspase-5 protein levels

increased in response to LPS but not IFNβ (Figure 4-6B). Thus, similar to murine caspase-11, caspase-4 is upregulated by both LPS and type I IFN.

Because caspase-4 is processed specifically in response to WT Lp and is upregulated by both LPS and type I IFN, we interrogated whether caspase-4 has a role in inflammasome activation. We used siRNA to knockdown caspase-4 expression in primary human MDMs and observed robust silencing of caspase-4 and no effect on caspase-1 (Figure 4-4B). Caspase-4 played a significant role in mediating cell death in MDMs from four out of five donors, and knockdown of caspase-4 significantly reduced IL-1α release for every set of donor cells tested (Figure 4-4C). However, in contrast to the role of caspase-11 in murine cells (Kayagaki et al., 2011), knockdown of caspase-4 did not significantly affect IL-1β secretion or maturation from primary MDMs (Figure 4-4D). Release of TNF, an inflammasome-independent cytokine, was unaffected by silencing caspase-4. Similar results were obtained in LPS-primed primary MDMs infected with *L. pneumophila* (Figure 4-7A and 4-7B). Additionally, both IL-1β maturation and caspase-1 processing did not require caspase-4 during infection of THP-1 cells, though IL-1α release was still dependent on caspase-4 (Figure 4-4E and 4-7C). In contrast, when we infected THP-1 cells that are deficient for the inflammasome adaptor ASC (ASC-def), IL-1β release was greatly diminished (Figure 4-8A and 4-8B), thus placing ASC upstream of caspase-1 activation and IL-1β release. These data support a key role for caspase-4 in non-canonical inflammasome activation in primary human macrophages during *L. pneumophila* infection. Furthermore, these data imply that non-canonical inflammasome activation in human macrophages in response to *L.*

pneumophila infection specifically regulates IL-1α release and cell death separately from IL-1β secretion.

Caspase-4 mediates cell death and IL-1 release in primary human macrophages in response to intracellular LPS

Intracellular LPS is a trigger for non-canonical, caspase-11-dependent inflammasome activation in murine macrophages (Hagar et al., 2013; Kayagaki et al., 2013). Both human caspase-4 and -5 directly bind LPS, and transfection of LPS induces caspase-4-dependent cell death in human monocytic, keratinocyte, and epithelial cell lines and IL-18 release from a human colonic epithelial cell line (Knodler et al., 2014; Shi et al., 2014). However, it is unknown whether primary human macrophages respond to cytosolic LPS by activating the non-canonical inflammasome and if so, whether caspase-4 is responsible. Therefore, we first determined whether transfecting LPS into primary human MDMs induces inflammasome activation. We observed that transfection of LPS into primary human macrophages induced cell death, whereas extracellular LPS treatment did not (Figure 4-9A). Silencing of caspase-4 prior to LPS transfection resulted in a significant reduction in cell death for every donor (Figure 4-9B and 4-9C), and both IL-1α and IL-1β release were also significantly reduced (Figure 4-9C). In agreement with prior studies (Martinon et al., 2002), we also found that primary MDMs treated with extracellular LPS for 20 hours induced cleavage of caspase-5, but LPS transfection did not increase this processing (Figure 4-10A). These data indicate that caspase-4 is primarily responsible for non-canonical inflammasome responses to intracellular LPS in primary human macrophages, but unlike the response to bacterial infection, caspase-4 appears to control both IL-1α and IL-1β release during LPS transfection (Figure 4-14).

Caspase-4 has a conserved role in non-canonical inflammasome activation against Gram-negative bacterial pathogens.

In murine macrophages, caspase-11 controls non-canonical inflammasome responses to a wide variety of Gram-negative bacteria, and caspase-11 is activated in response to bacteria that introduce bacterial products into the host cytosol via virulence-associated secretion systems (Broz et al., 2012; Casson et al., 2013; Gurung et al., 2012; Rathinam et al., 2012b). We thus hypothesized that caspase-4 has a conserved role in inflammasome activation against other pathogens that use specialized secretion systems to deliver bacterial components into the host cytosol. Similarly to the *L. pneumophila* T4SS, other Gram-negative pathogens, including *Salmonella enterica* serovar Typhimurium and *Yersinia pseudotuberculosis*, use a T3SS to inject bacterial effectors that modify host signaling (Galán et al., 2014). Though the T3SS is evolutionarily quite distinct from the T4SS, both secretion systems perform analogous functions to introduce bacterial products into the host cytosol. Therefore, we infected primary human MDMs with *S.* Typhimurium and *Y. pseudotuberculosis* to test whether these bacteria also activate non-canonical inflammasome responses in human cells. *S.* Typhimurium triggered robust cell death and IL-1 release in a manner requiring the *Salmonella* pathogenicity island I (SPI-1) T3SS, as bacteria lacking SPI-1 (T3SS- St) induced little inflammasome activation (Figure 4-11A). Because *Y. pseudotuberculosis* encodes effectors that block inflammasome activation (Brodsky et al., 2010; LaRock and Cookson, 2012), we infected macrophages with a strain of *Y. pseudotuberculosis* lacking the six known secreted effectors (Δ6 Yp), as Δ6 Yp induces robust caspase-11-dependent inflammasome activation in murine macrophages (Casson et al., 2013). For *Y. pseudotuberculosis,* robust inflammasome activation in human macrophages also

required the T3SS (Figure 4-11B). For both bacteria, the inflammasome-independent cytokine TNF was secreted independently of the presence of a T3SS.

We next tested whether caspase-4 has a conserved role in inflammasome responses against *Y. pseudotuberculosis* and *S.* Typhimurium by using siRNA to knockdown caspase-4 in primary human MDMs (Figure 4-12A and 4-12B). Indeed, caspase-4 silencing in MDMs significantly reduced IL-1α release (Figure 4-11D and 4-11E). Similar to infection with *L. pneumophila*, IL-1β secretion was caspase-4-independent during infection with *Y. pseudotuberculosis*, and IL-1β secretion and maturation and caspase-1 processing were also caspase-4-independent during *S.* Typhimurium infection (Figure 4-13). Additionally, infection of ASC-def THP-1 cells showed that ASC is required for IL-1β release during both *Y. pseudotuberculosis* and *S.* Typhimurium infection, again placing ASC upstream of caspase-1 (Figure 4-8C). Caspase-4 knockdown also significantly reduced cell death upon *Y. pseudotuberculosis* infection (Figure 4-12C), though cell death during *S.* Typhimurium infection was independent of caspase-4, implying that at least two distinct pathways that mediate cell death are activated in human cells during infection (Figure 4-12D). Furthermore, upon infection with wild-type *S.* Typhimurium (WT St), caspase-4 was processed into the p32 subunit and released into the supernatant (Figure 4-11C). Caspase-4 processing requires the presence of the T3SS, as infection with T3SS- St did not induce caspase-4 cleavage. Unlike caspase-4, caspase-5 was not processed during either *Y. pseudotuberculosis* or *S.* Typhimurium infection (Figure 4-10B). Thus, supporting our findings with *L. pneumophila*, caspase-4 mediates non-canonical, caspase-1-independent inflammasome activation during infection with *Y. pseudotuberculosis* and *S.* Typhimurium as well. Collectively, these data implicate

caspase-4 as a critical mediator of inflammasome activation against Gram-negative bacterial pathogens that translocate bacterial products into the cytosol of primary human macrophages.

E. Discussion

Our data demonstrate that caspase-4 regulates non-canonical inflammasome responses against Gram-negative bacterial pathogens in primary human macrophages. Recent findings also indicate a role for caspase-4 in non-hematopoietic cells, as caspase-4 mediates secretion of IL-18, another IL-1 family cytokine, in epithelial cell lines infected with *Salmonella* Typhimurium (Knodler et al., 2014). Interestingly, *Shigella flexneri* encodes an effector protein that blocks caspase-4 activity (Kobayashi et al., 2013), and overexpression of caspase-4 in cell lines restricts growth of *L. pneumophila* (Akhter et al., 2012), supporting a critical role for caspase-4 in defense against bacterial pathogens. Further studies will be important for understanding how caspase-4 responds specifically to virulent bacteria. As human caspase-4 can bind LPS directly and enhances inflammasome activation in response to LPS when ectopically expressed in mouse macrophages (Kajiwara et al., 2014; Shi et al., 2014), caspase-4 may respond to LPS that is somehow released into the cytosol during infection with virulent bacteria. In murine cells, IFN-inducible GBPs enhance disruption of phagosomes carrying bacterial cargo and allow bacterial products to enter the host cell cytosol, thus promoting caspase-11 activation (Meunier et al., 2014; Pilla et al., 2014). It would be of interest to determine if GBPs enhance caspase-4 activation in human macrophages as well.

As *L. pneumophila* and *S.* Typhimurium reside within pathogen-containing vacuoles and *Y. pseudotuberculosis* has an extracellular lifestyle, it is still unclear if caspase-4 is also activated by Gram-negative bacterial pathogens that reside within the macrophage cytosol. Presumably, as virulent strains of these pathogens reside and replicate within the cytosol, their LPS could be sensed directly via caspase-4. However, many of these pathogens may have also evolved to evade caspase-4-mediated sensing by blocking caspase-4 activity, as demonstrated by *S. flexneri* (Kobayashi et al., 2013), or by encoding LPS that is not readily detectable, as is the case for *Francisella novicida* in murine macrophages (Hagar et al., 2013).

We observed that although caspase-11 contributes to IL-1β release from murine macrophages in response to both cytosolic LPS and bacterial infection, caspase-4 contributes to IL-1β release in response to cytosolic LPS but does not play a major role in controlling IL-1β release from human macrophages during bacterial infection. We found that human ASC is upstream of caspase-4-independent caspase-1 activation and IL-1β secretion in response to bacterial infection. We speculate that ASC works in conjunction with NLRP3 to activate caspase-1 during Gram-negative bacterial infection, as NLRP3 is recruited to ASC foci during infection of THP-1 cells with *S.* Typhimurium (Man et al., 2014), and both ASC and NLRP3 contribute to IL-1β secretion (Li et al., 2008). It is possible that during bacterial infection, another cytosolic PAMP dominantly triggers a canonical NLRP3/ASC/caspase-1 inflammasome and IL-1β release independently of caspase-4. Alternatively, even though we do not detect caspase-5 processing, it is possible that caspase-5 contributes to caspase-1 activation and IL-1β secretion independently of caspase-4 during bacterial infection (Figure 4-14).

Intriguingly, in addition to our finding that caspase-4 is activated during bacterial infection, caspase-4 is also activated in response to ER stress (Hitomi et al., 2004) and ultraviolet B (UVB)-irradiation (Sollberger et al., 2012). This implies that both exogenous and endogenous stressors may trigger caspase-4 activation and that a common mechanism may be involved. Both UVB-irradiation and ER stress result in elevated cytoplasmic calcium, which has been linked to inflammasome activation (Feldmeyer et al., 2007; Murakami et al., 2012; Sano and Reed, 2013). As *L. pneumophila* intercepts ER-derived vesicles to establish its replicative vacuole (Horwitz and Silverstein, 1980; Swanson and Isberg, 1995), it is possible that perturbations to ER and calcium homeostasis during bacterial infection provide common signals that induce caspase-4 activation.

Overall, our data implicate caspase-4 as a critical mediator of host defense against virulent Gram-negative bacteria in primary human macrophages and reveal unexpected differences in the regulation of non-canonical inflammasome pathways in murine and human cells. Caspase-4 plays an important role as an innate immune effector for discrimination between pathogenic and nonpathogenic bacteria in humans, and further studies will examine the basis for differences in how non-canonical inflammasomes function in different organisms. Like caspase-11 in mice, caspase-4 may play a dual role in humans to both protect the host and mediate septic shock during bacterial infection. Therefore, studying caspase-4 is critical for our understanding of how the human immune system coordinates an appropriate response during bacterial infection.

F. Acknowledgements

We are grateful to the Human Immunology Core of the Penn Center for AIDS Research and Abramson Cancer Center for primary human PBMCs. We thank Igor Brodsky for critical reading of the manuscript, the Brodsky lab for valuable feedback on experimental design and for use of the *Y. pseudotuberculosis* and *S.* Typhimurium strains, and Leigh Knodler for valuable technical advice. This work was supported in part by National Institutes of Health grants K99/R00AI087963 (to SS) and T32GM007229 (to AMC), as well as grants from the American Lung Association (RG-268528-N, to SS), the University of Pennsylvania University Research Foundation (to SS), the American Heart Association (13BGIA14780070, to SS), and the University of Pennsylvania Institute for Immunology Pilot Grant (to SS). This material is based on work supported by the National Science Foundation under Grant No. DGE-0822 (to CNC, graduate research fellowship). Janet Yu was instrumental in designing experiments involving qRT-PCR and in performing immunoblot analysis. Valeria M. Reyes and Frances O. Taschuk performed infections in THP-1 cells and assisted in immunoblot analysis. Anjana Yadav and Ronald G. Collman provided critical reagents and instructions for how to isolate, culture, and maintain primary human macrophages. Alan M. Copenhaver assisted in the culture of primary human macrophages. Hieu T. Nguyen performed immunoblot analysis. Sunny Shin assisted in the design of experiments, interpretation of data, and writing of the manuscript.

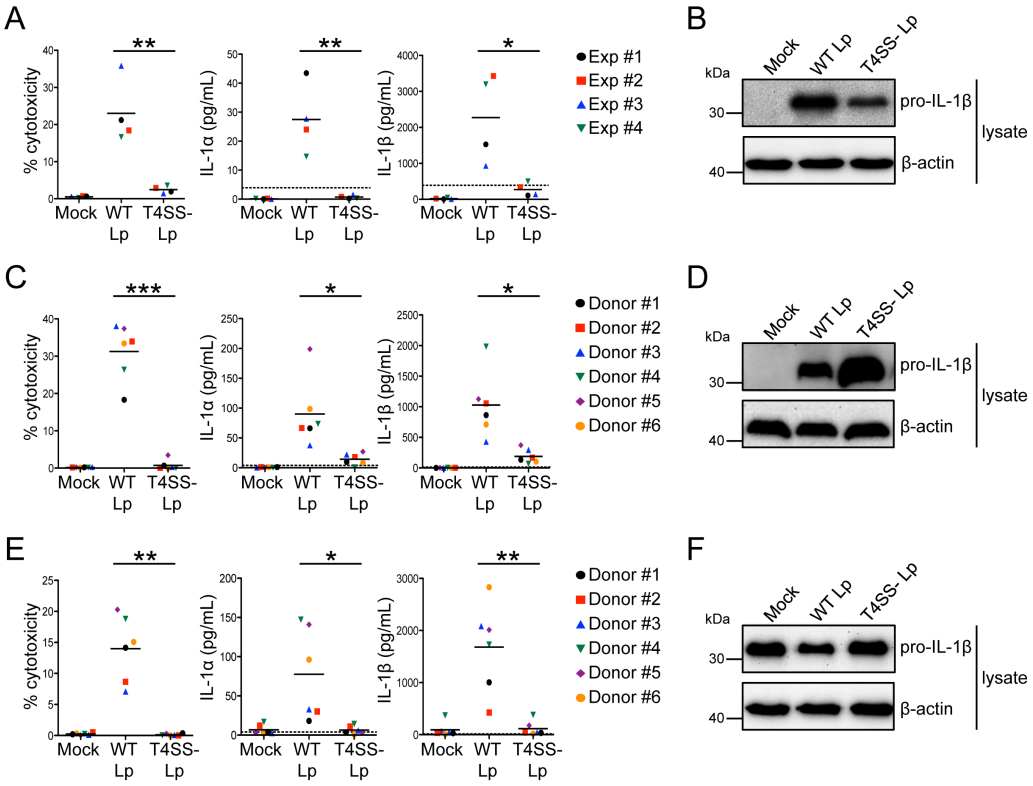

Figure 4-1. *L. pneumophila* induces both IL-1α and IL-1β release from human macrophages. (A-D) PMA-differentiated THP-1 cells (A and B) or primary human MDMs (C and D) were infected with wild-type *L. pneumophila* (WT Lp), T4SS-deficient *L. pneumophila* (T4SS- Lp), or mock infected with PBS for 20 hours. (E and F) Primary human MDMs were primed with LPS and infected with WT Lp, T4SS- Lp, or mock infected for 4 hours. Cell death (% cytotoxicity) was measured using LDH release assay and normalized to mock infected cells. IL-1α and IL-1β levels in the supernatants were measured by ELISA. Immunoblot analysis was performed on lysates for full-length IL-1β (pro-IL-1β), and blots were re-probed for β-actin as a loading control. Western blots (B, D, and F) are representative of at least 3 independent experiments. Shown are the pooled results of 4 independent experiments in THP-1 cells (A) or the pooled results of 6 independent infections of cells from different healthy human donors (C and E). Each data point shows the mean of triplicate infected wells. For (A), * is $p<0.05$ and ** is $p<0.01$ by unpaired t-test. For (C and E), *** is $p<0.001$, ** is $p<0.01$, and * is $p<0.05$ by paired t-test. Dashed line is the limit of detection.

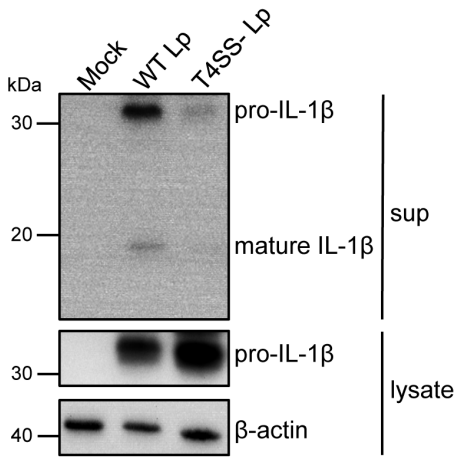

Figure 4-2. Primary human macrophages secrete mature IL-1β in response to WT *L. pneumophila*. Primary human MDMs were infected with wild-type *L. pneumophila* (WT Lp), T4SS-deficient *L. pneumophila* (T4SS- Lp), or mock infected with PBS for 20 hours. Immunoblot analysis was performed on supernatants for cleaved IL-1β (mature IL-1β) and lysates for pro-IL-1β, and blots were re-probed for β-actin as a loading control. Western blots are representative of at least 2 independent experiments.

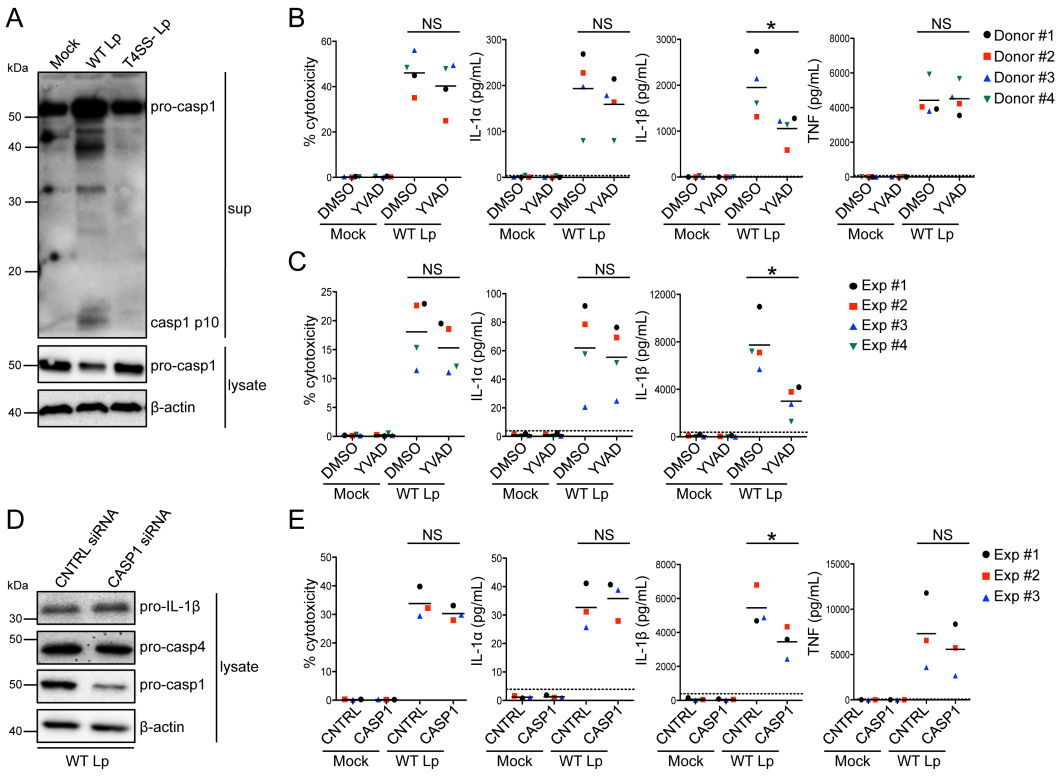

Figure 4-3. Caspase-1-dependent and -independent inflammasomes are activated during infection of human macrophages. (A) Primary human MDMs were infected with WT Lp, T4SS- Lp, or mock infected 20 hours. Immunoblot analysis was performed on supernatants for cleaved caspase-1 (casp-1 p10) and lysates for full-length caspase-1 (pro-casp-1). Lysates were re-probed for β-actin. (B and C) Primary human MDMs (B) or PMA-differentiated THP-1 cells (C) were pre-treated with 40μM caspase-1 inhibitor (YVAD) or vehicle control (DMSO) and infected with WT Lp or mock infected for 20 hours. (D and E) PMA-differentiated THP-1 cells were transfected with control siRNA or siRNA against caspase-1 and infected with WT Lp or mock infected for 20 hours. Immunoblot analysis was performed on lysates for pro-casp1, pro-casp4, and pro-IL-1β, and blots were re-probed for β-actin. Cell death was measured using LDH release assay and normalized to mock infected cells. IL-1α, IL-1β, and TNF levels in the supernatants were measured by ELISA. Western blots (A and D) are representative of 3 independent experiments. Shown are the pooled results of 4 independent infections of cells from different donors (B) or the pooled results of 4 (C) or 3 (E) independent experiments in THP-1 cells. Each data point shows the mean of triplicate infected wells. * is $p<0.05$ by paired t-test (B and E) or unpaired t-test (C). NS is not significant. Dashed line is the limit of detection.

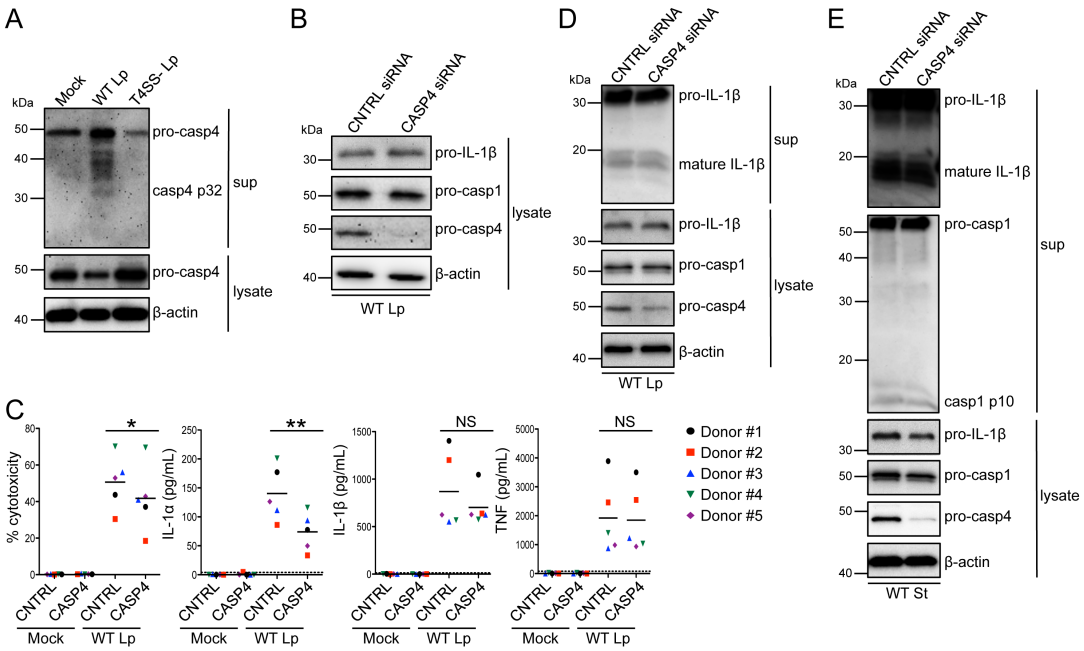

Figure 4-4. Caspase-4 contributes to non-canonical inflammasome activation in *L. pneumophila*-infected primary human macrophages. (A) Primary human MDMs were infected with WT Lp, T4SS- Lp, or mock infected for 20 hours. Immunoblot analysis was performed on supernatants for cleaved caspase-4 (casp4 p32) and lysates for pro-casp4. Lysates were re-probed for β-actin. (B-E) Primary human MDMs (B-D) or THP-1 cells (E) were transfected with control siRNA or siRNA against caspase-4 and infected with WT Lp or mock infected for 20 hours. Immunoblot analysis was performed on supernatants for cleaved IL-1β (mature IL-1β) and cleaved caspase-1 (casp-1 p10) and lysates for pro-IL-1β, pro-casp1, and pro-casp4, and blots were re-probed for β-actin. Cell death was measured using LDH release assay and normalized to mock infected cells. IL-1α, IL-1β, and TNF levels in the supernatants were measured by ELISA. Western blots (A, B, D, and E) are representative of at least 3 independent experiments. Shown are the pooled results of 5 independent infections of cells from different donors (C). Each data point shows the mean of triplicate infected wells. ** is $p<0.01$ and * is $p<0.05$ by paired t-test. NS is not significant. Dashed line is the limit of detection.

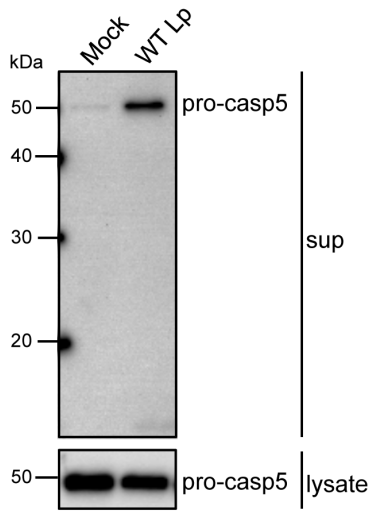

Figure 4-5. Pro-caspase-5 is found in the lysates but is not processed in response to WT *L. pneumophila*. Primary human MDMs were infected with WT Lp or mock infected 20 hours. Immunoblot analysis was performed on supernatants for cleaved caspase-5 and lysates for full-length caspase-5 (pro-casp5). Western blots are representative of at least 3 independent experiments.

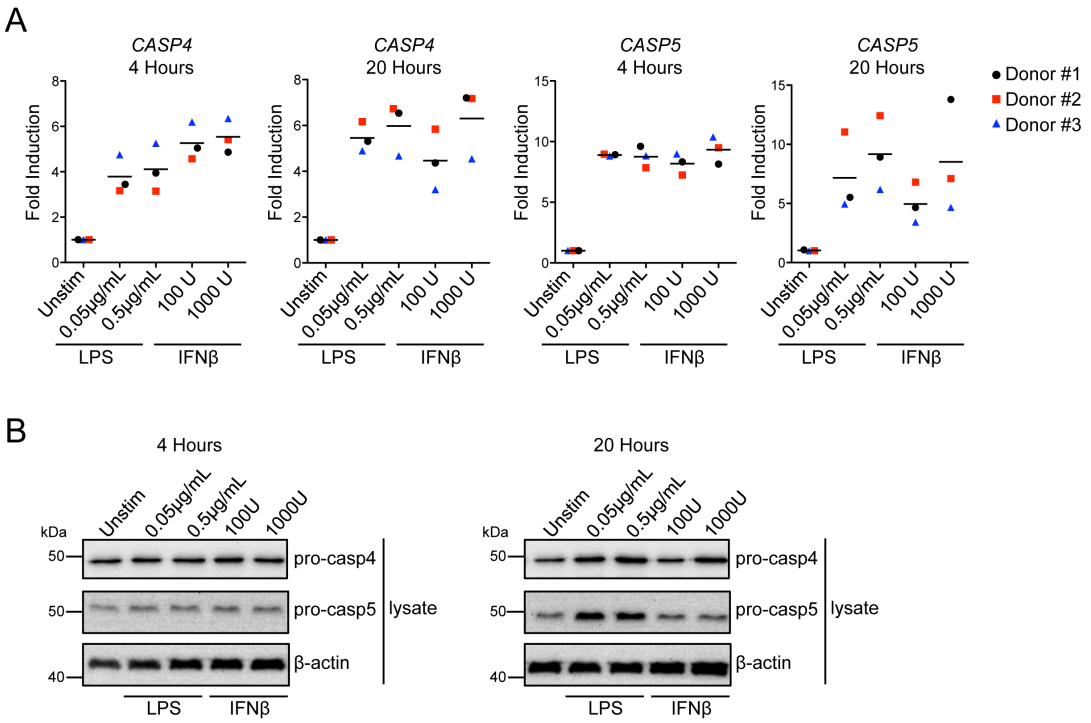

Figure 4-6. Caspase-4 is transcriptionally and translationally upregulated by LPS and IFNβ, whereas caspase-5 is transcriptionally upregulated by LPS and IFNβ but only translationally upregulated by LPS. (A and B) Primary human MDMs were either left untreated (unstim) or were treated with LPS or IFNβ at the indicated concentrations for either 4 or 20 hours. (A) Transcript levels were determined by qRT-PCR, and fold induction was calculated by normalizing to the HPRT housekeeping gene for each sample and then to the unstimulated sample for each time point. (B) Immunoblot analysis was performed on lysates for full-length caspase-4 (pro-casp4) and full-length caspase-5 (pro-casp5), and blots were re-probed for β-actin as a loading control. Western blots are representative of 3 independent experiments. Shown are the pooled results of 3 independent treatments of cells from different donors (A). Each data point shows the mean of duplicate treated wells.

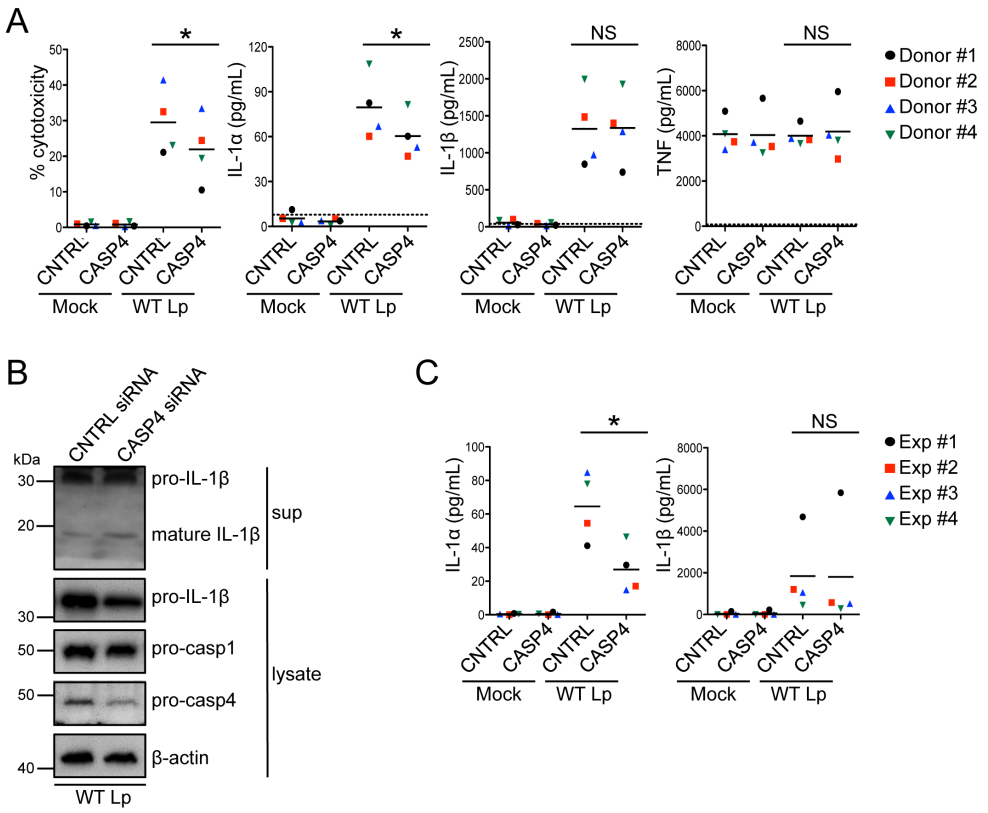

Figure 4-7. Caspase-4 contributes to non-canonical inflammasome activation in LPS-primed *L. pneumophila*-infected primary human macrophages and THP-1 cells. (A and B) Primary human MDMs were transfected with control siRNA or siRNA against caspase-4, primed with LPS, and infected with WT Lp or mock infected for 4 hours. (A) Cell death was measured using LDH release assay and normalized to mock infected cells. IL-1α, IL-1β, and TNF levels in the supernatants were measured by ELISA. (B) Immunoblot analysis was performed on supernatants for cleaved IL-1β (mature IL-1β) and lysates for pro-IL-1β, pro-casp1, and pro-casp4, and blots were re-probed for β-actin. Western blots are representative of 3 independent experiments. Shown are the pooled results of 4 independent infections of cells from different donors (A). Each data point shows the mean of triplicate infected wells. * is $p<0.05$ by paired t-test. NS is not significant. Dashed line is the limit of detection.
PMA-differentiated THP-1 cells were transfected with control siRNA or siRNA against caspase-4 and infected with WT Lp or mock infected for 20 hours. IL-1α and IL-1β levels in the supernatants were measured by ELISA. Shown are the pooled results of 4 independent experiments in THP-1 cells. Each data point shows the mean of triplicate infected wells. Control siRNA from Exp#1 is from the same experiment as Fig 2E. * is $p<0.05$ by unpaired t-test. NS is not significant. Dashed line is the limit of detection.

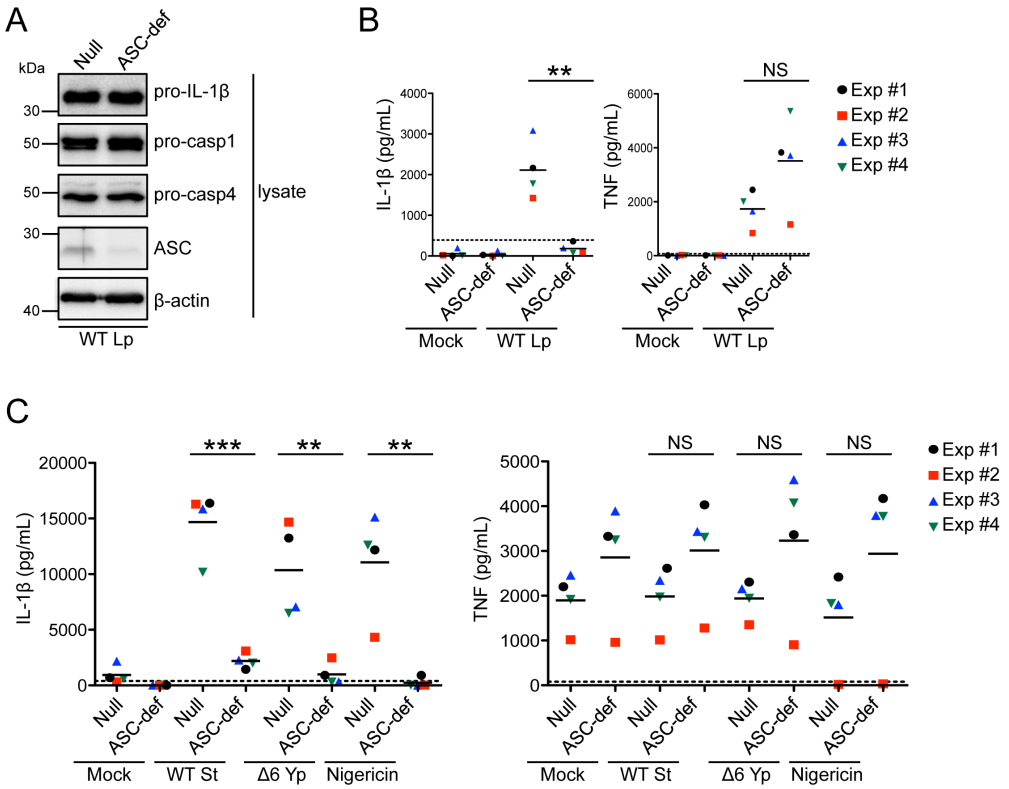

Figure 4-8. ASC is required for IL-1β release in response to Gram-negative bacterial infection. (A and B) ASC-deficient (ASC-def) or empty vector control (Null) PMA-differentiated THP-1 cells were infected with WT Lp or mock infected for 20 hours. (A) Immunoblot analysis was performed on lysates for pro-IL-1β, pro-casp1, pro-casp4, and ASC, and blots were re-probed for β-actin. (B) IL-1β and TNF levels in the supernatants were measured by ELISA. (C) ASC-def or Null PMA-differentiated THP-1 cells were primed with LPS and infected with wild-type *S.* Typhimurium (WT St), T3SS-expressing effectorless *Y. pseudotuberculosis* (Δ6 Yp), treated with 10μM nigericin for 2 hours as an ASC-dependent control, or mock infected for 4 hours. IL-1β and TNF levels in the supernatants were measured by ELISA. Western blots are representative of at least 2 independent experiments. Shown are the pooled results of 4 independent experiments in THP-1 cells (B and C). Each data point shows the mean of triplicate infected wells. ** is $p<0.01$ and *** is $p<0.001$ by unpaired t-test. NS is not significant. Dashed line is the limit of detection.

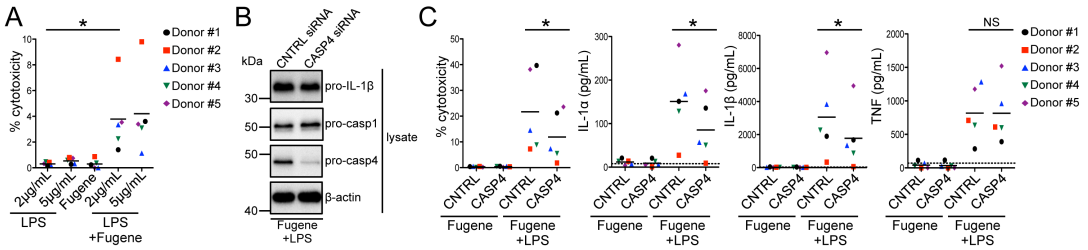

Figure 4-9. Caspase-4 mediates inflammasome activation in primary human macrophages in response to intracellular LPS. (A) Primary human MDMs were primed with Pam3CSK4 and either treated with extracellular LPS at the indicated concentrations, mock transfected with Fugene alone, or transfected with Fugene and LPS at the indicated concentrations for 20 hours. (B and C) Primary human MDMs were transfected with control siRNA or siRNA against caspase-4, primed with Pam3CSK4, and mock transfected with Fugene alone or transfected with Fugene and 2μg/mL LPS for 20 hours. Immunoblot analysis was performed on lysates for pro-casp1, pro-casp4, and pro-IL-1β, and blots were re-probed for β-actin. Cell death was measured using LDH release assay and normalized to LPS alone (A) or mock transfected cells (C). IL-1α, IL-1β, and TNF levels in the supernatants were measured by ELISA. Western blots (B) are representative of at least 4 independent experiments. Shown are the pooled results of 5 independent infections of cells from different donors (A and C). Each data point shows the mean of triplicate infected wells. * is $p<0.05$ by paired t-test. NS is not significant. Dashed line is the limit of detection.

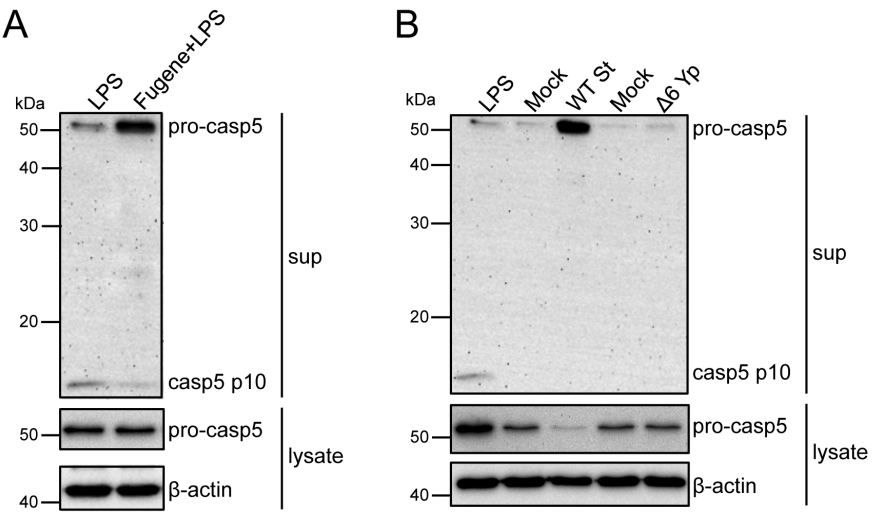

Figure 4-10. Caspase-5 does not undergo enhanced cleavage in response to intracellular LPS, *S.* Typhimurium, or *Y. pseudotuberculosis*. (A) Primary human MDMs were primed with Pam3CSK4 and either treated with 2μg/mL extracellular LPS or transfected with Fugene and 2μg/mL LPS for 20 hours. (B) Primary human MDMs were primed with LPS and infected with WT St, Δ6 Yp, mock infected for 4 hours, or treated with LPS alone for 20 hours. Immunoblot analysis was performed on supernatants for cleaved caspase-5 (casp5 p10) and lysates for full-length caspase-5 (pro-casp5), and blots were re-probed for β-actin. Western blots are representative of at least 2 independent experiments.

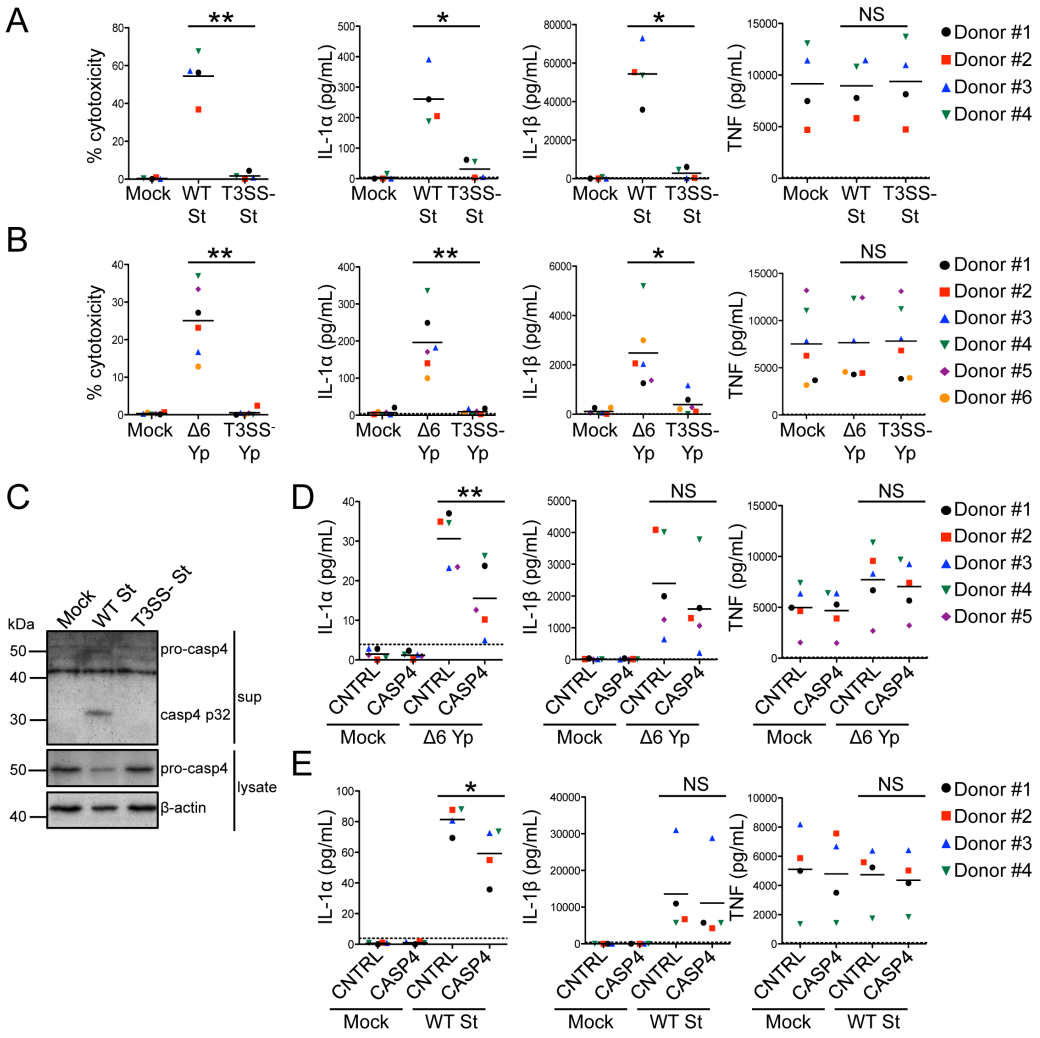

Figure 4-11. Caspase-4 has a conserved role in non-canonical inflammasome activation against Gram-negative bacterial pathogens. (A and C) Primary human MDMs were primed with LPS and infected with wild-type *S.* Typhimurium (WT St), T3SS-deficient *S.* Typhimurium (T3SS- St), or mock infected for 4 hours. Immunoblot analysis was performed on supernatants for casp4 p32 and lysates for pro-casp4. Blots were re-probed for β-actin. (B) Primary human MDMs were primed with LPS and infected with T3SS-expressing effectorless *Y. pseudotuberculosis* (Δ6 Yp), T3SS-deficient *Y. pseudotuberculosis* (T3SS- Yp), or mock infected for 4 hours. (D and E) Primary human MDMs were transfected with control siRNA or siRNA against caspase-4, primed with LPS, and infected with Δ6 Yp (D), WT St (E), or mock infected for 4 hours. Cell death was measured using LDH release assay and normalized to mock infected cells. IL-1α, IL-1β, and TNF levels in the supernatants were measured by ELISA. Western blots (C) are representative of at least 3 independent experiments. Shown are the pooled results of 4 (A and E), 5 (D), or 6 (B) independent infections of cells from different donors. Each data point shows the mean of triplicate infected wells. * is $p<0.05$ and ** is $p<0.01$ by paired t-test. NS is not significant. Dashed line is the limit of detection.

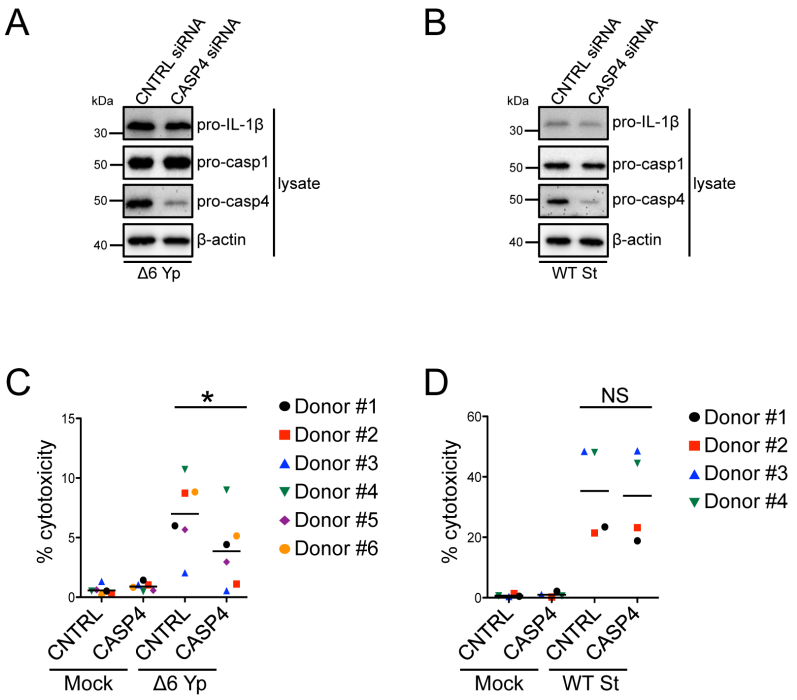

Figure 4-12. Caspase-4 contributes to cell death during infection with *Y. pseudotuberculosis* but not during infection with *S.* Typhimurium. Primary human MDMs were transfected with control siRNA or siRNA against caspase-4, primed with LPS, and infected with Δ6 Yp (A and C), WT St (B and D), or mock infected for 4 hours. (A and B) Immunoblot analysis was performed on lysates for pro-casp1, pro-casp4, and pro-IL-1β, and blots were re-probed for β-actin. (C and D) Cell death was measured using LDH release assay and normalized to mock infected cells. Western blots (A and B) are representative of at least 3 independent experiments. Shown are the pooled results of 6 (C) or 4 (D) independent infections of cells from different donors. Each data point shows the mean of triplicate infected wells. * is $p<0.05$ by paired t-test. NS is not significant. Dashed line is the limit of detection.

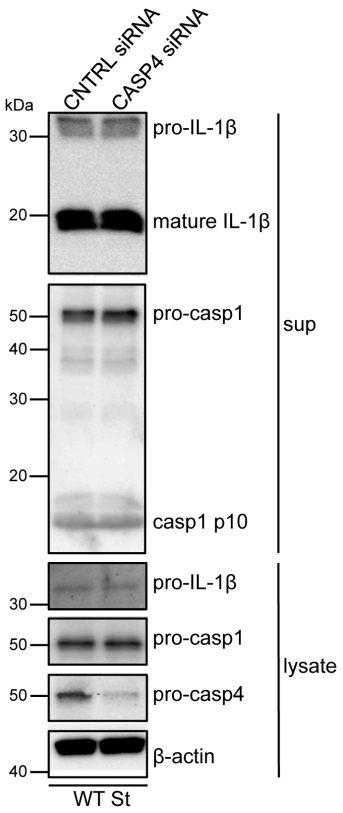

Figure 4-13. IL-1β maturation and caspase-1 cleavage are independent of caspase-4 during *S.* Typhimurium infection. Primary human MDMs were transfected with control siRNA or siRNA against caspase-4, primed with LPS, and infected with WT St for 4 hours. Immunoblot analysis was performed on supernatants for cleaved IL-1β (mature IL-1β) and cleaved caspase-1 (casp-1 p10) and lysates for pro-IL-1β, pro-casp1, and pro-casp4, and blots were re-probed for β-actin. Western blots are representative of at least 3 independent experiments.

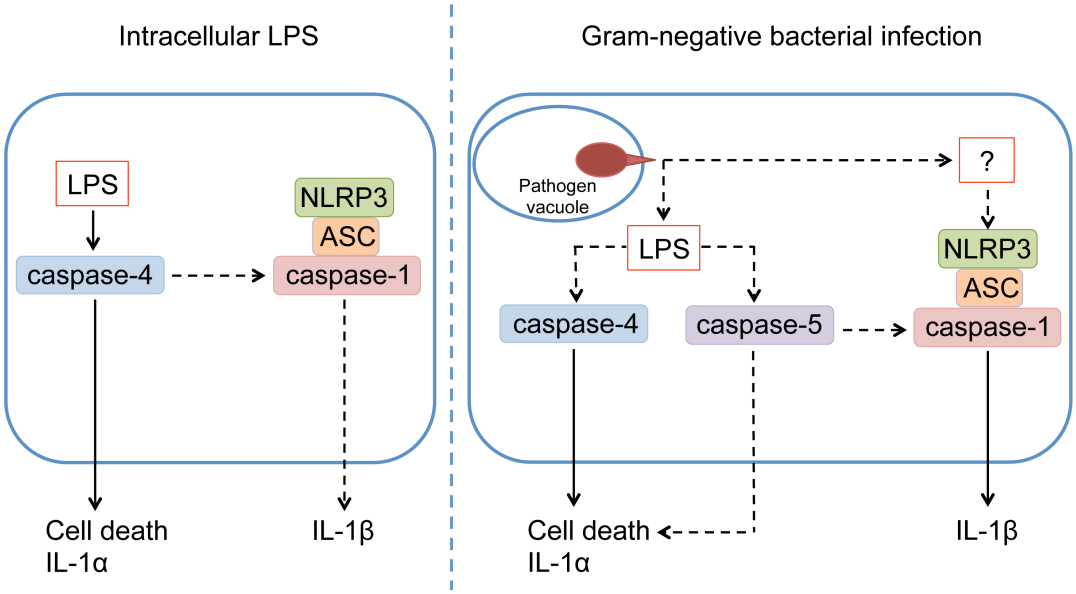

Figure 4-14. Model for inflammasome activation in primary human macrophages in response to intracellular LPS and Gram-negative bacterial infection. In the context of transfection of LPS into primary human MDMs, caspase-4 is required for maximal cell death and IL-1α and IL-1β release. Presumably, intracellular LPS binds directly to caspase-4 to induce activation. We speculate that caspase-1 is activated downstream of caspase-4 to mediate IL-1β release in response to intracellular LPS. In contrast, during infection of primary human MDMs with Gram-negative bacterial pathogens, IL-1β secretion appears to be independent of caspase-4. We speculate that the presence of a type III or type IV secretion system somehow allows bacterial LPS to access the macrophage cytosol, where LPS binds to and activates caspase-4 to induce cell death and IL-1α release. As IL-1β secretion is independent of caspase-4 but requires caspase-1 and ASC, we speculate that either LPS binds to caspase-5 to enhance caspase-1 activation or another PAMP is sensed via a canonical NLRP3 inflammasome to induce caspase-1 activation and IL-1β secretion during infection.

CHAPTER 5

SENSING OF FLAGELLIN INDUCES INFLAMMASOME ACTIVATION IN HUMAN MACROPHAGES

This chapter contains unpublished data generated by **Cierra N. Casson**, Valeria M. Reyes, Frances O. Taschuk, and Sunny Shin.

A. Introduction

PRRs are critical for initiating immune responses against invading microorganisms (Janeway and Medzhitov, 2002). Cytosolic PRRs, such as the NLRs, specifically detect microbial pathogens that have breached the host cell cytosol (Harton et al., 2002), a cellular compartment that is typically free of microorganisms. A subset of NLRs activate multi-protein complexes known as inflammasomes, which assemble in the host cytosol in response to a wide variety of pathogenic insults to activate the enzyme caspase-1 (Rathinam et al., 2012a). Caspase-1 has multiple effector functions, including the induction of a pro-inflammatory form of cell death known as pyroptosis (Cookson and Brennan, 2001). Caspase-1 also mediates the release of IL-1 family cytokines, including IL-1α and IL-1β (Kuida et al., 1995; Li et al., 1995).

In murine macrophages, the host enzyme caspase-11 also participates in inflammasome activation in response to infection by many Gram-negative bacterial pathogens (Broz et al., 2012; Case et al., 2013; Casson et al., 2013; Gurung et al., 2012; Kayagaki et al., 2011; Rathinam et al., 2012b). Caspase-11 is required for IL-1α release and cell death and contributes to NLRP3- and caspase-1-dependent IL-1β and IL-18 release as well (Kayagaki et al., 2011). However, humans do not encode caspase-11 (Martinon and Tschopp, 2007). Instead, humans encode two putative functional orthologs—caspase-4 and caspase-5 (Kamada et al., 1997; Kamens et al., 1995; Munday et al., 1995). Though caspase-5 was found to co-immunoprecipitate with caspase-1 when the inflammasome was first described in THP-1 cells (Martinon et al., 2002), recent studies have shown that human caspase-4 is a functional homolog of murine caspase-11 in that it mediates cytokine release and cell death during infection. For example, caspase-4 is required for

IL-18 release from epithelial cell lines during infection with *S.* Typhimurium (Knodler et al., 2014). Additionally, caspase-4 mediates IL-1α release from primary human macrophages in response to Gram-negative bacterial pathogens such as *L. pneumophila*, *Y. pseudotuberculosis,* and *S.* Typhimurium (Casson et al., 2015). Caspase-4 also mediates cell death in response to both transfection of intracellular LPS and Gram-negative bacterial infection in both immortalized cell lines and primary human macrophages (Casson et al., 2015; Knodler et al., 2014; Shi et al., 2014).

Currently, intracellular LPS is the only known bacterial ligand that triggers the non-canonical caspase-11-dependent inflammasome in murine cells and the non-canonical caspase-4-dependent inflammasome in human cells (Hagar et al., 2013; Kayagaki et al., 2013; Shi et al., 2014). However, multiple bacterial ligands are known to activate the canonical caspase-1-dependent inflammasome in both human and murine cells. Many bacterial ligands are sensed by host macrophages via a subset of NLRs known as the NAIP proteins. Though mice encode multiple NAIP paralogs, humans encode only one NAIP protein (Dietrich, 2001; Endrizzi et al., 2000). Each NAIP protein responds to a distinct bacterial stimulus. For example, the conserved inner rod component of the bacterial T3SS triggers murine NAIP2, while the needle protein of the T3SS triggers NAIP1 in murine cells and NAIP in human cells (Kofoed and Vance, 2011; Yang et al., 2013; Zhao et al., 2011). Additionally, in murine macrophages, intracellular bacterial flagellin is a robust trigger for the canonical NAIP5/NLRC4- and caspase-1-dependent inflammasome (Franchi et al., 2006; Molofsky et al., 2006; Ren et al., 2006; Zamboni et al., 2006). Flagellin associates with both NAIP5 and NAIP6 to induce inflammasome

activation, and NLRC4 appears to be an important adaptor downstream of the NAIP proteins (Kofoed and Vance, 2011; Zhao et al., 2011).

Human cells are not thought to respond to intracellular flagellin because human NAIP does not co-immunoprecipitate with flagellin, and the presence of cytosolic flagellin does not trigger the formation of a multi-meric complex containing NAIP and NLRC4 (Zhao et al., 2011). However, the assays examining inflammasome assembly were performed using overexpression of various NLRs in a heterologous cell expression system (Kofoed and Vance, 2011; Zhao et al., 2011). Therefore, it is possible that endogenous human NAIP could recognize flagellin during physiological infection conditions in primary cells. Interestingly, there is evidence that human NAIP responds to bacterial flagellin during infection with *L. pneumophila* and restricts bacterial growth (Vinzing et al., 2008). However, a role for NAIP in controlling inflammasome activation and release of IL-1 family cytokines in response to bacterial pathogens has not been examined. Additionally, it is unclear if flagellin can trigger inflammasome activation in human cells that is independent of sensing via NAIP. Therefore, we wanted to elucidate if flagellin is a trigger for IL-1 release during bacterial infection of human cells.

To determine if flagellin triggers inflammasome activation in human macrophages during Gram-negative bacterial infection, we infected human macrophages with flagellin-deficient strains of both *S.* Typhimurium and *L. pneumophila* and examined IL-1α and IL-1β release. Intriguingly, we find that both IL-1α and IL-1β release are reduced in response to infection with flagellin-deficient pathogens, suggesting that flagellin is a trigger for both the canonical and non-canonical inflammasomes in human

macrophages. Therefore, our data suggest that cytosolic flagellin is sensed in human macrophages and that flagellin, like LPS, may be an important trigger for caspase-4 activation during bacterial infection.

B. Results

IL-1 release from THP-1 cells in response to *S.* Typhimurium is dependent on the presence of bacterial flagellin.

To determine if bacterial flagellin is sensed in human macrophages to induce inflammasome activation, we first infected the immortalized monocytic THP-1 cell line with *S.* Typhimurium. Infection with WT St induces robust IL-1 release, while infection with flagellin-deficient *S.* Typhimurium (FliCFljB- St) induces significantly less IL-1α and IL-1β release (Figure 5-1). IL-1α and IL-1β release in response to *S.* Typhimurium require the presence of the bacterial SPI-1 T3SS (Casson et al., 2015). Therefore, the T3SS is required for flagellin-dependent IL-1 secretion, and cytosolic flagellin introduced by the presence of the T3SS is likely the trigger for inflammasome activation during infection. Secretion of TNF, an inflammasome-independent cytokine, was unaffected by flagellin-deficiency (Figure 5-1). Additionally, the decrease in IL-1β secretion in response to FliCFljB- St was not due to a general defect in priming, as expression of pro-IL-1β was not decreased during infection with FliCFljB- St (Figure 5-2).

IL-1 release from primary human macrophages in response to *L. pneumophila* is dependent on the presence of bacterial flagellin.

It remains unclear if flagellin triggers release of IL-1α and IL-1β in primary human macrophages. Therefore, we infected primary human MDMs with *L. pneumophila* and

examined inflammasome activation. For every set of donor cells tested, we find that both IL-1α and IL-1β release are significantly dependent on the presence of bacterial flagellin, as infection with flagellin-deficient *L. pneumophila* (FlaA- Lp) induces less IL-1 release than infection with WT Lp (Figure 5-3). However, secretion of TNF, an inflammasome-independent cytokine, was unaffected by the absence of flagellin during infection. Again, IL-1 release in response to *L. pneumophila* in primary MDMs requires the presence of the bacterial T4SS (Casson et al., 2015). Therefore, flagellin that gains access to the macrophage cytosol in the presence of the T4SS is likely the ligand that triggers IL-1 release during infection.

C. Discussion

Our data demonstrate that sensing of bacterial flagellin contributes to IL-1 release from human macrophages during Gram-negative bacterial infection. Contrary to previous studies (Yang et al., 2013; Zhao et al., 2011), our data imply that human cells are capable of responding to cytosolic flagellin and that cytosolic flagellin likely triggers inflammasome activation. Intriguingly, our data show that the presence of bacterial flagellin enhances both IL-1α and IL-1β release from human macrophages. Previous studies have demonstrated that caspase-4 controls IL-1α release in response to both *S.* Typhimurium and *L. pneumophila* (Casson et al., 2015). In contrast to caspase-11 in murine macrophages (Kayagaki et al., 2011), caspase-4 does not contribute to IL-1β release from human macrophages in response to Gram-negative bacterial pathogens (Casson et al., 2015). Instead, caspase-1 mediates IL-1β release separately from caspase-4. Therefore, our data place flagellin upstream of both caspase-1 and caspase-

4 as a trigger for both canonical and non-canonical inflammasome activation in human macrophages.

Although we have uncovered a role for flagellin in inducing IL-1α and IL-1β release from human macrophages, it is still unclear which NLR responds to cytosolic flagellin during infection. Presumably, human NAIP is involved in detecting flagellin in human cells, as NAIP restricts *L. pneumophila* growth (Vinzing et al., 2008), and the mouse NAIP paralogs NAIP5 and NAIP6 detect flagellin in murine cells (Kofoed and Vance, 2011; Zhao et al., 2011). However, as human NAIP is thought to specifically detect the needle protein of the bacterial T3SS (Yang et al., 2013), it is still unclear if NAIP senses flagellin during infection of human macrophages. Future studies interrogating a role for NAIP in mediating IL-1 release during infection with *S.* Typhimurium and *L. pneumophila* infection will be valuable for understanding how flagellin is sensed in human cells.

D. Acknowledgements

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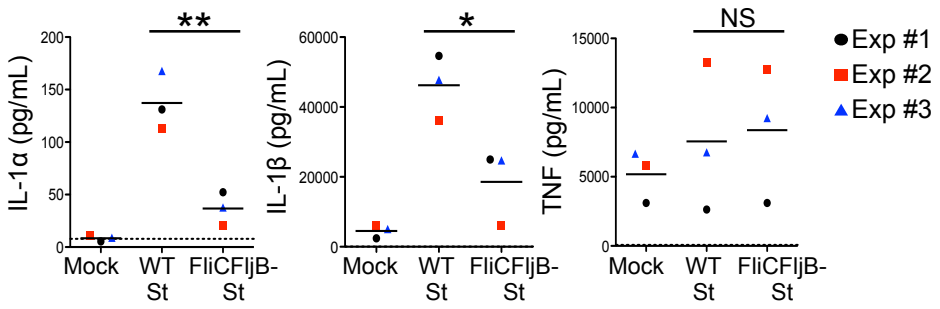

Figure 5-1. IL-1α and IL-1β release from THP-1 cells in response to *S.* Typhimurium infection is dependent on the presence of bacterial flagellin. PMA-differentiated THP-1 cells were primed with LPS and infected with wild-type *S.* Typhimurium (WT St), flagellin-deficient *S.* Typhimurium (FliCFljB- St), or mock infected with PBS for 4 hours. IL-1α and IL-1β levels in the supernatants were measured by ELISA. Shown are the pooled results of 3 independent experiments in THP-1 cells. Each data point shows the mean of triplicate infected wells. * is $p<0.05$ and ** is $p<0.01$ by unpaired t-test. Dashed line is the limit of detection.

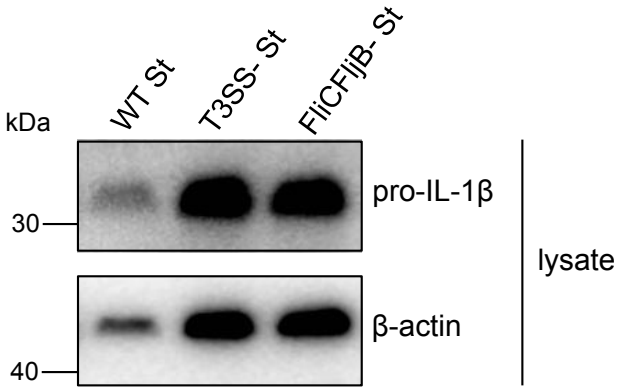

Figure 5-2. THP-1 cells do not have a defect in upregulating pro-IL-1β in response to flagellin-deficient *S.* Typhimurium. PMA-differentiated THP-1 cells were primed with LPS and infected with wild-type *S.* Typhimurium (WT St), T3SS-deficient *S.* Typhimurium (T3SS- St), or flagellin-deficient *S.* Typhimurium (FliCFljB- St) for 4 hours. Immunoblot analysis was performed on lysates for full-length IL-1β (pro-IL-1β), and blots were re-probed for β-actin as a loading control. Western blots are representative of 2 independent experiments.

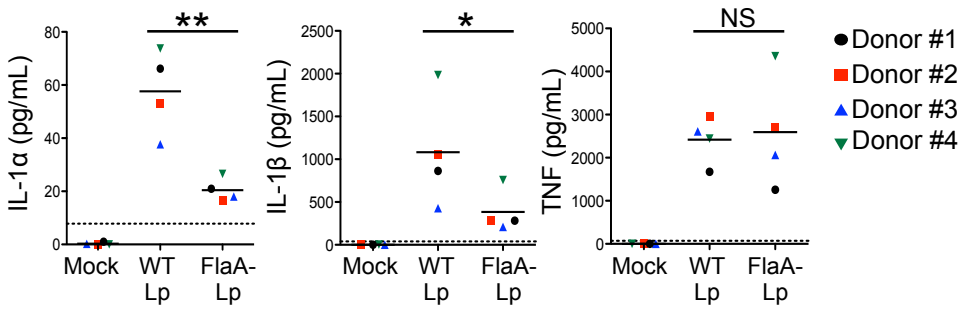

Figure 5-3. IL-1α and IL-1β release from primary human macrophages in response to *L. pneumophila* infection is dependent on the presence of bacterial flagellin. Primary human MDMs were infected with wild-type *L. pneumophila* (WT Lp), flagellin-deficient *L. pneumophila* (FlaA- Lp), or mock infected with PBS for 20 hours. IL-1α and IL-1β levels in the supernatants were measured by ELISA. Shown are the pooled results of 4 independent infections of cells from different healthy human donors. Each data point shows the mean of triplicate infected wells. * is $p<0.05$ and ** is $p<0.01$ by paired t-test. Dashed line is the limit of detection.

CHAPTER 6

DISCUSSION

Sections of this chapter have been adapted from a published peer-reviewed mini-review titled “Inflammasome-mediated cell death in response to bacterial pathogens that access the host cell cytosol: lessons from *Legionella pneumophila*” by **Cierra N. Casson** and Sunny Shin. Frontiers in Cellular and Infection Microbiology, 2013.

In the work presented in this dissertation, we have defined a critical role for caspase-11 in activating the inflammasome in murine macrophages in response to *L. pneumophila* and *Y. pseudotuberculosis*, two Gram-negative bacterial pathogens that use virulence-associated secretion systems to access the host cell cytosol during infection. We have also established a distinct role for IL-1α in driving neutrophil recruitment to the airway space independently of IL-1β during pulmonary *L. pneumophila* infection. Additionally, we have identified a conserved role for caspase-4 in human cells in controlling IL-1α release in response to *L. pneumophila*, *Y. pseudotuberculosis*, and *S.* Typhimurium. Finally, we have identified bacterial flagellin as a potential activator of both the canonical and non-canonical inflammasome in human macrophages.

A. Caspase-11 mediates inflammasome activation in murine macrophages in response to Gram-negative bacterial pathogens

In our studies, we used caspase-1-deficient and caspase-11-deficient macrophages to define the precise roles of caspase-1 and caspase-11 during infection with *L. pneumophila*. Previous studies have demonstrated that caspase-11 induces rapid cell death in response to bacterial pathogens that escape from the phagosome and enter the macrophage cytosol, such as *B. thailandensis* (Aachoui et al., 2013). Caspase-11 is also activated by *L. pneumophila* and *S.* Typhimurium that aberrantly enter the cytosol due to loss of vacuolar membrane integrity (Aachoui et al., 2013). However, a role for caspase-11 in generating an immune response against pathogens that remain outside of the cytoplasm but use secretion systems to introduce bacterial products into the host cell cytosol had not been investigated.

Through our work, we and others have determined that caspase-11 is robustly activated by *L. pneumophila* that remain within the pathogen-containing vacuole during infection, and this caspase-11 activation is independent of bacterial flagellin (Case et al., 2013; Casson et al., 2013). Though there is a moderate effect of a lack of caspase-11 on IL-1α release in response to WT Lp, there is a much more dramatic effect when the bacteria lack flagellin. We speculate that because the presence of cytosolic flagellin is a potent stimulus for canonical inflammasome activation, NAIP5/NLRC4-dependent caspase-1 activation is dominant during infection with WT bacteria. However, we speculate that caspase-11 may have evolved to respond rapidly and specifically to pathogens that access the host cell cytosol but lack or down-regulate flagellin during infection in an attempt to evade host immune recognition (Minnich and Rohde, 2007), as flagellin is an important conserved target for innate immune recognition (Vijay-Kumar and Gewirtz, 2009). In our studies, we find that after MyD88- and TRIF-dependent upregulation of caspase-11, host cells undergo rapid caspase-11-mediated cell death, occurring in less than 4 hours, in response to Δ*flaA* Lp. Non-canonical inflammasome activation in response to Δ*flaA* Lp requires T4SS-mediated cytosolic access, as Δ*dotA* Lp do not activate caspase-11. Like caspase-1-mediated pyroptosis, caspase-11-dependent cell death leads to release of important inflammatory mediators, such as IL-1α, IL-1β, and IL-18. Caspase-11 is required for cell death and IL-1α release and additionally enhances NLRP3-dependent caspase-1 activation and IL-1β and IL-18 secretion (Figure 6-1). NLRC4-independent caspase-1 activation and IL-1β and IL-18 secretion require ASC and NLRP3, although the identity of the *L. pneumophila*-derived signal sensed via NLRP3 is still unknown (Case et al., 2009; 2013; Casson et al., 2013).

Additionally, we extended the finding that caspase-11 responds to Gram-negative bacterial pathogens that use virulence-associated secretion systems to *Y. pseudotuberculosis*, a pathogen that uses a T3SS to access the host cytosol during infection. It is intriguing that both the presence of the T4SS and T3SS are sensed by murine macrophages to activate caspase-11, as the T4SS and T3SS are evolutionarily quite distinct. The pathogenic T4SS most closely resembles secretion systems used for conjugative transfer (Christie et al., 2014), while the pathogenic T3SS is evolutionarily related to the flagellar apparatus (Galán et al., 2014). However, the presence of either secretion system leads to caspase-11 activation, implying that it is probably not a structural component of the secretion systems that is recognized, as the proteins that make up the T4SS and T3SS are evolutionarily unrelated (Cambronne and Roy, 2006). Additionally, it is not likely that a particular conserved bacterial effector activates caspase-11, as the *Y. pseudotuberculosis* strain that we used (Δ6 Yp) lacks the six known secreted *Yersinia* effectors. Instead, our data raise two potential models for how caspase-11 is activated in murine macrophages: either a common virulence activity of the T4SS and T3SS, such as pore formation, is sensed by host cells, or a conserved PAMP is translocated by both the T4SS and T3SS to activate caspase-11.

B. Potential models for how caspase-11 is activated by virulence-associated secretion systems

Following from evidence that only Gram-negative and not Gram-positive bacterial species activate caspase-11 (Rathinam et al., 2012b), recent studies have shown that cytosolic LPS activates caspase-11 independently of extracellular sensing by TLR4 (Hagar et al., 2013; Kayagaki et al., 2013). Additionally, LPS isolated specifically from *L.*

pneumophila is capable of triggering caspase-11-dependent cell death in murine macrophages (Pilla et al., 2014). Therefore, the most likely current model for how caspase-11 is activated by bacteria that remain outside of the cytosol but use specialized secretion systems to deliver bacterial products into the host cell is that the presence of the T4SS or T3SS somehow allows LPS to gain access to the host cytosol. One possibility is that the T4SS physically translocates LPS from the bacterial cytoplasm into the host cytosol. Another possibility is that pores formed by the insertion of the T4SS into the phagosome membrane allow LPS that has somehow become dissociated from the bacterial cell wall or LPS from degraded bacteria to enter the cytosol. Yet a third possibility is that sensors in the host cytoplasm respond to the presence of the T4SS and induce host-mediate vacuolar disruption, which in turn delivers LPS into the macrophage cytosol. This last possibility is supported by evidence that a subset of IFN-inducible guanylate-binding proteins (GBPs) enhance disruption of pathogen-containing vacuoles during Gram-negative bacterial infection, which in turn leads to caspase-11 activation (Meunier et al., 2014). Interestingly, GBPs are also required for cell death in response to transfection of purified *L. pneumophila* LPS in IFNγ-primed macrophages, indicating that GPBs may play a role in activating caspase-11 independently of vacuolar disruption as well (Pilla et al., 2014). How GBPs are able to sense cytosolic LPS to enhance caspase-11 activation requires further investigation.

Though LPS from *L. pneumophila* is clearly capable of activating caspase-11 when transfected directly into the host cytosol (Pilla et al., 2014), it still remains unclear whether *L. pneumophila* LPS activates caspase-11 during physiological infection conditions. The lipid A component of *L. pneumophila* LPS is distinct from other common

Gram-negative bacterial pathogens in that it contains branched-chain fatty acids with few free hydroxyl groups that are nearly twice the length of the acyl chains from other pathogens (Wong et al., 1979; Zähringer et al., 1995). *L. pneumophila* LPS is a poor stimulator of TLR4, likely because it does not interact with the adaptor CD14 (Neumeister et al., 1998). Additionally, the effects of *L. pneumophila* LPS are not neutralized by incubation with Polymyxin B, an endotoxin-binding protein (Wong et al., 1979). Other pathogens that have lipid A components with long acyl chains that are poor stimulators of TLR4, such as *Helicobacter pylori*, possess LPS that does not activate caspase-11 (Kayagaki et al., 2013). Therefore, it is possible that the small amount of *L. pneumophila* LPS that may access the cytosol during physiological infection conditions is not very stimulatory to caspase-11 and is thus not sufficient to induce inflammasome activation, whereas transfection of large amounts of LPS directly into the cytosol could surpass a threshold needed to activate caspase-11 (Pilla et al., 2014). Currently, though, the only bacterial factor that has been shown to initiate non-canonical inflammasome activation is cytosolic LPS (Hagar et al., 2013; Kayagaki et al., 2013). However, we speculate that the T4SS and T3SS could certainly allow for other bacterial PAMPs, such as RNA, to enter the host cytosol as well. For some Gram-negative bacteria, it is thought that bacterial RNA may access the host cytosol to activate NLRP3 and caspase-11 (Kanneganti et al., 2006; Rathinam et al., 2012b). However, translocation of *L. pneumophila* RNA to initiate inflammasome activation has not been verified experimentally.

Alternatively, it is possible that caspase-11 is not activated by a PAMP at all but is activated by the host cell responding to pore formation induced by the T3SS or T4SS. As

NLRP3 is known to be activated by a wide variety of pathogen and host-derived stimuli, including changes in intracellular calcium levels (Brough et al., 2003; Murakami et al., 2012), it is possible that caspase-11 responds to changes in ion concentrations or other cellular stress signals induced by the T3SS or T4SS, which may feed forward into the caspase-11-dependent NLRP3 activation that we observe during infection. Further studies are needed to clarify what triggers the host response to Δ*flaA* Lp and to elucidate the molecular pathways that lead to caspase-11-mediated cell death and cytokine secretion in murine macrophages.

The model for how Δ*sdhAflaA* Lp activates caspase-11 is that *L. pneumophila* that aberrantly enter the macrophage cytosol expose their LPS directly to caspase-11. However, Δ*sdhAflaA* Lp are degraded and activate a type I IFN response when they enter the cytosol (Creasey and Isberg, 2012), and bacterial DNA is released, which activates AIM2 (Ge et al., 2012). Therefore, upon degradation, it is possible that other PAMPs, such as bacterial RNA and cell wall components, are released into the host cell cytosol and are capable of activating other cytosolic PRRs. Though caspase-11 is clearly required for cell death in response to Δ*sdhAflaA* Lp (Aachoui et al., 2013), it is not definitive that LPS is the bacterial component that is sensed upon entry of Δ*sdhAflaA* Lp into the macrophage cytosol. Therefore, we speculate that it is possible that another PAMP is detected in the cytosol to activate caspase-11 during infection with Δ*sdhAflaA* Lp. Whether LPS is the only trigger for caspase-11 or not, caspase-11 is clearly relevant for defense against many Gram-negative bacterial pathogens, especially those that lack or down-regulate flagellin during infection to avoid detection via caspase-1.

C. IL-1α and IL-1β have both distinct and overlapping roles during pulmonary *Legionella pneumophila* infection

Through our studies, we uncovered distinct intracellular mechanisms that lead to the release of IL-1α and IL-1β. We find that IL-1β secretion absolutely requires caspase-1, while IL-1α release is independent of caspase-1 and requires caspase-11. Therefore, because they are differentially regulated *in vitro*, we wanted to elucidate if IL-1α and IL-1β could have distinct roles *in vivo* during infection. Using antibodies that neutralize IL-1α and IL-1β, we have shown that IL-1α can act independently of IL-1β during a mouse model of Legionnaire's disease. As IL-1α and IL-1β bind the same receptor *in vivo* (Dower et al., 1986), it was largely assumed that they have redundant biological roles. However, previous studies have shown that IL-1α and IL-1β can play unique roles *in vivo*. For example, IL-1α and IL-1β are each required individually for survival during a mouse model of pulmonary infection with *M. tuberculosis* (Mayer-Barber et al., 2011), suggesting that they have non-redundant roles. Additionally, IL-1α can drive neutrophil recruitment into the peritoneal cavity upon introduction of MSU crystals in a mouse model of gout (Gross et al., 2012). However, it was unknown if IL-1R signaling is important for control of *L. pneumophila* replication in the lung and if IL-1α and IL-1β have non-redundant roles during a mouse model of Legionnaires' disease.

We have shown that IL-1R signaling *in vivo* is critical for host defense against pulmonary *L. pneumophila*, including neutrophil recruitment to the airway space and control of bacterial burden. Additionally, we find that neutralizing IL-1α alone has a dramatic effect on neutrophil recruitment, while neutralizing IL-1β alone has no effect. Therefore, IL-1β is not sufficient to drive maximal neutrophil recruitment during infection. However, we

find that neutralization of both IL-1α and IL-1β simultaneously has an even more striking effect on neutrophil recruitment than neutralization of IL-1α alone, implying that IL-1α and IL-1β share some overlapping roles during infection. Recent studies have shown that IL-1α is important for neutrophil recruitment during pulmonary infection with pathogenic *Pseudomonas aeruginosa* as well (Moussawi and Kazmierczak, 2014). Based on our *in vitro* data, we speculate that IL-1α release in response to Δ*flaA* Lp is caspase-11-dependent *in vivo*, though there are caspase-11-independent sources of IL-1α in response to WT Lp *in vivo* (Barry et al., 2013). Presumably, caspase-11-deficient mice would also have a defect in neutrophil recruitment to the airway space, though cell recruitment in caspase-11-deficient mice has not been examined experimentally.

As IL-1α and IL-1β both bind the IL-1R, it is unclear how they elicit different biological effects *in vivo*. Currently, we have two potential models for how IL-1α could modulate neutrophil recruitment independently of IL-1β. First, we speculate that IL-1α and IL-1β may engage the IL-1R in slightly different manners to elicit different downstream signaling cascades. For example, binding of IL-1α to the IL-1R may recruit different IL-1 receptor-associated kinases (IRAKs) than IL-1β (Flannery and Bowie, 2010), and differential recruitment of IRAKs could lead to differences in the production of chemokines important for neutrophil recruitment, such as C-X-C-motif ligand 1 (CXCL1)/keratinocyte-derived chemokine (KC) (Moser et al., 1990; Oquendo et al., 1989). Second, we speculate that total abundance or availability of each cytokine to access the IL-1R may have an effect on signaling *in vivo*. In general, we see higher total amounts of IL-1α protein in the BALF during infection, and IL-1α has a higher affinity for the IL-1R on certain cell types (Dower et al., 1986). Therefore, IL-1α could have a

dominant role in recruiting neutrophils during *L. pneumophila* infection because there is more cytokine available to engage the IL-1R.

In addition to its extracellular role in recruiting immune cells to the airway space during infection, IL-1α could potentially have a cell-intrinsic role in initiating immune responses that we did not uncover by using neutralizing antibodies. For example, it is known that pro-IL-1α translocates into the nucleus where it can interact with histone acetyltransferases and modulate the transcription of other pro-inflammatory cytokines (Buryskova et al., 2004; Cheng et al., 2008; Wessendorf et al., 1993). Experiments comparing infection of IL-1α-deficient mice to mice that have received IL-1α-neutralizing antibodies would elucidate any cell-intrinsic roles for IL-1α in response to *L. pneumophila*. However, in addition to its protective role, IL-1α can be pathological during bacterial infection as well. For example, IL-1α induces pathological intestinal inflammation during infection with the gastro-intestinal pathogen *Yersinia enterocolitica* (Dube et al., 2001). Therefore, studying the individual roles of IL-1α and IL-1β during disease could lead to the development of therapeutics that target individual cytokines rather than the IL-1R, which may ameliorate pathological symptoms while not completely compromising host defense.

D. Caspase-4 has a conserved role in inflammasome activation against *Legionella pneumophila*, *Yersinia pseudotuberculosis*, and *Salmonella* Typhimurium

In our studies, we used siRNA to knockdown caspase-4 to elucidate a role for caspase-4 in inflammasome activation in primary human macrophages. Previous data had demonstrated that LPS can bind directly to caspase-4 and caspase-5 in immortalized

macrophage, keratinocyte, and epithelial cell lines to induce cell death (Shi et al., 2014). Additionally, caspase-4 mediates IL-18 release in response to infection with *S. Typhimurium* in immortalized human epithelial cells (Knodler et al., 2014). However, a role for caspase-4 in mediating inflammasome activation, including cytokine release, in primary human cells had not been investigated.

In primary human MDMs, we find that *L. pneumophila* induces robust IL-1α and IL-1β release that is dependent on the presence of the T4SS. While IL-1β secretion requires caspase-1, IL-1α release is independent of caspase-1 catalytic activity. Thus, *L. pneumophila* activates both a canonical caspase-1-dependent and non-canonical caspase-1-independent inflammasome in human cells. We find that caspase-4 mediates cell death and IL-1α release in response to *L. pneumophila* in primary human MDMs. However, in contrast to murine caspase-11, human caspase-4 does not appear to play a significant role in mediating IL-1β secretion during infection, highlighting an important difference between non-canonical inflammasome activation in murine and human macrophages (Figure 6-2). Additionally, we find that caspase-4 plays a conserved role in non-canonical inflammasome activation via release of IL-1α in response to both *Y. pseudotuberculosis* and *S.* Typhimurium, Gram-negative bacterial pathogens that use an evolutionarily unrelated T3SS to access the host macrophage cytosol during infection. Interestingly, though caspase-4 contributes to cell death during infection with both *L. pneumophila* and *Y. pseudotuberculosis*, cell death is caspase-4-independent during infection with *S.* Typhimurium.

Though caspase-4 does not mediate IL-1β secretion during infection, caspase-4 contributes to both IL-1α and IL-1β release in response to transfection of LPS directly into the cytosol of primary human macrophages. Therefore, when LPS is the only PAMP present in the cytosol, it likely binds directly to caspase-4 and induces inflammasome activation, including IL-1α and IL-1β release and cell death. However, our data imply that in the context of infection with Gram-negative bacterial pathogens, a caspase-4-independent inflammasome pathway is triggered to induce IL-1β secretion. This caspase-4-independent inflammasome requires caspase-1 and ASC, and we speculate that NLRP3 is upstream of ASC for caspase-1 activation, as NLRP3 and ASC mediate IL-1β release in THP-1 cells in response to *S.* Typhimurium infection (Li et al., 2008) and NLRP3 is recruited to ASC foci during infection (Man et al., 2014). It is possible that a PAMP other than LPS, such as bacteria RNA (Kanneganti et al., 2006), is sensed via NLRP3 during infection of primary human macrophages. Conversely, it is possible that NLRP3 responds to cell stress induced by the presence of the T3SS or T4SS to activate caspase-1. Either way, our data suggest that caspase-1 activation is dominant over caspase-4 activation to control IL-1β secretion during Gram-negative bacterial infection of human macrophages. One explanation for how caspase-1 mediates IL-1β secretion during infection is that activation of caspase-1 somehow dampens the ability of caspase-4 to induce IL-1β release in response to binding LPS. For example, it is possible that activation of caspase-1 in human macrophages during infection could sequester IL-1β away from caspase-4. An alternative explanation for how caspase-1 mediates IL-1β secretion during infection is that LPS does not physically bind to caspase-4 in the context of bacterial infection and instead engages an upstream NLR that specifically activates caspase-4 for IL-1α release and cell death. Though LPS is clearly capable of

binding directly to caspase-4 (Shi et al., 2014), it has not been shown experimentally that LPS physically interacts with caspase-4 during actual bacterial infection. Therefore, it is possible that LPS could trigger caspase-4 in two manners: direct binding, which induces both IL-1α and IL-1β, or indirect activation via an as-yet-unidentified upstream NLR, which specifically induces IL-1α release.

E. Is LPS the only trigger for the non-canonical inflammasome?

As is the case for murine caspase-11, intracellular LPS is the only bacterial ligand that has been identified to activate human caspase-4 (Shi et al., 2014). However, unlike caspase-11, caspase-4 is activated by more stimuli than just Gram-negative bacterial infection. For example, caspase-4 cleavage is induced in neuroblastoma and epithelial cell lines in response to tunicamycin- and thapsigargin-induced ER stress (Hitomi et al., 2004). Tunicamycin induces ER stress by triggering the unfolded protein response (UPR) through blocking N-linked glycosylation that happens in the ER (Hong et al., 2004; Kuo and Lampen, 1974), while thapsigargin induces ER stress by depleting ER calcium stores (Jackson et al., 1988; Sano and Reed, 2013). Additionally, caspase-4 is activated by UVB irradiation in keratinocytes to control IL-1β and IL-18 release, though caspase-4 is upstream of caspase-1 activation in response to UVB irradiation (Sollberger et al., 2012). Interestingly, these data imply that caspase-4 is activated by a seemingly broad array of stimuli, ranging from bacterial infection to ER stress to irradiation. However, there may be commonalities between these triggers that induce caspase-4 activation.

As there is no bacterial LPS present during ER stress or UVB irradiation, there are two potential models for how caspase-4 is activated in human cells. First, caspase-4 could be activated by different ligands under different conditions. For example, it is known that LPS binds directly to the CARD domain of caspase-4 to induce activation (Shi et al., 2014). Therefore, it is possible that during bacterial infection, LPS binds to the CARD domain of caspase-4, but during ER stress and UVB irradiation, a host protein mimics the properties of LPS and also binds to the CARD domain of caspase-4 to induce activation. Alternatively, caspase-4 could be activated by different triggers under different conditions via activation of different upstream NLRs. For example, NLRP3 and ASC are known to be upstream of caspase-4-mediated inflammasome activation in keratinocytes (Feldmeyer et al., 2007; Sollberger et al., 2012).

The second hypothesis for how caspase-4 is activated in human cells is that host cells respond to a common stress signal induced by Gram-negative bacterial infection, ER stress, and UVB irradiation to induce caspase-4 activation. When *L. pneumophila* establishes its replicative niche within host cells, the bacterium hijacks ER-derived vesicles to form the LCV (Horwitz and Silverstein, 1980; Swanson and Isberg, 1995). This modulation of vesicle trafficking could presumably trigger an ER-related stress response within host cells. Both ER stress and UVB irradiation induce elevated cytoplasmic calcium levels, which has been linked to inflammasome activation in multiple cell types (Feldmeyer et al., 2007; Murakami et al., 2012; Sano and Reed, 2013). Therefore, perturbations of calcium homeostasis or ER function could potentially be a common mechanism downstream of all stimuli that lead to caspase-4 activation. In the context of Gram-negative bacterial infection, LPS could be a second signal required

for activation of caspase-4. However, we speculate that LPS might be the trigger for caspase-4 activation when bacterial pathogens reside and replicate within the cytosol, but endogenous stress signals, such as changes in calcium concentrations, might be the actual trigger for caspase-4 activation by pathogens that remain outside of the host cell cytosol but use specialized secretion systems to access the cytosol during infection. Additionally, our preliminary data provocatively suggest that flagellin may be a major trigger for both canonical and non-canonical inflammasome activation during infection with both *L. pneumophila* and *S.* Typhimurium.

F. Flagellin as a trigger for inflammasome activation in human macrophages

Through our preliminary studies, we have shown that bacteria that lack flagellin have a defect in triggering IL-1α and IL-1β release from human macrophages compared to wild-type bacteria. We find that both IL-1α and IL-1β secretion are reduced during infection with flagellin-deficient *L. pneumophila* and *S.* Typhimurium. The decrease in IL-1 secretion does not appear to be due to a general priming defect, as cells infected with flagellin-deficient bacteria have no defect in upregulating pro-IL-1β. However, the current paradigm is that human cells do not actually respond to cytosolic flagellin, as human NAIP does not associate with flagellin (Zhao et al., 2011). Instead, human NAIP is thought to associate specifically with the needle protein of the T3SS (Yang et al., 2013). However, the biochemical assays performed to assess the binding partners of human NAIP involved overexpression of inflammasome components in a heterologous system in 293T cells. Therefore, it is still possible that flagellin associates with endogenous NAIP during bacterial infection of macrophages. Additionally, there is evidence that human NAIP responds to flagellin during infection with *L. pneumophila*. Flagellin-

deficient *L. pneumophila* grow better than WT Lp in THP-1 cells, suggesting that recognition of flagellin restricts bacterial growth, and growth of WT Lp is equivalent to that of Δ*flaA* Lp when NAIP is knocked down using siRNA (Vinzing et al., 2008). Therefore, there is previous evidence that flagellin is sensed by human macrophages during bacterial infection.

As both IL-1α and IL-1β release are affected by flagellin deficiency, our data imply that both canonical and non-canonical inflammasome activation are triggered by flagellin. However, we find that IL-1α and IL-1β release are controlled by two separate inflammasome pathways during Gram-negative bacterial infection of human macrophages, with IL-1α requiring caspase-4 and IL-1β requiring caspase-1 for secretion. Therefore, our data potentially place flagellin as common inflammasome trigger upstream of both caspase-1 and caspase-4 (Figure 6-2). We speculate that flagellin could activate a single NLR, such as human NAIP, that then associates with both caspase-1 and caspase-4 to induce inflammasome activation. However, it is still unclear if NAIP is required for IL-1α and IL-1β release in human macrophages. If flagellin triggers non-canonical inflammasome activation via caspase-4, our data would introduce a paradigm shift in how we think about inflammasome activation during Gram-negative bacterial infection, as LPS is the only bacterial ligand currently thought to activate caspase-4 (Shi et al., 2014).

G. Potential for pathogenic inhibition of the inflammasome

Many viruses encode proteins that inhibit inflammasome activation. The first viral protein identified as a caspase-1 inhibitor is a serpin encoded by cowpox virus (Ray et al.,

1992). Subsequently, it was found that vaccinia virus encodes a homologous protein that blocks IL-1β secretion from human cells (Kettle et al., 1997). Additionally, other members of the poxvirus family encode proteins that mimic the cellular pyrin domain-only proteins (cPOPs) (Johnston et al., 2005), which block inflammasome activation by binding to ASC (Bedoya et al., 2007; Dorfleutner et al., 2007; Stehlik et al., 2003).

Though many bacteria have mechanisms to avoid or dampen inflammasome activation, few bacteria encode proteins analogous to the viral serpins that target caspase-1 directly (Taxman et al., 2010). Some strains of *Pseudomonas aeruginosa* encode a phospholipase, ExoU, which blocks activation of caspase-1, though it remains unclear if inhibition by ExoU occurs through a direct interaction with caspase-1 or by targeting of an upstream mediator of inflammasome activation (Sutterwala et al., 2007). Additionally, *Mycobacterium tuberculosis* encodes a zinc metalloprotease that blocks inflammasome activation through a poorly defined mechanism (Master et al., 2008). *Y. pseudotuberculosis* encodes an effector protein, YopK, that block recognition of the T3SS (Brodsky et al., 2010). However, *Y. pseudotuberculosis* also encodes the effector YopM, which physically interacts with murine caspase-1 to block inflammasome activation (LaRock and Cookson, 2012).

Currently, no bacterial effectors have been identified that counteract the activity of caspase-11. However, certain cytosolic Gram-negative bacterial pathogens may have evolved to modify their LPS so that it is not readily detected by caspase-11. For example, *Francisella novicida*, which replicates within the macrophage cytosol, evades caspase-11-mediated detection by encoding LPS with tetra-acylated lipid A that does not

stimulate caspase-11 (Hagar et al., 2013). Intriguingly, though no bacterial proteins have been identified that antagonize caspase-11, *Shigella flexneri* encodes an effector protein, OspC3, that blocks caspase-4 activity (Kobayashi et al., 2013). Whether other Gram-negative pathogens that live within the host cytosol, such as *Burkholderia thailandensis*, which robustly triggers caspase-11, have evolved to encode effectors to evade caspase-4-mediated detection in human cells remains an important area for further investigation.

H. A role for caspase-5 in inflammasome activation in human macrophages?

In our studies, we did not detect caspase-5 cleavage in response to infection with virulent *L. pneumophila*, *Y. pseudotuberculosis*, or *S.* Typhimurium. Though caspase-5 cleavage is indicative of activation, caspases do not need to be processed to perform all of their effector functions. For example, though caspase-1 requires cleavage to induce IL-1β secretion, caspase-1 can still mediate pyroptotic cell death independently of auto-processing (Broz et al., 2010). Therefore, it is possible that caspase-5 has a role in inflammasome activation against pathogens that use virulence-associated secretion systems that does not require caspase-5 cleavage. Notably, as caspase-4 is surprisingly not involved in IL-1β secretion during infection of human macrophages, we speculate that caspase-5 could have a role in enhancing caspase-1-dependent IL-1β release. It is possible that the two putative functional orthologs of murine caspase-11 have evolved to carry out distinct subsets of the functions of caspase-11. Specifically, caspase-4 may have evolved to direct IL-1α release, while caspase-5 may have evolved to direct IL-1β secretion. As both caspase-4 and caspase-5 bind directly to LPS, it is likely that LPS is a trigger for both caspase-4 and caspase-5 activation during Gram-negative bacterial

infection. Alternatively, both caspase-4 and caspase-5 could interact with NAIP to mediate the cytosolic sensing of bacterial flagellin. Elucidating a role for caspase-5 in inflammasome activation will be informative for understanding the biological roles of the two human homologs of murine caspase-11.

I. Future directions

There are many questions that remain to be answered to understand non-canonical inflammasome activation in both murine and human macrophages. In murine macrophages, it is unclear if LPS is the trigger for caspase-11 activation during infection with *L. pneumophila* and *Y. pseudotuberculosis*. Mutating the LPS synthesis genes in either bacterium would be challenging because defects in LPS synthesis could lead to membrane integrity issues in the bacteria. For examples, mutants of *E. coli* that are defective in acylation of lipid A fail to grow in rich media and lyse upon centrifugation (Vorachek-Warren et al., 2002). Therefore, LPS mutants may be more sensitive to lysis during infection of macrophages and could release additional PAMPs into the host cytosol. However, *Y. pseudotuberculosis* switches from making mostly penta-acylated LPS when grown at 21°C to making mostly tetra-acylated LPS when grown at 37°C (Rebeil et al., 2004). As the tetra-acylated LPS from the closely related *Yersinia pestis* does not trigger caspase-11 (Hagar et al., 2013), it is possible that forcing *Y. pseudotuberculosis* to express only tetra-acylated LPS would be a useful tool for determining if LPS is the only PAMP sensed via caspase-11 during actual bacterial infection.

In human macrophages, it is also unclear if LPS is the trigger for caspase-4 during infection. Additionally, it is unclear if caspase-5, the other putative functional ortholog of murine caspase-11, plays a role in inflammasome activation. As caspase-5 is difficult to target using siRNA, it is possible that clustered regularly interspaced short palindromic repeats (CRISPR) technology (Cong et al., 2013; Jinek et al., 2012; Wang et al., 2014) could be used to target caspase-5 in human macrophages to elucidate a role for caspase-5 in inflammasome activation during Gram-negative bacterial infection. Though siRNA knockdown of caspase-4 results in a significant decrease in IL-1α release, IL-1α release is not absolutely abrogated. Therefore, either the small amount of caspase-4 protein left in the cell after knockdown is enough to induce some inflammasome activation, or another enzyme, such as caspase-5, is involved in inflammasome activation as well. Targeting caspase-5 would be valuable for interrogating if caspase-5 enhances caspase-1-dependent IL-1β secretion from human macrophages, just as caspase-11 enhances caspase-1 activation in murine cells.

As flagellin-deficient *L. pneumophila* and *S.* Typhimurium show decreased inflammasome activation, flagellin is potentially a trigger for both canonical and non-canonical inflammasome activation in human macrophages. However, even though macrophages infected with flagellin-deficient bacteria do not show a defect in upregulation of pro-IL-1β, it is still possible that TLR5-mediated extracellular sensing of flagellin is involved in priming inflammasome activation in human macrophages. To test this possibility, we could use antibodies that bind and neutralize the ability of TLR5 to detect flagellin during infection with WT Lp and WT St to determine if TLR5-mediated sensing of flagellin is involved in regulating inflammasome activation. To assess if

intracellular flagellin is a trigger for inflammasome activation, we could use the established system of introducing purified flagellin into the cytosol by fusing flagellin to the N-terminal domain of anthrax lethal factor and delivering it into the cytosol by using protective antigen (Moltke et al., 2012). This system has been used to demonstrate that flagellin is a trigger for inflammasome activation in murine macrophages (Moltke et al., 2012). Alternatively, we could express *L. pneumophila* or *S.* Typhimurium flagellin in *Listeria monocytogenes*, a Gram-positive bacterial pathogen that does not encode LPS and ruptures the vacuole to replicate within the macrophage cytosol. Expression of flagellin by *L. monocytogenes* enhances inflammasome activation in murine macrophages (Warren et al., 2011).

J. Concluding remarks

Overall, the studies performed in this dissertation have revealed that both murine caspase-11 and its homolog human caspase-4 are critical for mediating inflammasome activation against Gram-negative bacterial pathogens. Additionally, our experiments have uncovered important differences between the regulation of IL-1 family cytokine release in murine and human macrophages. Studying the inflammasome pathways triggered by the pathogen *L. pneumophila* has shaped our knowledge of how host cells are poised to respond to intracellular pathogens, and the pathways triggered by *L. pneumophila* are applicable to infection by other Gram-negative bacterial pathogens, including *Y. pseudotuberculosis* and *S.* Typhimurium. Whether the bacterium utilizes a T3SS or T4SS to access the host cytosol, additionally delivers flagellin into the cytoplasm, or physically enters the cytosol itself, the host has evolved multiple ways to restrict replication of the pathogen and trigger an appropriate immune response.

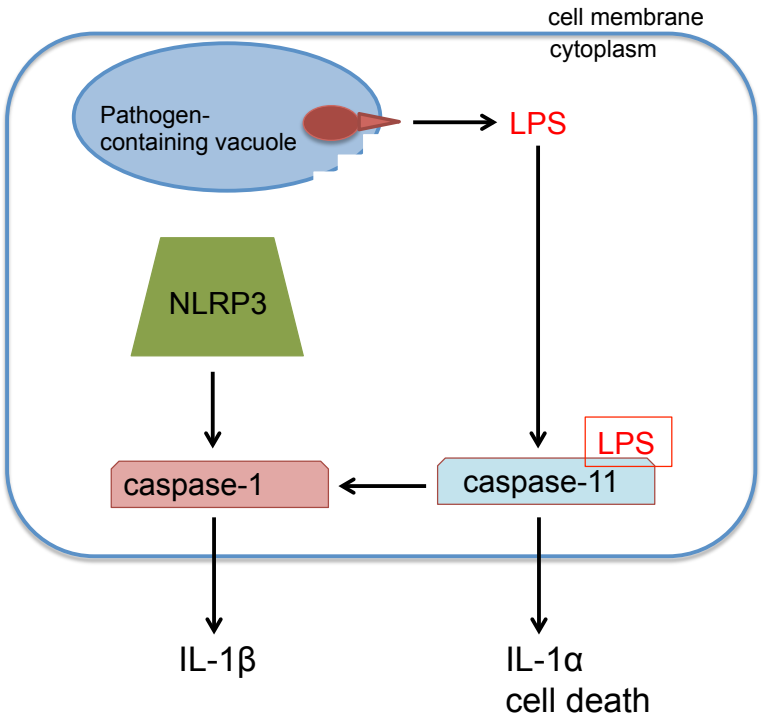

Figure 6-1. Model for non-canonical inflammasome activation by Gram-negative bacterial pathogens that use virulence-associated secretion systems in murine macrophages. Upon infection of murine macrophages, the use of the T3SS or T4SS to access the host cell cytosol to establish a replicative niche enhances vacuolar disruption, potentially in combination with murine GBPs, and bacterial LPS gains access to the cytosol. Once in the cytosol, LPS binds directly to caspase-11 to induce IL-1α release and cell death. Additionally, caspase-11 somehow enhances NLRP3- and caspase-1-dependent IL-1β secretion.

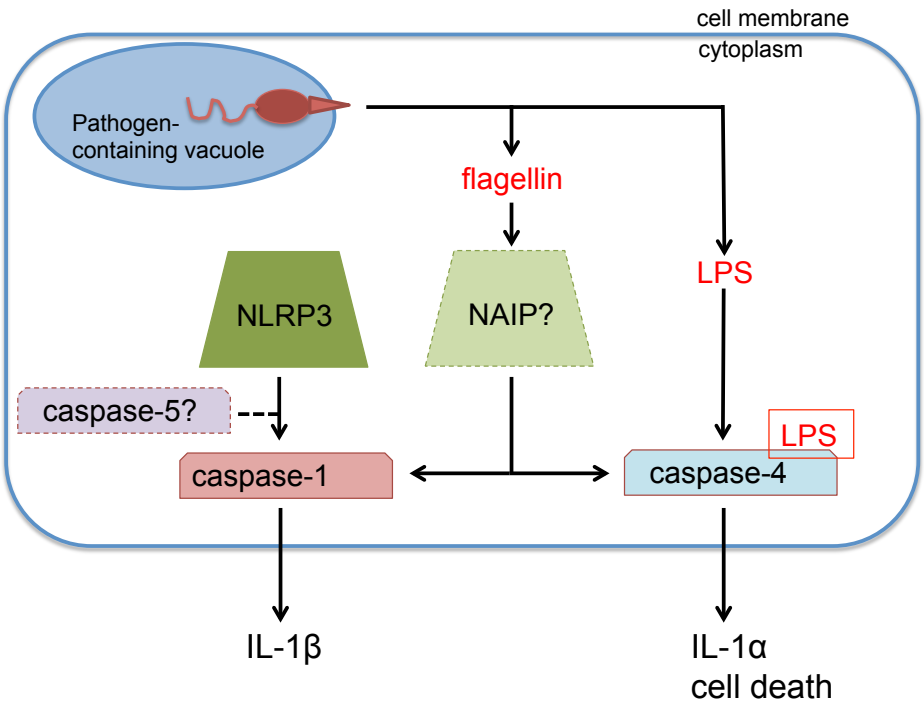

Figure 6-2. Model for non-canonical inflammasome activation by Gram-negative bacterial pathogens that use virulence-associated secretion systems in human macrophages. Upon infection of human macrophages, the use of the T3SS or T4SS to access the host cell cytosol to establish a replicative niche allows bacterial flagellin and LPS to gain access to the cytosol. Once in the cytosol, LPS binds directly to caspase-4 to induce IL-1α release and cell death. Additionally, flagellin is sensed, presumably through human NAIP, and somehow enhances both NLRP3/caspase-1-dependent IL-1β secretion and caspase-4-dependent IL-1α release and cell death. It remains unclear if caspase-5 responds to cytosolic flagellin to contribute to caspase-1-dependent IL-1β secretion.

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