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Investigating The Role Of The Human Naip/nlrc4 Inflammasome In Host Defense Against Gram-Negative Bacterial Infection

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Abstract

Inflammasomes are key multiprotein intracellular complexes that mediate host defense against pathogenic microorganisms by activating caspase-1-dependent cytokine secretion and cell death. In mice, specific nucleotide-binding domain, leucine-rich repeat-containing family, apoptosis inhibitory proteins (NAIPs) sense components of the type III secretion system (T3SS) and flagellar apparatus. Upon sensing of bacterial components, NAIPs recruit the nucleotide-binding domain, leucine-rich repeat-containing family, CARD domain-containing protein 4 (NLRC4). The resulting NAIP/NLRC4 inflammasome then recruits and activates caspase-1. Active caspase-1 mediates processing and secretion of IL-1 family cytokines and a proinflammatory cell death termed pyroptosis. In mice, bacterial ligands for four of seven distinct NAIPs are known: NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod protein, and both NAIP5 and NAIP6 recognize flagellin. In contrast, humans encode a single functional NAIP, raising the question of whether human NAIP senses one or multiple bacterial ligands. In this dissertation we show that, in contrast to murine NAIPs, promiscuous recognition of multiple bacterial ligands is conferred by a single human NAIP. We found that NAIP, but not NLRC4, appears to dictate the specificity or promiscuity of bacterial ligand recognition. In addition, our studies define a role for human NAIP in the inflammasome response to Salmonella Typhimurium infection. Overall, we provide a basis for understanding the mechanisms underlying human-specific innate immune responses against gram-negative bacterial infections.

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INVESTIGATING THE ROLE OF THE HUMAN NAIP/NLRC4 INFLAMMASOME IN HOST DEFENSE AGAINST GRAM-NEGATIVE BACTERIAL INFECTION

Valeria M. Reyes Ruiz

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ABSTRACT

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Inflammasomes are key multiprotein intracellular complexes that mediate host defense against pathogenic microorganisms by activating caspase-1-dependent cytokine secretion and cell death. In mice, specific nucleotide-binding domain, leucine-rich repeat-containing family, apoptosis inhibitory proteins (NAIPs) sense components of the type III secretion system (T3SS) and flagellar apparatus. Upon sensing of bacterial components, NAIPs recruit the nucleotide-binding domain, leucine-rich repeat-containing family, CARD domain-containing protein 4 (NLRC4). The resulting NAIP/NLRC4 inflammasome then recruits and activates caspase-1. Active caspase-1 mediates processing and secretion of IL-1 family cytokines and a proinflammatory cell death termed pyroptosis. In mice, bacterial ligands for four of seven distinct NAIPs are known: NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod protein, and both NAIP5 and NAIP6 recognize flagellin. In contrast, humans encode a single functional NAIP, raising the question of whether human NAIP senses one or multiple bacterial ligands. In this dissertation we show that, in contrast to murine NAIPs, promiscuous recognition of multiple bacterial ligands is conferred by a single human NAIP. We found that NAIP, but not NLRC4, appears to dictate the specificity or promiscuity of bacterial ligand recognition. In addition, our studies define a role for human NAIP in the inflammasome response to

Salmonella Typhimurium infection. Overall, we provide a basis for understanding the mechanisms underlying human-specific innate immune responses against gram-negative bacterial infections.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER 1: INTRODUCTION

Microorganisms colonizing our bodies consist of both commensal and harmful pathogens (Littman and Pamer, 2011). Our mucosal surfaces, such as the intestinal epithelium, form an important barrier against invading microbes. Innate immune cells at these surfaces have evolved to recognize pathogens and promote host defense (Belkaid and Artis, 2013). However, since innate immune defense relies on recognition of microbial structures that can be shared between commensals and harmful pathogens, discrimination between these two groups of microorganisms is needed to maintain homeostasis in our bodies or to eliminate the invading pathogen (Medzhitov, 2007).

1.1. Recognition of conserved structures in microbes by Toll-like receptors

Germline-encoded pattern recognition receptors (PRRs) coordinate the innate immune response against pathogens through recognition of conserved structures termed pathogen-associated molecular patterns (PAMPs). Many of these conserved structures are essential for the survival of the pathogen and are not expressed by eukaryotic cells (Janeway, 1989; Janeway and Medzhitov, 2002). They include bacterial components such as flagellin, lipopolysaccharide (LPS), peptidoglycan, and nucleic acids. Engagement of PRRs with PAMPs is essential to initiate defense mechanisms and to alert neighboring cells to the presence of a pathogen (Medzhitov, 2007; Kawai and Akira, 2011).

A subset of PRRs, termed Toll-like receptors (TLRs), has been extensively studied and can mediate extracellular sensing of microorganisms. TLRs are type I transmembrane proteins that can be encountered at the plasma membrane or in endosomal compartments. TLR1-TLR9 are conserved in both mice and humans. The TLRs that mediate recognition of nucleic acids are in endolysosomal compartments (TLR3, TLR7, TLR8, and TLR9), whereas the other members are found at the plasma membrane (TLR1,

TLR2, TLR4, TLR5, and TLR6) and can mediate recognition of extracellular components (Barton and Kagan, 2009; Kawai and Akira, 2010). TLR4 recognizes bacterial lipopolysaccharide (LPS), a major lipid component of the outer membrane from gram-negative bacteria (Poltorak et al., 1998; Hoshino et al., 1999). Interaction of TLR4 with LPS also requires the host proteins CD14 and MD-2 (Haziot et al., 1996; Shimazu et al., 1999; Moore et al., 2000). TLR2 can recognize a variety of ligands by forming heterodimers with TLR1 and TLR6. It can recognize bacterial lipoproteins (Aliprantis et al., 1999; Brightbill et al., 1999; Takeuchi et al., 2000), peptidoglycan (Schwandner et al., 1999; Takeuchi et al., 1999), and a component of yeast cell walls called zymosan (Underhill et al., 1999). TLR5 recognizes flagellin, the subunit that forms the bacterial flagellar apparatus (Hayashi et al., 2001).

Endosomal TLRs have been well-characterized for their role in recognition of nucleic acids to mediate immune defense against bacteria and viruses. TLR3 recognizes double-stranded RNA (dsRNA) that are produced during viral replication of single-stranded RNA (ssRNA) viruses (Liu et al., 2008; Kawai and Akira, 2010). TLR7 and TLR8 recognize ssRNA from viruses such as human immunodeficiency virus (Heil et al., 2004; Kawai and Akira, 2006). Finally, TLR9 recognizes unmethylated 2'-deoxyribo(cytidine-phosphate-guanosine) (CpG) DNA present in bacteria and viruses (Hemmi et al., 2000; Lund et al., 2003).

Recognition of microbial structures by TLRs mediates activation of adaptor molecules and downstream signaling pathways, resulting in the generation of a proinflammatory response. These include the production of inflammatory cytokines and chemokines, antimicrobial peptides, and other effectors needed for host defense (Janeway and Medzhitov, 2002). Upon binding to their cognate ligand, TLRs dimerize and recruit cytosolic TIR domain-containing adaptor molecules, such as myeloid differentiation

primary-response protein 88 (MyD88) and TIR-domain-containing adaptor protein inducing interferon-β (TRIF) (Mogensen, 2009). Several TLRs, including TLR1, TLR2, TLR5, TLR6, TLR4, and TLR7, can recruit the adaptor protein MyD88, resulting in activation of NF-κB and mitogen-activated protein kinases (MAPKs) to induce inflammatory cytokines (Akira and Takeda, 2004). In contrast, TLR3 and TLR4 can signal through TRIF, resulting in the activation of IRF3 and NF-κB to induce type I interferon and inflammatory cytokines (Yamamoto et al., 2003). Ultimately, activation of TLRs and downstream gene expression is critical to initiate antimicrobial response mechanisms to eliminate the pathogen and promote survival of the host. However, TLRs recognize structures shared by commensal and pathogenic microbes; therefore, they require additional innate immune signaling pathways to recognize and mediate efficient control of pathogenic infections.

1.2. Sensing of cytosolic access by NOD-like Receptors

Bacterial pathogens can use specialized secretion systems or pore-forming toxins to access the host cell cytosol. Our innate immune system has evolved to express cytoplasmic immune sensors of the nucleotide-binding domain leucine-rich repeat (NLR) superfamily that can mediate recognition of PAMPs and damage-associated molecular patterns (DAMPs) to promote host defense (Kim et al., 2016). NLRs are defined by a structure with three main domains, including: (a) a variable domain in the N-terminus important for protein-protein interactions (b) a central nucleotide-binding oligomerization (NOD) domain (NBD), and (c) a leucine-rich repeat (LRR) domain in the C-terminus. The human NLR family is composed of 22 members, whereas the NLR family in mice is composed of at least 34 members. NLRs are further divided into five categories (NLRA,

NLRB, NLRC, NLRP, and NLRX) depending on the type of N-terminal domain (Chen et al., 2009). Three major signaling events typically happen downstream of microbial product recognition by NLRs: NF-κB signaling, MAPK signaling, and inflammasome activation (Chen et al., 2009).

NOD1 and NOD2 were the first NLRs to be identified, and they sense bacterial peptidoglycan in the cytosol (Chamaillard et al., 2003; Girardin et al., 2003a; Girardin et al., 2003b; Inohara et al., 2003). Upon activation, NOD1 and NOD2 oligomerize to interact with the signaling adaptors receptor-interacting protein kinase 2 (RIPK2) and caspase-recruitment domain-containing adaptor protein (CARD9), which are essential for subsequent activation of the NF-κB and MAPK signaling pathways (Chin et al., 2002; Kobayashi et al., 2002; Hsu et al., 2007). In addition to recognizing peptidoglycan, NOD1 and NOD2 can sense a variety of danger signals (Keestra-Gounder and Tsolis, 2017). For example, the activation of small Rho GTPases by effector proteins from *Salmonella* Typhimurium and *Shigella flexneri* has been shown to be a danger signal detected by NOD1 (Fukazawa et al., 2008; Keestra et al., 2011; Keestra et al., 2013). NOD1 and NOD2 can also induce autophagy to control *S. flexneri* by recruiting ATG16L1 to the plasma membrane upon bacterial entry (Travassos et al., 2010).

A subset of NLRs can mediate the formation of the inflammasome, a multiprotein complex in the host cell cytosol that can lead to activation of the host enzyme caspase-1 (Martinon et al., 2002) (**Fig. 1.1**). Activated caspase-1 mediates processing and secretion of interleukin-1 (IL-1) family cytokines and a proinflammatory cell death known as pyroptosis (Kuida et al., 1995; Li et al., 1995; Bergsbaken et al., 2009). The NLR family, pyrin domain-containing 1 (NLRP1) inflammasome was the first to be described (Martinon et al., 2002). In humans, there is a single gene that encodes NLRP1 and it contains a pyrin

(PYD), a NBD, a LRR, a function-to-find (FIIND) domain, and a caspase activation and recruitment (CARD) domain. However, mice encode three polymorphic paralogues (Nlrp1a, Nlrp1b, and Nlrp1c) (Elinav et al., 2011). The mouse NLRP1B system has been well-characterized and is activated upon direct cleavage by the lethal factor protease, a subunit from the anthrax lethal toxin (**Fig. 1.1**) (Hellmich et al., 2012; Levinsohn et al., 2012). Recent findings demonstrate that the FIIND domain undergoes autoproteolysis but remains associated with the C-terminus of NLRP1B. The lethal factor from the anthrax toxin then cleaves an N-terminal fragment of NLRP1B that is targeted by ubiquitin ligases for its degradation. The degradation of the N-terminal fragment results in the release of the C-terminal portion from NLRP1B that contains a CARD domain which can now interact with caspase-1 (Okondo et al., 2018; Sandstrom et al., 2019; Xu et al., 2019). In addition, NLRP1B can be activated in response to infection by the apicomplexan parasite *Toxoplasma gondii* (Ewald et al., 2014; Gorfu et al., 2014); the identity of the precise stimulus leading to NLRP1B activation during *T. gondii* infection is unknown.

Interestingly, the anthrax lethal toxin is not an agonist of human NLRP1. In contrast, it has been suggested that the ligand for human NLRP1 is muramyl dipeptide, a product from the bacterial cell wall (Faustin et al., 2007). The murine NLRP1 lacks a functional PYD domain and is predicted to not interact with the adaptor protein apoptosis-associated speck-like protein containing a carboxy-terminal caspase recruitment domain (ASC), as NLRP1 activates caspase-1 in an ASC-independent manner (Van Opdenbosch et al., 2014). Despite these differences, the mechanism of activation seems to be somewhat conserved as proteolysis in the N-terminus is sufficient to activate NLRP1 in mice and humans (Chavarria-Smith et al., 2016). IpaH7.8, a ubiquitin ligase secreted by the bacterium *S. flexneri*, induces NLRP1B degradation and activation in mice, but not in humans (Sandstrom et al., 2019). These differences in inflammasome responses may be

a factor determining species specificity for *S. flexneri*, as it is human-adapted and not a natural pathogen of mice.

The NLRP3 inflammasome can be activated by several stimuli (**Fig. 1.1**). These include extracellular adenosine triphosphate (ATP), RNA, bacterial pore-forming toxins or secretion systems, ROS, uric acid crystals, and other agents that cause cell stress (Kanneganti et al., 2006; Mariathasan et al., 2006; Martinon et al., 2006; Dostert et al., 2008; Harder et al., 2009). A common step for NLRP3 activation involves potassium (K^+) efflux (Kahlenberg and Dubyak, 2004; Petrilli et al., 2007; Munoz-Planillo et al., 2013). Potassium ionophores such as nigericin can activate the NLRP3 inflammasome in LPS-stimulated macrophages (Mariathasan et al., 2006). The NLR family, pyrin domain-containing protein 6 (NLRP6) inflammasome is also implicated in innate immune signaling (Anand et al., 2012). Lipoteichoic acid (LTA) from gram-positive bacteria was recently found to activate the NLRP6 inflammasome (Hara et al., 2018).

The repertoire of NLRs varies between mice and humans (**Table 1.1**). Nod-like receptor pyrin domain-containing protein 7 (NLRP7) is absent in mice. However, it recognizes cytosolic microbial products in human macrophages (Radian et al., 2013). Bacterial acylated lipopeptides (acLP) activate an NLRP7-containing inflammasome to promote IL-1 maturation. Additionally, NLRP7 was required for restriction of bacterial replication in human macrophages upon infection with *Staphylococcus aureus* and *Listeria monocytogenes* (Khare et al., 2012). NLRP7 is also thought to negatively regulate inflammasome responses. Overexpression studies suggest that NLRP7 inhibits IL-1β processing and secretion (Kinoshita et al., 2005; Messaed et al., 2011). Future studies are needed to better understand the role of NLRP7 in human responses to bacterial infections.

Table 1.1: Inflammasome-related host proteins in mice and humans.

NLR family	Mice		Humans	
	Number of Members	Gene Nomenclature	Number of Members	Gene Nomenclature
NLRA	1	Ciita	1	CIITA
NLRB	7	Naip1, Naip2, Naip3, Naip4, Naip5, Naip6, Naip7	1	NAIP
NLRC	5	Nod1, Nod2, Nlrc3, Nlrc4, Nlrc5	5	NOD1, NOD2, NLRC3, NLRC4, NLRC5
NLRP	20	Nlrp1a, Nlrp1b, Nlrp1c, Nlrp2, Nlrp3, Nlrp4a, Nlrp4b, Nlrp4c, Nlrp4d, Nlrp4e, Nlrp4f, Nlrp4g, Nlrp5, Nlrp6, Nlrp9a, Nlrp9b, Nlrp9c, Nlrp10, Nlrp12, Nlrp14	14	NLRP1, NLRP2, NLRP3, NLRP4, NLRP5, NLRP6, NLRP7, NLRP8, NLRP9, NLRP10, NLRP11, NLRP12, NLRP13, NLRP14
NLRX	1	Nlrx1	1	NLRX1

The inflammasome can also be activated by receptors other than NLRs, as is the case for the absent in melanoma 2 (AIM2) inflammasome and the pyrin inflammasome. The AIM2 inflammasome recognizes dsDNA and mediates immune defense against several intracellular bacterial pathogens, including *Fransicella tularensis*, *L. monocytogenes*, and *Mycobacterium* species (**Fig. 1.1**) (Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Jones et al., 2010; Kim et al., 2010; Sauer et al., 2010; Warren et al., 2010; Saiga et al., 2012). The immune sensor pyrin activates the inflammasome when pathogens inactivate the small GTPase, RHOA (**Fig. 1.1**). Pyrin is encoded by the gene *MEFV,* and mutations in this gene lead to an autoinflammatory disease known as familial Mediterranean fever (FMF) (French, 1997). Interestingly, human pyrin has a C-terminal B30.2 domain that is absent in murine pyrin. Gain-of-function mutations in this domain can cause pathological inflammation in humans (Chae et al., 2011; Broz, 2019). Mice were genetically modified to express the human B30.2 domain harboring mutations related to FMF, and this was sufficient to induce severe autoinflammation in mice (Chae et al., 2011).

Finally, ASC often bridges the interaction of the NLR, AIM2, or pyrin with caspase-1 to promote the release of alarmins and cell death (Broz and Dixit, 2016).

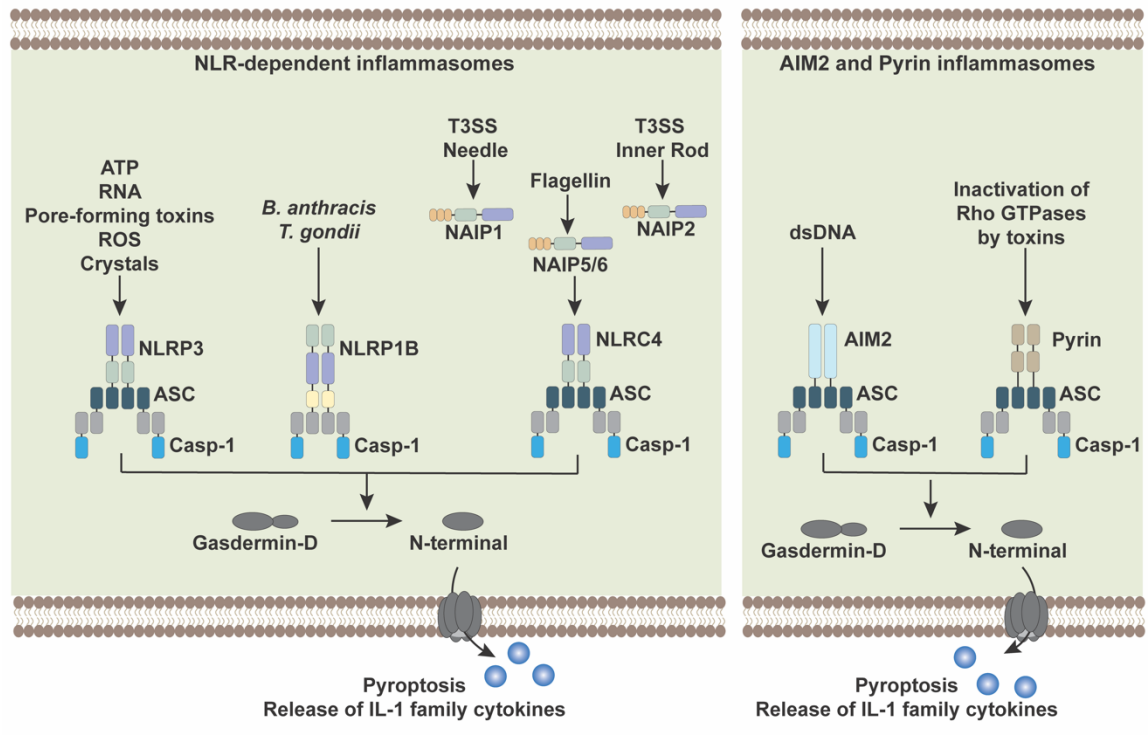

Figure 1.1: Canonical Caspase-1-dependent inflammasomes are activated by NLRs, AIM2, and Pyrin. The activation of caspase-1, secretion of IL-1 family cytokines, and pyroptosis requires the formation of the inflammasome in response to a variety of PAMPs and DAMPs. NLRP3 senses multiple stimuli including ATP, RNA, Pore-forming toxins, ROS, and crystals. The NLRP1B inflammasome can be activated by *Toxoplasma gondii* and *Bacillus anthracis*. NAIPs detect components from the T3SS and flagellin to recruit the adaptor protein NLRC4 for the formation of the inflammasome. AIM2 detects dsDNA and Pyrin detects the inactivation of Rho GTPases by bacterial toxins.

1.3. Innate immune recognition by the NAIP/NLRC4 inflammasome

A subfamily of NLRs, known as nucleotide-binding domain, leucine-rich repeat-containing family, apoptosis inhibitory proteins (NAIP), can also result in inflammasome activation. Initial studies with *NAIP* suggest a correlation of this gene with spinal muscular atrophy (SMA), as most patients with the disease have deletions in *NAIP*. However, a role

of *NAIP* in causing SMA or an underlying mechanism by which it can be related to this disease remains unknown (Roy et al., 1995). The human *NAIP* locus has a number of pseudogenes and has retained a single functional copy of the full-length *NAIP* gene (Romanish et al., 2007; Romanish et al., 2009). In contrast, rodents have an expansion in the number of *Naips*, with five copies in C57BL/6J mice and at least seven copies in the 129 mice strain (Growney and Dietrich, 2000; Growney et al., 2000).

A role of the NAIP inflammasome in immune defense against bacterial infections was first identified with *Legionella pneumophila*. Different inbred mouse strains have varying permissiveness to intracellular replication of *L. pneumophila,* and this permissiveness is controlled by a single genetic locus that maps to a region in the chromosome containing *Naip* genes (Yamamoto et al., 1991; Yoshida et al., 1991; Beckers et al., 1995; Dietrich et al., 1995). Additional studies showed that polymorphisms in *Naip5* (also known as *Birc1e*) are responsible for the differences in *L. pneumophila* permissiveness among mouse strains (Diez et al., 2003; Wright et al., 2003) and that Naip5-dependent caspase-1 activation contributes to the detection and control of *L. pneumophila* (Zamboni et al., 2006).

Gram-negative pathogens often employ specialized secretion systems, which are evolutionarily conserved virulence factors that promote bacterial colonization and disease. *S.* Typhimurium utilizes a type III secretion system (T3SS), a molecular syringe that injects bacterial effectors into the host cell cytosol, which promotes invasion into host cells and intracellular survival (Galan et al., 2014). Similarly, *L. pneumophila* uses a type IV secretion system (T4SS) to inject bacterial effectors into the host cell cytosol (Isberg et al., 2009). Different bacterial infections activate an NLR family, CARD domain-containing 4 (NLRC4) inflammasome and this activation is dependent on bacterial secretion

mechanisms such as the T3SS and T4SS (Mariathasan et al., 2004; Franchi et al., 2006; Miao et al., 2006; Zamboni et al., 2006; Sutterwala et al., 2007; Suzuki et al., 2007). In addition, the cytosolic presence of flagellin or the T3SS inner rod, which is the protein subunit that comprises the T3SS inner channel, activates the NLRC4 inflammasome in macrophages to induce caspase-1 activation and IL-1β secretion (Franchi et al., 2006; Miao et al., 2006; Miao et al., 2010b). However, it remained unclear how the NLRC4 inflammasome can mediate recognition of both the flagellar apparatus and the T3SS.

Inflammasome activation requires NAIP5 in response to cytosolic delivery of flagellin from *L. pneumophila* and *S.* Typhimurium (Lightfield et al., 2008). However, NLRC4 inflammasome activation by the T3SS inner rod is independent of NAIP5 (Lightfield et al., 2011). These results suggested that NAIP5 regulates the ligand specificity of the NLRC4 inflammasome. Additional studies showed that the NAIPs are the inflammasome sensors that lead to NLRC4 inflammasome activation. In mice, each NAIP has specificity to a single bacterial ligand from either the T3SS or flagellar apparatus. NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod protein, and NAIP5 and NAIP6 both recognize flagellin (**Fig. 1.1**) (Kofoed and Vance, 2011; Zhao et al., 2011; Rayamajhi et al., 2013; Yang et al., 2013; Rauch et al., 2016; Zhao et al., 2016). In contrast, humans encode a single *NAIP* (Romanish et al., 2007; Romanish et al., 2009). Initial studies with human monocytic cell lines suggested that the single human NAIP only recognizes the T3SS needle protein (Zhao et al., 2011; Rayamajhi et al., 2013; Yang et al., 2013). However, flagellin was also found to trigger NAIP inflammasome activation in primary human macrophages, and it was proposed that detection of flagellin was mediated by an alternate splice isoform of NAIP (Kortmann et al., 2015).

NAIP5 and NLRC4 both contain a nucleotide-binding domain (NBD), helical domain 1 (HD1), winged helix domain (WHD), and helical domain 2 (HD2) in the central portion of the protein, and a leucine-rich repeat (LRR) domain in the C-terminus (Tenthorey et al., 2014). At the N-terminus, NLRC4 contains a CARD domain for recruitment of caspase-1, whereas NAIP5 contains three baculovirus inhibitor-of-apoptosis repeat (BIR) domains (Chen et al., 2009). Upon binding their cognate ligand, the NAIPs recruit NLRC4. The resulting NAIP/NLRC4 inflammasome recruits caspase-1, resulting in caspase-1 activation (Martinon et al., 2002; Diebolder et al., 2015; Hu et al., 2015; Zhang et al., 2015). Studies in which chimeric NAIP proteins were generated mapped the region of murine NAIPs that confers ligand specificity to be an internal region composed of several nucleotide-binding domain (NBD)-associated α-helical domains (Tenthorey et al., 2014).

Recent studies employed cryo-electron microscopy to determine the structure of the flagellin-NAIP5-NLRC4 inflammasome and found that there is one bacterial ligand and one NAIP per inflammasome (Tenthorey et al., 2017). The single flagellin monomer is recognized by NAIP5 and does not directly interact with NLRC4. NAIP5 makes multiple contacts with flagellin using several regions, including the HD2, LRR, HD1, and BIR1 domains (Tenthorey et al., 2017; Yang et al., 2018). The current model for NAIP activation involves binding of a single flagellin monomer to NAIP5 and a subsequent conformational change in NAIP5 that unfurls the protein to recruit and activate NLRC4. Active NLRC4 can then recruit additional NLRC4 proteins for self-propagating oligomerization and recruitment of caspase-1 (Tenthorey et al., 2017).

The NAIP/NLRC4 inflammasome promotes host defense against *L. pneumophila* as well as other gram-negative bacterial infections. Mice deficient for *Nlrc4* are more

susceptible to *S.* Typhimurium and show decreased survival and increased bacterial loads. The increased susceptibility is exhibited by specific mouse strains and only during orogastric challenge, but not when *Salmonella* is administered intraperitoneally (Lara-Tejero et al., 2006; Carvalho et al., 2012; Franchi et al., 2012). Similarly, NLRC4 mediates protection against infection with *Klebsiella pneumonia* and *Burkholderia pseudomallei* (Ceballos-Olvera et al., 2011; Cai et al., 2012). Interestingly, NLRC4 protects against *Anaplasma phagocytophilum*, the causative agent of anaplasmosis, although *A. phagocytophilum* lacks both flagellin and a T3SS. In this case, *A. phagocytophilum* activates a functionally distinct NLRC4 inflammasome that requires production of the eicosanoid prostaglandin E_2 and subsequent signaling through one of its receptors, EP3 (Pedra et al., 2007; Wang et al., 2016).

In addition to IL-1 signaling downstream of inflammasome activation, distinct defense mechanisms have been proposed for the NAIP/NLRC4 inflammasome. Signaling by NAIP5 in macrophages infected with *Legionella* results in increased colocalization with lysosomal markers (cathepsin D and Lamp-1), suggesting a role of this inflammasome in promoting phagosome-lysosome fusion (Amer et al., 2006; Fortier et al., 2007). Future studies are needed to understand the mechanism by which NAIP5 may be regulating phagosome maturation. Activation of the NAIP5 inflammasome results in pyroptosis, therefore eliminating the replicative niche of intracellular bacteria. It was recently proposed that pyroptosis triggers a cellular structure termed the pore-induced intracellular trap (PIT). The PITs have a largely intact plasma membrane, thus trapping intracellular bacteria. Neutrophils can then be recruited to mediate efferocytosis of the PIT together with the intracellular bacteria (Jorgensen et al., 2016). Another means by which the NAIP inflammasome promotes host defense has been shown in the intestinal epithelium, where activation of the NAIP/NLRC4 inflammasome drives expulsion of infected enterocytes in

order to restrict *Salmonella* replication in the intestine (Sellin et al., 2014). Interestingly, caspase-8 can compensate for the loss of caspase-1 and mediate expulsion of epithelial cells downstream of inflammasome activation (Rauch et al., 2017). Sensing by the NAIP/NLRC4 inflammasome in enterocytes also results in the production of prostaglandin E_2 and fluid accumulation in the intestines. The authors proposed that this response may be important to eliminate the infected and expulsed enterocytes from the intestine (Rauch et al., 2017).

Activation of the NAIP/NLRC4 inflammasome can also result in pathological outcomes. Studies in mice have shown that activation of the NAIP5/NLRC4 inflammasome by a multidrug-resistant pathobiont can lead to lethal systemic inflammation resembling sepsis (Ayres et al., 2012). Additionally, patients with gain-of-function mutations in human *NLRC4* suffer from pathologic enterocolitis and Macrophage Activation Syndrome (MAS) (Canna et al., 2014; Romberg et al., 2014). The mutations were in amino acids 337 and 341 within the helical domain 1 (HD1). These patients showed elevated levels of peripheral IL-18. In one patient where IL-1 blockade was ineffective, blockade of IL-18 resulted in an improved clinical outcome (Canna et al., 2016). Another mutation in *NLRC4* has been described to result in more mild cases, where patients experience urticaria and arthritis after exposure to cold stimuli (Kitamura et al., 2014). Mutations for human *NAIP* have not yet been described, but perhaps gain-of-function mutations in *NAIP* confer similar pathological outcomes.

1.4. Inflammatory Caspases associated with inflammasome activation

Caspases are cysteine proteases that are synthesized as inactive zymogens. They are tightly controlled by proteolytic activation and cleave their substrates immediately following an aspartic residue (Schroder and Tschopp, 2010). Activation of inflammatory

caspases associated with the inflammasome can result in cell death and the release of inflammatory cytokines (Martinon et al., 2002). The canonical inflammasome is generally initiated by NLRs recognizing PAMPs and DAMPs and subsequent recruitment of caspase-1 (also known as ICE) through homotypic CARD-CARD interactions (Broz and Dixit, 2016). Caspase-1 contains an N-terminal CARD domain and consists of a large subunit and a small subunit separated by two linker domains. It was recently suggested that the predominant species of active caspase-1 elicited by the inflammasome are two dimers: full-length p46 and a transient species of p33/p10 (Boucher et al., 2018). Active caspase-1 can process IL-1β and IL-18 into their mature forms, but does not cleave IL-1α (Howard et al., 1991). In addition, active caspase-1 can cleave gasdermin-D and promote cell death (Kayagaki et al., 2015; Shi et al., 2015).

In contrast, the non-canonical inflammasome recruits and activates caspase-11 in mice and caspase-4 and 5 in humans (**Fig. 1.2**). The non-canonical inflammasome is activated upon recognition of gram-negative bacteria and LPS (Kayagaki et al., 2011; Hagar et al., 2013; Casson et al., 2015). The CARD domain from caspase-11, 4, and 5 can directly bind the lipid A moiety from LPS with high specificity and affinity (Shi et al., 2014). The acylation state of lipid A affects the activation of the murine non-canonical inflamamsome, as hexa-acylated and penta-acylated lipid A activates caspase-11, whereas tetra-acylated lipidA is not detected (Hagar et al., 2013) (Shi et al., 2014). In contrast, human caspase-4 appears to be able to recognize both tetra-acylated and hexa-acylated lipid A (Lagrange et al., 2018).

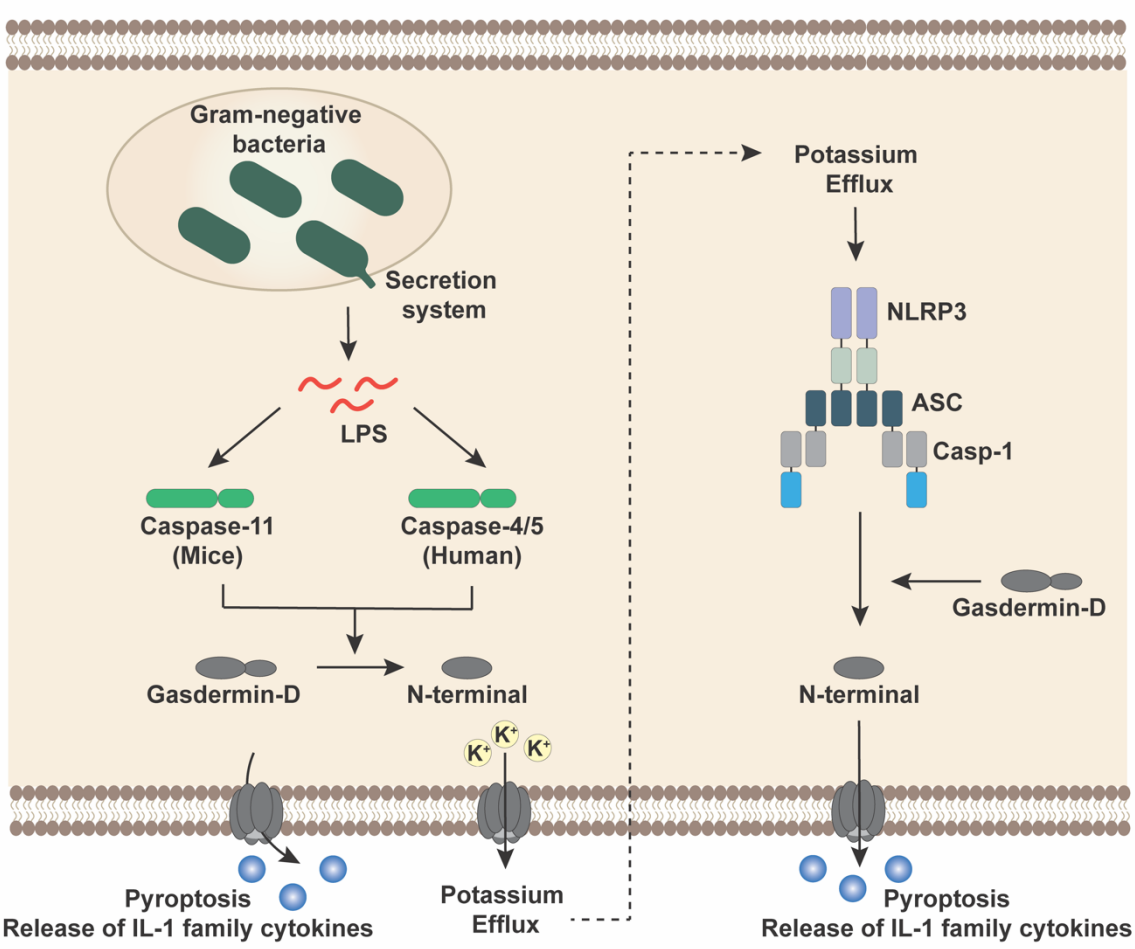

Figure 1.2: Non-canonical inflammasome activation requires caspase-11 in mice and caspase 4/5 in humans. Cytosolic access of LPS to the host cytosol can be sensed by inflammatory caspases resulting in inflammasome activation. Caspase-11 in mice and caspase-4 or caspase-5 in humans can directly bind LPS to induce caspase oligomerization and activation. Active caspase-11/4/5 cleave Gasdermin-D resulting in pore formation, the release of IL-1 family cytokines, and cell death. Potassium efflux downstream of non-canonical inflammasome responses can activate the NLRP3 inflammasome resulting in more pore formation by Gasdermin D and release of alarmins.

While both human caspase-4 and caspase-5 have been shown to bind LPS *in vitro*, it is possible that their functions may vary depending on the cell type and stimuli. For example, regulation of caspase-4 and 5 have been shown to be distinct. Caspase-4 is translationally upregulated by LPS and IFN-β, whereas caspase-5 is only translationally

induced by LPS (Casson et al., 2015). It has been reported that caspase-4, but not caspase-5, leads to inflammasome activation in response to LPS transfected into human THP-1 cells (Baker et al., 2015; Casson et al., 2015). Caspase-4 and caspase-5 can also play non-redundant roles, as they are both required for IL-1β secretion in monocytes infected with *S.* Typhimurium (Baker et al., 2015). In addition, caspase-4 was required in epithelial cells for control of bacterial replication, cell death, and IL-18 secretion during *Salmonella* Typhimurium infection (Knodler et al., 2014). Recent studies show that caspase-11 in mice can also be activated downstream of the NLRP6 inflammasome (Hara et al., 2018). In this model, lipoteichoic acid (LTA), a component of bacterial cell wall, can directly bind and activate NLRP6, which then recruits caspase-11 for its processing and activation (Hara et al., 2018). Downstream IL-18 production was detrimental for the host in response to *Listeria monocytogenes* infection and mice lacking caspase-11 were less susceptible to infection (Hara et al., 2018). It remains to be determined whether human NLRP6 has a similar mechanism for activation.

The non-canonical inflammasome can lead to septic shock and thus needs to be tightly regulated (Wang et al., 1998). The oxidized phospholipid 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (oxPAPC) inhibits the non-canonical inflammasome in macrophages. It can directly bind to caspase-4 and caspase-11 and compete with LPS to inhibit pyroptosis, IL-1β, and septic shock (Chu et al., 2018). Bacterial pathogens can also encode virulence factors to counteract these defense mechanisms. For example, the human pathogen enteropathogenic *Escherichia coli* (EPEC) encodes an effector NleF that is capable of binding to the catalytic domain of caspase-4 to inhibit its activity (Pallett et al., 2017).

The apoptotic caspase, caspase-8, can also be recruited to the NLRC4 inflammasome in response to *Salmonella* infection (Man et al., 2013). In addition, caspase-8 functions with the NLRP3 inflammasome to release IL-1β (Antonopoulos et al., 2015; Karki et al., 2015). Further studies showed that caspase-8 can compensate for the loss of caspase-1 in intestinal epithelial cells to mediate the expulsion of infected cells (Rauch et al., 2017). Inactivating mutations in human caspase-8 are linked to increased susceptibility to infection (Chun et al., 2002). More studies are warranted to determine the contribution of human caspase-8 or its close homolog, caspase-10, to inflammasome responses to bacterial pathogens.

Table 1.2: Family members in mice and humans of inflammasome-related host proteins.

Family	Mice		Humans	
	Number of Members	Gene Nomenclature	Number of Members	Gene Nomenclature
Inflammatory caspases	2	*Casp1, Casp11*	3	*CASP1, CASP4, CASP5*
Gasdermin	10	*Gsdma1, Gsdma2, Gsdma3, Gsdmc1, Gsdmc2, Gsdmc3, Gsdmc4, Gsdmd, Dfn5, Dfnb59*	6	*GSDMA, GSDMB, GSDMC, GSDMD, DFNA5, DFNB59*
Guanylate-binding proteins	11	*Gbp1, Gbp2, Gbp3, Gbp4, Gbp5, Gbp6, Gbp7, Gbp8, Gbp9, Gbp10, Gbp11*	7	*GBP1, GBP2, GBP3, GBP4, GBP5, GBP6, GBP7*
Immunity-related GTPases	23	*Irgc, Irgm1, Irgb8, Irgb9, Irgb1, Irgb2, Irgb3, Irgb4, Irgb5, Irgb6, Irgb7, Irgd, Irgb10, Irgm3, Irgm2, Irga1, Irga2, Irga3, Irga4, Irga5, Irga6, Irga7, Irga8*	2	*IRGC, IRGM*
PYD-only proteins	0		4	*PYDC1, PYDC2, POP3, NLRP2B*
CARD-only proteins	0		3	*CARD16, CARD17, CARD18*

1.5. Gasdermin family: Effectors of pyroptosis

Activation of the inflammatory caspases can result in the cleavage of the pore-forming protein gasdermin D, resulting in pyroptosis (Kayagaki et al., 2015; Shi et al., 2015). Gasdermin D is a substrate for caspase-1 and caspase 11/4/5 (Kayagaki et al., 2015; Shi et al., 2015). Upon cleavage, the N-terminus of gasdermin D can be incorporated into the host membrane, creating pores that result in cell swelling and osmotic lysis. The 10-15nm diameter gasdermin pore is sufficient to allow release of the IL-1 family cytokines (Aglietti et al., 2016; Ding et al., 2016; Liu et al., 2016; Sborgi et al., 2016).

Pyroptosis can defend against intracellular infection by eliminating the replicative niche of the bacteria and inducing an inflammatory response. For example, bacteria such as *Salmonella* and *Listeria* engineered to robustly activate the inflammasome remain trapped within a cellular structure termed the pore-induced intracellular trap (PIT). The bacteria trapped in these PITs are susceptible to other immune cells that are recruited to aid in controlling infection. (Jorgensen et al., 2016). Activation of gasdermin D can also drive downstream activation of the NLRP3 inflammasome due to potassium efflux caused by the gasdermin D pores in the membrane (Baker et al., 2015; Schmid-Burgk et al., 2015; Evavold et al., 2018). In addition, the gasdermin D pore can target bacterial membranes of *E. coli* and *Staphylococcus aureus in vitro* (Liu et al., 2016). Whether this occurs *in vivo* and what the consequences are for immune defense remain to be determined.

Humans have six gasdermin family members, including GSDMD, GSDMA, GSDMB, GSDMC, DFNA5, and DFNB59 (**Table 1.2**). While most mammals have one copy of each gasdermin, mice have three GSDMA (GSDMA1-3), four GSDMC (GSDMC1-C4), and have lost GSDMB (Kovacs and Miao, 2017; Shi et al., 2017). While we have some understanding of the function of gasdermin-D, the function of the other members

from the gasdermin family is unclear. These other gasdermin family members are not cleaved by inflammatory caspases, but the conserved N-terminus domain from some of them can induce pyroptosis in mammalian cells (Shi et al., 2015; Ding et al., 2016). Whether pyroptosis by other members from the gasdermin family, in addition to gasdermin D, have a role in innate immune defense remains to be determined.

1.6. Interferon-inducible GTPases: Guanylate-Binding Proteins

Guanylate-binding proteins (GBPs) are a group of interferon-inducible GTPases with a wide range of innate immune functions against intracellular pathogens. Humans encode seven GBPs, whereas the murine genome encodes 11 GBPs (**Table 1.2**). The seven human *Gbps* are located within one cluster on chromosome 1 (Olszewski et al., 2006). In contrast, the eleven murine *Gbps* are located within two gene clusters on chromosome 3 and chromosome 5 (Kresse et al., 2008). Many studies have shown that GBPs can act as cofactors important for inflammasome activation. The first observation that GBPs were involved in inflammasome activation was made by MacMicking and colleagues (Shenoy et al., 2012). In this study, human GBP5 and mouse Gbp5 were shown to be important for NLRP3 inflammasome responses, although the exact mechanism is not understood (Shenoy et al., 2012).

The defense mechanisms by GPBs against many pathogens *in vivo* have been explored using mice lacking the *Gbps* located on chromosome 3, which are *Gbp1*, *Gbp2, Gbp3, Gbp5,* and *Gbp7.* For example*,* GBPs on chromosome 3 are necessary for caspase-11-dependent pyroptosis in response to *Legionella pneumophila* or cytosolic delivery of LPS (Pilla et al., 2014; Liu et al., 2018). In addition, GBPs on chromosome 3 are necessary for inflammasome activation in response to the translocon proteins, YopB and YopD, from the T3SS of *Yersinia* (Zwack et al., 2017).

Several studies have aimed at understanding the mechanism by which GBPs can promote inflammasome responses. It has been proposed that GBPs mediate lysis of the pathogen-containing vacuole, which will then release bacteria into the cytosol to mediate caspase-11 inflammasome activation (Meunier et al., 2014). However, GBPs can also promote activation of caspase-11 independent of lysis of the vacuole, suggesting that there are other mechanisms for inflammasome activation (Finethy et al., 2015). In addition to targeting the vacuole, GBPs may also directly target bacteria that replicate in the cytosol. GBP2 and GBP5 are also important in promoting bacteriolysis of *Francisella*, releasing dsDNA, and activating the AIM2 inflammasome (Man et al., 2015; Meunier et al., 2015). Endogenous levels of GBPs are involved in the release of DNA from cytosol-accessible *L. pneumophila* and are required for canonical and non-canonical inflammasome responses to bacterial infection. Moreover, mice deficient for GBPs from chromosome 3 are more susceptible to *L. pneumophila* and have lower levels of IL-1 cytokines and the chemokine CXCL1 (Liu et al., 2018). Since GBPs from chromosome 3 have been shown to be necessary for pyroptosis in response to free LPS, it has been suggested that GBPs may facilitate translocation of LPS into the cytoplasm or oligomerization and activation of caspase-11 (Pilla et al., 2014).

Recently, more studies have been done to determine the role of human GBPs in defense against bacterial pathogens. Human GBP2 facilitates caspase-4 activation in response to *F. novicida* (Lagrange et al., 2018). In addition to their role in inflammasome activation, human GBPs also play a role in the control of cell-to-cell spread of cytosolic bacteria. Specifically, human GBP1 colocalizes with *S. flexneri* and *Burkholderia thailandensis*. The targeting of GBP1 to cytosolic bacteria recruits additional GBPs (GBP2, GBP3, GBP4, and GBP6). *Shigella* targeted by GBP1 have fewer actin tails and are deficient in cell-to-cell spread (Piro et al., 2017). Interestingly, the *Shigella* effector IpaH9.8

can induce degradation of GBP1 to counteract host defense (Li et al., 2017; Piro et al., 2017). Future studies are needed to better understand the mechanisms of action for human and mouse GBPs to mediate host defense against bacterial pathogens.

1.7. Interferon-inducible GTPases: Immunity-Related GTPases

The immunity-related GTPases (IRGs) are important for innate immune defense against intracellular pathogens (Meunier and Broz, 2016). There are many IRGs in mice, with 23 IRGs in the C57Bl/6 strain that are localized to chromosomes 7, 11, and 18 (Bekpen et al., 2005). In contrast, humans encode only two IRGs, named *IRGC* and *IRGM*, which are located on chromosomes 19 and 5, respectively (**Table 1.2**) (Bekpen et al., 2005; Bekpen et al., 2009). The expression of most of the IRGs in mice is upregulated by IFN-γ signaling, whereas the human IRGs are not interferon-inducible and are constitutively expressed (Boehm et al., 1998; Bekpen et al., 2005; Singh et al., 2006).

Murine IRGs have been shown to be important for resistance against several bacterial pathogens, such as *L. monocytogenes* and *Mycobacterium tuberculosis* (Collazo et al., 2001; MacMicking et al., 2003; Feng et al., 2004). They are thought to be involved in phagosomal maturation or induction of autophagy in response to intracellular bacteria (MacMicking et al., 2003; Singh et al., 2006). IRGB10 was shown to target *Chlamydia trachomatis* inclusions (Haldar et al., 2013). A role for IRGs in inflammasome responses was recently determined. Mice deficient in *Irgb10* have reduced IL-1 secretion and cell death in response to *F. novicida*. IrgB10 targets the cell membrane from cytosolic bacteria, leading to bacterial cell lysis and activation of the AIM2 inflammasome and the NLRP3 inflammasome downstream of caspase-11. Additionally, IRGB10 provides host protection against *F. novicida in vivo* (Man et al., 2016). Currently, there has not been an implication

for human IRGC or IRGM in inflammasome responses. There is a link between specific *IRGM* gene variants with susceptibility to Crohn's disease (Parkes et al., 2007). It is interesting to speculate that there may be a disadvantage for humans to contain IRGs, as they may lead to inflammatory diseases, thus providing an evolutionary pressure for the loss of genes in the IRG family. Future studies will expand our understanding of the mechanisms by which IRGs promote host defense.

1.8. PYD-only proteins (POPs) and CARD-only proteins (COPs)

PYD-only proteins (POPs) and CARD-only proteins (COPs) are small endogenous proteins that can inhibit inflammasome responses by binding to pyrin- or CARD-containing host proteins. POPs are proteins that only encode a pyrin domain (PYD) (**Table 1.2**). Humans encode four POPs named POP1-4 (Indramohan et al., 2018). POP1 (also known as PYDC1) can associate with ASC to inhibit its nucleation and caspase-1 activation downstream of ASC-containing inflammasomes (de Almeida et al., 2015). Using a transgenic mouse model, POP2 (also known as PYDC2) was found to affect both priming of macrophages and inflammasome activation (Periasamy et al., 2017; Ratsimandresy et al., 2017). POP3 interacts with AIM2 to inhibit inflammasome responses, and its depletion leads to increased IL-1 signaling in primary human macrophages infected with DNA viruses (Khare et al., 2014). Finally, POP4 (also known as NLRP2B) inhibits the activation of NF-κB, but does not seem to directly inhibit inflammasome activation (Porter et al., 2014). Interestingly, POPs are absent in mice, adding to the complexity of inflammasome regulation specifically in humans (Indramohan et al., 2018).

COPS are proteins that only encode a CARD domain. Other CARD-containing proteins include host caspases. Three COPs have been identified in humans: COP (also

named CARD16), INCA (also named CARD17), and ICEBERG (also named CARD18) (**Table 1.2**). They are located on chromosome 11 in close proximity to CASP1, CASP5, and CASP4. However, COPs are absent in the murine genome (Dorfleutner et al., 2015). COP, INCA, and ICEBERG interact with the CARD domain of caspase-1 (Humke et al., 2000; Druilhe et al., 2001; Karasawa et al., 2015). These studies have only been performed in the context of overexpression. Studies to understand the role of endogenous levels of these proteins are needed to further our understanding of COPs and regulation of inflammasome responses.

1.9. Immune signaling by Interleukin-1 cytokines

The Interleukin-1 (IL-1) family is composed of a variety of complex mediators with important roles in inflammation and immunity. IL-1 family cytokines are divided into three groups: secreted agonistic cytokines (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ), receptor antagonists (IL-1Ra, IL-36Ra, and IL-38), and one anti-inflammatory cytokine (IL-37) (Mantovani et al., 2019). IL-1 was originally described as a fever-inducing factor that was secreted by activated leukocytes and that could induce the stimulation and proliferation of T cells (Rosenwasser and Dinarello, 1981). It was later found that the biological activities of IL-1 were due to the presence of two distinct cytokines, IL-1α and IL-1β (March et al., 1985).

Inflammasome responses result in the activation and secretion of IL-1 cytokines such as IL-1α, IL-1β, and IL-18 (Martinon et al., 2002). In the cytoplasm, IL-1α and IL-1β are synthesized as 31kDa precursor peptides (pro-IL-1α and pro-IL-1β), whereas IL-18 is synthesized as a 23-kDa precursor (pro-IL-18). In contrast to most cytokines, IL-1α, IL-1β, and IL-18 lack signal peptides and are released by cells using an unconventional pathway

independent of the endoplasmic reticulum (ER) and Golgi apparatus (Mantovani et al., 2019). The precursors of IL-1β and IL-18 are biologically inactive and require cleavage by caspase-1 to become active (Cerretti et al., 1992; Thornberry et al., 1992; Ghayur et al., 1997). In contrast, IL-1α is not a substrate of caspase-1 and full-length IL-1α is biologically active (Mosley et al., 1987; Howard et al., 1991; Kim et al., 2013). Calpains are membrane-bound cysteine proteases that can cleave IL-1α, although with low frequency (Carruth et al., 1991; Mantovani et al., 2019). However, the consequences of calpain-mediated cleavage of IL-1α is not understood.

IL-1α and IL-1β cytokines bind the same IL-1 receptor (IL-1R) (Dower et al., 1986; Sims et al., 1988). IL-1R contains a cytoplasmic TIR (Toll IL-1R) domain that allows for interaction with the adaptor molecule MyD88. The N-terminal death domain (DD) of MyD88 interacts with the IRAK family members, promoting their phosphorylation and activation (Lin et al., 2010). The IRAKs recruit tumor necrosis factor receptor-associated factor 6 (TRAF-6), resulting in the recruitment and activation of the TAK1 kinase. TAK1 activates the IKK complex, releasing NF-κB for its translocation to the nucleus and regulation of its target genes (Chen, 2005; Dinarello, 2009). In addition, TAK1 activates mitogen-activated protein kinases (MAPKs), such as p38 and JNK (Deguine and Barton, 2014).

Activation of NF-κB and MAPKs downstream of IL-1 signaling results in the induction of a number of pro-inflammatory genes, including chemokines, cytokines, adhesion molecules and inducible nitric oxide synthase (iNOS) (Apte and Voronov, 2008; Mantovani et al., 2019). For example, IL-1β can induce the expression of adhesion molecules on endothelial cells, such as intercellular adhesion molecule-1 (ICAM-1) (Bevilacqua et al., 1985; Tamaru et al., 1998). In mice infected with gram-negative

bacteria, IL-1β derived from macrophages induce neutrophil-attracting chemokine production from non-hematopoietic cells (LeibundGut-Landmann et al., 2011). Even though IL-1α and IL-1β bind to the same receptor, distinct roles for innate immune defense against bacterial infection have been identified. IL-1α and IL-1β are both required for host resistance to *M. tuberculosis* (Mayer-Barber et al., 2011). In contrast, IL-1α but not IL-1β is required for the production of the chemokine CXCL1 and for neutrophil recruitment during pulmonary infection with *Pseudomonas aeruginosa* (Al Moussawi and Kazmierczak, 2014). In response to *L. pneumophila*, neutralizing antibodies that target IL-1α specifically result in decreased neutrophil recruitment and increased bacterial burdens. Even though neutralizing IL-1β alone had no effect, neutralizing both IL-1α and IL-1β had a stronger effect than IL-1α alone. These data suggest that IL-1α and IL-1β play both overlapping and non-redundant roles for neutrophil recruitment and bacterial clearance (Casson et al., 2013). In agreement with these studies, *Il1α* $^{-/-}$ mice infected with *L. pneumophila* have reduced neutrophil recruitment, whereas *Il1β* $^{-/-}$ mice have similar levels of neutrophils in the lung compared to WT mice. However, at later timepoints of infection, IL-1β can compensate for the loss of IL-1α (Barry et al., 2013).

IL-1 signaling also enables the production of inflammatory cytokines by uninfected bystander cells. *L. pneumophila* uses a T4SS to infect alveolar macrophages and to secrete effector molecules into the host cell (Isberg et al., 2009). Several of the secreted effector molecules can block host protein synthesis (Belyi et al., 2006; Belyi et al., 2008; Shen et al., 2009; Fontana et al., 2011; Barry et al., 2013). As a result, infected macrophages are poor inducers of other cytokines such as IL-6, TNF, and IL-12 (Copenhaver et al., 2015). However, despite the block in protein synthesis, infected macrophages produce IL-1α and IL-1β (Asrat et al., 2014; Copenhaver et al., 2015).

Instead, uninfected bystander cells robustly produce IL-6, TNF, and IL-12 and this bystander response requires IL-1 signaling (Copenhaver et al., 2015).

IL-18 signaling also promotes host defense against bacterial infections (Sahoo et al., 2011). IL-18 cooperates with IL-12 to induce Th1 immunity. Signaling through the IL-18R complex induces the production of IFN-γ from natural killer (NK) cells and T cells (Okamura et al., 1995; Chaix et al., 2008). IL-18 is important for resistance to systemic infection by *S.* Typhimurium, but dispensable for epithelial restriction of early bacterial infection (Raupach et al., 2006; Sellin et al., 2014). In addition, IL-18-deficient mice are more susceptible to *S. flexneri* and *B. pseudomallei* (Sansonetti et al., 2000; Ceballos-Olvera et al., 2011). Most of these phenotypes are potentially due to IL-18-dependent production of IFN-γ and subsequent induction of microbicidal effector functions. IFN-γ can induce enzymes important for the production of reactive nitrogen and oxygen species, such as iNOS and subunits that comprise the NADPH oxidase. In addition, it can lead to the production of complement proteins, chemokines, and adhesion molecules (Schroder et al., 2004). IFN-γ also induces NRAMP1 (natural resistance-associated macrophage protein) and idoleamine 2,3-dioxygenase (IDO). NRAMP1 is a divalent cation transporter that can mediate phagosome acidification and starve bacteria from important cofactors, whereas IDO depletes host tryptophan to inhibit bacterial replication (Carlin et al., 1989; Govoni et al., 1995; Wessling-Resnick, 2015). Finally, IFN-γ can also upregulate GBPs, which as discussed are important for innate immune defense.

1.10. *Salmonella*: pathogenesis and manipulation of innate immune pathways

Salmonella enterica is a gram-negative, flagellated, and facultative intracellular pathogen. The species of *S. enterica* include over 2,500 serovars, which are classified

into typhoidal or nontyphoidal. Typhoidal *Salmonella* include *S.* Typhi and *S.* Paratyphi, which are human-adapted and cause systemic disease (LaRock et al., 2015). Nontyphoidal *Salmonella* cause self-limiting gastroenteritis and is one of the leading causes of death from diarrheal disease, with ~93 million infections and 155,000 deaths each year (Ao et al., 2015). *Salmonella* is taken up via the fecal-oral route and can infect a broad range of hosts. *S.* Typhimurium causes gastroenteritis in humans and systemic disease in mice. Therefore, murine infections with *S.* Typhimurium are used as a model to study systemic disease by typhoidal Salmonella in humans (Broz et al., 2012).

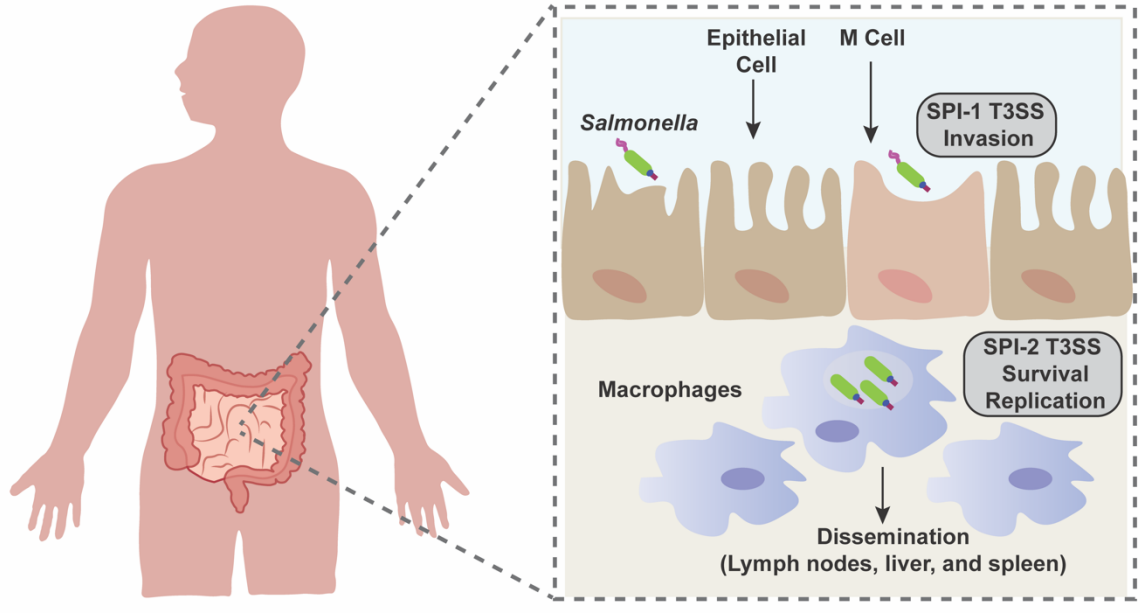

Figure 1.3: *Salmonella* employs two T3SS to inject virulence factors and cause disease. Upon colonization**,** *Salmonella* encodes the SPI-1 T3SS to invade M cells and enterocytes in the small intestine. Inside of the cell, the bacterium can sense the phagosomal environment and encode the SPI-2 T3SS to survive and replicate inside of the host cell. After crossing the epithelial barrier, the bacteria can be phagocytosed by immune cells where it can survive and disseminate to other sites of infection.

Once ingested, *Salmonella* invades epithelial cells in the small intestine (**Fig. 1.3**). The bacteria utilize several fimbrial and non-fimbrial adhesins to mediate adhesion to

epithelial cells (Wagner and Hensel, 2011). *Salmonella* can then invade the epithelial barrier through infection of M cells or non-phagocytic enterocytes (Jones et al., 1994). To invade epithelial cells, *Salmonella* employs a Type III Secretion System (T3SS) encoded by *Salmonella* pathogenicity island 1 (SPI-1). Several effector molecules that activate Rho GTPases, including SopB, SopE, and SopE2, are important for actin rearrangement in the host cell, membrane ruffling, and internalization of the bacteria into epithelial cells (Bakshi et al., 2000; Zhou et al., 2001; LaRock et al., 2015).

After crossing the epithelial barrier, *Salmonella* can be phagocytosed by innate immune cells, such as macrophages (Broz et al., 2012). To survive and replicate inside the host cell, *Salmonella* employs a second T3SS encoded by *Salmonella* pathogenicity island 2 (SPI-2) (Jennings et al., 2017). In order to induce the SPI-2 T3SS, *Salmonella* senses the environment within the acidified phagosome (Alpuche Aranda et al., 1992). Expression of the genes that encode the SPI-2 T3SS is tightly controlled by a two-component regulatory system involving the sensor kinase PhoQ and its cognate response regulator PhoP. PhoQ can be activated by low Mg^{2+} and low pH, which in turns activates PhoP. PhoP can repress the expression of *prg* (PhoP-repressed genes) and flagellar genes, whereas it activates the transcription of *pag* (PhoP-activated genes) (Prost and Miller, 2008). Therefore, once PhoQ is activated, the genes encoding the flagellar apparatus and the SPI-1 T3SS are repressed, whereas the genes encoding for the SPI-2 T3SS are induced. In fact, growth of *Salmonella* in minimal medium with low pH and low Mg^{2+} concentration promotes expression of the SPI-2 T3SS, but not the SPI-1 T3SS or flagellin. Conversely, growing the bacteria in a rich medium with low aeration and high NaCl concentration leads to expression of the SPI-1 T3SS and flagellin, but not the SPI-2 T3SS (Deiwick et al., 1999; Yu et al., 2010).

Following phagocytosis, the SPI-2 T3SS injects at least 28 effectors to establish an intracellular compartment named the *Salmonella*-containing vacuole (SCV) and inhibit phagosomal maturation (Jennings et al., 2017). Some of the important effectors secreted by the SPI-2 T3SS include SifA, SseJ, SseF, SseG, and PipB2. Bacterial mutants deficient in each of these effectors are attenuated in mice, highlighting their role in pathogenesis (Hensel et al., 1998; Beuzon et al., 2002; Freeman et al., 2003; Henry et al., 2006). SseF and SseG are important in tethering the SCV with the Golgi network and contribute to the formation of *Salmonella*-induced filaments (SIFs), which may promote nutrient acquisition for the bacteria (Jennings et al., 2017). The effector SseJ modifies the composition of cholesterol from the SCV (LaRock et al., 2012). PipB2 recruits kinesin-1 to the SCV, and SifA is important for vacuolar membrane integrity (Beuzon et al., 2000; Henry et al., 2006). SifA-deficient *Salmonella* leads to the rupture of the SCV and release of bacteria into the cytosol (Beuzon et al., 2000).

Colonization resistance to *Salmonella* is in part provided by the commensal microbiota, as treatment with antibiotics prior to infection increases susceptibility to infection (Que and Hentges, 1985). *S.* Typhimurium causes inflammation of the gut by using both SPI-1 and SPI-2 to invade epithelial cells and survive inside the host cell. This inflammation is important for allowing *Salmonella* to outcompete the intestinal microbiota (Stecher et al., 2007). Immune cells infected by *Salmonella* release reactive oxygen species (ROS) that can react with endogenous thiosulphate to form tetrathionate. Tetrathionate can then be used by *S.* Typhimurium as an alternate respiratory electron acceptor and outcompete the microbiota (Winter et al., 2010a). Furthermore, *S.* Typhimurium can also induce the production of host-derived nitrate and mediate anaerobic nitrate respiration to enhance its growth in the inflamed intestine (Lopez et al., 2012).

Several host PRRs engage with *Salmonella*-derived PAMPs and DAMPs to promote innate immune defense. However, *Salmonella* employs several SPI-2 T3SS effectors that manipulate innate immune signaling pathways. For example, SpvC irreversibly dephosphorylates p38 and JNK MAPKs to inhibit transcription of proinflammatory cytokines (Mazurkiewicz et al., 2008). GgtA can directly cleave the NF-κB transcription factors p65 and RelB, and the SseK family of effectors also inhibit NF-κB signaling (Sun et al., 2016; Jennings et al., 2017). The effector SopE activates small RHO GTPases, therefore activating NF-κB through NOD1 signaling (Keestra et al., 2013). In addition, SopE seems to have a role in the activation of caspase-1, although the mechanism is unknown (Muller et al., 2009).

1.11. Dissertation Aims

Salmonella is one of the leading causes of death from diarrheal disease. Increasing antibiotic resistance among *Salmonella* strains is highly concerning. Thus, there is an urgent need for a better understanding of the innate immune response to *Salmonella*, as this information is critical for developing novel therapeutics that can bolster host anti-microbial control. Most studies of *Salmonella* interactions with the immune system are conducted in mice. However, there are key differences in innate immune genes encoded by mice and humans, and the human immune response to *Salmonella* is poorly understood. To address this key knowledge gap, I have been investigating human-specific innate immune responses to *Salmonella* infection.

The inflammasome activates the protease caspase-1, which cleaves host substrates that enable secretion of IL-1 family cytokines and death of the infected cell. The NAIP inflammasome is crucial for host defense against *Salmonella* infection in mice. Mice

encode several NAIPs, each specific for a different bacterial ligand: NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod protein, and NAIP5 and NAIP6 recognize flagellin. In contrast, humans encode a single NAIP, and it was unclear what human NAIP senses and its role in antimicrobial responses to *Salmonella*. To better understand NAIP inflammasome responses to *Salmonella,* this dissertation will focus on the following aims (Fig. 1.4):

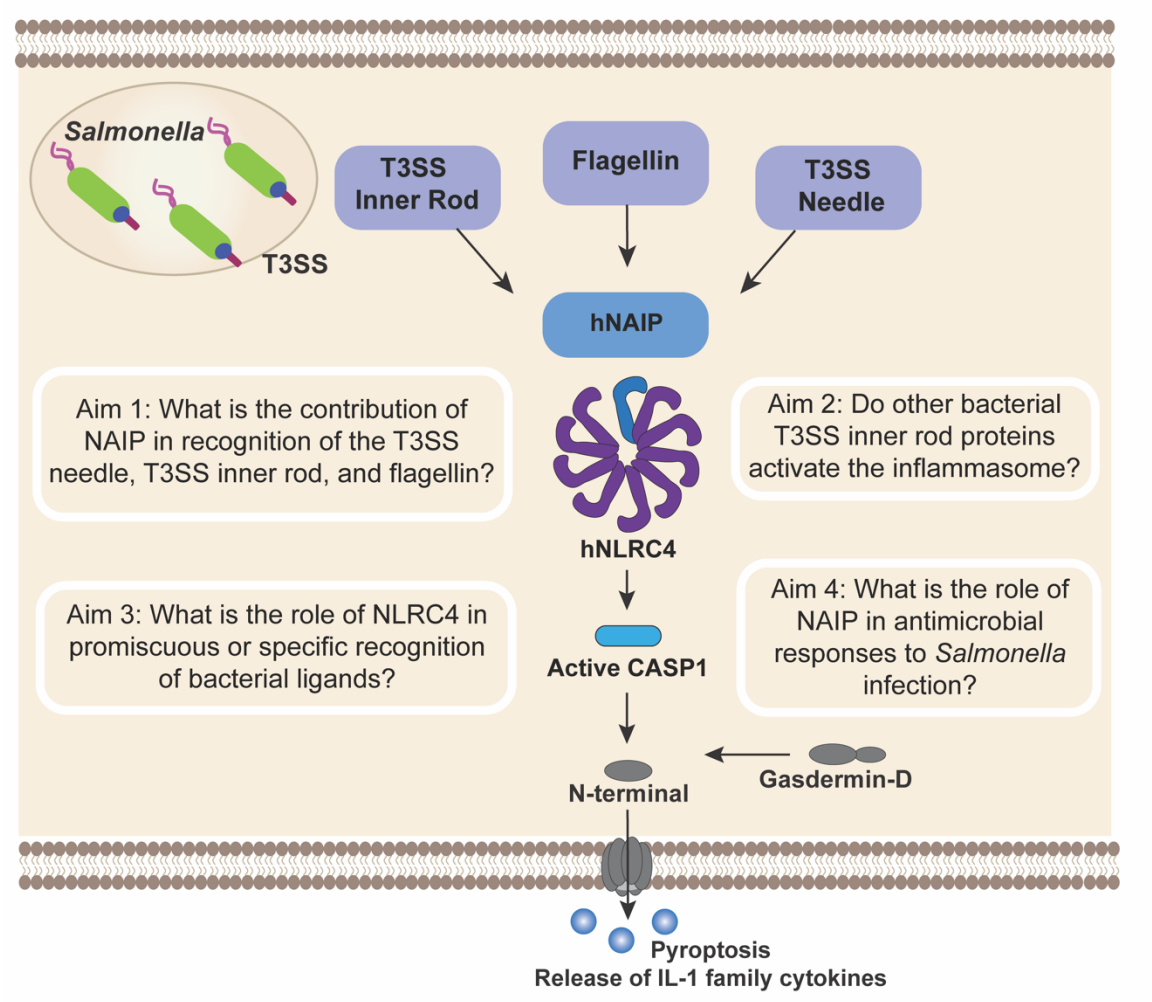

Figure 1.4: Human NAIP inflammasome responses to *Salmonella* infection. This dissertation will focus on defining the role of human the NAIP/NLRC4 inflammasome in recognition of components from the Type III secretion system and flagellar apparatus from *Salmonella* Typhimurium.

Aim 1: Define the contribution of hNAIP to inflammasome responses against different bacterial ligands

In contrast to mice, humans encode a single copy of the functional *NAIP* gene. Previous studies using biochemical approaches and immortalized monocytic cell lines determined that hNAIP is functionally similar to the murine NAIP1, as they both recognize the T3SS needle protein. However, studies in macrophages also suggested a role for hNAIP in restriction of flagellated bacteria. Preliminary data from our lab also show that *L. pneumophila* infection of primary human monocyte-derived macrophages (hMDMs) induces robust flagellin-dependent inflammasome activation. These data suggest that flagellin may be recognized by the hNAIP inflammasome. Indeed, a recent study found that flagellin triggers NAIP inflammasome responses in primary human monocyte-derived macrophages. In CHAPTER 2, we sought to determine inflammasome responses to *S.* Typhimurium and found that hNAIP promiscuously recognizes multiple bacterial ligands, which include flagellin, T3SS needle, and T3SS inner rod.

Aim 2: Define inflammasome responses to PrgJ homologs from other bacterial species

As our data showed that the T3SS inner rod from *Salmonella* is sensed by the human NAIP inflammasome, we also tested whether PrgJ homologs from other bacterial species similarly activate hNAIP in macrophages. In CHAPTER 2, we show that hNAIP broadly recognizes the T3SS inner rod from multiple bacterial species.

Aim 3: Determine the role of the adaptor protein NLRC4 in broad recognition of multiple bacterial ligands

Our data demonstrate that hNAIP mediates promiscuous recognition of multiple bacterial ligands. In CHAPTER 3, we sought to determine if the interaction between human NAIP and NLRC4 is important for promiscuous recognition. To test the role of NLRC4 in promiscuous or specific recognition of bacterial ligands, we reconstituted the inflammasome in HEK293 cells with different combinations of NAIPs and NLRC4s from mice or humans. Our preliminary data suggest that NAIP, rather than NLRC4, dictates ligand recognition specificity or promiscuity.

Aim 4: Determine the role of hNAIP in antimicrobial responses to *Salmonella* infection

Most studies aimed at determining the role of NAIP in anti-microbial responses are conducted in mice. In CHAPTER 3, we sought to determine the role of hNAIP in anti-microbial responses to *S*. Typhimurium infection. We employed CRISPR/Cas9 to knock out the single *NAIP* gene in THP-1 cell lines. Our preliminary data suggest that hNAIP is required for maximal inflammasome responses to *S.* Typhimurium. Additionally, NLRP3 may not be required for inflammasome responses to *S.* Typhimurium in human macrophages. Future studies are needed to validate these results and to test whether hNAIP mediates restriction of bacterial replication.

CHAPTER 2

Broad detection of bacterial type III secretion system and flagellin proteins by the human NAIP/NLRC4 inflammasome

This chapter appeared as a published peer-reviewed article titled “Broad detection of bacterial type III secretion system and flagellin proteins by the human NAIP/NLRC4 inflammasome” by **Valeria M. Reyes Ruiz**, Jasmine Ramirez, Nawar Naseer, Nicole M. Palacio, Ingharan J. Siddarthan, Brian M. Yan, Mark A. Boyer, Daniel A. Pensinger, John-Demian Sauer, and Sunny Shin. *PNAS*, 2017.

2.1. Abstract

Inflammasomes are cytosolic multi-protein complexes that initiate host defense against bacterial pathogens by activating caspase-1-dependent cytokine secretion and cell death. In mice, specific NAIPs (NLR family, apoptosis inhibitory proteins) activate the NLRC4 (NLR family, CARD domain-containing protein 4) inflammasome upon sensing components of the type III secretion system (T3SS) and flagellar apparatus. NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod protein, and NAIP5 and NAIP6 recognize flagellin. In contrast, humans encode a single functional NAIP, raising the question of whether human NAIP senses one or multiple bacterial ligands. Previous studies found that human NAIP detects both flagellin and the T3SS needle protein, and suggested that the ability to detect both ligands was achieved by multiple isoforms encoded by the single human *NAIP* gene. Here, we show that human NAIP also senses the *Salmonella* Typhimurium T3SS inner rod protein PrgJ, and that T3SS inner rod proteins from multiple bacterial species are also detected. Furthermore, we demonstrate that a single human NAIP isoform is capable of sensing the T3SS inner rod, needle, and flagellin. Our findings indicate that in contrast to murine NAIPs, promiscuous recognition of multiple bacterial ligands is conferred by a single human NAIP.

2.2. Significance Statement

Inflammasomes are cytosolic multiprotein complexes that initiate innate immune responses to microbial infection. Inflammasome specificity is determined by cytosolic innate immune sensors, including the NLR family, apoptosis inhibitory proteins (NAIPs). In mice, which encode seven different NAIPs, individual NAIPs recognize specific components of the structurally related bacterial type III secretion system (T3SS) and flagellar apparatus. Humans encode a single functional NAIP, raising the question of

whether human NAIP recognizes the same repertoire of bacterial ligands. Here, we find that in contrast to the ligand specificity exhibited by the murine NAIPs, the single human NAIP broadly detects multiple T3SS and flagellin proteins. These findings provide a basis for understanding the mechanisms underlying human-specific innate immune responses against bacterial infection.

2.3. Introduction

In response to pathogenic bacteria, the innate immune system is required for inflammatory responses that promote host defense. Host defense is initiated by the engagement of pattern recognition receptors (PRRs) by pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). Cytosolic PRRs detect pathogens that introduce products into host cells as a consequence of bacterial virulence activities, such as specialized secretion systems. A subset of cytosolic PRRs, termed the NLR (nucleotide-binding domain, leucine-rich repeat-containing) family, is composed of 22 members in humans and 34 members in mice. A subfamily of NLRs, known as NLR family, apoptosis inhibitory proteins (NAIPs), recognize bacterial proteins that are translocated into the host cell by gram-negative bacteria. One such pathogen is *Salmonella,* which employ a virulence-associated type III secretion system (T3SS) to inject effector proteins into the host cell cytosol that promote bacterial invasion and survival (Galan et al., 2014). These secretion systems also translocate structurally related components of the T3SS or closely related flagellar apparatus, enabling cytosolic detection of bacteria by NAIPs (Sun et al., 2007). In mice, ligands for four of the seven distinct NAIPs are known: NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod protein, and both NAIP5 and NAIP6 recognize flagellin (Kofoed and Vance, 2011; Zhao et al., 2011; Rayamajhi et al., 2013; Yang et al., 2013; Rauch et al., 2016; Zhao et al., 2016). Upon

binding their cognate ligands, the NAIPs recruit the adaptor NLRC4 (Diebolder et al., 2015; Hu et al., 2015; Zhang et al., 2015). The resulting NAIP/NLRC4 inflammasome then recruits and activates caspase-1 (Martinon et al., 2002). Active caspase-1 mediates processing and secretion of IL-1 family cytokines and a proinflammatory cell death termed pyroptosis (Kuida et al., 1995; Li et al., 1995; Bergsbaken et al., 2009), which promote anti-microbial functions critical for controlling bacterial infection (Miao et al., 2010a; Sellin et al., 2014; Jorgensen et al., 2016; Rauch et al., 2017). This inflammasome also plays a protective role in mouse models of colitis-associated colorectal cancer and may be a useful strategy in tumor immunotherapy (Garaude et al., 2012; Lin et al., 2016). However, the NLRC4 inflammasome can cause sepsis-like disease following antibiotic disruption of the microbiota, and activating NLRC4 mutations can lead to human auto-inflammatory diseases (Ayres et al., 2012; Canna et al., 2014; Kitamura et al., 2014; Romberg et al., 2014; Canna et al., 2016). Defining the mechanisms of human NAIP sensing of bacterial ligands may therefore provide insight into therapeutic approaches for diverse infectious and autoinflammatory diseases.

Unlike mice, the human *NAIP* locus has a number of pseudogenes and gene duplications and has retained a single functional copy of the full-length *NAIP* gene (Romanish et al., 2007; Romanish et al., 2009). Initial studies with human monocytic cell lines suggested that human NAIP could only sense the T3SS needle protein (Zhao et al., 2011; Rayamajhi et al., 2013; Yang et al., 2013). However, a recent study found that flagellin also triggers NAIP inflammasome activation in primary human macrophages, and indicated that detection of flagellin was mediated by an alternate splice isoform of NAIP (Kortmann et al., 2015). These findings suggested that in humans, specificity for different bacterial ligands is encoded by distinct splicing variants of the single *NAIP* gene.

Here, we demonstrate that in addition to the T3SS needle protein and flagellin, primary human macrophages also mount NAIP inflammasome responses against T3SS inner rod proteins from multiple bacterial pathogens. In addition, our data show that the *Salmonella* Typhimurium SPI-2 T3SS inner rod protein, SsaI, which is required for intracellular bacterial replication, does not activate the inflammasome in human macrophages, suggesting that intracellular *Salmonella* evade NAIP recognition in both humans and mice. Intriguingly, we find that a single human NAIP isoform is sufficient for NLRC4 inflammasome responses to the T3SS needle, inner rod, and flagellin. Overall, our findings suggest that unlike mice, which express multiple NAIPs that each possesses exquisite ligand specificity, the single human NAIP has evolved to broadly recognize multiple bacterial ligands. These findings provide important insight into distinct mechanisms of innate immune sensing of gram-negative bacteria by mice and humans.

2.4. Results

2.4.1. *Salmonella* Typhimurium induces flagellin-independent inflammasome responses in primary human macrophages

In murine macrophages, the NAIPs induce inflammasome activation upon direct recognition of proteins from the T3SS and the structurally related flagellar apparatus. The relative contribution of these components to the inflammasome response in human macrophages is still unclear. Thus, we examined cell death as well as secretion of IL-1α and IL-1β following infection of human monocyte-derived macrophages (hMDMs) with wild type (WT), SPI-1 T3SS-deficient (Δ*sipB*), or flagellin-deficient (Δ*fliCfljB*) *Salmonella* Typhimurium strains. Compared to WT *Salmonella*-infected macrophages, Δ*fliCfljB*

Salmonella-infected macrophages exhibited a slight, but not statistically significant decrease in inflammasome activation as measured by IL-1α and IL-1β secretion, IL-1β processing, and cell death (Fig. 2.1 A-D). In contrast, inflammasome activation was abrogated in Δ*sipB*-infected macrophages (Fig. 2.1 A-C). Immunoblot analysis indicated

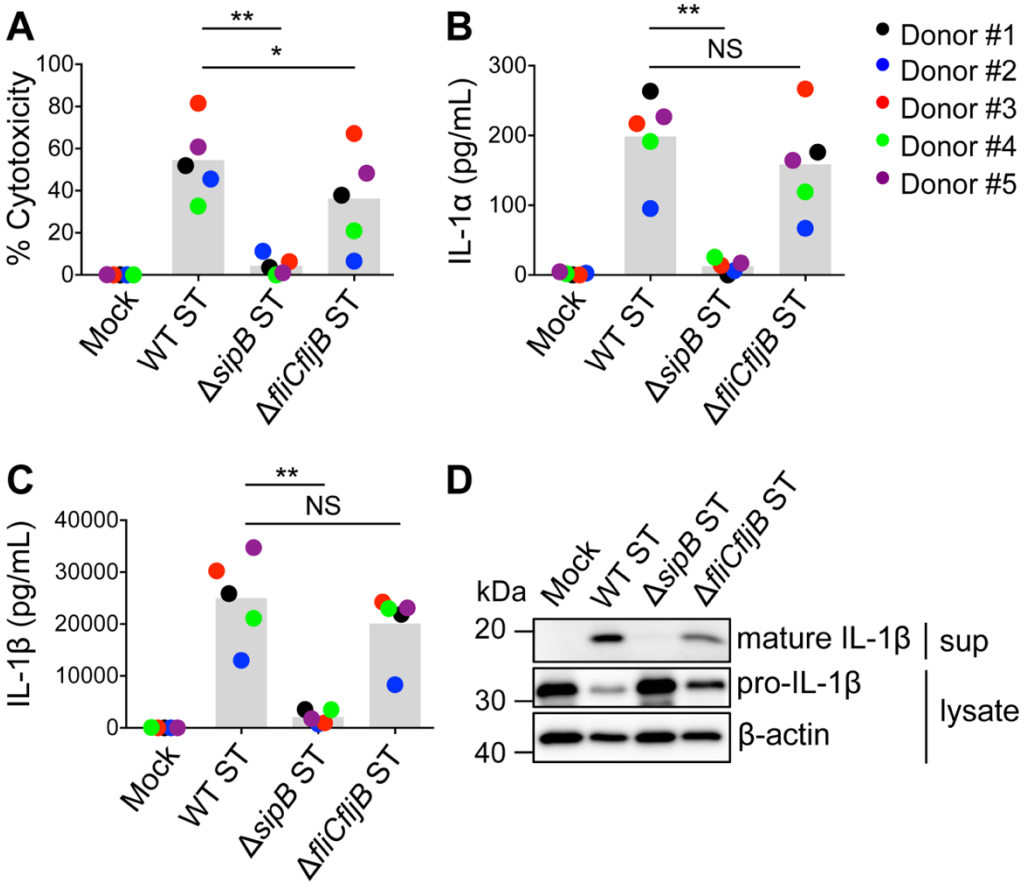

Figure 2.1: *S.* Typhimurium induces T3SS-dependent, flagellin-independent inflammasome responses in primary human macrophages. hMDMs were primed with LPS for 3 hours and treated with PBS (Mock), wild type *Salmonella* (WT ST), Δ*sipB* ST, or Δ*fliCfljB* ST at an MOI of 20 for 4 hrs. (A) Cell death (% cytotoxicity) was measured by LDH release assay and normalized to mock infected cells. (B-C) IL-1α and IL-1β supernatant levels were measured by ELISA. (D) Immunoblot analysis was performed on supernatants for mature IL-1β and on lysates for pro-IL-1β and β-actin as a loading control (representative of two donors). Each data point represents the mean of triplicate infected wells for each of five different human donors. Shaded bars represent the overall mean of the donors. * $p<0.05$, ** $p<0.01$ by paired *t* test.

no defect in pro-IL-1β production in Δ*sipB*-infected hMDMs, but inflammasome-mediated cleavage of pro-IL-1β into its active form was not observed (Fig. 2.1 D). These results suggest that *Salmonella* infection of primary human macrophages induces robust flagellin–independent inflammasome activation that requires the SPI-1 T3SS.

2.4.2. *S.* Typhimurium T3SS inner rod protein PrgJ activates the inflammasome in primary human macrophages

Previous studies using immortalized human monocyte cell lines found that the NAIP inflammasome could be activated by the T3SS needle protein, but not flagellin or the T3SS inner rod (Zhao et al., 2011; Rayamajhi et al., 2013; Yang et al., 2013). However, another study found that NAIP played a role in restricting the intracellular replication of flagellated bacteria (Vinzing et al., 2008). Recently, it was shown that flagellin can activate the NAIP inflammasome in primary hMDMs (Kortmann et al., 2015). As our data suggested that there is a robust flagellin-independent, T3SS-dependent inflammasome response to *Salmonella*, we sought to determine whether in addition to the T3SS needle protein PrgI, the T3SS inner rod protein PrgJ could induce inflammasome activation in primary hMDMs. We utilized the gram-positive pathogen *Listeria monocytogenes,* which does not encode a T3SS apparatus, to directly deliver PrgJ or PrgI into host cells (Sauer et al., 2011). Following infection, *Listeria* uses the pore-forming toxin Listeriolysin O (LLO) to escape into the cytosol, where it expresses the protein ActA on the bacterial surface to polymerize actin (Gaillard et al., 1987; Tilney and Portnoy, 1989). We utilized strains that ectopically express PrgJ or PrgI translationally fused to the N-terminus of ActA and under control of the *actA* promoter. This approach of delivering flagellin into the host cell cytosol robustly activates the mouse NAIP5 inflammasome (Sauer et al., 2011). Indeed, as

expected, hMDMs infected with *Listeria* expressing PrgI induced robust IL-1α and IL-1β secretion, IL-1β processing, and cell death above that of WT *Listeria*-infected cells (Fig. 2.2 A-D). Surprisingly, infection with PrgJ-expressing *Listeria* also induced robust IL-1α and IL-1β release, IL-1β processing, and cell death (Fig. 2.2 A-D). Importantly, cytosolic

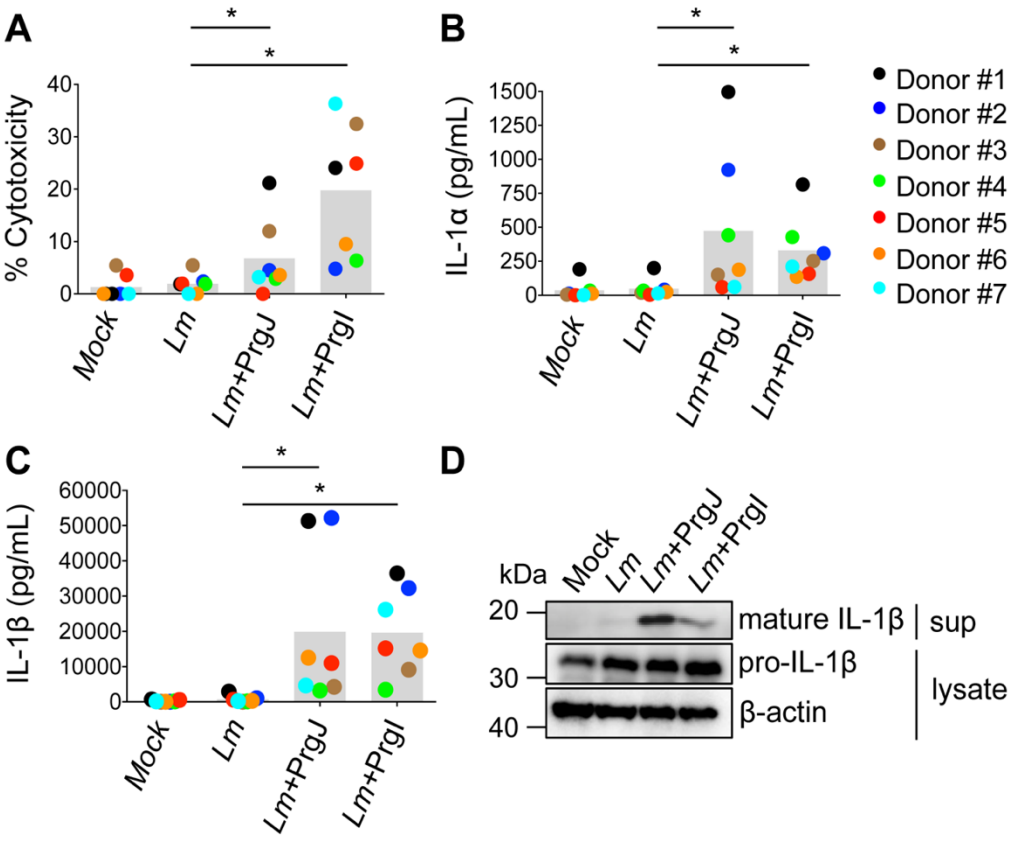

Figure 2.2: *Listeria monocytogenes*-mediated delivery of the T3SS inner rod protein PrgJ activates the inflammasome in primary human macrophages. hMDMs were primed with Pam3CSK4 for 3 hours and infected with PBS (Mock), WT *Listeria (Lm)*, or strains expressing PrgJ and PrgI at an MOI of 5 for 16 hours. (A) Cell death (% cytotoxicity) was measured by LDH release assay and normalized to mock infected cells. (B-C) IL-1α and IL-1β supernatant levels were measured by ELISA. (D) Immunoblot analysis of supernatants for mature IL-1β and lysates for pro-IL-1β and β-actin as a loading control (representative of two donors). Each data point represents the mean of triplicate infected wells for each of seven different human donors. Shaded bars represent the overall mean of the donors. * $p<0.05$ by paired Wilcoxon signed-rank test

access was required for inflammasome activation, as PrgJ-expressing *Listeria* lacking *hly*, the gene encoding LLO, did not induce IL-1β secretion (Fig. 2.3).

To determine whether PrgJ alone could induce inflammasome activation independently of bacterial infection, we employed an anthrax toxin-based delivery system (Zhao et al., 2011; von Moltke et al., 2012; Rauch et al., 2016). In this system, bacterial ligands are translationally fused to the N-terminal domain of *Bacillus anthracis* lethal factor (LFn). The LFn domain enables ligand translocation into the host cell cytosol through a membrane channel formed by the anthrax protective antigen (PA) protein. We employed a translational fusion of LFn and PrgJ (LFn-PrgJ), as well as LFn fused to flagellin as a positive control for NAIP inflammasome activation. To avoid potential confounding effects of TLR5 detection of flagellin, we used a truncated *Legionella pneumophila* flagellin that

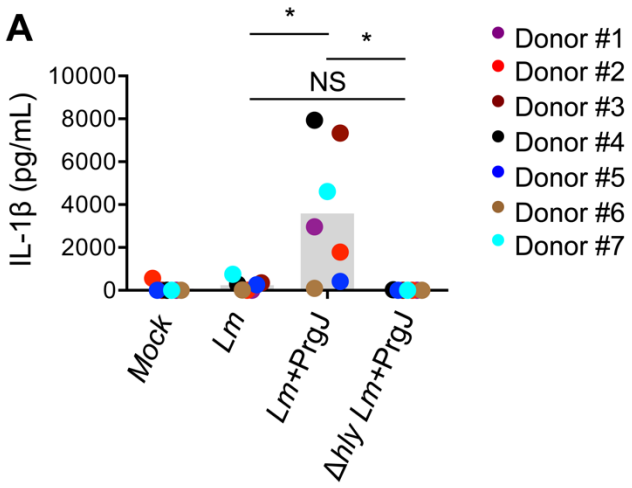

Figure 2.3: *Listeria monocytogenes*-mediated delivery of the T3SS inner rod protein PrgJ requires Listeriolysin O for inflammasome activation in primary human macrophages. hMDMs were primed with Pam3CSK4 for 3 hours and infected with PBS (Mock), WT *Listeria* (*Lm*) expressing PrgJ, or Δ*hly Lm* expressing PrgJ at an MOI of 5 for 16 hours. IL-1β supernatant levels were measured by ELISA. Each data point represents the mean of triplicate infected wells for each of seven different human donors. Shaded bars represent the overall mean of the donors. * $p<0.05$, NS=not significant by paired Wilcoxon signed-rank test.

lacks the TLR5-activating region but retains the C-terminal 166 amino acids detected by murine NAIP5 (Lightfield et al., 2008; Lightfield et al., 2011). In agreement with previous findings (Kortmann et al., 2015), hMDMs treated with PA+LFn-FlaA$^{310-475}$ (referred to as FlaTox) induced robust inflammasome activation, as measured by significantly increased

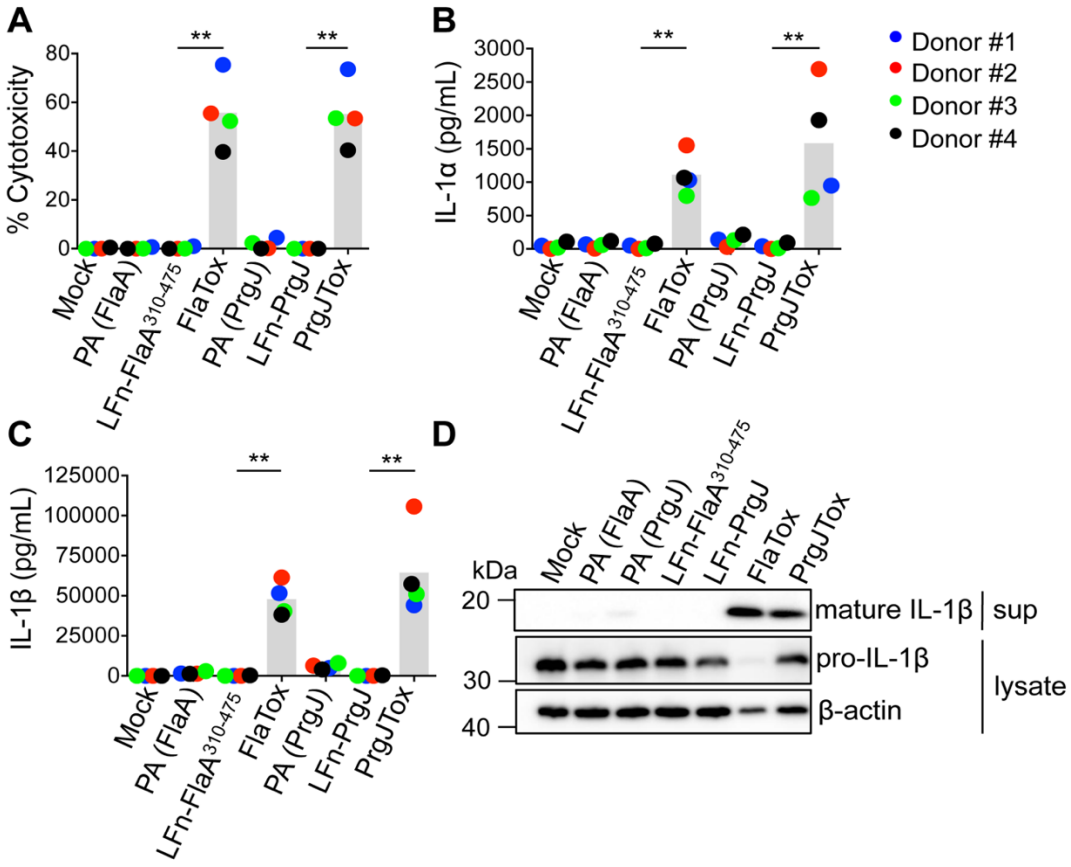

Figure 2.4: Anthrax toxin-mediated delivery of the T3SS inner rod protein PrgJ induces robust inflammasome activation in primary human macrophages. hMDMs were primed with Pam3CSK4 for 4 hours and treated with: PA alone, LFn-FlaA$^{310-475}$ alone, LFn-PrgJ alone, PA+LFn-FlaA$^{310-475}$ (FlaTox), or PA+LFn-PrgJ (PrgJTox) for 16h. (A) Cell death (% cytotoxicity) was measured by LDH release assay and normalized to mock infected cells. (B-C) IL-1α and IL-1β supernatant level were measured by ELISA. (D) Immunoblot analysis of supernatants for mature IL-1β and lysates for pro-IL-1β and β-actin as a loading control (representative of two donors). Each data point represents the mean of triplicate infected wells for each of four different human donors. Shaded bars represent the overall mean of the donors. * $p<0.05$, ** $p<0.01$ by paired *t* test.

IL-1α and IL-1β cytokine release, IL-1β processing, and cell death (Fig. 2.4 A-D). Treatment with PA+LFn-PrgJ (referred to as PrgJTox) also induced robust IL-1α and IL-1β cytokine secretion, IL-1β processing, and cell death (Fig. 2.4 A-D). In contrast, treatment with PA, LFn-FlaA$^{310-475}$, or LFn-PrgJ alone did not activate, indicating that FlaA and PrgJ induce inflammasome activation only when delivered into the host cell cytosol via PA. Altogether, these results show that primary human macrophages undergo inflammasome activation upon cytosolic sensing of the *S.* Typhimurium T3SS inner rod protein.

2.4.3. Human NAIP is required for maximal inflammasome responses to the T3SS inner rod protein PrgJ

Human NAIP is required for inflammasome responses to flagellin and the T3SS needle protein (Zhao et al., 2011; Yang et al., 2013; Kortmann et al., 2015). To test whether NAIP is also necessary for detecting PrgJ, we used siRNAs to silence *NAIP* in primary hMDMs (Fig. 2.5). As expected (Kortmann et al., 2015), anti-*NAIP* siRNA treatment resulted in significantly decreased IL-1α and IL-1β secretion following FlaTox administration compared to control siRNA treatment. Anti-*NAIP* siRNA treatment also led to significantly decreased IL-1α and IL-1β secretion in response to PrgJTox administration relative to control siRNA treatment, suggesting that NAIP is required for maximal inflammasome responses to PrgJ (Fig. 2.6 A and B). Importantly, siRNA-mediated silencing of NAIP did not significantly affect inflammasome responses to LPS+Nigericin, which specifically activates the NLRP3 inflammasome and does not engage NAIP (Mariathasan et al., 2006) (Fig. 2.6 C and D). These results indicate that NAIP is required for maximal inflammasome responses to the T3SS inner rod.

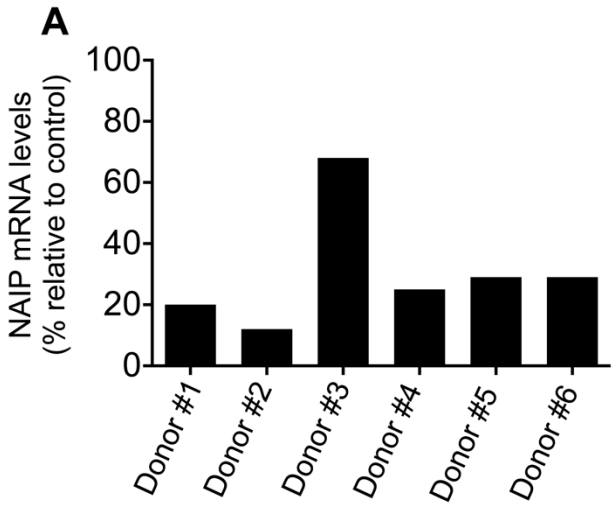

Figure 2.5: *NAIP* siRNA knockdown efficiency in primary human macrophages. qRT-PCR was performed to quantitate *NAIP* mRNA levels in hMDMs treated with either control siRNA or *NAIP* siRNA. For the *NAIP* siRNA-treated cells from each donor, *NAIP* mRNA levels were normalized to human HPRT mRNA levels, and each sample was normalized to control siRNA-treated cells from the same donor.

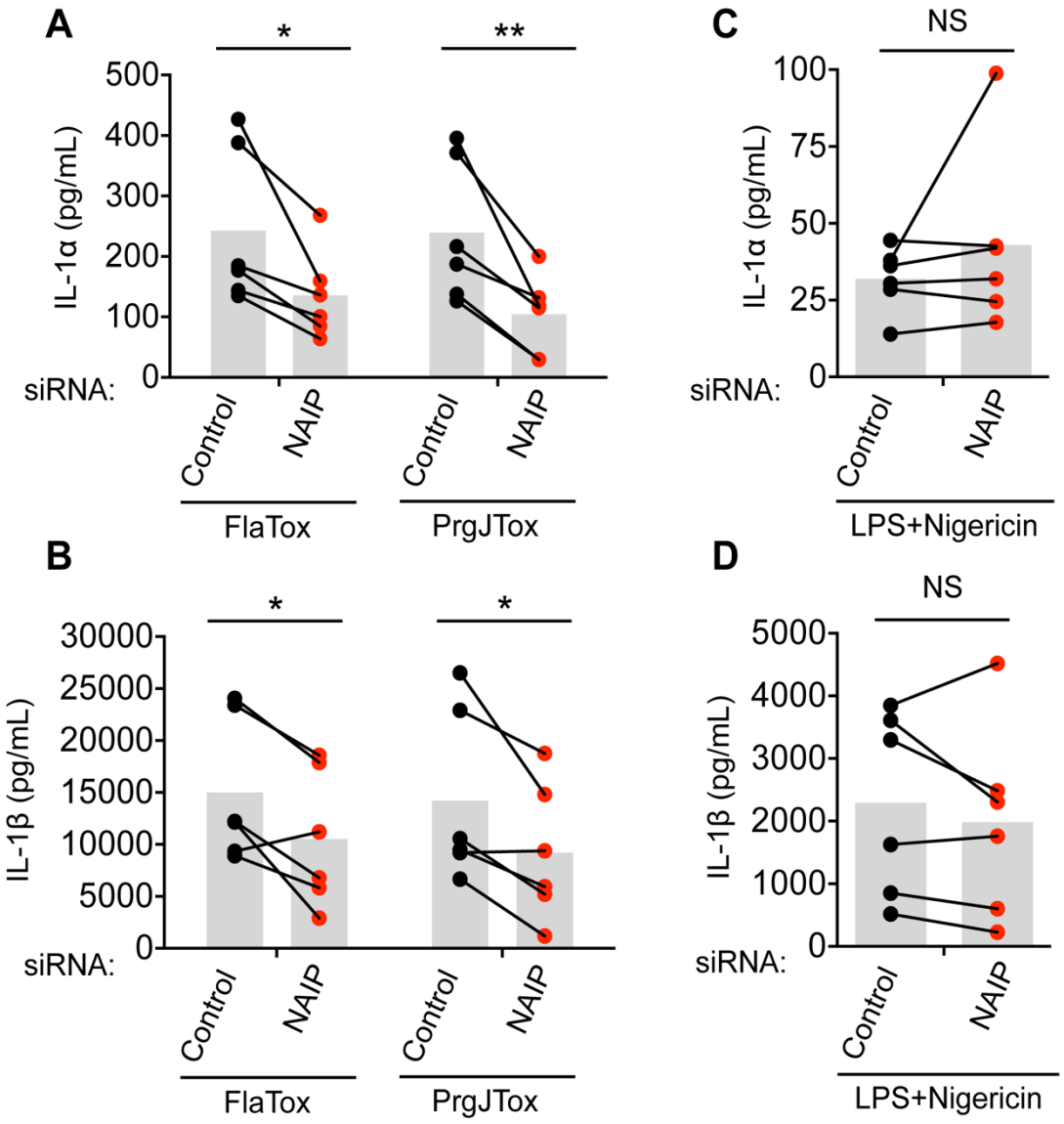

Figure 2.6: Human NAIP is required for maximal inflammasome responses to flagellin and the T3SS inner rod protein PrgJ. (A-D) Primary hMDMs were transfected with control siRNA or siRNA against NAIP for 48h and primed with Pam3CSK4 for 4h. (A-B, E-F) Cells were treated with PA+LFn-PrgJ (PrgJTox) or PA+LFn-FlaA$^{310-475}$ (FlaTox) for 5h. (C-D) Cells were treated with LPS+Nigericin for 5h. IL-1α and IL-1β supernatant levels were measured by ELISA. Each data point represents the mean of triplicate infected wells for six different human donors. Shaded bars represent the overall mean of the donors. * $p<0.05$, ** $p<0.01$ by paired *t* test.

2.4.4. T3SS inner rod proteins from other bacterial species induce inflammasome activation in human macrophages

As T3SS inner rod proteins from multiple bacterial species activate the mouse NAIP2 inflammasome (Miao et al., 2010b), we next examined whether other bacterial T3SS inner rod homologs similarly activate human cells. We engineered *Listeria* strains expressing the T3SS inner rod proteins from *Burkholderia thailandensis* (BsaK), *Shigella flexneri* (MxiI), and *Chromobacterium violaceum* (CprJ). In agreement with previous findings (Miao et al., 2010b), mouse macrophages infected with *Listeria* expressing these inner rod homologs robustly secreted IL-1β (Fig. 2.7). hMDMs infected with *Listeria* expressing PrgI, PrgJ, BsaK, and MxiI resulted in robust IL-1β secretion and processing well above that of WT *Listeria*-infected cells (Fig. 2.8 A and B). In contrast, CprJ-expressing *Listeria* induced relatively low levels of IL-1β secretion and processing. These

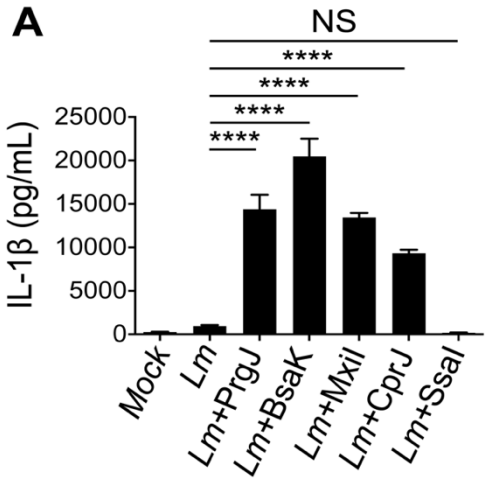

Figure 2.7: *Listeria monocytogenes* strains ectopically expressing T3SS inner rod homologs induce inflammasome activation in mouse macrophages. Bone marrow-derived macrophages were primed with Pam3CSK4 for 16 hours and infected with WT *Listeria* (*Lm*) or strains ectopically expressing PrgJ, PrgI, BsaK MxiI, CprJ, or SsaI at an MOI of 5 for 6 hours. Cells were treated with PBS for the Mock control. IL-1β levels in the supernatants was measured by ELISA. Bar graphs display the mean ± SD of triplicate wells. Representative of two independent experiments. **** $p<0.0001$ by Tukey's multiple comparisons test.

findings show that human macrophages broadly detect and activate the inflammasome in response to T3SS inner rod proteins from multiple bacterial species.

2.4.5. *S.* Typhimurium SPI-2 T3SS inner rod protein SsaI evades immune detection by human macrophages

S. Typhimurium employs two different T3SS, termed SPI-1 and SPI-2. The SPI-1 T3SS plays a role in bacterial invasion, whereas the SPI-2 T3SS is required for intracellular survival and replication (Galan, 2001; Figueira and Holden, 2012; LaRock et al., 2015), suggesting a need to evade host recognition of the SPI-2 T3SS. Indeed, while the SPI-1 T3SS inner rod protein, PrgJ, robustly activates the mouse NAIP2 inflammasome, the SPI-2 T3SS inner rod protein, SsaI, evades detection (Miao et al., 2010b). We therefore asked whether SsaI also evades human NAIP by expressing SsaI in *Listeria*. Consistent with previous findings (Miao et al., 2010b), mouse macrophages infected with *Listeria* expressing SsaI secreted negligible levels of IL-1β (Fig. 2.7). Infection of hMDMs with SsaI-expressing *Listeria* also resulted in negligible IL-1β secretion and cleavage compared to infection with *Listeria* expressing PrgJ or PrgI (Fig. 2.8 C and D). These data suggest that the SPI-2 T3SS inner rod protein SsaI has evolved to evade NAIP recognition in both mice and humans.

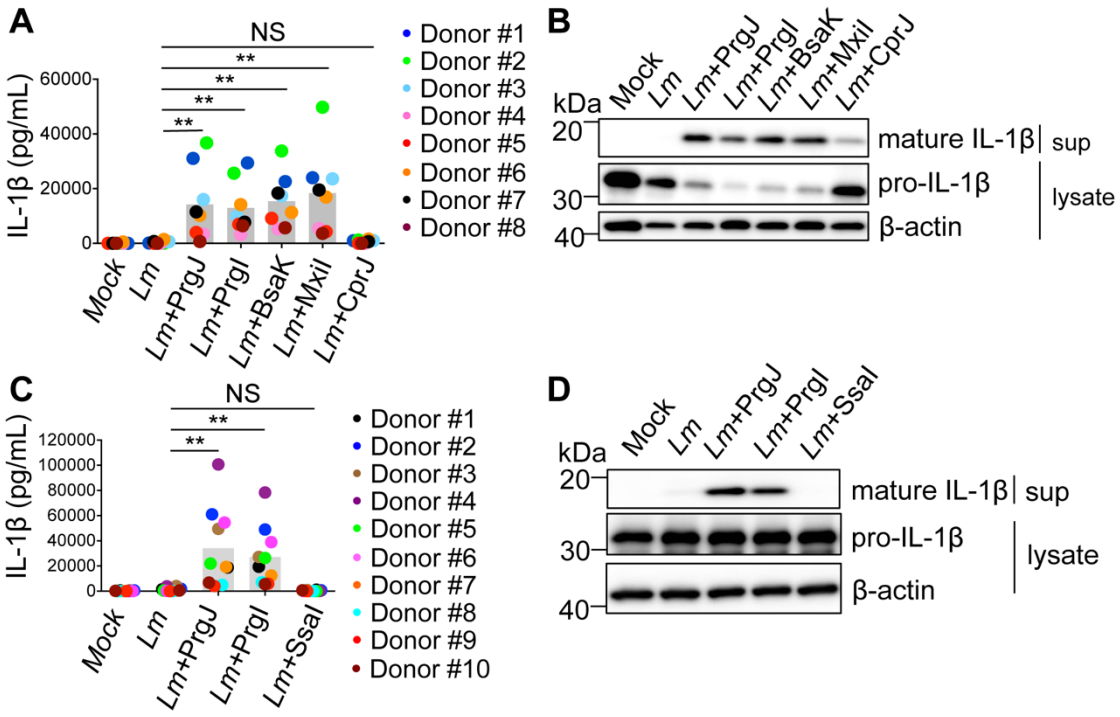

Figure 2.8: The T3SS from multiple bacterial species activate the human inflammasome whereas the *S.* typhimurium SPI-2 T3SS inner rod protein, SsaI, evades immune recognition. hMDMs were primed with Pam3CSK4 for 3 hours and infected with WT *Listeria (Lm)* or strains ectopically expressing PrgJ, PrgI, BsaK MxiI, CprJ, or SsaI at an MOI of 5 for 16 hours. Cells were treated with PBS for the Mock control. (A, C) IL-1β supernatant levels were measured by ELISA. (B, D) Immunoblot analysis of supernatants for mature IL-1β and lysates for pro-IL-1β and β-actin as a loading control. Each data point represents the mean of triplicate infected wells for each of eight to ten different human donors. Shaded bars represent the overall mean of the donors. ** $p<0.01$ by paired Wilcoxon signed-rank test

2.4.6. The THP-1 monocytic cell line undergoes inflammasome activation in response to T3SS inner rod and flagellin proteins

Previous studies using immortalized U937 and THP-1 monocyte cell lines found that anthrax toxin-mediated delivery of flagellin or inner rod proteins did not induce inflammasome activation (Zhao et al., 2011; Yang et al., 2013). Transfection of purified PrgJ protein into these cells also failed to activate the inflammasome (Rayamajhi et al., 2013). In contrast, recent findings and the data presented here demonstrate that hMDMs

mount robust inflammasome responses to flagellin (Kortmann et al., 2015) and the T3SS inner rod. A previously proposed explanation for these discrepant findings is that distinct *NAIP* splicing isoforms possess differing ligand specificities, and that primary human macrophages and immortalized cells express differing levels of particular isoforms (Kortmann et al., 2015). Alternatively, human NAIP may recognize all three bacterial ligands regardless of isoform type. As THP-1 cells express lower levels of NAIP and NLRC4 than primary macrophages (Kortmann et al., 2015), the method of ligand delivery or specific bacterial proteins previously employed may not have been sufficient for inflammasome activation in this cell type. Previous studies utilized the *C. violaceum* inner rod protein CprJ (Zhao et al., 2011; Yang et al., 2013), which we found to be a poor inflammasome activator in hMDMs relative to other T3SS inner rod homologs (Fig. 2.8 A and B). Another study used transfection-based delivery of PrgJ protein (Rayamajhi et al., 2013), which is likely not as efficient at delivering proteins into host cells as the anthrax toxin system.

Thus, we next tested whether THP-1 cells activate inflammasome responses to PrgJ delivered via *Listeria* or the anthrax toxin system. Although PrgI-expressing *Listeria* induced IL-1α and IL-1β secretion in THP-1 cells, PrgJ-expressing *Listeria* failed to do so (Fig. 2.9), despite robustly activating hMDMs (Fig. 2.2). In contrast, THP-1 cells treated with PrgJTox robustly secreted IL-1α and IL-1β (Fig. 2.10 A and B). Consistent with previous findings (Zhao et al., 2011; Yang et al., 2013), anthrax toxin-mediated delivery of full-length flagellin failed to activate THP-1 cells (Fig. 2.11). Intriguingly, anthrax toxin-mediated delivery of a truncated version of flagellin robustly triggered IL-1α and IL-1β secretion (Fig. 2.10 A and B), likely due to more efficient delivery of truncated flagellin. These data demonstrate that THP-1 cells are capable of detecting the T3SS needle, inner

rod, and flagellin, but are less responsive than hMDMs, as the type of bacterial ligand and route of delivery influences the extent of inflammasome activation.

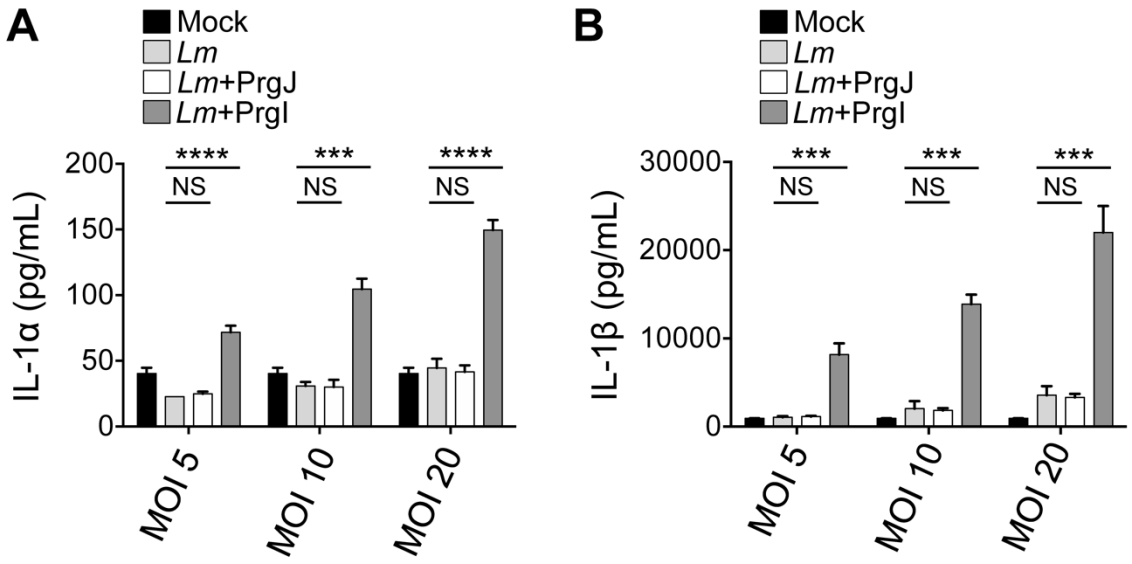

Figure 2.9: *Listeria monocytogenes*-mediated delivery of PrgJ does not induce inflammasome activation in THP-1 cells. THP-1 cells were primed with Pam3CSK4 for 16 hours and infected with WT *Listeria* (*Lm)* or strains ectopically expressing PrgJ or PrgI at an MOI of 5, 10, or 20 for 6 hours. Cells were treated with PBS for the Mock control. (A-B) IL-1α and IL-1β supernatant levels were measured by ELISA. Bar graphs display the mean ± SD of triplicate wells. Representative of three independent experiments. *** $p<0.001$ and **** $p<0.0001$ by unpaired *t* test.

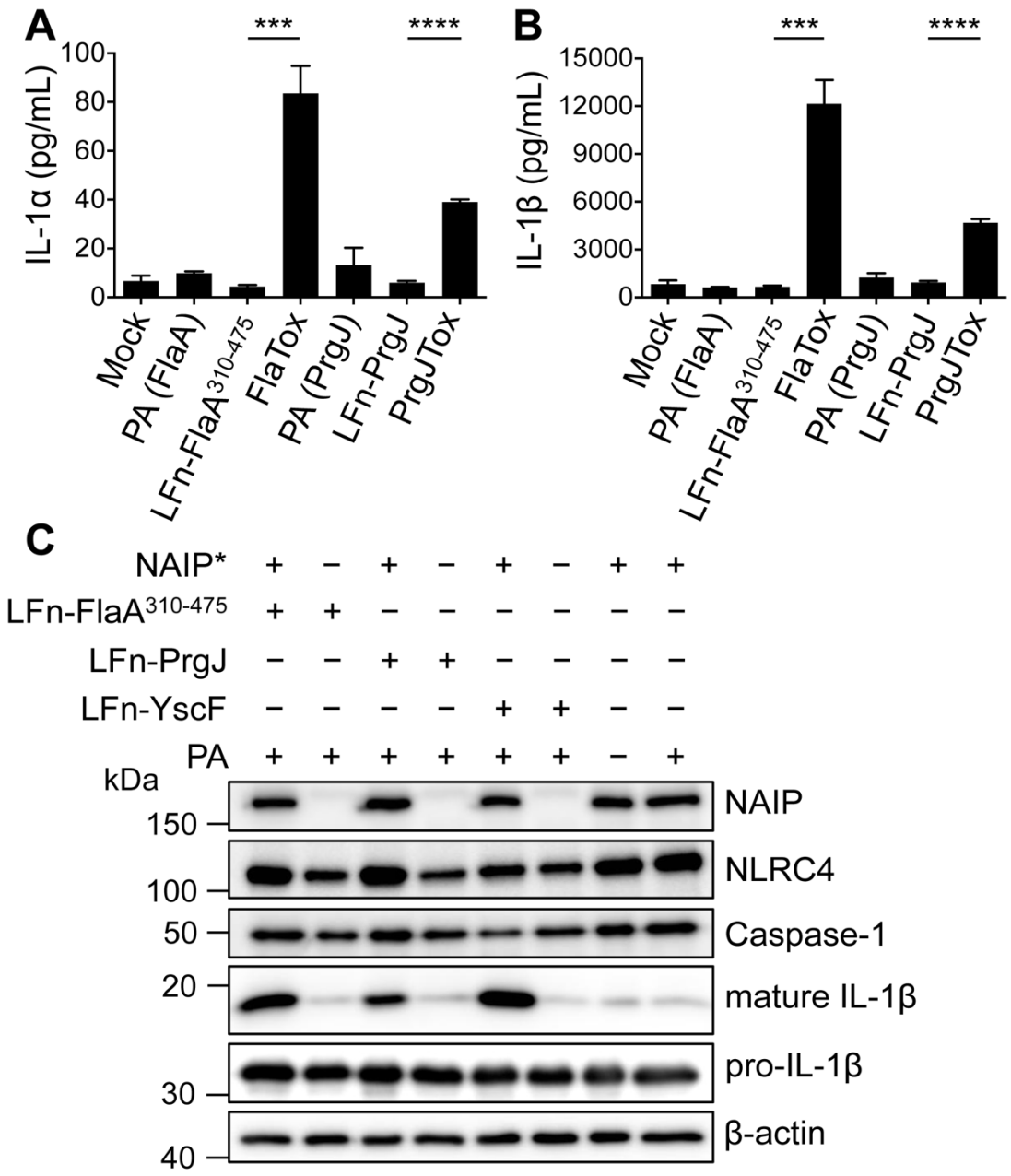

Figure 2.10: A single NAIP isoform is sufficient for inflammasome responses to flagellin, the T3SS inner rod protein, and the T3SS needle protein. (A-B) THP-1 cells were primed with Pam3CSK4 and treated with PA alone, LFn-FlaA$^{310-475}$ alone, LFn-PrgJ alone, PA+LFn-FlaA$^{310-475}$ (FlaTox), or PA+LFn-PrgJ (PrgJTox) for 5h. IL-1α and IL-1β supernatant levels were measured by ELISA. Bar graphs display the mean ± SD of triplicate wells. Representative of three independent experiments. *** $p<0.001$, **** $p<0.0001$ by unpaired *t* test. (C) HEK293 cells were transfected with expression vectors encoding NLRC4, caspase 1, and IL-1β. Where indicated, cells were also transfected with vectors encoding NAIP* (+) or empty vector control (-). After 18 hours, cells were treated

with PA+LFn-PrgJ, PA+LFn-FlaA$^{310-475}$, PA-LFn-YscF, or PA alone for 9 hours. Immunoblot analysis was performed on cell lysates for mature and pro-IL-1β, NAIP*, NLRC4, caspase-1, and β-actin as a loading control. Representative of three independent experiments.

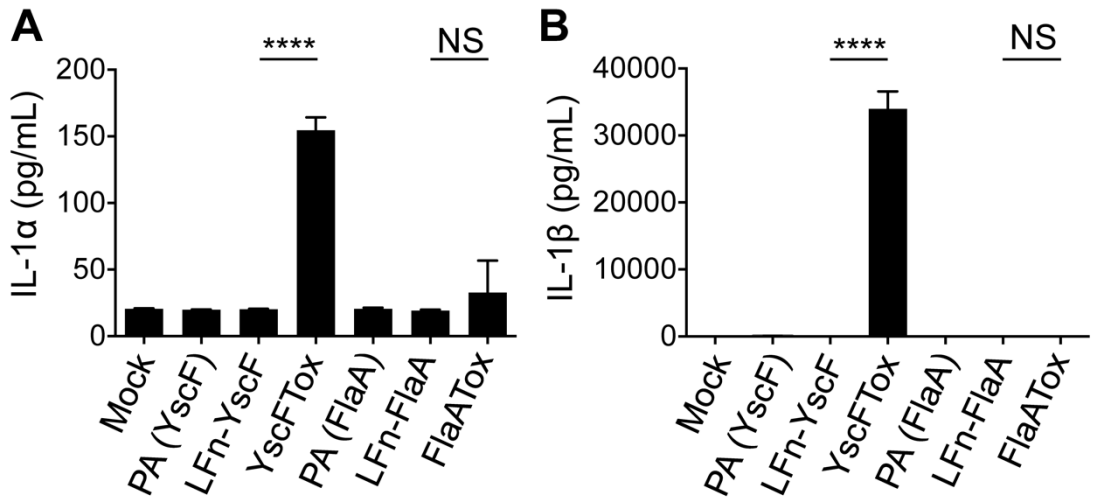

Figure 2.11: Anthrax toxin-mediated delivery of full-length flagellin fails to induce inflammasome activation in THP-1 cells. (A-B) THP-1 cells were primed with Pam3CSK4 for 4h and treated with PA alone, LFn-FlaA (full-length FlaA) alone, LFn-YscF alone, PA+LFn-FlaA (FlaTox) (full-length FlaA), or PA+LFn-YscF (YscFTox) for 16h. IL-1α and IL-1β supernatant levels were measured by ELISA. Bar graphs display the mean ± SD of triplicate wells. Representative of two independent experiments. **** $p<0.0001$ by unpaired *t* test

2.4.7. A single NAIP isoform mediates inflammasome responses to T3SS needle, inner rod, and flagellin proteins

Our data demonstrate that both THP-1 cells and hMDMs recognize the T3SS needle, inner rod, and flagellin, and that NAIP contributes to ligand detection. We next sought to understand how a single human *NAIP* gene could confer recognition of all three ligands, in contrast to mice, which utilize distinct NAIPs to recognize each ligand. Interestingly, studies in which chimeric mouse NAIPs were generated to define the ligand

specificity domain identified a chimeric mouse NAIP capable of recognizing multiple ligands, suggesting the possibility that human NAIP might function as a broad receptor (Tenthorey et al., 2014). Human monocytic cell lines express lower levels than hMDMs of a particular full-length *NAIP* splicing isoform (termed *NAIP**) that enables sensing of flagellin (Kortmann et al., 2015). We therefore sought to test whether a single NAIP isoform possesses specificity for a given bacterial ligand or is capable of detecting all three bacterial ligands. We ectopically expressed the NAIP* isoform previously shown to recognize flagellin (Kortmann et al., 2015), along with other NLRC4 inflammasome components in HEK293 cells, and then used the anthrax toxin system to deliver bacterial ligands into these cells. As expected, HEK293 cells expressing the NAIP* isoform robustly processed IL-1β in response to flagellin (Fig. 2.10 C). Unexpectedly, delivery of PrgJ or the *Burkholderia* T3SS needle protein (YscF) also induced robust IL-1β processing (Fig. 2.10 C). Critically, inflammasome activation by FlaA, PrgJ, and YscF required NAIP, as delivery of bacterial ligands into cells only expressing NLRC4, caspase-1, and IL-1β did not result in IL-1β processing. Inflammasome activation required delivery of the bacterial ligands, as untreated cells or PA treatment alone did not process IL-1β. Altogether, these data indicate that a single human NAIP isoform is sufficient to mediate inflammasome responses to the T3SS needle, inner rod, and flagellin proteins.

2.5. Discussion

Our data demonstrate that, like murine cells, human macrophages sense multiple bacterial ligands from the T3SS and flagellar apparatus. In addition to the T3SS needle and flagellin, T3SS inner rod proteins from multiple bacterial species activate the human NAIP inflammasome. Furthermore, a single human NAIP isoform can mediate inflammasome responses to all three bacterial proteins, in contrast to mouse NAIPs, which

are highly selective for recognition of individual flagellin or T3SS proteins (Kofoed and Vance, 2011; Zhao et al., 2011; Rayamajhi et al., 2013; Yang et al., 2013; Rauch et al., 2016; Zhao et al., 2016). Consistent with our findings, a recent study found that the *Pseudomonas aeruginosa* T3SS inner rod also activates the human NAIP inflammasome (Teddy et al., 2017). The conserved region of murine NAIPs that confers ligand specificity has been mapped to an internal region composed of several NBD-associated α-helical domains (Tenthorey et al., 2014). This region has evolved under positive selection in both rodents and primates (Tenthorey et al., 2014), suggesting that this domain mediates ligand detection in human NAIP as well. How NAIP achieves broad recognition of multiple ligands, and whether NAIP binds these ligands with similar or differing affinities or binding kinetics, is unclear. The T3SS inner rod, needle, and flagellin proteins exhibit low sequence conservation, but have some structural conservation, as the T3SS is thought to have evolved from the flagellar apparatus (Saier, 2004). Thus, human NAIP may recognize structural elements common to all three ligands. It will be of interest to determine whether NAIP detection of these three ligands is functionally redundant or distinct in the initiation of anti-microbial activities.

Our study raises intriguing questions about the evolution of the NAIP/NLRC4 inflammasome. It is likely that a single *NAIP* progenitor was present in the last common ancestor of primates and rodents (Growney et al., 2000). In mice, there has been an expansion of *NAIP* genes as a consequence of several gene duplication events (Endrizzi et al., 2000); interestingly, the murine NAIPs are specialists, as they each recognize only one of three bacterial proteins derived from the evolutionarily related T3SS and flagellar apparatus. In contrast, the single human NAIP is a generalist, as it is capable of functionally detecting all three bacterial proteins. The promiscuity displayed by human

NAIP may provide a selective advantage, as it may be more difficult for pathogens to simultaneously evade recognition of all three ligands by human NAIP.

Promiscuous ligand recognition may be a general strategy used by the innate immune system to diversify protein functionality as a means of promoting responses against different pathogenic stimuli. For example, the natural killer (NK) activating receptor NKG2D broadly recognizes several major histocompatibility complex (MHC) class I-related proteins, in contrast to other NK receptors, which typically recognize a single ligand. The ability of NKG2D to recognize a broad array of stress-inducible host ligands may provide an evolutionary advantage against viruses that employ mechanisms to downregulate NKG2D ligands as well as rapidly evolving cancers (Eagle and Trowsdale, 2007). Furthermore, the TLR sorting adaptor TIRAP promiscuously detects multiple lipids, which diversifies subcellular sites of TLR signaling and thus enables responses to both extracellular and endosomal pathogens (Bonham et al., 2014). However, one possible tradeoff with a more promiscuous mode of sensing is that human NAIP may possess weaker affinities or altered binding kinetics for its bacterial ligands and hence decreased signaling potency. In contrast, a given mouse NAIP may possess higher affinity or binding kinetics for its particular ligand and thus confer heightened immune responses. Indeed, compared to mouse macrophages, human macrophages do not seem to be as responsive to cytosolic flagellin, as they are more permissive for intracellular replication of flagellated bacteria (Vinzing et al., 2008). While the precise basis for this difference is unknown, one possibility is that human NAIP detects flagellin with lower affinity or altered binding kinetics than mouse NAIP5.

It will be of interest to examine how co-evolution with gram-negative bacteria shaped the *NAIP* genes in humans and other mammals, and whether pathogens have evolved strategies for evading human NAIP. Functional NAIP copy number varies among

human populations, and increased copy number has been postulated to confer a selective advantage in anti-bacterial defense (Boniotto et al., 2012). Studies in mice have shown that inappropriate activation of the NAIP/NLRC4 inflammasome can lead to lethal systemic inflammation resembling sepsis (Ayres et al., 2012; von Moltke et al., 2012). Moreover, gain-of-function mutations in human NLRC4 result in pathologic enterocolitis and autoinflammation (Canna et al., 2014; Kitamura et al., 2014; Romberg et al., 2014; Canna et al., 2016). Perhaps gain-of-function mutations in human NAIP confer similar pathological outcomes.

Our results provide new insight into human NAIP detection of bacterial proteins from the T3SS and flagellar apparatus. The data presented here provide an important basis for elucidating the mechanisms underlying human NAIP inflammasome responses to bacterial infection, which could prove crucial to understanding how the NAIP/NLRC4 inflammasome contributes to human health and disease.

2.6. Acknowledgements

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2.7. Materials and Methods

2.7.1. Primary Human Samples

All studies involving human monocyte-derived macrophages (hMDMs) 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.

2.7.2. Bacterial Strains and Growth Conditions

Salmonella enterica serovar Typhimurium WT, Δ*sipB* (Lawley et al., 2006), and Δ*fliCfljB* (Wynosky-Dolfi et al., 2014) isogenic strains on the SL1344 background were used. Three hours before infection, *Salmonella* were diluted into Luria-Bertani (LB) broth containing 300 mM NaCl and grown for 3 h standing at 37°C to induce SPI-1 expression (Lee and Falkow, 1990). *Listeria monocytogenes* WT and isogenic strains on the 10403S background were cultured in brain heart infusion (BHI) medium (Sauer et al., 2011). *Listeria* strains encoding heterologous bacterial ligands (*Legionella pneumophila* FlaA, *S.* Typhimurium PrgJ, and *S.* Typhimurium PrgI) translationally fused to the truncated N-terminus of ActA and under the control of the *actA* promoter were used (Sauer et al., 2011). The pPL2 vector encoding PrgJ was introduced into Δ*hly Listeria* as previously described (Lauer et al., 2002; Sauer et al., 2011). *Listeria* strains expressing *S.*

Typhimurium SsaI, *Burkholderia thailandensis* BsaK, *Shigella flexeri* MxiI, and *Chromobacterium violaceum* CprJ were constructed using codon-optimized gene fragments (IDT) cloned into the pPL2 vector and introduced into *Listeria* as previously described (Lauer et al., 2002; Sauer et al., 2011).

2.7.3. Cellular Assays

Purified human monocytes from de-identified healthy human donors were obtained from the University of Pennsylvania Human Immunology Core. Monocytes were cultured in RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/ml streptomycin, and 50 ng/ml recombinant human M-CSF (Gemini Bio-Products) for 6 days to promote differentiation into hMDMs. One day prior to infection, adherent hMDMs were replated in media with 25 ng/ml human M-CSF lacking antibiotics at 1.0 x 10^5 cells/well in a 48-well plate. Pam3CSK4 (100 ng/ml) and LPS (500 ng/ml) pretreatments, bacterial infections, anthrax toxin-mediated delivery of bacterial ligands, siRNA experiments, cytotoxicity assays, ELISA, immunoblot analyses, quantitative RT-PCR analyses, HEK293 inflammasome reconstitution assays, and statistical analyses were performed as described in SI Materials and Methods.

2.7.4. Human Monocyte-Derived Macrophage Experiments

In experiments where macrophages were primed with Pam3CSK4, cells were pretreated with 100 ng/mL or 400ng/mL Pam3CSK4 (Invivogen) for 3 hours prior to bacterial infections or 4 hours before anthrax toxin treatments, respectively. For experiments involving LPS, cells were pretreated with 500 ng/mL LPS (Sigma-Aldrich).

For infections with *S.* Typhimurium, bacterial cultures were pelleted at 6,010 x *g* for 3 minutes and washed with PBS. Bacteria were then resuspended in PBS and added to the cells at a multiplicity of infection (MOI) of 20. The infected cells were then centrifuged at 290 x *g* for 10 min and incubated at 37d. After 1 hour of infection, 100 µg/mL of gentamicin was added to each well to prevent extracellular growth. Infections proceeded at 37°C for a total of 4hrs. For infections with *L. monocytogenes,* bacterial cultures were backdiluted on the day of infection and grown until OD_{600}=0.8. Cultures were pelleted at 6,010 x *g* for 3 minutes and resuspended in PBS. Cells were infected with *L. monocytogenes* at an MOI of 5, 10, 20 or 75 and incubated at 37°C. After 1 hour of infection, 50 µg/mL of gentamicin was added to each well. Infections proceeded for a total of 16 hrs. For all experiments, control cells were mock-infected with PBS.

2.7.5. Mouse Bone Marrow-Derived Macrophage Experiments.

All experiments performed with mouse bone marrow-derived macrophages were done so in accordance with the Animal Welfare Act (AWA), the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all procedures (protocol #804928).

Bone marrow was collected from the femurs and tibiae of C57BL/6J mice (Jackson Laboratory). 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 100 µg/mL streptomycin at 37˚C. One day before infection, macrophages were replated in RPMI containing 15% L929 cell supernatant and 10% FBS at a concentration of 1.25 x 10^5 cells/well in a 48-well plate. Cells were pretreated with 100 ng/mL Pam3CSK4

(Invivogen) for 16 hours prior to infection, then either mock-infected with PBS or infected with *L. monocytogenes* at an MOI of 5. After 1 hour of infection, 50 µg/mL of gentamicin was added to each well. Infections were continued for a total of 6 hours.

2.7.6. THP-1 Monocytic Cell Line Experiments

THP-1 cells (TIB-202; American Type Culture Collection) were maintained in RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, 0.05 mM β-mercaptoethanol, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified incubator. One day before infection, cells were replated in media lacking antibiotics at a concentration of 2.0 x 10^5 cells/well in a 48-well plate. THP-1 cells were differentiated into macrophages with 200 nM phorbol 12-myristate 13-acetate (PMA) for 24 hours.

2.7.7. Anthrax Toxin-Mediated Delivery of FlaA, PrgJ and YscF

Recombinant proteins (PA, LFn-FlaA, LFn-PrgJ, and LFn-YscF) were kindly provided by R. Vance, University of California, Berkeley (Rauch et al., 2016). In experiments with THP-1 and hMDMs, cells were plated in a 48-well plate at a concentration of 2.0 x 10^5 and 1.0 x 10^5 cells per well, respectively. PA and LFn doses for *in vitro* delivery were 1 µg/ml PA (for FlaTox), 4 µg/ml PA (for PrgJTox and YscFTox), 500ng/ml LFn-FlaA$^{310-475}$ (truncated C-terminus of *L. pneumophila* flagellin), 8 ng/ml LFn-PrgJ, 200 ng/mL LFn-YscF, and 2 µg/mL LFn-FlaA (full-length flagellin).

2.7.8. Expression Plasmids Encoding Human Inflammasome Components

pCMV6-XL5 plasmids encoding NAIP (NM_004536), IL-1β (NM_000576), or empty vector were purchased from Origene. The pCI plasmid encoding human caspase-

1 (NM_033292.3) was a gift from Kate Fitzgerald (Addgene plasmid # 41552) (Hornung et al., 2009). The NLRC4 (NM_021209) ORF was amplified from an expression vector (GeneCopoeia), between flanking BamHI and NotI sites, and a Kozak sequence (GCCACC) was engineered to precede the start codon. The following primers were used (5'-3'):

NLRC4 forward: AAAAGGATCCGCCACCATGAATTTCATAAAGGACAATAGCC

NLRC4 reverse: TTTTTGCGGCCGCTTAAGCAGTTACTAGTTTAAAATCACC

The digested NLRC4 PCR product was cloned into a BglII/NotI digested MSCV2.2 vector, which was a gift from Russell Vance (Addgene plasmid #60206) (Kofoed and Vance, 2011). Plasmids were prepared with the Qiagen EndoFree Plasmid Maxi Kit.

2.7.9. Reconstitution of the NAIP/NLRC4 Inflammasome in HEK293 cells

HEK293 cells were maintained in DMEM supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C. Cells were replated at $7x10^4$ cells/well in 500 µL replating media (DMEM + 10% FBS + 2 mM L-glutamine) in a 24-well plate. Transfection of expression plasmids (described above) was performed using Lipofectamine 2000 (Thermo Fisher Scientific). The amounts of plasmids used were 20 ng of NAIP, 20 ng of NLRC4, 10 ng of caspase-1, and 400 ng of pro-IL-1β. 18 hours later, cells were treated with anthrax toxin components for cytosolic delivery of FlaA, PrgJ, or YscF. Cells were harvested 9h later and subjected to immunoblot analysis.

2.7.10. siRNA Knockdown Experiments

All Silencer Select siRNA oligos were purchased from Ambion (Life Technologies). For NAIP, the siRNAs used were siRNA ID# s9262, s9263, and s9264. The siRNAs used for NLRC4 were siRNA ID# s33828, s33829, and s33830. To knockdown NAIP or NLRC4, 10 nM each of the three oligos were used per well. As a control, Silencer Select negative control siRNAs (Silencer Select Negative Control No. 1 siRNA 4390843 and Silencer Select Negative Control No. 2 siRNA 4390846) were used at 15 nM each per well. Transfection of the pooled siRNAs into macrophages was performed using HiPerfect transfection reagent (Qiagen) following the manufacturer's protocol for "Transfection of Differentiated Macrophage Cell Lines, Including THP-1." Treatment with appropriate siRNAs was performed for 48 hours. After 24 hours, fresh media lacking antibiotics was added to each well. After a total of 48 hours, treatment with anthrax toxin components was performed as described above. In parallel, siRNA-transfected hMDMs were treated with LPS + Nigericin (500 ng/mL and 10 μM, respectively).

2.7.11. Quantitative RT-PCR Analysis

Cells were lysed and RNA was isolated using the RNeasy Plus Kit (Qiagen). Synthesis of the first strand cDNA was performed using Superscript II reverse transcriptase and oligo (dT) primer (Invitrogen). Quantitative PCR was performed with the CFX96 real-time system (Bio-Rad) using the SsoFast EvaGreen Supermix with LOW ROX kit (Bio-Rad). The following primers from PrimerBank (Wang and Seed, 2003; Spandidos et al., 2008; Spandidos et al., 2010) were used. The PrimerBank IDs are *NAIP* (119393877c3), *NLRC4* (312433959c2), and *HPRT* (164518913c1) (all 5'-3'):

NAIP forward: CCCATTAGACGATCACACCAGA

NAIP reverse: GGAGTCACTTCCGCAGAGG

NLRC4 forward: TGCATCATTGAAGGGGAATCTG

NLRC4 reverse: GATTGTGCCAGGTATATCCAGG

HPRT forward: CCTGGCGTCGTGATTAGTGAT

HPRT reverse: AGACGTTCAGTCCTGTCCATAA

For analysis, mRNA levels of siRNA-treated cells were normalized to control siRNA-treated cells using the $2^{-\Delta\Delta CT}$ (cycle threshold) (Livak and Schmittgen, 2001) method to calculate fold induction.

2.7.12. Cytotoxicity Assays

Cells were infected as described above and were assayed for cell death, as determined by measuring loss of cellular membrane integrity via lactate dehydrogenase (LDH) activity in the supernatant. LDH release was quantified using an LDH Cytotoxicity Detection Kit (Clontech) and normalized to mock infected cells.

2.7.13. ELISA

Harvested supernatants from infected cells were assayed using ELISA kits for human IL-1α (R&D Systems) and IL-1β (BD Biosciences).

2.7.14. Immunoblotting

Infected or treated cells were lysed directly with 1X SDS-PAGE sample buffer, and low-volume (90 µL per well of a 48-well plate) supernatants were mixed 1:1 with 2X SDS-PAGE buffer containing Complete Mini EDTA-free Protease Inhibitor Mixture (Roche). Protein samples were boiled for 5 minutes, separated by SDS-PAGE, and transferred to

PVDF Immobilon-P membranes (Millipore). Samples were then probed with antibodies specific for IL-1β (8516; R&D Systems), NAIP (ab25968; Abcam), NLRC4 (12421S; Cell Signaling), and caspase-1 (2225S; Cell Signaling). As a loading control, all blots were probed with anti-β-actin (4967L; Cell Signaling). Detection was performed with HRP-conjugated anti-mouse IgG (F00011; Cell Signaling) or anti-rabbit IgG (7074S; Cell Signaling).

2.7.15. Statistical analysis

Prism 6.0 (GraphPad Software) was utilized for the graphing of data and all statistical analyses. Statistical significance for human monocyte-derived macrophages was determined using the paired two-way *t* test in experiments delivering bacterial ligands via anthrax toxin and infections with *S.* Typhimurium, and the paired Wilcoxon signed-rank test in experiments delivering bacterial ligands via engineered *L. monocytogenes*. All data are graphed such that each data point represents the mean of triplicate infected wells for a given donor. Individual experiments in figures were performed using primary human monocyte-derived macrophages from at least four different donors. Statistical significance for experiments with THP-1 cells was determined using the unpaired two-way *t* test. Statistical analyses for experiments with mouse bone marrow-derived macrophages were determined using the one-way ANOVA test and the Tukey's multiple comparisons test. Differences were considered statistically significant if the *P* value was <0.05.

CHAPTER 3

NAIP is essential for recognition of bacterial type III secretion system and flagellin proteins and mediates inflammasome responses to *Salmonella* infection

This chapter contains unpublished data generated by **Valeria M. Reyes Ruiz**, Marisa Egan, and Brian Yan.

3.1. Abstract

Inflammasomes are cytosolic multiprotein complexes that initiate antimicrobial responses through recognition of bacterial ligands and activation of the host protease caspase-1. Activated caspase-1 cleaves and activates IL-1 family cytokines and the host protein gasdermin-D. The N-terminal domain of gasdermin-D creates pores in the host cell membrane and results in the proinflammatory type of cell death known as pyroptosis. In mice, specific nucleotide-binding domain, leucine-rich repeat-containing family, apoptosis inhibitory proteins (NAIPs) recognize components of the type III secretion system (T3SS) and flagellin to recruit the nucleotide-binding domain, leucine-rich repeat-containing family, CARD domain-containing protein 4 (NLRC4), leading to caspase-1 activation. NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod protein, and NAIP5 and NAIP6 recognize flagellin. In contrast, the single human NAIP is capable of sensing multiple bacterial ligands. However, whether NAIP is essential for sensing of bacterial ligands from the T3SS and flagellar apparatus and the precise mechanism by which human NAIP achieves promiscuous recognition of multiple bacterial ligands is unclear. Here, we sought to determine if NLRC4 plays a role in promiscuous recognition of the T3SS inner rod protein, T3SS needle protein, and flagellin by the NAIP/NLRC4 inflammasome. In addition, we generated *NAIP, NLRC4, CASP1,* and *NLRP3* knockout cells using Clustered Regularly Interspersed Palindromic Repeat (CRISPR)/Cas9 to test if NAIP is essential for sensing of bacterial ligands and what inflammasome components are essential for inflammasome responses to *Salmonella* Typhimurium infection. Our findings suggest that NAIP, but not NLRC4, dictates the specificity or promiscuity of bacterial ligand recognition, and that NAIP appears to be essential for inflammasome responses to bacterial ligands. Additionally, our preliminary data suggest that *Salmonella* induces caspase-1-dependent inflammasome responses in

human macrophages. Human NAIP, but not NLRP3, appear to be required for maximal IL-1β secretion during infection with *Salmonella* Typhimurium. Our preliminary data provide insight into human-specific responses to *Salmonella* infection and are an important basis for elucidating antimicrobial mechanisms to other gram-negative bacteria.

3.2. Significance Statement

Salmonella is one of the leading causes of death from diarrheal disease. Antibiotic resistance among *Salmonella* strains is on the rise. Thus, there is an urgent need for alternative therapeutics and a better understanding of the innate immune response to *Salmonella*. Most studies of *Salmonella* interactions with the immune system are conducted in mice. However, there are key differences between the innate immune responses in mice and in humans. In fact, the human immune response to *Salmonella* is poorly understood. The studies presented in this chapter examine a role for the NAIP/NLRC4 inflammasome in macrophage responses to bacterial ligands and to *Salmonella* infection. Our data indicate that the human NAIP/NLRC4 inflammasome is essential for recognition of components from the T3SS inner rod protein, T3SS needle protein, and flagellin. Furthermore, the human NAIP/NLRC4 inflammasome appears to be required for inflammasome responses to *Salmonella* infection in macrophages. These findings provide a foundation for understanding human-specific innate immune responses to Gram-negative bacterial infection.

3.3. Introduction

Recognition of microbial products by pattern recognition receptors (PRRs) is critical to initiate host defense against bacterial pathogens (Medzhitov, 2007). A subfamily

of cytosolic PRRs termed Nod-like receptors (NLRs) mediate the formation of the inflammasome, a multiprotein complex that results in the activation of the host enzyme caspase-1 (Martinon et al., 2002). Activated caspase-1 can then lead to the secretion of pro-inflammatory cytokines from the IL-1 family as well as pyroptotic cell death, resulting in host defense and bacterial clearance (Kuida et al., 1995; Li et al., 1995; Martinon et al., 2002; Bergsbaken et al., 2009). Intracellular bacterial pathogens such as *Salmonella* Typhimurium employ virulence-associated secretions systems to survive and cause disease (Galan et al., 2014; LaRock et al., 2015). Bacterial products from the type III Secretion System (T3SS) and flagellin activate a subfamily of NLRs termed the nucleotide-binding domain, leucine-rich repeat-containing family, apoptosis inhibitory proteins (NAIPs). In mice, NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod protein, and NAIP5 and NAIP6 each recognize flagellin (Kofoed and Vance, 2011; Zhao et al., 2011; Rayamajhi et al., 2013; Yang et al., 2013; Rauch et al., 2016; Zhao et al., 2016). Upon binding their cognate ligand, the NAIPs recruit the adaptor nucleotide-binding domain, leucine-rich repeat-containing family, CARD domain-containing protein 4 (NLRC4) (Diebolder et al., 2015; Hu et al., 2015; Zhang et al., 2015; Tenthorey et al., 2017). The resulting NAIP/NLRC4 inflammasome recruits and activates caspase-1 (Martinon et al., 2002). In contrast to mice, which possess multiple *NAIP* genes that encode NAIP receptors with strict ligand specificity, humans encode a single *NAIP* gene that encodes a NAIP receptor with promiscuous recognition of the T3SS inner rod protein, T3SS needle protein, and flagellin (Zhao et al., 2011; Rayamajhi et al., 2013; Yang et al., 2013; Kortmann et al., 2015; Reyes Ruiz et al., 2017). The precise molecular basis that allows for specific recognition by the murine NAIPs and promiscuous recognition by human NAIP is not yet understood.

The region of the murine NAIPs that dictates ligand specificity has been mapped to an internal region composed of α-helical domains associated with the nucleotide-binding domain (NBD). In addition, this region has evolved under positive selection in both primate and rodent genomes (Tenthorey et al., 2014). The cryo-electron microscopic structure of the murine flagellin-NAIP5-NLRC4 inflammasome complex shows that there is one bacterial ligand and one NAIP per inflammasome (Tenthorey et al., 2017). Furthermore, the flagellin monomer is recognized by NAIP5 and does not appear to directly bind NLRC4 (Tenthorey et al., 2017; Yang et al., 2018). The model for NAIP5 activation therefore includes the binding of a flagellin monomer to NAIP5 and a subsequent conformational change in NAIP5 that unfurls the protein to recruit and activate NLRC4. Active NLRC4 can then recruit additional NLRC4 promoters for self-propagating oligomerization (Tenthorey et al., 2017). However, the model of activation of the human NAIP/NLRC4 inflammasome in response to the T3SS needle protein, T3SS inner rod protein, or flagellin by human NAIP is unclear. In addition, the role of the adaptor protein NLRC4 in promiscuous recognition of multiple bacterial ligands has not been tested. Thus, we sought to determine the role of human NLRC4 in the promiscuous recognition of bacterial ligands from the T3SS and flagellin. Here, our preliminary data suggest that, human NAIP and not NLRC4 dictates the broad recognition of multiple bacterial ligands.

Our studies using siRNA-mediated silencing of human *NAIP* suggest that NAIP is required for maximal inflammasome responses to components of the T3SS and flagellar apparatus (Reyes Ruiz et al., 2017). However, whether residual human NAIP in siRNA-treated cells or an additional NLR is also involved in cytokine secretion upon cytosolic delivery of these bacterial ligands is unclear. Additionally, the importance of NAIP in antimicrobial protection against *Salmonella* infection is not yet understood. Here, we used

CRISPR/Cas9 to generate *NAIP* and *NLRC4* knockout THP-1 cells and tested the role of human NAIP and NLRC4 in inflammasome responses to both intracellular delivery of T3SS components and flagellin, as well as *in vitro Salmonella* infection. Our preliminary data suggest that human NAIP is essential for recognition of the T3SS inner rod, T3SS needle, and flagellin, as well as for maximal inflammasome responses to *Salmonella* infection.

During systemic infection, *Salmonella* downregulates flagellin and expresses the SPI-2 T3SS, which is thought to evade immune recognition by the NAIP/NLRC4 inflammasome (Cummings et al., 2006; Miao et al., 2010b). *Salmonella* induces delayed NLRP3 inflammasome activation independently of both the SPI-1 and SPI-2 T3SSs (Broz et al., 2010). The delayed inflammasome response is due to evasion of the NLRP3 inflammasome via control of oxidative metabolism by *Salmonella* (Wynosky-Dolfi et al., 2014). In murine macrophages, both NLRC4 and NLRP3 are required for maximal inflammasome responses to *S.* Typhimurium (Broz et al., 2010). Consistent with NLRC4 and NLRP3 being important for inflammasome responses *in vitro*, mice lacking both NLRs are more susceptible to infection as compared to the WT or single knockout mice (Broz et al., 2010). However, the role of the NLRP3 inflammasome in human inflammasome responses to *Salmonella* is not yet understood. Here, we used CRISPR/Cas9 to target NLRP3 and determined that the NLRP3 inflammasome appears to be dispensable for the response to *Salmonella* in human macrophages. Overall, our preliminary data provides insight into human-specific immune responses to *Salmonella* infection in macrophages.

3.4. Results

3.4.1. NAIP appears to dictate the specific or broad recognition of bacterial ligands

The cryo-electron microscopic structure of the murine NAIP5 inflammasome shows that flagellin binds to NAIP5 and not NLRC4 (Tenthorey et al., 2017; Yang et al., 2018). We sought to determine whether human NAIP could also bind flagellin by overexpressing human NAIP alongside c-Myc-tagged flagellin (c-Myc-FlaA) in HEK293 cells and investigating their interaction via co-immunoprecipitation analysis. Untransfected HEK293 cells are not natively responsive to cytosolic delivery of flagellin, as they do not express any inflammasome components. Our data show that human NAIP co-immunoprecipitates with c-Myc-FlaA (**Fig. 3.1**). Critically, uncoated beads bound to neither NAIP nor flagellin (**Fig. 3.1**). These data suggest that human NAIP binds flagellin in the absence of NLRC4.

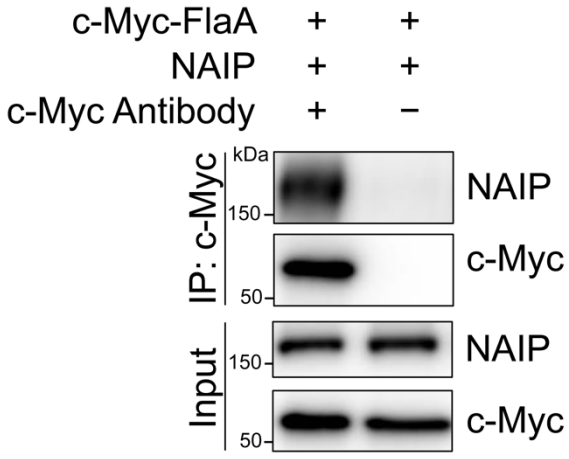

Figure 3.1: NAIP appears to bind flagellin independently of NLRC4. HEK293 cells were transfected with pCMV6-XL5 human NAIP and c-Myc-FlaA. The lysates were analyzed by co-immunoprecipitation analysis with Dynabeads coated with the c-Myc antibody. Uncoated beads were used as control (–). Immunoblot analysis after immunoprecipitation was performed on samples for human NAIP and c-Myc.

However, HEK293 cells may have low levels of endogenous NLRC4 that stabilize the interaction of human NAIP and flagellin. Therefore, an alternative approach such as biolayer-interferometry (BLI), or fluorescence polarization is needed to test whether flagellin or the T3SS inner rod and needle directly bind to human NAIP in the absence of an adaptor protein.

It is possible that the interaction of flagellin with human NAIP is of lower affinity than flagellin binding to murine NAIP5, and that human NLRC4 may have higher affinity for NAIP, thus allowing for NLRC4 oligomerization and inflammasome activation. If this is the case, human NLRC4 may play a role in the promiscuous recognition of multiple bacterial ligands by human NAIP. We therefore sought to understand the role of NLRC4 in promiscuous recognition of multiple bacterial ligands by the human NAIP/NLRC4 inflammasome. To this end, we reconstituted the NAIP inflammasome in HEK293 cells with either mouse or human NLRC4, human caspase-1, and human IL-1β. We then delivered the T3SS inner rod protein from *S.* Typhimurium (PrgJ), the T3SS needle protein from *B. thailandensis* (YscF), or flagellin from *L. pneumophila* (FlaA) using an anthrax-toxin based delivery system (von Moltke et al., 2012; Rauch et al., 2016). In this system, bacterial ligands are translationally fused to the N-terminal domain of *Bacillus anthracis* lethal factor (LFn). The LFn domain mediates cytosolic delivery of bacterial ligands by translocation through a membrane channel formed by the anthrax protective antigen (PA) protein. We used a translational fusion of LFn and PrgJ (LFn-PrgJ), LFn and FlaA (LFn-FlaA), and LFn and YscF (LFn-YscF). To avoid confounding effects of TLR5 detection of flagellin, we used a truncated flagellin that lacks the TLR5-stimulating region but retains the ability to activate murine NAIP5 (Lightfield et al., 2008; Lightfield et al., 2011).

We first tested the ability of human NAIP and murine NAIP2 to form a functional inflammasome complex with human NLRC4 or murine NLRC4 in response to PrgJ. As shown previously, HEK293 cells ectopically expressing human NAIP and human NLRC4 robustly processed IL-1β into the mature p17 form in response to PrgJ (**Fig. 3.2 A**) (Reyes Ruiz et al., 2017). Also, in agreement with previous findings, HEK293 cells expressing murine NAIP2 and murine NLRC4 in conjunction with human caspase-1 and IL-1β robustly processed IL-1β in response to PrgJ (**Fig. 3.2 A**) (Kofoed and Vance, 2011; Zhao et al., 2011). HEK293 cells expressing murine NAIP2 and human NLRC4 were able to mediate inflammasome responses to PrgJ, as seen by the presence of processed IL-1β (**Fig. 3.2 A**). However, HEK293 cells expressing both human NAIP and murine NLRC4 failed to process IL-1β in response to PrgJ (**Fig. 3.2 A**). These data suggest that murine NAIP2 can functionally interact with both murine and human NLRC4, whereas human NAIP can only work in concert with its natural counterpart, human NLRC4, to mediate inflammasome responses to the T3SS inner rod.

As our data suggested that murine NAIP2 can mediate functional inflammasome responses to the T3SS inner rod when coupled with human NLRC4, we next tested whether human NLRC4 could confer upon murine NAIP2 the ability to promiscuously recognize multiple bacterial ligands. In agreement with previous findings, ectopic expression of murine NAIP2 with murine NLRC4 results in inflammasome activation specifically in response to the T3SS inner rod PrgJ, as seen by the presence of processed IL-1β only in response to cytosolic delivery of PrgJ, but not in response to cytosolic delivery of flagellin or the T3SS needle protein YscF (**Fig. 3.2 B**) (Kofoed and Vance, 2011; Zhao et al., 2011). Murine NAIP2 co-expressed with human NLRC4 similarly resulted in robust

processing of IL-1β in response to PrgJ, but not in response to flagellin or YscF (**Fig. 3.2 B**), indicating that the murine NAIP2/human NLRC4 inflammasome still exhibits specificity

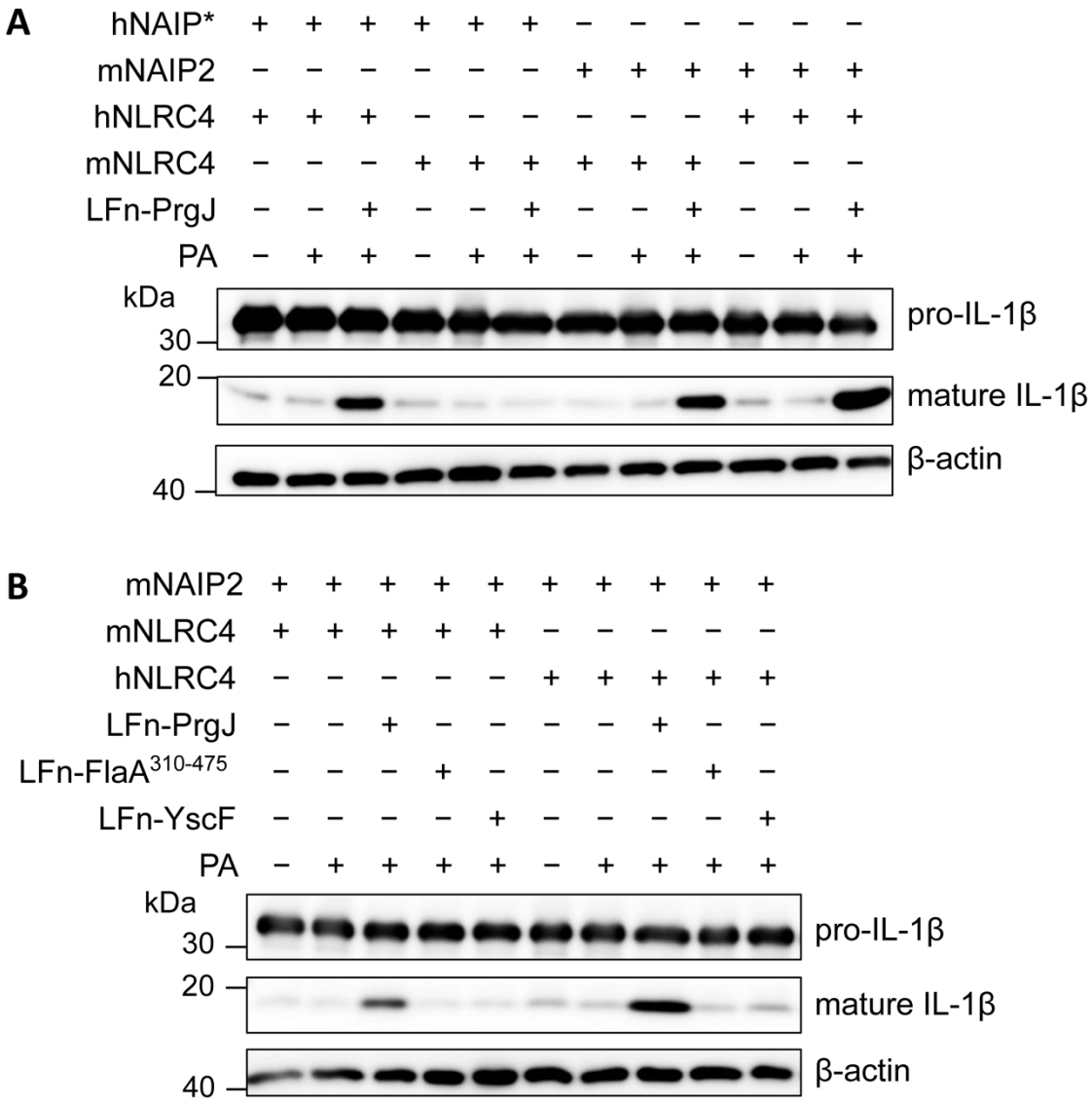

Figure 3.2: NAIP appears to dictate ligand specificity and subsequent inflammasome activation. (A-B) HEK293 cells were transfected with expression vectors encoding human caspase-1 and IL-1β. Where indicated, cells were also transfected with human NAIP, murine NAIP2, human NLRC4, murine NLRC4, or empty vector controls (–). After 18 h, cells were treated with PA+LFn-PrgJ, PA+LFn-FlaA$^{310-475}$, PA+LFn-YscF, or PA alone for 9 h. Immunoblot analysis was performed on cell lysates for mature and pro-IL-1β, and β-actin as a loading control.

for the T3SS inner rod. Inflammasome activation in cells expressing each of the tested combinations of murine NAIP2 and murine or human NLRC4 required cytosolic delivery of bacterial ligands, as untreated cells or PA treatment alone did not contain high levels of processed IL-1β (**Fig. 3.2 A-B**). Our preliminary data suggest that NAIP, rather than NLRC4, dictates the specificity or promiscuity of bacterial ligand recognition.

3.4.2. Generation of NLRC4- or NAIP-deficient THP-1 cells using CRISPR/Cas9

In mouse macrophages, multiple NAIPs are required for inflammasome responses to the T3SS inner rod protein, the T3SS needle protein, and flagellin (Rauch et al., 2016; Zhao et al., 2016). In addition, both NAIPs and NLRC4 are required for the inflammasome response to *S.* Typhimurium infection (Miao et al., 2010b; Zhao et al., 2016). In human macrophages, the single hNAIP is required for maximal inflammasome responses to the T3SS inner rod, T3SS needle, and flagellin (Reyes Ruiz et al., 2017). However, it is still unclear whether human NAIP is completely essential for sensing these bacterial ligands or whether other NLRs can also mediate sensing of the T3SS inner rod, T3SS needle, and flagellin. Additionally, the roles of human NAIP and NLRC4 in anti-microbial responses to bacterial infections are poorly understood. Previous studies using siRNA-mediated silencing of hNAIP indicate that hNAIP is required for maximal restriction of *Legionella pneumophila* (Vinzing et al., 2008).

Here, we used the Clustered Regularly Interspersed Palindromic Repeat (CRISPR) system, in conjunction with the RNA-guided exonuclease Cas9, to disrupt the *NAIP* and *NLRC4* genes and test their roles in inflammasome responses to T3SS components and flagellin, as well as during *Salmonella* infection. THP-1 cells were infected with lentiviral particles encoding Cas9 and gRNAs specific to either *NAIP* or

NLRC4 and a gene encoding puromycin resistance. After puromycin selection, single cell clones were expanded and sequenced to determine whether genome editing of *NAIP* and *NLRC4* disrupted these two genes and resulted in a functional knockout. The *NLRC4* gRNA targets exon 3 and the targeted sequence is highlighted in **Fig. 3.3 A**. Out of 24 clones tested for NLRC4 expression, we sequenced four clones that exhibited reduced NLRC4 expression. Of these four clones, two clones showed efficient CRISPR/Cas9 editing of the *NLRC4* gene. The sequencing chromatograms for the DNA region surrounding the target sequence of both *NLRC4* alleles in each clone are shown (**Fig. 3.3 B-C**). Additionally, sequence alignments of the WT THP-1 and *NLRC4*$^{-/-}$ THP-1 clones are shown for both *NLRC4* alleles (**Fig. 3.3 B-C**). The *NLRC4* alleles in clone 4 present with deletions of 31 or 13 nucleotides, resulting in premature stop codons in the translated protein sequence (**Fig. 3.3 B**). In clone 7, one allele for *NLRC4*$^{-/-}$ shows a small insertion and the other allele shows a deletion of 20 nucleotides. Both changes in the DNA result in protein products with premature stop codons. Western Blot analyses of both *NLRC4*$^{-/-}$ clone 4 and clone 7 showed abrogated NLRC4 expression as compared to WT THP-1 cells (**Fig. 3.4**). Future experiments are needed to phenotypically validate these clones and to test the role of NLRC4 in responses to components from the T3SS and flagellar apparatus as well as antimicrobial responses to *S.* Typhimurium and other Gram-negative bacterial pathogens.

To study the role of NAIP in recognition of different bacterial ligands from the T3SS and flagellar apparatus as well as antimicrobial responses to infection, THP-1 cells were transduced with lentiviral particles encoding Cas9 and gRNA against human NAIP. The targeted sequence for *NAIP* is in exon 4 and is highlighted in **Fig. 3.5 A**. After infection with lentiviral particles, we mediated puromycin selection. Single cell clones were then

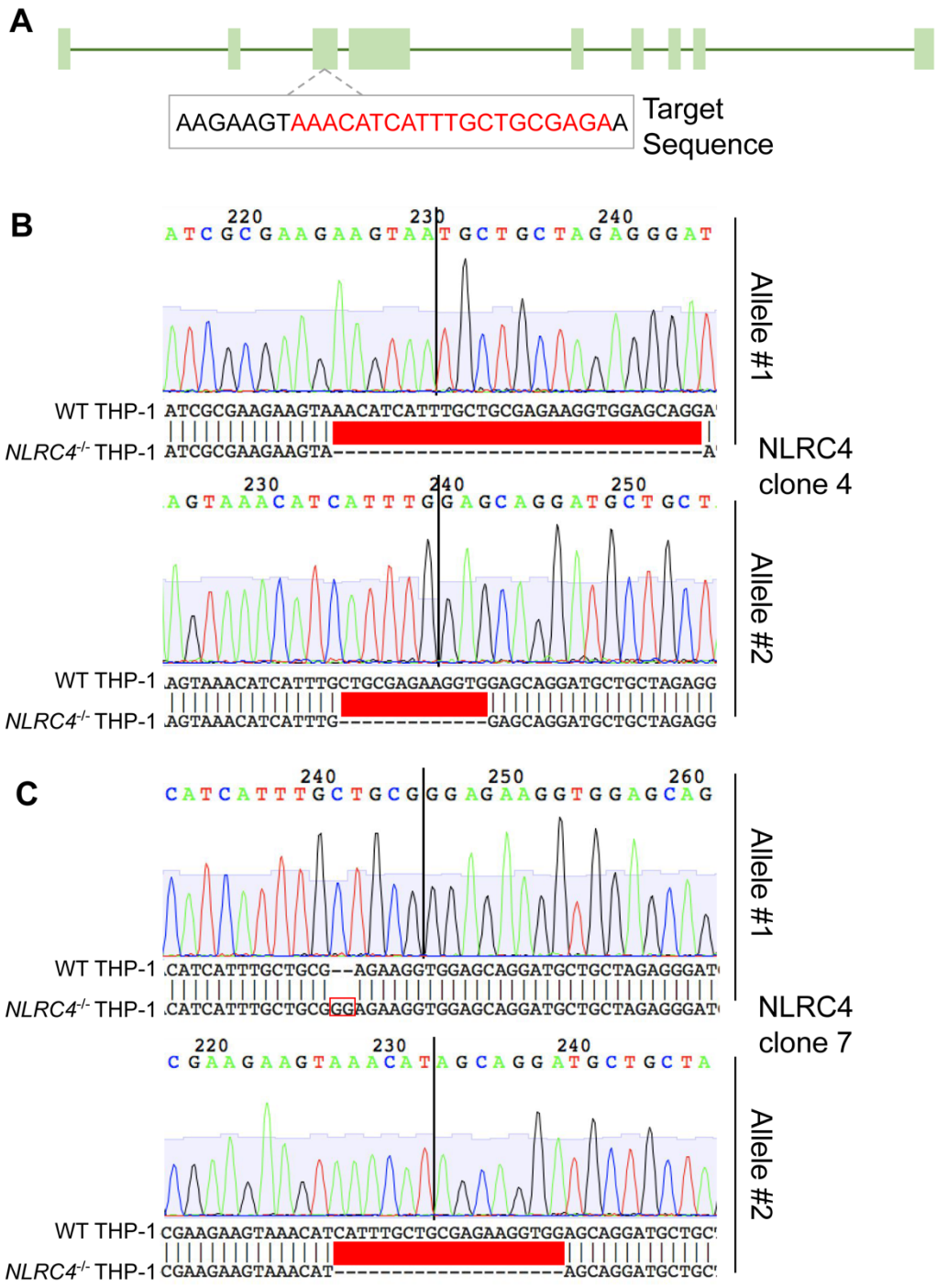

Figure 3.3: Validation of *NLRC4* mutant THP-1 clones generated with CRISPR/Cas9-mediated genome editing. (A) Schematic representation of the *NLRC4* gene with exons (filled boxes) and introns (lines). gRNA target sequence is highlighted in red. (B-C) Sequencing chromatograms and sequence alignments of WT THP-1 and *NLRC4*$^{-/-}$ clones are shown for both alleles per clone. Red boxes highlight the mutated region.

expanded and sequenced. We saw reduced mRNA levels in six clones and sequenced three of these. One clone showed efficient CRISPR/Cas9 editing of both *NAIP* alleles. The chromatogram for both alleles shows the sequencing results for the region of DNA containing the target sequence. Additionally, sequence alignments between WT THP-1 and *NAIP*$^{-/-}$ THP-1 clone 12 are shown for both alleles (**Fig. 3.5 B**). The *NAIP* alleles in clone 12 show small deletions of 1 or 2 nucleotides and they both result in premature stop codons (**Fig. 3.5 B**). qRT-PCR analysis shows that the NAIP mRNA levels in *NAIP*$^{-/-}$ THP-1 cells are severely diminished in comparison to those in WT THP-1 cells (**Fig. 3.5 C**). The other two clones sequenced also have mutations in one allele, but we are still in the process of determining the sequence of the second allele.

Our previous experiments used siRNA-mediated silencing of NAIP and determined that NAIP was required for maximal inflammasome responses to the components from the T3SS and flagellar apparatus (Reyes Ruiz et al., 2017). We next sought to determine whether NAIP is completely essential for recognition of the T3SS inner rod protein, T3SS needle protein, and flagellin or whether an additional NLR is responsible for residual inflammasome responses in siRNA-treated cells. The bacterial ligands were delivered into WT THP-1 cells or *NAIP*$^{-/-}$ THP-1 cells using the anthrax delivery system. In agreement with previous findings, WT THP-1 cells treated with PA+LFn-FlaA$^{310-475}$ (referred to as FlaTox), PA+LFn-PrgJ (referred to as PrgJTox), or PA+LFn-YscF (referred to as YscFTox) exhibited robust inflammasome activation, as measured by IL-1β cytokine release (**Fig. 3.5 D**) (Reyes Ruiz et al., 2017). In contrast, treatment of *NAIP*$^{-/-}$ THP-1 cells with FlaTox, PrgJTox, or YscFTox resulted in almost completely abrogated levels of IL-1β secretion (**Fig. 3.5 D**). As expected, *NAIP* deficiency had little effect on inflammasome responses to LPS+Nigericin, a known activator of the NLR pyrin domain-containing protein 3 (NLRP3)

inflammasome that does not engage NAIP (**Fig. 3.5 D**) (Mariathasan et al., 2006). Importantly, treatment with PA, LFn-FlaA$^{310-475}$, LFn-PrgJ, or LFn-YscF alone did not activate the inflammasome, indicating that FlaA, PrgJ, and YscF induce inflammasome activation only when delivered into the host cell cytosol via PA. These results suggest that NAIP is essential for inflammasome responses to the T3SS inner rod, T3SS needle, and flagellin. Additionally, the *NAIP*$^{-/-}$ THP-1 cells have proven an appropriate tool with which to study NAIP-dependent antimicrobial responses to *Salmonella* infection and other Gram-negative bacterial pathogens.

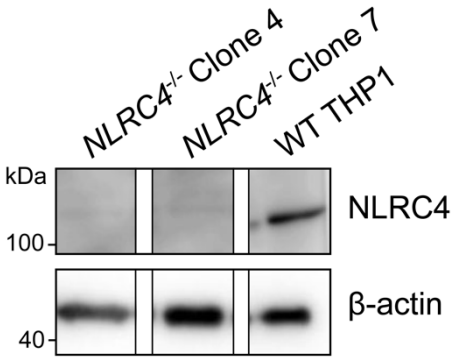

Figure 3.4: Expression of NLRC4 in *NLRC4* mutant THP-1 single cell clones generated by CRISPR/Cas9-mediated editing. Immunoblot analysis was performed on cell lysates for human NLRC4, and β-actin as a loading control.

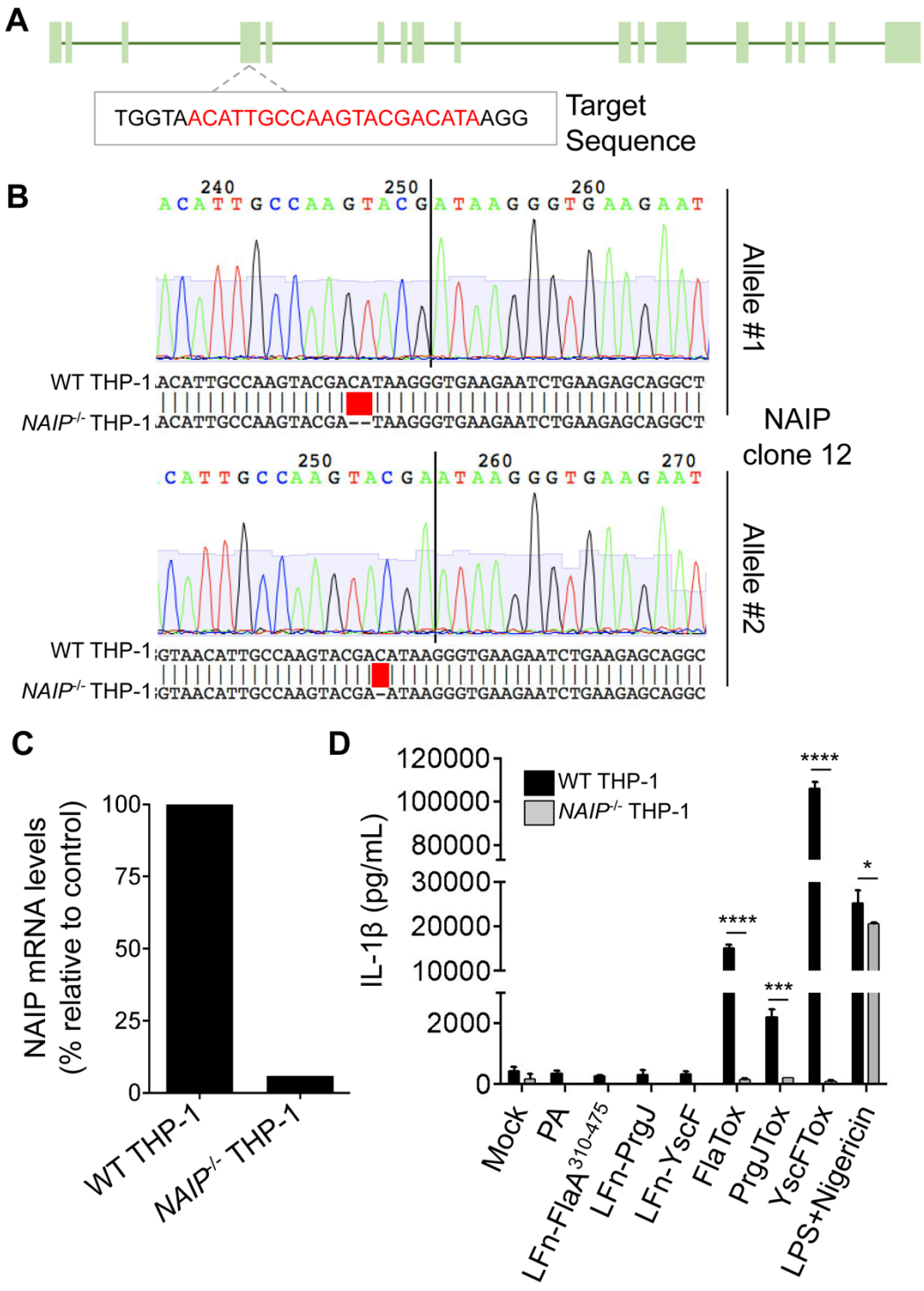

Figure 3.5: Genetic and phenotypic validation of *NAIP* mutant THP-1 cells generated with CRISPR/Cas9 genome editing. (A) Schematic representation of the *NAIP* gene with exons (filled boxes) and introns (filled lines). gRNA target sequence is highlighted in red.

(B) Sequencing chromatograms and sequence alignments of WT THP-1 and *NAIP*$^{-/-}$ clone 12 are shown for both alleles. Red boxes represent the mutated region. (C) qRT-PCR was performed to quantitate *NAIP* mRNA levels in WT THP-1 and *NAIP*$^{-/-}$ THP-1 cells. For the *NAIP*$^{-/-}$ THP-1 cells, *NAIP* mRNA levels were normalized to human HPRT mRNA levels and WT THP-1 cells. (D) THP-1 cells were primed with Pam3CSK4 for 16h and treated with PA alone, LFn-FlaA$^{310-475}$ alone, LFn-PrgJ alone, LFn-YscF alone, PA+LFn-FlaA$^{310-475}$ (FlaTox), PA+LFn-PrgJ (PrgJTox), PA+LFn-YscF (YscFTox), or LPS+Nigericin for 6h. IL-1β supernatant levels were measured by ELISA. Bar graphs display the mean ± SD of triplicate wells. *p<0.05, *** p<0.001, **** p<0.0001 by unpaired *t* test.

3.4.3 NAIP appears to be required for maximal inflammasome responses to *Salmonella* Typhimurium

Our studies show that human NAIP is essential for inflammasome responses to components from the T3SS and flagellar apparatus. However, the requirement of NAIP for inflammasome responses to infection with *Salmonella* is unknown. To test the role of human NAIP in inflammasome responses to *Salmonella* infection, WT or *NAIP*$^{-/-}$ THP-1 cells were infected with wild type (WT) or SPI-1 T3SS-deficient (Δ*sipB*) *Salmonella* Typhimurium (ST) strains. As expected, infection of WT THP-1 cells with WT ST results in robust IL-1β secretion, and this secretion is dependent on the T3SS, as infection with Δ*sipB* ST results in decreased inflammasome responses (**Fig. 3.6 A**). Interestingly, WT *Salmonella*-infected *NAIP*$^{-/-}$ THP-1 cells exhibited abrogated IL-1β secretion (**Fig. 3.6 A**). In contrast, *NAIP* deficiency had little effect on inflammasome responses to LPS+Nigericin (**Fig. 3.6 B**). Release of TNF, an inflammasome-independent cytokine, was not affected in *NAIP*$^{-/-}$ THP-1 cells when compared to WT THP-1 cells (**Fig. 3.6 C**). These preliminary data suggest that NAIP is required for maximal inflammasome responses to *Salmonella*. However, the data additionally suggest that there exists NAIP-independent inflammasome activity in response to *Salmonella,* as seen by residual IL-1β secretion in *NAIP*$^{-/-}$ THP-1 cells.

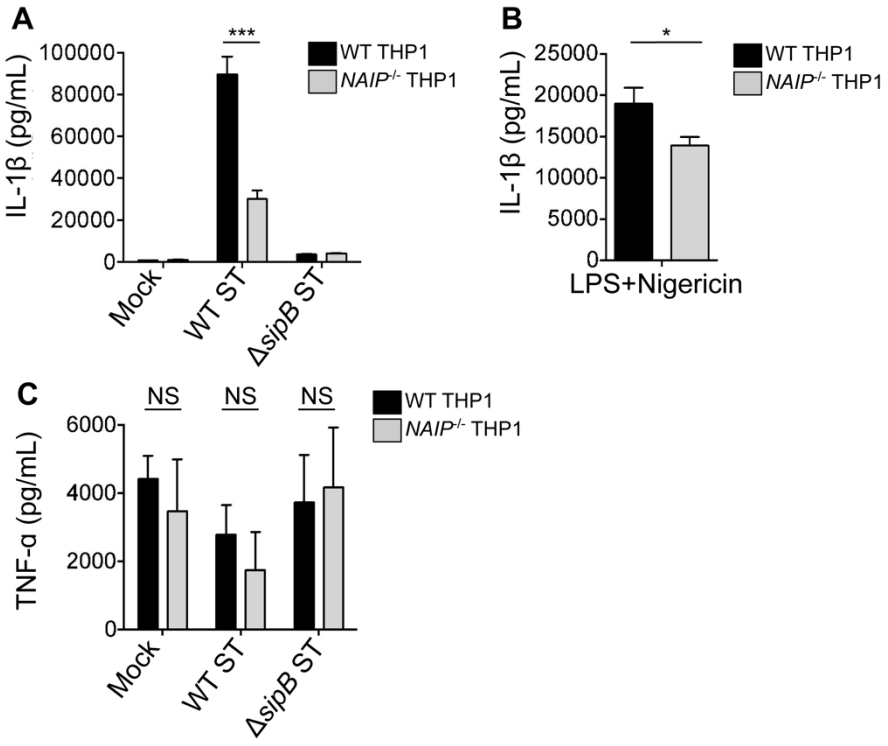

Figure 3.6: NAIP appears to be required for maximal inflammasome responses to *Salmonella* Typhimurium. (A and C) WT and *NAIP*$^{-/-}$ THP-1 cells were primed with Pam3CSK4 for 16 hours and treated with PBS (Mock), wild type *Salmonella* (WT ST), or Δ*sipB* ST at an MOI of 20 for 6 hrs. (B) Cells were treated with LPS+Nigericin for 6h. (A-B) IL-1β supernatant levels were measured by ELISA. (C) TNF supernatant levels were measured by ELISA. Bar graphs display the mean ± SD of triplicate wells. Representative of two independent experiments. NS $p>0.05$, * $p<0.05$, *** $p<0.001$ by unpaired *t* test.

3.4.4. CASP1 but not NLRP3 may be required for inflammasome responses to *Salmonella*

Studies in murine macrophages suggest that both the NAIP/NLRC4 and NLRP3 inflammasomes are required for maximal secretion of IL-1 cytokines in response to *S.* Typhimurium infection (Broz et al., 2010). As our preliminary data suggested that *Salmonella*-infected THP-1 cells undergo a NAIP-independent inflammasome response,

we next sought to determine the role of the NLRP3 inflammasome in antimicrobial responses to *Salmonella* infection. THP-1 cells were infected with lentiviral particles encoding Cas9 and gRNAs specific to *CASP1* or *NLRP3*. After 48 hours, the bulk population was selected in puromycin. Western Blot analysis of *CASP1*$^{-/-}$ THP-1 and *NLRP3*$^{-/-}$ THP-1 bulk populations showed decreased CASP1 and NLRP3 expression, respectively. WT THP-1 cells expressed high levels of both CASP1 and NLRP3 (**Fig 3.7A**). These cells were then infected with WT ST, flagellin-deficient (Δ*fliCfljB*) ST, or Δ*sipB* ST. *CASP1*$^{-/-}$ THP-1 cells infected with WT ST or Δ*fliCfljB* ST exhibited reduced IL-1β secretion as compared to infected WT THP-1 cells (**Fig. 3.7B**). In contrast, *NLRP3*$^{-/-}$ THP-1 cells had similar levels of IL-1β secretion as compared to WT THP-1 cells in response to Δ*fliCfljB* ST (**Fig. 3.7B**). However, a small but significant decrease in IL-1β secretion was observed in *NLRP3*$^{-/-}$ THP-1 cells as compared to WT THP-1 cells in response to WT ST (**Fig. 3.7B**). As expected, both *CASP1*$^{-/-}$ THP-1 and *NLRP3*$^{-/-}$ THP-1 cells showed abrogated IL-1β secretion when treated with LPS+Nigericin, a known activator of the NLRP3 inflammasome that results in CASP1 activation and subsequent IL-1β secretion (**Fig. 3.7C**). These preliminary data suggest that CASP1, but not NLRP3, is indispensable for inflammasome responses to *Salmonella* infection. Additional experiments with sequence-validated *CASP1*$^{-/-}$ and *NLRP3*$^{-/-}$ single cell clones are needed to confirm these results and further understand the role of the NLRP3 inflammasome in antimicrobial responses to *Salmonella* infection. Altogether, these preliminary data suggest that *Salmonella* induces CASP1-dependent inflammasome responses that may involve the NAIP and NLRP3 inflammasomes in human macrophages.

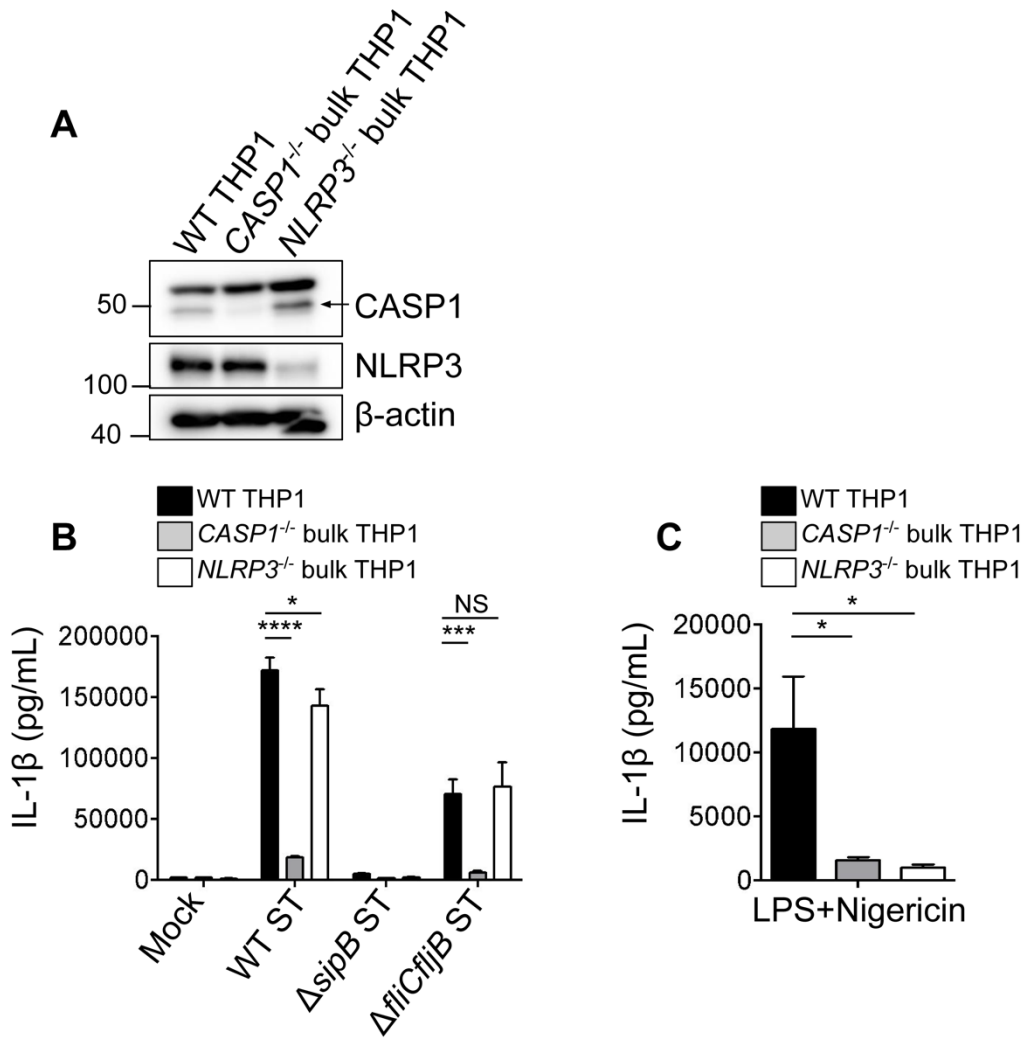

Figure 3.7: CASP1, but not NLRP3, may be required for inflammasome responses to *Salmonella*. (A) Immunoblot analysis was performed on cell lysates for human NLRC4, human CASP1, and β-actin as a loading control. (B) WT THP-1, *CASP1*$^{-/-}$ bulk THP-1, and *NLRP3*$^{-/-}$ bulk THP-1 cells were primed with Pam3CSK4 for 16 hours and treated with PBS (Mock), wild type *Salmonella* (WT ST), Δ*sipB* ST, or Δ*fliCfljB* ST at an MOI of 20 for 6 hrs. (C) Cells were treated with LPS+Nigericin for 6h. (B-C) IL-1β supernatant levels were measured by ELISA. Bar graphs display the mean ± SD of triplicate wells. Representative of two independent experiments. NS $p>0.05$, * $p<0.05$, *** $p<0.001$, ****$p<0.0001$ by unpaired *t* test.

3.5. Discussion

Our data suggest that specific or broad detection of bacterial ligands is dictated by NAIP rather than NLRC4. Additionally, we tested the requirement of human NAIP in recognition of the T3SS inner rod, T3SS needle, and flagellin by knocking out *NAIP* and *NLRC4* in THP-1 cells with CRISPR/Cas9 technology. Our data suggest that human NAIP is essential for recognition of these bacterial ligands. We next tested the role of NAIP, caspase-1, and NLRP3 in inflammasome responses during *S.* Typhimurium infection. Our data suggest that *Salmonella* induces CASP-1 dependent inflammasome responses and that the NAIP inflammasome, rather than the NLRP3 inflammasome, appears to be the main inflammasome activated in human macrophages during *Salmonella* infection. In mice, bacterial ligands bind to specific NAIPs and do not bind to NLRC4 (Tenthorey et al., 2017). Furthermore, specific recognition of bacterial ligands has been mapped to NBD-associated α-helical domains in the murine NAIPs (Tenthorey et al., 2014). Our preliminary data suggest that flagellin binds human NAIP in HEK293 cells where NLRC4 is not overexpressed. However, HEK293 cells may have low levels of endogenous NLRC4 or a human-specific adaptor that can interact with human NAIP and flagellin. Thus, a different approach such as biolayer-interferometry (BLI) or fluorescence polarization is needed to test whether flagellin, the T3SS inner rod, or T3SS needle can directly bind to human NAIP. How human NAIP achieves broad recognition of multiple bacterial ligands and whether NAIP has differing affinities or binding kinetics remains to be determined. It is possible that human NAIP mediates recognition of multiple bacterial ligands through the NBD-associated α-helical domains, as this region has evolved under positive selection in both humans and primates (Tenthorey et al., 2014).

Although human NAIP can mediate sensing of the T3SS inner rod, T3SS needle, and flagellin, genetic evidence of its role in antimicrobial responses to *Salmonella* infection is lacking. In an effort to more closely analyze immune responses to *Salmonell*a infection, we generated *NAIP* or *NLRC4* mutant THP-1 cells using CRISPR/Cas9 technology and sequence-validated single cell clones deficient for NAIP or NLRC4 expression. Our data suggest that NAIP appears to be essential for maximal inflammasome responses to *Salmonella* infection. In mice, the NAIP5/NLRC4 inflammasome is critical for defense against *L. pneumophila* infection (Growney and Dietrich, 2000; Wright et al., 2003; Zamboni et al., 2006; Kofoed and Vance, 2011; Zhao et al., 2011). The involvement of human NAIP in the restriction of *L. pneumophila* infection has also been shown via overexpression of NAIP or siRNA-mediated silencing of human *NAIP* (Vinzing et al., 2008). It will be of interest to utilize our NAIP-deficient THP-1 cells and determine whether human NAIP is critical for restriction of *S.* Typhimurium, *L. pneumophila,* or other Gram-negative bacterial infections. The mechanisms by which human NAIP may be defending against these bacterial infections are still unclear. In addition, our study raises intriguing evolutionary questions about NAIP/NLRC4 inflammasome activation. Promiscuous recognition may provide an evolutionary advantage to the host in restricting bacterial pathogens due to the host's ability to recognize rapidly evolving microbial structures. In contrast, promiscuous recognition may be an evolutionary disadvantage to the host due to weaker affinities for given ligands, thereby decreasing signaling potency.

Salmonella infection in human macrophages can also lead to activation of the non-canonical inflammasome, which is dependent on human caspase-4 or caspase-5 (Casson et al., 2015). Interestingly, under our experimental conditions, most of the inflammasome responses to *Salmonella* appear to be caspase-1-dependent. In agreement with these results, human monocyte-derived macrophages infected with *Salmonella* exhibited IL-1β

secretion independently of caspase-4 (Casson et al., 2015). Future studies are needed to better understand the kinetics and respective consequences of the activation of the canonical and non-canonical inflammasomes. Even though human NAIP recognizes the T3SS inner rod, the T3SS needle, and flagellin, when these ligands are delivered into the host cytosol, studies are needed to determine whether all bacterial components are detected under physiological conditions. A mouse model of systemic infection with *Salmonella* engineered to ectopically express bacterial ligands was used to understand the role of murine NAIPs in host defense. NAIP2- and NAIP5-mediated recognition of the inner rod and flagellin, respectively, result in host protection and survival. NAIP1 recognition of the T3SS needle appears to be dispensable for host defense (Zhao et al., 2016). It would be of interest to determine whether human NAIP detection of these three bacterial ligands is functionally redundant or distinct in antimicrobial responses and host defense against *Salmonella* infection.

In murine macrophages, both NLRC4 and NLRP3 are required for maximal inflammasome responses to *S.* Typhimurium (Broz et al., 2010). Additionally, mice lacking both NLRs are more susceptible to infection compared to WT or single knockout mice (Broz et al., 2010). However, the contribution of the human NLRP3 inflammasome in inflammasome responses to *Salmonella* is not yet understood. As our data suggested that there are NAIP-independent inflammasome responses in human macrophages, we also tested the role of NLRP3 in the inflammasome response to *Salmonella* infection. Interestingly, NLRP3 does not appear to be critical for inflammasome responses to *Salmonella* infection. However, we still need to determine whether the NLRP3 inflammasome is involved in inflammasome responses during later stages of infection during which flagellin is downregulated and *Salmonella* expresses the SPI-2 T3SS. It would be of interest to test whether NLRP3 is important in other cell types or during later

stages of infection to mediate restriction of *Salmonella*. Additionally, other NLR family members may be involved in inflammasome responses to *Salmonella* infection as there are 22 known NLRs in humans. Our preliminary data provide insight into human-specific responses to *Salmonella* infection. The data presented here provide an important basis for elucidating antimicrobial mechanisms to other gram-negative bacteria that also utilize specialized secretion systems to cause disease. This understanding could provide a foundation for future therapeutics aimed at controlling bacterial infection or dampening pathological immune responses by the NAIP/NLRC4 inflammasome.

3.6. Acknowledgements

We thank Paul Bates and Stephen Bart for helpful discussions, protocols, and reagents. We also thank Randilea Nichols, Isabella Rauch, Jeannette Tenthorey, and Russell Vance for generously providing the anthrax toxin-based reagents. We also thank Igor Brodsky and members from the Brodsky laboratory and the laboratory of Sunny Shin for helpful discussions. This work is supported in part by R01AI118861 (SS), R01AI123243 (SS), T32GM07229 (VRR), a Burroughs-Wellcome Fund Investigators in the Pathogenesis of Infectious Diseases Award (SS), and an NSF Graduate Fellowship (VRR).

3.7. Materials and Methods

3.7.1. Expression Plasmids Encoding Human Inflammasome Components

For human inflammasome components, pCMV6-XL5 plasmids encoding NAIP (NM_004536), IL-1β (NM_000576), or empty vector were purchased from Origene. The NAIP (NM_004536) ORF was amplified from the pCMV6-XL5 vector, between flanking

sites XhoI and SalI, and a Kozak sequence (GCCACC) was engineered to precede the start codon. The following primers were used (5'-3'):

NAIP forward: AATTAGATCTCTCGAGGCCACCATGGCCACCCAGCAGAAAG

NAIP reverse: TATCGATACCGTCGACTTATTTCTGAATGATTGGAGAGAAC

The NAIP PCR product was cloned into XhoI and SalI digested MSCV2.2 vector (Addgene plasmid #60206) using In-Fusion HD Cloning purchased from Takara Bio USA, Inc. (Cat # 638909).

The pCI plasmid encoding human caspase-1 (NM_033292.3) was a gift from Kate Fitzgerald (Addgene plasmid # 41552) (Hornung et al., 2009). The human NLRC4 (NM_021209) ORF was amplified from an expression vector (GeneCopoeia), between flanking BamHI and NotI sites, and a Kozak sequence (GCCACC) was engineered to precede the start codon. The following primers were used (5'-3'):

NLRC4 forward: AAAAGGATCCGCCACCATGAATTTCATAAAGGACAATAGCC

NLRC4 reverse: TTTTTGCGGCCGCTTAAGCAGTTACTAGTTTAAAATCACC

The digested NLRC4 PCR product was cloned into a BglII/NotI digested MSCV2.2 vector. The MSCV2.2 vector was a gift from Russell Vance (Addgene plasmid #60206) (Kofoed and Vance, 2011).

For mouse inflammasome components, mscv2.2-NAIP2 was a gift from Russell Vance (Addgene plasmid # 60201; http://n2t.net/addgene:60201; RRID:Addgene_60201) and mscv2.2-NLRC4 was also a gift from Russell Vance (Addgene plasmid # 60199; http://n2t.net/addgene:60199; RRID:Addgene_60199) (Kofoed and Vance, 2011). All plasmids were prepared with the Qiagen EndoFree Plasmid Maxi Kit.

3.7.2. Reconstitution of the Inflammasome in HEK293 cells

HEK293 cells were maintained in DMEM supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C. Cells were replated at $7x10^4$ cells/well in 500 µL replating media (DMEM + 10% FBS + 2 mM L-glutamine) in a 24-well plate. Transfection of expression plasmids (described above) was performed using Lipofectamine 2000 (Thermo Fisher Scientific). The amounts of plasmids used were 20 ng of human NAIP, 20ng murine NAIP2, 20 ng of human NLRC4, 20ng of murine NLRC4, 10 ng of human caspase-1, and 400 ng of human pro-IL-1β. 18 hours later, cells were treated with anthrax toxin components for cytosolic delivery of FlaA, PrgJ, or YscF. Cells were harvested 9 hours later and subjected to immunoblot analysis.

3.7.3. Anthrax Toxin-Mediated Delivery of FlaA, PrgJ and YscF

Recombinant proteins (PA, LFn-FlaA, LFn-PrgJ, and LFn-YscF) were kindly provided by Russell Vance (Rauch et al., 2016). In experiments with THP-1, cells were plated in a 48-well plate at a concentration of 2.0×10^5 per well. PA and LFn doses for *in vitro* delivery were 1 µg/ml PA for FlaTox, 4 µg/ml PA for PrgJTox and YscFTox) 500ng/ml LFn-$FlaA^{310-475}$ (truncated C-terminus of *L. pneumophila* flagellin), 8 ng/ml LFn-PrgJ, and 200 ng/mL LFn-YscF.

3.7.4. Co-Immunoprecipitation assay

For co-immunoprecipitation assays, the MSCV2.2 plasmid encoding c-Myc-FlaA was purchased from Addgene (Addgene plasmid #60203). The pCMV6-XL5 plasmid encoding NAIP (NM_004536) or empty vector were purchased from Origene. HEK293 cells were plated at 2.0×10^6 cells/10 cm dish in 10mL of DMEM supplemented with 10%

(vol/vol) heat-inactivated FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin. After 24 hours, plasmids were transfected using Lipofectamine 2000. The amounts of plasmids used were 4µg of c-Myc flagellin and 2µg of human NAIP. The amount of Lipofectamine 2000 used was 40µl per 10cm dish. After 18 hours, HEK293 cells were resuspended in 10mL of growth media and centrifuged at 2000rpm for 10 minutes at 4°C. For crosslinking of proteins, the pellet was resuspended in 500µl of 5mM BS^3 (Thermo Fisher Scientific Cat # 21580) in Conjugation Buffer (20mM Sodium Phosphate, 0.15M NaCl, pH 7-9) and incubated for 30 minutes in ice. Quench solution (1M Tris, pH 7.5) was added to a final concentration of 20mM Tris and incubated for 15 minutes at room temperature. The solution was spun down at 1200rpm for 10 minutes and the cell pellet was lysed in 500µL of NP40 cell lysis buffer (Thermo Scientific Cat # FNN0021) supplemented with a protease inhibitor cocktail (Sigma Aldrich Cat # 11836170001) for 30 minutes on ice and vortexing at 10-minute intervals. The extract was spun down at 13,000rpm for 10 minutes at 4°C. 50µL of the supernatant was saved as the input sample.

To conjugate the antibody with the beads, 50µL of magnetic Dynabeads Protein G (Thermo Fisher Scientific Cat # 10003D) were placed on a magnet, the supernatant was removed, and the beads were resuspended in the c-Myc antibody solution. The c-Myc antibody (Clone 9E10) was purchased from Takara (Cat # 631206) and 5µg of antibody was diluted in 200µl of PBS + 0.02% Tween-20 per sample. The beads and antibody were incubated for 1 hour at 4°C. The beads conjugated with antibody were then washed and incubated with 5mM BS^3 in Conjugation Buffer for 30 minutes at room temperature with rotation. Quenching Buffer was added and incubated for 15 minutes at room temperature. The sample with the target antigen was incubated with the conjugated beads overnight at

4°C with rotation. The next morning, the tube was placed in the magnet and the beads were washed 3 times with PBS. For elution of target antigen, the beads were resuspended in 50μl of 1X SDS/PAGE Sample Buffer and boiled for 5 minutes. The samples were then removed from the Dynabeads using the magnet and assayed for Western Blot.

3.7.5. Generation of CRISPR Cas9 knockouts in THP-1 cells

To mediate *NAIP*, *NLRC4, PYCARD,* or *CASP1* knockout in THP-1 cells, plasmids encoding the desired guide RNA (gRNA) and Cas9 in the pLentiCRISPR v2 plasmid were purchased from GenScript. The following target sequences were used:

NAIP: gRNA 1 (ACATTGCCAAGTACGACATA)

NLRC4: gRNA 1 (AAACATCATTTGCTGCGAGA)

NLRP3: gRNA 1 (CGAAGCAGCACTCATGCGAG)

CASP1: gRNA 1 (GACAGTATTCCTAGAAGAAC)

For the production of lentiviral particles, pCMV-VSV-G and psPAX2 plasmids were kindly provided by Paul Bates at the University of Pennsylvania. HEK293T cells were plated at $2.5X10^6$ cells per 10cm dish in 10mL of DMEM supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. After 24 hours, plasmids were transfected using the Lipofectamine 2000 protocol. The amounts of plasmids used were 1μg of pCMV-VSV-G, 2.5μg of psPAX2, and 8μg of pLentiCRISPR v2 with appropriate gRNA. The amount of Lipofectamine 2000 used was 50μL per dish. Transfected HEK293T cells were incubated for 18 hours at 37°C, and the media was then aspirated and replaced with 6mL of fresh growth media. After 16-18 hours, the supernatant containing lentiviral particles was harvested and filtered using 0.22μM filter. THP-1 cells were infected in 1mL of viral-containing media with 8μg/mL of

polybrene. The cells were spin-infected at 1250 × *g* for 90 min at 25°C, then incubated at 37°C for 48 hours. After 48 hours, puromycin was added to a final concentration of 1.0μg/mL. The cells were maintained in puromycin for 3 weeks and then harvested for Western Blot analysis and clonal selection. For clonal selection, cells were plated in 96-well plates at 0.5 cell per well or 2 cells per well in 200μl of growth media and were incubated for 4-8 weeks until single clones were visible in the bottom of the well. Single clones were then expanded from a 96-well plate through a series of multi-well plates ending in 10cm dishes. Cells were then plated in 48-well plates at a concentration of 2.0 x 10^5 cells per well in 500μl of media and harvested for purification of RNA, DNA, and Western Blot assays.

3.7.6. Validation of CRISPR Cas9 THP-1 single clones for NAIP and NLRC4 knockouts

To validate single cell clones after CRISPR Cas9 editing, DNA was purified using the DNeasy Blood and Tissue kit (Qiagen). The genomic region containing the target sequence was then amplified by PCR using the following primers (all 5' to 3'):

NAIP forward: CCGTACAGCTCATGGATACCACAG

NAIP reverse: GTACCTGTAAAGACAAAGCCAGCC

NLRC4 forward: CCCAGCCGGATATGCACATT

NLRC4 reverse: TCTGCCATGGGGAAGATGGAT

The PCR product was purified using the PCR Cleanup kit (Qiagen). A poly A-tail was added to the purified PCR product by adding together 7μl of PCR product, 5 Units of Taq DNA polymerase, 1X PCR Buffer containing $MgCl_2$, and 0.2mM dATP. The reaction was then incubated at 70°C for 30 minutes. 2μL of this product was then ligated into the

pGEM-T vector and transformed into DH5α competent cells using the protocol in the pGEM-T Vector System Protocol (A1360; Promega). Positive colonies were sequenced using a T7 Promoter Primer (5′ TAATACGACTCACTATAGGG 3′).

3.7.7. Bacterial Strains and Growth Conditions

Salmonella enterica serovar Typhimurium WT, Δ*sipB* (Lawley et al., 2006), and Δ*fliCfljB* (Wynosky-Dolfi et al., 2014) isogenic strains on the SL1344 background were used. Three hours before infection, *Salmonella* were diluted into Luria-Bertani (LB) broth containing 300 mM NaCl and grown for 3 h standing at 37°C to induce SPI-1 expression (Lee and Falkow, 1990).

3.7.8. THP-1 Monocytic Cell Line Experiments

THP-1 cells (TIB-202; American Type Culture Collection) were maintained in RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, 0.05 mM β-mercaptoethanol, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified incubator. Two days before infection, cells were replated in media lacking antibiotics at a concentration of 2.0 x 10^5 cells/well in a 48-well plate. THP-1 cells were differentiated into macrophages with 200 nM phorbol 12-myristate 13-acetate (PMA) for 24 hours. After 24 hours, cells were primed with 100ng/mL of Pam3CSK4. The next day, THP-1 cells were infected with *Salmonella*. Bacterial cultures were pelleted at 6,010 × *g* for 3 min and washed with PBS. Bacteria were then resuspended in PBS and added to the cells at a multiplicity of infection (MOI) of 20. The infected cells were then centrifuged at 290 × *g* for 10 min and incubated at 37°C. After 1 h of infection, 100µg/mL of gentamicin was added to each well to prevent extracellular bacterial growth. Infections proceeded at 37°C for a total of 6 h. Control cells

were mock-infected with PBS. For the LPS+Nigericin control, cells were primed with 500ng/mL of LPS for 4 hours and treated with 10μM Nigericin for 6 hours.

3.7.9. Quantitative RT-PCR Analysis

Cells were lysed and RNA was isolated using the RNeasy Plus Kit (Qiagen). Synthesis of the first strand cDNA was performed using Superscript II reverse transcriptase and oligo (dT) primer (Invitrogen). Quantitative PCR was performed with the CFX96 real-time system (Bio-Rad) using the SsoFast EvaGreen Supermix with LOW ROX kit (Bio-Rad). The following primers were used (all 5'-3'):

NAIP forward: GCATTCTCCTCTATTAGACTAG

NAIP reverse: GCCAACTGAACTGCATCTAG

HPRT forward: CCTGGCGTCGTGATTAGTGAT

HPRT reverse: AGACGTTCAGTCCTGTCCATAA

For analysis, mRNA levels of CRISPR-modified THP-1 cells were normalized to control THP-1 cells using the $2^{-\Delta\Delta CT}$ (cycle threshold) (Livak and Schmittgen, 2001) method to calculate fold induction.

3.7.10. ELISA

Harvested supernatants from infected cells were assayed using ELISA kits for IL-1β (BD Biosciences Cat # 557953) and TNF-α (Biolegend Cat # 430201).

3.7.11. Immunoblotting

Infected or treated cells were lysed directly with 1X SDS-PAGE sample buffer. Protein samples were boiled for 5 minutes, separated by SDS-PAGE, and transferred to

PVDF Immobilon-P membranes (Millipore). Samples were then probed with antibodies specific for IL-1β (8516; R&D Systems), NAIP (ab25968; Abcam), NLRC4 (12421S; Cell Signaling), caspase-1 (2225S; Cell Signaling), c-Myc (631206; Takara), and NLRP3 (15101S; Cell Signaling). As a loading control, all blots were probed with anti-β-actin (4967L; Cell Signaling). Detection was performed with HRP-conjugated anti-mouse IgG (F00011; Cell Signaling) or anti-rabbit IgG (7074S; Cell Signaling).

3.7.12. Statistical analysis

Prism 6.0 (GraphPad Software) was utilized for the graphing of data and all statistical analyses. Statistical significance for experiments with THP-1 cells was determined using the unpaired two-way *t* test. Differences were considered statistically significant if the *P* value was <0.05.

CHAPTER 4

A. Data Summary

Inflammasomes are critical for host defense against bacterial pathogens (Broz and Dixit, 2016; Broz, 2019). Most studies of bacterial interactions with the innate immune system are conducted in mice. However, there are key differences between the innate

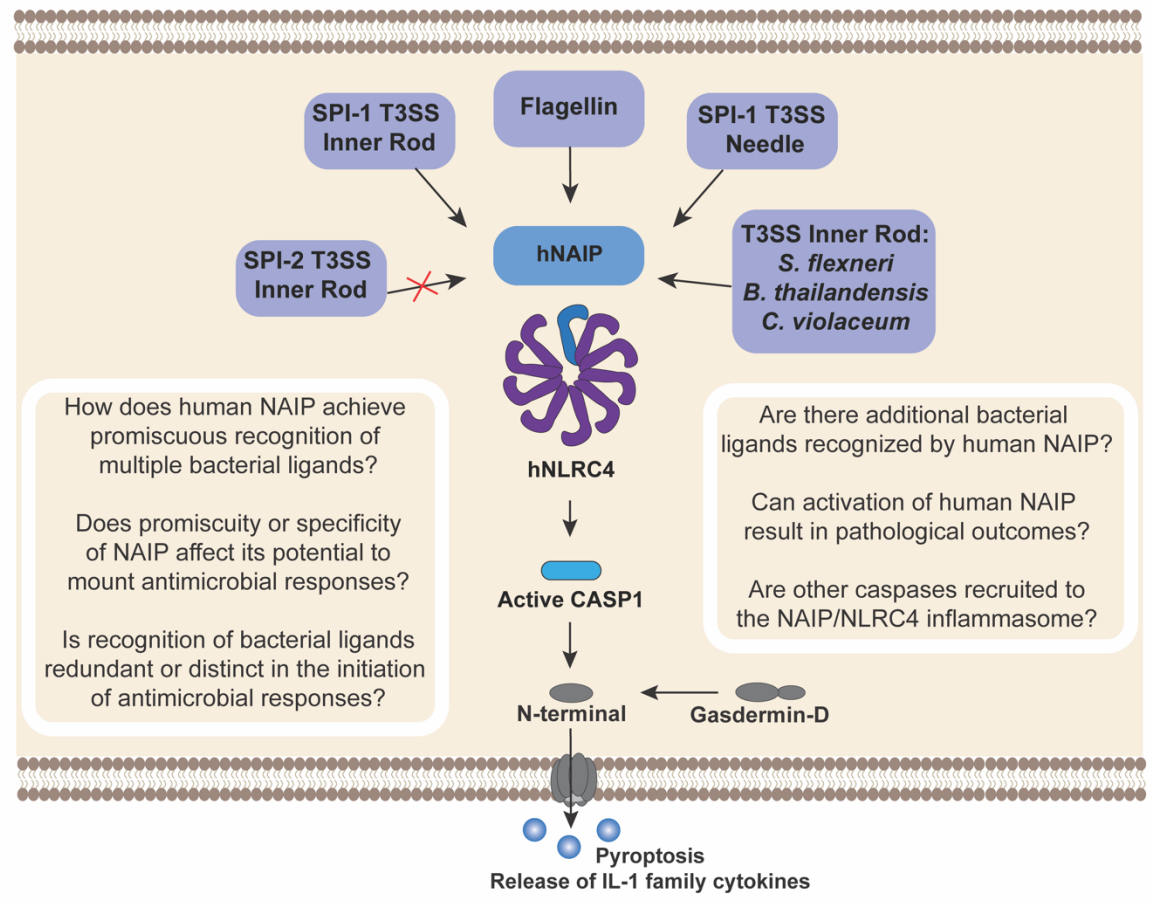

Figure 4.1: Model for human NAIP inflammasome responses to bacterial T3SS and flagellin proteins. Human NAIP mediates promiscuous recognition of the SPI-1 T3SS inner rod, SPI-1 T3SS needle, and flagellin. The T3SS inner rods from multiple bacterial species can also activate the human NAIP/NLRC4 inflammasome. However, the SPI-2 T3SS from S. Typhimurium evades immune recognition by human NAIP. Future directions for this project are highlighted and discussed in this chapter.

immune genes encoded by mice and those encoded by humans (**Tables 1.1 and 1.2**). Therefore, a better understanding of inflammasome responses in humans is necessary to inform the development of novel therapeutics for bacterial infections. To address this knowledge gap, we have been investigating human innate immune responses to Gram-negative bacterial infections. The work presented in this dissertation defined the human-specific innate immune recognition of bacterial T3SS and flagellin proteins by the NAIP/NLRC4 inflammasome (**Fig. 4.1**).

In **Chapter 2**, we found that in addition to the T3SS needle protein and flagellin, human NAIP can also sense the T3SS inner rod from multiple bacterial species. Furthermore, our data indicate that the *S*. Typhimurium SPI-2 T3SS inner rod, SsaI, which is required for intracellular bacterial replication, does not activate the inflammasome in human macrophages. The basis for this evasion of human NAIP is not understood, but studies in murine macrophages suggest that divergent sequences in the C-terminus of SsaI allow for evasion of NAIP2-mediated immune detection in mice. Furthermore, we determined that a single isoform of human NAIP is sufficient to activate inflammasome responses to the T3SS inner rod, needle, and flagellin. It remains to be determined whether other NAIP isoforms have specificity for a given bacterial ligand. Our findings indicate that, in contrast to the requirement for multiple NAIPs in the murine model, promiscuous recognition of multiple bacterial ligands is conferred by a single NAIP in humans.

In **Chapter 3**, we utilized CRISPR/Cas9 technology to generate *NAIP* and *NLRC4* knockout cell lines and tested the role of NAIP in recognition of bacterial ligands from the T3SS and flagellar apparatus as well as the role for inflammasome responses to *Salmonella* infection. Our preliminary data suggest that NAIP is essential for inflammasome responses to the T3SS inner rod, T3SS needle, and flagellin. In addition,

NAIP, but not the adaptor protein NLRC4, dictates the specificity or promiscuity of ligand recognition. We also found that human NAIP does not interact with murine NLRC4. Similar to the murine model where NAIPs, but not NLRC4, bind bacterial ligands, human NAIP appears to bind flagellin in the absence of NLRC4. However, one caveat of this experiment is that HEK293 cells may be expressing low endogenous levels of NLRC4 or a human-specific adaptor protein that also interacts with human NAIP and flagellin. Therefore, we will be taking additional approaches such as Biolayer Interferometry (BLI) and fluorescence polarization to test whether human NAIP directly binds the T3SS inner rod, T3SS needle, and flagellin. To understand the role of the NAIP/NLRC4 inflammasome in antimicrobial responses to *S.* Typhimurium, we infected our THP-1 cell lines deficient in *NAIP* or *NLRC4*. Our data suggest that NAIP is necessary for maximal inflammasome responses against *S*. Typhimurium. However, there are NAIP-independent inflammasome responses to *Salmonella* infection. In addition, caspase-1 appears to be required whereas NLRP3 may be dispensable for inflammasome responses.

Overall, our studies raise intriguing questions about the evolution and mechanisms of NAIP/NLRC4 inflammasome activation. In this chapter, I will be discussing open questions and proposing future studies to better understand the NAIP/NLRC4 inflammasome and its role in antimicrobial responses to gram-negative bacterial infections and human health.

B. Future Directions

How does human NAIP achieve broad recognition of multiple bacterial ligands?

Our data show that human NAIP is a generalist, as it is capable of functionally detecting the T3SS inner rod, T3SS needle, and flagellin. In contrast, the murine NAIPs are specialists as they each recognize only one bacterial protein (Kofoed and Vance,

2011; Zhao et al., 2011; Rayamajhi et al., 2013; Yang et al., 2013; Rauch et al., 2016; Zhao et al., 2016). The bacterial ligands recognized by the NAIP/NLRC4 inflammasome are structurally related, as they all belong to the T3SS and flagellar apparatus, which are thought to be of a common evolutionary origin (Saier, 2004). The T3SS inner rod, needle, and flagellin proteins exhibit low sequence conservation, but have some structural homology. Therefore, it is possible that human NAIP may recognize structural elements common to all three ligands. In mice, both NAIP5 and NAIP2 recognize two conserved structures in flagellin and the T3SS inner rod, respectively. These two conserved surfaces are located at the central portion and at the C-terminus of the bacterial ligands (Miao et al., 2010b; Tenthorey et al., 2017). Specifically, in flagellin they correspond to residues 31-33 (RLS motif) and 470-473, and in the T3SS inner rod, PrgJ, they correspond to residues 32-34 (RLS motif) and 95-101. These two surfaces in flagellin and the T3SS inner rod are highly conserved among bacterial species (Miao et al., 2010b; Tenthorey et al., 2017). Despite recognizing similar motifs in flagellin and the T3SS inner rod, NAIP5 and NAIP2 are highly specific to their cognate ligand. Interestingly, NAIP5 divergence from NAIP2 is concentrated in the regions where flagellin binds (Tenthorey et al., 2017). The conserved motifs in flagellin and the T3SS inner rod may be important for recognition by NAIPs, but variable regions surrounding these conserved motifs may additionally contribute to the specificity of ligand recognition by the murine NAIPs. Sequence divergence in human NAIP may allow for recognition of these variable regions in all three bacterial ligands, therefore allowing for promiscuous recognition. Another possibility is that the sequence differences in human NAIP may allow for the presence of multiple binding pockets for the bacterial ligands. In this case, the T3SS inner rod, T3SS needle protein, and flagellin may bind to different regions of the single human NAIP.

To test what portion of human NAIP allows for its broad recognition of bacterial ligands, we will use chimeric proteins made from the murine and human NAIPs. Reconstitution of the inflammasome with the different chimeric proteins and delivery of bacterial ligands will allow for the identification of regions in human NAIP critical for the recognition of bacterial ligands. This approach was used to determine the domain responsible for specific recognition of bacterial components by the different murine NAIPs (Tenthorey et al., 2014). We hypothesize that, similar to the murine NAIPs, the internal region composed of the NBD and adjacent α-helical domains will be required for specificity or promiscuity in NAIP. An additional approach would be to determine the function of NAIP in different host species and gain insights into the features that dictate NAIP to be a generalist or specialist. NAIP from multiple eukaryotic species can be cloned and used to reconstitute the inflammasome in HEK293 cells. We will perform sequence analysis of generalist versus specialist NAIPs and search for conserved motifs between the two modes of recognition. We can then perform mutagenesis analyses to confirm the importance of the same motifs in broad or specific recognition.

To test if the same conserved residues in the bacterial ligands are required for immune recognition by human NAIP, as they are required for recognition by the murine NAIPs, we will be performing alanine scanning in the T3SS needle, T3SS inner rod, and flagellin. We performed sequence alignments of all three bacterial ligands recognized by human NAIP and identified three highly conserved amino acids present in the three bacterial ligands. Future studies will determine if these amino acids are important for immune recognition by human NAIP. Additionally, cryo-electron microscopy of the NAIP/NLRC4 inflammasome bound to bacterial ligands will shed light into the mechanisms of activation by multiple bacterial ligands.

The affinities and binding kinetics of multiple NLRs to their cognate ligands have been determined (Schaefer et al., 2017; Hara et al., 2018). However, the affinities and binding kinetics of both murine and human NAIPs to components of the T3SS and flagellin are still unknown. We hypothesize that broad recognition by human NAIP will lead to a weaker binding affinity to each bacterial ligand compared to the specific murine NAIPs. To test this, we will perform Bio-layer interferometry and fluorescence polarization studies to determine the affinity and binding kinetics of each NAIP to their cognate ligands. Overall, the proposed studies will define the mechanism by which human NAIP achieves broad recognition of multiple bacterial ligands.

What is the role of NAIP in antimicrobial responses to Gram-negative bacterial infections?

Innate immunity mediated by murine NAIPs can defend against a variety of Gram-negative bacterial pathogens through several mechanisms. Flagellin-mediated activation of NAIP5 in mice can restrict *L. pneumophila* infection *in vivo* and *in vitro* (Growney and Dietrich, 2000; Wright et al., 2003; Zamboni et al., 2006; Kofoed and Vance, 2011; Zhao et al., 2011). A/J mice expressing a hypomorphic allele of *NAIP5* are more susceptible to *L. pneumophila* infection (Diez et al., 2003). The NAIP5/NLRC4 inflammasome contributes to the control of bacterial replication by enhancing fusion of the *Legionella*-containing vacuole (LCV) with the lysosome (Amer et al., 2006; Fortier et al., 2007). Activation of the NAIP/NLRC4 inflammasome and IL-1 signaling are also important for host defense against *S.* Typhimurium, as *Nlrc4*$^{-/-}$ and *Il1r1*$^{-/-}$ mice are more susceptible to orogastric infection (Franchi et al., 2012). In addition, pyroptosis leads to the production of pore-induced intracellular traps (PITs), which can trap viable bacteria and recruit neutrophils to mediate clearance through efferocytosis (Jorgensen et al., 2016).

Most studies looking at the role of NAIP in antimicrobial responses to bacterial infection have been performed in mice. We sought to determine the role of human NAIP in antimicrobial responses to *S.* Typhimurium infection. Our data suggest that human NAIP and caspase-1 are both important for inflammasome responses to *Salmonella*. Future studies are needed to determine whether human NAIP promotes restriction of intracellular bacterial replication. In addition, by assessing the necessity of gasdermin-D in THP-1 cells, we will determine whether cell death downstream of the NAIP/NLRC4 inflammasome is important for the control of *Salmonella*. Microscopy studies looking at lysosomal markers and the Salmonella-containing vacuole (SCV) are needed to test whether NAIP activation in humans promotes fusion with the lysosome and further degradation of the bacteria.

Activation of the NAIP/NLRC4 inflammasome in intestinal epithelial cells is also critical for host defense against *S.* Typhimurium in a mouse model of infection. Inflammasome activation drives expulsion of infected enterocytes to control *Salmonella* replication in the intestine (Sellin et al., 2014; Rauch et al., 2017). Studies in our laboratory are currently defining the role of human NAIP in intestinal epithelial cells for antimicrobial responses to *Salmonella* infection. Human intestinal organoid cultures can be used to study expulsion of enterocytes as a consequence of NAIP/NLRC4 inflammasome activation. Overall, a better understanding is needed to determine if human NAIP is important for control of bacterial infection, as well as elucidate the mechanism NAIP uses to promote host defense. A humanized mouse can be generated to understand *in vivo* responses downstream of human NAIP activation. In this case, both human NAIP and NLRC4 should be incorporated in the mouse, as our data indicate that human NAIP does not interact with murine NLRC4 (**Figure 3.1**).

Does promiscuity or specificity in NAIP affect its potential to mount antimicrobial responses?

Promiscuous recognition by human NAIP may provide a selective advantage, as it may be more difficult for pathogens to simultaneously evade the recognition of all three bacterial ligands by human NAIP. NAIP5 contacts flagellin at residues that are typically buried within the flagellar filament when flagellin is polymerized. Mutations in flagellin that disrupt the interaction with NAIP5 therefore disrupt the flagellar filament formation, which results in a lack of motility (Tenthorey et al., 2017). Perhaps similar residues are targeted for recognition by human NAIP in order to limit immune evasion by pathogens. Additionally, similar studies are needed to determine whether the mutation of residues recognized by human NAIP in the T3SS inner rod and needle proteins leads to a non-functional T3SS. Mutations can be generated in *Salmonella,* and we can take advantage of a reporter system to validate the functionality of the T3SS. *Salmonella* will be transformed with a plasmid containing β-lactamase fused to an effector protein known to be secreted through the T3SS. After infection, cells are loaded with a membrane-permeant β-lactamase substrate. In the presence of a functional T3SS, the translocation of β-lactamase will lead to substrate cleavage and a shift in emission fluorescence (Copenhaver et al., 2015). Another selective advantage for promiscuous recognition may be to diversify protein functionality, allowing for responses to multiple pathogenic stimuli.

In contrast, promiscuous recognition by human NAIP may only provide a selective advantage in certain circumstances, as it may affect the potency of its antimicrobial activities. One possible tradeoff of a promiscuous mode of recognition by human NAIP may be weaker binding affinities or altered binding kinetics to its bacterial ligands, which may decrease signaling potency. In contrast, each murine NAIP may possess a higher affinity or half-life in binding to its cognate ligand and thus confer heightened antimicrobial

activities. Indeed, compared to mouse macrophages, human macrophages do not seem to be as responsive to flagellin, as they are more permissive for intracellular bacterial replication of flagellated bacteria (Vinzing et al., 2008). The basis for these differences is unknown, but one possibility is that murine NAIP5 detects flagellin with a higher affinity than does human NAIP. Now that we have generated THP-1 cell lines deficient in human NAIP, we can add a copy of human NAIP or murine NAIP5 and compare the ability of each NAIP to mount antimicrobial responses against *L. pneumophila* infection. *L. pneumophila* would be a good model since it does not contain a T3SS, and, therefore, it is expected to only activate human NAIP through recognition of cytosolic flagellin.

Is recognition of bacterial ligands redundant or distinct in the initiation of antimicrobial properties?

During infection, several Gram-negative bacteria downregulate or halt the expression of flagellin (Chain et al., 2004; Tominaga et al., 2005; Cummings et al., 2006; Winter et al., 2010b). The relative contribution of recognition of the T3SS inner rod, needle, and flagellin by human NAIP for antimicrobial responses is unknown. In mice, it has been suggested that detection of the T3SS inner rod, but not the needle protein, by the NAIP/NLRC4 inflammasome leads to the clearance of *S.* Typhimurium. This observation was made by infecting WT or NLRC4 deficient mice with *Salmonella* overexpressing the T3SS inner rod or the needle protein (Miao et al., 2010b). In recent studies, the relative contribution of the murine NAIPs in host defense against *S.* Typhimurium was determined. *In vitro* studies suggest that there is redundancy among various NAIPs for inflammasome responses, as *Naip1*$^{-/-}$, *Naip2*$^{-/-}$, and *Naip5*$^{-/-}$ bone marrow-derived macrophages (BMDMs) infected with *S.* Typhimurium had similar levels of pyroptosis compared to WT bone marrow-derived macrophages. In contrast, BMDMs deficient in all murine NAIPs (*Naip1-*

$6^{\Delta/\Delta}$) had a defect in cell death when infected with *Salmonella* (Rauch et al., 2016). Infection of BMDMs with a flagellin-deficient strain of *Salmonella* or WT *S. flexneri* suggests that the recognition of the T3SS inner rod by NAIP2 is required for optimal inflammasome responses, whereas the recognition of the T3SS needle protein by NAIP1 is dispensable (Zhao et al., 2016). However, this phenotype may be due to lower NAIP1 expression levels. Additionally, *S.* Typhimurium was engineered to mediate ectopic expression of each of the bacterial ligands from the SPI-1 T3SS and flagellin and to secrete each bacterial ligand using the SPI-2 T3SS. Mice infected with *Salmonella* ectopically expressing flagellin or the T3SS inner rod have increased survival as compared to mice infected with WT *Salmonella* or *Salmonella* expressing the T3SS needle protein (Zhao et al., 2016). These results suggest that the recognition of bacterial ligands by murine NAIPs can be redundant or specific during different stages of infection.

To test whether recognition of bacterial ligands by human NAIP is redundant or distinct in the initiation of antimicrobial responses, WT or *NAIP*-deficient THP-1 cells can be infected with *Salmonella* engineered to express and deliver distinct bacterial ligands using the SPI-2 T3SS. Additionally, the knowledge of residues required for immune recognition by human NAIP can be utilized to mutate the T3SS inner rod, T3SS needle, or flagellin in *S.* Typhimurium to abolish specific recognition of distinct bacterial components. Recognition of bacterial ligands by human NAIP leading to redundancy in antimicrobial activities would limit evolutionary immune evasion mechanisms by pathogens, as it would be less likely to simultaneously mutate several distinct bacterial components. *S.* Typhimurium has evolved to express a second T3SS (SPI-2 T3SS) to survive and replicate inside of host cells. Our data indicate that the SPI-2 T3SS inner rod, SsaI, evades immune recognition by human NAIP. In mice, it is suggested that SsaI

evades immune recognition by NAIP2 through amino acid changes in its C-terminus (Miao et al., 2010b). It remains to be determined whether similar amino acid changes in SsaI result in evasion of immune recognition by human NAIP. Some Salmonella serovars, including *S.* Typhi, are human-adapted and cause systemic disease (Broz et al., 2012). It will be of interest to determine whether *S.* Typhi can secrete effector molecules that specifically target the NAIP/NLRC4 inflammasome, as well as whether inflammasome responses play any role in host tropism.

Are there additional bacterial ligands recognized by the NAIP/NLRC4 inflammasome?

In mice, ligands for four of the seven distinct NAIPs are known: NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod protein, and both NAIP5 and NAIP6 recognize flagellin (Kofoed and Vance, 2011; Zhao et al., 2011; Rayamajhi et al., 2013; Yang et al., 2013; Rauch et al., 2016; Zhao et al., 2016). It will be of interest to determine if additional bacterial ligands can be detected by the remaining three NAIPs or by the single human NAIP. As the T3SS and flagellar apparatus are evolutionarily related, it remains to be determined whether bacterial ligands from the flagellar apparatus basal body are recognized. Interestingly, BMDMs infected with a strain of *Salmonella* lacking a hook-associated protein (FlgK) from the flagellar apparatus show reduced cell death compared to WT-infected macrophages (Miao et al., 2006). We will be using different approaches to mediate cytosolic delivery of FlgK and test whether it can be recognized by the NAIP/NLRC4 inflammasome. Additionally, we can perform pull-down assays for human NAIP and mass-spectrometry to identify potential binding partners during infection with *S.* Typhimurium.

What are the roles of other caspases in inflammasome responses downstream of human NAIP activation?

The apoptotic caspase, caspase-8, can bind ASC and be recruited to the NLRC4 inflammasome-dependent ASC speck during *Salmonella* infection of macrophages (Masumoto et al., 2003; Man et al., 2013). Caspase-8 compensates for the loss of caspase-1 in intestinal epithelial cells and mediates expulsion of infected enterocytes downstream of NAIP/NLRC4 inflammasome activation (Rauch et al., 2017). Therefore, caspase-8 contributes to NAIP/NLRC4 responses against *S.* Typhimurium infection in mice. However, the roles of caspase-8 and its close ortholog, caspase-10, in human NAIP/NLRC4 inflammasome responses are unknown. Human patients with inactivating mutations in caspase-8 are more susceptible to infections (Chun et al., 2002). Studies in our laboratory will use siRNA-mediated silencing and CRISPR-Cas9 technology to target caspase-8 and caspase-10 and test their role in inflammasome responses to *Salmonella* infection. Our preliminary data suggest that most inflammasome responses to *Salmonella* require *CASP1*. However, it is possible that caspase-8 and caspase-10 have a role in other host cells or stages of infection.

In *L. pneumophila* or S. Typhimurium-infected primary human macrophages, secretion of IL-1β requires caspase-1, whereas IL-1α secretion does not. Instead, secretion of IL-1α requires caspase-4 (Casson et al., 2015). Our data suggest that infection with *S.* Typhimurium and delivery of bacterial ligands leads to IL-1α secretion which is dependent on activation of the NAIP/NLRC4 inflammasome. It will be of interest to determine whether activation of the NAIP/NLRC4 inflammasome can also recruit human caspase-4 or caspase-5 to the same inflammasome to aid the secretion of IL-1α. We can perform microscopy analyses of the NAIP/NLRC4 inflammasome, co-

immunoprecipitation, or native gels and immunoblot analyses to determine which host caspases are being recruited to the same macromolecular complex.

Can other NLRs mediate recognition of bacterial ligands and host defense against Gram-negative bacterial infections?

In mice there are 35 NLRs whereas there are 23 in humans (**Table 1.1**). There has been an expansion of NAIP and NLRP genes in mice. However, there are NLRs specific to humans, such as NLRP7, NLRP8, NLRP11, and NLRP13. It will be of interest to mediate an siRNA or CRISPR/Cas9 screen for the known human NLRs to determine whether additional NLRs mediate the recognition of components from the T3SS and flagellin or unknown bacterial ligands to mediate host defense against *S.* Typhimurium infection. Additionally, it would be of interest to determine whether mutations in any of these genes in humans increases the risk of infection or results in autoinflammation.

Can activation of human NAIP result in excessive inflammation and pathological outcomes?

Gram-negative bacteria cause about 70% of hospital-acquired infections in intensive care units (Peleg and Hooper, 2010). Infected cells use innate immune mechanisms to control bacterial infection. However, in many cases, uncontrolled immune responses to bacterial infection can lead to pathological outcomes such as sepsis. Annually, there are approximately 300,000 cases of Gram-negative septicemia in the US with a >30% mortality rate (Martin et al., 2003; Mayr et al., 2014). These infections are increasingly difficult to treat with the rise of multidrug-resistant Gram-negative bacteria. Additionally, alternative approaches to treat sepsis developed in mouse models failed during clinical trials in humans (Fink and Warren, 2014; Marshall, 2014). The basis for

these failures is unclear, but differences between mouse and human innate immune responses may play an important role. Therefore, understanding human-specific innate immune responses to pathogens is needed to identify more effective approaches for treating bacterial infections or sepsis in humans.

Although inflammasome responses are protective against multiple bacterial pathogens, excessive inflammation caused by the inflammasome has the potential to be pathological. In mice, gut injury and disruption of the microbiota results in lethal systemic inflammation resembling sepsis. This sepsis-like disease has been associated with the systemic spread of a multidrug-resistant pathobiont and required activation of the NAIP5/NLRC4 inflammasome (Ayres et al., 2012). It remains to be determined whether disruption in intestinal homeostasis in humans can result in members of the microbiota leading to a sepsis-like disease that is dependent on the human NAIP/NLRC4 inflammasome. As mentioned previously, promiscuous recognition of human NAIP may result in decreased signaling potency as compared to the murine NAIPs. This potential decrease in signaling potency may be a disadvantage when defending against bacterial pathogens; however, in some cases it may be beneficial to avoid excessive inflammation and pathological outcomes such as in sepsis.

Other examples of pathological outcomes associated with the human NAIP/NLRC4 inflammasome have been defined in human patients. Gain-of-function mutations in human *NLRC4* can result in pathologic enterocolitis and Macrophage Activation Syndrome (MAS) (Canna et al., 2014; Romberg et al., 2014; Canna et al., 2016). Other mutations in NLRC4 result in more mild cases where patients experience urticaria and arthritis after exposure to cold stimuli (Kitamura et al., 2014). Future studies are needed to determine whether mutations in human NAIP also confer similar pathological outcomes.

Out of the eight orthologs from the inhibitor of apoptosis protein (IAP) family, human *NAIP* has undergone the most extensive genomic rearrangements during mammalian evolution (Romanish et al., 2007). The *NAIP* gene is located in a genomic region of 500-kb containing the gene responsible for spinal muscular atrophy (*SMN1*). This 500-kb region has undergone an inverted duplication specifically in humans. In contrast, other primates have a pericentromeric inversion and a translocation to a different chromosome that has repositioned *NAIP* (Romanish et al., 2007). In contrast to mice, the human *NAIP* locus has a number of pseudogenes and gene duplications and has retained only a single functional copy of the full-length *NAIP* gene (Romanish et al., 2009). *NAIP* also possesses multiple promoters that are not shared between humans and mice. Long terminal repeats (LTRs) of endogenous retroviral elements provide *NAIP* promoter function in humans and rodents. However, these LTRs functioning as promoters for *NAIP* were independently acquired during mammalian evolution (Romanish et al., 2007). Another level of regulation in human *NAIP* is conferred by the presence of intragenic retrotransposons, resulting in 5' truncated transcripts (Romanish et al., 2009). Future studies are needed to determine if any of the pseudogenes or 5' truncated transcripts from the human *NAIP* gene are important for innate immune defense. Interestingly, African populations have a duplication of the full-length human NAIP at a higher frequency than Europeans and Asians. The higher amount of NAIP results in increased cell death upon infection with *L. pneumophila* (Boniotto et al., 2012). These data suggest that a gene duplication in Africans may confer an advantage for host defense against flagellated bacteria or against bacteria that utilize a T3SS to cause disease. The presence of pseudogenes and gene duplications events in the human *NAIP* locus will make the discovery of potential single-nucleotide polymorphism associated with pathology in humans difficult. Therefore, a better understanding of the composition and evolution of the

NAIP locus is needed to further our understanding of NAIP in bacterial defense and human health.

Concluding remarks

Our results provide insight into human NAIP detection of bacterial proteins from the T3SS and flagellar apparatus. In contrast to mice having specialist NAIPs, human NAIP mediates promiscuous recognition of multiple bacterial ligands. In addition, our data defined a role for human NAIP in antimicrobial responses to *S.* Typhimurium infection. Our study raises intriguing questions about the evolution of the NAIP/NLRC4 inflammasome. It will be of interest to understand how coevolution with Gram-negative bacteria has shaped the *NAIP* genes in humans and other mammals. Perhaps pathogen-induced evolutionary pressure on rodents caused the expansion of specialist NAIPs. In contrast, it is possible that the single human NAIP may have decreased signaling potency, which could be beneficial in cases of NAIP-mediated pathological inflammation. It will be of interest to understand whether human NAIP plays a role in species tropism for human adapted pathogens or whether human NAIP can also defend against other bacterial pathogens that use a T3SS to cause disease.

Overall, our studies provide an important basis for elucidating the mechanisms underlying human NAIP inflammasome responses, which could prove crucial to understanding how the NAIP/NLRC4 inflammasome contributes to human health and disease. In addition, we defined a role for human NAIP in antimicrobial responses to *Salmonella* infection. Future therapeutics may be developed to target this immune sensor as an alternative mechanism to treat bacterial infections or pathological outcomes. Future work is needed to further our knowledge of human-specific innate immune responses to Gram-negative bacterial infections.

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