Microbial Pathogen-Induced Necrosis Mediated By NLRP3 and ASC

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ABSTRACT

STEPHEN B. WILLINGHAM: Microbial Pathogen-Induced Necrosis Mediated By NLRP3 and ASC
(Under the direction of Dr. Jenny P-Y. Ting)

NLRP3 and ASC are important components of the inflammasome, a multi-protein complex required for caspase-1 activation and IL-1β production. *NLRP3* mutations underlie autoinflammation characterized by excessive IL-1β secretion. Disease-associated NLRP3 also causes a program of necrosis-like cell death in macrophages, the mechanistic details of which are unknown. We find that patient monocytes carrying disease-associated *NLRP3* mutations exhibit excessive necrosis-like cell death by a process dependent on ASC and cathepsin B, resulting in spillage of the proinflammatory mediator HMGB1. *Shigella flexneri* and *Klebsiella pneumoniae* infection also cause NLRP3-dependent macrophage necrosis with features similar to the death caused by mutant *NLRP3*. This necrotic death is independent of caspase-1 and IL-1β, and thus independent of the inflammasome. While similar proteins mediate pathogen-induced cell death in plants, this report identifies NLRP3 as an important host regulator of pathogen-induced necrosis in animals, a process we term pyronecrosis.

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Chapter 1: Jenny P.-Y. Ting, Stephen B. Willingham, and Daniel T. Bergstralh. NLRs at the Intersection of Cell Death and Immunity. Nature Reviews Immunology 2008, Manuscript in press.

Chapter Two: Stephen B. Willingham, Daniel T. Bergstralh, William O'Connor, Amy C. Morrison, Debra J. Taxman, Joseph A. Duncan, Shoshana Barnoy, Malabi M. Venkatesan, Richard A. Flavell, Mohanish Deshmukh, Hal M. Hoffman, and Jenny P.-Y. Ting**.** Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyin and ASC. Cell Host & Microbe 2007 Sep 13;2(3):147-59.

Chapter Three: Stephen B. Willingham, Irving C. Allen, Daniel T. Bergstralh, W. June Brickey, Joseph A. Duncan, and Jenny P.-Y. Ting. *Klebsiella pneumoniae* induces IL-1β release and pyronecrosis through NLRP3. J Ex Med 2008 Manuscript in preparation.

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LIST OF ABBREIVATIONS

ASC	Apoptosis-associated speck-like protein containing a CARD
BIR	Baculovirus inhibitory repeat
CARD	Caspase Recruitment Domain
CATERPILLER	CARD, transcription enhancer, R(purine)-binding, pyrin, lots of leucine repeats
CIAS1	Cold-Induced Autoinflammatory Syndrome 1
CINCA	Chronic Infantile Neurologic Cutaneous and Articular syndrome
FCAS	Familial Cold Autoinflammatory Syndrome
IAP	Inhibitor of apoptosis
ICE	Interleukin Converting Enzyme
IL-1β	Interleukin-1β
IPAF	ICE-Protease Activating Factor
LRR	Leucine Rich Repeat
MWS	Muckle-Wells Syndrome
NACHT	NAIP, CIITA, HET-E, and TP-1
NOMID	Neonatal-Onset Multisystem Inflammatory Disease
NBD	Nucleotide Binding Domain
NBS-LRR	Nucleotide binding site–leucine rich repeats (aka NB-LRR)
NLR	Nucleotide-binding, leucine-rich repeat
PYPAF	Pyrin-containing APAF1-like proteins
RAIDD	RIP-associated ICH-1/CED-3 homologous protein with a death domain

CHAPTER I

INTRODUCTION

Portions of this chapter have been adapted from: Jenny P.-Y. Ting, Stephen B. Willingham, and Daniel T. Bergstralh. NLRs at the Intersection of Cell Death and Immunity. Nature Reviews Immunololgy. 2008 *Manuscript in press.*

1.1 Summary

Inflammation is a crucial element of the host response to cellular insult. Pathogen-induced inflammation includes a molecular pathway which proceeds through activation of the protease caspase-1 to the release of the inflammatory cytokines IL-1β and IL-18. Importantly, pathogens may also induce forms of cell death with inherently pro-inflammatory features. Here I review recent evidence demonstrating that NLR family proteins serve as a common component of both caspase-1-activated apoptotic pathways and caspase-independent necrotic pathways. Parallels are drawn between NLR protein function and the activity of structurally similar proteins involved in cell death: the apoptotic mediator APAF1 and the plant disease resistance NBS-LRR proteins.

1.2 Introduction

	NLR Nomenclature	Alternative Nomenclature	GenBank Accession
AD	NLRA	CIITA, MHC2TA	NM_000246
BIR	NLRB1	BIRC1, NAIP	NM_004536
CARD	NLRC1	CARD4, NOD1	NM_006092
	NLRC2	CARD15, NOD2, BLAU, CD, PSORAS1, IBD1	NM_002162
	NLRC4	CLR2.1,CARD12, IPAF, CLAN	NM_021209
PYR	NLRP1	CLR17.1, CARD7, DEFCAP, NALP1, NAC	NM_033004
	NLRP2	PYPAF2, NBS1, NALP2, CLR19.9	NM_017852
	NLRP3	CLR1.1, CIAS1, PYPAF1, NALP3	NM_004895
	NLRP7	PYPAF3, NOD12, NALP7, CLR19.4	NM_206828
	NLRP10	NALP10, PYNOD, NOD8, CLR11.1	NM_176821

Table 1.1 - NLRs discussed in this dissertation, classified by effector domain

The NLR (nucleotide-binding domain, leucine-rich repeat containing) family of proteins (previously known as CATERPILLERs, NODs, NACHT–LRRs; see the HUGO Gene Nomenclature webpage on the NLR family) is rapidly emerging as critical regulators of immunity. For NLRs discussed in this dissertation, see Table 1.1 Members of this family are distinguished by their domain architecture (Fig. 1.1), which consists of a variable N-terminal effector domain, a central nucleotide binding domain (NBD), and C-terminal leucine-rich repeats (LRRs). To date, work on NLR proteins in animals has focused largely on their ability to mediate the initial immune response to pathogenic insult, particularly with regard to inflammation. However, a number of recent papers show that NLR proteins also represent a surprising intersection between innate immunity and cell-death signaling. Intriguingly, this signaling is not confined to apoptosis, but instead extends to two newly recognized cell-death programs: pyroptosis and pyronecrosis.

Clues to the cell-death-related functions of the NLR proteins can be drawn from the structural relationship between NLRs and molecules that are known cell death effectors; the apoptotic protease activating factor-1 (APAF1) and the NBS-LRR (nucleotide binding site–leucine rich repeats, also known as NB-LRR) plant disease resistance proteins (Belkhadir et al., 2004). APAF1 has an important role in triggering mitochondrial-dependent apoptosis. Similar to the NLRs, APAF1 has an N-terminal effector domain and a central NBD. APAF1 also contains C-terminal repeats, though these differ from the NLRs (Fig. 1.1). Members of the NLR family have an even closer structural resemblance to the NBS-LRR subset of plant resistance proteins, which are characterized by a variable N-terminal domain, a central NBD, and C-terminal LRRs (Fig. 1.1). NBS–LRR proteins function in part by helping to induce the hypersensitive response, a form of programmed cell death with necrotic features (Lam et al., 2001). In this dissertation, I discuss the emerging theme that mammalian NLRs, similar to APAF1 and the NBS–LRRs, act in the regulation of cell death and inflammation.

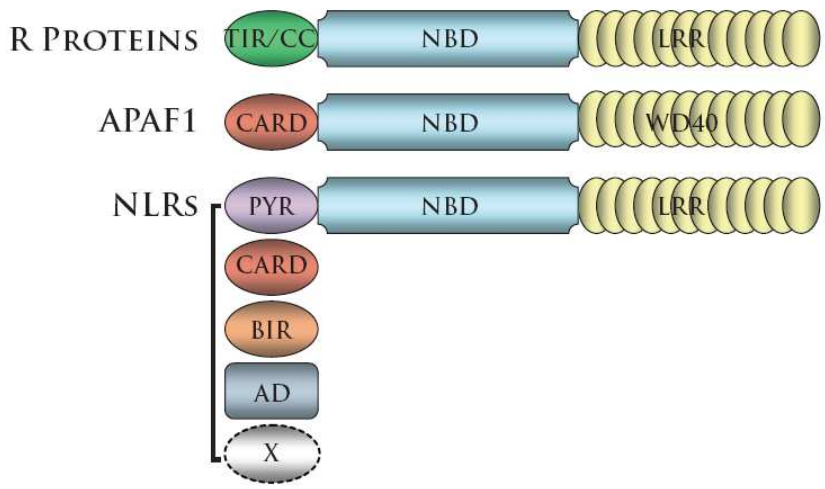


Figure 1.1 - NLR proteins are structurally similar to the pro-apoptotic protein APAF1 and the plant cell-death mediating NBS-LRR proteins. NLR proteins are defined by three characteristics: an N-terminal effector domain, a central NBD (nucleotide binding domain), and C-terminal LRRs (leucine-rich repeats). When defined, NLR effector domains consist of either a pyrin domain (PYR), caspase recruit domain (CARD), baculovirus inhibitor of apoptosis repeat (BIR) domains, or a transactivation domain (AD). One NLR has an undefined or uncharacterized effector domains (X). APAF1 (apoptotic protease activating factor 1) also has an N-terminal effector CARD and a central NBD. However, its C-terminal repeats differ from those of the NLRs. NBS-LRR (nucleotide binding site, leucine-rich repeats) proteins are characterized by a Toll-IL-1 receptor (TIR) or coiled-coil (CC) N-terminal effector domains, a central NBD, and C-terminal LRRs.

1.3 NLR Domain Organization

NLR Effector Domains

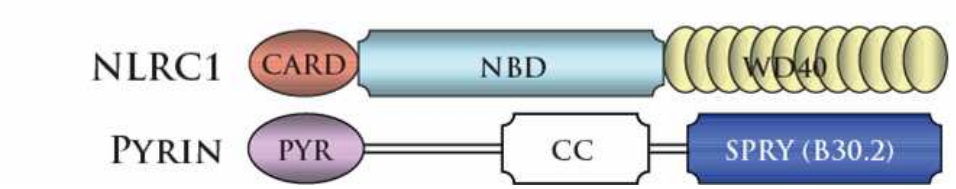
NLR effector domains, when defined, consists of an N-terminal CARD, pyrin, BIR or Activation Domain, all of which engage in homotypic interactions to mediate signaling downstream of NLR molecule activation. Though the overall domain organization of NLRs has been conserved from the plant NBS-LRR proteins, the R proteins utilize both TIR (Toll-IL-1 receptor) and CC (coiled-coil) domains as effectors. Much insight can be gained through analysis of the individual effector domains, namely the potential involvement of NLRs in the regulation of inflammation and cell death.

NLR Effector Domains: Caspase Recruitment Domain (CARD)

Of the possible NLR effector domains, the CARD has the deepest roots in cell death. Predictive secondary structure analysis indicates CARDs contain a six-α-helix bundle characteristic of the death domain-fold superfamily (Fairbrother et al., 2001). Members of this superfamily are well established mediators of protein–protein interactions between molecules involved in cell death and inflammation (Tibbetts et al., 2003). Amongst the NLRs, CARDs can be found at both the N- and C-terminus, as in NLRC1-5 and NLRP1. The caspase recruitment domain (CARD) was defined by Jurg Tschopp over 10 years ago in a screen for domains similar to the N-terminus of RAIDD, ICH-1(Caspase-2), and Ced-3 (Hofmann et al., 1997). At the time, RAIDD was a newly described bipartite adapter

involved in signaling downstream of TNFR1 (Duan and Dixit, 1997)1 . Binding to RIP through its death domain, RAIDD recruits and activates ICH-1 (Duan and Dixit, 1997). More recently, a similar function has been described in which RAIDD recruits ICH-1 to the PIDDosome, an apoptotic death initiating complex initiated by p53 in response to DNA damage (Tinel and Tschopp, 2004). Interestingly, RAIDD itself was identified in a search for motifs similar to the N-terminus of ICH-1. Within the original report the authors repeatedly note the resemblance of RAIDD and ICH-1 to *ced-3*, the C. *elegans* gene required for cell death during development. (Ellis and Horvitz, 1986). Indeed, Tschopp utilized the similarity within the N-terminus of these same 3 proteins to define and identify CARD in other caspases (Caspase-4, Mch6, ICE), homologues of the viral apoptosis inhibitor lAP (c-lAP1, c-lAP2), and the nematode cell death protein ced-4 (Hofmann et al., 1997). Shortly thereafter, CARDs were found in many proteins involved in cell death and inflammation. Amongst the more notable examples, the CARDs of APAF1 and caspase-9 interact during formation the apoptosome to initiate apoptosis after cytochrome c release from the mitochondria (Li et al., 1997; Qin et al., 1999). Homotypic CARD interactions are also utilized by NRLC1 and NLRC2 to bind the serine-threonine kinase RICK (Seth et al., 2005). Interestingly, CARDs are not just used by NLRs for cell death purposes, many CARD-containing NLRs use their CARDs to directly engage and activate caspase-1 to cause inflammation (Mariathasan et al., 2004). Even NLR molecules lacking CARDs can engage caspases by interacting with CARD containing adapter proteins such as ASC and TUCAN (Agostini et al., 2004; Yu et al., 2005). (For more, see chapter 1.6, NLR Inflammasomes).

NLR Effector Domains: Pyrin Domain



Like CARDs, the pyrin domain also belongs to the death-domain-fold superfamily. The pyrin domain was initially described by *Bertin et al* in late 2000 (Bertin and DiStefano, 2000). Based on the resemblance of NLRC1 to plant R-proteins and APAF1, this group reasoned that though NLRC1 contains an identifiable N-terminal CARD, similar proteins may contain novel, non-CARD signaling domains. To identify these, *Bertin et al* searched for proteins possessing regions similar to the central nucleotide binding domain of NLRC1. Amongst the results were pyrin, NBS1, CARD7, zebrafish caspase-13, and ASC (Bertin and DiStefano, 2000). Ultimately, the term "pyrin domain" was coined to represent the ~95 residue region of shared homology between pyrin, NBS1, CARD7. Within weeks, *Pawloski et al* also reported the existence of this domain (Pawlowski et al., 2001) . Unlike *Bertin et al*, *Pawlowski et al* searched for proteins with sequence similarity to the first 100 amino acids of the N terminus of the pyrin molecule. The term "PAAD" domain reflects the families of proteins recovered in their search, namely Pyrin, AIM (absent in melanoma), ASC, and death domain (DD)-like. Interestingly, seven proteins were identified containing both the PAAD and newly-defined NACHT domain. Accordingly, these proteins were named PAN, for PAAD and nucleotide-binding. Pyrin domains are found in at least 14 NLR proteins, many of

which utilize the domain to engage in homotypic interactions with ASC to both positively and negatively regulate the activation of caspase-1 (For more information, see chapter 1.6, NLR Inflammasomes)

NLR Effector Domains: Baculovirus Inhibitory Repeats (BIR) Domain

NLRB1 (formerly NAIP5) is unique within the NLR family as the only member containing BIR (Baculovirus IAP Repeat) effector domain (Listen et al., 1996). The ~70 amino acid BIR domain can occur in up to three tandem copies and function as a zinc binding domain (Crook et al., 1993). The BIR domain was identified in 1993 by Lois Miller and her colleagues following a series of experiments involving the *Autographa californica* virus (Crook et al., 1993). Infection of SF-21 cells with p35 mutant *A. californica* viruses results in cellular apoptosis before completion of viral replication. Specifically, these mutants are unable to delay cell death sufficiently to allow the formation of polyhedral occlusion bodies. To identify baculovirus genes with the ability to inhibit host cell apoptosis, *Crook et al* infected SF-21 cells with p35 mutant *A. californica* while concurrently transfecting a cosmid library prepared from the genomic DNA of the *Cydia pomonella granulosis virus* (CpGV). Subcloning of cosmids demonstrating successful rescue of p35 mutant (as determined by polyhedra formation) ultimately yielded a single ORF encoding a ~31 kDa protein the authors name IAP, Inhibitor of Apoptosis. Interestingly, IAP has no significant homology to p35, but rather the conserved distribution of cysteines and histidine within the BIR suggests the presence of a zinc finger-like motif commonly seen in many proteins

involved in the regulation of cell death. Subsequent identification and analysis of similar anti-apoptotic baculovirus genes elucidated a common repeating sequence ($GX_2YX_4DX_3CX_2CX_6WX_9HX_{6-10}C$) the authors termed the BIR (Baculovirus IAP Repeat) (Birnbaum et al., 1994). The BIR was subsequently identified in species ranging from viruses to mammals and now is the defining characteristic of a whole family of anti-apoptotic proteins known as the IAPs (Inhibitors of Apoptosis) (Deveraux and Reed, 1999). Both *in vitro* and *in vivo*, NLRB1 has been shown to inhibit apoptosis in response to several stimuli by BIR-dependent inhibition of effector caspases (Diez et al., 2000; Maier et al., 2002). NLRB1 also limits the intracellular replication of *Legionella pneumophila*, though this function is independent of its ability to inhibit apoptosis and the role of the BIR domains in this process is unclear (Diez et al., 2000; Wright et al., 2003).

NLR Effector Domains: Transactivation Domain (AD)

Amongst NLRs, the activation domain has only been identified in the founding member, NLRA. This acidic domain comprises the first 125 residues of NLRA and is required for MHC class II gene-specific transcription activation by NLRA (Chin et al., 1997; Harton and Ting, 2000). Here, the AD required interact with many proteins involved in transcriptional machinery including CREB binding protein (CBP), TFIIB, and TAFIIs (Fontes et al., 1997; Fontes et al., 1999; Kretsovali et al., 1998). Interestingly, part of the AD

resembles a CARD, but this region lacks caspase recruitment capabilities (Nickerson et al., 2001). Currently, the AD has no known involvement in initiating or regulating cell death.

NLR Nucleotide Binding Domains

Similar to APAF1, NLRs possess a large central nucleotide binding domain (NBD) which regulates activation and oligomerization of the molecule. Though APAF1 has been shown to bind adenosine-based nucleotides, the nucleotide specificity of few NLRs has been characterized (Kim et al., 2005; Zou et al., 1999). ATP binding by APAF1 is required for caspase-3 activation, thus nucleotide binding and hydrolysis are likely required for NLR activity. Indeed, the binding of ATP is necessary for NLRP3 and NLRC4-mediated cell death and inflammation. (Duncan et al., 2007; Lu et al., 2005). ATP is also preferred by NLRP12 and is required for NLRP12 to inhibit NFκB signalling (Ye et al., 2008). Not all NLRs bind adenosine-based nucleotides, NLRA requires GTP binding for its oligomerization and transactivation functions (Linhoff et al., 2001). Interestingly, some NLRs may bind nucleotides without preference. Such is the case with NLRP1, which binds nucleotide triphosphates indiscriminately while activating caspase-1. (Faustin et al., 2007). NLR reliance on nucleotide binding suggests nucleotide analogs may have use in modulating NLR cell death and inflammatory pathways and in treatment of NLR associated inflammatory disorders.

NLR Leucine Rich Repeats

The C-terminus of NLR molecules is comprised of a varying number of leucine rich repeats, which are defined by repeating units of LxxRxxL. When assembled, the individual

beta strand-turn-alpha helix units form a horseshoe shaped domain with the α-helicies arrayed outward (Kobe and Deisenhofer, 1994). Similar to the WD40 repeats found in APAF1, LRRs are thought to be involved autoregulation, ligand recognition and protein-protein interactions. In both plant R-proteins and NLRs, truncation of the LRRs can yield constitutively active molecules, suggesting the LRRs keep the NLR in an autoinhibited state (Dowds et al., 2004; Harton et al., 2002b; Tanabe et al., 2004; Tao et al., 2000; Weaver et al., 2006). Intramolecular inhibitory contacts between the WD40 repeats and NBD of APAF1 are relieved by cytochrome C. (Hu et al., 1999; Schafer and Kornbluth, 2006; Srinivasula et al., 1998). Similarly, NLR autoinhibition is presumptively disrupted upon LRR interaction with an activating stimulus. However, evidence of a direct interaction between the LRRs and pathogens or pathogen associated products is sparse. While no evidence yet supports the direct interaction of an NLR LRR and a pathogen, yeast two-hybrid experiments have detected an interaction between the LRR-like region of *Pi-ta*, a rice *R* gene, and the cognate AVR effector from the rice blast fungus *Magnaporthe grisea* (Jia et al., 2000).

1.4 Disease Associated Mutations in NLRs

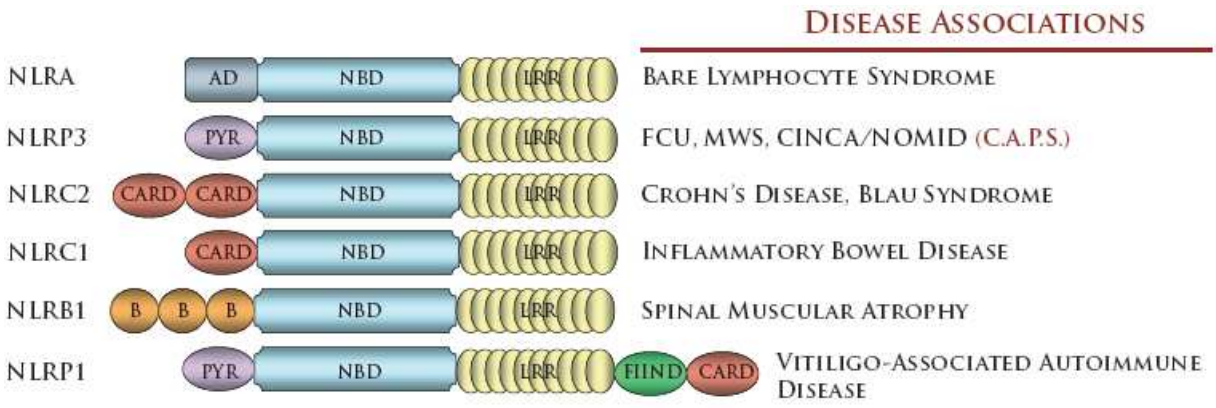


Figure 1.2 - Mutations in NLRs have been identified in several human diseases.

An understanding of the *in vivo* role of NLR proteins is aided by the remarkable association of NLR genes with human immune disorders. A summary of diseases associated with NLR mutations is shown in Figure 1.2. NLR molecules were defined based on their resemblance to NLRA. Mutations in NLRA result the absence of MHC class II expression on immune cells, a condition known as Bare Lymphocyte Syndrome (Steimle et al., 1993). Mutations in NLRP3 have been identified in a trio of dominantly inherited autoiflammatory disorders collectively referred as CAPS (CIAS1 Associated Periodic Syndromes) (Aksentijevich et al., 2002; Feldmann et al., 2002; Hoffman et al., 2001b). These disorders are characterized by fever, rash, and excessive IL-1β production (Discussed in detail, chapter 1.5, Cryopyrin Associated Periodic Syndromes). Both NLRC1 and NLRC2 have been linked to inflammatory conditions of the gastrointestinal tract. NLRC1 mutations can result in inflammatory bowel disease, while NLRC2 mutations have been identified in Crohn's Disease, a chronic condition involving intestinal inflammation.(Hugot et al., 2001; Ogura et al., 2001) Mutations in NLRC2 have also been linked to a second inflammatory condition called Blau Syndrome which is characterized by uveitis, arthritis, and skin rash (Miceli-

Richard et al., 2001). Positional cloning has established NLRB1 as a candidate gene involved in Spinal Muscular Atrophy (Roy et al., 1995). Finally, SNP analysis has identified mutations in NLRP1 which are observed in vitiligo-associated autoimmune disease, a chronic condition involving the loss of pigment in the skin (Jin et al., 2007). While future studies may yet reveal additional diseases associated NLRs, this association firmly establishes NLR molecules as critical regulators of human inflammation and immunity worthy of further investigation.

1.5 Cryopyrin Associated Periodic Syndromes (CAPS)

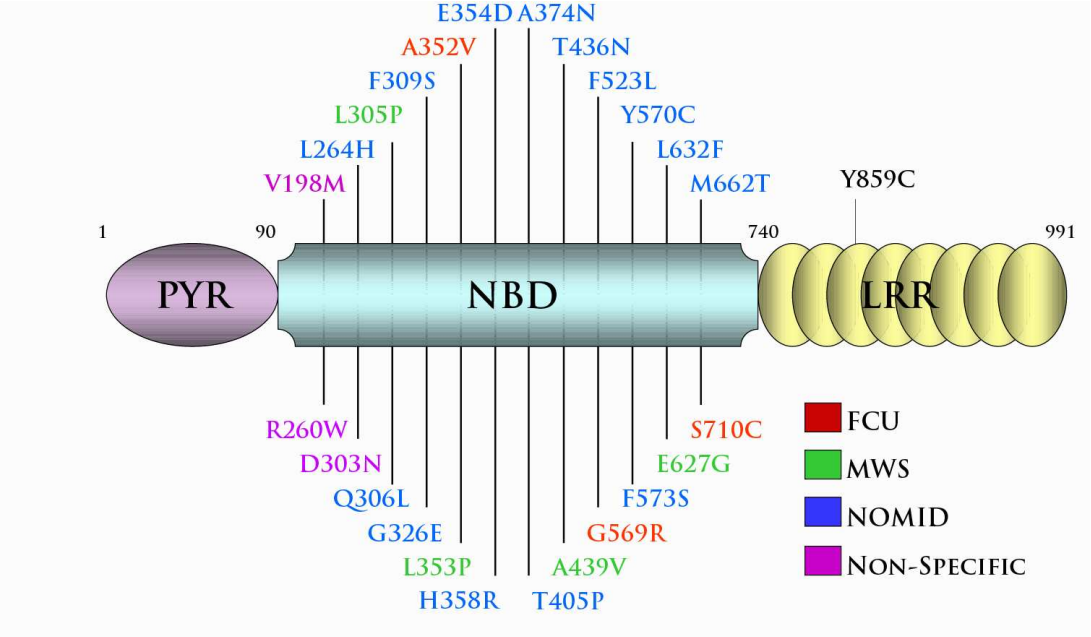


Figure 1.3- NLRP3 disease associated mutations cluster within or proximal to the nucleotide binding domain. No correlation between location of the mutation and resulting severity of disease. Few mutations span the CAPS spectrum; these are labeled as "non-specific."

Significant attention has been focused on one NLR family member, NLRP3, which is mutated in a trio of dominantly-inherited periodic fevers: FCAS, Muckle-Wells Syndrome, (Hoffman et al., 2001a), and CINCA/NOMID.(Feldmann et al., 2002) These disorders are characterized by spontaneous, yet recurrent, outbreaks of fever, rash, and the excessive production of IL-1β in the absence of high titers autoantibodies or antigen-specific T cells.

Currently, over 100 NLRP3 disease associated mutations have been identified, the vast majority of which cluster within or proximal to the NBD (Touitou et al., 2004). Interestingly, no correlation is observed between the location of the mutation and the corresponding severity disease (Fig. 1.3).

Since several *NLRP3* point mutations and clinical symptoms have been identified as overlapping between FCAS, MWS, and CINCA/NOMID, it has been proposed that these fevers comprise a continuum of disease severity collectively referred to as CAPS (Cryopyrin Associated Periodic Syndromes). A summary of symptoms associated with each disorder is presented in Table 1.2. FCAS, the mildest of the three, is characterized by recurring outbreaks of fever, urticaria rash, and conjunctivitis following generalized cold exposure. Each outbreak typically last less than 24 hours, with symptoms often peaking in the evening and resolving before morning. These attacks begin before 6 months of age and persist through adulthood (Hoffman et al., 2001b). Muckle-Wells syndrome represents an intermediate phenotype. In addition to chronic arthritis and episodic outbreaks of rash and fever which present at infancy, MWS also features AA amyloidosis and sensorineural hearing loss developing during adolescence (Muckle, 1979). Though FCAS was first described in 1940 and Muckle-Wells in 1962, it was not until 2001 that Hoffman *et al* first identified mutations in NLRP3 were responsible for both disorders (Hoffman et al., 2001a). In 2002, *Feldmann et al*. and *Aksentijevich et al*. identified NLRP3 mutations in a third autoinflammatory disorder, CINCA/NOMID (Aksentijevich et al., 2002; Feldmann et al., 2002). These patients exhibit the most severe symptoms on the CAPS spectrum and are typically diagnosed at birth with recurrent outbursts of migratory rash and fever. Headaches are often reported due to chronic aseptic meningitis. Over time, crippling joint deformities may develop as a result of massive tumor-like cartilage overgrowth.

Symptoms	FCU	MWS	NOMID
Onset	Infancy	Infancy	Infancy
Cutaneous	Cold Induced Urticaria	Spontaneous Urticaria	Spontaneous Urticaria
Auditory	Normal	Hearing Loss	Hearing Loss
Musculoskeletal	Arthralgia	Arthralgias	Deforming Arthropathy
Systemic	Fever, Chills	Fever, Amyloidosis	Fever, Chronic Aseptic Meningitis

Table 1.2 – Summary of symptoms characteristic of CAPS

To date, hyperactive formation of the inflammasome (discussed in detail, chapter 1.6, NLR Inflammasomes) has been identified as the predominant defect underlying the excessive IL-1β and inflammation associated with CAPS. On a molecular level, NLRP3 mutations likely weaken the auto-inhibitory interactions between the NBD and LRRs, thereby yielding a hyperactive form of NLRP3 more readily able to form the inflammasome (Fig. 1.4) (Aksentijevich et al., 2007). Indeed, expression of pro-IL-1 β is increased in resting monocytes from CAPS patients and these cells release more IL-1β following stimulation with LPS than mutation-negative controls (Agostini et al., 2004; Aksentijevich et al., 2002; Janssen et al., 2004). Furthermore, the remarkable response of CAPS patients to IL-1β neutralization highlights the fundamental role of IL-1β in the inflammation associated with CAPS. Daily injections of the IL-1 receptor antagonist Anakinra (Kineret) has resulted in a rapid reduction in inflammatory symptoms all along the CAPS spectrum (Goldbach-Mansky et al., 2006; Hawkins et al., 2004). However, the short half life of Anakinra has spurred interest in alternative treatments. These next generation options include a humanized IL-1β neutralizing antibody, a soluble IL-1 receptor accessory protein (IRAP), and an "IL-1 Trap," an IL-1 receptor antagonist coupled with an IRAP (Church et al., 2008; Smith et al., 2003). The use

of an oral caspase-1 inhibitor has also proven effective in blocking the activation and secretion of IL-1β in CAPS patients (Stack et al., 2005).

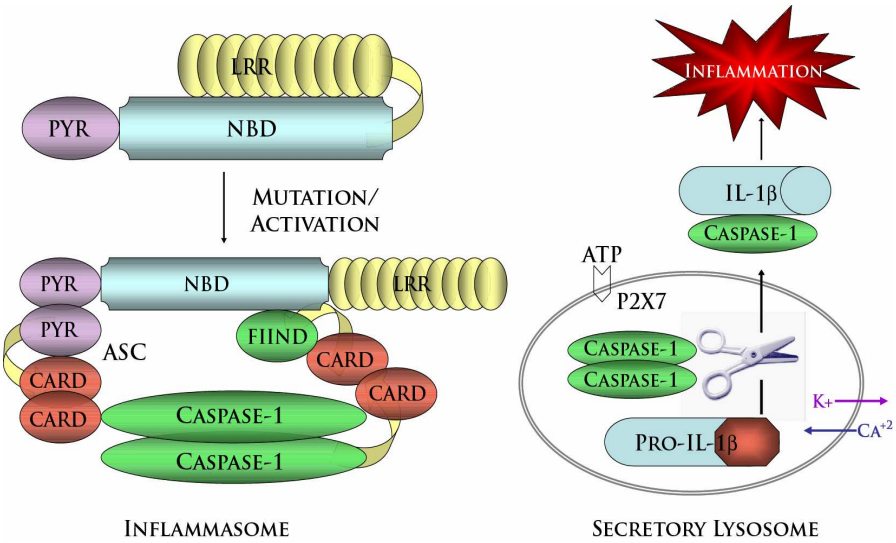


Figure 1.4 – Depiction of the NLRP3 inflammasome

1.6 NLR Inflammasomes

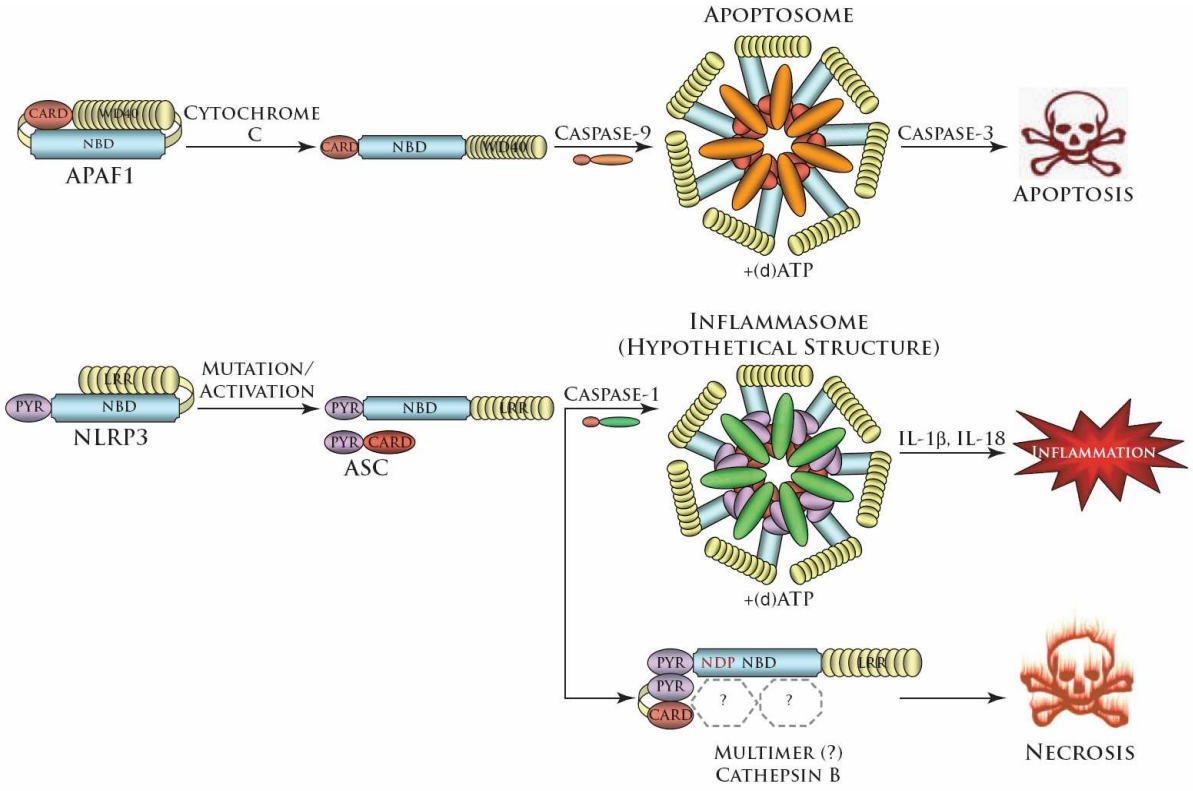


Figure 1.5 - Comparison of APAF1 and NLRP3 Cell Death Pathways. Release of cytochrome c from the mitochondria initiates apoptosome-dependent apoptosis. Cytochrome c induces a conformational change in APAF1 to relieve the intramolecular interactions holding the molecule in an auto-inhibited state. Once activated, APAF1 and cytochrome c undergo dATP dependent-oligomerization into a heptameric wheel-like structure termed the apoptosome. Pro-caspase-9 molecules aggregate through homotypic interactions between their CARDs and those of APAF1. Subsequent homodimerization of pro-caspase-9 generates active caspase-9 molecules which cleave and activate the effector caspases -3 and -7 to induce apoptotic cell death. Similarly, NLRP3 activation or disease associated mutations may weaken inhibitory intramolecular interactions between the NLRP3 NBD and its C-terminal LRRs. Utilizing the adaptors ASC and CARDINAL, activated NLRP3 aggregates caspase-1 molecules through the formation of an inflammasome complex. Complex formation potentiates subsequent caspase-1 cleavage and activation. Based on studies of the NLRP1 inflammasome, the complex is likely comprised of 5-7 subunits, each with the ability to recruit pro-caspase-1 molecules. IL-1β is activated by caspase-1 mediated cleavage of the inactive pro-IL-1 β precursor molecule. However, formation of the inflammasome is not the only function of NLRP3. Activated NLRP3 also initiates pyronecrosis, a molecular pathway of necrotic cell death which is dependent on ASC and proceeds through cathepsin B. This cell death pathway does not rely on caspase-1 or IL-1 β, and thus is independent of inflammasome function.

Due to their structural similarities, the apoptotic mediator APAF1 has been used as a model to understand NLR function. Whereas APAF1 activates a caspase-dependent program

of cell death, several NLR proteins act to promote a caspase-dependent program of inflammation. Research on the latter has mainly focused on formation of protein complexes called inflammasomes (Martinon et al., 2002; Srinivasula et al., 2002). In some respects the inflammasomes resemble the apoptosome, which includes APAF1 (Fig. 1.5). APAF1 is composed of an N-terminal CARD, a central NBD, and C-terminal WD-40 repeats. It is thought to be held inactive by intramolecular contact between its WD-40 repeats and N-terminal regions until cytochrome c and dATP relieve this inactive conformation. This enables assembly of the apoptosome, leading to activation of pro-caspase-9 (Schafer and Kornbluth, 2006). The inflammasome NLR proteins appear to act in an analogous manner, in that intramolecular interaction mediated by the C-terminal LRRs is proposed to hold these proteins in an inactive formation until stimulation promotes inflammasome assembly and activating cleavage of pro-caspase-1. (Fig 1.4)

Biochemical studies have identified a number of inflammasomes, which promote inflammation by activating caspase-1, resulting in the release of the pyrogenic cytokine IL-1β and IL-18 from cells treated with different stimuli (Agostini et al., 2004; Duncan et al., 2007) (Fig 1.6). Though differing slightly in their makeup, each inflammasome includes the IL-1β-converting enzyme pro-caspase-1, as well as one of four NLR proteins: NLRP1, NLRP2, NLRP3, or NLRC4. Given that over 20 human NLR genes have been recognized, it is likely that more NLR inflammasomes exist. A potential fourth inflammasome containing the related molecule pyrin has already been identified (Yu et al., 2005).

The inflammasome complexes appear to differ from each other in respect to their activating stimuli (Fig. 1.6). The NLRC4 inflammasome is activated in response to pathogens including *Salmonella typhimurium* and *Legionella pneumophila* (Franchi et al.,

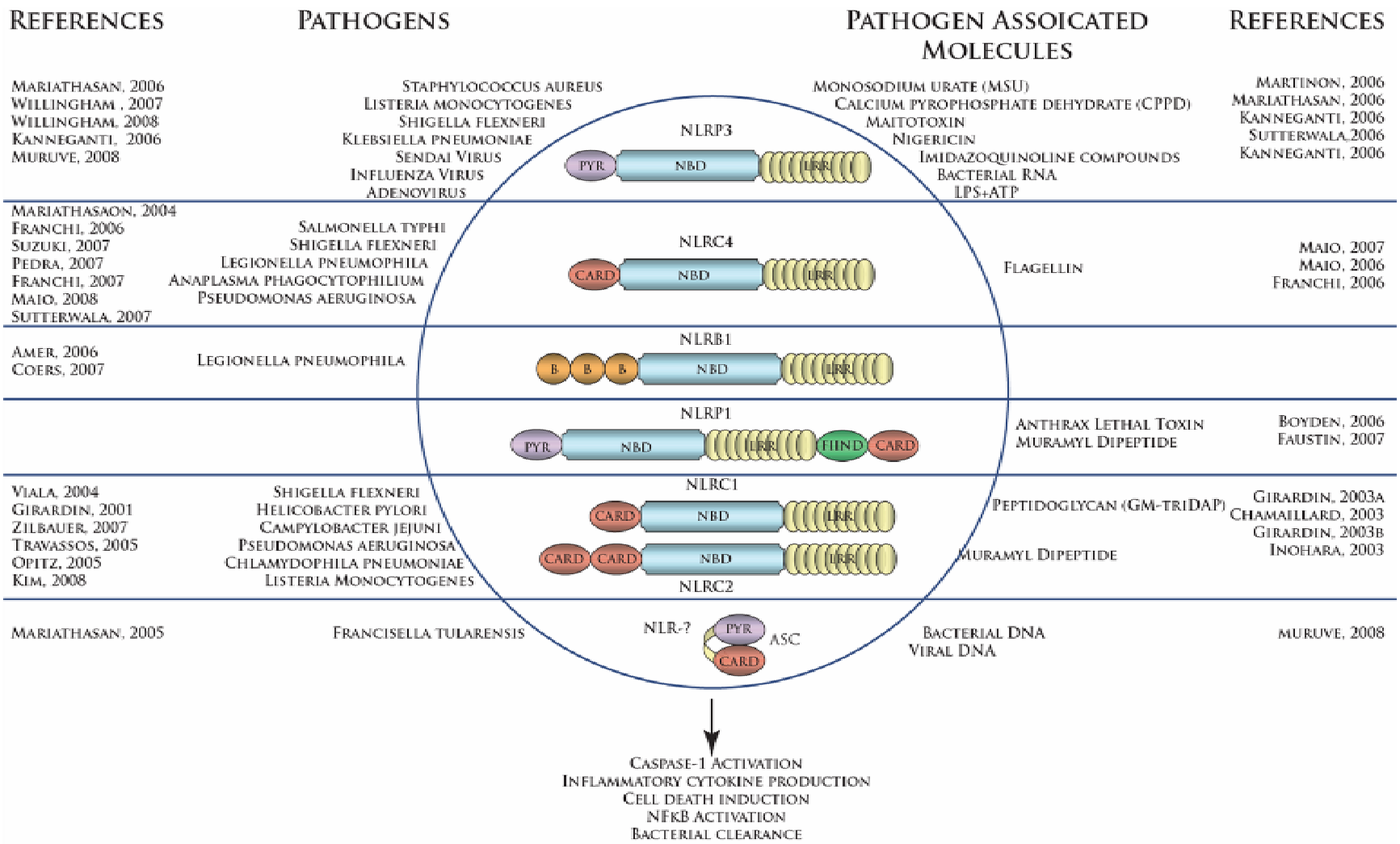
2007; Mariathasan et al., 2004; Miao et al., 2006). The NLRP1 inflammasome is required for caspase-1 activation in response to anthrax lethal toxin (Boyden and Dietrich, 2006). To date, the NLRP3 inflammasome is associated with the widest range of stimuli, including LPS in the presence of ATP, uric acid crystals, poly I:C, bacterial and viral RNA, and both gram-positive and gram-negative bacteria (Kanneganti et al., 2006a; Kanneganti et al., 2006b; Mariathasan and Monack, 2007; Mariathasan et al., 2006a; Martinon et al., 2006; Sutterwala et al., 2006; Willingham et al., 2007). Though the pathogen recognition steps leading to inflammasome activation continue to be elucidated, recent work has shown that cytosolic bacterial molecules can induce NLRP3-mediated caspase-1 activity independently of TLR signaling (Kanneganti et al., 2007) (For extensive list of activators of NLR inflammasomes, see Fig. 1.6)

Two recent papers describe biochemical stages in inflammasome activation (Agostini et al., 2004; Faustin et al., 2007). Using purified components of the NLRP1 inflammasome, Faustin *et al.* demonstrated that assembly of this inflammasome complex required the microbial product muramyl dipeptide (MDP) as well as the presence of nucleotide (Faustin et al., 2007). Surprisingly, and in contrast to the apoptosome, the NLRP1 inflammasome exhibited little nucleotide specificity. However, this does not appear to be true for the NLRP3 inflammasome. Duncan *et al.* showed that NLRP3 binds specifically to ATP or dATP and acts as an ATPase. NLRP3-catalyzed nucleotide hydrolysis was shown to be vital for NLRP3 self-association, interaction with the inflammasome adaptor protein ASC, caspase-1 activation and IL-1 release (Duncan et al., 2007).

Several CARD or pyrin domain containing proteins also regulate the various inflammasomes. Amongst the CARD regulators, COP and Iceberg inhibit inflammasome

function by interfering with the recruitment and activation of caspase-1 (Druilhe et al., 2001; Humke et al., 2000; Lee et al., 2001). The pyrin-only proteins POP1 and POP2 also inhibit inflammasome formation through their ability competitively interact with ASC, thereby blocking inflammasome formation (Bedoya et al., 2007; Lee et al., 2001). Finally, several NLR molecules also negatively regulate the inflammasomes. NLRP10 has the potential to inhibit both caspase-1-dependent IL-1β secretion as well as ASC-mediated NF-κB activation. Together, NLRP2 and NLRP7 accomplish the same function. However, NLRP7 is restricted to blocking caspase-1 activation while NLRP2 blocks ASC-mediated NF-κB activation without influencing caspase activity. (Kinoshita et al., 2005)

Figure 1.6 - Specificity of pathogens and pathogen components amongst NLRs involved in the induction of cell death and inflammation. The immune response to an ever growing number of pathogens and/or pathogen derived molecules relies in part on specific NLR molecules involved in the initiation of cell death, inflammation, and NFκB activation. In some cases, a single NLR molecule has been currently implicated in regulating a particular facet of the immune response. However, other stimuli activate several NLR molecules, creating a NLR swarm which cooperates to initiate several downstream pathways. Future studies will no doubt expand this list and reveal further cooperation amongst NLRs.


REFERENCES
PATHOGENS
PATHOGEN ASSOICATED MOLECULES
REFERENCES
MARIATHASAN, 2006
WILLINGHAM , 2007
WILLINGHAM, 2008
KANNEGANTI, 2006
MURUVE, 2008
STAPHYLOCOCCUS AUREUS
LISTERIA MONOCYTOGENES
SHIGELLA FLEXNERI
KLEBSIELLA PNEUMONIAE
SENDAI VIRUS
INFLUENZA VIRUS
ADENOVIRUS
NLRP3
PYR
NBD
LRR
MONOSODIUM URATE (MSU)
CALCIUM PYROPHOSPHATE DEHYDRATE (CPPD)
MAITOTOXIN
NIGERICIN
IMIDAZOQUINOLINE COMPOUNDS
BACTERIAL RNA
LPS+ATP
MARTINON, 2006
MARIATHASAN, 2006
KANNEGANTI, 2006
SUTTERWALA,2006
KANNEGANTI, 2006
MARIATHASAON, 2004
FRANCHI, 2006
SUZUKI, 2007
PEDRA, 2007
FRANCHI, 2007
MAIO, 2008
SUTTERWALA, 2007
SALMONELLA TYPHI
SHIGELLA FLEXNERI
LEGIONELLA PNEUMOPHILA
ANAPLASMA PHAGOCYTOPHILIUM
PSEUDOMONAS AERUGINOSA
NLRC4
CARD
NBD
LRR
FLAGELLIN
MAIO, 2007
MAIO, 2006
FRANCHI, 2006
AMER, 2006
COERS, 2007
LEGIONELLA PNEUMOPHILA
NLRB1
B
NBD
LRR
NLRP1
PYR
NBD
LRR
FIIND
CARD
ANTHRAX LETHAL TOXIN
MURAMYL DIPEPTIDE
BOYDEN, 2006
FAUSTIN, 2007
VIALA, 2004
GIRARDIN, 2001
ZILBAUER, 2007
TRAVASSOS, 2005
OPITZ, 2005
KIM, 2008
SHIGELLA FLEXNERI
HELICOBACTER PYLORI
CAMPYLOBACTER JEJUNI
PSEUDOMONAS AERUGINOSA
CHLAMYDOPHILA PNEUMONIAE
LISTERIA MONOCYTOGENES
NLRC1
NLRC2
PEPTIDOGLYCAN (GM-TRIDAP)
MURAMYL DIPEPTIDE
GIRARDIN, 2003A
CHAMAILLARD, 2003
GIRARDIN, 2003B
INOHARA, 2003
MARIATHASAN, 2005
FRANCISELLA TULARENSIS
NLR-?
PYR
ASC
CARD
BACTERIAL DNA
VIRAL DNA
MURUVE, 2008
CASPASE-1 ACTIVATION
INFLAMMATORY CYTOKINE PRODUCTION
CELL DEATH INDUCTION
NFKB ACTIVATION
BACTERIAL CLEARANCE

1.7 NLRs at the Intersection of Cell Death and Immunity

Several routes to cell death

Though cell death is known to play an important role in the immune system, the majority of studies have focused on the role of apoptosis in cell death. Emerging evidence suggests that additional cell-death pathways are crucial for triggering of inflammation and immunity. To begin to understand the contribution of cell death to immunity, it is useful to highlight a number of key differences between these types of death (Table 1.3).

Apoptosis is a programmed form of cell death, in that it is a deliberate activity on the part of the cell and requires specific molecular mediators, most importantly the apoptotic caspases. Two caspase-dependent pathways, the intrinsic and extrinsic pathways, regulate the final stages of apoptosis. The intrinsic pathway relies on the release of cytochrome *c* from mitochondria to induce formation of the apoptosome, a large protein complex comprised of cytochrome *c*, procaspase-9, APAF1, and deoxyribonucleic ATP (Zou et al., 1997). Several models have been proposed concerning the mechanisms by which apoptosome formation results in caspase-9 activation (Riedl and Salvesen, 2007). The current favored model suggests that proximity induced homodimerization of procaspase-9 within the apotosome holoenzyme creates an active site, allowing caspase-9 to become an initiator caspase that in turn cleaves and activates downstream effector caspases (caspase-3, caspase-6, and caspase-7) (Zimmermann et al., 2001). The extrinsic pathway of apoptosis begins on the cell surface, where death receptors – proteins that contain an intracellular death domain (DD) – are activated by ligand binding. Receptor-triggered intracellular events result in the proteolytic activation of initiator caspase-8 and caspase-10, leading to cleavage of effector caspases (caspase-3, caspase-6, and caspase-7) (Zimmermann et al., 2001). Substrates of the effector caspases include poly(ADP) ribose

polymerase (PARP), DNA-PK, and other regulatory and structural proteins that maintain cellular and genomic integrity (Nicholson, 1999). Cumulatively, the cleavage of these substrates leads to the death and breakdown of the cell.

In contrast to apoptosis, necrosis has been considered by some to be a passive, and therefore unprogrammed, form of cell death. In general, apoptosis relies on the protease activity of caspases, while necrosis is caspase-independent. Whereas apoptosis is an energy expensive process, necrosis has been described as bioenergetic failure, meaning that the cell lacks sufficient energy resources to maintain its metabolism. This condition may be triggered by the loss of ion pump activity or overconsumption of ATP (Zong and Thompson, 2006). At the nuclear level, necrosis is distinguished from apoptosis by the persistence of DNA content, which remains uncondensed. Perhaps the most striking difference between these forms of cell death is at the plasma membrane. Apoptosis is a slow process marked by membrane blebbing and the packaging of cellular material for recycling, but necrosis is characterized by rapid loss of plasma membrane integrity with the resultant release of cellular contents into the extracellular medium (Edinger and Thompson, 2004).

This last feature is central to the importance of necrosis in an immune and inflammatory context. Predictably, the release of cellular components has a drastic effect on the local environment. Some of these components, including uric acid, adenine phosphate, purine metabolites, and heat-shock proteins, become pro-inflammatory effectors (Zong and Thompson, 2006). Necrotic macrophages can release proinflammatory cytokines such as tumour necrosis factor (TNF) and interleukin (IL)-1 (Chen et al., 2007; Dinarello, 1996). In addition, significant attention has been paid to another protein released from necrotic cells, the nuclear DNA-binding protein HMGB1 (high-mobility group box protein 1). Once

released, HMGB1 becomes an agonist for RAGE (receptor for advanced glycosylation endproducts) and the Toll-like receptors (TLRs) TLR2 and TLR4, all of which are expressed by monocytes and some other cell types (Lotze and Tracey, 2005; Park et al., 2004). Activation of these receptors results in the exacerbation of inflammation in the microenvironment through the induction of additional pro-inflammatory cytokines (Sunden-Cullberg et al., 2006). Recent work has identified two more forms of cell death, pyroptosis and pyronecrosis, which appear to exploit the pro-inflammatory features of necrosis within the context of immunity. However, the extent to which each resembles apoptosis and necrosis is different.

Pyroptosis is a cell-death pathway activated by microbial pathogens, including *Salmonella* and *Listeria* (Brennan and Cookson, 2000; Cervantes et al., 2008). Pyroptosis is similar to apoptosis in that DNA damage occurs and the process is caspase-dependent (Fink and Cookson, 2005). However pyroptosis does not rely on the classical pro-apoptotic initiator and effector caspases (caspases 3, 8 and 9), but rather on caspase-1. In addition to its apoptotic qualities, pyroptosis exhibits some features of necrosis. Similar to necrosis, pyroptosis is characterized by plasma membrane breakdown. Moreover, mitochondrial membrane integrity is maintained during pyroptosis (Cervantes et al., 2008; Fink and Cookson, 2005). Ongoing studies are aimed at identifying additional molecular mediators of pyroptosis. Very recent work has described the pyroptosome, a large complex comprised of ASC dimers that assembles as a feature of this process (Fernandes-Alnemri et al., 2007). ASC is a common binding partner for NLR family proteins. Though it has been demonstrated that a large ASC complex can assemble *in vitro* in the absence of NLR proteins, it has also been suggested that NLRs are required for pyroptosome formation *in*

vivo (Fernandes-Alnemri et al., 2007). From an inflammation standpoint, the two outstanding features of pyroptosis are the activation of caspase-1 and the breakdown of the plasma membrane.

Two very recent studies have identified another NLR-dependent pathway of pro-inflammatory cell death, termed pyronecrosis, which has primarily necrotic features (Fujisawa et al., 2007; Willingham et al., 2007). This form of cell death is found in genetic autoinflammatory diseases involving mutations in the *NLRP3* gene and is also associated with microbial pathogens such as *Shigella flexneri* (Willingham et al., 2007). Unlike pyroptosis, pyronecrosis is caspase-independent; neither the activating cleavage of effector caspase-3 nor its substrate PARP occur during pyronecrosis, and cell death proceeds in the presence of caspase-1-specific inhibitor and pan-caspase-inhibitor (Fujisawa et al., 2007; Willingham et al., 2007). However, cell death is abrogated in the presence of an inhibitor of the lysosomal protease cathepsin B, implicating lysosome activity in the pathway (Fujisawa et al., 2007). Additional hallmarks of apoptosis are not observed. Pyronecrotic cells demonstrate neither DNA fragmentation nor the loss of mitochondrial membrane potential (Willingham et al., 2007). As determined by electron microscopy, the morphological changes characteristic of pyronecrosis are consistent with necrosis and include membrane degradation and uncondensed chromatin (Fujisawa et al., 2007; Willingham et al., 2007). Similar to classical necrosis, pyronecrosis is accompanied by release of the pro-inflammatory cytokine HMGB1 (Willingham et al., 2007) Recent work suggests an intriguing connection between cell death pathways and the NLR proteins, which are early mediators of inflammation in response to cellular insult.

	PHYSIOLOGICAL CONSEQUENCE	MORPHOLOGICAL FEATURES	BIOCHEMICAL FEATURES	ACTIVATING FACTORS	PATHOGENIC STIMULI	REFERENCES
APOPTOSIS	NON-INFLAMMATORY CELL DEATH AFFECTS LIMITED TO DYING CELL	BLEBBING OF PLASMA MEMBRANE RECYCLING OF CELLULAR CONTENTS CHROMATIN CONDENSATION	CASPASE DEPENDENT PARP CLEAVAGE MITOCHONDRIAL PERMEABILIZATION DNA LADDERING HMGB1 MAINTAINED IN NUCLEUS	CASPASE-8, -10 (EXTRINSIC) APAF-1 — INTRINSIC (APOPTOSOME) CYTOCHROME C — INTRINSIC (APOPTOSOME) CASPASE-9 — INTRINSIC (APOPTOSOME) CASPASE-3, -6, -7 (EFFECTORS)		NICHOLSON ZIMMERMAN KORNBLUTH
NECROSIS	ELICITS SUBSTANTIAL INFLAMMATION AFFECTS LOCAL ENVIRONMENT	LOSS OF PLASMA MEMBRANE INTEGRITY RELEASE OF CELLULAR CONTENTS NO CHROMATIN CONDENSATION	CASPASE INDEPENDENT NO PARP CLEAVAGE MITOCHONDRIAL SWELLING HMGB1 RELEASE FROM NUCLEUS			ZONG EDINGER
PYROPTOSIS	ELICITS SUBSTANTIAL INFLAMMATION AFFECTS LOCAL ENVIRONMENT	LOSS OF PLASMA MEMBRANE INTEGRITY MITOCHONDRIA MEMBRANE MAINTAINED NO CHROMATIN CONDENSATION	CASPASE-1 DEPENDENT ASC DIMERIZATION INTO PYROPTOSOMES	NLRC4 NAIP5 NLRP1 ASC CASPASE-1	SALMONELLA SHIGELLA LISTERIA ANTHRAX LETHAL TOXIN	BRENNAN CERVANTES FERNANDES-ALNEMRI
PYRONECROSIS	ELICITS SUBSTANTIAL INFLAMMATION AFFECTS LOCAL ENVIRONMENT	LOSS OF PLASMA MEMBRANE INTEGRITY MITOCHONDRIAL MEMBRANE MAINTAINED NO CHROMATIN CONDENSATION	REQUIRES ASC CASPASE INDEPENDENT HMGB1 RELEASE FROM NUCLEUS	NLRP3 ASC CATHEPSIN B	SHIGELLA NIGERICIN (?) MAITOTOXIN (?)	FUJISAWA WILLINGHAM MARIATHASAN SUTTERWALA

Table 1.3 – A comparison of cell death pathways

NLR proteins, apoptosis, and pyroptosis

Although a comparison between inflammasome and apoptosome activation may help to illuminate steps in the induction of inflammation, similarities extend to function as well. Much attention has been paid to the role of the NLR protein NLRB1 in determining susceptibility to *Legionella pneumophila* (Wright et al., 2003). In addition to inducing the release of IL-1β through the NLRC4 inflammasome, cytosolic *L. pneumophila* flagellin also activates a caspase-1-dependent form of cell death in macrophages which requires NLRB1 (Suzuki et al., 2007; Zamboni et al., 2006). One characteristic of this cell-death pathway is nuclear condensation, which is typical of apoptosis (Molofsky et al., 2006). Moreover, membrane blebbing, another feature of apoptosis, was observed in NLRB1-expressing HEK293 cells following infection with *L. pneumophila* (Zamboni et al., 2006). However, unlike classical apoptosis, *L. pneumophila* flagellin-induced macrophage cell death has been reported to be independent of caspase-3 activity (Molofsky et al., 2006). Thus the observed apoptotic features may instead be related to pyroptosis. Notably, caspase-1 and NLRC4-dependent cell death has been observed at timepoints of less than three hours in *Shigella flexneri* infected cells (Suzuki et al., 2007). NLRC4 is also required for cell death in *Salmonella typhimurium*-infected macrophages, which is mediated by bacterial flagellin (Franchi et al., 2007; Mariathasan et al., 2004). Though features of the mechanism remain to be determined, these results demonstrate that an NLR protein is required to mediate an apoptosis-like cell death induced by a bacterial component.

The above mentioned work suggests a pro-apoptotic function for NLR proteins. Intriguingly, evidence supporting a functional relationship between NLRs and anti-apoptotic signaling factors has been provided in another system. In a cell-free system, the NLRP1

inflammasome is activated by the bacterial product MDP, resulting in the maturation of IL-1β. This process is regulated by two members of the anti-apoptotic BCL2 (B-cell CLL/lymphoma 2) family of mitochondrial membrane proteins. Both BCL2 and $BCLX_L$ bind to NLRP1 directly to suppress its activity (Bruey et al., 2007). These data illustrate a surprising cross-talk between inflammatory and anti-apoptotic signaling, though the influence of this interaction on cell survival or death has not yet been determined. Although the regulation of inflammation is an unexpected role for BCL2 family members, the interface between mitochondrial membrane factors and innate immunity is not unprecedented. Recent work has established the mitochondrial outer membrane as a critical staging area for anti-viral signaling through MAVS (mitochondrial antiviral signaling, also called IPS-1, VISA, and CARDIF) the RIG-I (retinoic acid inducible gene-I)-like RNA helicases, and the NLR protein NLRX1 (Moore et al., 2008; Seth et al., 2005; Yoneyama and Fujita, 2007).

Additional work points to NLRP1 as a mediator for toxin-induced cell death. Boyden and Dietrich dissected mouse genetics to implicate NLRP1 as the primary mediator of mouse macrophage susceptibility to the anthrax lethal toxin (Boyden and Dietrich, 2006). In cells expressing functional NLRP1, anthrax lethal toxin elicited a form of cell death that is caspase-1-dependent (thus suggestive of pyroptosis). Without functional NLRP1, macrophages do not undergo this form of cell death and fail to activate caspase-1 in the presence of anthrax lethal toxin. These findings suggest that NLRP1 mediates macrophage cell death as a deliberate response to anthrax lethal toxin, and raise the interesting possibility that anti-apoptotic signaling factors may regulate NLRP1-induced death as well as NALP1 inflammasome activity.

NLR proteins, necrosis, and pyronecrosis

The induction of necrotic cell death as a crucial component of immunity is well established across phylogenic kingdoms. The plant NBS-LRR disease resistance proteins act in the defense against pathogens by helping to mediate the hypersensitive response, a form of rapid programmed cell death, and it has recently been shown that the NLR family protein NLRP3 mediates a similar pathway in monocytes (Fujisawa et al., 2007; Willingham et al., 2007). *NLRP3* was first identified through its association with two dominantly inherited periodic fevers: FCAS (Familial Cold Autoinflammatory Syndrome) and Muckle-Wells Syndrome (Aganna et al., 2002; Hoffman et al., 2001a). It has since been identified as the genetic locus for a third fever syndrome, CINCA/NOMID. (Aksentijevich et al., 2002; Feldmann et al., 2002). These three diseases are now considered to be a spectrum of severity for one single condition, cryopyrin-associated periodic syndrome (CAPS), which is characterized by spontaneous inflammation (Ting et al., 2006). This suggests that disease-associated variants of *NLRP3* may encode a hyperactive version of NLRP3 that promotes excessive production of IL-1β, a possibility that is consistent with the gain-of-function phenotype typically associated with dominant inheritance. Indeed, following stimulation, monocytes isolated from patients with *NLRP3* mutations demonstrate hyperactivation of IL-1β {Agostini}(Janssen et al., 2004; Stack et al., 2005).

However, this is not the extent of the phenotypic changes associated with mutant *NLRP3*. Peripheral blood mononuclear cells isolated from patients with *NLRP3* mutations lose viability when exposed to lipopolysaccharide (LPS) (Saito et al., 2008; Willingham et al., 2007). To identify more precisely the cellular consequences of mutant *NLRP3* expression, two groups developed constructs encoding known disease-associated variants of

NLRP3 (Fujisawa et al., 2007; Willingham et al., 2007). The expression of these variants in the monocytic cell line THP-1 induces excessive IL-1β release, as expected, but also an inflammatory necrosis that we termed pyronecrosis (Willingham et al., 2007). Intriguingly, pyronecrosis is not dependent on IL-1β signaling or caspase-1, though it requires the presence of the inflammasome component ASC and intact cathepsin B (Fujisawa et al., 2007; Willingham et al., 2007). The binding of ATP to NLRP3 is also necessary for this pathway to proceed (Duncan et al., 2007). Because NLRP3 and ASC appear to act together in a function that is independent of procaspase-1 activation, and hence independent of the inflammasome, we suggest that these two factors comprise an alternate complex to promote pyronecrosis (Fig. 1.5). Cumulatively, these observations offer insight into the consequence of NLRP3 hyperactivity. The inherent function of the protein and its relationship to pathogen resistance merit further consideration.

Necrosis has long been observed in monocytic cells infected with intracellular bacteria or exposed to toxins. Though in some cases pathogen-induced cell death is almost certainly passive, the active and programmed process of pyronecrosis might be a critical feature of macrophage function. Notable among the necrosis-inducing pathogens is the gram negative bacteria *Shigella flexneri*. At early timepoints, *S. flexneri*–induced macrophage cell death exhibits apoptotic and pyroptotic features (Navarre and Zychlinsky, 2000; Suzuki et al., 2007). However, *S. flexneri* induces caspase-1-dependent IL-1β release and caspase-1-independent necrosis in human monocyte-derived macrophages (Fernandez-Prada et al., 1997; Koterski et al., 2005; Suzuki et al., 2005). These characteristics closely mirror those of mutant NLRP3-induced cell death, suggesting that native NLRP3 may mediate *S. flexneri* -induced necrotic death. Through the use of knock out and knock down techniques, *S.*

flexneri-induced necrotic cell death was shown to be dependent on NLRP3 in both mouse macrophages and in the human monocytic cell line THP-1. Similar to mutant NLRP3-induced cell death, this process also depends on ASC and requires the protease cathepsin B (Willingham et al., 2007). These findings demonstrate that NLRP3 mediates pyronecrosis as part of its native function within the cell, and show that programmed necrosis can be activated in response to pathogen invasion of macrophages. We suggest that this program is an adapted response to bacterial invasion. Not only does rapid cell death deny the pathogen an environment in which to replicate, the process of necrosis is inherently pro-inflammatory, leading to release of IL-1β and other factors from surrounding cells. Thus, pyronecrosis is likely to contribute substantially to the disease state in patients with CAPS, who suffer from spontaneous inflammation characterized by IL-1β production.

In its more severe forms, CAPS is also characterized by joint deformities and arthralgias (Ting et al., 2006). NLRP3 expression is not limited to monocytic cells but extends to osteoblasts as well, and the joint-related symptoms of CAPS are likely due to excessive NLRP3 activity in these cells. As with *Shigella* in macrophages, wild type NLRP3 appears to also regulate pathogen-induced cell death in osteoblasts. Though *Salmonella typhimurium* activates the NLRC4 inflammasome in macrophages, mouse primary osteoblasts do not express NLRC4. In these cells NLRP3 is partly required for maximal *S. typhimurium*-induced cell death (McCall et al., 2008). This finding suggests that pyronecrosis may contribute to the joint-related symptoms of CAPS Moreover, it demonstrates an additional level of complexity to NLR-mediated cell death. In the absence of NLRC4, NLRP3 assumes a role in the response to *Salmonella* that it would not otherwise play.

The induction of necrosis by *Shigella, Salmonella,* and other microbial pathogens may be mediated through toxins. Some of these toxins have been examined directly with respect to caspase-1 activation and cell death. Nigericin is a toxin produced by *Streptomyces hygroscopicus*. This molecule functions as a potassium ionophore and is a potent inducer of both IL-1β release and necrosis in monocytes (Hentze et al., 2003; Perregaux et al., 1992). As with pyronecrosis, both functions are dependent on the activity of cathepsin B (Hentze et al., 2003). Another potent toxin, maitotoxin, is produced by the dinoflagellate *Gambierdiscus toxicus.* Maitotoxin has been demonstrated to induce necrosis in a manner dependent on the calcium-activated cysteine protease calpain and also promotes IL-1β release by mouse macrophages (Verhoef et al., 2004; Zhao et al., 1999). Similar to *S. flexneri*, both nigericin and maitotoxin activate the NLRP3 inflammasome (Mariathasan et al., 2006a; Sutterwala et al., 2006). Moreover, these toxins also alter the levels of intracellular potassium (Mariathasan et al., 2006a; Sutterwala et al., 2006). While molecular mediators of nigiricin and maitotoxin continue to be identified, the work outlined above indicates the participation of NLRP3 or another NLR family protein in macrophage response to these toxins.

1.8 NLRC1 and NLRC2 Function in Immunity and Inflammation

Perhaps the most well studied NLRs are NLRC1 and NLRC2 (formerly NOD1 and NOD2). In 1990, Yuan and Horvitz described a simple pathway in the nematode *C. elegans* wherein CED-4 interacts with and proteolytically activates CED-3, resulting in cell death (Yuan and Horvitz, 1990). Subsequently, a number of serine proteases, including caspase-1 and caspase-3, had been identified as human orthologs of CED-3, while no ortholog had been found for CED-4 (Fernandes-Alnemri et al., 1994; Xue et al., 1996; Yuan et al., 1993). That changed in 1997 when *Zou et al* isolated APAF1, a mammalian CED-4 homolog responsible for the cytochrome-C dependent activation of caspase-3 and cell death. (Zou et al., 1997). Upon these reports, NRLC1 was identified near simultaneously in 1999 by two groups searching for proteins resembling the CARD domain of APAF1 (Bertin et al., 1999; Inohara et al., 1999). In turn, NLRC2 identified based on genome search for proteins similar to NLRC1 (Ogura et al., 2001). Interest in NLRC1 and NLRC2 was further intensified when mutations these genes were identified in inflammatory bowel syndrome, Crohn's disease, and Blau Syndrome (Hugot et al., 2001; Miceli-Richard et al., 2001; Ogura et al., 2001). More than 90% of NLRC2 mutations identified in Crohn's disease occur within or proximal to the LRR region suggesting a crucial role of for this domain the development of disease (Lesage et al., 2002).

Unlike APAF1, neither NLRC1 nor NLRC2 induce cell death on their own, though both interact with caspase-9 to promote apoptosis when concurrently overexpressed (Bertin et al., 1999; Inohara et al., 1999). Instead, NRLC1 and NLRC2 function as cytosolic sensors which initiate NFκB signaling in response to conserved structures within the bacterial cell wall (Girardin et al., 2003a; Inohara et al., 2003). Peptidoglycan (PGN) is a major structural

component in the cell wall of Gram positive bacteria, while only existing as a thin layer in the periplasmic space of Gram negative bacteria. Hydrolases constantly degrade PGN into small glycan chains containing alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) sugars. These sugar moieties are linked together by small peptides. Importantly, the composition of gram positive and gram negative PGN differ from each other. In Gram-positive bacteria, the amino acid lysine is found at the third position in the linker peptide, whereas diaminopimelic acid (DAP) is found in most Gram-negative bacteria (Girardin et al., 2003c). Both NLRC1 and NLRC2 recognize naturally occurring PGN degradation products, albeit through distinct mechanisms. The minimal natural structure recognized by NLRC1 is GlcNAc-MurACc-LAla-c-D-Glu-*meso*-DAP (GM-triDAP) (Girardin et al., 2003a). The presence of the terminal meso-DAP implicates NLRC1 in the detection of Gram negative bacteria. In contrast, NLRC2 responds to the muropeptide GlcNAc-MurNAc-LAla-D-isoGln (GM-Di), and thus can detect both Gram-positive and Gram-negative bacteria. NLRC2 also responds to muramyl dipeptide (MurNAc-L-Ala-D-isoGln) (Girardin et al., 2003b; Inohara et al., 2003). While this is not a naturally occurring bacterial product, it also can be isolated from both Gram-negative and Gram-positive bacteria, suggesting NLRC2 may have a broad sensing range. Importantly, as is the case with all NLR-pathogen interactions, no direct binding between NLRC1 or NLRC2 and the PGN derivative has been detected.

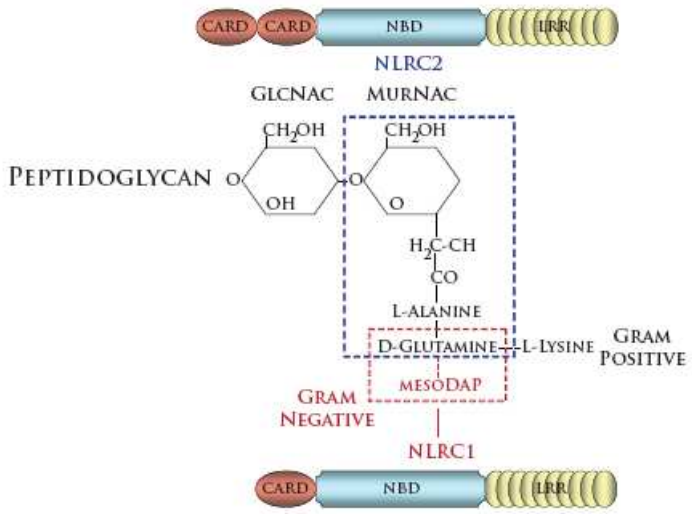


Figure 1.7 - Structure of peptidoglycan derivatives recognized by NLRC1 and NLRC2

Initial reports demonstrated that both NRLC1 and NLRC2 utilize homotypic CARD interactions to bind the serine-threonine kinase RICK. RICK interacts with IKKγ, resulting in IκB phosphorylation, and ultimately the activation of NκFB (Bertin et al., 1999; Inohara et al., 1999; Inohara et al., 2000). Indeed, RICK is essential for NLRC1 and NLRC2 activation, as NLRC1 and NLRC2-dependent NFκB activation was abolished in RICK deficient mouse embryonic fibroblasts (Kobayashi et al., 2002). Furthermore, the interaction of NLRC2, RICK, and IKK has also been shown to be essential for the activation of NFκB and JNK pathways following *Shigella flexneri* infection. Either NLRC1 or NLRC2 also been shown to mediate NFκB signaling in response to several pathogens including *Heliobacter pylori*, *Pseudomonas aeruginosa*, *Campylobacter jejuni*, *Chlamydophila pneumoniae*, and *Listeria monocytogenes* (Fig. 1.6) (Cervantes et al., 2008; Opitz et al., 2005; Travassos et al., 2005; Viala et al., 2004; Zilbauer et al., 2007).

1.9 Conclusions

Emerging evidence reveals that NLR proteins contribute to the host cell response to insult by not only facilitating the maturation of IL-1β, but also by mediating cell death (Fig. 1.5). Both processes have a strong impact on immunity. IL-1β release is a well-established signal for the onset of inflammation and initiation of the adaptive immune response. The consequences of pathogen-induced cell death in the context of immunity have not been studied as thoroughly, although a plethora of reports have now shed light on this topic. One obvious result is that invading bacteria are denied an environment in which to replicate. However, cell-death programs which result in a loss of plasma-membrane integrity can also exacerbate inflammation through the discharge of such intracellular inflammatory cytokines and factors such as IL-1β, TNFα, and HMGB1.

Two such modes of cell death, pyroptosis and pyronecrosis, have been recently identified. Though there are significant differences between the two, such as their differential dependence on caspase-1, they are evidently both pathways which respond to pathogen by promoting the inherently pro-inflammatory release of cellular contents. NLR proteins have emerged as important regulators of both of these pathways. Future studies should aim to find additional mediators of pyroptosis and pyronecrosis. Recent work has described inhibitory interaction of mitochondrial anti-apoptotic proteins with the NLR family protein NLRP1. Accordingly, pro-pyroptotic and pro-pyronecrotic factors may be found within the pool of recognized pro-apoptotic cell-death proteins. Given that the activity of the NLR proteins extends beyond caspase-1 activation to cell death, it will be interesting to see if known death regulatory factors contribute to this new role.

CHAPTER II

MICROBIAL PATHOGEN-INDUCED NECROTIC CELL DEATH MEDIATED BY CIAS1 (NLRP3) AND ASC

Portions of this chapter have been adapted from: Stephen B. Willingham, Daniel T. Bergstralh, William O'Connor, Amy C. Morrison, Debra J. Taxman, Joseph A. Duncan, Shoshana Barnoy, Malabi M. Venkatesan, Richard A. Flavell, Mohanish Deshmukh, Hal M. Hoffman, and Jenny P.-Y. Ting**.** Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyin and ASC. Cell Host & Microbe 2007 Sep 13;2(3):147-59.

2.1 Abstract

Cryopyrin/CIAS1/NLRP3 and ASC are important components of the inflammasome, a multi-protein complex required for caspase-1 activation and cytokine IL-1β production. *CIAS1* mutations underlie autoinflammation characterized by excessive IL-1β secretion. Disease-associated cryopyrin also causes a program of necrosis-like cell death in macrophages, the mechanistic details of which are unknown. We find that patient monocytes carrying disease-associated *CIAS1* mutations exhibit excessive necrosis-like cell death by a process dependent on ASC and cathepsin B, resulting in spillage of the proinflammatory mediator HMGB1. *Shigella flexneri* infection causes cryopyrin-dependent macrophage necrosis with features similar to the death caused by mutant *CIAS1*. This necrotic death is independent of caspase-1 and IL-1β, and thus independent of the inflammasome. Furthermore, necrosis of primary macrophages requires the presence of *Shigella* virulence genes. While similar proteins mediate pathogen-induced cell death in plants, this report identifies cryopyrin as an important host regulator of pathogen-induced necrosis in animals, a process we term pyronecrosis.

2.2 Introduction

The NLR family (Harton et al., 2002a) (formerly CATERPILLER) is comprised of proteins involved in the regulation of innate immunity (Inohara and Nunez, 2003; Martinon and Tschopp, 2005). Functionally similar to the evolutionarily conserved Toll-like receptors (TLRs), increasing evidence suggests that NLRs may serve as intracellular molecules that sense pathogen-derived products (Hoffmann and Reichhart, 2002; Poltorak et al., 1998). Significant attention has been focused one NLR family member, Cryopyrin, which is encoded by the gene *CIAS1*. *CIAS1* is mutated in a trio of dominantly inherited periodic fevers: FCAS (Familial Cold Autoinflammatory Syndrome), MWS (Muckle-Wells Syndrome), and CINCA/NOMID (Chronic Infantile Neurological Cutaneous and Articular syndrome / Neonatal Onset Multisystemic Autoinflammatory Disease), which are proposed to represent a continuum of severity for a single condition, *CIAS1*-associated periodic syndrome (CAPS) (Aksentijevich et al., 2002; Feldmann et al., 2002; Hoffman et al., 2001a; Hoffman et al., 2001b).

Recent investigations have highlighted an essential role for IL-1β in the development of mutant-*CIAS1*-associated periodic fevers. Mutant *CIAS1* causes elevated levels of spontaneous and induced IL-1β both *in vitro* and *in vivo*. Indeed, FCAS, MWS, and CINCA/NOMID have all been successfully treated with daily doses of the IL-1β receptor antagonist Anakinra® (Kineret) (Goldbach-Mansky et al., 2006; Hawkins et al., 2004; Hoffman et al., 2004). Cryopyrin participates in the regulation of IL-1β through involvement in a multimolecular complex called the inflammasome (Agostini et al., 2004). This complex, which also includes ASC (Apoptotic Speck protein containing a CARD) and TUCAN, promotes activation of caspase-1/ICE. In turn, caspase-1 then cleaves pro-IL-1β to produce

mature IL-1β, which is released from the cell. Mutations in cryopyrin result in the hyperactivation of this pathway, causing excessive IL-1β production and the severe episodes of inflammation.

The functions of cryopyrin in the immune system are not limited to autoinflammatory disorders. Several recent reports have established cryopyrin as an important adaptor capable of organizing the inflammasome to elicit IL-1β release in response to bacterial, viral, and other pro-inflammatory stimuli (Kanneganti et al., 2006a; Kanneganti et al., 2006b; Mariathasan et al., 2006a; Mariathasan et al., 2006b; Martinon et al., 2006; Sutterwala et al., 2006). However, it is not yet known if the protein has additional biologic functions in the containment of pathogens.

Clues regarding an additional role for cryopyrin in response to pathogen may lie within its makeup. Cryopyrin consists of an amino terminal pyrin domain, a central NACHT (NAIP, CIITA, HET-E, TP1) domain, and seven carboxy terminal LRRs (Leucine Rich Repeats). This architecture is conserved in plants, where similar proteins comprise a subfamily of disease-resistant (R) proteins called NB-LRRs (Ausubel, 2005; Chisholm et al., 2006). The NB-LRR proteins respond to microbial pathogen by eliciting a hypersensitive death response in infected cells, thus resulting in elimination of the pathogen (Greenberg et al., 1994; Nimchuk et al., 2003). Similarly, one mammalian host response to microbial pathogen is macrophage/monocyte necrotic-like death, which can lead to pathogen elimination, but also to exacerbated inflammation and sepsis (Krysko et al., 2006). The participation of cryopyrin in initiating necrosis has been hinted at previously, as cryopyrin deficient macrophages demonstrate reduced levels of cell death in response to the gram positive *Staphylococcus* bacteria (Mariathasan et al., 2006a). However, the molecular players

that mediate such a process and the mechanism of this form of cell death have yet been defined.

We report here that cryopyrin and ASC are required for a process of necrotic-like cell death. We furthermore expand the capabilities of cryopyrin by demonstrating that it mediates both the IL-1β and cell death response to a gram-negative bacterium, *S. flexneri*, resulting in cellular necrosis and the exacerbation of inflammation. The observation that *Shigella*-induced cell death is independent of caspase-1 and IL-1β indicates that this process occurs independently of inflammasome formation. It further suggests that disease-associated cryopyrin represents a hyperactive form of the protein, while the function of the normal counterpart is to induce cell death only upon stimulation with bacteria or other pathogens.

2.3 Materials & Methods

Cell lines and reagents – THP-1 cells purchased from American Type Culture Collection (ATCC) and cultured as described previously (Williams et al., 2005). Anti-caspase-3 antibody purchased from Cell Signaling; anti-PARP, anti-ipaB, anti-Actin, and HRP-conjugated secondary antibodies from Santa Cruz Biotechnology; anti-HMGB1 antibody from Abcam; anti-ASC antibody from Immuno Diagnostic Oy; anti-Cryopyrin antibody from Alexis Biochemicals; Super Signal ECL reagent from Biorad; E. Coli LPS from Chemicon. Detailed methods for preparation of retroviral shuttle vectors, transduction, and sorting to generate THP-1 cell lines stably expressing shRNA have been described (Taxman et al., 2006). The shRNA target sequences are as follows: shASC-GCTCTTCAGTTTCACACCA, shCtrl-GCTCTTCctggcCACACCA, shCIAS-GGATGAACCTGTTCCAAAA. Stable expression of shRNA did not induce interferon response as assessed by OAS1 expression (not shown).

Generation of recombinant adenoviruses - Recombinant adenovirus expressing *CIAS1* or *LacZ* was generated using Adeno-X Expression System (Clontech). Briefly, genes were subcloned into pShuttle2 intermediate vector and ligated into the modified Type 5 human adenoviral genome vector Adeno-X following excision with PI-*Sce* and I-*Ceu* enzymes. Recombinant adenovirus was then amplified in HEK293 cells and purified using Adeno-X Virus Purification Kit (Clontech). Viral titers were determined by UNC Viral Vector Core Facility (UNC-Chapel Hill).

Adenovirus transduction of THP-1 cells – THP-1 cells were aliquoted into Falcon 2059 polypropylene tubes at a density of 10^6/ml in RPMI 1640 containing 10% FBS. After addition of adenovirus (MOI=1 unless otherwise noted), cells were centrifuged at 2000*g for 2 hours at 37^O C. Immediately after centrifugation, cells were resuspended and incubated at $5*10^5$/ml following the addition of fresh RPMI 1640 containing 10% FBS.

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XTT assay – Cells were plated into 96-well plates at 20,000 cells per well 24 hours after adenovirus transduction. 50 µl of serum-free media containing 25µM phenazine methosulfate and 1mg/ml XTT was added to each well. Plates were read at 450nM after four hours of incubation.

Mitochondrial membrane potential staining – Cells were stained with Tetramethylrhodamine ethyl ester, perchlorate (TMRE) for 25 minutes at 37°C at a final concentration of 5nM. After staining, cells were rinsed in PBS, resuspended in 0.5 ml PBS and analyzed using a FACScan (Becton Dickinson) in FL2.

Immunoblotting – Immunoblots were performed as described previously (Williams et al., 2005). Cryopyrin was immunoprecipitated with rabbit anti-CIAS-1 peptide IgG. Expression was confirmed by probing immunoblots with anti-CIAS1. HMGB1 blots were performed directly on culture supernatants and developed as indicated.

Quantitative PCR - Total RNA was isolated, cDNA was reversed transcribed, and quantitative PCR was performed using Absolute SYBR green mix (ABgene, UK) to assess

ASC mRNA expression as described (Taxman et al., 2006). Realtime values were standardized to the expression of 18s rRNA and normalized to 100 in control (untransduced) cells. Primers used for real-time PCR are as follows: ASC-[AACCCAAGCAAGATGCGGAAG, TTAGGGCCTGGAGGAGCAAG], 18s-[CGGCTACCACATCCAAGG, GCTGCTGGCACCAGACTT].

Viaprobe and 7-AAD cell staining – Cells were collected and rinsed twice in cold PBS. Pellets were resuspended in 0.5ml PBS with 3 µl Viaprobe (Becton Dickinson) or 1 µl 7-AAD (BD Pharmingen). Cells were incubated in the dark for 15 minutes before analysis on a FACScan (BD).

Propidium Iodide staining – Following treatment, cells were collected and pelleted via centrifugation. Pelleted cells were fixed in 70% ethanol for a minimum of 2 hours, rinsed once in PBS, then resuspended in PBS containing 1% Triton, 20 µg/ml propidium iodide (Sigma Chemical Co.), and 200 µg/ml RNAse A (Qiagen). The cells were allowed to stain for 15 minutes or longer then analyzed using a FACScan (BD).

ELISA – THP1 Samples were harvested 24 hours post transduction and assayed with BD OptEIA Human IL-1β ELISA Set (BD Biosciences) and Human IL-18 ELISA (MBL International). Mouse samples were taken at indicated times post infection and assayed with BD OptEIA Mouse IL-1β ELISA Set (BD Biosciences).

Electron microscopy – THP-1 cells were infected with adenoviruses at MOI=1 and fixed in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.15M sodium phosphate, pH 7.4 2 or 6 hours post infection. Electron microscopy was performed at the UNC Microscopy Services Laboratory.

Patient cells – Two female patients (ages 62 and 71) with FCAS were included in the study. Both FCAS patients had classic clinical presentation and met diagnostic criteria, as described previously (Hoffman et al., 2001b). Neither FCAS subject was experiencing significant inflammatory symptoms at the time of study, nor on regular anti-inflammatory medications. Two female controls (ages 39 and 41) were studied simultaneously with the FCAS patients. However, one control was later found to have significantly abnormal inflammatory responses and therefore was not included. An additional 4 male controls aged 27-34 were subsequently studied. PBMCs were isolated and prepared as previously described (Stack et al., 2005).

***CIAS1* and caspase-1 deficient mice -** *CIAS1*$^{-/-}$ and Caspase-1$^{-/-}$ mice were described previously and were respectively produced by Millenium Inc. and Dr. Richard Flavell, Yale University (Sutterwala et al., 2006). They were backcrossed for a minimum of six generations to C57BL/6**.** Macrophages were obtained by peritoneal lavage 5 days after intraperitoneal injection with 4% thioglycollate and cultured in DMEM supplemented with 10% fetal calf serum and 50 ug/ml penicillin and streptomycin. Bone marrow macrophages were harvested from 6-8 week old mice and cultured for 7 days in 30% M-CSF conditioned media.

Bacterial infections – *Shigella flexneri* strain 12022 was obtained from ATCC. 2457T and BS103 have been described previously (Fernandez-Prada et al., 1997). THP-1 cells were cultured at 10^6/ml in antibiotic free RPMI. All samples were infected with *S. flexneri or S. typhi* bacteria at a MOI of 50 at 37^o for the indicated amount of time. Samples were centrifuged at 650*g for 10 minutes immediately following addition of bacteria. 50 □g/ml gentamicin was added to cultures 2 hours post infection.

2.4 Results

Expression of disease-associated *CIAS1* mutants induces a necrotic-like cell death

Mutations in *CIAS1* are associated with the periodic fever syndromes FCAS, MWS, and CINCA/NOMID. Adenoviral constructs were transduced at a MOI=1 to promote efficient exogenous expression of wild type *CIAS1* or *CIAS1* containing mutations encoding the disease-associated amino acid changes A439V or R260W (Fig. 2.8A). A fourth construct encoding *LacZ* was designed as a negative control. Expression of the disease-associated mutants dramatically decreased cell viability in the THP-1 monocytic cell line in three separate assays: the XTT assay (Fig. 2.1A), trypan blue (Fig. 2.8B) and Viaprobe (Fig. 2.8C). Staurosporine was used to induce apoptosis in all of these assays. To determine the mode of cryopyrin-induced cell death, we examined the activation of caspase-3. During apoptosis, caspase-3 undergoes activating cleavage. In turn, caspase-3 cleaves PARP and other downstream substrates. Neither caspase-3 nor PARP were cleaved in cells expressing a disease-associated mutant cryopyrin, though both were cleaved in staurosporine-treated cells (Fig. 2.1B). Further, pretreatment of cells with the pan-caspase inhibitor (zVAD-fmk) failed to abrogate cell death (Fig. 2.1C). These results indicate that mutant cryopyrin-induced cell death does not require nor proceed *via* caspase activation. DNA fragmentation, another hallmark of apoptosis, was not observed in mutant cryopyrin expressing cells (Fig. 2.1D), though the positive control, staurosporine, induced DNA fragmentation in a caspase-dependent manner (Fig. 2.1D and Fig. 2.9A). Moreover, in contrast to apoptotic cells, mutant-cryopyrin expressing cells did not demonstrate an increase in mitochondrial membrane permeability at two timepoints (summarized in Fig. 2.1E, and shown in detail in Fig. 2.9B). Finally, electron microscopy shows that mutant-cryopyrin expressing cells

exhibit morphological features consistent with necrosis. Cells expressing mutant cryopyrin demonstrate several of these features: a) degradation of the plasma membrane, b) dysmorphic/swollen mitochondria, and c) lack of chromatin condensation (Fig 2.1F, middle panel). Staurosporine caused a typical apoptotic morphology (Fig. 2.1F, right panel). Taken together, our results support previous data indicating that disease-associated variants of cryopyrin induce cell death consistent with necrosis (Fujisawa et al., 2006).

Disease-associated cryopyrin mutants induce enhanced IL-1β release, but cell death is independent of caspase-1 and IL-1β signaling

CIAS1-associated periodic fevers are characterized by excessive IL-1β production. To explore the mechanism by which disease-associated cryopyrin causes cell death, we first determined if this process is dependent on caspase-1 or IL-1β. In agreement with previous observations, substantially more IL-1β was released from cells expressing mutant cryopyrin than cells expressing wild-type cryopyrin (Fig. 2.2A) (Agostini et al., 2004; Dowds et al., 2004). IL-18 is also regulated by caspase-1 and levels of IL-18 are greatly induced by disease-associated cryopyrin (Fig. 2.2B). Treatment with YVAD-CHO, a specific peptide inhibitor of caspase-1 abrogated mutant cryopyrin-induced release of IL-1β and IL-18 (Figs. 2.2A and 2.2B.). However, while YVAD-CHO successfully blocked IL-1β and IL18, mutant-cryopyrin induced cell death was unaffected (Fig. 2.2C). Kineret® (Anakinra), an IL-1 receptor antagonist (IL-1Ra) also failed to diminish mutant-cryopyrin induced cell death (Fig. 2.2D). To assure the concentration of Kineret® was adequate to block the biologic function of IL-1β, we measured its effect on IL-1β -mediated induction of IL-8 (Fig. 2.2E). Even at a concentration a log lower than that used in the cell viability assay (Fig. 2.2D), the

induction of IL-8 by IL-1β was abolished by Kineret®. Together, these results demonstrate that mutant cryopyrin-induced cell death occurs independently of caspase-1 activity and IL-1 β mediated signaling. As previously reported, inhibition of cathepsin B with 50 μM Ca-074-Me substantially blocked cell death caused by disease-associated cryopyrin, but had no effect on staurosporine-induced death (Fujisawa et al., 2006). Of note, Fig. 2.1C shows that the pan-caspase inhibitor caused a slight reversal of disease variant cryopyrin-mediated cell death. This slight improvement in viability may be attributed to cross inhibition of cathepsin B by the zVAD peptide (Schotte et al., 1999).

Disease-associated mutant cryopyrin induced cell death is ASC dependent

To further explore the mechanism by which disease-associated *CIAS1* causes cell death, the role of ASC was examined. Short hairpin RNA molecules (shRNAs) were designed to promote the degradation of ASC mRNA (shASC). A control shRNA with a mutated target ASC sequence was also prepared (shCtrl). These shRNAs were incorporated into retrovirus and stably transduced into THP-1 cells, resulting in stable reduction of both ASC protein and mRNA (Figs. 2.3A and 2.3B). A second ASC shRNA generated the same results (not shown). Consistent with a role for ASC in the inflammasome, shASC diminished spontaneous production of IL-1β induced by wildtype and mutant cryopyrin (Fig. 2.3C). More importantly, shASC reverted cell death induced by the A439V disease-associated cryopyrin mutant (Fig. 2.3D). These results demonstrate that ASC is required for mutant cryopyrin-induced cell death as well as IL-1β production in monocytic/macrophage cell types.

Disease-associated cryopyrin mutants induce HMGB1 release

HMGB1 is emerging as an important therapeutic target for sepsis, cancer, and other conditions. Normally maintained as a nuclear factor within the healthy cell, HMGB1 takes on the role of a strong pro-inflammatory factor when released from cells undergoing necrosis (Scaffidi et al., 2002). This prompted us to examine the release of HMGB1 in the presence of disease-associated cryopyrin. As measured by western analysis, HMGB1 release from cells transduced with the wildtype *CIAS1*-adenovirus is barely detectable, but a high level is released by cells expressing either of two disease-associated forms of *CIAS1* (Fig. 2.4A). Though ASC is essential for HMGB1 release (Fig. 2.4B), caspase-1 activity is not (Fig. 2.4C). HMGB1 release following the induction of apoptosis with staurosporine is not observed at the 6 hr timepoint, but is only observed 24 hr post-treatment. This is likely the consequence of secondary necrosis caused by longer treatment time (Fig. 2.9C). Collectively, the results presented in Figs. 2.3 and 2.4 suggest that mutant cryopyrin causes necrotic-like cell death and subsequent HMGB1 release in an ASC-dependent but caspase-1 independent fashion.

LPS induces death of FCAS patient cells.

We next sought to determine the effects of *CIAS1* mutation on cell viability in peripheral blood mononuclear cells (PBMCs) from FCAS patients with confirmed *CIAS1* mutations. Samples were obtained from patients with *CIAS1* mutations who have not undergone anti-inflammatory treatment. The endotoxin lipopolysaccharide (LPS) has been used by others to induce monocytic cell death (Karahashi and Amano, 1998). LPS is also known to induce *CIAS1* mRNA and protein expression, both of which are very low in resting

mononuclear cells (O'Connor et al., 2003). LPS challenge resulted in a dose-dependent decrease in cell viability in patient PBMCs but not healthy controls, supporting the conclusions made from exogenous expression of disease-associated cryopyrin (Fig. 2.5A). As disease-associated cryopyrin variants are generally accepted as gain-of-function mutants, properties observed with disease-associated cryopyrin are expected to be observed with wildtype cryopyrin, either at a reduced level or under stimulated conditions. While adenovirus with disease-associated CIAS1 caused substantial cell death at MOI=1, transduction of adenovirus containing wildtype cryopyrin into THP-1 cells also resulted in cell death when a higher MOI was used, (Fig. 2.5B). These findings prompted us to determine whether wildtype cryopyrin plays a role in cell necrosis associated with bacterial pathogenesis.

***Shigella flexneri* induced cell death requires cryopyrin and ASC, but not caspase-1.**

Necrosis of monocytes and macrophages is a documented response to pathogenic bacteria, although proteins that control this process are not well defined (Golstein and Kroemer, 2007; Zong and Thompson, 2006) Significant evidence indicates *Shigella* causes a necrotic-like cell death, although apoptosis has also been reported (Koterski et al., 2005; Nonaka et al., 2003; Suzuki et al., 2005; Zychlinsky and Sansonetti, 1997). This led us to examine the potential of wildtype cryopyrin to mediate necrosis in response to *Shigella flexneri*. Stable reduction of cryopyrin protein in THP-1 cells was achieved utilizing retroviral- transduced shRNAs specific for *CIAS1* which caused a near-ablation of targeted gene expression (Fig. 2.6A). The induction of IL-1β by *S. flexneri* was nearly abolished in cryopyrin-deficient cells as measured by ELISA, providing a biologic assay to assure that the

CIAS1 shRNA caused the intended biologic effect (Fig. 2.6B). More importantly, *S. flexneri*-induced death was substantially abrogated in shCIAS1 cells (Fig. 2.6C). The presence of shCIAS1 did not affect staurosporine-induced cell death, indicating specificity of cryopyrin for *S. flexneri*-induced death. To confirm that cryopryin mediated cell death requires ASC, we tested the ability of *Shigella* to elicit cell death and IL-1β in ASC deficient THP1 cells. As expected, both cell death and IL-1β release were substantially abrogated in the shASC cells (Fig. 2.6D and 2.6E).

To examine the physiologic importance of these results, we utilized macrophages isolated from wildtype and *CIAS1* gene-ablated mice. In wildtype bone-marrow derived macrophages, *Shigella* caused a 2.5-3 fold increase in cell death compared to uninfected macrophages. However, in *CIAS1*$^{-/-}$ macrophages, *Shigella* failed to initiate cell death above the level of uninfected cells (Fig. 2.6F). Moreover, the level of IL-1β secretion was reduced approximately 15-fold in peritoneal macrophages from *CIAS1*$^{-/-}$ mice (Fig. 2.6G). The requirement of *CIAS1* for both cell death and IL-1β release in response to *S. flexneri* establishes wildtype cryopyrin as a critical host adaptor capable of responding to a gram negative bacterial pathogen. In contrast, bone marrow-derived macrophages isolated from mice lacking caspase-1 demonstrated no differential cell death response to *S. flexneri* (Fig. 2.6H), though caspase-1 remained essential for IL-1β activation (Fig. 2.6I). These results indicate that cryopyrin and ASC, but not caspase-1, are required for *S. flexneri*-induced macrophage necrosis.

Cryopyrin-dependent cell death is not observed in cells infected with another intracellular bacteria, *Salmonella typhi,* thus indicating that the role of cryopyrin cannot be generalized to all intracellular bacteria (Fig. 2.10) (Mariathasan et al., 2006a). To assess if

cryopyrin-induced cell death is caused by virulence factors expressed by *S. flexneri*, we compared avirulent, plasmid-cured *S. flexneri* (BS103) and the virulent parental strain (2457T). The former lacks a 230-kb virulence plasmid which encodes the invasion plasmid antigens IpaB, IpaC, and IpaD. These antigens are essential for *S. flexneri* virulence and entrance into the host cell (Menard et al., 1993). Immunoblot analysis was used to confirm the plasmid-cured *Shigella* strain lacks the IpaB protein, while this protein is detected in the parental strain (Fig 2.6J, left panel, inset). Cell death was assayed at two (2 and 4 hr) different time points. At both timepoints, virulent *Shigella* (2457T) caused cell death, and this process was reduced in macrophages lacking the *CIAS1* gene (Fig 2.6J, right and left panels). As expected, when macrophages were infected with the plasmid-cured BS103 strain, cell death is reduced by >80%. This residual level of cell death was not affected by the absence of CIAS1. These results indicate that virulence factors expressed by *S. flexneri* causes *CIAS1*-dependent cell death.

Cryopyrin mediates *Shigella* induced necrotic-like cell death with properties similar to mutant cryopyrin-induced death.

Having established that cryopyrin was essential for *Shigella*-mediated cell death, we sought to determine the nature of cell death and whether it was consistent with the necrotic-like death induced by mutant cryopyrin. Infection of shCTRL THP-1 cells with *S. flexneri* for six hours resulted in cell death with features morphologically consistent with necrosis (Fig. 2.7A, compare Panels i and ii). To verify that the necrotic cells contain bacteria, we performed electron microscopy at an earlier timepoint (2 hours after infection) when the cell morphology is less fragmented. Cells with a negative control shRNA that are infected with

bacteria demonstrate the initial loss of cytoplasm, indicating necrosis (Fig. 2.7A, Panel iii). In contrast, cellular morphology consistent with cell death was not observed in shCIAS1 cells, despite the presence of several intracellular *Shigella* bacteria (Fig. 2.7A, Panel iv). Next, we examined biochemical properties of cryopyrin initiated IL-1β and cell death response to *Shigella*. In contrast to the apoptotic control staurosporine, *Shigella*-induced cell death did not result in the cleavage of PARP after 6 hours (Fig. 2.7B). Earlier reports have suggested that *S. flexneri* induces apoptosis in macrophages at early timepoints, while necrosis is observed at later points (Zychlinsky et al., 1992; Koterski et al., 2005). We observed that treatment with the pan-caspase inhibitor zVAD slightly diminished cell death after 2 hours of infection, but not after 6 hours of infection. The caspase-1 specific inhibitor YVAD had no effect. Attempts to inhibit cell death and IL-1β release with glycine, previously shown to reduce *Shigella*-induced apoptosis, were also ineffective (Fig. 2.11) (Edgeworth et al., 2002). However, the cathepsin B inhibitor Ca-074-Me substantially blocked cell death, further validating that mutant cryopyrin and *Shigella*-induced necrosis occurred via the same pathway (Fig. 2.7C). Although the caspase-1 inhibitor YVAD failed to block cell death, it substantially abrogated both IL-1β and IL-18 in response to *Shigella* in shCTRL cells, indicating that cell death is predominantly caspase-1, IL-1β, and IL-18 independent (Fig. 2.7D and 2.7E). As expected, shCIAS1 nearly abolished both IL-1β and IL-18. Finally, *Shigella*-mediated cell death was associated with the release of HMGB1 (Fig. 2.7F). In contrast, treatment with apoptosis inducing staurosporine for 2 and 6 hrs did not cause HMGB1 release.

Thus *S. flexneri*-induced death shared multiple characteristics associated with the necrotic-like cell death observed with disease-associated cryopyrin, suggesting that this

process is mediated by cryopyrin and ASC, proceeds through cathepsin B independent of either caspase-1 or IL-1β, resulting in HMGB1 release.

2.5 Discussion

We report a necrotic-like cell death caused by disease-associated mutants of *CIAS1* which is dependent on ASC but not on caspase-1 or IL-1β. This process results in release of the proinflammatory mediator, HMGB1, which likely propagates the inflammatory response. Additionally, we show that ASC and native, wildtype cryopyrin are required for *S. flexneri*-induced cell death, which proceeds in a manner identical to that induced by disease-associated cryopyrin. These results implicate cryopyrin as a crucial regulator of pathogen-induced necrotic-like death, a process proposed to play an important role in the pathogenesis of a myriad of infectious diseases, which we propose to call pyronecrosis.

From the perspective of cryopyrin-associated periodic fever, these results and the accumulated data in the literature suggest that cryopyrin mutants directly regulate disease progression through at least two distinct signaling pathways. It is known that cryopyrin participates in the activation of caspase-1, the maturation of IL-1β, and subsequent hyperactivation of the inflammatory process *via* an ASC-dependent process (Agostini et al., 2004). Previous reports have indicated that disease-associated mutants demonstrate a gain-of-function phenotype with respect to these properties leading to enhanced IL-1β release (Dowds et al., 2004). We report a second gain-of-function, the induction of necrotic-like cell death. The manner of death is particularly important. While it is generally believed that apoptotic cells die an orderly death with minimal impact on inflammation, necrosis involves the spilling of cellular contents into the environment, intensifying local inflammation and damaging neighboring cells (Krysko et al., 2006). It is important to keep in mind that the IL-1 receptor antagonist Anakinra® has been successfully used to treat patients suffering from the *CIAS1* associated periodic syndrome, indicating that excessive IL-1β production

underlies disease. This would seem to suggest that the inflammasome function of cryopyrin is solely responsible for periodic fever in these patients. However, given that necrosis allows for the release of pro-inflammatory factors such as HMGB1, which in turn promote further release of IL-1β, it is likely that cryopyrin-mediated cell death also contributes to disease-state in patients.

Both disease-associated cryopyrin expression and *S. flexneri* infection triggered the release of HMGB1, a chromatin-associated protein released by necrotic cells. Once HMGB1 is released, it acts as a potent danger indicator, inducing several pro-inflammatory cytokines by signaling through the RAGE, TLR2, and TLR4 receptors to elicit a severe inflammatory response (Andersson et al., 2000; Hori et al., 1995; Park et al., 2004). Serum HMGB1 is increased during both endotoxin exposure in mice, and in septic patients who succumbed to infection (Wang et al., 1999). HMGB1 neutralization has been shown to significantly reduce inflammation and improve survival in animal models of established sepsis (Yang et al., 2004). The release of HMGB1 elicited by bacterial-induced cell death further supports the use of HMGB1 antagonists to reduce inflammation during sepsis.

The results shown here demonstrate a requirement for ASC in cryopyrin induced cell death. ASC was initially identified as a cytosolic protein aggregated into specks in myeloid cells undergoing apoptosis and has since been implicated in several cell death pathways in non-myeloid cells (Masumoto et al., 1999). Functionally ASC is a bipartite adapter protein comprised of N-terminal pyrin domain (PD) and C-terminal CARD. It is proposed that these domains each engage in homotypic interactions, thereby linking the PD of cryopyrin to the CARD region of caspases. Constitutive interactions between several transfected disease-associated cryopyrin mutants and ASC have been reported when overexpressed in a

HEK293T cells (Dowds et al., 2004). Overexpressed ASC has also been shown to interact with caspases-8 and-9, and several studies have implicated ASC in the progression of apoptosis. The concurrent over-expression of a related protein, Ipaf, with ASC in HEK293T cells results in caspase-8 dependent apoptosis, while over-expression of ASC alone induces caspase-9 mediated cell death in HEK293 cells (Masumoto et al., 2003; McConnell and Vertino, 2000). These observations suggest the involvement of apoptotic caspases in ASC-induced cell death in non-monocytic cells (Dowds et al., 2004; Masumoto et al., 2003; Wang et al., 2004). However, co-immunoprecipitation experiments in monocytic THP-1 cells indicate that ASC/caspase interaction is limited to caspase-1, suggesting that ASC is not involved in the initiation of apoptotic caspases in these cells (Stehlik et al., 2003). Our results in a monocytic cell type demonstrate that ASC is important in a necrotic-like cell death pathway that is caspase-independent.

The results here delineate an endogenous program of necrosis initiated by cryopyrin and ASC. This pathway proceeds through cathepsin B, yet occurs independent of caspase-1, IL-1β, and the inflammasome. Recently, ASC has also been implicated in the initiation of another rapid form of inflammatory cell death called pyroptosis. In contrast to the necrosis initiated by cryopyrin and ASC, pyroptosis requires caspase-1 activation by ASC following the dimerization of ASC into speck-like pyroptosomes (Hersh et al., 1999). Pyroptosomes per se do not contain detectable cryopyrin, however the role of cryopyrin in the formation of this structure was not tested. This form of cell death has features of both apoptosis and oncosis. Unlike the cryopyrin/ASC necrosis pathway described here, pyroptosis can be blocked by exogenous caspase inhibitors however the requirement for cathepsin B has not been investigated (Fernandes-Alnemri et al., 2007). The involvement of ASC in the induction

of both pyronecrosis and pyroptosis establish ASC as a key component in the host defense arsenal to invading pathogens.

Several recent reports establish cryopyrin as a pivotal regulator of IL-1 and IL-18 release to both bacterial and viral challenges. Utilizing macrophages isolated from *CIAS1*$^{-/-}$ mice, components of the immune response to gram positive bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes*, as well as Sendai and Influenza viruses have been shown to require cryopyrin (Kanneganti et al., 2006a; Mariathasan et al., 2006b). However, infection with gram negative *Salmonella typhimurium* or *Francisella tularensis* elicits caspase-1 activation and IL-1β release in a cryopyrin-independent fashion (Mariathasan et al., 2005; Sutterwala et al., 2006). Macrophage cell death induced by *Salmonella* and *Francisella* are similarly unaffected by *CIAS1* deficiency (Mariathasan et al., 2005). Alternate proteins mediate the response to these pathogens. Ipaf, which participates in its own inflammasome, is responsible for initiating inflammation in response to *Salmonella typhimurium* (Mariathasan et al., 2004). It has been suggested that cryopyrin does not mediate the recognition of gram negative bacteria. However, our results indicate that cryopyrin mediates both IL-1β release and cell death in response to the gram negative bacteria *S. flexneri* in THP-1 cells and mouse macrophages (Fig. 2.7). Thus, cryopyrin mediates IL-1β processing and secretion in response to specific gram positive and negative bacteria.

Despite substantial progress made in understanding pathogen-induced host cell death, the mechanisms governing *Shigella* induced host cell death have not been conclusively delineated (Haimovich and Venkatesan, 2006). This study illuminates a necrotic host cell-death pathway detonated by *Shigella* requiring both cryopyrin and ASC. While the strain

M90T (Serogroup 5) has been reported to induce both necrosis and caspase-1 dependent apoptosis (Francois et al., 2000; Raqib et al., 2002; Zychlinsky et al., 1992), many others have observed caspase-1 independent necrosis initiated by strains 2457T (Serogroup 2A), YSH6000 (Serotype 2A), and now 12022 (Serogroup 2B) (Fernandez-Prada et al., 1997; Fernandez-Prada et al., 2000; Koterski et al., 2005; Nonaka et al., 2003). These differences have been attributed to differences in species and infected cell types, *Shigella* strains, duration and multiplicity of infection, and conclusions regarding the nature of cell death drawn from incomplete methodology (Nonaka et al., 2003). The results presented here verify and define cryopyrin and ASC as key components of a caspase-1 independent mechanism by which *Shigella* induces necrosis and should do much to advance the understanding of *Shigella* pathogenesis.

Further resolved is the putative involvement of caspase-1 in mediating *Shigella* induced cell death. Previous reports have suggested that the virulence factor IpaB is secreted by *Shigella* via a type III secretion system upon contact with host cells and subsequently binds directly to caspase-1 to induce cell death.(Blocker et al., 1999; Chen et al., 1996; Menard et al., 1994) Our results clearly demonstrate a caspase-1 independent pathway as both caspase-1 specific inhibitors and macrophages lacking caspase-1 were equally susceptible to *Shigella*-induced cell death as wildtype controls, a result consistent with other reports (Suzuki et al., 2005). It should be noted that *Suzuki et al*. observed an early apoptotic event requiring caspase-1and IpaB preceded a later necrotic event which occurs independent of caspase-1 or IpaB, and thus both outcomes are possible. While we show here that cryopyrin and ASC are required for *Shigella* initiated necrosis, the activation of NFκB and JNK in epithelial cell line following *Shigella* infection has been reported to require the

overexpression of NOD1/CARD4 (Girardin et al., 2001). Although verification of this finding using primary epithelial cells from mice lacking NOD1/CARD4 has not been performed to confirm the physiologic relevance of this data, taken together, these data suggest that several NLR proteins might mediate different aspects of the immune response to *Shigella* in a cell-type specific fashion.

In summary, we report that disease-associated cryopyrin mediates a form of necrotic-like cell death that is replicated when normal monocytes encounter the bacterial pathogen *S. flexneri*. These findings suggest that the gain-of-function mutant cryopyrin in patients might propagate an inflammatory response without the normal stimulation caused by pathogens. These findings also show a parallel with pathogen-induced, NB-LRR-mediated cell death found in plants. A long history of elegant studies in plants have shown that cell death is a major mechanism by which plant R proteins mediate host response to multiple microbial pathogens (Belkhadir et al., 2004). It will be important to determine if other NBD-LRR proteins also cause the induction of cell death in myeloid and non-myeloid cells, and if these proteins mediate both apoptotic and necrotic death in a pathogen-specific fashion.

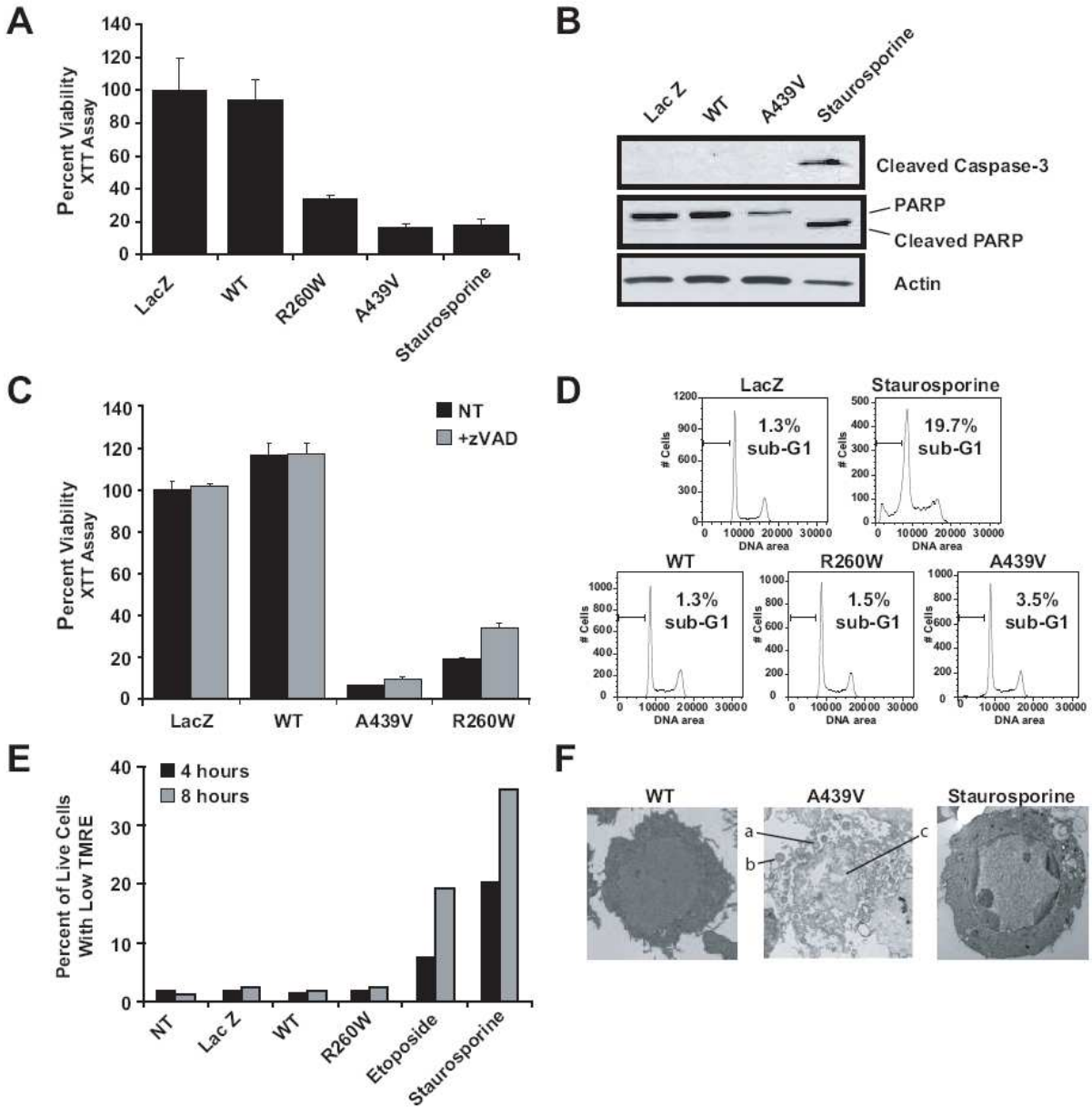

A
Percent Viability
XTT Assay
140
120
100
80
60
40
20
0
LacZ
WT
R260W
A439V
Staurosporine
B
Lac Z
WT
A439V
Staurosporine
Cleaved Caspase-3
PARP
Cleaved PARP
Actin
C
Percent Viability
XTT Assay
NT
+zVAD
LacZ
WT
A439V
R260W
D
LacZ
1.3%
sub-G1
Staurosporine
19.7%
sub-G1
WT
1.3%
sub-G1
R260W
1.5%
sub-G1
A439V
3.5%
sub-G1
Cells
DNA area
E
Percent of Live Cells
With Low TMRE
4 hours
8 hours
NT
Lac Z
WT
R260W
Etoposide
Staurosporine
F
WT
A439V
Staurosporine
a
b
c

Figure 2.1 – Disease-associated cryopyrin causes necrotic-like cell death. A) Cell viability is diminished in THP-1 cells expressing disease-associated *CIAS1* mutants. XTT reduction was measured 24 hours after adenoviral transduction. B) Mutant *CIAS1* induced cell death does not cause caspase-3 or PARP cleavage. Immunoblots for caspase-3 or its substrate PARP were performed on lysates made from cells infected with the indicated adenoviral constructs or treated with staurosporine. Cleaved caspase-3 and PARP are observed in staurosporine-treated cells but not in THP-1 cells expressing wildtype or disease-associated A439V *CIAS1*. C) Incubation with 100 μM pan-caspase (zVAD-fmk) inhibitor does not substantially block cell death. D) THP-1 cells expressing the R260W or A439V disease-associated mutant die without exhibiting DNA fragmentation. DNA content was measured by PI staining followed by flow cytometry. The percentage of cells with sub-G1 content, indicating the DNA-fragmentation characteristic of apoptosis, is shown. E) Disease-associated cryopyrin expression does not promote the loss of mitochondrial membrane potential. THP-1 cells were treated as indicated. Mitochondrial membrane potential was measured with the potential sensitive dye TMRE. Data summarized here is shown in Figure 2.9B. F) Representative EM images of wildtype cryopyrin (left), A439V transduced (middle) and staurosporine treated (right) THP-1 cells. A439V transduced cells demonstrate necrotic features including: a) degradation of the plasma membrane, b) dysmorphic/swollen mitochondria, and c) the lack of chromatin condensation. Staurosporine treated cells exhibit a typical apoptotic morphology.

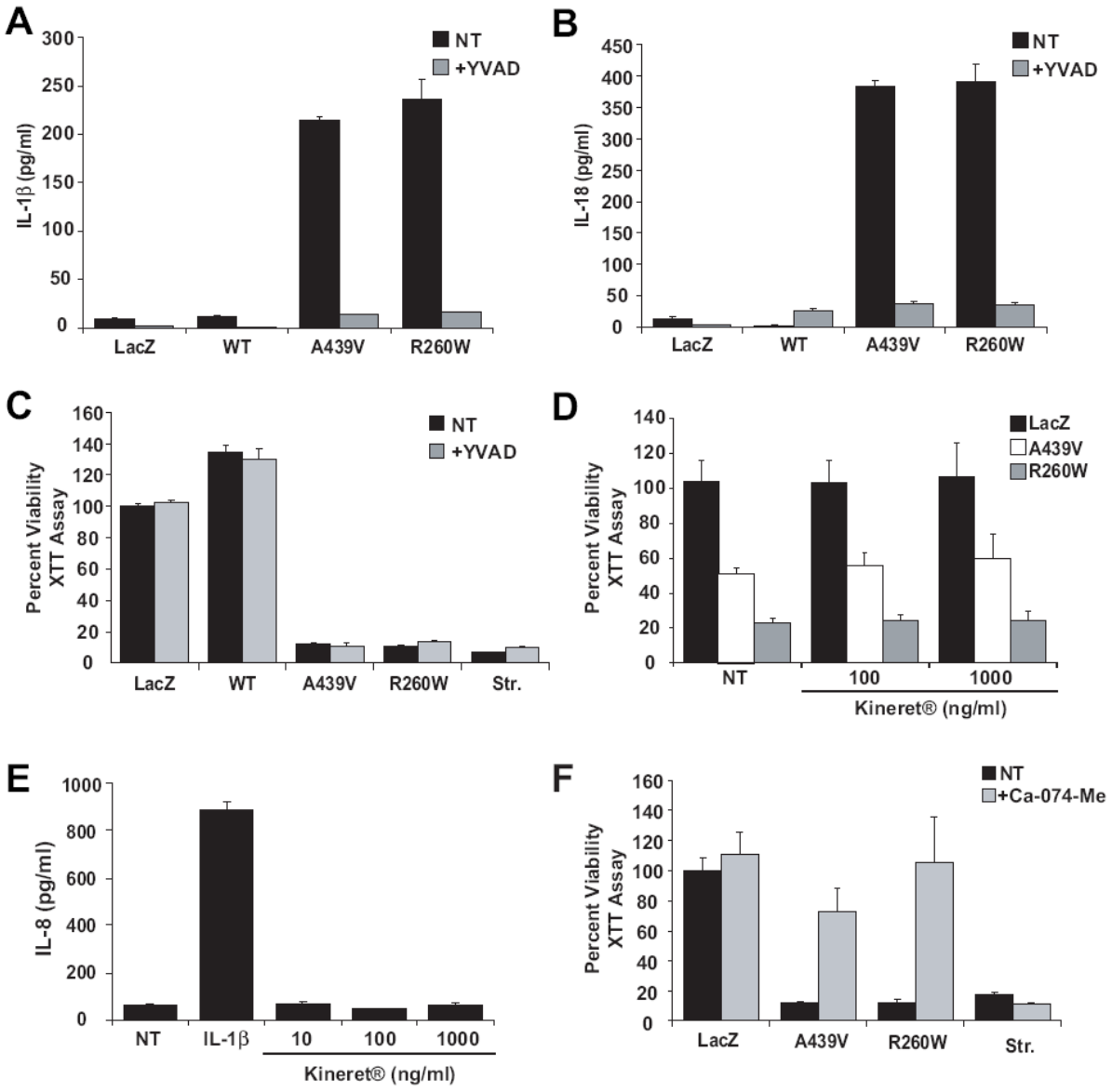

A
IL-1β (pg/ml)
300
250
200
150
100
50
0
NT
+YVAD
LacZ
WT
A439V
R260W
B
IL-18 (pg/ml)
450
400
350
300
250
200
150
100
50
0
NT
+YVAD
LacZ
WT
A439V
R260W
C
Percent Viability XTT Assay
160
140
120
100
80
60
40
20
0
NT
+YVAD
LacZ
WT
A439V
R260W
Str.
D
Percent Viability XTT Assay
140
120
100
80
60
40
20
0
LacZ
A439V
R260W
NT
100
1000
Kineret® (ng/ml)
E
IL-8 (pg/ml)
1000
800
600
400
200
0
NT
IL-1β
10
100
1000
Kineret® (ng/ml)
F
Percent Viability XTT Assay
160
140
120
100
80
60
40
20
0
NT
+Ca-074-Me
LacZ
A439V
R260W
Str.

Figure 2.2 – Disease-associated CIAS1 induces IL-1β release, but cell death is IL-1β independent. A) IL-1β is released from THP-1 cells infected with two mutant forms of *CIAS1* as measured by ELISA. IL-1β release is abrogated with 100 μM YVAD. B) IL-1β is released from THP-1 cells infected with two mutant forms of *CIAS1* as measured by ELISA. IL-1β release is abrogated with 100 μM YVAD. C) Cell death induced by *CIAS1* mutants is not inhibited by 100 μM YVAD. Viability was measured by XTT reduction 24 hours post transduction. D) Kineret®, the IL-1 receptor antagonist, does not prevent cryopyrin-induced cell death. THP-1 cells were infected with the indicated adenovirus for 24 hours in the presence or absence of Kineret®. NT, not treated with Kineret®. E) IL-8 induction in THP-1 cells by recombinant IL-1β is inhibited by Kineret®. IL-1β induced a significant level of IL-8 production; this biologic effect of IL-1β was completely abrogated by Kineret®. F) Cell death induced by cryopyrin mutants is blocked by a cathepsin B inhibitor, Ca-074-Me. THP-1 cells were infected with the indicated adenovirus for 24 hours in the presence or absence of Ca-074-Me. Viability was measured by XTT reduction after 24 hours

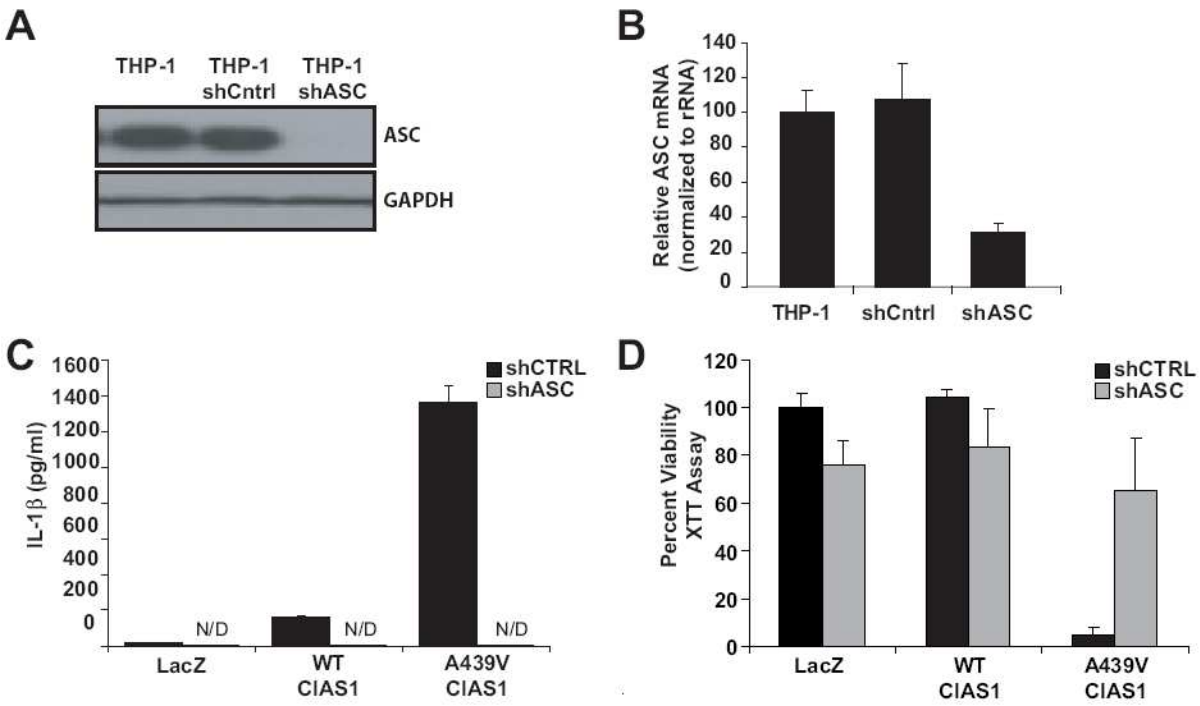

A
THP-1
THP-1 shCntrl
THP-1 shASC
ASC
GAPDH
B
Relative ASC mRNA (normalized to rRNA)
140
120
100
80
60
40
20
0
THP-1
shCntrl
shASC
C
IL-1β (pg/ml)
1600
1400
1200
1000
800
600
400
200
0
shCTRL
shASC
N/D
N/D
N/D
LacZ
WT CIAS1
A439V CIAS1
D
Percent Viability XTT Assay
120
100
80
60
40
20
0
shCTRL
shASC
LacZ
WT CIAS1
A439V CIAS1

Figure 2.3 - Mutant CIAS1-induced THP-1 cell death is ASC dependent. A and B) Expression of ASC is markedly decreased in cells stably transduced with shRNA designed to promote the degradation of ASC mRNA (shASC). Immunoblot analysis and real-time PCR of the indicated stable cell lines verify a decrease of ASC protein (A) and mRNA (B). C) IL-1β release following infection with *CIAS1*-containing adenovirus is abrogated in cells with shASC. IL-1β was determined by ELISA. Values <10 pg/ml are considered not detectable ("N/D"). D) Mutant *CIAS1*-induced cell death is abrogated in cells with decreased expression of ASC caused by shASC. XTT reduction was measured 24 hours after adenoviral infection.

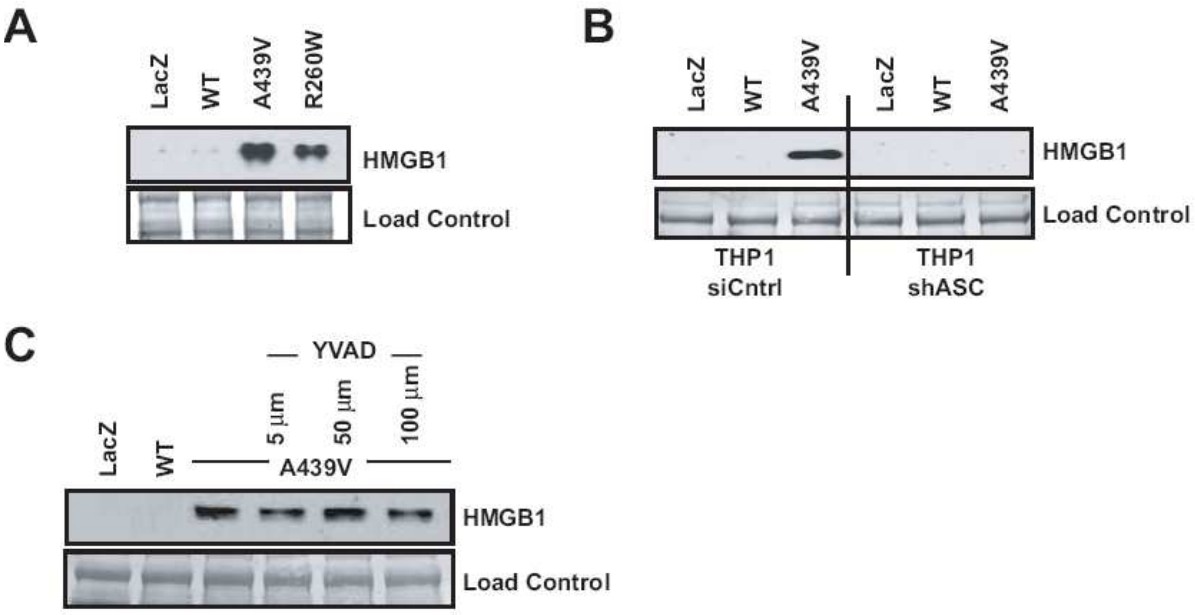


Figure 2.4 - HMGB1 is released from THP-1 cells following expression of disease-associated CIAS1 mutants. A) Two disease-associated *CIAS1* mutants induce substantially more HMGB1 release than the wild type gene. B) HMGB1 release is abrogated in cells with shASC which reduced ASC expression. C) Inhibition of caspase-1 with YVAD-CHO does not substantially affect HMGB1 release. Nitrocellulose membranes stained with amido black are provided as loading controls.

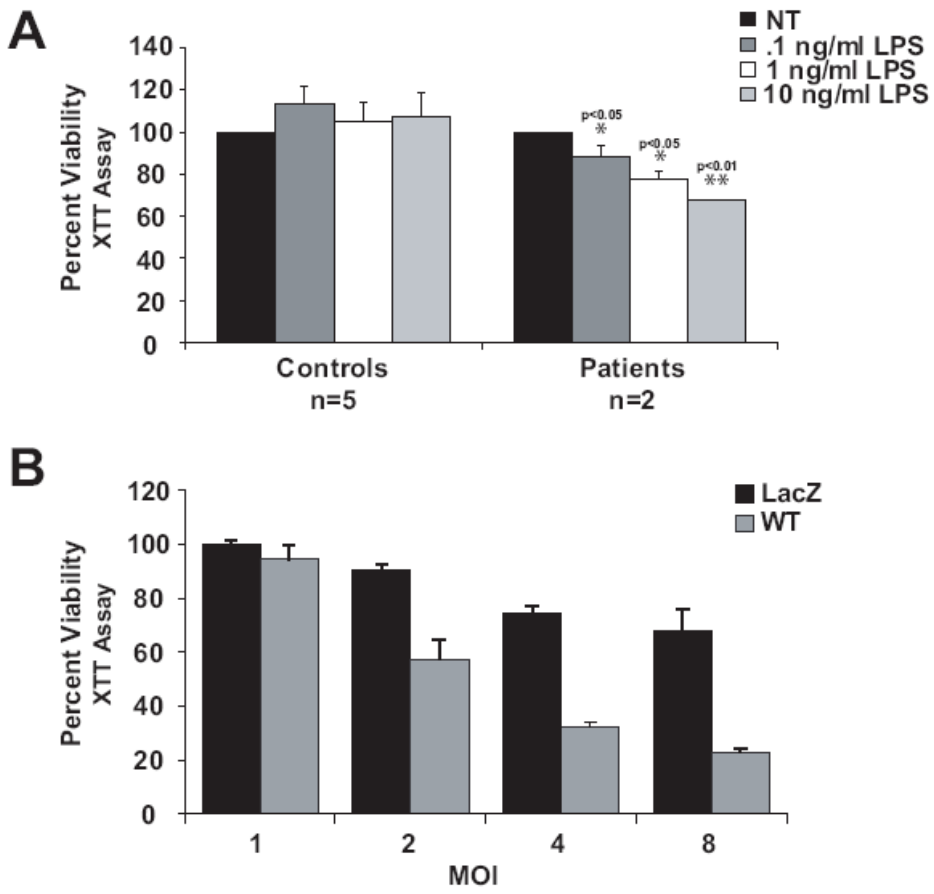


Figure 2.5 – Cell death in cells isolated from CAPS patients and in cells expressing wildtype *CIAS1*. A) Cell viability is decreased in PBMCs from FCAS patients in response to LPS. XTT reduction was used to assay cell viability, and was measured 72 hours after stimulation. *$p<0.05$, ** $p<0.01$ when compared to controls. B) Cell viability is diminished in THP-1 cells expressing wild type *CIAS1* at higher multiplicities of infection. XTT reduction was measured 48 hours after adenoviral infection.

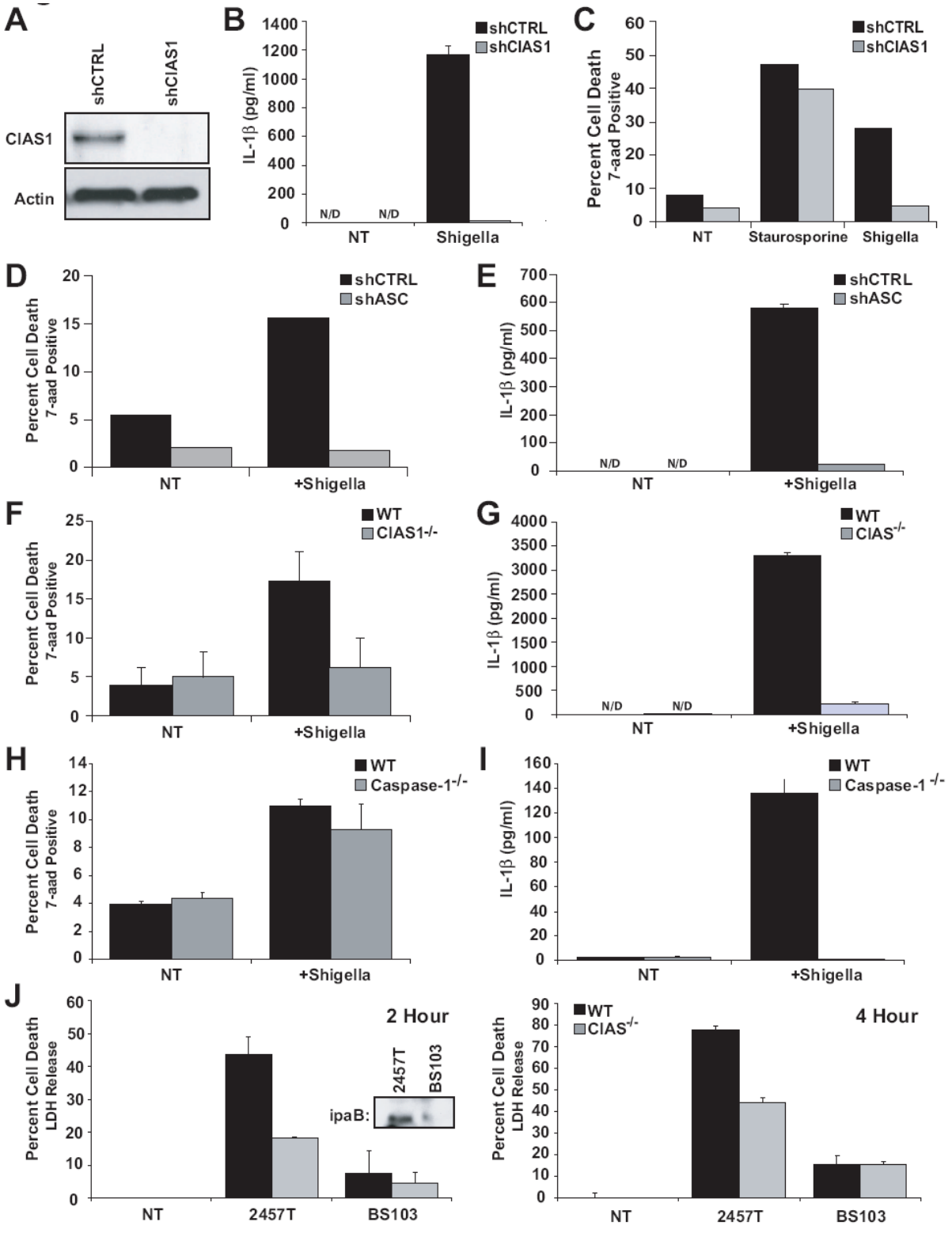

A
shCTRL
shCIAS1
CIAS1
Actin
B
IL-1β (pg/ml)
shCTRL
shCIAS1
N/D
N/D
NT
Shigella
C
Percent Cell Death
7-aad Positive
shCTRL
shCIAS1
NT
Staurosporine
Shigella
D
Percent Cell Death
7-aad Positive
shCTRL
shASC
NT
+Shigella
E
IL-1β (pg/ml)
shCTRL
shASC
N/D
N/D
NT
+Shigella
F
Percent Cell Death
7-aad Positive
WT
CIAS1-/-
NT
+Shigella
G
IL-1β (pg/ml)
WT
CIAS-/-
N/D
N/D
NT
+Shigella
H
Percent Cell Death
7-aad Positive
WT
Caspase-1-/-
NT
+Shigella
I
IL-1β (pg/ml)
WT
Caspase-1 -/-
NT
+Shigella
J
Percent Cell Death
LDH Release
2 Hour
2457T
BS103
ipaB:
NT
2457T
BS103
WT
CIAS-/-
4 Hour
Percent Cell Death
LDH Release
NT
2457T
BS103

Figure 2.6 – *Shigella flexneri* induced cell death and IL-1β require CIAS1 and ASC but not caspase-1. A) Cryopyrin protein expression is decreased in THP-1 cells transduced with cryopyrin-specific shRNA (shCIAS1). B) *S. flexneri* induced IL-1β release is diminished in CIAS1 deficient THP1 cells C) THP-1 cells with shCIAS1 resist *S. flexneri*-induced death but not staurosporine-induced death. D and E) ASC is required for *S. flexneri*-induced cell death (D) and IL-1β release (E) in THP-1 cells. F) *CIAS1*$^{-/-}$ bone marrow-derived macrophages exhibit decreased levels of cell death in response to *S.flexneri*. G) *S. flexneri*-induced IL-1β release from thioglycolate-elicited peritoneal macrophages is reduced in *CIAS1*$^{-/-}$ macrophages. H) *S. flexneri* does not require Caspase-1 to initiate cell death. I) *S. flexneri* requires caspase-1 to induce IL-1β production in bone marrow-derived macrophages. J) Cryopyrin initiates cell death in response to virulent *Shigella*. Bone marrow derived macrophages were infected with either 2457T (virulent) or BS103 (avirulent) *S. flexneri* at a MOI of 50 for 2 or 4 hours. Absence of virulence plasmid in BS103 was verified by ipaB immunoblot (inset). In all cases, cell death was measured by 7-aad uptake or LDH release and IL-1β determined by ELISA.

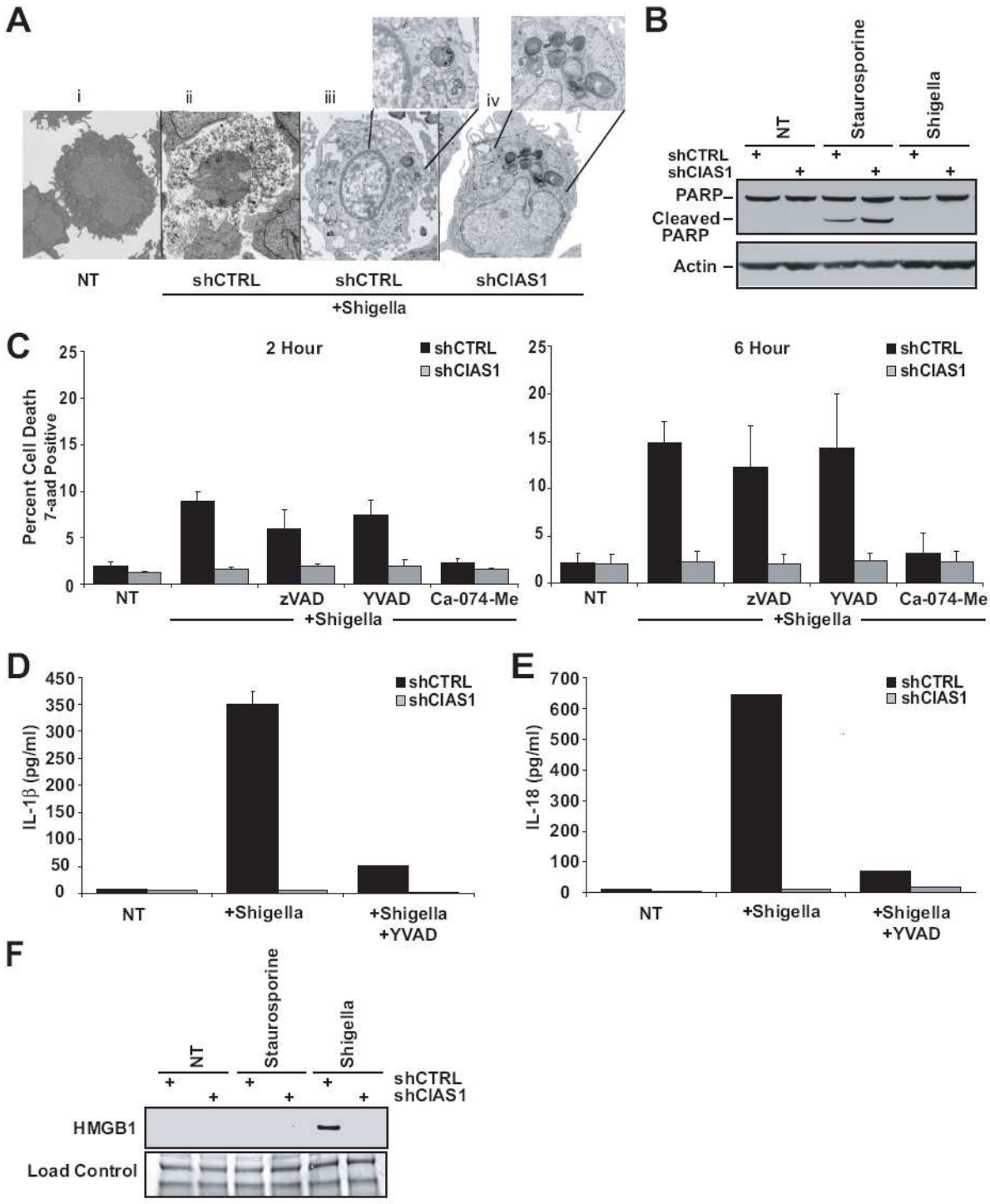

A
i
ii
iii
iv
NT
shCTRL
shCTRL
shCIAS1
+Shigella
B
NT
Staurosporine
Shigella
shCTRL
shCIAS1
PARP
Cleaved PARP
Actin
C
2 Hour
6 Hour
shCTRL
shCIAS1
Percent Cell Death 7-aad Positive
NT
zVAD
YVAD
Ca-074-Me
+Shigella
D
IL-1β (pg/ml)
NT
+Shigella
+Shigella +YVAD
E
IL-18 (pg/ml)
F
HMGB1
Load Control

Figure 2.7 - *Shigella flexneri* induces cryopyrin-dependent necrosis. A) Infection with *S. flexneri* for six hours induced cell death that is morphologically consistent with necrosis (see panels i and ii). To detect intracellular bacteria a shorter infection (2 hr) time was used so that the cells are just entering the initial phase of cell death. Cells with shCTRL, but not shCIAS1 exhibited a lost of cytoplasmic content as determined by EM imaging. Insets show the presence of bacteria (opaque round or oblong structures). B) PARP is not cleaved following *S. flexneri* infection. C) 50 μM cathepsin B inhibitor (Ca-074-Me) substantially abrogates *S. flexneri* cell death. In contrast, 100 μM pan-caspase (zVAD-fmk) and 100 μM caspase-1 specific (YVAD-CHO) inhibitors fail to block *S. flexneri*-induced cell death in shCTRL and shCIAS1 cells at 6 hours. D and E) *S. flexneri* induced IL-1β (D) and IL-18 (E) release is reduced in shCIAS1 THP-1 cells and in cells treated with 100 μM YVAD-CHO. F) *S. flexneri*-induced HMGB1 release is abrogated by shCIAS1, and thus is cryopyrin dependent. In all cases, cell death was measured by 7-aad uptake. IL-18 and IL-1β release were determined by ELISA

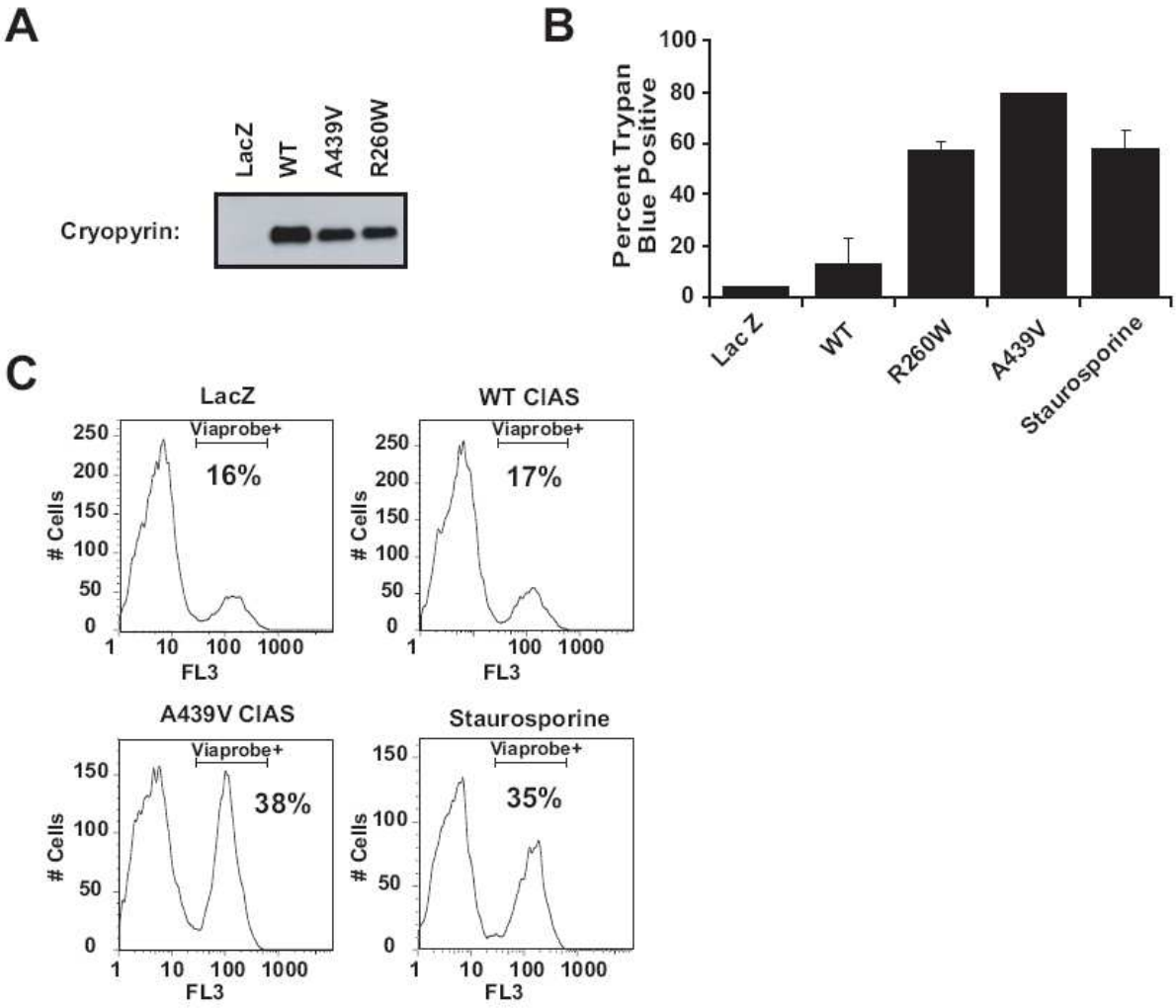

A
LacZ
WT
A439V
R260W
Cryopyrin:
B
Percent Trypan Blue Positive
100
80
60
40
20
0
Lac Z
WT
R260W
A439V
Staurosporine
C
LacZ
WT CIAS
A439V CIAS
Staurosporine
Viaprobe+
16%
17%
38%
35%
Cells
FL3
250
200
150
100
50
0
1
10
100
1000

Figure 2.8 – Immunoblot of CIAS1 and supplemental cell death assays. A) Adenoviral constructs promote similar expression of FLAG-tagged *CIAS1* variant and wildtype proteins in the 293T cell line as revealed by immunoblotting. B and C) Increased cell-permeability to two viability dyes indicates that disease-associated cryopyrin reduced cell viability. Cell-permeability following 48 hours of staurosporine treatment or infection with the indicated construct was determined by (B) trypan blue and (C) flow cytometry performed on Viaprobe stained cells.

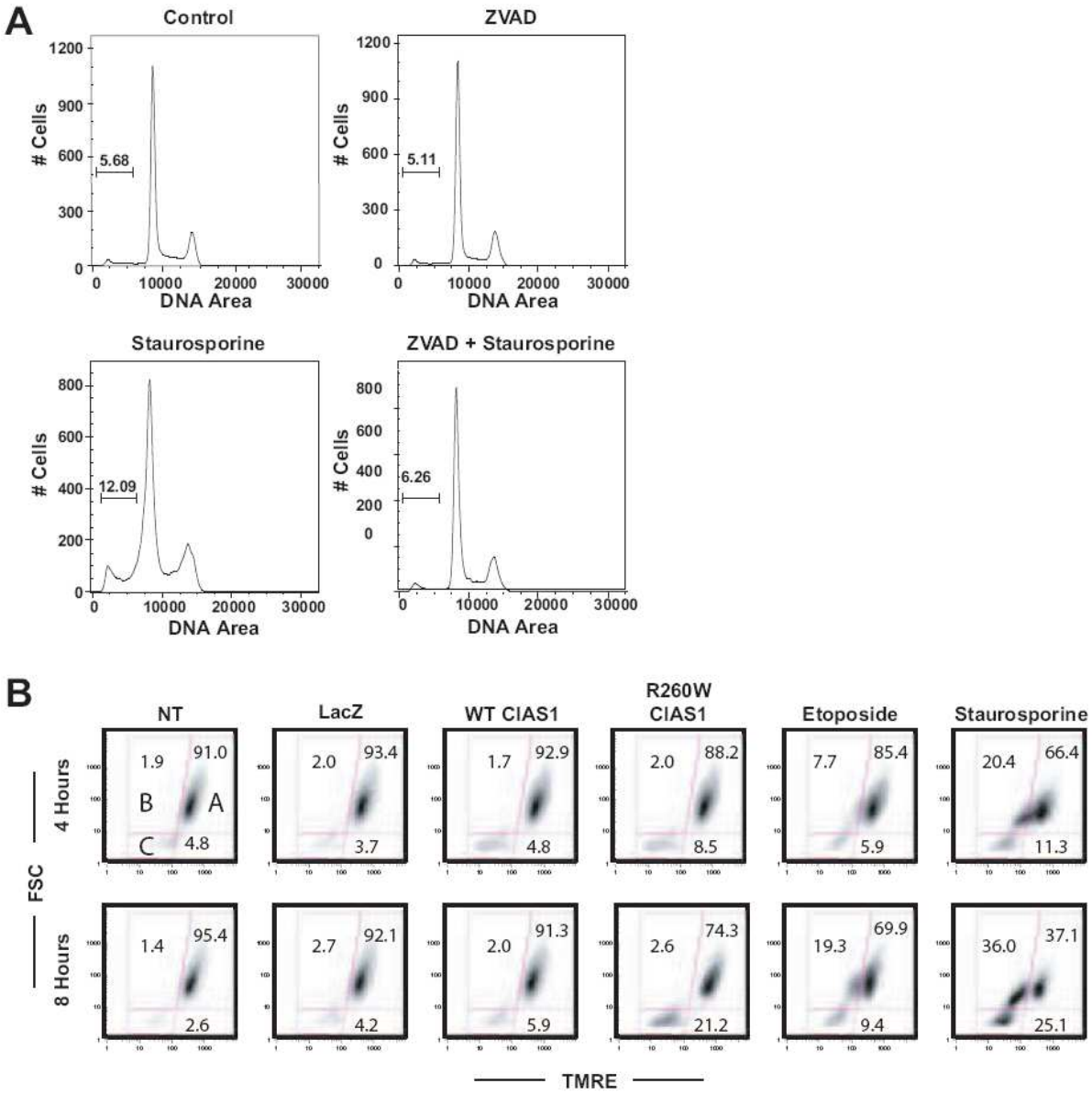

A
Control
ZVAD
Staurosporine
ZVAD + Staurosporine
Cells
DNA Area
5.68
5.11
12.09
6.26
B
NT
LacZ
WT CIAS1
R260W CIAS1
Etoposide
Staurosporine
4 Hours
8 Hours
FSC
TMRE
A
B
C
C
NT
+Staurosporine
2
6
24
HMGB1:
Load Control

Figure 2.9 – Staurosporine induced cell death and mitochondrial membrane permeability transition controls. A) Staurosporine-induced cell death causes DNA fragmentation that is sensitive to a pan-caspase inhibitor. One hour pretreatment with 100 μM zVAD-fmk blocks DNA fragmentation induced by 1 μM staurosporine. B) Cells expressing disease-associated cryopyrin do not undergo a mitochondrial membrane permeability transition. Cells were treated as indicated for 4 or 8 hours before staining with TMRE to measure membrane permeability. Results are presented along two axes, forward scatter (*y* axis) and TMRE (*x* axis). Healthy cells are positive along both axes (Gate A, see first panel). Cells which have lost both forward scatter and membrane potential are considered dead (Gate C). Cells which maintain forward scatter while losing membrane potential are undergoing a transition state (Gate B). Only cells treated with the pro-apoptotic agents etoposide and staurosporine show an accumulation in Gate B. However, cells expressing mutant cryopyrin or treated with pro-apoptotic agents show an accumulation in gate C, indicating that all three treatments result in cell death. C) HMGB1 is not released from THP1 at the 2 and 6 hr time points after treatment with 1 μM staurosporine, but it is released upon prolonged (24 hr) treatment, likely after secondary necrosis reported in the literature.

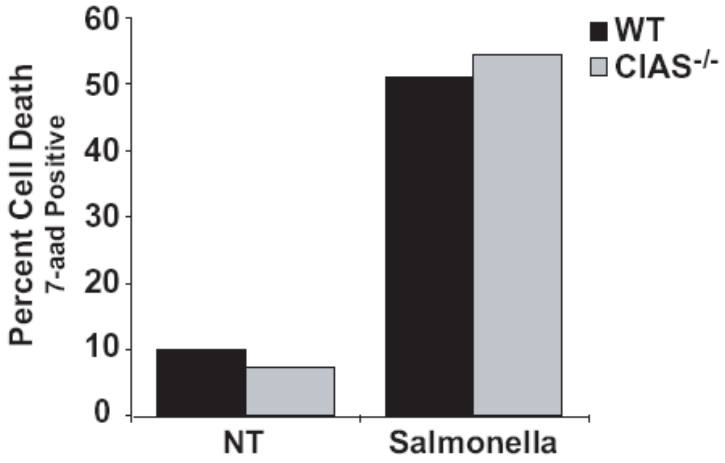


Figure 2.10 - Salmonella induced cell death does not require *CIAS1*. Bone marrow derived macrophages were infected with *Salmonella typhi* at a MOI of 50 for 2 hours. Cell death was determined by flow cytometry performed on 7-aad stained cells

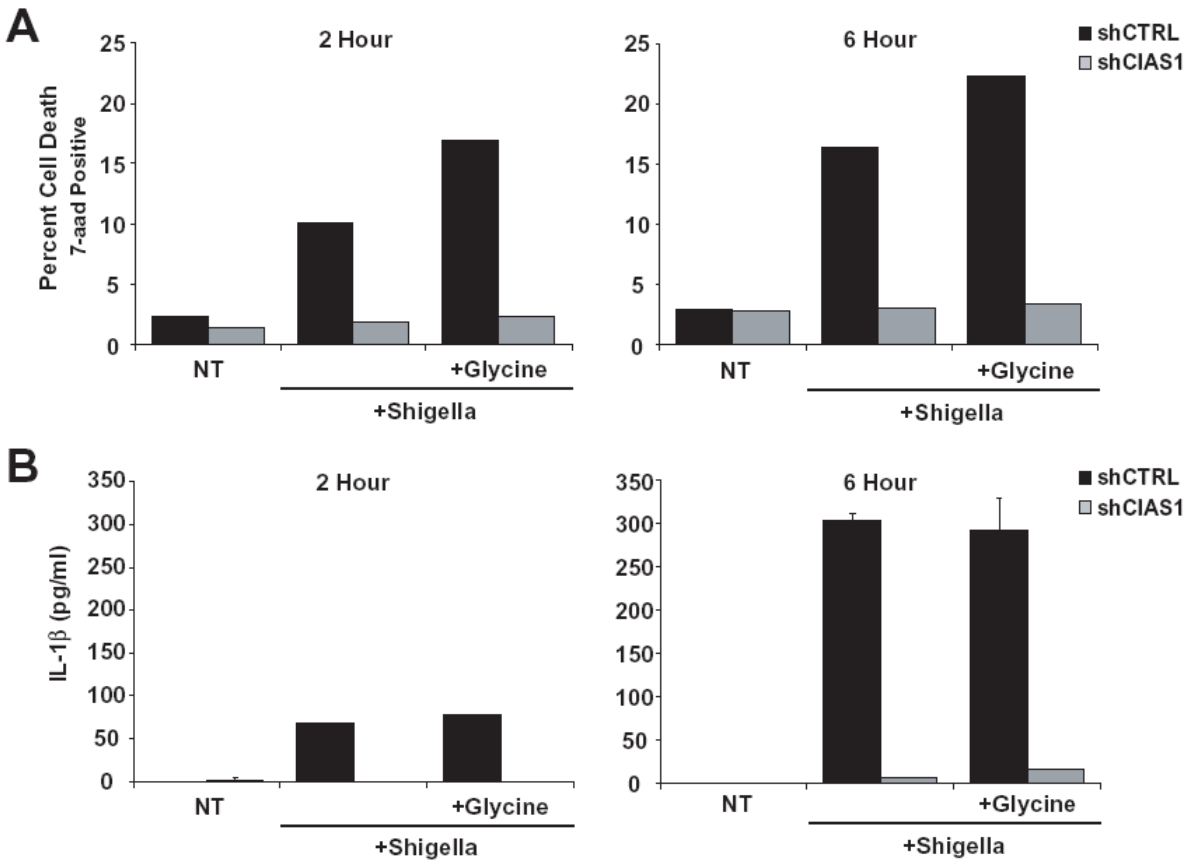


Figure 2.11 - Glycine fails to abrogate *S.flexneri* initiated cell death or IL-1β release.

A) Glycine did not reduce cryopyrin mediated cell death. THP-1 cells were pretreated with 10mM glycine for 1 hour before infection with *S. flexneri* at a MOI of 50 for the indicated time period. B) Glycine does not affect *Shigella* induced IL-1β at the indicated time period.

CHAPTER III

KLEBSIELLA PNEUMONIAE INDUCES IL-1β RELEASE AND PYRONECROSIS THROUGH NLRP3.

Portions of this chapter have been adapted from: Stephen B. Willingham, Irving C. Allen, Daniel T. Bergstralh, Willie June Brickey, Joseph A. Duncan, and Jenny P.-Y. Ting. *Klebsiella pneumoniae* induces IL-1β release and pyronecrosis through NLRP3. J Ex Med 2008 Manuscript in preparation

3.1 Abstract

NLRP3 has emerged as an important regulator of pathogen-induced inflammation. It not only participates in one of several caspase-1-activating inflammasome complexes, which mediate maturation of the pro-inflammatory cytokine IL-1β, but is also a critical mediator of pyronecrosis, an inflammatory cell death program with necrotic features. Both processes have been shown to be activated by pathogens *in vitro*, but the consequence of these processes to the host organism remains undetermined. Here we show that the extracellular pathogen *Klebsiella pneumoniae* induces pyronecrosis and NLRP3-dependent IL-1β processing in the human monocytic cell line THP-1 and in bone marrow derived macrophages. Consistent with these results, mice lacking NLRP3 exhibit significant decreases in lung inflammation following pulmonary infection with *K. pneumoniae.* However, these mice are more susceptible to *K. pneumoniae* induced lethality compared to controls. Cumulatively, these results demonstrate that NLRP3 activity is beneficial to the host in defense against *K. pneumoniae.*

3.2 Introduction

Klebsiella pneumoniae is a non-motile, non-flagellated, gram negative, rod-shaped bacterium which normally resides within the mouth, skin, and intestines. Pathogenic *K. pneumoniae* invades the lungs where it is capable of inducing severe bacterial pneumonia that is often complicated with bacteremia and sepsis (Sahly and Podschun, 1997). Airway infection typically leads to extensive lung injury resulting from increased inflammation, hemorrhage, and the necrotic destruction of lung tissue. This process results in thick, blood-laced mucous known as "currant jelly" sputum, which is characteristic of *K. pneumoniae*-induced pneumonia. Worldwide, *K. pneumoniae* is amongst the most common gram negative bacteria encountered by clinicians and is a leading cause of community-acquired and hospital-associated respiratory infection (Ko et al., 2002). Its frequency in the latter context is particularly alarming. *K. pneumoniae* is responsible for up to 23% of nosocomial infections, and the difficulty in treatment of elderly or otherwise compromised patients results in a mortality rate of up 50% (Feldman et al., 1995). Moreover, the growing prevalence of antibiotic resistant strains in this species has led to increased attention and concern (Keynan and Rubinstein, 2007; Paterson et al., 2004). Though recent work has identified innate mechanisms underlying the initial host response to bacterial infection, little work has yet been done to examine the contribution of these mechanisms to *Klebsiella* pathogenesis or immunity.

Initially described in our laboratory, the NLR (nucleotide binding – leucine rich repeats, formerly CATERPILLER) family of genes/proteins is increasingly implicated in the regulation of immunity (Harton et al., 2002a; Ting and Davis, 2005). The NLR family

member NLRP3 (formerly cryopyrin), which is expressed abundantly in neutrophils and macrophages, is emerging as a critical mediator of inflammation. *CIAS1/NLRP3*, the gene encoding this protein, was first identified through its association with the periodic fever condition CAPS (CIAS1-associated periodic syndromes), which comprises a wide range of severity (Ting et al., 2006). This condition is believed to result from gain-of-function mutations in NLRP3. Using disease-associated variants, our lab and others confirmed the importance of NLRP3 in mediating inflammation directly, by participating in IL-1β maturation, and demonstrated an unexpected role for NLRP3 in promoting inflammation indirectly, by inducing a necrotic pathway of cell death (Fujisawa et al., 2007; Willingham et al., 2007).

A role for NLRP3 in caspase-1 maturation is also well established. Following stimulation, NLRP3, ASC (Apoptotic Speck-like protein containing a Card), Cardinal/TUCAN, and pro-caspase-1 combine to form one of several known inflammasome complexes. Within this complex, pro-caspase-1 is activated, which in turn cleaves and activates the pyrogenic cytokines IL-1β and IL-18 (Agostini et al., 2004). Currently, activation of the NLRP3 inflammasome is associated with the widest spectrum of stimuli. Among these are gram-positive and gram-negative bacteria, including *Staphylococcus aureus, Listeria monocytogenes,* and *Shigella flexneri* (Fig 1.6). To date, no studies have examined inflammasome activation in response to *K. pneumoniae.*

Recent work from our laboratory has demonstrated a second pro-inflammatory function for NLRP3 (Willingham et al., 2007). Necrosis has been shown to occur in monocytic cells infected with intracellular bacteria or following exposure to toxins. Though in some cases pathogen-induced death is likely to be a passive response, programmed

necrotic cell death pathways are potentially critical to macrophage function. Recently, our lab has identified a novel mechanism of necrotic-like cell death, termed pyronecrosis (Willingham et al., 2007). Pyronecrosis requires the activity of NLRP3, its partner protein ASC, and the lysosomal protease cathepsin B (Willingham et al., 2007). Though pyronecrosis is entirely independent of caspase-1, it is inherently pro-inflammatory. One defining feature of necrosis is the loss of plasma membrane integrity and subsequent spilling of intracellular contents. Certain intracellular components, notably the nuclear factor HMGB1, elicit strong pro-inflammatory effects when released into the microenvironment (Scaffidi et al., 2002).

Pyronecrosis serves as an interesting contrast to pyroptosis, another form of pathogen-induced cell death. Both pyronecrotic and pyroptotic cells demonstrate morphological features characteristic of necrosis. However, the two pathways are readily distinguishable at the molecular level. Unlike pyronecrosis, pyroptosis requires the activity of caspase-1. These two pathways also appear to be induced by different stimuli. Pyronecrosis has been observed in monocytic cells infected with *Shigella flexneri*(Willingham et al., 2007). Pyroptosis was first observed in monocytic cells infected with *Salmonella*, which activates signaling through a different NLR protein, NLRC4 (formerly IPAF) (Brennan and Cookson, 2000; Mariathasan et al., 2004). NLRC4 has been implicated in pyroptotic cell death in response to *Salmonella typhimurium* and *Pseudomonas aeruginosa* (Brennan and Cookson, 2000; Franchi et al., 2007; Mariathasan et al., 2004; Sutterwala et al., 2007). Interestingly, though ASC is required for activation of caspase-1, deletion of ASC does not abrogate caspase-1 dependent pyroptosis initiated by *P. aeruginosa* (Sutterwala et al., 2007).

Despite the wealth of *in vitro* data implicating the NLR family in pathogen-induced inflammation, *in vivo* evidence for the importance of these proteins remains elusive. We show here that NLRP3 carries out both its IL-1β processing and pyronecrotic functions in response to *K. pnuemoniae*, and that the absence of NLRP3 decreases the rate of survival in mice infected with the bacteria. This is the first demonstration that NLRP3 activity is protective to the organism in vivo, and reveals an important component of the host immune response to *K. pneumoniae.*

3.3 Materials and Methods

Experimental Animals - All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. Mice deficient in NLRP3, ASC, NLRC4, and Caspase-1 were generated as previously described (Mariathasan et al., 2004; Sutterwala et al., 2006). WT animals were obtained from NCI. All animals were maintained in specific pathogen-free animal facilities at The University of North Carolina at Chapel Hill.

Cell lines and reagents – THP-1 cells purchased from American Type Culture Collection (ATCC) and cultured as described previously (Williams et al., 2005). Anti-caspase-3 antibody purchased from Cell Signaling; anti-PARP, anti-Actin, and HRP-conjugated secondary antibodies from Santa Cruz Biotechnology; anti-HMGB1 antibody from Abcam; Super Signal ECL reagent from BioRad. Detailed methods for preparation of retroviral shuttle vectors, transduction, and sorting to generate THP-1 cell lines stably expressing shRNA have been described (Taxman et al., 2006). The shRNA target sequences are as follows: shASC-GCTCTTCAGTTTCACACCA, shCtrl-GCTCTTCctggcCACACCA, shNLRP3-GGATGAACCTGTTCCAAAA.

Bacteria – *Klebsiella pneumoniae* 43816, serotype 2 was obtained from the ATCC and propagated in LB for approximately 2 hours at 37^{o}C. Bacteria density was estimated by

measuring the absorbance at 600 nm (1 OD^{600} = 3 X 10^8 bacteria/ml). Accurate CFUs were determined for each experiment by plating an aliquot onto LB agar plates.

***Klebsiella pneumoniae* induced airway inflammation.** OD readings were determined on actively growing cultures of *K. pneumoniae*. Bacteria were pelleted, washed twice with PBS, and resuspended in an equal volume of PBS. Mice were anesthetized and challenged via intratracheal (i.t.) instillation with 4 X 10^4 CFUs of *K. pneumoniae* in 50 µl of PBS. Mock challenged mice received 50 µl of PBS. THP-1 cells were with infected with *Salmonella* and *Klebsiella* at a MOI=50 for 1 or 6 hours, respectively. Bone marrow derived macrophages were infected with *Klebsiella* (MOI=200, 6 hours) or *Salmonella* (MOI=50, 1 hour). Samples were centrifuged at 650*g for 10 minutes immediately following addition of bacteria. Gentamicin (50 µg/ml) was added to cultures 1 hour post infection.

Assessment of bacteria burden. Mice were euthanized via i.p. injection with 2,2,2 tribromoethanol (avertin). Whole liver (gal bladder removed), spleen and lungs were removed, wet weight assessed, homogenized in 500 µl HBSS with a Tissue Master 125 hand held tissue homogenizer (Omni International) and centrifuged. The resulting supernatants were serially diluted and plated on LB plates and grown for 24 hours at 37^{o}C.

Assessment of airway inflammation. Survival, body weight and body temperature were assessed over the course of 48 hours post *K. pneumoniae* infection. Mice were euthanized via lethal i.p. injection with avertin and serum was harvested via cardiac puncture. The liver, kidney and spleen were harvested, weighed and either homogenized in 500 µl HBSS or fixed

in 4% paraformaldehyde (PFA). Mice were then perfused with HBSS and a tracheal cannula was inserted below the larynx. The lungs were lavaged 5 times with 1 ml HBSS. The recovered bronchoalveolar lavage fluid (BALF) was pooled and red blood cells were lysed via hypotonic saline treatment. Total BALF cellularity was assessed with a hemacytometer and trypan blue staining. Aliquots of each BALF were cytospun onto slides and Diff-Quik (Dade Behring) stained for differential cell counts. Leukocytes were identified based on morphological criteria of no fewer than 200 cells per BAL sample. An aliquot of BALF was serially diluted, plated on LB agar plates, and incubated for 24 hours at 37^{o}C to assess bacteria burden. The remaining BALF was centrifuged and the supernatant was collected. Following BALF harvest, the lungs were fixed by inflation (20-cm pressure) and immersion in 4% PFA.

Histopathology. Whole inflated lungs were embedded in paraffin wax. 4-μm sections were cut and stained with hematoxylin and eosin (H&E). Serial sections of the left lobes of the lungs that yield maximum longitudinal visualization of the intrapulmonary main axial airway were examined and the degree of inflammation was scored by one of the authors (I.C.A.) who was blinded to genotype and treatment.

Cytokine and chemokine assessment. Cell free supernatants were harvested from *K. pneumoniae* (MOI=200, 6 hour) or *S. typhi* (MOI=50, 1 hour) infected thioglycolate-elicited peritoneal macrophages. Supernatants were analyzed using RayBio® Mouse Cytokine Antibody Array G Series 3 (RayBiotech Inc. Axon scanner 4000B with GenePix software was used to collect fluorescence intensities from cytokine-bound antibody spots. These

values were normalized to the ratio of positive control values for each sample. Afterward, the total normalized florescence values of replicate spots were averaged and expressed as fold increase over the non-infected sample for each respective genotype. “N/D” indicates cytokines where the raw fluorescence values of replicate spots deviated more than 2 fold from each other and were thus dismissed. If this occurred in the non-treated sample, the cytokine was removed from the data set. Cytokine concentrations were determined directly by RayBiotech Inc. using their Quantibody processing service.

Preparation of Macrophages – Bone marrow was harvested from 6-8 week old mice and cultured for 7 days in antibiotic free DMEM supplemented with 10% FCS and 30% M-CSF conditioned media. Bone marrow derived macrophages were washed and cultured in antibiotic free DMEM+10% FCS immediately prior to bacterial infection.

ELISA – Samples were harvested at indicated times and assayed with OptEIA Human IL-1β ELISA Set or OptEIA Mouse IL-1β ELISA Set (BD Biosciences).

Cell Viability – Cell viability was assayed per manufacturer protocol using either CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega), ToxiLight® BioAssay Kit (Lonza Bioscience), or 7-AAD (BD Pharmingen) staining as indicated. In the case of 7-AAD cell staining, cells were collected and rinsed twice in cold PBS. Pellets were resuspended in 0.5ml PBS with 1 µl 7-AAD. Cells were incubated in the dark for 15 minutes before analysis on a FACScan (BD).

3.4 Results

K. pneumoniae-induced cell death and IL-1β release require NLRP3 and ASC.

To date, the participation of NLR proteins in host immune response to *K. pneumoniae* has not been examined. We observed both cell death and IL-1β release following infection of THP-1 human monocytic cells with *K. pneumoniae* (Fig. 3.1A and 3.1B). Both processes were abrogated in the absence of NLRP3 (Fig. 3.1A and 3.1B). Reduction of NLRP3 was achieved through stable integration of retroviruses encoding shRNAs designed to promote the targeted degradation of NLRP3 mRNA, as described previously (Taxman et al., 2006).

Previously, we demonstrated that ASC, a partner protein of NLRP3, is required for *Shigella flexneri*–induced cell death in THP-1 cells (Willingham et al., 2007). Therefore, we tested the ability of *Klebsiella* to elicit cell death and IL-1β in ASC-deficient THP-1 cells. Both IL-1β release and cell death were substantially abrogated in the shASC cells (Fig. 3.1C and 3.1D). To expand the physiologic importance of these results, bone marrow derived macrophages were isolated from wild-type mice and mice deficient for NLRP3, ASC, or NLRC4. Deletion of NLRP3 or ASC resulted in a near complete inhibition of IL-1β induced by *Klebsiella* as measured by ELISA, whereas NLRC4 null macrophages demonstrated no substantial difference from wild type (Fig. 3.1E). Importantly, this phenomenon is not common to all pathogenic bacteria. In agreement with previous work, IL-1β release from macrophages infected with *Salmonella typhi* was unaffected by NLRP3 deletion, whereas deletion of NLRC4 eliminated the inflammatory response (Fig. 3.1E). The NLRP3 inflammasome was previously reported to be activated by a combination of *E. coli* lipopolysaccharide (LPS) and ATP (Agostini et al., 2004). To determine if the NLRP3

inflammasome is also activated by *Klebsiella* LPS, bone marrow derived macrophages were challenged with 50 ng/ml LPS isolated from *K. pneumoniae* for 16 hours followed by stimulation with 5 mM ATP for 20 min. In contrast to wild type and NLRC4 deficient macrophages, deletion of ASC or NLRP3 eliminated *Klebsiella* LPS-induced IL-1β release (Fig. 3.1F). Together, these results indicate that NLRP3 is the predominant NLR activated by *Klebsiella* and that deletion of either NLRP3 or ASC substantially abrogates the hosts inflammatory and cell death responses.

NLRP3 mediates *Klebsiella* induced pyronecrosis.

Having established that NLRP3 is essential for *Klebsiella*-induced cell death, we sought to determine the nature of this phenomenon. Indicative of pyronecrosis, *K. pneumoniae*-induced cell death was markedly reduced in shControl THP-1 cells treated with the cathepsin-B inhibitor Ca-074-Me (Fig. 3.2A). Pan-caspase inhibitor (zVAD-fmk) also reduced *Klebsiella* induced cell death, likely reflecting a well-documented off target inhibition of cathepsin B by the zVAD peptide (Schotte et al., 1999). The caspase-1 specific inhibitor YVAD-cho had no effect (Fig. 3.2A). Both pan-caspase and caspase-1 specific inhibitors were used at concentrations sufficient to inhibit caspase activity, as evidenced by the attenuation of *Klebsiella* induced IL-1β release (Fig. 3.2B). Interestingly, the cathepsin B inhibitor not only blocked *Klebsiella* induced cell death in THP1 cells, but also prevented IL-1β release in response to the pathogen (Fig. 3.2B). This effect was also observed in bone marrow derived macrophages (Fig. 3.2C).

Additional features of *Klebsiella*-induced cell death are also consistent with pyronecrosis. As measured by western analysis, HMGB1 is released from *K. pneumoniae*

infected shCTRL cells but not shNLRP3 cells (Fig. 3.2D). HMGB1 is not released by THP-1 cells treated with staurosporine, a well-established inducer of apoptotic cell death (Fig 3.2D). During apoptosis, caspase-3 undergoes activating cleavage. In turn, caspase-3 cleaves PARP and other downstream substrates. Neither caspase-3 nor PARP were cleaved in shCTRL or shNLRP3 cells infected with *Klebsiella*, though both were cleaved in staurosporine-treated cells (Fig. 3.2E).

K. pneumoniae **induces chemotactic and inflammatory cytokine production in primary mouse macrophages**

The processing and release of proinflammatory cytokines and chemokines is fundamental to proper innate immune response to pathogens. Cell free supernatants prepared from *Klebsiella*- or *Salmonella*-infected macrophages were analyzed on anti-cytokine antibody arrays containing antibodies to 62 inflammatory mediators (For mediators induced ≥3 fold, see Fig. 3.3A. For complete set, see Table 3.1). Production of G-CSF, IL-1β, IL-1α, and IFNγ was markedly decreased in both ASC and NLRP3 deficient macrophages (Fig 3.3A). Modest decreases in CD62L, Fractalkine, and IL-3 receptor beta were also observed (Fig. 3.3A). IL-1β was not decreased in NLRP3 deficient macrophages treated with *Salmonella*, which activates the NLRC4 inflammasome. Of note, several inflammatory cytokines including MIP-1α, MIP-2, TNFα, and IL-6 were induced to a greater extent in macrophages lacking NLRP3, perhaps to compensate for the loss of IL-1β (Fig. 3.3A). To confirm the results obtained by the antibody array, a subset of inflammatory mediators were measured using quantitative multiplexed anti-cytokine arrays. Cytokine measurements of IL-

1β, IFNγ, and KC support the data obtained by the antibody arrays (Fig 3.3B, for complete list of quantified cytokines, see Table 3.2).

Mice lacking NLRP3 and ASC demonstrate significantly increased mortality following *K. pneumoniae* airway infection.

To determine whether NLRP3 and ASC are involved in mediating the host immune response to *K. pneumoniae in vivo*, we used a mouse airway infection model. Mice were intratracheally challenged with (4×10^4) CFUs of *K. pneumoniae* and survival was assessed over the course of 4 days. Mice lacking NLRP3 demonstrated significantly increased mortality compared with wild type mice ($p < 0.05$, Logrank Test) (Fig. 3.4). ASC deficient mice demonstrated similar increases in mortality. This increase in lethality was not associated with significant increases in either local or systemic bacterial burden as only subtle differences were detected (Fig. 3.7). No significant difference in survival was observed between mice lacking NLRC4 and wild type animals.

NLRP3 deficient mice demonstrate significantly attenuated airway inflammation following *K. pneumoniae* infection.

Our *in vitro* evidence suggested that NLRP3 reduction prevents *K. pneumoniae*-induced inflammation. To determine if inflammation was also reduced *in vivo*, NLRP3 deficient mice were challenged with (7.4×10^4) CFUs of *K. pneumoniae* for 48 hours and histology analysis was performed on lung sections cut to reveal the main bronchi of the large lobe. Representative sections from the apical region of the main bronchi of the large lobe (10x magnification) are shown from indicated genotypes (Fig. 3.5A). In comparison to WT and $NLRC4^{-/-}$ mice, $NLRP3^{-/-}$ mice show decreased inflammatory cell recruitment and less

occlusion of the alveolar spaces. These findings are consistent with a decrease in overall inflammation (Fig. 3.5A). To quantify this change each of 6 histology parameters was scored as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe): mononuclear cell infiltration; polymorphonuclear cell infiltration; airway epithelial cell hyperplasia/injury; extravasation; perivascular cuffing; and percent of lung involved with inflammation. The scores of the parameters were averaged for a total histology score. All mice demonstrated a significant increase in airway inflammation following *K. pneumoniae* challenge. However, a significant attenuation in airway inflammation was only observed in mice lacking NLRP3 (Fig. 3.5B).

In vivo levels of IL-1β and cell death are reduced in _K. pneumonia_ challenged NLRP3$^{-/-}$ mice.

NLRP3 is required for pyronecrosis in both human and mouse cells challenged with *Klebsiella*. To determine if NLRP3 was also required for the induction of pyronecrosis *in vivo*, we measured IL-1β levels in the bronchoalveolar lavage fluid (BALF) and serum of *K. pneumoniae* infected mice. In both cases, deletion of NLRP3 caused a reduction in IL-1β, with larger differences observed in the serum (Fig. 3.6A-B). This decrease in IL-1b was accompanied by an increase in IL-6 in serum and BALF samples (Fig 3.6C-D). Deletion of ASC also demonstrated marked decreases in circulating levels of IL-1β (Fig. 3.6A). Deletion of NLRP3 not only reduced IL-1β, but also decreased overall levels of cell death in BALF samples as determined by lactate dehydrogenase release (Fig. 3.6E). To examine whether NLRP3 is responsible for the induction of necrosis *in vivo*, HMGB1 levels were measured in serum samples of *K.pneumoniae* challenged mice. As measured by western blot analysis,

Klebsiella-induced HMGB1 release was substantially abrogated in NLRP3$^{-/-}$ mice (Fig. 3.6F).

3.5 Discussion

Several recent reports have established a role for NLR proteins in mediating pathogen-induced inflammation *in vitro*, but *in vivo* confirmation of these results has been lacking. Here, we identify NLRP3 as a critical effector of the host immune response to *K. pneumoniae*, a major cause of community-acquired bacterial pneumonia. This is the first demonstration that an NLR molecule participates in IL-1β maturation and cell death in response to *K. pneumoniae*. Furthermore, these results demonstrate the *in vivo* consequences of NLRP3 activity on host survival and inflammation. Despite substantial decreases in lung inflammation, mice lacking NLRP3 demonstrate increased susceptibility to *Klebsiella*-induced lethality. This finding confirms that NLRP3 activity contributes to protective host responses to bacterial pathogens.

Following *Shigella flexneri*, *K. pneumoniae* is the second gram negative bacterial pathogen identified which activates the NLRP3-dependent cell death program termed pyronecrosis (Willingham et al., 2007). This pathway of cell death has morphological features characteristic of necrosis, and like necrosis is inherently pro-inflammatory. Cellular components spill out from the pyronecrotic cell into the microenvironment. Among these components is HMGB1, a nuclear protein which takes on the role of a powerful pro-inflammatory cytokine when released from the cell (Sunden-Cullberg et al., 2006; Willingham et al., 2007). HMGB1 stimulates the RAGE, TLR2, and TLR4 receptors on neighboring monocytes and macrophages and results in the induction of several inflammatory cytokines, including TNFα, IL-1β, and IL-6 (Andersson et al., 2000; Hori et al., 1995; Park et al., 2004). *K. pneumoniae* induced a significant increase in the systemic levels of HMGB1 in the wild type mice, while no HMGB1 was observed in the serum from

NLRP3$^{-/-}$ animals. This NLRP3-dependent release of HMGB1 was also observed in human THP-1 monocytic cells challenged with *Klebsiella*. Previously, we demonstrated that caspase-1 inhibitors failed to abrogate NLRP3 mediated HMGB1 release, suggesting that this phenomenon does not require inflammasome activity (Willingham et al., 2007). It should be noted that HMGB1 levels are significantly increased in human septic patients, including those with *K. pneumoniae* sepsis (Wang et al., 1999). Neutralization of HMGB1 is currently under investigation as a therapeutic target for the intervention of sepsis, bacteremia, and induced acute respiratory distress syndrome (Abraham et al., 2000; Mantell et al., 2006). Though inhibition of NLRP3-dependent pyronecrosis may be detrimental to host survival, neutralization of HMGB1may provide an opportunity to abrogate NLRP3-mediated inflammation without increasing host mortality.

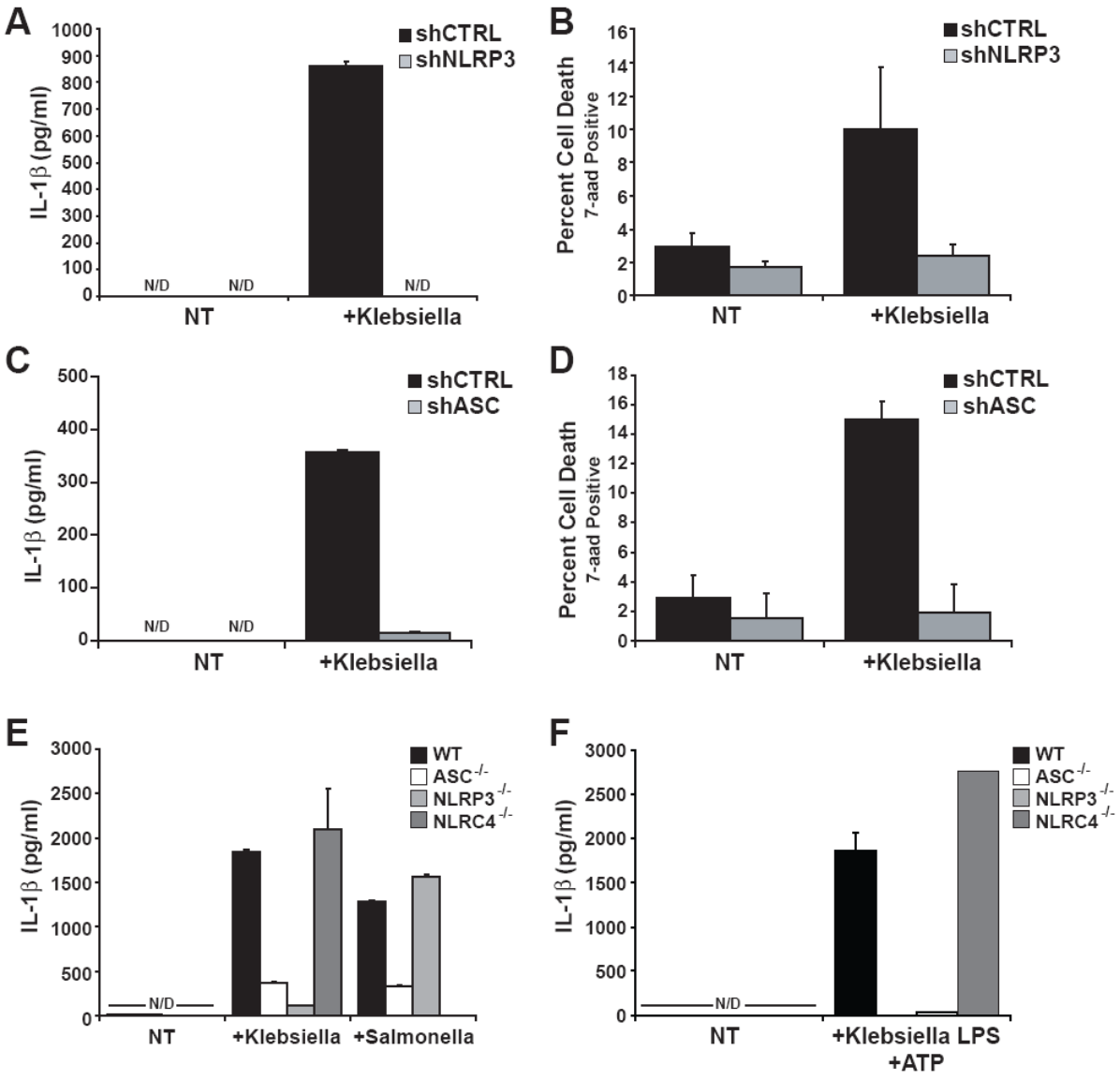
Our results indicate that NLRP3-dependent pyronecrosis is the predominant cell death and inflammation pathway induced by *Klebsiella*. In contrast to the *Salmonella* induced pyroptosis pathway, ablation of NLRC4 or caspase-1 has minimal effect on inflammation or cell death induced by *K. pneumoniae*. In some instances, pyronecrosis may be attenuated by high concentrations of pan caspase inhibitor (ZVAD-fmk) due to a known off target inhibition of cathepsin B (Schotte et al., 1999). Interestingly, cathepsin B inhibitors not only block NLRP3-mediated pathogen induced cell death, but also block the induction of IL-1β maturation by *K. pneumoniae*. This finding suggests that IL-1β release is primarily downstream of pyronecrosis, which is not surprising given the strong pro-inflammatory activity of HMGB1.

In addition to its clinical relevance, *Klebsiella* airway infection is one of the most thoroughly characterized mouse models of gram negative bacterial pneumonia and acute lung

injury with secondary bacteremia. (Lau et al., 2007; Lawlor et al., 2005). Amongst *K. pneumoniae* virulence factors identified in these models, production of a prominent capsule is the most well-characterized determinant of pathogenesis (Cortes et al., 2002; Favre-Bonte et al., 1999). Capsule production has been shown to enhance virulence primarily through protecting the pathogen from phagocytosis by macrophages and the initiation of an immune response. Currently, over 75 different capsule serotypes of *Klebsiella* have been classified based on the variable expression of 2 surface antigens, the lipopolysaccharide "O" antigen and the capsular polysaccharide "K" antigen. Approximately 9 different O antigens and over 75 K antigens have been identified, both of which contribute to pathogenicity (Podschun and Ullmann, 1998). In particular, *K. pneumoniae* serotype K2 is amongst the most clinically relevant serotypes due to its high incidence in both urinary tract infections and pneumonia cases and its marked virulence in mouse pneumonia models (Lau et al., 2007; Yu et al., 2007). Several previous reports have noted that serotype K2 lacks the mannose-α2/3-mannose sequence recognized by the macrophage mannose receptor, thereby increasing its resistance to phagocytosis (Kabha et al., 1995). As NLRP3 is expected to function as a cytosolic pathogen sensor, internalization of the bacteria or bacterial LPS is likely required for initiation of pyronecrosis. Thus, this increased resistance to phagocytosis likely contributes to the relatively high MOI and longer timepoints required for the NLRP3-mediated induction of IL-1β and our inability to detect *Klebsiella* induced cell death in bone marrow derived macrophages. In the future, it will be interesting to determine the influence of other capsule serotypes and mannose composition in triggering pyronecrosis. Though capsule K1 and K2 serotypes are most frequently associated with *in vivo* virulence, it must be

noted that capsule serotype is not an absolute determinant of virulence (Lau et al., 2007; Yu et al., 2007).

In summary, our results indicate that NLRP3 and ASC are key regulators of necrosis and inflammation associated with *K. pneumoniae* infections. The induction of NLRP3-dependent pyronecrosis by *K. pneumoniae* results in the extracellular release of HMGB1, further propagating host inflammation. Mice deficient in NLRP3 demonstrated significant reductions in both local and systemic inflammation and cell death when challenged with *Klebsiella*. However, these mice demonstrate increased mortality upon *Klebsiella*, indicating that pyronecrosis helps protect the host from pathogen induced death. Though future attempts to neutralize NLRP3-dependent pyronecrosis *in vivo* may result in decreased inflammation and necrotic tissue destruction, these studies suggest strong consideration of the detrimental consequences on host survival.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IL-1β (pg/ml)
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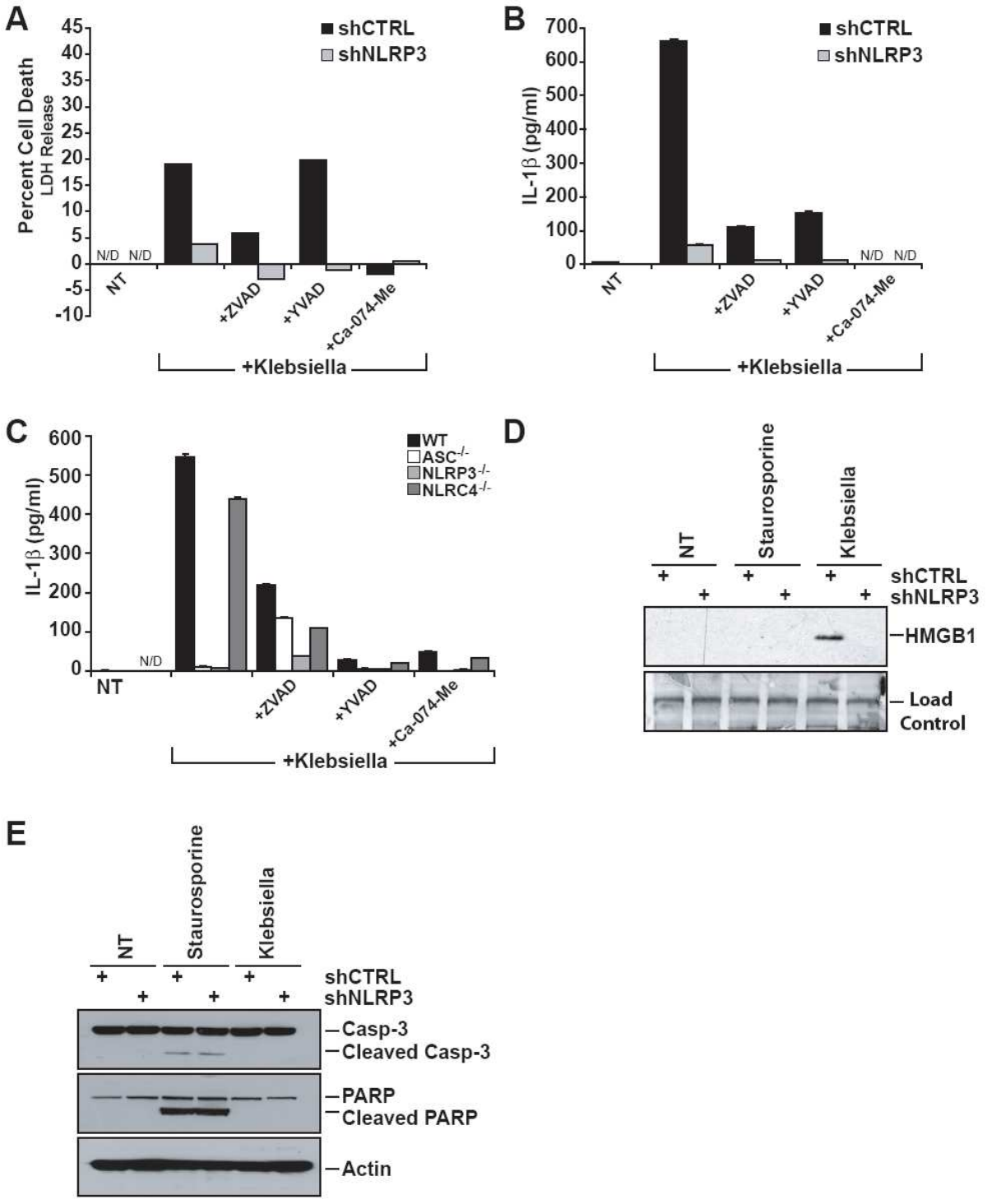
ASC-/-
NLRP3-/-
NLRC4-/-
N/D
NT
+Klebsiella
+Salmonella
F
IL-1β (pg/ml)
WT
ASC-/-
NLRP3-/-
NLRC4-/-
N/D
NT
+Klebsiella LPS
+ATP

Figure 3.1 - *Klebsiella pneumoniae* induced cell death and IL-1β require NLRP3 and ASC. A) *K. pneumoniae* induced IL-1β release is decreased in THP-1 cells stably transduced with NLRP3-specific shRNA (shNLRP3). B) shNLRP3-THP-1 cells are resistant to *K. pneumoniae*-induced death. C and D) ASC is required for *K. pneumonia*-induced IL-1β release (C) and cell death (D) in THP-1 cells. THP-1 cells were with infected with *Salmonella* and *Klebsiella* at a MOI=50 for 1 or 6 hours, respectively. E) *NLRP3*$^{-/-}$ and *ASC*$^{-/-}$ bone marrow-derived macrophages exhibit decreased levels of IL-1β release in response to *K. pneumoniae* but not *Salmonella typhi*.. *NLRC4*$^{-/-}$ macrophages demonstrate no defect in *K. pneumoniae* induced IL-1β release, but IL-1β release is attenuated in response to *S. typhi*. G) *K. pneumoniae* LPS (50 ng/ml, 16 hours) in combination with ATP (5 mM, 20 min.) stimulates NLRP3 and ASC-dependent, but NLRC4-independent, IL-1 β release from bone marrow derived macrophages. Bone marrow derived macrophages were infected with *Klebsiella* at a MOI=200 (6 hours) or *Salmonella* at a MOI=50 (1 hour).


A
Percent Cell Death
LDH Release
shCTRL
shNLRP3
N/D N/D
NT
+ZVAD
+YVAD
+Ca-074-Me
+Klebsiella
B
IL-1β (pg/ml)
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+ZVAD
+YVAD
+Ca-074-Me
+Klebsiella
C
IL-1β (pg/ml)
WT
ASC-/-
NLRP3-/-
NLRC4-/-
N/D
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shCTRL
shNLRP3
HMGB1
Load Control
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Casp-3
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PARP
Cleaved PARP
Actin

Figure 3.2 - *Klebisella pneumoniae* induces NLRP3 and Cathepsin B dependent pyronecrosis. A) 50 µM cathepsin B inhibitor (Ca-074-Me) substantially abrogates *K. pneumoniae* induced cell death (A) and IL-1β release (B) in shCTRL and shNLRP3 cells. In contrast, 100 µM pan-caspase (zVAD-fmk) and 100 µM caspase-1 specific (YVAD-CHO) inhibitors fail to block *K. pneumoniae*-induced cell death but do inhibit IL-1β release. C) 50 µm cathepsin B inhibitor (Ca-074-Me) also attenuates NLRP3- dependent IL-1β release in bone marrow derived macrophages stimulated by *K. pneumoniae*. D) *K. pneumoniae*-induced HMGB1 release is abrogated by shNLRP3. E) Caspase-3 and PARP are cleaved in response to an apoptotic stimulus (staurosporine), but not in THP-1 cells following *K. pneumoniae* infection. Cell death was measured by LDH release and IL-1β release was determined by ELISA.

A

>3 Fold Induction Over NT

	+Klebsiella			+Salmonella	
	WT	ASC $^{-/-}$	NLRP3$^{-/-}$	WT	NLRP3$^{-/-}$
MIP-1α	126	264	293	108	80
IL-1α	97	40	31	69	38
MIP-2	85	89	103	80	96
IL-6	82	80	156	19	22
G-CSF	82	44	29	4	1
TNFα	79	93	93	20	11
MCP-1	58	66	46	20	N/D
KC	55	38	66	49	46
RANTES	26	28	57	21	16
IL-12 P40/P70	14	18	19	2	2
IL-1β	12	1	3	11	10
TIMP1	8	4	5	2	1
IP10	5	4	5	1	1
FAS L	4	3	N/D	3	2
IL-17α	4	6	3	2	2
IL-3 Rβ	4	3	N/D	1	N/D
TPO	3	2	2	2	2
MCP-5	3	2	3	1	1
FRACTALKINE	3	2	1	1	1
VCAM1	3	N/D	3	2	2
IFNγ	3	1	1	1	1
CD30	3	1	2	1	2
CXCL4	3	4	4	1	1
CCL25	3	3	2	1	1
VEGF	3	2	3	1	0
IL-2	3	1	1	N/D	1

B

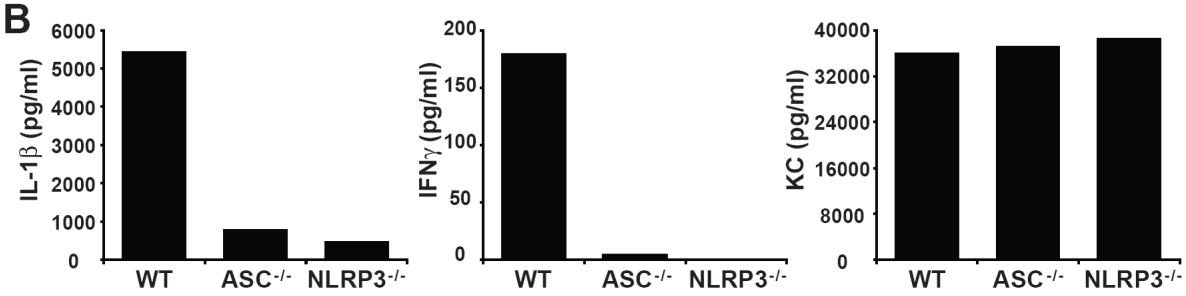


Figure 3.3 - *Klebsiella pneumoniae* induces inflammatory cytokines and chemokines in primary macrophages. A) Thioglycolate-elicted peritoneal macrophages of indicated genotypes were infected with *K. pneumoniae* (MOI=200, 6 hours) or S. typhi (MOI=50, 1 hour). Cell free supernatants were analyzed on RayBiotech G Series 3 cytokine antibody arrays. Cytokines and chemokines induced ≥3 fold over the non-treated control of each genotype are shown. For complete list of results, see Table 3.1) "N/D" designates samples which were not reliably measured. Quantification of IL-1β, IFNγ, and KC support trends observed on cytokine arrays. Cytokine levels were determined using RayBiotech custom Quantibody service. For the complete list of results, see Table 3.2.

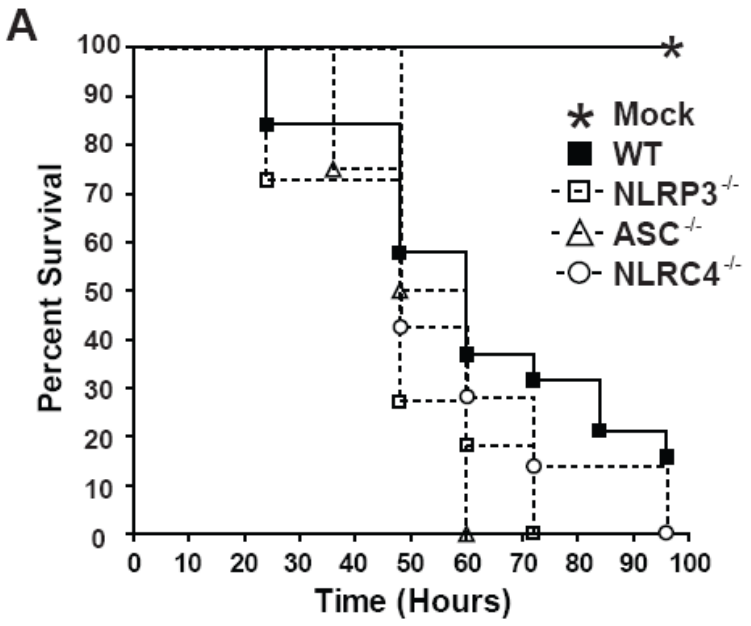
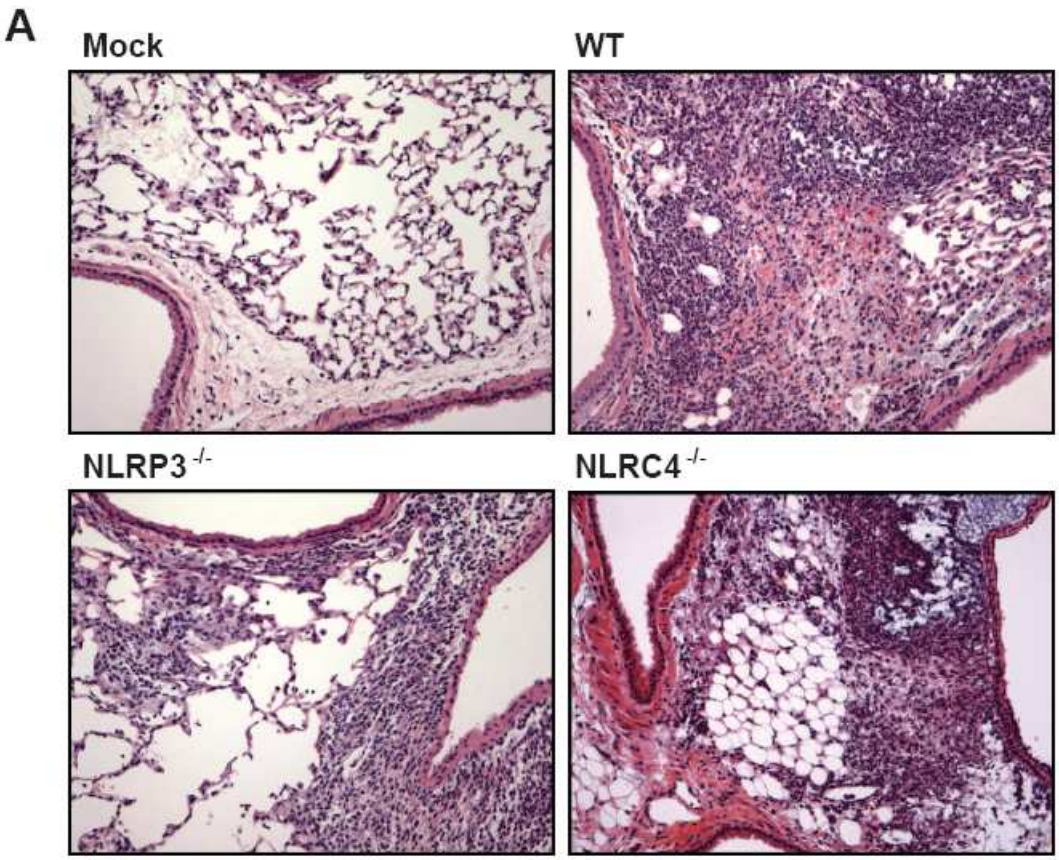


Figure 3.4 - NLRP3 and ASC deficient mice demonstrate significantly increased mortality following *K. pneumoniae* airway infection. Mice were intratracheally challenged with (7.5×10^4) CFUs of *K. pneumoniae* and survival was assessed over the course of 4 days. Mice lacking NLRP3 or ASC demonstrated significantly increased mortality compared with wild type mice ($p < 0.05$, Logrank Test). No significant difference in survival was observed between mice lacking NLRC4 and wild type animals.


A
Mock
WT
NLRP3 -/-
NLRC4 -/-

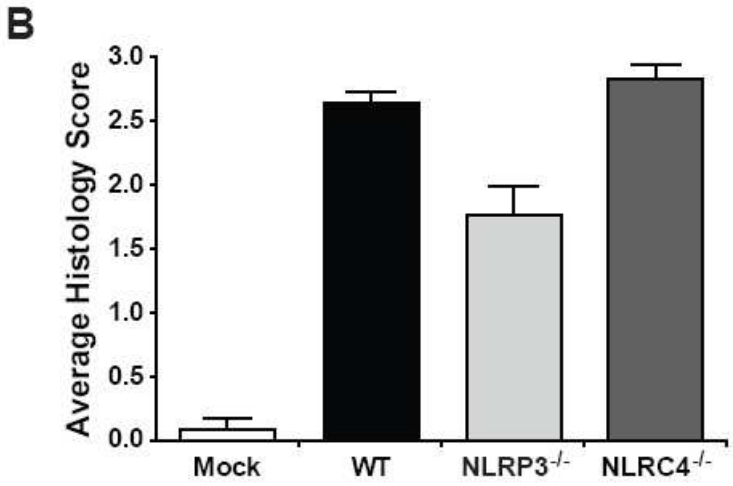

B
Average Histology Score
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Mock
WT
NLRP3-/-
NLRC4-/-

Figure 3.5: NLRP3 deficient mice demonstrate significantly attenuated airway inflammation following *Klebsiella pneumoniae* infection. A) In comparison to WT and NLRC4$^{-/-}$ mice, NLRP3$^{-/-}$ mice show decreased inflammatory cell recruitment and less occlusion of the alveolar spaces following K. pneumoniae infection. Mice were challenged with (7.4 x 10^6) CFUs of *K. pneumoniae* for 48 hours. Representative histology sections from the apical region of the main bronchi of the large lobe (10x magnification) are shown. B) Significant attenuation in airway inflammation is observed in *K. pneumoniae* challenged NLRP3$^{-/-}$ mice. Histology images were evaluated and seach of the following inflammatory parameters were scored between 0 (absent) and 3 (severe): mononuclear cell infiltration; polymorphonuclear cell infiltration; airway epithelial cell hyperplasia/injury; extravasation; perivascular cuffing; and percent of lung involved with inflammation. The scores of the parameters were averaged for a total histology score.

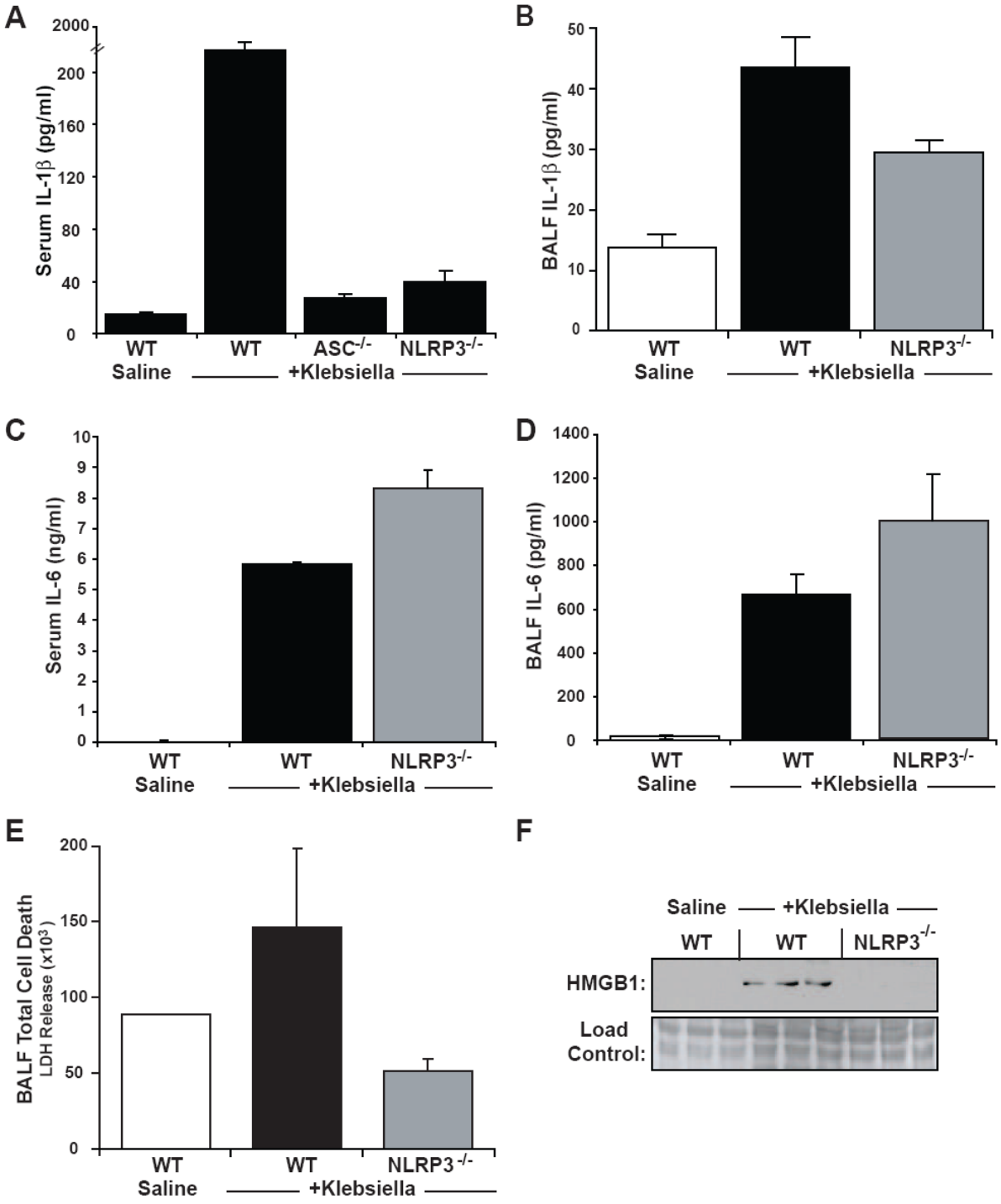

A
Serum IL-1β (pg/ml)
2000
200
160
120
80
40
0
WT
Saline
WT
ASC-/-
NLRP3-/-
+Klebsiella
B
BALF IL-1β (pg/ml)
50
40
30
20
10
0
WT
Saline
WT
NLRP3-/-
+Klebsiella
C
Serum IL-6 (ng/ml)
10
9
8
7
6
5
4
3
2
1
0
WT
Saline
WT
NLRP3-/-
+Klebsiella
D
BALF IL-6 (pg/ml)
1400
1200
1000
800
600
400
200
0
WT
Saline
WT
NLRP3-/-
+Klebsiella
E
BALF Total Cell Death
LDH Release (x10³)
200
150
100
50
0
WT
Saline
WT
NLRP3-/-
+Klebsiella
F
Saline
+Klebsiella
WT
WT
NLRP3-/-
HMGB1:
Load
Control:

Figure 3.6 - NLRP3 regulates *Klebsiella pneumoniae* induced IL-1β and necrotic cell death *in vivo*. A) Serum levels of IL-1β are substantially reduced in NLRP3$^{-/-}$ or ASC$^{-/-}$ mice challenged with *K. pneumoniae*. B) A modest, yet significant, decrease in K. pneumoniae induced IL-1β is observed in the bronchoalveolar lavage fluid (BALF) of NLRP3$^{-/-}$ mice compare to wildtype. In contrast, IL-6 levels are elevated in the serum (C) and BALF (D) of NLRP3-/- mice following. *K pneumoniae* infection. IL-1β and IL-6 were both measured by ELISA. E) Decreased levels of cell death are detected in the BALF of *K. pneumoniae* infected NLRP3 deficient mice as determined by LDH release. F) Serum levels of HMGB1 are dramatically reduced in NLRP3$^{-/-}$ mice as determined by western blot analysis.

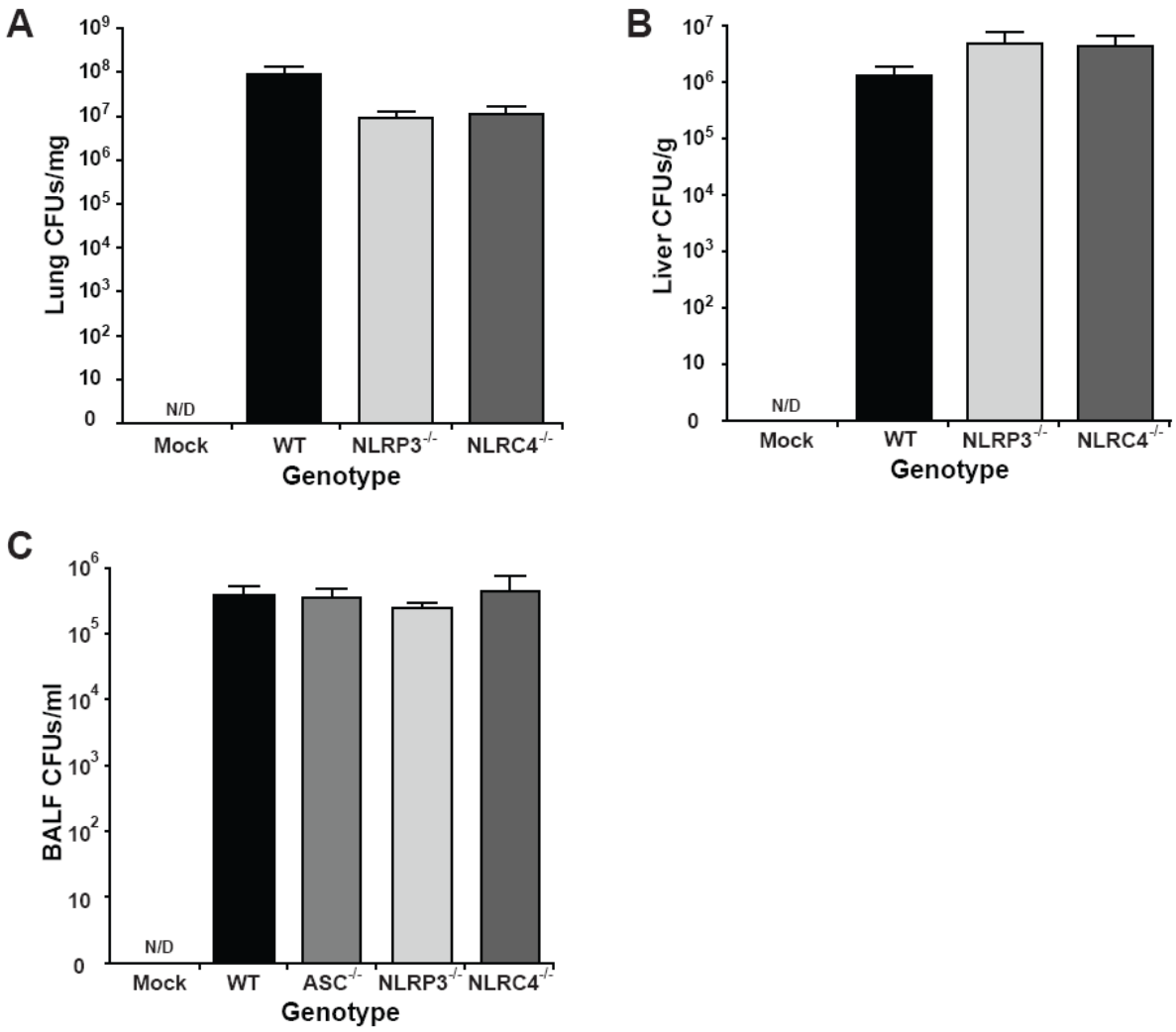


Figure 3.7 - Increased mortality of *Klebsiella pneumoniae* infected NLRP3$^{-/-}$ mice is not due to increased bacterial burden. Mice were euthanized via intraperitoneal (i.p.) injection with 2,2,2 tribromoethanol (avertin). Whole liver (gal bladder removed), spleen and lungs were removed, wet weight was assessed and the tissues were homogenized in 500 µl of Hanks balanced salt solution (HBSS) with a Tissue Master 125 hand held tissue homogenizer (Omni International). Homogenized tissues were centrifuged and the resulting supernatants were serially diluted and plated on LB agar plates and grown for 24 hours at 37^{o}C.

Fold Induction Over NT

ID	+Klebsiella			+Salmonella	
	WT	ASC$^{-/-}$	NLRP3$^{-/-}$	WT	NLRP3$^{-/-}$
AXL	1	1	1	1	1
CCL1	2	1	1	1	1
CCL17	1	1	1	1	1
CCL25	3	3	2	1	1
CCL27	1	1	1	1	1
CD30	3	1	2	1	2
CD30L	2	1	N/D	1	1
CD40	2	2	3	2	2
CXCL12	1	1	2	1	1
CXCL13	1	0	1	0	1
CXCL16	1	1	1	0	0
CXCL4	3	4	4	1	1
CXCL5	2	3	3	N/D	1
EOTAXIN	1	1	1	1	2
EOTAXIN-2	2	2	2	1	2
FAS L	4	3	N/D	3	2
FRACTALKINE	3	2	1	1	1
G-CSF	82	44	29	4	1
GM-CSF	1	1	1	1	1
IFNγ	3	1	1	1	1
IGFBP-6	1	2	1	1	1
IL-10	1	1	2	1	1
IL-12 P40/P70	14	18	19	2	2
IL-12 P70	1	1	1	1	1
IL-13	1	2	2	1	1
IL-17α	4	6	3	2	2
IL-1α	97	40	31	69	38
IL-1β	12	1	3	11	10
IL-2	3	1	1	N/D	1
IL-3	1	3	1	1	1
IL-3 Rβ	4	3	N/D	1	N/D
IL-4	1	1	1	1	1
IL-5	1	1	1	1	1
IL-6	82	80	156	19	22
IL-9	1	1	1	1	1
IP10	5	4	5	1	1
KC	55	38	66	49	46
LEPTIN R	2	1	1	2	1
LYMPHOTACTIN	2	1	N/D	1	N/D
MCP-1	58	66	46	20	N/D
MCP-5	3	2	3	1	1
M-CSF	2	2	1	1	1
MIP-1α	126	264	293	108	80
MIP-1γ	1	1	1	1	1
MIP-2	85	89	103	80	96
MIP-3α	1	1	1	1	1
MIP-3β	1	N/D	2	2	1
RANTES	26	28	57	21	16
SCF	1	1	1	1	1
SELP	1	1	1	1	1
sTNF RI	1	1	1	1	1
sTNF RII	2	2	3	2	2
TIMP1	8	4	5	2	1
TNFα	79	93	93	20	11
TPO	3	2	2	2	2
VCAM1	3	N/D	3	2	2
VEGF	3	2	3	1	0

Table 3.1 Results of all cytokines and chemokines profiled using RayBiotech G Series 3 cytokine antibody array. Thioglycolate-elicted peritoneal macrophages of indicated genotypes were infected with *K. pneumoniae* (MOI=200, 6 hours) or S. typhi (MOI=50, 1 hour). Cell free supernatants were analyzed on RayBiotech G Series 3 cytokine antibody arrays. “N/D” designates samples which were not reliably measured.

A

Cytokine (pg/ml)	+Klebsiella		
	WT	ASC $^{-/-}$	NLRP3 $^{-/-}$
GM-CSF	0	35	0
IFNγ	179	4	0
IL-1α	6258	6859	4687
IL-1β	5455	777	460
IL-2	2	20	13
IL-3	0	0	0
IL-4	0	1	0
IL-5	0	0	0
IL-6	13042	15840	7901
IL-9	9	25	0
IL-10	289	78	76
IL-12	169	231	0
IL-13	0	0	1
IL-17α	195	519	68
KC	36031	37240	38640
MCP-1	3709	5183	3679
M-CSF	1	54	0
RANTES	22593	27708	25863
TNFα	3582	4964	2256
VEGF	850	786	751

Table 3.2: All cytokines and chemokines quantified by RayBiotech custom Quantibody service.

CHAPTER IV

CONCLUSIONS & FUTURE DIRECTIONS

4.1 Conclusions & Future Directions

The discovery of NLR-regulated cell death and inflammatory pathways has opened several interesting opportunities for future research. No doubt, additional bacterial, viral, and environmental NLR activators will continue to be identified and will further our understanding of the interplay that occurs between NLRs in immunity. Given over 20 NLR molecules have been identified, both with pro- and anti-inflammatory properties, some degree of cooperation and antagonism is expected, as is some functional redundancy. Beyond the characterization of the NLRs and their activating stimuli, three critical questions remain unanswered: 1) What signaling pathways involved in pyronecrosis and pyroptosis? 2) Why do cathepsin B inhibitors block NLRP3 inflammasome activity? and 3)Why are the NLRP3 deficient mice more susceptible to pathogen induced lethality?

1 – What signaling pathways involved in pyronecrosis and pyroptosis?

The greatest opportunity raised by this work is the characterization of the pyronecrosis and pyroptosis pathways. At this time, the two cell death pathways are distinguished only by the requirement of caspase-1 and much work is needed to fully elucidate pyronecrosis and pyroptosis signaling. We've shown that NLRP3, ASC, and the lysosomal protease cathepsin B are required to initiate pyronecrosis, a caspase-1 independent form of necrotic cell death. However, it is unclear how and where cathepsin B interacts with NLRP3 or ASC. Our preliminary data suggests that NLRP3 is required for pathogen-induced cathepsin B activation. It's possible that cathepsin B interacts with ASC and NLRP3 within a complex similar to the apoptosome or inflammasome, which we term the necrosome. The

existence of this complex could be evaluated by co-immunoprecipitation assays and/or the purification of high molecular weight complexes containing NLRP3, ASC, and cathepsin B. Given the ability of disease-associated NLRP3 mutants to induce pyronecosis, novel components of the necrosome and pyronecrosis pathway could be identified by mass spectrometry analysis following immunoprecipitation of mutant NLRP3. Finally, the use of small molecule inhibitors of known cell death mediators may be effective in identifying components of the pyronecrosis and pyroptosis pathways. This approach has proven useful in identifying cathepsin B and caspase-1 as a mediators of pyronecrosis and pyroptosis and may yield additional results.

Several opportunities are available in the characterization of pyroptosis also. Though caspase-1 inhibitors fail to abrogate pyronecrosis, the ability of cathepsin B inhibitors to block pyroptosis has not been investigated. This could be evaluated by determining if cathepsin B inhibitors block *Salmonella* or *Pseudomonas* induced cell death, two known activator of NLRC4-dependent pyroptosis (Brennan and Cookson, 2000; Franchi et al., 2007; Sutterwala et al., 2007). Our lab and others have confirmed the requirement of caspase-1 in pyroptosis initiated by *Salmonella,* however the events leading to caspase-1 activation are worthy of additional investigation. Both NLRC4 and ASC deficient macrophages fail to activate caspase-1 in response to *Salmonella typhimurium* and *Pseudomonas aeruginosa***,** an event presumably required to initiate caspase-1 dependent cell death (Mariathasan et al., 2004; Sutterwala et al., 2007). However, $ASC^{-/-}$ macrophages demonstrate no defect in *Pseudomonas* induced cell death and only a partial decrease in *Salmonella* induced pyroptosis. This suggests that in the absence of ASC, NLRC4 initiates cell death in a

caspase-1 independent manner. It's possible that this involves another caspase, such as caspase-11, but this backup mechanism is currently unknown.

2 - Why do cathepsin B inhibitors block NLRP3 inflammasome activity?

Biochemical studies have identified a number of inflammasomes, which promote inflammation by activating caspase-1, resulting in the release of the pyrogenic cytokine IL-1β and IL-18 from cells treated with different stimuli (Agostini et al., 2004; Duncan et al., 2007) (Fig 1.6). The NLRP3 inflammasome consists of NLRP3, ASC, and CARDINAL, which aggregate and activate pro-caspase-1 in an induced proximity model analogous to the activation of caspase-8 and caspase-9 (Agostini et al., 2004; Martinon et al., 2002). We have demonstrated that cathepsin B inhibitors not only inhibit pyronecrosis, but also NLRP3 mediated IL-1β release in response to pathogens. This suggests a previously unrecognized role for cathepsin B in regulating inflammasome activity. The mechanism of this inhibition is currently unknown. The simplest explanation is that cathepsin B is required for cleavage and activation of caspase-1, though this is untested. Also unknown is whether this effect is specific for the NLRP3 inflammasome or if cathepsin B inhibitors regulate the NLRC4 and NLRP1 inflammasomes as well. Answering these questions will significantly impact our understanding of inflammasome function and may fundamentally alter our perception of how the NLRs elicit inflammation and cell death.

3 - Why are the NLRP3 deficient mice more susceptible to *K. pneumoniae* induced lethality?

Few others have attempted to evaluate the *in vivo* relevance of NLRs in response to pathogens, often with lackluster results. The dramatic defects associated with NLR

deficiency *in vitro* are typically much more subtle when evaluated in animal models, perhaps reflecting NLR functional redundancy and cell type specific functionality. We have demonstrated that mice lacking NLRP3 exhibit significant decreases in lung inflammation following pulmonary infection with *K. pneumoniae.* However, these mice are more susceptible to *K. pneumoniae* induced lethality compared to controls. Cumulatively, these results demonstrate that NLRP3 activity is beneficial to the host in defense against *K. pneumoniae,* though the cause of the increased NLRP3$^{-/-}$ mortality is currently unknown.

In plants, the hypersensitive response initiated by R genes is required for the inhibition of pathogen growth, thus bacterial burden was expected to be substantially higher in NLR deficient mice (Mackey et al., 2002; Morel and Dangl, 1997). Indeed, NLRC1 and NLRC2 are required for *L. monocytogenes* clearance and host survival (Kim et al., 2008). Similar modest increases in pathogen expansion were observed in NLRC4$^{-/-}$ mice injected with *P. aeruginosa*, though this was not associated with a loss of viability *(Franchi et al., 2007; Sutterwala et al., 2007).* However, NLRP3 deficient were not more permissive to *K. pneumoniae* growth or systemic dissemination, indicating that pathogen replication is unlikely responsible for this effect.

While NLRP3$^{-/-}$ mice demonstrated dramatic decreases in IL-1β and HMGB1, they also had significant increases in IL-6 as well as several other inflammatory mediators after *K. pneumoniae* challenge. This may be a compensatory response for the loss of IL-1β and may contribute to the increased mortality, though this has not been evaluated. Finally, it's possible that the 24-36 hour delay *in K. pneumoniae* induced lethality is simply a combination of several minor factors. This could include the bacterial growth, cytokine production, and the actions of other NLRs. In the future, it will be interesting to revisit these *in vivo* pathogen

experiments in mice deficient for several NLRs, thereby eliminating any confounding functional redundancy.

4.2 Final Acknowledgements

I sincerely thank my mentor, Dr. Jenny Ting for her support and guidance. My graduate experience owes everything to the time, effort, and resources she's put into my training. The skills and experience I take from her lab have made me a better person and better scientist and have prepared me well for the future ahead. I thank her for everything she has done for me, without her none of this was possible.

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