The Role of Cell Death in Interleukin-1beta Activation and Secretion

a thesis submitted in total fulfilment of the degree of Doctor of Philosophy

by

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Abstract

The innate immune system can detect the presence of danger and pathogens through receptors that activate both inflammatory cytokine production and cell death. These programmed cell death pathways therefore play critical roles in protecting the host through driving protective inflammation and innate immunity. This thesis focuses on the cell death pathways of pyroptosis, apoptosis and necroptosis; the ways in which these signalling pathways overlap, as well as mechanisms by which these pathways activate inflammation and innate immunity through the cytokine interleukin-1β (IL-1β).

Pyroptosis is a lytic form of cell death activated upon innate immune receptor sensing of pathogens or damage to host cells. This caspase dependent form of cell death is driven by the activation of inflammatory caspases upon multiprotein platforms termed inflammasomes. These same caspases simultaneously process and activate inflammatory cytokines such as IL-1β and interleukin-18 (IL-18). Pyroptosis is therefore associated with the release of cytokines and other cellular proteins through rupture of the plasma membrane. What remains under contention is whether cell death is required for IL-1β release or whether active secretion of IL-1β can occur. I address this topic using molecular tools that allowed direct activation of the inflammatory caspase, caspase-1, limiting simultaneous activation of inflammatory signalling pathways. In doing so I experimentally separate the two functions of caspase-1, pyroptotic cell death and cytokine activation, providing evidence that IL-1β can be actively secreted from cells.

The same molecular tools allow me to directly examine whether the apoptotic caspase, caspase-8, is capable of activating IL-1β through enzymatic processing. This is important as it is usually assumed that apoptotic caspases kill cells in an immunologically silent manner. By demonstrating the ability of caspase-8 to directly activate pro-inflammatory IL-1β I am contributing to a growing field of literature highlighting the inflammatory potential of caspase-8 signalling.

Finally, this thesis explores the molecular mechanisms through which mixed lineage kinase-like (MLKL) mediated necroptotic signalling activates innate immunity via the activation of caspase-1. Like pyroptosis, necroptosis is an inflammatory and lytic form of

cell death that results in the release of pro-inflammatory cellular contents. In addition, it has been shown to induce the formation of the NOD (nucleotide-binding oligomerization domain)-like receptor family pyrin domain containing protein-3 (NLRP3) inflammasome to drive caspase-1 activation. I investigate the mechanisms by which necroptosis activates this inflammasome, demonstrating that this activation is a cell intrinsic process, activated prior to the total rupture of the plasma membrane and death of cells. In addition, I examine the contribution of NLRP3-caspase-1 activation of IL-1β to innate immune responses that are triggered in healthy bystander cells following necroptotic cell death. Notably, these results reveal that MLKL-dependent activation of IL-1β has a greater role to play in activating neighbouring innate immune cells then MLKL-driven necroptosis alone.

The pathways of apoptosis, pyroptosis and necroptosis all contribute to immunity. In addition, they are each implicated in a range of inflammatory diseases. As such, understanding the crosstalk between these pathways of programmed cell death, as well as the ways in which these pathways can activate inflammation, will be necessary to be able to accurately therapeutically target these pathways in distinct inflammatory diseases.

Declaration

This is to certify that:

This thesis comprises only my original work towards the Doctor of Philosophy except where indicated in the preface.

Due acknowledgment is made for the work of other authors.

This thesis is fewer than 100,000 words, exclusive of tables and bibliographies.

Stephanie A Conos

Preface

All of the work presented in this thesis was performed at the Walter and Eliza Hall Institute of Medical Research, University of Melbourne, Melbourne Australia.

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My overall contribution of work presented in this thesis is 95%.

The following authors contributed data within Chapter 4: Kaiwen Chen and Kate Schroder contributed data to Figures 4.3F and 4.3G, S 4.2A and S 4.2B. Dominic De Nardo assisted with protocol design for ASC speck visualisation and took confocal microscopy images for Figures 4.4F and 4.4G, S 4.4A and S 4.4B, S 4.5A and S 4.5B. Lachlan Whitehead quantified ASC speck formation for the same figures. Hideki Hara and Gabriel Núñez performed mass spectrometry for Figures 4.2E and S 4.1D.

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I belonged to 2 different lab groups, but spent time (and stole reagents) from at least 5, I'd therefore like to thank all the great people that I've worked with over my years at WEHI for their generosity with reagents, ideas and their friendships.

Finally, I'd like to thank my family, for never doubting that I could or should do a PhD. My friends for being a great distraction when I needed it. And my partner Michael, for his patience and support.

Abbreviations

4HB	4 helical bundle
AIM2	absent in melanoma 2
AP-1	activator protein 1
APAF1	apoptotic protease activating factor 1
ASC	apoptosis-associated speck-like protein containing a CARD
ATP	adenosine triphosphate
BCA	bicinchoninic acid assay
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BIR	baculovirus inhibitor of apoptosis repeat domains
BMDM	bone marrow derived macrophages
CA	constitutively active MLKL mutant
CAPS	cryopyrin-associated periodic syndrome
CARD	caspase activation and recruitment domain
Cp.A	Smac-mimetic Compound A
CYLD	cylindromatosis
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	danger associated molecular pattern
DD	death domain
DED	death effector domain
DISC	death inducing signalling complex
DNA	deoxyribonucleic acid
DME	Dulbecco's modified Eagle's medium
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinase 1/2
FADD	Fas associated death domain
FasL	Fas Ligand
FCAS	familial cold auto-inflammatory syndrome
FCS	foetal calf serum
FMF	familial mediterranean fever
GFP	green fluorescent protein
GSDM-D	Gasdermin-D
h	hour
HCl	hydrochloric acid
HMGB1	high mobility group box 1
HSP	heat shock protein
IAP	inhibitor of apoptosis protein
IFNγ	Interferon-gamma
IL	Interleukin
IL-18BP	Interleukin-18 binding protein
IL-1RI and IL-1RII	type I and type II Interleukin-1 receptor
IL-1Ra	interleukin-1 receptor antagonist
IL-1R-AcP	interleukin-1 receptor accessory protein
IL-1R	interleukin-1 receptor

IPAF	Interleukin converting enzyme (ICE) protease-activating factor
IPS-1	Interferon promoter stimulator-1
IRAK	IL-1 receptor–associated kinase
IRF-3	Interferon regulatory factor 3
JNK	c-Jun NH2-terminal kinase
Gp130	glycoprotein 130 receptor
L	litre
LB	Luria broth
LDH	lactate dehydrogenase
LGP2	laboratory of genetics and physiology 2
LPS	lipopolysaccharide
LRR	leucine rich repeat domain
LUBAC	linear ubiquitin chain assembly complex (LUBAC)
m-	milli
M	molar
min	minute
MAL	MyD88-adaptor-like
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signalling
MEF	mouse embryonic fibroblast
MDA5	melanoma differentiation-associated gene 5
MHC	major histocompatibility complex
MLKL	mixed lineage kinase-like
MyD88	myeloid differentiation primary-response protein 88
MWS	Muckle-Wells syndrome
NACHT	domain present in Naip, CIITA (MHC class II transactivator), *HET-E* and TP-1 (telomerase-associated protein 1)
NAIP	neuronal apoptosis inhibitor protein
NBT	nitroblue tetrazolium chloride
NF-κB	nuclear factor-κB
NLR	nucleotide-binding oligomerization domain (NOD)-Like receptor
NLRC	NLR family CARD domain containing protein
NLRP	NLR family pyrin domain containing protein
NOD	nucleotide-binding oligomerization domain
NOMID/CINCA	neonatal onset multi-systemic inflammatory disease/ chronic infantile neurological cutaneous articular syndrome
NTD	N-terminal domain
P3C	Pam3CysK
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
PMA	phorbol 12-myristate 13-acetate
PMS	phenazine methosulfate
PYD	pyrin domain
RIG-I	retinoic acid-inducible gene I
RHIM	RIP homotypic interaction motif
RIPK	receptor interacting protein kinase
RNA	ribonucleic acid

ROS	reactive oxygen species
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SYK	spleen tyrosine kinase
TA	Tris-acetate
TICAM-1	TIR-domain-containing adaptor molecule 1
TIRAP	TRIF-related adaptor protein
TNF	tumour necrosis factor
TNFSF	TNF superfamily member
TNFR	tumour necrosis factor receptor
TRADD	TNF receptor associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
TRAM	TRIF-related adaptor molecule
TRAPS	TNF receptor-associated periodic syndrome
TRIF	TIR domain-containing adaptor inducing IFN-β
Tx	Triton X-100
WT	Wild-type

Publications

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Chapter 1: Introduction

The immune system is the body's defence mechanism against pathogens. It consists of two related programmes, innate and adaptive immunity (Janeway et al., 2001). Programmed cell death pathways contribute to immunity by activating inflammatory signalling and aiding clearance of microbial infected host cells. This introduction chapter covers the ways in which the programmed cell death pathways of apoptosis, pyroptosis and necroptosis activate innate immunity and host defence against pathogens.

Firstly, a brief summary of these cell death pathways will be covered. Following this, there will be an exploration of innate immunity, including critical receptors and signalling pathways (section 1.2.1). Particular focus will be given to the NLRP3 receptor (section 1.3.7), and the cytokine IL-1β (section 1.5), as these are the key focus of experiments within chapters 3 and 4. Finally, a deeper analysis will be given to the ways in which the cell death pathways of pyroptosis, apoptosis and necroptosis can activate NLRP3 and IL-1β and in doing so drive innate immune responses (section 1.7). The conclusion of this literature review will come with a discussion of the thesis statement, hypotheses and aims.

1.1 Programmed cell death

As recently as 15 years ago, cell death was defined as a dichotomous system, cells either died through a programmed form of cell death coined apoptosis, or via necrosis (Cookson and Brennan, 2001). Although necrosis is still defined as a passive and lytic form of cell death new pathways of programmed necrotic cell death have since been defined. The pathways of pyroptosis and necroptosis differ from apoptosis in key ways (Table 1.1).

Table 1.1 Different forms of programmed cell death

	Apoptosis	**Pyroptosis**	**Necroptosis**
Programmed pathway?	Yes	Yes	Yes
Caspase mediated?	Yes	Yes	No
Lytic plasma membrane rupture?	No	Yes	Yes
Considered to be inflammatory?	No	Yes	Yes

1.1.1 Apoptosis

Apoptotic cell death is required for both embryonic development, homeostasis of the immune system and prevention of autoimmune diseases (Lamkanfi and Dixit, 2010). Two pathways activate apoptosis, the first is triggered within a cell and is termed cell intrinsic and the second is triggered from outside the cell, for example by death receptor signalling, termed cell extrinsic (Strasser et al., 2000). Each of these pathways converge upon the activation of apoptotic caspases and the destruction of the cell in an immunologically silent manner. Apoptotic cell death also represents a major mechanism by which pathogens are controlled (Lamkanfi and Dixit, 2010), in that it promotes the phagocytosis of infected cells, thereby limiting pathogenic infections (Ebert et al., 2015, Speir et al., 2016).

Morphologically, apoptotic cells break down into membrane bound fragments that are rapidly cleared through phagocytosis. The coordinated dismantling of the cell during apoptosis, and the production of ‘eat-me’ signals that are displayed upon apoptotic bodies, means that apoptosis proceeds without incurring secondary inflammatory events. In fact, the processes activated during apoptosis may be actively immunosuppressive and limit inflammatory cytokine production (Savill et al., 2002, Rongvaux et al., 2014, White et al., 2014).

1.1.2 Apoptotic caspases, roles and activation

Caspases are a family of cysteine proteases which initiate cell death signalling by modifying protein functions through cleavage (Riedl and Salvesen, 2007). They are broadly categorized as either apoptotic or inflammatory caspases, although the apoptotic caspase-8 has functions in both categories. Apoptotic caspases can be divided into two subgroups; initiators caspases, and their substrates the effector caspases. Apoptotic stimuli activate the initiator caspases-2, -8, -9 and -10 by dimerization induced conformational changes (Boatright et al., 2003). Once activated these initiator caspases then mediate apoptosis through the proteolytic processing of downstream executioner caspases, caspase-3, -6 and -7. This second subgroup of caspases exist as inactive dimers and require processing into a small and large subunits to become catalytically active (Boatright and Salvesen, 2003). Once activated, executioner caspases cleave a large range of substrates which ultimately drives the destruction of the cell (Taylor et al., 2008). Although apoptosis is usually considered immunologically silent this thesis focuses on ways in which

apoptotic and pyroptotic activation of caspase-8 can be inflammatory (covered in section 1.10).

1.1.3 Inflammatory caspases and pyroptosis

Like apoptosis, pyroptosis is a programmed cell death pathway that results from the activation of caspases (Fink and Cookson, 2005). First described to occur following bacterial infection (Zychlinsky et al., 1992), pyroptosis is activated as part of an innate immune response to pathogenic infection or tissue injury and is inherently inflammatory (Cookson and Brennan, 2001). Unlike apoptosis, pyroptosis results in the activation of the inflammatory caspases, caspase-1 and caspase-11 in mice and caspase-1, -4 and -5 in humans. Innate immune receptors that form inflammasome protein complexes mediate the aggregation and activation of caspase-1 to cause pyroptotic killing (covered in detail in section 1.3.6). On the other hand, caspase-11, and the human orthologues, caspase -4 and -5, are unique amongst caspases. These caspases have been shown to directly bind cytosolic bacterial lipopolysaccharide (LPS) (Shi et al., 2014) or host derived oxidized phospholipids (Zanoni et al., 2016) and undergo auto-activation. The complexes formed during this process have been termed non-canonical inflammasomes (Broz and Dixit, 2016). Both active caspase-1 and -11 can process gasdermin-D (GSDM-D) at the same cleavage site, resulting in an active GSDM-D amino-terminal fragment that forms pores in the plasma membrane to induce a lytic cell death phenotype (He et al., 2015, Kayagaki et al., 2015, Shi et al., 2015, Liu et al., 2016).

The formation of plasma membrane pores, cell swelling and rupture are all features of inflammatory-caspase and GSDM-D mediated pyroptosis, which results in the inflammatory and non-specific release of cell components (Bergsbaken et al., 2009). In addition, caspase-1 activates pro-inflammatory IL-1 family cytokines, IL-1β and IL-18, through proteolysis (Fantuzzi and Dinarello, 1999). These two processes of lytic cell death and cytokine activation and release occur simultaneously under most experimental conditions. Despite this, there is no clear consensus on whether cell death is required for the release of IL-1β. One of the aims of this thesis was to address this question of whether IL-1β can be actively secreted.

1.1.4 Necroptosis

Necroptosis occurs in the absence of caspase activity; however; similar to inflammatory caspase mediated pyroptosis, necroptosis is characterised by plasma membrane disruption and rapid cell lysis. The pathway of programmed necrosis was first described with the finding that TNF could activate cell death that was either apoptotic and necrotic in morphology (Laster et al., 1988). TNF induced necroptosis is triggered following receptor interacting protein kinase 3 (RIPK3) phosphorylation of MLKL, which results in a conformational change, oligomerisation and translocation of the MLKL to cellular membranes (Murphy et al., 2013, Hildebrand et al., 2014) (Covered in detail in section 1.11.1). Subsequent cell death involves membrane disruption and cellular rupture, although the exact mechanism of this process remains undefined.

Like pyroptosis, necroptosis is considered inflammatory due to the release of cellular components. Despite this, there are few studies that investigate the release of DAMPs and the affect that this has on the immune system (Kaczmarek et al., 2013). In fact, one study concluded that it was not the necroptotic death of cells, but RIPK3-dependent upregulation of inflammatory signalling pathways, such as nuclear factor-κB (NF-κB) prior to death, which activated an immune response (Yatim et al., 2015). It has also been reported that necroptotic signalling mediates the activation of IL-1β through the NLRP3 inflammasome (Lawlor et al., 2015, Kang et al., 2013, Kang et al., 2015). This crosstalk between necroptosis and innate immunity, in particular the mechanism by which necroptotic signalling can activate NLRP3, is the focus of chapter 4 within this thesis.

Ultimately apoptosis, necroptosis and pyroptosis all contribute to immunity by eliminating infected cells, reducing the burden of infection and thus restricting the growth of pathogens. They simultaneously contribute to immune defences through the activation of inflammatory cytokines and chemokines. Necrotic cell death programmes such as pyroptosis and necroptosis further contribute to immune activation by releasing cell debris that acts as danger signals to activate innate immune receptors, while simultaneously promoting cross presentation and adaptive immune activation.

1.2 Immunity

The first line of response against invasive microbes is the innate immune system. This is mediated by tissue resident immune cells that detect pathogens and clear them through engulfment (Janeway et al., 2001). It is non-specific, mediated by local and systemic inflammation that limits bacterial growth, enhances removal and activates host wound healing. Innate immunity also plays a crucial role in facilitating the activation and targeting of the adaptive immune system. This second arm of immunity, adaptive immunity, provides a more refined, albeit slower, defence against pathogens. It targets pathogens in a specific manner, and contributes to the development of long term immune memory that protects against subsequent reinfection. Crucial to the coordination of both innate and adaptive immunity are cytokines, which mediate the communication between the first cells to detect the presence of danger and the broader immune system.

1.2.1 Innate Immunity, inflammation and cytokines.

The first defence infecting pathogens must overcome are physical barriers such as the skin and epithelial cell lining of the internal respiratory, gastrointestinal and urogenital tracts. Once this barrier is breached microbes come in contact with tissue resident innate immune cells such as macrophages and dendritic cells (Figure 1.1), which are able to detect the microorganisms through surface and cell-internal receptors that activate immune signalling (Sims and Smith, 2010). They further mediate the clearance of pathogens through engulfing and destroying them in digestive phagolysosomes. A critical part of this acute immune response is the activation and release of cytokines from innate immune cells. Cytokines released alongside other proteins and chemokines from cells at the site of injury or infection mediate a protective process of inflammation both on a local and systemic level (Gruys et al., 2005).

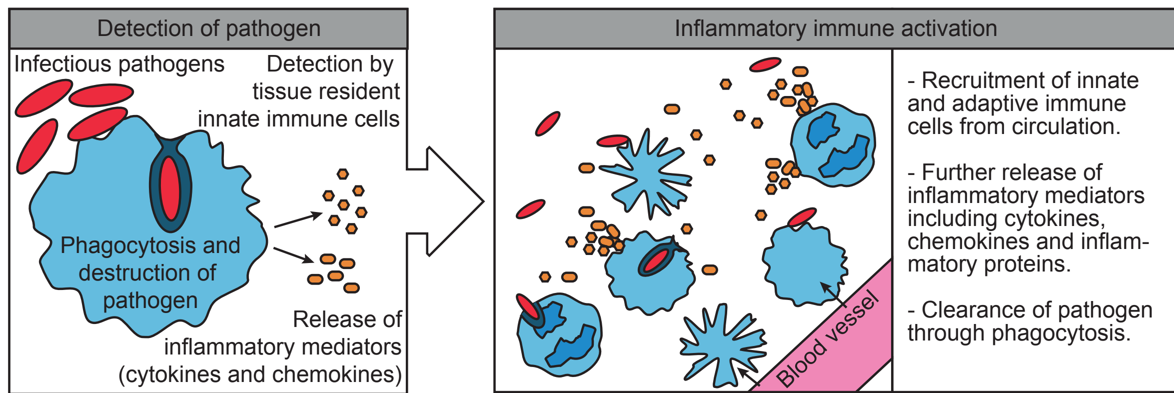

Figure 1.1 Activation of an inflammatory immune response

Inflammatory responses are activated by tissue resident innate immune cells following detection of infectious microorganism. The response can include secretion of inflammatory mediators such as cytokines and chemokines, recruitment of innate and adaptive immune cells and clearance of pathogen through phagocytosis.

Broadly, the symptoms of inflammation include redness, swelling, heat, pain and stiffness. Pyrogenic cytokines such as IL-1β drive these processes during innate immune responses in order to limit the growth of pathogens and aid the immunological destruction of microbes (Dinarello, 2009). Increased blood flow allows the migration of innate and adaptive immune cells into the site of injury, but causes the symptoms of redness and swelling. This swelling of tissue and joints is also a function of an increased concentration of plasma proteins that accumulate at the site of injury, mediating immune recognition of pathogens and activation of tissue repair mechanisms. This inflammation further heightens the innate immune response by driving the activation of local innate immune cells and recruitment of additional innate immune cells such as macrophages, neutrophils and monocytes (Gruys et al., 2005). Cytokine mediated inflammation further coordinates the activation of adaptive immunity against pathogens in cases where innate immunity alone cannot control the infection (Sims and Smith, 2010).

1.3 Pattern Recognition Receptors

Pathogens are incredibly diverse and have high mutation rates (Inohara et al., 2005). Defence against these microbes is coordinated by a series of germline-encoded receptors termed pattern-recognition receptors (PRRs), which mediate activation of innate immune responses (Akira et al., 2006). This section will cover the role of toll-like receptors (TLR) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) in innate immunity. The NLR family pyrin-domain containing protein (NRLP) NLRP3 will be covered in greater detail as it is activated downstream of cell death pathways, such as apoptosis and necroptosis, and drives innate immune responses following host cell death.

1.3.1 Danger and pathogen associated molecular patterns

There are two broad categories of molecular signals that are detected by PRRs that activate innate immune responses. The first are pathogen-associated molecular patterns (PAMPs). These molecular moieties are derived from components of pathogenic microbes required for their survival, such as cell wall components or double-stranded RNA (Akira et al., 2006). The second category are damage-associated molecular patterns (DAMPs), which are defined as molecules with an intracellular physiological role that have additional functions when released into the extracellular space. Some of the first DAMPs to be characterised include high mobility group box 1 (HMGB1) and uric acid crystals both of which contribute to inflammation and mediate activation of immune responses when released from cells (Shi et al., 2003, Scaffidi et al., 2002). There have since been a wide range of DAMPs identified that are released from cells both passively and through active secretion mechanisms in times of stress (Venereau et al., 2015). Some common examples of DAMPs include deoxyribonucleic acid (DNA), ribonucleic acid (RNA), adenosine triphosphate (ATP), heat shock proteins (HSP) and cytokines, such as IL-1α and IL-33.

1.3.2 Families of pattern recognition receptors

PRRs are expressed on both innate and adaptive immune cells and can be divided into families based on conserved domains. In many cases, members of these families are activated by similar sets of DAMPs and PAMPs. For example, retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA-5) are both cytosolic sensors of dsRNA and hence are classed as RIG-I-like receptors (RLRs). Similarly, C-type lectin receptors (CLRs) are cell surface receptors of glycoproteins that recognise a diverse range

of proteins from viruses, bacteria and fungi. Two further categories of PRRs, TLRs and NLRs will be covered in detail below. Broadly, detection of DAMPs or PAMPs by PRRs activates signal transduction cascades which result in gene transcription through the transcription factors nuclear factor-κB (NF-κB), activator protein 1 (AP1), and interferon regulatory factors (IRFs) (Takeuchi and Akira, 2010). This, in turn, drives the production of pro-inflammatory cytokines, chemokines, interferons, as well as genes with anti-microbial or wound healing functions (Schroder and Tschopp, 2010). A further commonality between receptor families is the induction of signalling cascades through large oligomeric protein platforms such as the myddosome for TLR signalling (Ferrao et al., 2012), inflammasome in NLR signalling (Schroder and Tschopp, 2010) and the adaptor (MAVS) downstream of RLRs (Hou et al., 2011).

1.3.3 Toll-Like receptors

The TLR family of transmembrane receptors detect the presence of extracellular pathogens, as well as phagocytosed pathogens in endosomes and lysosomes (Akira et al., 2006) (Figure 1.2). 12 TLR family members have been identified in mammals, these are grouped into subfamilies based on their localisation (Akira et al., 2006). Intracellular TLRs, TLR3, TLR7, TLR8 and TLR9, are found in compartments such as endosomes and detect the presence of viral, bacterial nucleic acids, as well as endogenous DNA that may be degraded in endolysosomes during times of stress (Takeuchi and Akira, 2010, Kawai and Akira, 2006). TLR1, TLR2, TLR4, TLR5 and TLR6 are localised on the plasma membrane and detect extracellular PAMPs derived from bacteria, fungi and protozoa, as well as, host-derived DAMPs such as HMGB1 (Kumar et al., 2011).

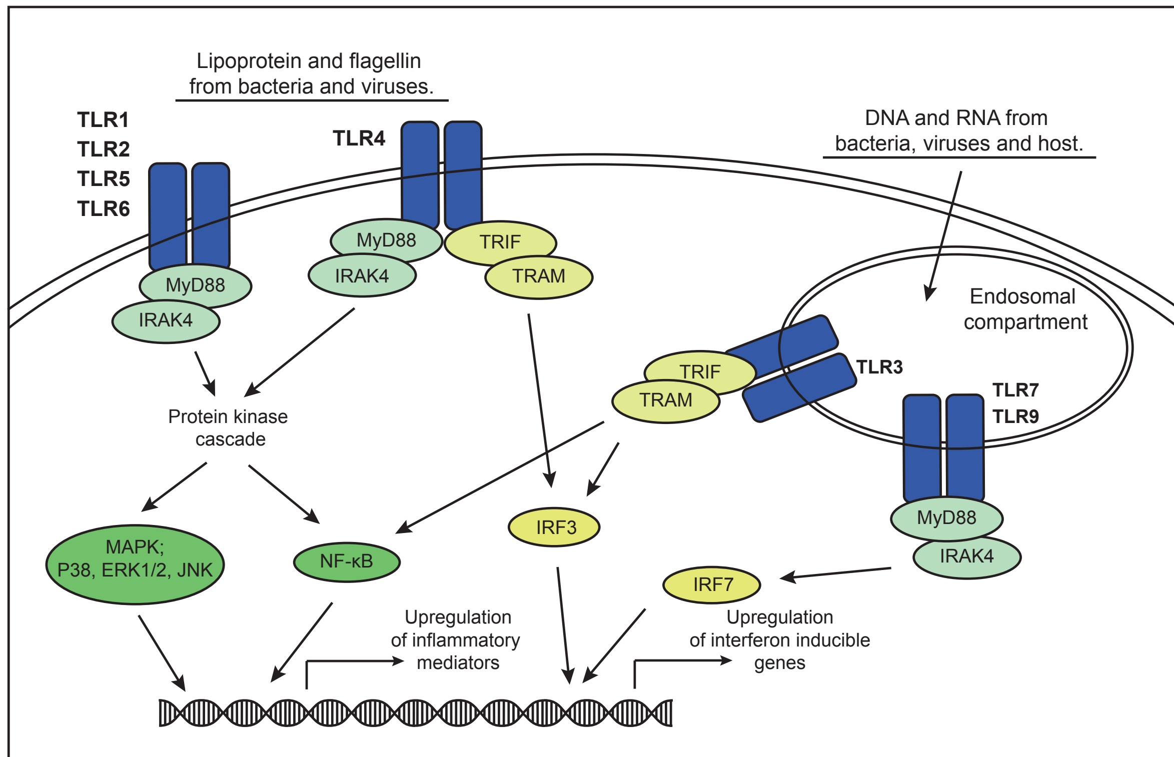

Figure 1.2 Mammalian TLR signalling pathways

Ligand recognition by TLR family members triggers inflammatory and anti-pathogenic immune defences. The signalling pathways activated downstream of TLRs include nuclear factor κB (NF-κB), and mitogen-activated protein kinase (MAPK), including c-Jun NH2-terminal kinase (JNK), which upregulate the production of pro-inflammatory mediators. A subset of TLR receptors also induce the upregulation of interferon-inducible genes and type-I interferons.

TLRs bind to their specific ligand through N-terminal leucine-rich repeats (LRRs) (Bell et al., 2003). This results in receptor dimerization and signal transduction through a cytoplasmic signalling region termed the Toll/IL-1R homology (TIR) domain (Slack et al., 2000, Gay and Keith, 1991a). Two key adaptors are recruited to this domain, TIR domain-containing adaptor inducing IFN-β (TRIF), also known as TIR-domain-containing adaptor molecule 1 (TICAM-1), and myeloid differentiation primary-response protein 88 (MyD88), and are responsible for triggering two independent signalling pathways (Takeda and Akira, 2005). The majority of TLRs signalling through Myd88 and IL-1 receptor–associated kinase-4 (IRAK-4), while TLR3 signals through TRIF (Figure 1.2). TLR4 has the unique ability to activate both signalling pathways concurrently (Lu et al., 2008).

Recruitment of IRAK4 and other IRAK family members by MyD88 activates both NF-κB signalling and the mitogen-activated protein kinase (MAPK) signalling cascade, which causes the formation of the activator protein 1 (AP-1) transcription factor complex (Takeuchi and Akira, 2010). The end result of which is the transcriptional upregulation of pro-inflammatory genes, including cytokines and chemokines. Significantly, this includes upregulation of NLRP3 and IL-1β thereby priming cells for activation inflammasome-mediated secretion of IL-1β (section 1.5.5). Alternatively TRIF mediates transcriptional upregulation of interferon-inducible genes, along with production of type I interferons (Takeuchi and Akira, 2010).

1.3.4 NOD-like receptors

NLRs are complementary to TLRs, as they are cytosolic proteins that can detect intracellular PAMPs and DAMPs. They function to induce apoptosis, pyroptosis and pathogen resistance (Inohara et al., 2005). The NLR family is comprised of 22 genes in humans, although gene duplication events have resulted in a greater number in mice (Schroder and Tschopp, 2010). They can be divided into 3 subfamilies based on their phylogenetic relationships. The first subfamily is the nucleotide-binding oligomerization domains (NODs) including NOD1-5. Secondly, the NLR family pyrin-domain containing proteins (NRLPs), is comprised of NLRP1-14. Finally, the ICE protease-activating factor (IPAF) subfamily includes NLR family CARD-domain containing protein-4 (NLRC4), which is also known as IPAF, and neuronal apoptosis inhibitor protein (NAIP) (Schroder and Tschopp, 2010).

Activation of NLRs results in the aggregation of large signalling platforms such as the NOD signalosome or inflammasomes (Martinon et al., 2009) (Figure 1.3). NLRs are characterised by their central nucleotide-binding domain, NACHT (domain present in Naip, CIITA (MHC class II transactivator), *HET-E* and TP-1 (telomerase-associated protein 1)) (Ting et al., 2008). They also contain a C-terminal LRR domain which is thought, although often not demonstrated, to enable NLR ligand recognition (Latz et al., 2013). Additionally, protein binding domains such as the caspase activation and recruitment domain (CARD), Pyrin domain or baculovirus inhibitor of apoptosis repeat (BIR) domains facilitate protein interactions and activation of downstream signalling cascades (Akira et al., 2006).

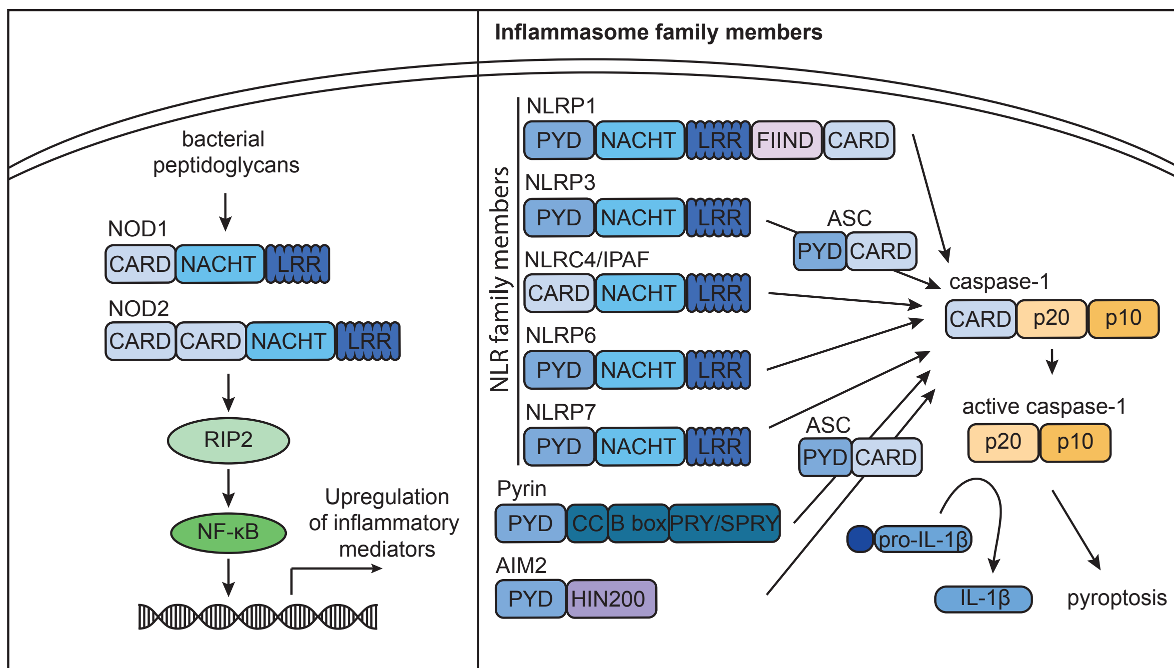

Figure 1.3 Mammalian NLR signalling pathways

A subset of NLR proteins including NOD1 and NOD2 activate pro-inflammatory signalling pathways and the upregulation of inflammatory genes through NF-κB signalling. Inflammasome forming NLR family members, along with pyrin and AIM2 which do not belong to the NLR family of proteins, form multiprotein platforms termed inflammasomes. These bind to caspase-1 either directly, or through the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC). They drive activation of caspase-1, resulting in the cleavage and activation of the pro-inflammatory cytokines IL-1β and IL-18 and caspase-1 driven pyroptotic cell death.

1.3.5 Pattern recognition functions within the NLR family

NOD1 and NOD2 were the first NLRs shown to have PRR function. They sense pathogenic peptidoglycan from bacteria cell walls and activate NF-κB signalling (Kufer et al., 2006) (Figure 1.3). In addition a subset of NLRs, including NLRP1, NLRP3, NLRP6, NLRP7 and NLRC4, activate large multimeric protein complexes termed inflammasomes (Martinon et al., 2002) (Figure 1.3). Inflammasomes activate the inflammatory caspases, caspase-1 or caspase-11, and hence are named after the apoptosome, a high molecular weight platform that activates apoptotic caspase-9 to induce cell death (Shi, 2002, Li et al., 1997).

1.3.6 Inflammasome activating NLR family members

Molecules which trigger inflammasome formation are derived from pathogenic microbes, are DAMPs derived from host cells, or are environmental molecules (Table 1.2). These bind NLR family members initiating self-oligomerisation of NLR inflammasome receptors through their internal NACHT domains (Schroder and Tschopp, 2010). Two additional PRRs can form inflammasomes despite not belonging to the NLR family of proteins (Figure 1.3). Absent in melanoma 2 (AIM2) is capable of forming an inflammasome following its binding to dsDNA (Hornung et al., 2009, Fernandes-Alnemri et al., 2009, Burckstummer et al., 2009) (Table 1.2). Similarly, Pyrin can form an inflammasome following inactivation of Rho-GTPases by bacterial toxins (Xu et al., 2014) (Table 1.2). In contrast the NLRP3 inflammasome has the broadest range of activating stimuli and it is unlikely that NLRP3 can directly bind to all of these molecules (Wen et al., 2013) (Table 1.2).

Caspase-1 is recruited into inflammasome complexes through CARD-CARD homotypic interactions following receptor activation (Figure 1.3). Inflammasome receptors NLRC4 and NLRP1 can directly bind caspase-1 through their CARD domains (Latz et al., 2013). Other receptors such as NLRP3, Pyrin, and AIM2 recruit the adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC) through homotypic pyrin domain (PYD) interactions (Figure 1.3). ASC then self-aggregates into microscopic ‘specks’ which further increases caspase-1 aggregation and allows multiple caspase-1 proteins to become activated simultaneously (Fernandes-Alnemri et al., 2007). Once aggregated caspase-1 becomes activated driving pyroptosis and activation of inflammatory cytokines.

Table 1.2 Inflammasomes and selective activating stimuli

DAMP/PAMP	Detected pathogen
NLRP1	
Lethal toxin	*B. anthracis* (Boyden and Dietrich, 2006)
Pyrin	
Bacterial inactivation of Rho-GTPase	*C. difficile, C. botulinum, V. parahaemolyticus, H. somni,* (Xu et al., 2014)
NLRC4	
Flagellin Bacterial type III or IV secretion systems	*S. typhimurium* (Franchi et al., 2006, Miao et al., 2006) *P. aeruginosa* (Sutterwala et al., 2007, Franchi et al., 2007, Miao et al., 2008) *L. pneumophila* (Amer et al., 2006, Lamkanfi et al., 2007) *S. flexneri* (Suzuki et al., 2007)
NLRP6	
Undefined	Gut microbiota (Chen et al., 2011, Elinav et al., 2011)
NLRP7	
Bacterial acylated lipopeptides	Numerous gram-positive and -negative bacteria (Khare et al., 2012)
AIM2	
Cytosolic dsDNA	
Synthetic and mammalian DNA	(Hornung et al., 2009, Burckstummer et al., 2009, Fernandes-Alnemri et al., 2009)
Bacterial DNA	*F. tularensis* (Fernandes-Alnemri et al., 2010, Jones et al., 2010) *L. monocytogenes* (Sauer et al., 2010)
DNA viruses	vaccinia virus and cytomegalovirus (Rathinam et al., 2010)
NLRP3	
Endogenous and environmental DAMPs	Necrotic cells (Iyer et al., 2009) ATP (Mariathasan et al., 2006) Uric acid crystals (Martinon et al., 2006) β-amyloid (Halle et al., 2008) Cholesterol crystals (Duewell et al., 2010) Asbestos (Dostert et al., 2008)
Bacterial PAMPs	*N. gonorrhoeae* (Duncan et al., 2009) *C. pneumoniae* (He et al., 2010, Shimada et al., 2011) *V. cholerae, E. coli, C. rodentium* (Kayagaki et al., 2011)
Fungal PAMPs	*C. albicans* (Gross et al., 2009, Hise et al., 2009, Joly et al., 2009) *A. fumigatus* (Said-Sadier et al., 2010)
Viral PAMPs	Viral RNA (Allen et al., 2009) Influenza (Ichinohe et al., 2010, Thomas et al., 2009) encephalomyocarditis virus, and vesicular stomatitis virus (Rajan et al., 2011)
Parasitic PAMPs	*S. mansoni* (Ritter et al., 2010) *D. pteronyssinus* (Dai et al., 2011)

1.3.7 NLRP3 inflammasome

As with other inflammasomes, NLRP3 acts as a scaffold for the aggregation of caspase-1 through the adaptor protein ASC. Activation of this inflammasome occurs through a two-step process (Latz et al., 2013) (Figure 1.4).

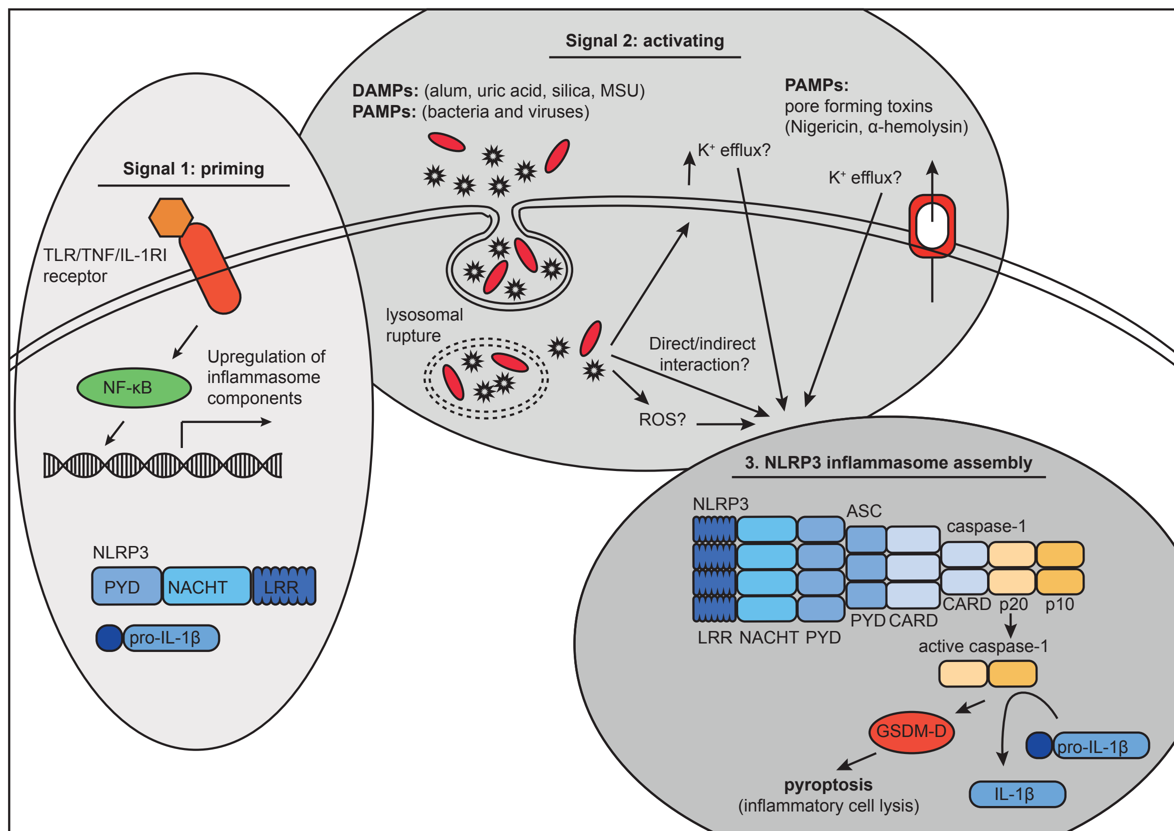

Figure 1.4 Activation mechanisms of the NLRP3 inflammasome

Activation of NLRP3 requires a priming signal that drives upregulation of inflammasome components NLRP3 and precursor IL-1β. Signalling pathways such as TLR, TNF or IL-1RI receptor signalling each activate NF-κB transcription, thus providing this priming. A secondary stimulus is then needed to activate the NLRP3 receptor. Proposed mechanisms of NLRP3 activation include direct binding of NLRP3 to DAMP and PAMP molecules, or binding through an unidentified adaptor protein. Other indirect mechanisms include generation of reactive oxygen species (ROS) or potassium efflux which are presumed to trigger NLRP3 aggregation. Active, aggregated NLRP3 can then recruit caspase-1 through homotypic domain interactions with the adaptor protein ASC. Caspase-1 auto-activates due to the forced proximity within this inflammasome complex. Active caspase-1 then processes and activates inflammatory cytokines such IL-1β and IL-18. In addition, it processes GSDM-D to cause pyroptosis.

Firstly, a priming signal such as TLR stimulation or TNF signalling is required (see section 1.5.5 for a more detailed description). This activates NF-κB induction of NLRP3 gene expression, as the basal expression levels of NLRP3 are usually not sufficient for inflammasome formation (Bauernfeind et al., 2009). A second activating stimuli is then required to initiate the aggregation of NLRP3 to form a functional inflammasome complex (Figure 1.4).

The structurally diversity of the stimuli (Table 1.2) that activate NLRP3 has led to its categorisation as a sensor of metabolic homeostasis, rather than a direct receptor for pathogen and host derived motifs (Lawlor and Vince, 2014). The mechanism by which NLRP3 is activated remains undetermined (Figure 1.4). Production of reactive oxygen species (ROS) has been hypothesised as a common feature of NLRP3 agonists (Martinon, 2010). Despite the fact that many NLRP3 stimuli induce ROS, not all ROS-inducing agents (for example cytokines) or cellular processes activate the NLRP3 inflammasome. In addition, it has been shown that many of the pharmacological ROS inhibitors block the upstream priming of NLRP3, rather than the activation of NLRP3 (Bauernfeind et al., 2011). A further proposed commonality between NLRP3 agonists is that they each trigger the efflux of potassium from cells, although how NLRP3 can sense this flux remains undetermined (Munoz-Planillo et al., 2013). There have since been reports of NLRP3 activating stimuli that are not blocked by high levels of extracellular potassium, suggesting this may not be a universal activation mechanism (Gaidt et al., 2016, Wolf et al., 2016, Zanoni et al., 2016). NLRP3 has a unique position as a sensor of both infections as well as host cell stress and damage. In addition, the NLRP3 inflammasome has been implicated to drive inflammation and pathology in a wide range of diseases (Menu and Vince, 2011), including a family of inherited auto-inflammatory disorders (Broderick et al., 2015). A better understanding of the pathways regulating its activation would be beneficial to developing anti-inflammatory therapeutic interventions.

1.4 Immunity and cytokines

Cytokines regulate a number of complex processes from embryonic development and stem cell differentiation, through to activating immune responses following injury or infection. Although there exist a wide range of diverse cytokines, they often have overlapping functionalities as a result of shared receptors, adaptor proteins and signalling pathways. As a result of this, cytokines are often pleiotropic (Table 1.3), with diverse and contradictory functions that are dependent on cellular context. The timing of activation, localisation, secreting cell type, receptive cell type and concentration of a cytokine can all modify its functionality. For example, IL-6 can activate both pro- and anti-inflammatory signalling by binding to two different receptor complexes (Scheller et al., 2011) (Table 1.3). In addition, cytokines rarely function in isolation, and display various synergistic or antagonistic functions in combination with each other.

Table 1.3 Cytokines and their function in immunity.

Cytokine families	Functions in immunity
IL-6 family	• Anti-inflammatory and regenerative signalling through 'classic' signalling pathways • Resolves the inflammatory acute phase of an immune reaction • Promotes adaptive immunity following pathogenic infection • IL-6 also activates pro-inflammatory signalling through an alternative soluble receptor
IL-10 family	• Immunoregulatory and anti-inflammatory; limits inflammatory cytokine production and upregulates production of cytokine antagonists • Drives tissue repair and wound healing • Mediates antiviral immunity
TNF family (Covered in section 1.9.3)	• Activates extrinsic apoptosis • Drives pro-survival functions; cell proliferation, differentiation, immune cell activation • Induces expression of pro- and anti-inflammatory cytokines
IL-1 family (Covered in section 1.5)	• Pro-inflammatory; upregulates pro-inflammatory genes including cytokines, cytokine receptors, growth factors and other chemokines • Recruitment of peripheral immune cells • Activation and differentiation of adaptive immune cells

In this literature review the IL-1 family, and IL-1β in particular, will be covered in greater detail as the processing, secretion and activity of this cytokine is a key focus in the aims and hypotheses of this thesis.

1.5 IL-1 family of cytokines

The IL-1 family of cytokines is comprised of 11 members. They drive pro-inflammatory innate immune responses to tissue injury following either pathogenic infections or trauma (Weber et al., 2010). The less well defined members of the IL-1 family include a set of three pro-inflammatory cytokine family members and one antagonist, all of which signal through the same receptor complex, and are grouped under the common name of IL-36; these are IL-36α, IL-36β, IL-36γ and IL-36 receptor antagonist (IL-36Ra) (Dinarello et al., 2010). This group of cytokines activates NF-κB signalling through MAPK pathway, similar to the signalling of IL-1α, IL-1β or IL-18 (Dunn et al., 2001, Debets et al., 2001). Additional members of the IL-1 family are IL-33, IL-37, and IL-38. IL-33 is pro-inflammatory, activating Th2 cells and allergic responses (Schmitz et al., 2005, Dinarello, 2009). Both IL-33 and IL-1α can function as a nuclear transcription factors, in addition to activating a cell surface receptor (Baekkevold et al., 2003). The anti-inflammatory IL-37 supresses activation of innate immune responses (Nold et al., 2010). Finally, IL-38 was recently proposed to have anti-inflammatory functions (Mora et al., 2016). The best characterized cytokines from this family IL-1α, IL-1β and IL-18 will be the focus of the remainder of this review.

1.5.1 IL-1β

Along with IL-1α, IL-1β is the founding member of the IL-1 family of cytokines. Although inflammasomes and caspase-1 drive inflammation through the activation and release of a range of IL-1 family cytokines most inflammatory caspase-1 functions have been attributed to its activation of IL-1β. Further, specific blockade of IL-1β have proven effective in treating diseases caused by auto-activating mutations in NLRP3 (Masters et al., 2009). Therefore, the focus of research within this thesis is on IL-1β and its activating pathways.

IL-1α and IL-1β signal through a receptor heterodimer composed of type I IL-1 receptor (IL-1RI) and the IL-1 receptor accessory protein (IL-1R-AcP) (Greenfeder et al., 1995) (Figure 1.5). The IL-1R belongs the IL-1R/TLR receptor superfamily (Gay and Keith, 1991b) and drives four protein kinase cascades to promote increased gene transcription and stabilisation of mRNA; NF-κB signalling, and the three MAPK pathways; p38, extracellular signal-regulated kinase 1/2 (ERK1/2), as well as the c-Jun NH2-terminal kinase (JNK) pathway (O'Neill, 2000) (Figure 1.5). A key component within the IL-1R complex is the adaptor protein MyD88, which recruits the kinase IRAK-4 (Brikos et al., 2007, Wesche et al., 1997). Loss of either of these components of the signalling complex severely limits IL-1 signalling (Suzuki et al., 2002, Adachi et al., 1998).

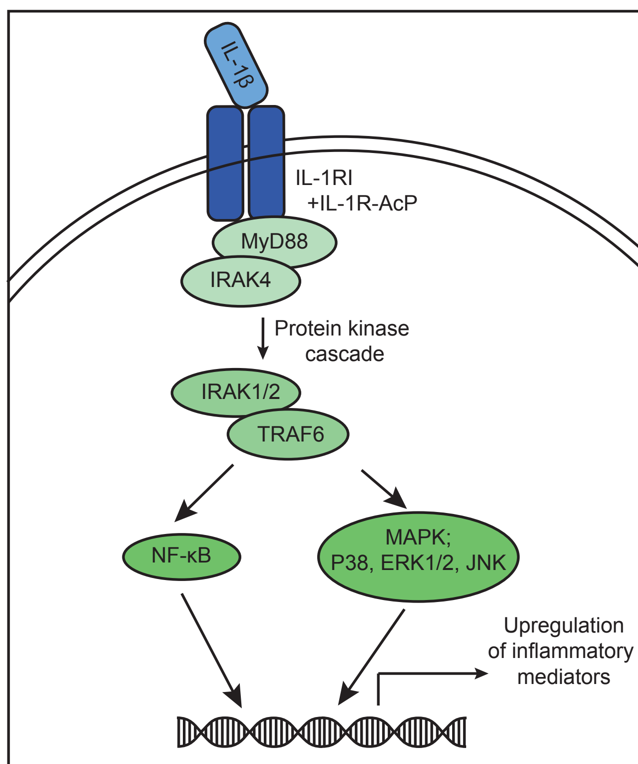

Figure 1.5 IL-1 receptor signalling

Binding of IL-1β to the IL-1RI complex induces MyD88 and IRAK4 adaptor proteins to activate nuclear factor κB (NF-κB), and mitogen-activated protein kinase (MAPK) signalling pathways; c-Jun NH2-terminal kinase (JNK), p38 and ERK1/2. These pathways culminate in transcriptional upregulation of inflammatory mediators such as cytokines and their receptors, chemokines and other genes involved in the proliferation and survival of immune cells.

1.5.2 IL-18

IL-18 and IL-1β must both be cleaved into active forms. This is commonly as result of caspase-1 activation within the inflammasome (section 1.3.6) (Fantuzzi and Dinarello, 1999, Ghayur et al., 1997, Gu et al., 1997), but can also occur following caspase-8 activation (section 1.10) (Bossaller et al., 2012). Although IL-18 binds through a unique and specific IL-18 receptor heterodimer, it activates the same downstream adaptors and signalling pathway as IL-1β, leading to activation of NF-κB (Akira, 2000, Weber et al., 2010). Another similarity shared between IL-1β and IL-18 is that neither are secreted

through the classical ER-Golgi pathway, and instead their secretion is linked to their processing by caspase-1 (Fantuzzi and Dinarello, 1999) (see section 0). In addition, the antagonist IL-18 binding protein (IL-18BP) plays a similar role to the IL-1RI antagonist (IL-1RIa), by binding with high affinity to the IL-18R and limiting IL-18 signalling (Kim et al., 2000).

Despite these many shared features with IL-1β, IL-18 displays a number of distinct biological functions. These include activating cytotoxic T cells (Nakanishi et al., 2001b, Nakanishi et al., 2001a) and driving interferon-gamma (IFNγ) production (Kohno et al., 1997, Nakamura et al., 1989), which activates natural killer (NK) cells (Micallef et al., 1996, Okamura et al., 1995). Induction of IFNγ by IL-18 also activates B-cell mediated allergic responses (Yoshimoto et al., 1997). In addition IL-18 can induce expression of Fas ligand (FasL), a death receptor ligand that can induce apoptosis (Dinarello et al., 2013) (see section 1.9.2).

1.5.3 IL-1α

Although the majority of studies have focused on IL-1β as the major extracellular form of IL-1, both Il-1α and IL-1β are regulators of inflammation (Sims and Smith, 2010). IL-1α can be released from cells both in a caspase-1 dependent manner (Keller et al., 2008, Li et al., 1995, Kuida et al., 1995), but also in an inflammasome-independent manner (Gross et al., 2012), likely as a result of cell death. These two cytokines share structural similarity (Graves et al., 1990, Priestle et al., 1989), and both bind to the IL-1R heterodimer in a similar manner (Labriola-Tompkins et al., 1993, Grütter et al., 1994). In doing so they activate the same signalling pathways (Figure 1.5).

Despite this redundancy, the comparison of IL-1$\alpha^{-/-}$ and IL-1$\beta^{-/-}$ animals has demonstrated distinct and independent functions for these two cytokines downstream of inflammasome activation (Duewell et al., 2010, Kamari et al., 2011, Vonk et al., 2006, Yazdi et al., 2010), and in inflammatory diseases (Horai et al., 1998, Nakae et al., 2001, Nakae et al., 2003). This likely reflects differences in expression and activation of IL-1α compared with IL-1β. For example, IL-1α, unlike IL-1β, is constitutively expressed in primary cells (Hacham et al., 2002), biologically active in its expressed pro-form (Mosley et al., 1987), and not cleaved by caspase-1 (Howard et al., 1991).

Unlike IL-1β, IL-1α may also exist as a bioactive membrane bound form that can cause local inflammation through the IL-1R (Stevenson et al., 1993, Kurt-Jones et al., 1985, Brody and Durum, 1989, Dinarello, 2009). Ultimately this has meant that IL-1α can act as an endogenous danger signal or DAMP, triggering sterile inflammation following its release from dying cells (Chen et al., 2007). Intriguingly, IL-1α also contains nuclear localisation signal and may directly influence the transcriptional upregulation of pro-inflammatory genes involved in processes such as cell proliferation and migration (Buryskova et al., 2004, Werman et al., 2004)

1.5.4 Biological activity of IL-1β

Early evidence of IL-1β's ability to drive systemic inflammation came from studies where low doses of recombinant IL-1β were administered to humans. IL-1β caused headache, fatigue and drowsiness, nausea and vomiting, muscle and joint aches and pains (Dinarello, 1996) (Table 1.4). Comparably, mice with increased IL-1RI signalling due to IL-1R antagonist (IL-Ra) deficiency reproduce at a lower rate, have stunted growth, and develop spontaneous diseases (Dinarello, 2009). Despite this, IL-1β deficient mice are born at Mendelian ratios, are healthy and fertile and develop without major histopathological defects, demonstrating that there is little function for IL-1β in development or tissue homeostasis (Zheng et al., 1995). However, demonstrating a clear role in innate immunity, IL-1β deficient mice have increased susceptibility to live infections (Miller et al., 2007, Labow et al., 1997, Ichinohe et al., 2009), and show reduced pathology in models of inflammatory driven disease (Netea et al., 2010, Fantuzzi and Dinarello, 1996).

Table 1.4 Innate and adaptive immune effects of IL-1β

Whole body effects	Effect on innate immune cells	Effect on adaptive immune cells
Headaches Fatigue and drowsiness Nausea and vomiting Muscle and joint aches and pains	Increased cytokine production Increased expression of cytokine receptors Differentiation and maturation Increased survival Increased phagocytosis	Recruitment and activation of adaptive immune cells Promotes T cell responses including differentiation of T_H17 cells Enhanced B cell proliferation

In addition to recruiting and activating innate immune cell proliferation, differentiation and survival, IL-1β can also regulate B and T cell functions (Sims and Smith, 2010, Lichtman et al., 1988) (Table 1.4). For example, IL-1β drives the expansion and survival of naïve T cells (Ben-Sasson et al., 2009), while also promoting the development of the TH17 cell subset (Chung et al., 2009, Kryczek et al., 2007). Similarly IL-1β promotes antibody production from B cells (Falkoff et al., 1983, Lipsky et al., 1983) and enhances B cell proliferation in synergy with stimuli that promote B cell expansion such as immunoglobulin or CD40 ligation (Maliszewski et al., 1990, Rousset et al., 1991).

IL-1β signalling is tightly controlled on a number of levels; from gene transcription to post-translational activation and regulation of its biological activities (Figure 1.6). These regulatory processes limit the inflammatory activity of IL-1β.

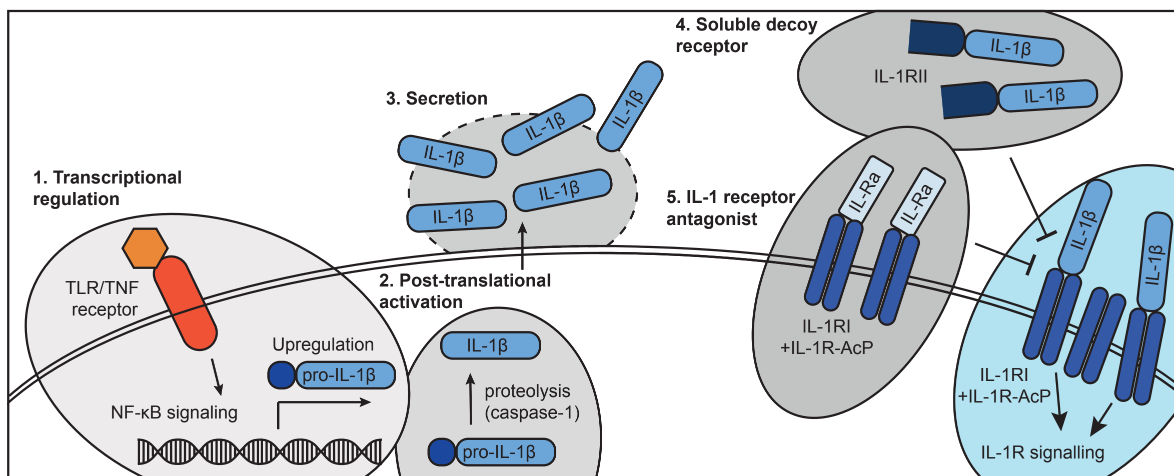

Figure 1.6 Transcriptional and post-translational regulation of IL-1β

The activation of IL-1β is highly regulated. (**1**) IL-1β is expressed at low levels and with a short protein half-life. Expression of pro-IL-1β must first be upregulated by NF-κB following TNF or TLR signalling, prior to its activation and release from cells. (See section 1.5.5) (**2**) IL-1β is expressed in an inactive pro-form. This must be cleaved by a caspase in order to activate its biological activity. (See section 1.5.6) (**3**) IL-1β is not constitutively secreted from cells, and is only released following activation of inflammatory caspases within the inflammasome (see section 0). Once secreted, IL-1β signalling is modulated by both the (**4**) soluble IL-1R decoy receptor (see section 1.5.7) and (**5**) IL-1R antagonist (see section 1.5.8). These hinder the binding of IL-1β to its receptor and hence can limit IL-1R signalling.

1.5.5 Transcriptional and post-translational priming of IL-1β

IL-1β activation is firstly controlled through a transcriptional process that 'primes' cells (Figure 1.6). IL-1β has a short half-life, with its turnover mediated by proteosomal degradation (Moors and Mizel, 2000), or possibly autophagic destruction (Harris et al., 2011). Inactive pro-IL-1β expression is upregulated following induction of NF-κB transcriptional activity (Hiscott et al., 1993) (Figure 1.6). This upregulation can occur following detection of DAMPs and PAMPs by TLRs, or through signalling of inflammatory cytokines such as TNF, IL-18, IL-1α or even IL-1β itself (Schroder and Tschopp, 2010, Dinarello et al., 1987). This process simultaneously induces the expression of receptors such as NLRP3 that are required to allow the processing and activation of IL-1β by caspase-1 (Bauernfeind et al., 2009) (section 1.3.7).

In addition, post-translational events also augment inflammasome activation (Schroder et al., 2012). NLRP3 deubiquitination (Py et al., 2013), TLR-dependent production of ROS (Fernandes-Alnemri et al., 2013), ERK1 phosphorylation and proteasome activity (Ghonime et al., 2014) have all been proposed to play a role in the rapid post-translational priming of inflammasomes.

1.5.6 IL-1β activation by proteolytic cleavage.

Early studies established that IL-1β was inactive in it's expressed form and only had biological activity as a smaller p17 fragment (Matsushima et al., 1986, Auron et al., 1987). Although it was established that this process required the activity of proteases (Black et al., 1988) it took a further 5 years before caspase-1 was identified as the main protease capable of cleaving and activating IL-1β (Thornberry et al., 1992, Nett et al., 1992, Cerretti et al., 1992). The process of IL-1β activation by caspase-1 within the inflammasome has since been well described (Martinon and Tschopp, 2004, Schroder and Tschopp, 2010). Caspase-1 is activated following inflammasome formation (section 1.3.6), allowing it to processes IL-1β into its biologically active p17 form (Figure 1.6). IL-1β is then released from cells, although the mechanism for this secretion remains controversial and is discussed in more detail below in section 1.6.

1.5.7 Type II IL-1 receptor acts as a decoy receptor

Once IL-1β is activated and released from cells it is still subject to regulatory processes that limit its binding to the IL-1R, hence restraining receptor signalling (Figure 1.6). Although two receptors for IL-1β were originally identified, it was demonstrated that the IL-1RI was responsible for signal transduction following IL-1β stimulation (Sims et al., 1994, Stylianou et al., 1992, Sims et al., 1993, Burch and Mahan, 1991). The type II IL-1 receptor (IL-1RII) was identified as a decoy receptor that can bind IL-1β, but cannot activate signalling as it does not contain a cytosolic tail (Giri et al., 1990, Sims et al., 1994). IL-1RII can be detected in circulation in healthy subjects (Dinarello, 1996), and binds to both IL-1α and IL-1β with low dissociation rates (Arend et al., 1994), hence moderating the ability of IL-1 to signal through the active receptor complex (Symons et al., 1991, Colotta et al., 1993).

1.5.8 IL-1 Receptor antagonist

The IL-1 receptor antagonist (IL-1Ra) shares structural similarities with both IL-1α and IL-1β (Graves et al., 1990, Priestle et al., 1989, Schreuder et al., 1995) and binds to the IL-RI complex with similar affinity as both the active cytokines (Hannum et al., 1990). Despite this, the IL-1Ra does not activate IL-1RI signalling (Dripps et al., 1991). Instead the IL-1Ra, which has a lower dissociation rate than either of the active cytokines (Arend et al., 1994), limits binding of IL-1β to its receptor (Seckinger et al., 1987) (Figure 1.6). The IL-1Ra acts in synergy with the decoy receptor IL-1RII (Smith et al., 2003), as they each bind tightly to either IL-1β or the IL-1RI complex with little interaction between themselves (Sims and Smith, 2010). The presence of multiple regulatory mechanisms that can modify the activity of IL-1β, demonstrates the potentially dangerous pro-inflammatory nature of this cytokine.

1.6 Cytokine Secretion

Most pro- and anti-inflammatory cytokines, such as TNF, IL-6 and IL-10 are transported constitutively to the cell surface following expression through the canonical ER-Golgi secretory pathway (Stow et al., 2009, Lacy and Stow, 2011). These cytokines are targeted onto or into ER membranes through an N-terminal signal peptide (Walter et al., 1984), after which they undergo vesicular transport through the Golgi apparatus to the plasma membrane where they can be released into the extracellular environment (Nickel, 2003).

IL-1β belongs to a family of secreted proteins that are not transported through the ER-Golgi pathway. These proteins are said to undergo non-conventional secretion as they lack the N-terminal signal sequence that targets them to the ER, and do not accumulate in the ER or Golgi apparatus (Nickel, 2003). Despite this there is no one pathway through which these proteins are secreted, with numerous independent, mechanistically distinct pathways of non-conventional protein secretion proposed. Caspase-1 is a known regulator of unconventional secretion as it has been shown to cause the release of a range of leaderless proteins including IL-1α, IL-1β, IL-18, IL-33 and FGF-2 (Keller et al., 2008). The mechanism by which IL-1β is secreted from cells remains controversial and this next section will summarise a few of the more recent hypotheses on this topic.

1.6.1 Non-canonical secretion of IL-1β

Many mechanisms have been proposed for the secretion of IL-1β although a consensus amongst researchers has yet to be reached (Monteleone et al., 2015). IL-1β does not undergo ER-Golgi mediated secretion, as it lacks the signal peptide required for ER targeting (March et al., 1985, Auron et al., 1984), and it does not localize to secretory organelles (Singer et al., 1988, Matsushima et al., 1986). In addition, Brefeldin A, a potent inhibitor of ER-Golgi transport which blocks the release of ER targeted cytokines such as TNF does not impact its secretion (Rubartelli et al., 1990). Alternative mechanisms for IL-1β release include non-specific release via cell lysis, inclusion and secretion in multivesicular bodies or autolysosomes, as well as secretion directly across the plasma membrane potentially with the involvement of protein transporters.

1.6.2 Passive secretion of IL-1β

Fundamental to the release of IL-1β from cells is the requirement for caspase-1 cleavage induced activation, which invariably coincides with pyroptotic killing (section 1.8). IL-1β is often reported to be released in parallel with DAMPS and other cellular components such as lactate dehydrogenase (LDH) following pyroptosis (Hogquist et al., 1991), so much so that it has been proposed that IL-1β itself is a DAMP (Martin, 2016). A number of recent studies support the conclusions that the pyroptotic death of macrophages following caspase-1 activation is inevitable. For example, two related studies demonstrated using time-lapse imaging of cells that caspase-1 activation in peritoneal macrophages was a binary event; cells with active caspase-1 were committed to pyroptosis (Liu et al., 2014, Shirasaki et al., 2014). This link between pyroptosis and IL-1β secretion has been further demonstrated both *in vitro* (Cullen et al., 2015) and *in vivo* (Sagoo et al., 2016).

Despite these reports, it remains difficult to experimentally discern whether cell death is essential for IL-1β secretion, or if cell death simply occurs in parallel to its release. NLRP3 inflammasome stimuli such as Nigericin, ouabain, valinomycin, streptolysin O, ATP, are inherently toxic to cells even when caspase-1 function is compromised (Cullen et al., 2015). In addition, many experimental techniques do not efficiently differentiate between the inactive pro-form of IL-1β and its cleaved active form, both of which can be released following plasma membrane rupture.

Recent studies have documented that caspase-1 cleavage and activation of GSDM-D is required for pyroptotic cell death (He et al., 2015, Kayagaki et al., 2015, Shi et al., 2015) and that GSDM-D undergoes membrane translocation and forms plasma membrane pores (Ding et al., 2016). It has also been shown that cleavage of GSDM-D is required for maximal secretion of IL-1β from macrophages following both caspase-11 and caspase-1 activation (He et al., 2015, Kayagaki et al., 2015, Shi et al., 2015). Although this suggests that GSDM-D mediated plasma membrane disruption may be required for the release of IL-1β from macrophages, it has also been proposed that GSDM-D participates in distinct parallel pathways of pyroptosis and non-lytic IL-1β secretion (Russo et al., 2016).

1.6.3 Active Secretion of IL-1β

Several groups have published evidence in direct conflict with the hypothesis that IL-1β is passively released upon plasma membrane rupture. For example, it has been shown that IL-1β secretion can occur prior to the loss of plasma membrane integrity (Brough and Rothwell, 2007, Martin-Sanchez et al., 2016). In addition, blocking either cell lysis with the osmoprotectant glycine (Fink and Cookson, 2006, Weinberg et al., 2016) or pore function with non-specific channel inhibitors (Russo et al., 2016), does not prevent IL-1β secretion. Similarly, it has been documented that *Salmonella* infected neutrophils activate NLRC4-caspase-1 to process and secrete IL-1β in the absence of cell death (Chen et al., 2014a). In addition, *Streptococcus pneumoniae* infected neutrophils show all the signs of NLRP3 inflammasome activation, including detectable ASC speck formation, caspase-1 enzymatic activity and IL-1β secretion, in the absence of cell lysis (Karmakar et al., 2015). Similarly, dendritic cells have been reported to activate NLRP3, form ASC specks and secrete IL-1β, in the absence of cell death (Zanoni et al., 2016). Recent reports have also highlighted possible cell death independent non-canonical inflammasome pathways in human monocytes (Gaidt et al., 2016) and murine BMDM (Wolf et al., 2016, Duong et al., 2015), which also drive the secretion of IL-1β.

Several mechanisms for active IL-1β secretion have been proposed. For example, protein transport channels have been hypothesized to play a role in IL-1β secretion (Singer et al., 1995). The ATP Binding Cassette transporter (ABC) in particular has been implicated (Hamon et al., 1997, Marty et al., 2005, Zhou et al., 2002), although later studies attributed this to non-specific inhibitor activity (Lamkanfi et al., 2009) and no further protein transporters have been implicated since (Monteleone et al., 2015). In contrast, a number of pathways of IL-1β secretion have been proposed that involve the inclusion of IL-1β into membrane bound vesicles prior to export. They include secretion through lysosomes (Wewers, 2004, Andrei et al., 2004, Andrei et al., 1999, Qu et al., 2007, Carta et al., 2006), microvesicles (MacKenzie et al., 2001, Bianco et al., 2005, Pizzirani et al., 2007), and exosomes (Qu et al., 2007). Each of these pathways have since been shown not to be required for the secretion of IL-1β (Qu et al., 2007, Brough et al., 2003, Brough and Rothwell, 2007, Verhoef et al., 2003), or in the case of exosome secretion IL-1β was never isolated within exosomes (Qu et al., 2007, Ohman et al., 2014). Finally, there have been a number of conflicting roles proposed for autophagy in IL-1β secretion. Firstly

inflammasome components have been shown to be sequestered in autophagic lysosomes and undergo autophagic turnover of that suppresses inflammasome activation (Shi et al., 2012, Harris et al., 2011). In opposition, autophagy has been suggested to contribute positively to IL-1β secretion (Dupont et al., 2011, Saitoh et al., 2008, Zhang et al., 2015).

Although the above studies suggest that IL-1β can be actively secreted from cells, they examined the amount of LDH released into the cell supernatant as the read out of cell death or pyroptosis. The LDH release assay is a population based assay that measures the non-specific release of the cellular protein LDH, but it does not determine on a single cell level whether cell death is occurring. Given the difficulty of experimentally separating the activation of caspase-1 from pyroptosis it remains controversial whether IL-1β can be secreted from cells in the absence of cell death. This controversy forms the basis of aim 1 within this thesis, and is addressed with the results in chapter 3.

1.7 The role of programmed cell death in regulating innate immune responses

This section explores how different cell death pathways regulate inflammation and immunity, in particular through the activation of IL-1β. As necrosis is an unregulated process of cell death it will not be covered in this section.

1.8 Pyroptosis: caspase-1 activation

As previously mentioned, the inflammatory caspases, caspase-1 and -11, kill cells via pyroptosis when activated by inflammasome sensor proteins or LPS, respectively (see sections 1.1.3 and 1.3.6) (Figure 1.7). How caspase-1 activation resulted in pyroptosis was recently clarified by several studies. These documented GSMD-D as a novel substrate of caspase-1 and -11, required for pyroptotic cell death (He et al., 2015, Kayagaki et al., 2015, Shi et al., 2015). Caspase-1 or -11 processing of GSDM-D releases its autoinhibition (Ding et al., 2016), and allows its 30 kDa N-terminal fragment to localized with plasma membrane and form circular pores within liposomes (Liu et al., 2016). In addition, although GSDM-D is required for killing downstream of caspase-11, NLRP3 can induce both GSDM-D dependent pyroptosis and GSDM-D independent cell death, possibly through its ability to activate both caspase-1 and caspase-8 (He et al., 2015).

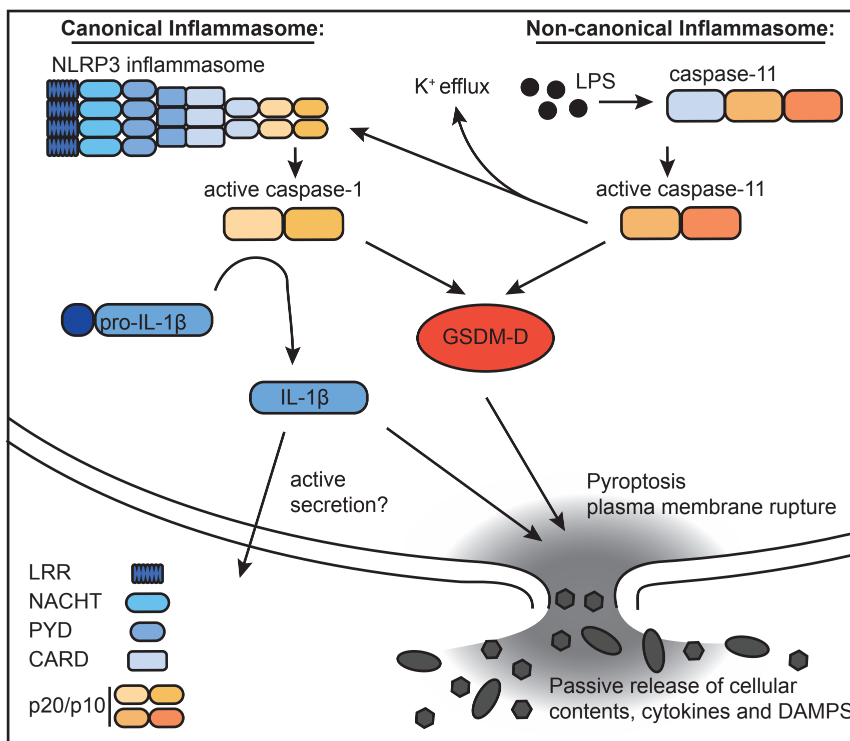

Figure 1.7

Inflammasome activation of pyroptosis

Pyroptosis is triggered following activation of either canonical (receptor and caspase-1) or non-canonical (caspase-11) inflammasomes. Both caspase-1 and caspase-11 process the substrate gasdermin-D (GSDM-D) to expose the active N-terminal fragment that forms pores in the plasma membrane resulting in lytic cell rupture. Caspase-1 can also process and activate inflammatory cytokines such as IL-1β and IL-18. These are then either actively secreted from cells or released in a GSDM-D dependent manner as a result of plasma membrane disruption.

1.8.1 Activation of caspase-1

As with other caspases, caspase-1 is expressed in cells as an inactive, monomeric zymogen. The processing of caspase-1 into a large and small subunit (p10 and p20) is seen as a read-out for its catalytic activity. Despite this, recent studies have shown that that it's auto-proteolysis is not required for its activity (Jorgensen and Miao, 2015). Differential patterns of caspase-1 activation downstream have been reported. For example, caspase-1 processing isn't required for pyroptosis downstream of NLRC4 in the absence of ASC (Case et al., 2009, Mariathasan et al., 2004). One hypothesis was that NLRC4 could induce two inflammasome structures, one in the absence of ASC and one where ASC was recruited and mediated caspase-1 auto-processing, which in turn enhanced caspase-1 processing of IL-1β (Broz et al., 2010). Ultimately different inflammasomes may mediate caspase-1's activation and activities in different ways, and caspase-1 processing may not be a true read out for caspase-1 activity.

1.8.2 Substrates of caspase-1

In addition to processing GSDM-D and the cytokines IL-1β and Il-18, caspase-1 induces the release of potentially lethal eicosanoids (von Moltke et al., 2012), cleaves enzymes involved in glycolysis (Shao et al., 2007) and activates apoptotic caspase-7 (Lamkanfi et al., 2008); however, the full physiological relevance of each of these events remains to be determined (Sollberger et al., 2014). Activation of caspase-1 also results in the release of other leaderless proteins along with a number of pro-inflammatory DAMPs, although this may be a passive process due to pyroptotic cell lysis (Gross et al., 2012, Keller et al., 2008).

1.8.3 Pyroptosis as an immune response

An inherent feature of pyroptosis is the non-specific release of DAMPs that can mediate communication between neighbouring cells about the presence of damage or danger (Fink and Cookson, 2005). In addition, the lytic death of a cell exposes intracellular pathogens that could otherwise avoid immune surveillance (Jorgensen et al., 2016). By simultaneously mediating the release of pathogens, cytokines and DAMPs pyroptosis alerts neighbouring cells to the presence of danger, activates pro-inflammatory, feedforward loops of cytokine production, and recruits and activates innate immune cells that can destroy pathogens through phagocytosis. Mice with activating mutations in NLRP3 provide a clear example of the ability of inflammasomes to activate immunity. These mutations drive constitutive inflammasome activity, causing toxic inflammation and activation of innate immunity, which is only partially rescued by IL-1RI or IL-18R deficiency (Brydges et al., 2009, Brydges et al., 2013).

In many experimental systems pyroptosis occurs simultaneously with IL-1β and IL-18 activation and secretion; therefore the use of IL-1β and IL-18 deficient mice, or mice where both IL-1α and IL-1β signalling is blocked due to loss of the IL-1R, has proved valuable in allowing the study of inflammation driven by pyroptosis in the absence of cytokine signalling (Jorgensen and Miao, 2015). A growing number of studies have shown that pyroptosis alone mediates an immune response to infections in the absence of IL-1R1, IL-1β and Il-18 (Miao et al., 2010a, Warren et al., 2011). For example, in a sepsis model with *E. coli* caspase-1/caspase-11 knock out animals were protected independently of either IL-1β or IL-18 (Sarkar et al., 2006, Wang et al., 2005). This has since been shown to

be a caspase-11 specific effect, as caspase-11 deficient mice and not caspase-1, NLRP3 or ASC deficient mice are resistant to LPS-induced endotoxic shock (Kayagaki et al., 2011). It's important to note that caspase-11 doesn't directly process cytokines such as IL-1β or IL-18 (Kayagaki et al., 2011) and instead activates the NLRP3-caspase-1 inflammasome (Baker et al., 2015) potentially through GSMD-D mediated potassium efflux (Ruhl and Broz, 2015, Yang et al., 2015). Therefore, the dependence of this sepsis model on caspase-11 further reinforces that in some circumstances pyroptosis and not cytokine production can drive inflammatory pathology.

In addition to driving inflammation host cell death is critical in mediating the release and exposure of pathogens, as host cells provide pathogens with a protective, intracellular niche in which they can replicate protected from immune exposure. Some pathogens have even evolved systems to avoid activating pyroptosis, for example *Salmonella typhimurium* represses expression of flagellin and variant type-III secretion system machinery so as not to trigger NLRC4 activation (Miao et al., 2006, Miao et al., 2010b, Cummings et al., 2006). Interestingly, pyroptosis is not always beneficial to the host. For example, despite the fact that pyroptotic death of T cells during HIV infection might prevent viral replication within these cells, it contributes to damaging inflammation and the depletion of T cells (Doitsh et al., 2014, Monroe et al., 2014). It was recently shown that intracellular microbes were damaged and trapped within cellular debris during pyroptosis, limiting their ability to reinfect host cells and coordinating their clearance by secondary phagocytes such as neutrophils (Jorgensen et al., 2016).

Although neutrophils contribute to immunity by phagocytosing and degrading pathogens, they also have functional inflammasomes and produce IL-1β in vivo (Cho et al., 2012). Interestingly, reports so far suggest they do not undergo caspase-1 mediated pyroptosis (Chen et al., 2014a, Karmakar et al., 2015). Instead neutrophils, which are short lived and highly microbicidal, can both kill intracellular pathogens and drive chronic inflammation through their ability to continuously secrete IL-1β. It has since been reported that the caspase-1 activation does not always activate pyroptosis in monocytes (Gaidt et al., 2016), dendritic cells (Zanoni et al., 2016, Kang et al., 2013) and BMDMs (Wolf et al., 2016). This raises the interesting question of whether in some cases pyroptosis may in fact limit chronic inflammation by preventing continuous cytokine secretion.

1.9 Apoptosis

Apoptosis is a process of coordinated dismantling of a cell. It can be activated either cell intrinsically or extrinsically (Figure 1.8), but ultimately results in features such as nuclear condensation, chromatin cleavage, an intact plasma membrane, but formation of smaller membrane bound blebs and exposure of 'eat me' signals on the external cell surface (Fink and Cookson, 2005).

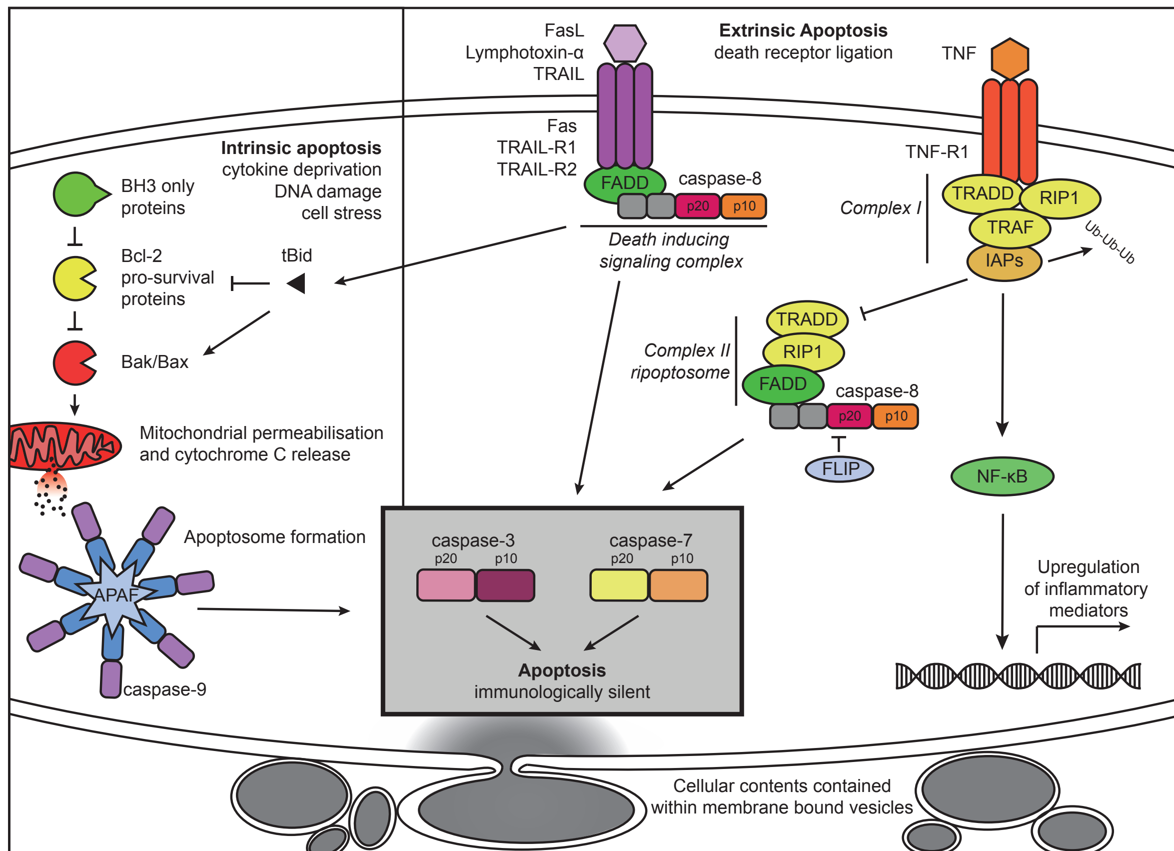

Figure 1.8 Caspase mediated apoptosis

Two pathways of apoptosis converge upon the activation of executioner caspases, caspase-3, -6 and -7. Intrinsic apoptosis is activated by BH3 only proteins which antagonise the Bcl-2 pro-survival proteins. This releases their inhibition on Bak and Bax, allowing Bax and Bak to oligomerise resulting in mitochondrial membrane permeabilisation. Release of Cytochrome C from the mitochondria seeds formation of the APAF/Caspase-9 apoptosome. This activates caspase-9, which can then drive apoptosis by activating the effector caspases, caspase-3 and -7. Caspase-8 is activated downstream of death receptor signalling via the adaptor protein FADD within the multi-protein platform; the death inducing signalling complex. It can also be activated downstream of TNF-R1 signalling. TNF activates complex I driving pro-inflammatory signalling through NF-κB. Disruption of this signalling complex results in the formation of complex II, a caspase-8 activating platform. Both pathways culminate in the apoptotic dismantling of the cell.

1.9.1 Intrinsic apoptosis

The intrinsic pathway of apoptosis (Figure 1.8) is triggered in response to cellular stressors including growth factor withdrawal, chemotherapeutic drugs and microbial infection (Lamkanfi and Dixit, 2010). This pathway is ordinarily inhibited through binding of anti-apoptotic (pro-survival) members of the BCL-2 protein family to Bax and Bak, keeping them in an inactive state (Strasser et al., 2000) (Figure 1.8). Apoptotic stimuli activate Bcl-2 homology 3 (BH3)-only proteins. This pro-apoptotic class of proteins directly antagonise anti-apoptotic Bcl2 proteins (Figure 1.8). Bax and Bak can then become activated, following which they oligomerise on the mitochondrial outer membrane resulting in its permeabilisation. The release of cytochrome C from mitochondria then stimulates the formation of the apoptosome, a multi-protein complex consisting of apoptotic protease activating factor 1 (APAF1) and caspase-9 (Figure 1.8). This pathway converges with the extrinsic pathway of apoptosis with the activation of the same executioner caspases.

1.9.2 Extrinsic death receptors apoptosis signalling

Extrinsic apoptosis is activated through plasma membrane death receptors (DR) (Figure 1.8). These receptors belong to the TNF superfamily of receptors, and each contain a death domain (DD) on their cytoplasmic tail and can activate both apoptotic and non-apoptotic signalling pathways. Members of the family include TNFR1, Fas, and TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and 2 (TRAIL-R2). These receptors primarily activate apoptosis upon binding of their respective ligands, TNF/lymphotoxin-α, Fas ligand and TRAIL, by signalling through the adaptor protein Fas associated death domain (FADD) (Wertz and Dixit, 2009). This results in the formation of the death inducing signalling complex (DISC) and recruitment and activation of the apical caspases, caspase-8 and caspase-10 (Figure 1.8).

1.9.3 TNFR1 signalling and apoptosis

TNFR1 signalling is able to activate two distinct signalling pathways, one of cell survival through NF-κB signalling, and the second of caspase dependent apoptosis (Micheau and Tschopp, 2003) (Figure 1.8). TNF firstly acts as a pro-inflammatory cytokine, a function it shares with 20 other members within the TNF family of cytokines (Locksley et al., 2001). This function is mediated by complex I downstream of TNFR1. This complex is comprised of TNF Receptor Associated Death Domain (TRADD), TNF receptor-associated factor

(TRAF), RIPK1 and inhibitor of apoptosis proteins (IAPs). Signalling through this complex initiates pro-survival and pro-inflammatory signalling pathways through NF-κB signalling (Figure 1.8).

The formation and stability of this complex is mediated by ubiquitination of key complex members through the ubiquitin ligase activity of cIAP1 and cIAP2 (Silke and Meier, 2013) (Figure 1.9). These E3 ligases ubiquitinate RIPK1, promoting NF-κB signalling, and preventing RIPK1 association and activation of caspase-8 (Silke and Meier, 2013) (Figure 1.9). When IAP function is compromised, either through deletion or inhibition, complex I becomes destabilised. Experimentally this can be achieved through the use of Smac mimetics such as compound A (Cp. A) (Vince et al., 2007) or birinipant (Benetatos et al., 2014), which were designed to antagonise IAPs proteins in a similar manner to mammalian Smac protein. RIPK1 then detaches from TNFR1 and forms a secondary complex in the cytosol, complex II (Figure 1.9). Under these circumstances RIPK1 interacts with TRADD, FADD and caspase-8 causing apoptosis.

1.10 Apoptosis, caspase-8 and inflammatory signalling

Like pyroptosis, apoptosis induced by host cells can act to both eliminate infected host cells and mediate the phagocytosis of apoptotic bodies containing microbes, which subsequently induces an adaptive immune response through MHC I presentation (Ashida et al., 2011). Although death receptor signalling and caspase-8 are best studied for their role in driving apoptosis, there has been recent interest in non-apoptotic and inflammatory signalling roles for caspase-8. Caspase-8 activity restricts the signalling of some inflammatory machinery, such as RIPK3, which otherwise drives lethal necroptosis and inflammation (Kaiser et al., 2011, Oberst et al., 2011). Caspase-8 and RIP kinases also play a key role in the transcription of cytokines, including IL-1β, downstream of TLRs (Su et al., 2005, Lemmers et al., 2007, Allam et al., 2014, Lawlor et al., 2015, Weng et al., 2014). Most importantly for this thesis, caspase-8 has been proposed to activate IL-1β through a number of distinct signalling pathways (Figure 1.9).

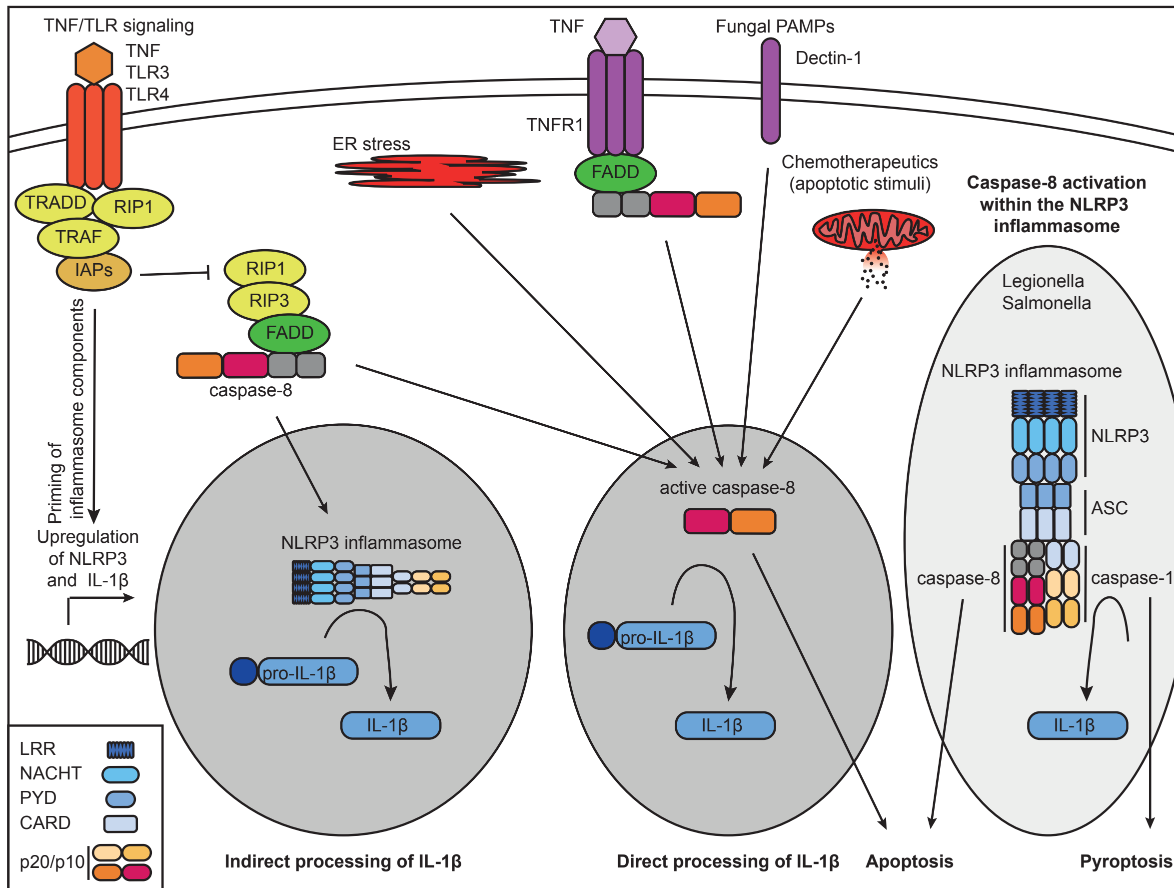

Figure 1.9 Interactions between apoptotic caspase-8, IL-1β and the NLRP3 inflammasome

Caspase-8 can directly and indirectly mediate the activation of IL-1β. TNF and TLR signalling both drive the priming of inflammasome components through NF-κB. When the receptor signalling complex controlling this process is compromised, for example by inhibition of IAP proteins, a second complex forms that activates caspase-8. This can cause the processing and activation of IL-1β either by direct caspase-8 processing of IL-1β, or indirectly by activating the NLRP3 inflammasome. Caspase-8 has also been shown to directly process IL-1β downstream of a variety of signals that activate apoptosis such as ER stress, death receptor signalling, detection of fungal PAMPs through Dectin-1 or treatment with chemotherapeutics. Caspase-8 has further been shown to interact and become activated by ASC within the NLRP3 inflammasome. Caspase-8 can drive apoptotic cell death in this pathway when caspase-1-mediated pyroptosis in inhibited.

1.10.1 NLRP3 activation of caspase-8

Caspase-8 mediated apoptotic cell death can be observed downstream of inflammasomes in the absence of caspase-1, although the full biological implications of this pathway are yet to be determined (Vince and Silke, 2016) (Figure 1.9). Inflammasomes have been shown to activate apoptotic caspases, caspase-3 and -7 following infection by *Legionella* or *Salmonella* in an ASC dependent manner (Abdelaziz et al., 2011, Puri et al., 2012). These effector caspases are likely activated by caspase-8, which can interact via its DED with the pyrin domain of ASC (Masumoto et al., 2003) and co-localise with ASC and caspase-1 within ASC specks (Man et al., 2013, Pierini et al., 2012) (Figure 1.9). In addition, the AIM2 inflammasome can activate caspase-8-apoptosis following *Fransicella* infection (Pierini et al., 2012). Similarly, activation of canonical inflammasomes NLRP3 and AIM2 with either nigericin or DNA transfection respectively, activates ASC-caspase-8 dependent apoptosis in the absence of caspase-1 (Sagulenko et al., 2013). In some circumstances canonical NLRP3-ASC activation of caspase-8, in the absence of caspase-1, induces not only apoptosis but also processing of IL-1β (Antonopoulos et al., 2015, Chen et al., 2015). As caspase-8 is activated often in the absence of caspase-1 and has slower kinetics of killing it has been proposed this is a secondary pathway of inflammasome mediated cell death (Antonopoulos et al., 2015, Puri et al., 2012, Man et al., 2013). Notably lower levels of activating stimuli shifted the balance of the pathways towards apoptosis, perhaps revealing a preference for the immunologically silent cell death pathway under more physiologically relevant conditions (Sagulenko et al., 2013).

1.10.2 Caspase-8 activation of NLRP3 and caspase-1

Caspase-8 has also been proposed to act upstream of the NLRP3 inflammasome. For example, TLR signalling drives RIPK1-RIPK3-caspase-8 to activate NLRP3 when IAPs are inhibited (Lawlor et al., 2015) (Figure 1.9). IL-1β processing and secretion downstream of this pathway can occur in both an NLRP3-caspase-1 dependent and independent manner through the ability of caspase-8 to directly process IL-1β (Vince et al., 2012) (Figure 1.9). It is likely that this pathway is activated in a tissue and cell specific manner, as TRIF-RIPK1-FADD-caspase-8 signalling drives NLRP3 activation in human monocytes in response to TLR signalling alone without an additional activating stimuli or IAP inhibition (Gaidt et al., 2016). Further, a TLR4-caspase-8 mediated pathway of IL-1β activation has been proposed in a model of retina ischemia (Chi et al., 2014).

An alternative role for FADD-RIPK3-caspase-8 within the NLRP3 inflammasome was proposed following evidence that loss of caspase-8 inhibited activation of both canonical (NLRP3-caspase-1) and non-canonical (caspase-11) driven inflammasome activation (Gurung et al., 2014); however it has been shown that cells deficient in caspase-8 have reduced priming (Allam et al., 2014, Weng et al., 2014, Lawlor et al., 2015). Later studies have since confirmed that both canonical and non-canonical inflammasome activation is normal when caspase-8 deficient cells are sufficiently primed (Kang et al., 2013, Lawlor et al., 2015, Antonopoulos et al., 2015). Therefore, although caspase-8 contributes to priming and non-canonical activation of inflammasomes it is less likely that it contributes to caspase-1 activation within canonical inflammasomes.

1.10.3 Caspase-8 proteolysis of IL-1β

Although caspase-8 drives the activation of IL-1β through NLRP3-caspase-1, it has also been shown that caspase-8 can directly process IL-1β into its active form (Figure 1.9). The first study to describe this found the combination of protein synthesis inhibitors (to inhibit NF-κB upregulation of pro-survival proteins) combined with TLR3 or TLR4 stimulation resulted in caspase-8 processing of pro-IL-1β at the same cleavage site as recombinant caspase-1 (Maelfait et al., 2008). Direct caspase-8 processing of IL-1β has since been reported under similar circumstances of TLR priming in combination with apoptotic stimuli. For example, numerous studies have shown the combination of TLR priming with chemotherapeutic compounds that promote apoptosis results in non-canonical processing of IL-1β via caspase-8. These compounds include doxyrubicin and staurosporin (Antonopoulos et al., 2013, England et al., 2014), Smac-mimetic compounds that inhibit IAP proteins (Vince et al., 2012), and histone deactylase inhibitors (Stammler et al., 2015). Similarly combining TLR4 stimulation with ER-stress inducing agents also promotes caspase-8 processing of IL-1β (Shenderov et al., 2014). Further, in dendritic cells stimulation of TLR4 with LPS alone results in caspase-8 dependent processing of IL-1β (Moriwaki et al., 2015). Although the exact mechanisms behind each of these pathways is not fully elucidated one hypothesis is that apoptotic stimuli limit the activity of pro-survival proteins that would otherwise repress TLR induced apoptosis, hence resulting in caspase-8 activation. In support of this genetic deletion of proteins that supress caspase-8 following TLR or TNF stimulation can promote caspase-8 mediated Il-1β activation, for

example deletion of c-FLIP (Wu et al., 2014b) or upon inhibition or deletion of IAP proteins (Vince et al., 2012, Lawlor et al., 2015, Yabal et al., 2014).

Consistent with a role for caspase-8 in activating inflammatory cytokines, activation of the Fas death receptor results in the secretion of IL-1 family cytokines (Tsutsui et al., 1999) (Fukui et al., 2003). Fas signalling has been shown to drive caspase-8 processing of IL-1β independent of caspase-1 (Bossaller et al., 2012, Uchiyama et al., 2013). Similarly, stimulation of death receptor 3 by TNF superfamily member 15 (TNFSF15) also drives caspase-1 independent processing of IL-1β by caspase-8 (Hedl and Abraham, 2014). Further, caspase-8 is responsible for the activation of IL-1β following detection of fungus by the extracellular pathogen sensor Dectin-1 (Gringhuis et al., 2012, Ganesan et al., 2014). This pathway is complicated by reports that pathogenic fungi can also activate canonical inflammasomes (Gross et al., 2009) as well as non-canonical NLRP3-ASC-Caspase-8 inflammasomes (Chen et al., 2015). Further studies into the activation and regulation of caspase-8 cytokine processing pathways are required to define its physiological relevance.

1.11 Necroptosis

Necroptosis is characterised by membrane disruption, rapid cell lysis and release of inflammatory DAMPs and can contribute to the clearance of infected host cells, as well as the activation of innate and adaptive immune responses (Mocarski et al., 2014). This caspase-independent form of cell death is activated downstream of death receptor signalling (Cho et al., 2009, He et al., 2009, Zhang et al., 2009) and TLRs (Kaiser et al., 2013a, He et al., 2011, Feoktistova et al., 2011), as well as T cell receptors (Ch'en et al., 2011), interferon signalling (Thapa et al., 2013, Mandal et al., 2014, McComb et al., 2014) and genotoxic stress induced depletion of IAPs (Tenev et al., 2011). Of these pathways the activation of necroptosis downstream of TNF signalling is the most well defined (Murphy and Vince, 2015) (Figure 1.10).

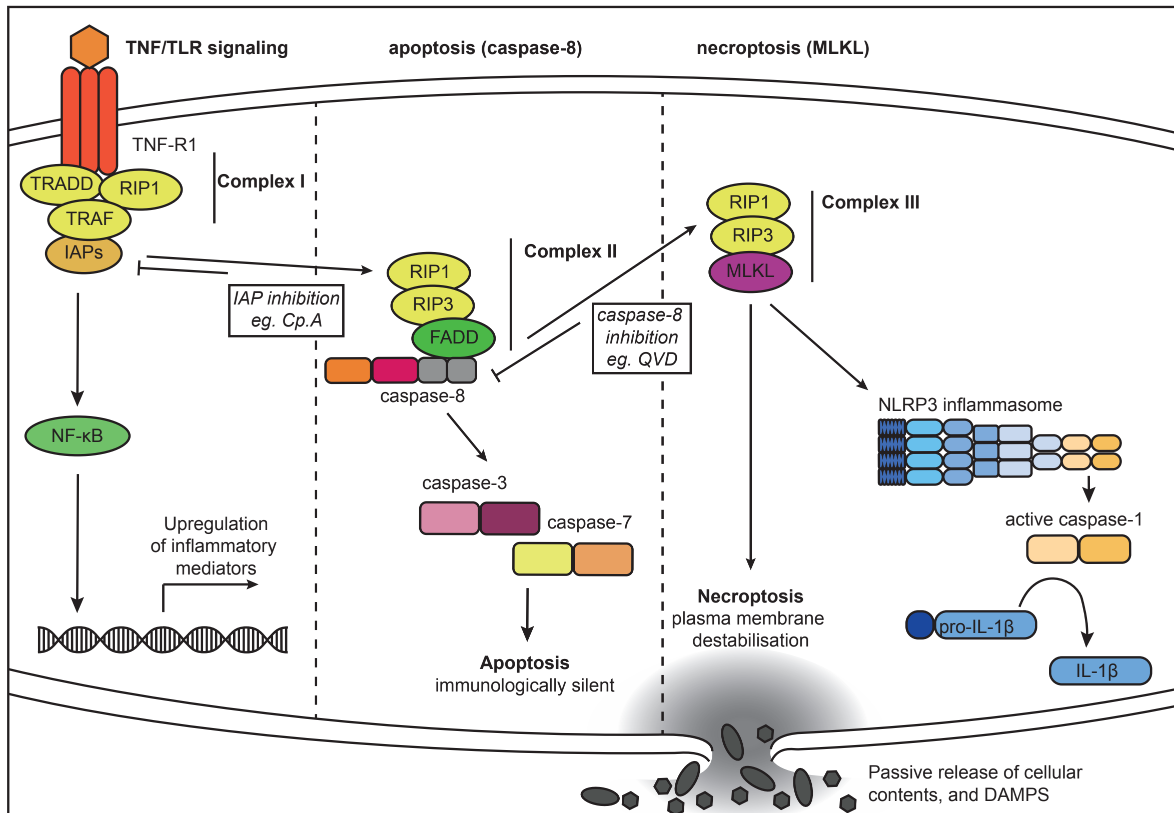

Figure 1.10 Activation of necroptosis downstream of TNF or TLR receptor signalling

Activation of TLRs or the death receptor TNFR1 activates pro-inflammatory NF-κB signalling. TRADD, TRAF, RIPK1 and the IAP proteins cIAP1 and 2 mediate this within the receptor associated complex I. Disruption of this complex by inhibiting IAP proteins activates a second signalling complex that is capable of driving apoptotic cell death through caspase-8. In the absence of caspase-8 activity RIPK3 can phosphorylate and activate MLKL. MLKL then oligomerises and translocates to cellular membranes, resulting in plasma membrane disruption and lytic cell death. Recent reports have shown that active MLKL can also mediate IL-1β activation by activating the NLRP3 inflammasome.

1.11.1 TNFR1 signalling and necroptosis

Under normal circumstances the binding of TNF to its receptor TNFR1 activates pro-survival signalling pathways that upregulate transcription of inflammatory cytokines and cell survival genes. This is mediated through a receptor associated complex containing TRADD, RIPK1, TRAF2, linear ubiquitin chain assembly complex (LUBAC) and IAPs, termed complex I (Micheau and Tschopp, 2003) (Figure 1.10). Inhibition of IAP proteins destabilises this complex, resulting in the formation of complex II, comprised of RIPK1, RIPK3, FADD and caspase-8 (Figure 1.10).

Within the apoptotic complex II caspase-8 not only drives apoptosis through activation of executioner caspases, but also limits activation of necroptosis by inactivating key necroptotic proteins such as RIPK1, RIPK3 and cylindromatosis (CYLD) (Chan et al., 2015, Feng et al., 2007, O'Donnell et al., 2011). When the activity of caspase-8 is low complex III, also known as the necrosome, is formed (Figure 1.10). This complex is detected within cells as an amyloid-like filamentous structure composed of RIPK1 and RIPK3, which interact through their RIP homotypic interaction motif (RHIM) domains (Li et al., 2012). Other signalling pathways that activate necroptosis also do so through RHIM-RHIM interactions between RIPK3 and adaptor proteins such as TRIF (TLR3 and TLR4 signalling) and the DNA receptor, DNA-dependent activator of IFN-regulatory factors (DAI) (Murphy and Vince, 2015). Within this complex the oligomerization of RIPK3 with itself has been shown to be critical in driving its auto-phosphorylation (Wu et al., 2014a). This phosphorylation of RIPK3 is required for the recruitment of MLKL by RIPK3 into complex III and the subsequent activation of MLKL via phosphorylation of the activation loops within MLKL's pseudokinase domain (Murphy et al., 2013, Rodriguez et al., 2016, Sun et al., 2012).

1.11.2 Activation and necroptotic activity of MLKL

MLKL has no kinase activity, instead its phosphorylation by RIPK3 is thought to result in a conformational change, obstructing the auto-inhibition of the pseudokinase domain, and releasing the 'killer' N-terminal domain (Hildebrand et al., 2014, Murphy and Vince, 2015). Following these events activated MLKL can be detected as a high molecular weight oligomer that is associated with cellular membranes (Cai et al., 2014, Chen et al., 2014b, Wang et al., 2014, Dondelinger et al., 2014).

Despite ongoing research the mechanism by which MLKL drives membrane rupture and cell death remains undetermined (Murphy and Vince, 2015). Numerous models have been proposed, hypothesizing either direct membrane permeabilisation by MLKL (Dondelinger et al., 2014, Su et al., 2014, Wang et al., 2014) or a requirement for a downstream effector molecule (Cai et al., 2014, Chen et al., 2014b). Studies supporting this later hypothesis propose that MLKL regulates the function of ion channels to cause cell lysis, although follow-up studies have shown necroptosis occurs in ion-free media, disputing this model (Wang et al., 2014). Further, a number of independent studies have shown that activated

MLKL interacts with membrane phospholipids and has the ability to directly disrupt *in vitro* generated liposomal membranes, suggesting that MLKL may directly damage the plasma membrane (Dondelinger et al., 2014, Wang et al., 2014, Xia et al., 2016, Tanzer et al., 2016). Regardless, the necroptotic death of a cell has a number of defining features including osmotic swelling, lysosome membrane permeabilisation and plasma membrane rupture (Vandenabeele et al., 2010). This lytic form of cell death shares many similarities to pyroptosis, and both are categorized as highly inflammatory forms of programmed necrosis (Table 1.1). Despite this, relatively few studies have examined whether necroptosis is associated with the release of specific DAMPs, and whether the necroptotic release of DAMPs have immunomodulatory functions (Kaczmarek et al., 2013).

1.11.3 Role for necroptosis in inflammatory diseases

Necroptosis has been implicated in a number of models of tissue damage, including atherosclerosis, pancreatitis, heart attack, as well as liver, retina and kidney injury (Silke et al., 2015). The exact role of MLKL in many of these models remains unclear, as an involvement for necroptosis is often inferred following inhibition of RIPK1 activity, or the deletion of RIPK3. This is complicated by the growing evidence that both RIPK1 and RIPK3 have necroptotic independent functions (Silke et al., 2015). A recent study made a direct comparison of mice lacking either RIPK3, RIPK1 catalytic activity or MLKL in a number of inflammatory disease models (Newton et al., 2016). This highlighted the importance of separating the inflammatory functions of the RIP kinases from MLKL-dependent cell lysis when evaluating the contribution of necroptosis to inflammatory phenotypes (Newton et al., 2016). This study documented that while MLKL deficiency improved survival following kidney ischemia–reperfusion injury and in a TNF-induced model of SIRS, its loss had no impact on several models that necroptosis (i.e. RIPK3 deficiency) had previously been implicated in (Newton et al., 2016). Despite this, the recent development of antibodies that detect the active phosphorylated form of MLKL has implicated necroptosis in diseases such as drug induced liver injury (Wang et al., 2014) and toxic epidermal necrolysis (Kim et al., 2015, Panayotova-Dimitrova et al., 2015).

1.11.4 Necroptosis as an immune response

It has been proposed that necroptosis evolved as an immune strategy to counteract pathogenic avoidance of apoptosis (Kaiser et al., 2013b). Consistent with this necroptosis can drive immunity against a range of viral and bacterial pathogens, including those that encode caspase inhibitors (Guo et al., 2015). For example, RIPK3 deficient mice are unable to activate necroptosis in response to *Vaccinica* virus, and therefore cannot mount an efficient immune response and succumb to the infection (Cho et al., 2009). In addition, viruses have been shown to express proteins that suppress RIPK1 activation, blocking activation of host immune response through the necroptotic cell death pathway (Upton et al., 2008).

Necroptotic cells are immunomodulatory, activating T-cell priming, adaptive immune responses and anti-tumour immunity (Aaes et al., 2016). However, it has been reported that RIPK3 can also activate adaptive immune responses via activation of RIPK1 and the pro-inflammatory NF-κB pathway, prior to necroptotic cellular disruption (Yatim et al., 2015). Together these studies highlight the complex interaction between cell death and inflammatory pathways that ultimately result in activation of immune defences.

1.11.5 Crosstalk between MLKL-driven necroptosis and the NLRP3 inflammasome.

Although necroptosis is considered inflammatory in its own right, it has also been reported to activate IL-1β through the NLRP3 inflammasome (Figure 1.10). As covered in section 1.10.2, activation of this inflammasome can be triggered through RIPK1 and RIPK3 in a caspase-8 dependent manner (Gaidt et al., 2016, Moriwaki et al., 2015, Lawlor et al., 2015). In addition, activation of MLKL following inhibition of caspase-8, can also result in the processing of caspase-1 and IL-1β via the NLRP3 inflammasome (Lawlor et al., 2015, Kang et al., 2013, Kang et al., 2015) (Figure 1.10).

Experimentally, necroptosis acts as an activating stimulus for the inflammasome in primed cells. In caspase-8 deficient dendritic cells the stimulation of TLR4 alone is enough to activate NLRP3 in a RIPK3 dependent manner (Kang et al., 2013). While in BMDM the combination of caspase-8 inhibition with either a priming signal through TLR3 (Kang et al., 2015), or a priming signal through TLR4 and IAP inhibition (Lawlor et al., 2015), mediates NLRP3 activation via RIPK3-MLKL signalling. Although it is hypothesised that

necroptosis may activate NLRP3 through MLKL-mediated membrane disruption and potassium efflux, none of the above studies confirmed this. It has further been suggested that necrotic cells can induce the activation of the NLRP3 inflammasome in neighbouring macrophages (Iyer et al., 2009). Similarly, recent work has suggested that ASC specks released from pyroptotic cells can be taken up by neighbouring phagocytes to trigger NLRP3 activation (Franklin et al., 2014, Baroja-Mazo et al., 2014). This raises the question of whether MLKL-mediated activation of NLRP3 is a cell-intrinsic process, or reliant on DAMP release.

There are a number of inflammatory diseases within which both necroptosis and IL-1β signalling have been separately implicated. These include atherosclerosis (Lin et al., 2013, Paramel Varghese et al., 2016, Sheedy and Moore, 2013), multiple sclerosis (Ofengeim et al., 2015, Jha et al., 2010, Inoue and Shinohara, 2013), and ischemia reperfusion injury of the kidney (Lau et al., 2013, Linkermann et al., 2013, Bakker et al., 2014), heart (Smith et al., 2007, Toldo et al., 2012) and brain (Degterev et al., 2005, Northington et al., 2011, Brough et al., 2011). There have already been reports of simultaneous activation of RIPK3-MLKL necroptosis and caspase-1 mediated IL-1β activation in a model of *Staphylococcus aureus* infection (Kitur et al., 2015). It is therefore important to better understand how the cross-talk between these pathways drives inflammation and immune responses.

1.12 Thesis statement

This thesis examines the ways in which three distinct pathways of programmed cell death can trigger activation of the potent pro-inflammatory cytokine IL-1β to drive an innate immune response. The cell death pathways covered are caspase-1 driven pyroptosis, caspase-8 driven apoptosis and finally RIPK3 and MLKL-dependent necroptosis.

1.13 Aims:

1 – Determine whether caspase-1-mediated pyroptosis is required for the release of activated IL-1β.

2 – Examine whether direct activation of caspase-8 can enable its processing of IL-1β in the absence of secondary signalling pathways.

3 – Study the mechanism by which necroptotic MLKL drives the activation of the NLRP3 inflammasome and caspase-1 processing of IL-1β.

Aims 1 and 2 are relevant to Chapter 3, while aim 3 is explored in Chapter 4. A broad discussion in Chapter 5 will explore the complex crosstalk between these pathways.

Chapter 2: Materials and Methods.

2.1 Cell culture maintenance and treatment.

2.1.1 MEF cells

WT and *Caspase-8*$^{-/-}$ MEF cells immortalized with SV40 large T antigen were cultured in Dulbecco's modified Eagle's medium (DME) containing 10% foetal calf serum (FCS) (37°C, 10% CO_2). MEFs were seeded at 1.5 x 10^5 cells per well (6 well) and treated 24 h later as indicated in the figure legends. Following treatment non-adherent cells were collected, while remaining adherent cells were typsinised and resuspended in media, after which both fractions were combined.

2.1.2 THP1 cells

THP1 cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 containing 8% FCS (37°C, 10% CO_2). Cells were seeded at 3 x 10^5 per mL prior to treatment.

2.1.3 Differentiation of THP1 cells

Differentiation of THP1 cells was achieved by incubating cells in 500 nM phorbol 12-myristate 13-acetate (PMA) in RPMI 1640 media containing 8% FCS for 3 hours (37°C), before washing twice with RPMI 1640 media, seeding at 1.5 x 10^6 per well (6 well) or 3 x 10^5 per well (24 well plate) and incubating for 48 hours (37°C, 10% CO_2) prior to treatment. Cells were harvested for flow cytometry by collecting non-adherent cells, while remaining adherent cells were typsinised and resuspended in media and both fractions combined.

2.1.4 NF-κB reporter cells

NF-κB reporter THP1 monocytic cells were made by infection with the lentiviral reporter vector (pTRH1-NF-κB-dscGFP, TR503PA; System Bioscience) and sorted for GFP expression by flow cytometry.

2.1.5 HEK293T cells

HEK293T cells were maintained in DME containing 8% FCS (37°C, 10% CO_2).

2.1.6 Bone Marrow Derived Macrophages.

Bone marrow derived macrophages (BMDM) were generated from C57BL/6, *Ripk3*$^{-/-}$ (Newton et al., 2004), *Mlkl*$^{-/-}$ (Murphy et al., 2013), *Caspase-1*$^{-/-}$ (*ICE*$^{-/-}$*,* with a naturally occurring inactivating caspase-11 deletion (Kuida et al., 1995, Kayagaki et al., 2011)) and *Nlrp3*$^{-/-}$ (Brydges et al., 2009) mice. Macrophages were generated from bone marrow cells harvested from femoral and tibia bones, cultured on bacterial Petri dishes for 6 days in DME containing 10% foetal calf serum, 50 U/mL penicillin and 50 mg/mL streptomycin and supplemented with 20% L929 conditioned media (37 °C, 10% CO2). BMDM were plated at 4 x 10^5 per well (24 well) and incubated for 24-48 h prior to treatment. The Walter and Eliza Hall Institute (WEHI) Animal Ethics Committee approved all animal experiments.

BMDM were seeded on non-tissue culture treated plates for flow cytometry viability assays. Non-adherent cells and supernatant were collected and remaining adherent cells harvested using 5 mM Ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS), both fractions were combined. BMDM were seeded on tissue culture treated plates for immunoblotting and cytokine ELISAs.

2.1.7 Preparation of treatments:

Salt solutions (lithium chloride, sodium chloride, potassium chloride, rubidium chloride, cesium chloride) were made up at 2 M in H_2O, diluted into DME and filtered (0.2 μM syringe filter). Glycine was dissolved in DME and filtered (0.2 μM syringe filter).

Table 2.1 Reagents

Reagent	Catalogue number	Company
ATP	A2383	Sigma
Doxycycline	D3447	Sigma
Compound A	-	TetraLogic Pharmaceuticals
Coumermycin	C9270	Sigma
Etoposide	1846841	Clifford Hallam
Glycine	G7126	Sigma
human Fc-TNF	(Bossen et al., 2006)	produced in house
MCC950	(Coll et al., 2015)	Provided by A. Roberson and M. Cooper
Nigericin	N1743	Sigma
Pam3Csk4	tlrl-pms	Invivogen
Polybrene	107689	Sigma
Propydium iodide	81845	Sigma
Puromycin	p8833	Sigma
Q-VD-OPh	03OPH109	MP biomedicals
Ultrapure lipopolysaccharide from *E. coli* K	tlrl-peklps	Invivogen
ZVAD-fmk	V116	Sigma
lithium chloride	10374	BDH
sodium chloride	465	Univar
potassium chloride	104936	Merck
rubidium chloride	R2252	Aldrich
cesium chloride	15507	Invitrogen

2.2 Generation of DNA constructs

2.2.1 Cloning of caspase constructs

Murine caspases were cloned into a pre-existing pFTRE 3G rtTA puro vector containing an N-terminal FLAG tag, C-terminal fragment of *E. coli* gyrase B (residues 2–220) and green fluorescent protein (GFP) generated previously by Dr. John Silke. Murine caspase-1 and caspase-8 were cloned from existing vectors using primers that introduction an N-terminal BamHI site with ATG start codon and a C-terminal NheI while removing any stop codons. Murine caspase-9 was cloned from a Gblock® within which internal BamH1 sites were mutated (all primers and sequences are listed in Table 2.2 and 2.3).

Caspase-1 mutations were introduced via Gblock® sequences with N-terminal BstEII and C-terminal HindIII which are naturally occurring restriction sites within caspase-1. Caspase-1 was cloned into a pcDNA vector to allow subcloning of Gblock® sequences prior to cloning into the original pFTRE 3G rtTA puro gyrase vector.

2.2.2 Polymerase Chain Reaction

PCR Reaction Mix

1 µL	Taq DNA polymerase
10 µL	10x master mix buffer
1 µL	dNTPs (10 mM)
1 µL	DNA (0.2-1 µg)
1 µL	Forward primer (40 mM)
1 µL	Reverse primer (40 mM)

Filled to 100 µL with water

Thermocycling conditions

1.	95 °C	5 min
2.	95 °C	1 min
3.	68 °C *	2 min
4.	72 °C	5 min
Steps 2-4 cycled 30 times		
5.	72 °C	5 min

*temperature optimised for primer pair

Polymerase Chain Reaction (PCR) products were separated by agarose gel electrophoresis on a 1.5% agarose Tris-acetate (TA) gel with ethidium bromide. Gels were visualised on a UV trans-illuminator and DNA extracted using Wizard® SV Gel and PCR Clean-Up System (Promega) according to manufacturer’s instructions.

2.2.3 Digestion and ligation to insert fragments into bacterial expression vectors

Full length caspase inserts and pFTre vector were digested with BamHI and NheI according to manufacturer's instructions, while caspase-1 mutation Gblocks® and pcDNA vector were digested with BstEII and HindIII. Both digested vectors and inserts were separated by agarose gel electrophoresis and cleaned up as described above (Section 2.2.2).

Ligations were set up as follows and incubated at room temp for 3 h. Ratios were altered if the first ligation was unsuccessful.

Ligation Reaction Mix

1 µL T4 DNA ligase

1 µL 10x T4 DNA ligase buffer

~150 ng Vector

~300 ng Insert

Filled to 20 µL with water

2.2.4 Transformation of ligated construct into Stbl3 E. coli

Ligations were transformed into chemically competent E. coli by combining 50 µL of thawed bacteria with 10 µL of ligation reaction, the mixture was incubated on ice for 5 min, at 42°C for 30 seconds, then ice for 5 min. Bacteria were incubated in 250 µL of Luria broth (LB) for 20 min at 37°C. Bacteria were pelleted at 5,000 revolutions per minute (rpm) and all but 20 µL of the supernatant discarded. Bacterial pellets were then resuspended and spread evenly on plates filled with LB Agar with 100 µg/mL ampicillin. Plates were incubated at 37°C overnight.

Colonies were confirmed positive for insert ligation through colony PCR and visualisation on 1.5% agarose TA gel with ethidium bromide.

Colony PCR Reaction Mix

10 µL	GoTaq green master mix
.25 µL	Forward primer
.25 µL	Reverse primer

Filled to 20 µL with water

Thermocycling conditions

1. 95 °C 5 min
2. 95 °C 1 min
3. 68 °C * 2 min
4. 72 °C 5 min

Steps 2-4 cycled 30 times

5. 72 °C 5 min

*temperature optimised for primer pair

Positive colonies were picked and grown in 5 mL LB with 100 µg/mL ampicillin overnight at 37°C. DNA was extracted with QIAGEN midi-prep kit according to manufacturer's instructions. All constructs were confirmed through sequencing.

Table 2.2 Primers used for cloning

Caspase-1 forward	GGTGGTGGATCCATGGCTGACAAGATCCTGAGGGC
Caspase-1 reverse	GCTAGCGCTAGCATGTCCCGGGAAGAGGGTAG
Caspase-8 forward	GGTGGTGGATCCATGGATTTCCAGAGTTGTCTTTA
Caspase-8 reverse	AGCAGCGCTAGCGGGAGGGAAGAAG
Caspase-9 forward	GATAAGGGATCCATGGACGAGG
Caspase-9 reverse	GGAACCGCTAGCTGAAGTTTT
Caspase-1 Gblock forward	TTTTGTCAGGGTCACCCTATCA
Caspase-1 Gblock reverse	GAAGTGCCCAAGCTTGAAAGAC

Table 2.3 Gblock sequences

Insert	Sequence
Caspase-9	GATAAGGGATCCATGGACGAGGCGGACCGGCAGCTCCTGCGGCGAT GCAGGGTGCGCCTAGTGAGCGAGCTGCAAGTCGCGGAGCTCTGGGAC GCTCTGCTGAGTCGAGAGCTCTTCACGCGCGACATGATCGAGGATAT TCAGCAGGCAGGATCTGGGTCTCGGCGGGATCAGGCCAGGCAGCTGG TCACAGACCTTGAGACCCGAGGGAGGCAGGCCCTTCCTCTCTTCATCT CCTGCTTAGAGGACACAGGCCAAGGCACCCTGGCTTCACTCTTGCAA AGCGGTCGGCAAGCAGCCAAGCAGGACCCAGAGGCTGTTAAACCCC TAGACCACCTGGTGCCTGTGGTCCTGGGACCAATGGGACTCACAGCA AAGGAGCAGAGAGTAGTGAAGCTGGACCCGTCACAGCCTGCCGTGG GAAACCTCACCCCAGTGGTGCTGGGGCCAGAAGAGCTCTGGCCTGCT CGGCTCAAGCCAGAGGTTCTCAGACCAGAAACACCCAGGCCCGTGGA CATTGGTTCTGGCGGAGCTCATGATGTCTGTGTTCCAGGGAAGATCA GGGGACATGCAGATATGGCATACACCCTGGATTCGGACCCCTGTGGC CACTGCCTCATCATCAACAATGTGAACTTCTGCCCTTCCTCGGGGCTC GGCACACGCACGGGCTCCAACTTGGACCGTGACAAACTCGAGCACCG ATTCCGCTGGCTGCGCTTCATGGTGGAGGTGAAGAACGACCTGACTG CCAAGAAAATGGTCACGGCTTTGATGGAGATGGCACACCGGAACCAC CGTGCCCTGGACTGCTTTGTGGTGGTCATCCTCTCTCATGGCTGCCAG GCCAGCCACCTCCAGTTCCCGGGTGCTGTCTATGGGACAGATGGATG CTCCGTGTCCATTGAGAAAATTGTGAATATCTTCAACGGGAGCGGCT GCCCCAGCCTGGGAGGGAAGCCCAAGCTCTTCTTCATCCAGGCCTGC GGTGGTGAGCAGAAAGACCATGGCTTTGAGGTGGCCTGCACTTCCTC TCAAGGCAGGACCTTGGACAGTGACTCTGAGCCAGATGCTGTCCCCT ATCAGGAAGGCCCAAGGCCCTTGGACCAGCTGGATGCTGTGTCAAGT TTGCCTACCCCCAGTGACATCCTTGTGTCCTACTCCACCTTCCCAGGT TTTGTCTCCTGGAGGGACAAGAAAAGTGGCTCCTGGTACATCGAGAC CTTGGATGGCATTCTGGAGCAGTGGGCTCGCTCTGAAGACCTGCAGT CCCTCCTTCTCAGGGTTGCCAATGCTGTTTCTGCGAAAGGGACTTACA AGCAGATTCCTGGCTGTTTTAACTTCCTCCGGAAAAAGCTGTTTTTA AAACTTCAGCTAGCGGTTCC
Caspase-1 cysteine dead mutant Gblock®	GAAGTGCCCAAGCTTGAAAGACAAGCCCAAGGTGATCATTATTCAGG CAGCTCGTGGAGAGAAACAAGGAGTGGTGTTGTTAAAAGATTCAGTA AGAGACTCTGAAGAGGATTTCTTAACGGATGCAATTTTTGAAGATGA TGGCATTAAGAAGGCCCATATAGAGAAAGATTTATTGCTTTCTGCTC TTCAACACCAGATAATGTGTCTTGGAGACATCCTGTCAGGGGCTCAC TTTTCATTGAGTCACTCATCAAACACATGAAAGAATATGCCTGGTCTT GTGACTTGGAGGACATTTTCAGAAAGGTTCGATTTTCATTTGAACAAC CAGAATTTAGGCTACAGATGCCCACTGCTGATAGGGTGACCCTGACA AAA
Caspase-1 Uncleavable mutant Gblock®	GAAGTGCCCAAGCTTGAAAGACAAGCCCAAGGTGATCATTATTCAGG CATGCCGTGGAGAGAAACAAGGAGTGGTGTTGTTAAAAAATTCAGTA AGAAACTCTGAAGAGAATTTCTTAACGAATGCAATTTTTGAAAATAA TGGCATTAAGAAGGCCCATATAGAGAAAGATTTATTGCTTTCTGCTC TTCAACACCAGATAATGTGTCTTGGAGACATCCTGTCAGGGGCTCAC TTTTCATTGAGTCACTCATCAAACACATGAAAGAATATGCCTGGTCTT GTGACTTGGAGGACATTTTCAGAAAGGTTCGATTTTCATTTGAACAAC CAGAATTTAGGCTACAGATGCCCACTGCTGATAGGGTGACCCTGACA AAA

2.2.5 MLKL constructs.

Inducible MLKL constructs were provided by James Murphy (Hildebrand et al., 2014, Murphy et al., 2013, Tanzer et al., 2016).

2.2.6 Lentiviral infection.

5 μg total DNA was transfected onto 293Ts; 2.5 μg pCMVδR8.2, 1.5 μg plasmid of interest, 2 μg VSV-G or ENV. DNA was prepared using the Effectine® Transfection Reagent kit (QIAGEN) according to manufacturer's instructions and dropped over the 293Ts. After 24 h the media on the 293Ts was removed and replaced with fresh media.

Viral supernatant was collected at 48 and 72 hours filtered through a 0.45 μm filter, it was used immediately or stored at -80°C. Cells were incubated in viral supernatant containing polybrene (5 μg/mL), either undiluted or diluted 2-fold with fresh media. Media was changed after 8 h. 24 h after infection cells were incubated in puromycin (2-5 μg/mL) alongside uninfected control cells until selection was complete (24-48 h).

2.3 Cell Viability assays

2.3.1 Propidium iodide uptake

Resuspended cells were incubated with propidium iodide (PI, 1 μg/mL) and analysed for uptake by flow cytometry on a FACSCalibur or FACSCanto (BD Immunocytometry Systems). Data was analysed using FlowJo software version 7.6.5.

2.3.2 Quantification of cell number using flow cytometry particles

Cells were seeded and treated 24 h later as indicated in the figure legends. Equal numbers of blank calibration beads (6.3×10^4 beads/well) (Spherotech) were added to each culture well just before harvesting. Adherent and non-adherent cells were harvested and incubated with 1 μg/mL PI in PBS prior to analysis by flow cytometry. The number of PI negative cells per 1000 beads was measured for each sample using a FACSCalibur (Becton Dickinson), data was analysed using FlowJo software version 7.6.5.

2.3.3 LDH release cytotoxicity assay

Supernatants were removed from cells and pelleted at 1,500 rpm to remove any debris. Control wells were left untreated or lysed in 1% Triton X-100 (Tx, Sigma-Aldrich, T9284)

to establish maximum LDH release. %LDH activity was analysed by Cytotoxicity Detection Kit (LDH) according to manufacturer's instructions (Roche; 11644793001). Briefly 25 μL of supernatant from each condition was incubated with 25 μL of kit reagent in the dark for 5-15 minutes until a colour change was visible in the Tx lysed positive control. The reaction was stopped with 25 μL of 1 M hydrochloric acid (HCl). Absorbance was measured at 490 nm and % cytotoxity calculated as:
[(experimental value – untreated control)/(Tx lysed control – untreated control)].

2.3.4 MTS-PMS cell proliferation assay

MTS assays were conducted as described in (Rickard et al., 2014). Although this assay is a measure of cell metabolism it was used as an indirect measure of cell proliferation, changes in cell metabolism could therefore also account for any differences observed between treatments. Briefly, phenazine methosulfate (PMS; 0.92 mg/ml in PBS; Sigma-Aldrich) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; 2 mg/ml in PBS; Promega) were combined in a 1:20 ratio. The mixture was added directly to treated cells at a 1:5 ratio and incubated for 1–4 hr (37°C, 10% CO_2). Supernatants were transferred to a flat bottom 96 well plate and absorbance was measured at 490 nm. Cell proliferation was calculated relative to the untreated sample.

2.4 Cytokine release assays

2.4.1 Measurement of cytokines by enzyme-linked immunosorbent assay

Cells were treated as indicated. Supernatants were removed from cultured cells and pelleted at 1,500 rpm to remove any debris. Lysates were made in DISC buffer [20 mM Tris pH 7.5, 150 mM NaCl, 2mM EDTA, 10% Glycerine, 1% Triton X-100 including protease inhibitors (Roche, 11697498001)] or by subjecting cells to three rounds of freeze thawing and centrifuging lysates at 13,000 rpm to remove cellular debris.
Supernatants and lysates were assay by enzyme-linked immunosorbent assay (ELISA) for mouse IL-1β (R&D Systems; DY401), human IL-1β (R&D Systems; DY201), mouse TNF (Ebioscience; 88-7324), human TNF (Ebioscience; 88-7346) and mouse IL-1α (BioLegend; 433401) according to manufacturer's instructions.

2.4.2 IL-1β activity assay using NF-κB reporter cell line

MEF and THP1 cells were treated as indicated. Supernatants were removed from cultured cells and pelleted to remove any debris. Duplicate seedings of THP1 cells were subjected to three rounds of freeze thawing and supernatants were cleared by pelleting at 13,000 rpm. THP1 cells bearing the NF-κB reporter construct were cultured in the harvested supernatant for 8-24 h prior to measuring GFP expression of THP1 cells relative to those in media alone using a FACSCalibur (Becton Dickinson).

2.5 Preparation of cell lysates for immunoblot assays

2.5.1 ONYX buffer.

Resuspended MEF and THP1 cells were washed 1x in cold PBS. Cell pellets were resuspended in ONYX buffer (20 mM Tris, pH 7.5, 135 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 10% glycerol, 1% Triton X-100 (Sigma-Aldrich, T9284) including protease inhibitors (Roche, 11697498001). Lysates were incubated on ice for 20 minutes and centrifuged at 13,000 rpm to remove debris. Protein concentration was quantified using the pierce bicinchoninic acid assay (BCA) protein assay kit (Thermo scientific) according to manufacturer's instructions. Up to 20 μg of protein from each sample was diluted with Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) sample buffer and boiled for 5 minutes prior to immunoblotting.

2.5.2 SDS-PAGE sample buffer.

Resuspended THP1 cells were pelleted and lysed in SDS-PAGE sample buffer, while adherent BMDM and differentiated THP1 cells were lysed directly in SDS-PAGE sample buffer (50 mM Tris pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol, 0.01% (v/v) bromophenol blue, 0.05% (v/v) 2-mercaptoethanol). Supernatants were diluted with 5x SDS-PAGE sample buffer. Samples were boiled for 20 minutes prior to immunoblotting.

2.5.3 Membrane fractionation for Blue Native PAGE.

Fractionation of cells into cytoplasmic and membrane fractions was performed as previously published (Hildebrand et al., 2014). Briefly, WT BMDM and MEFs were treated as indicated in figure legends. Cells were harvested by scraping in PBS and lysed in

MELB buffer (20 mM Hepes, 100 mM KCl, 2.5 mM $MgCl_2$, 100 mM sucrose, 0.025% digitonin) containing .025% digitonin including protease inhibitors (Complete protease inhibitor tablet (Roche, 11697498001), 5 mM beta-glycerophosphate disodium (Sigma, G9422), 1 mM sodium molybdate (sigma, 221848), 1 mM tetra-sodium pyrophosphate (Sigma, P8010) and 100 µM sodium fluoride (Sigma, S7920), 2 µM N-ethyl maleimide (NEM, Sigma, E3876)). A fraction of each sample was lysed in ONYX buffer as whole cell lysates. Lysates were incubated on ice for 10 minutes and centrifuged at 13,000 rpm to separate the cytosolic fraction from the crude membrane fraction. The pellet was washed twice in MELB buffer and resuspended in MELB buffer containing 1% digitonin (20 mM Hepes, 100 mM KCl, 2.5 mM $MgCl_2$, 100 mM sucrose, 1% digitonin) including protease inhibitors (Complete protease inhibitor tablet (Roche, 11697498001), 5 mM beta-glycerophosphate disodium (Sigma, G9422), 1 mM sodium molybdate (Sigma, 221848), 1 mM tetra-sodium pyrophosphate (Sigma, P8010) and 100 µM sodium fluoride (Sigma, S7920), 2 µM N-ethyl maleimide (NEM, Sigma, E3876)). Lysates were incubated on ice for 20 minutes and centrifuged at 13,000 rpm to separate the digitonin soluble membrane fraction from the insoluble fraction.

2.6 Immunoblotting

2.6.1 SDS-PAGE

Reduced and denatured cell lysates and supernatants were separated on NuPAGE Novex 4-12% Bis-Tris gels (Life Technologies), and transferred onto nitrocellulose (Amersham) or PVDF (Millipore) membranes.

2.6.2 Blue-Native PAGE

Samples were diluted with 10x blue native loading dye (5% Coomassie blue G, 500mM ε-amino n-caproic acid, 100mM Bis-Tris pH 7.0) and separated on a NativePAGE™ Novex™ 4-16% Bis-Tris Protein Gel (Thermo Fisher Scientific, BN1002BOX), alongside a native molecular weight marker (Thermo Fisher Scientific, LC0725) using both Novex running buffer Native PAGE (Thermo Fisher Scientific, BN2001) and blue cathode buffer (Thermo Fisher Scientific, BN2002). Samples were transferred onto PVDF membrane (Millipore) and destained (50% (v/v) methanol, 25% (v/v) acetic acid). Samples were

incubated in denaturing buffer at room temperature for 1-2 h (6 M GuHCl, 10 mM Tris-Cl pH 7.0, 5 mM 2-ME) prior to immunoblotting.

2.6.3 Immunoblotting

Membranes were blocked in 5% skim milk/0.1% Tween/PBS. Primary antibodies diluted in 5% skim milk/0.1% Tween/PBS were incubated on membranes overnight at 4°C. All secondary antibodies used were conjugated to HRP, diluted in 5% skim milk/0.1% Tween/PBS and incubated on membranes for 1-2 h at room temperature. Minimum of 3 washes were carried out between antibody incubations (0.1% Tween/PBS). Membranes were developed using ECL reagent (Amersham, RPN2232, Millipore, WBKLS0).

2.7 ELISpot assay

Capture antibody (R&D systems; MAB401) was incubated at 50 μg/mL in 50 μL PBS overnight on a 96 well filtration plate (Millipore, MAHAS4510). Plates were subsequently blocked with 100 μL Dulbecco's Modified Eagle's Medium (DME) supplemented with 8% FCS at 37°C for 1 h. MEF cells bearing FLAG-caspase-1-gyrase-GFP and pro-IL-1β GFP constructs were then seeded on the plates as indicated and treated for 16 h. As a control media with recombinant IL-1β (standard from mouse IL-1β duoSet ELISA, R&D systems; DY401) at concentrations between 20,000 pg/mL to 1,000 pg/mL was incubated on the plate for 16 hours. An AID ELISpot Reader (Autoimmun Diagnostika GMBH) was used to take fluorescent images of GFP positive live cells.

Plates were immediately washed 3x with PBS/0.05%Tween 20 then 3x with PBS alone. Plates were incubated with biotin-conjugated detection antibody (R&D systems; BAF401) at 5 μg/mL in 50 μL PBS 1%FCS for 2 h at room temperature. After washing, streptavidin-alkaline phosphate (ALP) (Mabtech; 3310) diluted 1/1000 in PBS/1%FCS was added to the plates for 1 h at room temperature.

After washing, ELISpots were developed using 50 μL of 5-bromo-4-chloro-3- indolyl phosphate (BCIP) / Nitroblue tetrazolium chloride (NBT)-plus substrate (Mabtech; 3650-10). Plates were rinsed with room temperature water to stop the reaction and dried. GFP positive cells and ELISpot numbers were analysed using the AID ELISpot software.

The brightness and contrast of figure images were adjusted using Fiji (ImageJ) and Adobe Illustrator in a linear fashion, with settings applied equally to every image. Pseudo-colouring was applied to merged images using Adobe Illustrator to allow overlay of ELISpots and GFP positive cells. Tissue culture plates were also seeded simultaneously to ELISpots to allow for microscopy imaging using the Opera Phenix, taken at 10x with 2x2 binning.

2.8 Immunofluorescence of endogenous ASC specks by confocal microscopy.

Immunofluorescence of ASC specks was performed similarly as described (Beilharz et al., 2016). Briefly, 8-well chamber μ-slides (Ibidi) were coated with retronectin, before wildtype (Ly5.1) and *Mlkl*$^{-/-}$ (Ly5.2) BMDMs were seeded in either mixed populations at 0.5 x10^5 cells per genotype, or ~1x10^5 of either wildtype or *Mlkl*$^{-/-}$ BMDMs per well. Cells treated as described in figure legends were fixed with 4% PFA, before blocking (PBS, 10% FCS) for 60 min. Cells were then stained for Ly5.2 surface expression (BD Pharmingen, 561874, 1:100) overnight at 4°C. The following day cells were washed and permeabilised (PBS, 10% FCS, 0.5% Triton-X100) for 60 min, before staining for intracellular ASC (N-15, Santa Cruz, 1:500) overnight at 4°C. Cells were then washed and stained with secondary antibodies staining for ASC with goat anti-rabbit-Alexa647 (Invitrogen, A-21245, 1:1000) or Ly5.2 with goat anti-mouse-Alexa488 (Invitrogen, A-11029, 1:300) for 60 min, before nuclear staining with DAPI for 10 min.

Cells were imaged using a Zeiss LSM 780 confocal microscope; 3x3 tile scans with Z-stacks were obtained for each experimental condition using a 40x oil objective with Immersol 518 F (Zeiss) and acquired with ZEN 2012 v8.1 software (Zeiss). Image channels were merged and displayed as maximum projection before conversion to TIFF using FIJI software. Quantification of ASC specks was performed by detecting local maxima in the Red channel (ASC stain), the resulting objects were then determined to be either inside (*Mlkl*$^{-/-}$ cells) or outside (wildtype cells) the Green channel (Ly5.2 stain; *Mlkl*$^{-/-}$) through morphological filtering. Total cell number (wildtype and *Mlkl*$^{-/-}$) per image was determined from the DAPI channel. Quantification was automated using a custom written FIJI macro (Schindelin et al., 2012).

Table 2.4 Antibodies

Antibody	Species reactivity	Source	Catalogue No	Company
ASC	Human, Mouse, Rat	Rabbit	sc-22514-R	Santa Cruz Biotechnology
β-actin	Human, Mouse, others	Mouse	A-1978	Sigma
Caspase-1 (full length and p10 fragment)	Mouse	rabbit	sc-514	Santa Cruz
Caspase-1 (full length and p20 fragment)	Mouse	Mouse	AG-20B-0042	Adipogen
caspase-1 (full length and p20 fragment)	Human	Rabbit	3866	Cell Signaling Technology
caspase-3 (19 and 17 kDa fragments)	Human, Mouse, others	Rabbit	9661	Cell signalling
caspase-8 (full length)	Mouse	Rabbit	4927	Cell Signaling Technology
caspase-8 (43 and 18 kDa fragments)	Mouse	Rabbit	8592	Cell Signaling Technology
caspase-9 (full length and cleaved fragments)	Human, Mouse	Mouse	ADI-905-686	Enzo life sciences
GAPDH	Human, Mouse, others	Rabbit	2118	Cell Signaling Technology
Goat Anti-Mouse Ig-Horseradish peroxidase	Mouse	Goat	1010-05	Southern Biotech
Goat Anti-Rabbit Ig-Horseradish peroxidase	Rabbit	Goat	4010-05	Southern Biotech
Goat Anti-Rat Ig-Horseradish peroxidase	Rat	Goat	3010-05	Southern Biotech
Horseradish peroxidase-conjugated anti-Goat IgG	Goat	Rabbit	HAF017	R&D
HMGB1	Human, Mouse, others	Rabbit	ab18256	Abcam
HSP 60	Human, Mouse, others	Goat	sc-1052	Santa Cruz
HSP 90	Human, Mouse, others	Rat	ADI-SPA-835	Enzo
IL-1β (full length and p17 fragment)	Mouse	Goat	AF-401-NA	R&D
MLKL	Human, Mouse	Rat	clone 3H1	in-house
NLRP3	Human, Mouse	Mouse	AG-20B-0014	Adipogen
RIPK3	Mouse, Rat	Rabbit	PSC-2283	Axxora
VDAC1	Human, Mouse, others	Rabbit	AB10527	EMD Millipore

Chapter 3: Cell death is not essential for caspase-1-mediated interleukin-1β activation and secretion

3.1 Preface

This chapter contains the following publication:

Conos S A, Lawlor K E, Vaux D L, Vince J E, Lindqvist L M. Cell death is not essential for Caspase-1 mediated Interleukin-1β activation and secretion. Cell Death and Differentiation, 23, 1827-1838. 2016.

As the first and primary author of this paper I played a leading role in the development and realisation of the project, along with analysis of results. I completed 100% of the experiments within the project and wrote the first draft of the manuscript, including compiling figures.

The remaining co-authors on this manuscript contributed to the conception, planning and design of the project, and provided important input into the analysis of results. They contributed to editing of the manuscript and responding to reviewers.

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Cell death is not essential for caspase-1-mediated interleukin-1β activation and secretion

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Caspase-1 cleaves and activates the pro-inflammatory cytokine interleukin-1 beta (IL-1β), yet the mechanism of IL-1β release and its dependence on cell death remains controversial. To address this issue, we generated a novel inflammasome independent system in which we directly activate caspase-1 by dimerization. In this system, caspase-1 dimerization induced the cleavage and secretion of IL-1β, which did not require processing of caspase-1 into its p20 and p10 subunits. Moreover, direct caspase-1 dimerization allowed caspase-1 activation of IL-1β to be separated from cell death. Specifically, we demonstrate at the single cell level that IL-1β can be released from live, metabolically active, cells following caspase-1 activation. In addition, we show that dimerized or endogenous caspase-8 can also directly cleave IL-1β into its biologically active form, in the absence of canonical inflammasome components. Therefore, cell death is not obligatory for the robust secretion of bioactive IL-1β.
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Interleukin-1β (IL-1β) is a pro-inflammatory cytokine that is activated by proteolytic cleavage to a mature 17 kD form before it is released from the cell. After its release, IL-1β binds to the IL-1 receptor to coordinate immune responses that are important for host protection against microbial organisms, and regulate the wound-healing response following tissue damage.[1,2] In some circumstances the pathological production of IL-1β results in auto-inflammatory diseases, such as cryopyrin-associated periodic syndromes (CAPS), which are successfully treated by blocking IL-1β signaling.

Caspase-1 cleaves and activates the precursor of IL-1β (pro-IL-1β) within large cytosolic protein complexes termed inflammasomes. Inflammasome sensor proteins include NOD-like receptor (NLR) family members, such as NLRP1, NLRP3 and NLRC4, AIM2-like receptors and the tripartite motif family member pyrin. Of the NLRs, NLRP3 is the most widely studied as it responds to a diverse range of microbial products, host cell derived danger molecules and environmental irritants.[3]

Pyrin domain-containing inflammasome sensor proteins, such as NLRP3 and AIM2, drive caspase-1 recruitment and activation via the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD). Once activated, caspase-1 can process inflammatory cytokines, such as IL-1β and IL-18. Cleaved, mature IL-1β is rapidly released from the cell, often alongside processed caspase-1 and ASC. IL-1β lacks a conventional secretory signal sequence and hence is secreted in a non-conventional manner.[4] How IL-1β is secreted from cells and whether other caspases can directly substitute for caspase-1 in this process remains of outstanding interest.

Recent research has highlighted cross-talk between inflammasome-associated caspase-1 and the death receptor apoptotic initiator caspase, caspase-8.[5] For example, like caspase-1, caspase-8 can directly process pro-IL-1β into its bioactive form.[6–9] Caspase-8 has also been proposed to directly cleave caspase-1 following bacterial detection[10,11] or trigger caspase-1 by activating the NLRP3 inflammasome following toll-like receptor (TLR) or TNF ligation.[12,13] In addition, recent studies have reported that NLRP3 and AIM2 inflammasome-associated ASC can directly bind to caspase-8 to cause apoptotic cell death in the absence of caspase-1.[14–16] Despite these reported novel roles for caspase-8, it remains unclear how caspase-8 activates IL-1β.

It has been suggested that IL-1β is released passively when a cell dies, and that cell death is required for IL-1β release. This idea stems from the observation that caspase-1 activation can trigger a lytic pro-inflammatory cell death program termed pyroptosis.[17] Recent studies have postulated that most, if not all, inflammasome activators cause either necrosis or pyroptosis, thus allowing the passive release of active IL-1β.[18–20] However, several groups have suggested that IL-1β secretion may occur prior to the loss of plasma membrane integrity in macrophages and dendritic cells.[21,22] Therefore, we sought to resolve whether caspase-1 must induce cell death for IL-1β to be secreted.

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Abbreviations: ASC, apoptosis-associated speck-like protein containing a CARD; BMDM, bone marrow-derived macrophages; CAPS, cryopyrin-associated periodic syndromes; Cp.A, smac-mimetic compound A; DME, Dulbecco's modified eagle's medium; dox, doxycycline; IAP, inhibitor of apoptosis protein; IL-1β, interleukin-1β; IL-1R, interleukin-1 receptor; MEF, murine embryonic fibroblast; NLR, NOD-like receptor; NLRP3, NLR protein 3; PI, propidium iodide; pro-IL-1β, precursor interleukin-1β; TLR, toll-like receptor; WT, wild type; TNF, tumor necrosis factor; AIM2, absent in melanoma 2
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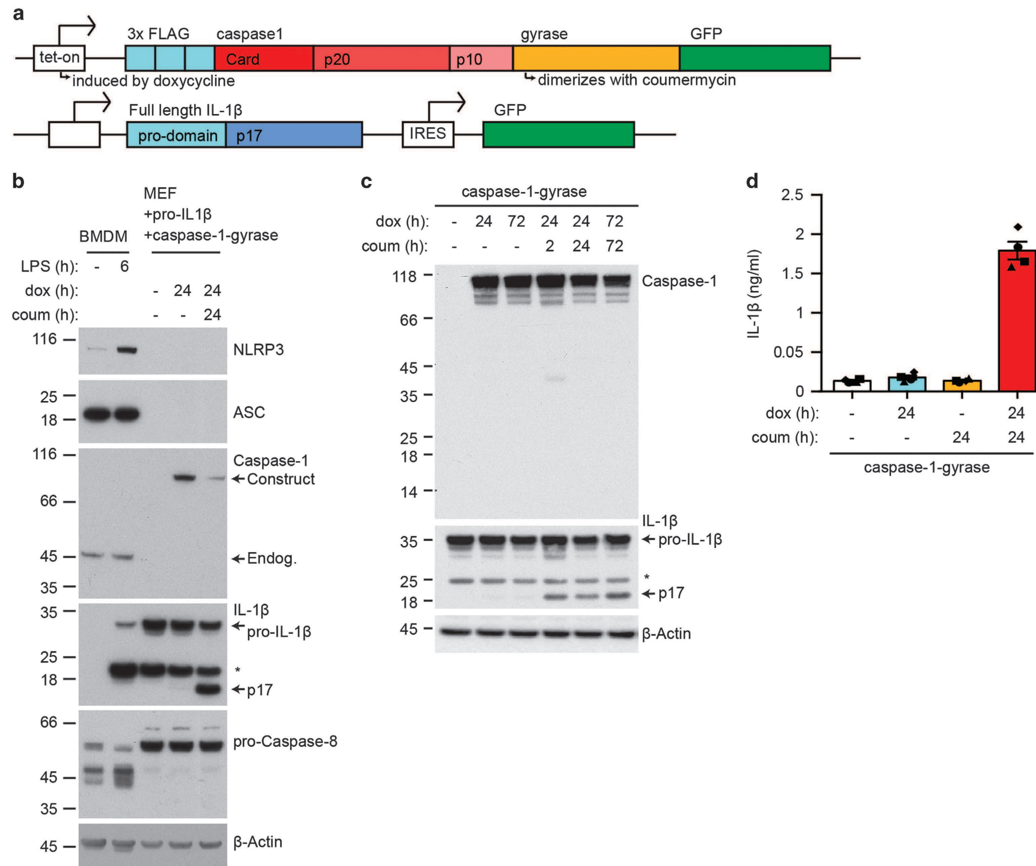

Figure 1 Forced dimerization of caspase-1 causes IL-1β cleavage in the absence of an intact inflammasome pathway. (**a**) A schematic of the doxycycline inducible fusion protein FLAG-caspase-1-gyrase-GFP (caspase-1-gyrase) vector system and pro-IL-1β retroviral vector system. Doxycycline treatment induces expression of caspase-1-gyrase fusion protein, whereas coumermycin binds to the gyrase domain to cause caspase-1 dimerization. (**b**) MEFs lack expression of the inflammasome components NLRP3, ASC and caspase-1. WT BMDMs were treated with 100 ng/ml LPS for 6 h to induce NLRP3 expression. WT MEFs, previously infected with caspase-1-gyrase and a pro-IL-1β, were treated with 1 μg/ml doxycycline to induce caspase-1-gyrase expression and 700 nM coumermycin to dimerize caspase-1-gyrase for the indicated times. Cells lysates were analyzed by western blot for the indicated proteins. (**c**) Dimerization of caspase-1-gyrase with coumermycin caused rapid cleavage of pro-IL-1β. Western blot of lysates from MEFs containing caspase-1-gyrase and pro-IL-1β, which were treated with 1 μg/ml doxycycline and 700 nM coumermycin for the indicated times. Blot is representative of three independent experiments. (**d**) Dimerization of caspase-1-gyrase causes secretion of IL-1β. MEFs stably infected with caspase-1-gyrase vector and pro-IL-1β were treated with 1 μg/ml doxycycline and 700 nM coumermycin. Supernatants were analyzed by ELISA. $n=4$ independent experiments. Error bars represent the S.E.M. Asterisks denote non-specific bands in western blots

Results

Dimerization of a caspase-1-gyrase fusion protein leads to activation and secretion of IL-1β in mouse embryonic fibroblasts, in the absence of inflammasome machinery. In order to study caspase-1-mediated activation of IL-1β directly, we designed a FLAG- and GFP-tagged dimerizable doxycycline (dox)-inducible caspase-1 construct, referred to as caspase-1-gyrase (Figure 1a). By expressing caspase-1 as a fusion protein with *Escherichia coli* gyrase, we can dimerize the protein using the divalent compound coumermycin.[23,24] Hence, this system allows induction of caspase-1 expression, which can be monitored at a single cell level by GFP fluorescence, followed by dimerization.

Using a lentiviral vector, the caspase-1-gyrase construct was stably infected into murine SV40 large T immortalized mouse embryonic fibroblasts (MEFs) bearing a second construct that constitutively expresses untagged mouse pro-IL-1β and GFP separately (Figure 1a). We took advantage of our observation that, unlike bone marrow-derived macrophages (BMDMs), MEFs do not endogenously express inflammasome components that could confound the

interpretation of data, including the sensor NLRP3, adaptor protein ASC, caspase-1 and pro-IL-1β (Figure 1b). Induction of caspase-1-gyrase expression by dox alone did not cause significant amounts of pro-IL-1β cleavage. However, when caspase-1-gyrase was dimerized by co-treatment with coumermycin, cleavage of pro-IL-1β into the active p17 fragment was readily detected in cell lysates within 2 h (Figure 1c). Interestingly, we were not able to detect robust capsase-1-gyrase cleavage of itself, suggesting that processing of caspase-1 may not be required for it to cleave pro-IL-1β, as previously reported.[25] Importantly, caspase-1 dimerization also induced secretion of IL-1β, as measured in supernatants by ELISA (Figure 1d).

Western blot analysis confirmed that the IL-1β detected in the supernatants was cleaved to the mature bioactive p17 fragment (Figure 2a). As measured by densitometry from three independent experiments, 32.3% (±0.02 S.E.M.) of the processed IL-1β was released into the supernatant after 24 h. Measurements of IL-1β levels by ELISA (Mouse IL-1β/IL-F2 ELISA DY401, R&D) did not distinguish clearly between pro-IL-1β and the active p17 fragment, although they appeared to be more specific for cleaved IL-1β (Figure 2b). Despite this caveat, the ELISA results largely agreed with the western blot data, indicating that at least one-third of cleaved cellular IL-1β is released into the supernatant upon caspase-1-gyrase dimerization (Figure 2b). Notably, the mature p17 fragment of IL-1β, but not cellular proteins such as precursor IL-1β, caspase-1, HSP60, HMGB1, GAPDH or β-actin, were released into the supernatant upon caspase-1 dimerization (Figure 2a). The absence of these abundant intracellular components in the supernatant, other than cleaved IL-1β, implies that caspase-1-gyrase-induced secretion of mature IL-1β may occur prior to, or in the absence of, significant cell lysis.

To formally demonstrate that the cleaved IL-1β detected in the supernatant by western blot was biologically active, we used Thp1 cells that express GFP specifically upon NF-κB activation.[26] As expected, these Thp1 reporter cells exhibited robust GFP fluorescence, indicating NF-κB activation, when they were treated with recombinant mature IL-1β or LPS (Supplementary Figure S1a). Similar NF-κB activity was observed when the Thp1 reporter cells were treated with supernatants from dox- and coumermycin-treated caspase-1-gyrase MEFs, as indicated by strong GFP expression (Figure 2c, Supplementary Figure S1b). In contrast, supernatants prepared by freeze-thawing MEFs to release cytosolic proteins, such as monomeric caspase-1-gyrase, pro-IL-1β and β-actin, did not cause robust NF-κB reporter activity in Thp1 cells, unless caspase-1-gyrase cleavage of IL-1β was induced before freeze/thaw lysis (Supplementary Figure S1c, S1d and S1e). These data demonstrate that dimerization of caspase-1 is sufficient to cause cleavage and secretion of bioactive IL-1β; even in the absence of other inflammasome components or obvious cell lysis.

In order to demonstrate that the secretion of IL-1β was dependent on the proteolytic activity of caspase-1-gyrase, we used the broad-spectrum caspase inhibitor Z-VAD-fmk. Pretreating MEFs for 1 h with Z-VAD-fmk, before inducing and activating caspase-1-gyrase, prevented IL-1β cleavage and secretion, as seen by both ELISA and western blot (Figures 2d and e). To confirm and extend this finding we generated caspase-1-gyrase constructs with mutations to render caspase-1 either catalytically inactive (caspase-1-gyrase catalytically inactive; C385G), or unable to be processed between the p10 and p20 domains (caspase-1-gyrase uncleavable; D296N, D300N, D304N, D308N, D313N, D314N) (Supplementary Figure S2a).[27–29] As expected, the catalytically inactive caspase-1-gyrase did not cleave IL-1β (Figure 2f). On the other hand, we observed that uncleavable caspase-1-gyrase was still capable of cleaving IL-1β as efficiently, or even more so, than wild-type caspase-1-gyrase (Figures 2f and g). These data demonstrate that the dimerization of capase-1 is sufficient to induce catalytic activity and the cleavage of IL-1β in the absence of caspase-1 processing between the p20 and p10 subunits. This is in agreement with our results with the wild-type caspase-1-gyrase (Figure 1c) and a recent report indicating that some inflammasomes (that is, NLRP1b) activate caspase-1 directly, causing secretion of processed IL-1β in the absence of caspase-1 auto-processing.[25]

Dimerized caspase-1-gyrase activates IL-1β in the absence of cell death. Caspase-1-gyrase-mediated cleavage of IL-1β and its secretion appeared to occur in the absence of cell death, as we could not detect other intracellular proteins (that is, HSP60, HMGB1, GAPDH, β-actin and caspase-1) in supernatants upon caspase-1-gyrase dimerization (Figure 2a). To confirm that IL-1β secretion could occur in the absence of cell death, we monitored individual cells. Flow cytometry was used to follow dox-induced expression of caspase-1-gyrase by measuring fluorescence of its GFP tag in cells that did not contain the GFP-expressing pro-IL-1β construct (Figure 3a). Despite caspase-1-gyrase being expressed in 72–85% of all cells, caspase-1 dimerization for 24 h did not cause cell death, as measured by propidium iodide (PI) uptake (Figures 3a and b). Nevertheless, at this time mature IL-1β was detected at high levels in the supernatant (Figures 1d and 2a).

To determine whether cells were undergoing a complete cellular rupture that could, in theory, prevent PI staining of cells, we measured the absolute number of PI-negative cells using calibration beads. Etoposide treatment, which induces intrinsic apoptosis, reduced the number of viable cells by more than 95%. In contrast, the expression and dimerization of caspase-1-gyrase caused no reduction in the total number of viable cells (Figure 3c). Similar to the wild-type caspase-1-gyrase, the catalytically inactive mutant did not induce cell death, whereas the uncleavable mutant induced a small amount of cell death after 24 h of dimerization (Supplementary Figure S2b).

As an alternative readout for cellular health, we used the MTT-PMS assay to measure cell replication and metabolism. Consistent with our previous results, MEFs showed comparable colorimetric change after 72 h regardless of pro-IL-1β or caspase-1-gyrase expression and dimerization, in contrast to etoposide treatment (Figure 3d). Therefore, despite efficient caspase-1-gyrase expression at a single cell level, and high levels of IL-1β detectable in the supernatant, cells with dimerized caspase-1-gyrase are metabolic activity and viable.

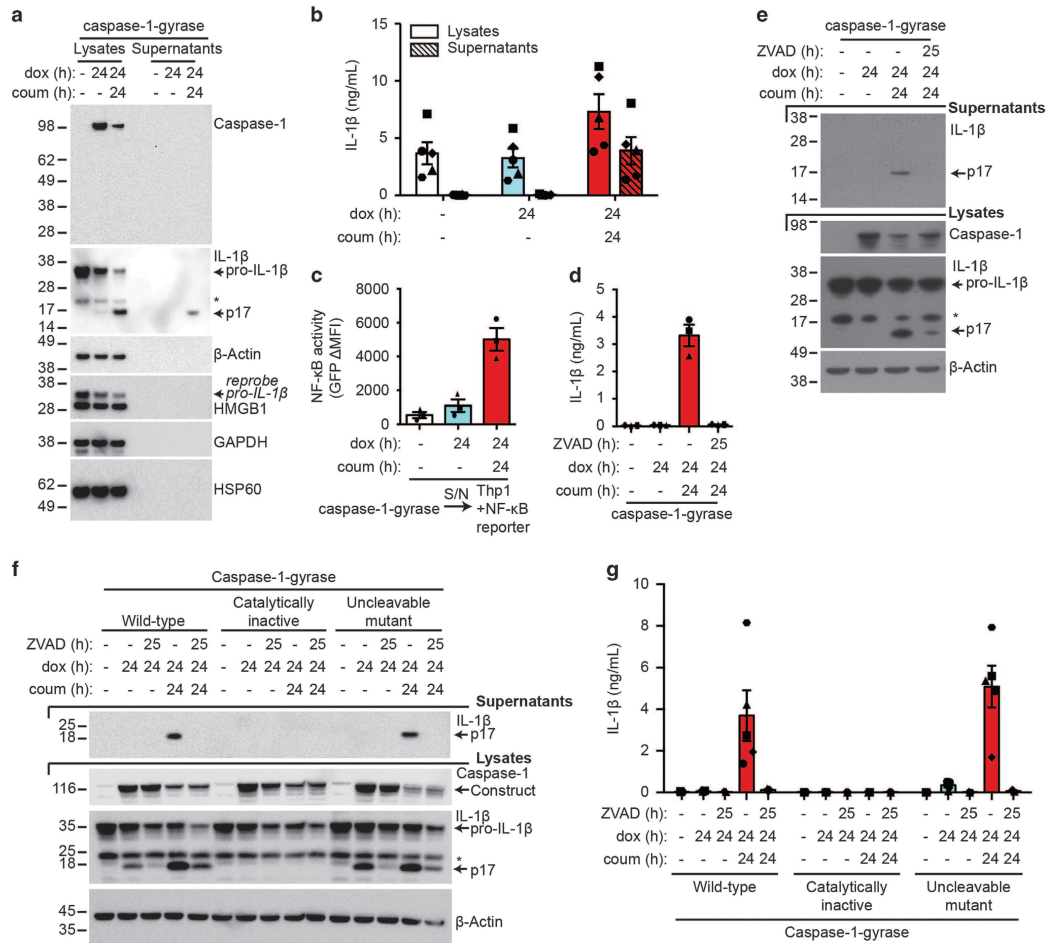

Figure 2 Biologically active IL-1β is secreted from MEFs following caspase-1 dimerization. (**a** and **b**) Dimerization of caspase-1-gyrase causes cleavage and secretion of IL-1β. MEFs stably infected with caspase-1-gyrase vector and pro-IL-1β were treated with 1 μg/ml doxycycline and 700 nM coumermycin. Lysates and supernatants were adjusted to be of equal volume when harvested. (**a**) Equal volumes of lysates and supernatants were analyzed by western blot. Blot is representative of three independent experiments. (**b**) One-third of cleaved cellular IL-1β is released into the supernatant. IL-1β detected from equal volumes of lysates and supernatants by ELISA. $n=5$ independent experiments. (**c**) IL-1β secreted from MEFs is biologically active. Supernatants (S/N) from MEFs stably infected with caspase-1-gyrase and pro-IL-1β, treated for 24 h with 1 μg/ml doxycycline and 700 nM coumermycin, were transferred onto Thp1 cells bearing an NF-κB GFP reporter construct. After 24 h incubation, GFP expression was quantified by flow cytometry. Data are expressed as the change in mean fluorescence intensity (MFI) relative to Thp1 cells in media alone. $n=3$ independent experiments. (**d** and **e**) Secretion of IL-1β following caspase-1 activation requires catalytic caspase activity. MEFs containing pro-IL-β and caspase-1-gyrase were treated with 1 μg/ml doxycycline, 700 nM coumermycin and 25 μM pan-caspase inhibitor Z-VAD-fmk for the indicated times. Supernatants were analyzed by (**d**) ELISA ($n=3$ independent experiments) and (**e**) western blot, whereas lysates were analyzed by (**e**) western blot. (**f** and **g**) Cleavage and secretion of IL-1β requires catalytic activity of caspase-1 but not auto-processing. MEFs stably infected with pro-IL-1β and wild-type caspase-1-gyrase, or a catalytically inactive mutant (C385G), or an uncleavable mutant (D296N, D300N, D304N, D308N, D313N, D314N) were treated with 1 μg/ml doxycycline, 700 nM coumermycin and 25 μM Z-VAD-fmk. (**f**) Lysates were analyzed by western blot, whereas supernatants were analyzed by (**f**) western blot and (**g**) ELISA. $n=3$-5 independent experiments. Error bars represent the S.E.M. in all graphs. Asterisks denote non-specific bands in western blots. See also Supplementary Figure S1 and S2

Caspase-8-gyrase dimerization can induce cleavage and secretion of IL-1β. Recent studies have reported that caspase-8 may (i) process caspase-1,[10,11] (ii) activate NLRP3,[12,30] or (iii) cleave and activate IL-1β directly.[6–9] Therefore, to determine whether dimerization of caspase-8 can directly activate IL-1β and induce its secretion in the absence of other inflammasome components (that is NLRP3 and caspase-1), we generated IL-1β-expressing MEF lines

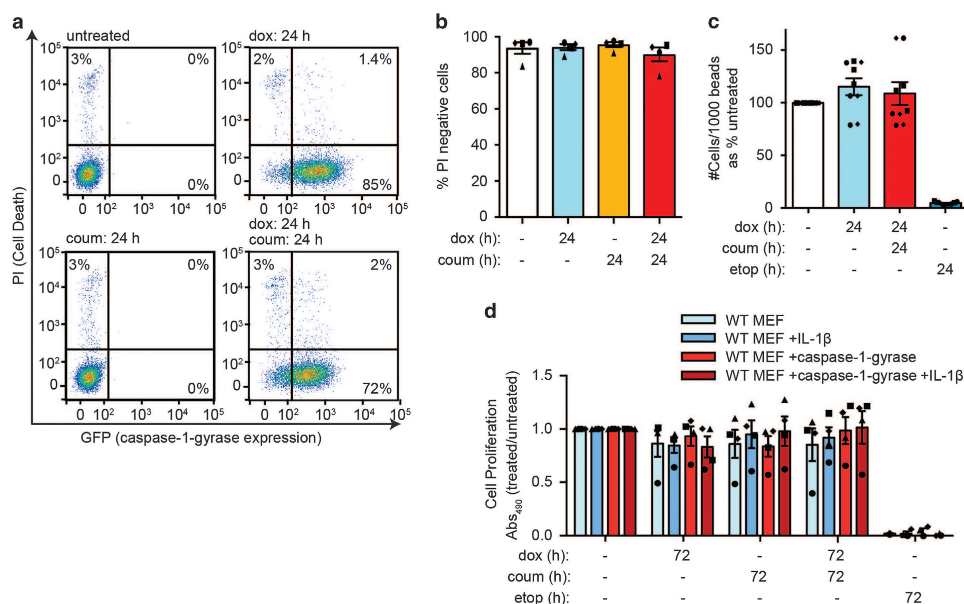

Figure 3 Dimerization of caspase-1-gyrase in MEFs does not induce cell death. (**a** and **b**) Caspase-1-gyrase was induced in MEFs with 1 μg/ml doxycycline and dimerized with 700 nM coumermycin for the indicated times. Cell viability was measured by PI uptake and flow cytometric analysis. (**a**) Representative flow cytometry data. (**b**) Quantification of cell viability from four independent experiments. (**c**) Dimerization of caspase-1-gyrase does not alter the total number of viable cells. MEFs containing caspase-1-gyrase were treated with 1 μg/ml doxycycline and 700 nM coumermycin, or 34 μM etoposide as specified for the indicated times and analyzed by flow cytometry. The absolute number of PI-negative cells was measured as a ratio to unstained flow cytometry beads. $n=3$ independent experiments, each performed in triplicate. (**d**) Cell proliferation and metabolism is not altered by caspase-1-gyrase dimerization. MEFs containing caspase-1-gyrase and pro-IL-1β constructs were treated as in (**c**) for the indicated times and cellular proliferation was measured by the MTT-PMS viability assay. $n=4$ independent experiments. Error bars represent the S.E.M. in all graphs

bearing dox-inducible FLAG- and GFP- tagged dimerizable caspase-8 (caspase-8-gyrase) or control caspase-9 (caspase-9-gyrase). Direct comparison of caspase-8- and caspase-9-gyrase constructs to caspase-1-gyrase MEFs revealed that dimerized caspase-8, but not caspase-9, induced cleavage and secretion of IL-1β (Figure 4a). In agreement with our previous work,[6] caspase-8 was less efficient than caspase-1.

To determine whether in contrast to caspase-1-gyrase, caspase-8-gyrase or caspase-9-gyrase dimerization was capable of inducing cell death we measured the cleavage of the apoptotic effector caspase, caspase-3, as well as the release of cellular proteins into the supernatant by western blot. Intriguingly, in contrast to dimerized caspase-1-gyrase, we observed cleavage of caspase-3 to the active p19 fragment upon expression and dimerization of apoptotic caspase-8 and caspase-9 (Figure 4a). We confirmed apoptotic cell death occurred, albeit less efficiently than etoposide, by analyzing PI uptake after 24 h in dimerized caspase-8-gyrase and caspase-9-gyrase MEFs (Figure 4b). This cell death was clearly apoptotic in nature, as it was blocked by pre-treatment with the pan-caspase inhibitor Z-VAD-fmk (Supplementary Figure S3a). Consistent with this low level of caspase-dependent death upon caspase-8- and caspase-9-gyrase dimerization, both β-actin and pro-IL-1β were released into the supernatants (Figure 4a). This was in contrast to the absence of these proteins in the supernatants of cells expressing dimerized caspase-1-gyrase (Figures 2a and 4a, Supplementary Figure S1c).

Analysis of supernatants by ELISA revealed that IL-1β was released upon dox-induction and dimerization of all three constructs, as well as with etoposide treatment (Figure 4c). The release of IL-1β by the caspase-8-gyrase and caspase-9-gyrase was dependent on the catalytic activity of caspase-8 and caspase-9, respectively, as it was inhibited by Z-VAD-fmk (Supplementary Figure S3b). To establish whether, as with caspase-1, the caspase-8-gyrase construct was causing the release of biologically active IL-1β, as suggested by our western blot analysis (Figure 4a), we transferred supernatants from each of the caspase-gyrase constructs onto the NF-κB GFP reporter Thp1 cells. Notably, only supernatants from caspase-1-gyrase or caspase-8-gyrase transferred from MEFs treated with both dox and coumermycin were able to increase the activation of NF-κB above the level of untreated cells, as indicated by increased GFP fluorescence (Figure 4d). In contrast, despite releasing detectable amounts of IL-1β by ELISA (Figure 4c,Supplementary Figure S3b and S3c), supernatants from MEFs-expressing caspase-9-gyrase were not able to further activate NF-κB (Figure 4d). This is consistent with western blot results showing that dimerized

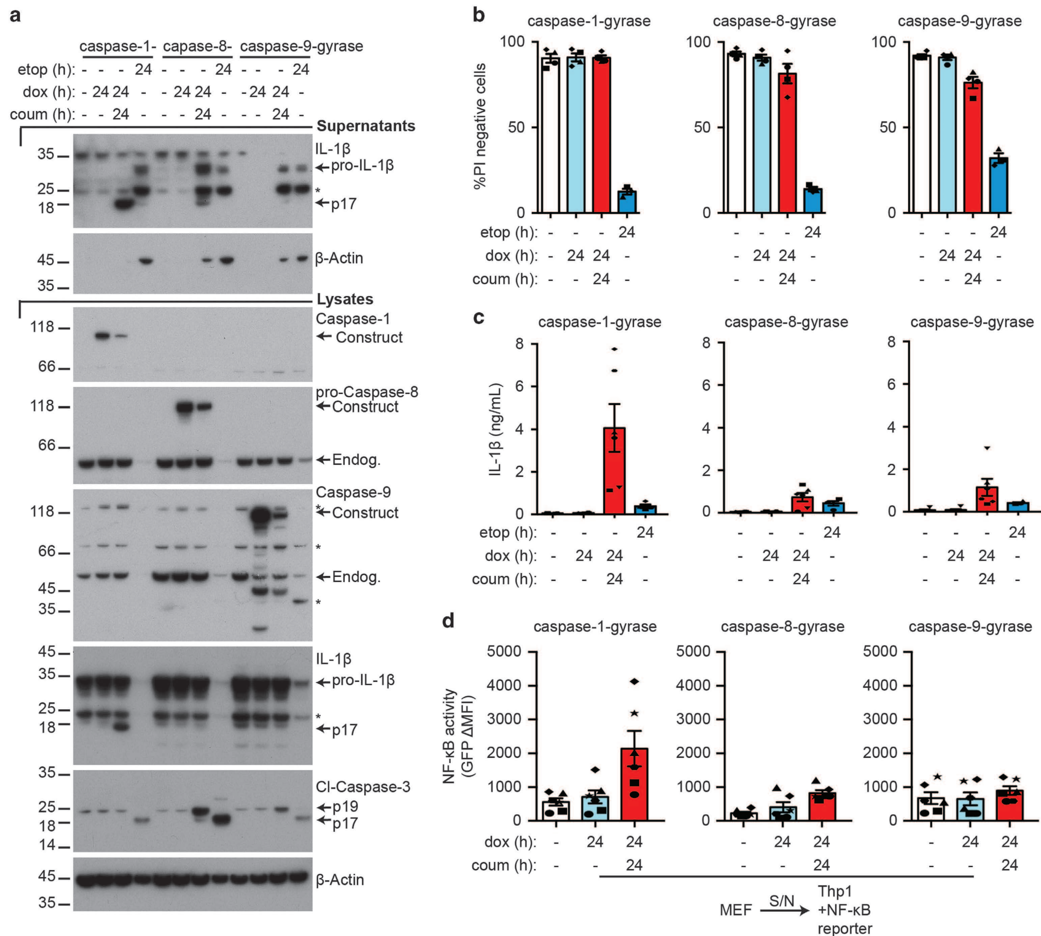

Figure 4 Caspase-8-gyrase dimerization can induce low levels of IL-1β secretion. (**a**) Caspase-8-gyrase dimerization causes IL-1β cleavage and secretion. Western blot analysis of lysates and supernatants from MEFs containing overexpressed pro-IL-1β and either caspase-1-gyrase, caspase-8-gyrase or caspase-9-gyrase treated as specified with 1 μg/ml doxycycline and 700 nM coumermycin, or 34 μM etoposide, for the indicated times. Asterisks denote non-specific bands. (**b**) Caspase-8-gyrase and caspase-9-gyrase dimerization induces small amounts of cell death. Cells were treated as in (**a**) and analyzed by flow cytometry for uptake of PI. $n=4$ independent experiments. (**c**) IL-1β is secreted upon dimerization and activation of caspases. MEFs were treated as in (a) and IL-1β released into the supernatant was measured by ELISA. $n=4$–6 independent experiments. (**d**) IL-1β secreted following caspase-1-gyrase or caspase-8-gyrase dimerization is biologically active. Thp1 cells bearing an NF-κB GFP reporter were incubated for 24 h with supernatants from caspase-1-gyrase, caspase-8-gyrase or caspase-9-gyrase MEFs treated for 24 h with 1 μg/ml doxycycline and 700 nM coumermycin. GFP expression indicative of NF-κB activation was quantified as the change in mean fluorescence intensity (MFI) relative to Thp1 cells in media alone. $n=6$ independent experiments. Error bars represent the S.E.M. in all graphs. Asterisks denote non-specific bands in western blots. Data for caspase-1, -8 and -9 were collected in parallel. See also Supplementary Figure S3

caspase-1-gyrase induced the cleavage and release of the mature p17 form of IL-1β, dimerized caspase-8-gyrase stimulated the release of both the pro and cleaved forms of IL-1β, whereas caspase-9-gyrase and etoposide treatment only caused the release of inactive pro-IL-1β into the supernatant (Figure 4a).

In order to determine whether the caspase-8-gyrase construct would be more efficient at killing cells via endogenous death receptor signaling, in comparison with dimerization-induced activation, we stimulated cells with TNF and induced the degradation/inhibition of inhibitor of apoptosis proteins using Smac-mimetic compound A (Cp.A).[26] As expected based on our previous work,[26,31] TNF and Cp.A killed about half of the wild-type (WT) MEFs after 16 h of treatment (40–50% PI positive; Supplementary Figure S4a, S4b). Similarly, *Caspase-8*$^{-/-}$ MEFs were sensitive to

TNF and Cp.A treatment when reconstituted with caspase-8-gyrase, either when induced with dox or dimerized with coumermycin (Supplementary Figure S4c). In contrast, *Caspase-8*$^{-/-}$ MEFs-expressing caspase-1-gyrase did not die following TNF and Cp.A treatment (Supplementary Figure S4d). Hence, the caspase-8-gyrase protein, but not the caspase-1-gyrase, can function in place of endogenous caspase-8 to kill cells when activated by death receptor signaling.

Given that forced dimerization of caspase-8 causes cleavage and secretion of IL-1β (Figures 4a and c), we asked if activated endogenous caspase-8 could do the same. In fact, endogenous caspase-8 exhibited greater efficiency in inducing IL-1β cleavage and secretion in WT MEFS treated with TNF and Cp.A, compared with caspase-8-gyrase dimerization (Figures 5a–c). Furthermore, as *Caspase-8*$^{-/-}$ MEFs lack both expression of caspase-8, as well as expression of endogenous inflammasome machinery, including caspase-1 (Figure 1b), cleavage and secretion of IL-1β could only occur if caspase-1-gyrase was exogenously expressed and dimerized (Figures 5a and d), or if caspase-8-gyrase was exogenously expressed and activated either with TNF and Cp.A or by dimerization (Figures 5a and e). Therefore, activated endogenous caspase-8, or dimerized overexpressed caspase-8, is capable of directly cleaving IL-1β and causing its secretion in the absence of other inflammasome components, including ASC and caspase-1.

Collectively, these results demonstrate that endogenous caspase-8 activation, or the dimerization of caspase-8-gyrase or caspase-1-gyrase (but not caspase-9-gyrase) can cleave and activate IL-1β and induce its secretion. This may explain why some viral proteins, such as CrmA, have evolved to inhibit both caspase-1 and caspase-8.

IL-1β secretion occurs in viable cells. Recent studies propose that caspase-1 killing invariably coincides with IL-1β secretion, and/or that cell death is the dominant mechanism by which IL-1β is released.[18–20] However, our data on populations of cells suggest that dimerized caspase-1 is capable of inducing active IL-1β secretion without loss of viability or impacting cell growth. To extend these results to a single cell level, we visualized dimerized caspase-1-gyrase-induced IL-1β secretion from individual living cells by ELISpot (Experimental schematic Supplementary Figure S5a).

The vector that expresses pro-IL-1β is a bicistronic construct that also expresses GFP (Figure 1a). Comparably strong GFP expression from this construct was maintained whether caspase-1-gyrase was expressed or not, as seen both by flow cytometry (Figure 6a) and by single cell imaging on the ELISpot plate (Figure 6b). In contrast, when MEFs were killed with etoposide, GFP was no longer detectable in the PI-positive dead cells (Figure 6a), indicating that in this system GFP acts as marker of live cells. Following overnight incubation of caspase-1-gyrase MEFs on ELISpot plates and treatment with etoposide, or dox with or without coumermycin, live cells were imaged by GFP fluorescence (top row Figure 6b). As expected, there were 70% less GFP-positive cells detected when the cells were treated with 34 μM etoposide, indicating significant cell death. In contrast, whether the caspase-1-gyrase cells were untreated, treated with dox alone (caspase-1-gyrase expression), or treated with dox plus coumermycin (caspase-1-gyrase expression and dimerization), we observed no differences in the number of GFP-positive cells (top row Figure 6b, c), as we showed previously in viability assays (Figure 3).

To ensure the ELISpot-seeded cells were not aggregating, which would make it difficult to define if individual GFP-positive live cells could secrete IL-1β, we plated duplicate experiments in parallel on tissue culture treated plates of the same well size. This allowed for simultaneous analysis of cell density by bright light microscopy and GFP fluorescence (Supplementary Figure S5b). These images confirmed that cells treated with dox and coumermycin maintain their GFP positivity equivalent to untreated or dox alone treated cells. Importantly, we failed to detect aggregated cell clusters; the cell densities used to seed the ELISpot plates resulted in clearly distinguishable individual GFP-positive cells. Moreover, dox and coumermycin-treated cells exhibited equivalent density and morphology to control treated wells (Supplementary Figure S5b).

Immediately after imaging the plates for GFP fluorescence, we developed the IL-1β ELISpots (second row Figure 6b). In wells where MEFs-expressing caspase-1-gyrase were dimerized, hundreds of discrete dark purple spots were detected, indicating IL-1β released from single cells (second row Figure 6b). All other conditions yielded ~10-fold fewer spots or only had background staining (Figure 6b). The position of the IL-1β spots typically corresponded to the position of live GFP cells imaged prior to ELISpot processing (Figure 6b, bottom two rows), identifying that live cells are capable of secreting IL-1β. Incubating ELISpot plates with mature recombinant IL-1β resulted in diffuse uniform purple staining across the entire well, with the staining intensity being dependent on the dose of recombinant IL-1β added (Supplementary Figure S5c). This demonstrates that IL-1β is quickly captured by the coating antibody after being released from a single cell, and that a small number of cells releasing diffuse IL-1β into the supernatant cannot explain the presence of ELISpots.

Quantitation of IL-1β ELISpots as a ratio to the number of cells seeded, indicated that 30–40% of caspase-1-gyrase-dimerized MEF cells secreted IL-1β (Figure 6d), despite no significant cell death occurring at the population level (Figure 3). To accurately quantify the per cent live (GFP-positive) cells secreting IL-1β, we evaluated the ELISpots as a ratio of the GFP-positive cells measured for each well. Importantly, when caspase-1-gyrase was dimerized, 60–70% of GFP-positive cells were also positive for IL-1β secretion (Supplementary Figure S5d). Taken together, these data provide strong evidence that caspase-1 can cleave and cause the secretion of IL-1β without simultaneously causing cell death.

Discussion

IL-1β can be activated and released from cells in a caspase-1 or caspase-8 dependent manner.[1] However, because it lacks a canonical signal sequence IL-1β is not secreted via the conventional ER/Golgi secretory pathway.[4] Considering caspase-1-dependent IL-1β release occurs simultaneously

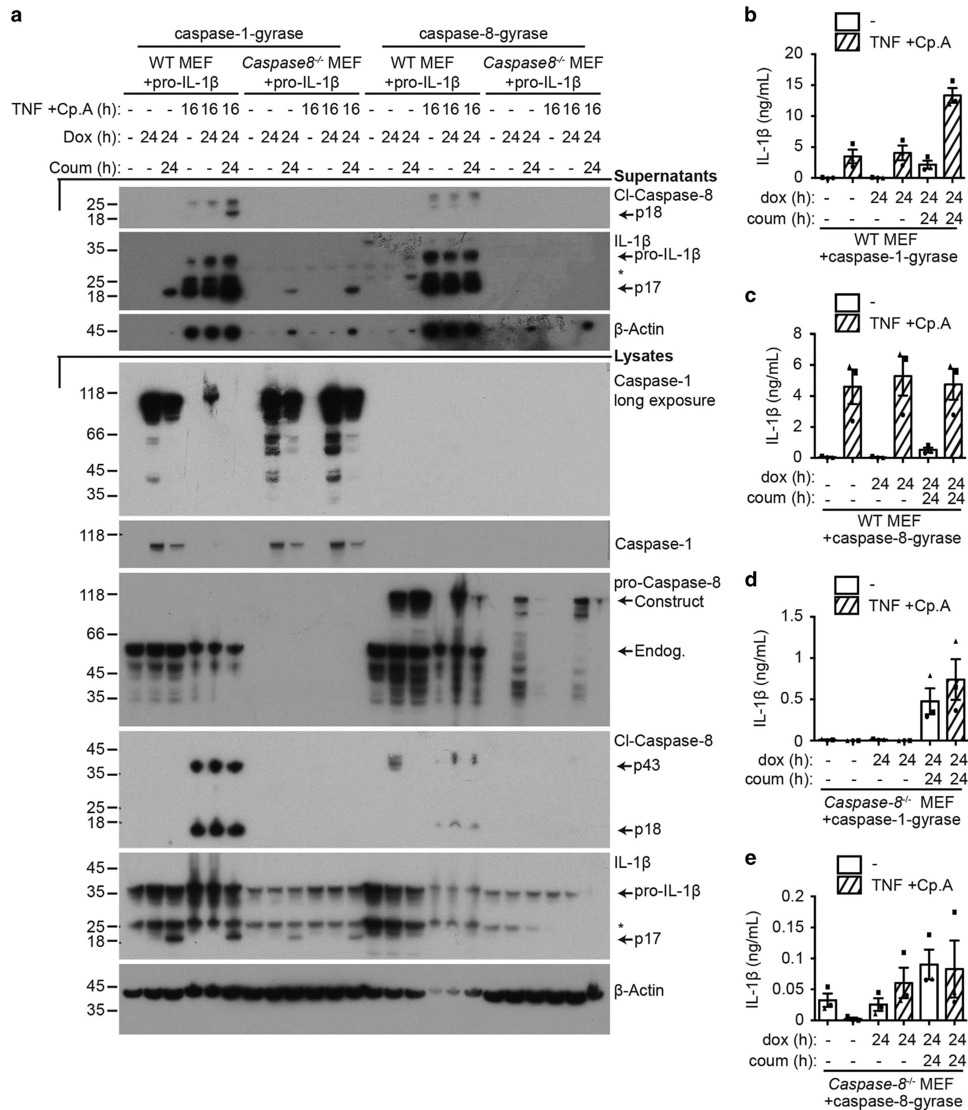

Figure 5 Both endogenous caspase-8 and caspase-8-gyrase can cleave IL-1β and cause its secretion. MEFs containing pro-IL-1β and either caspase-1-gyrase (**a**, **b** and **d**) or caspase-8-gyrase (**a**, **c** and **e**) vectors were treated with 1 μg/ml doxycycline and 700 nM coumermycin, as well as 100 ng/ml TNF and 1 μM Cp.A when indicated by cross-hatched columns. (**a**) Representative western blot of lysates and supernatants blotted for the indicated proteins. Asterisks denote non-specific bands. (**b, c, d** and **e**) Activation of endogenous caspase-8, caspase-8-gyrase or caspase-1-gyrase causes secretion of IL-1β. Supernatant of indicated cells was analyzed by IL-1β ELISA after treatment. Error bars represent S.E.M. $n = 3$–5 independent experiments. See also Supplementary Figure S4

with pyroptosis or cellular necrosis, it has been proposed that these two caspase-1-dependent functions cannot be separated and therefore that lysis of the plasma membrane is the mechanism by which mature IL-1β leaves the cell.[18–20] However, recent studies have reported that active IL-1β can be released from cells without the release of cellular LDH, which occurs upon cell death.[13,21,32–36] Using multiple experimental parameters we now demonstrate on both the population and single cell levels that cell death is not required for IL-1β secretion.

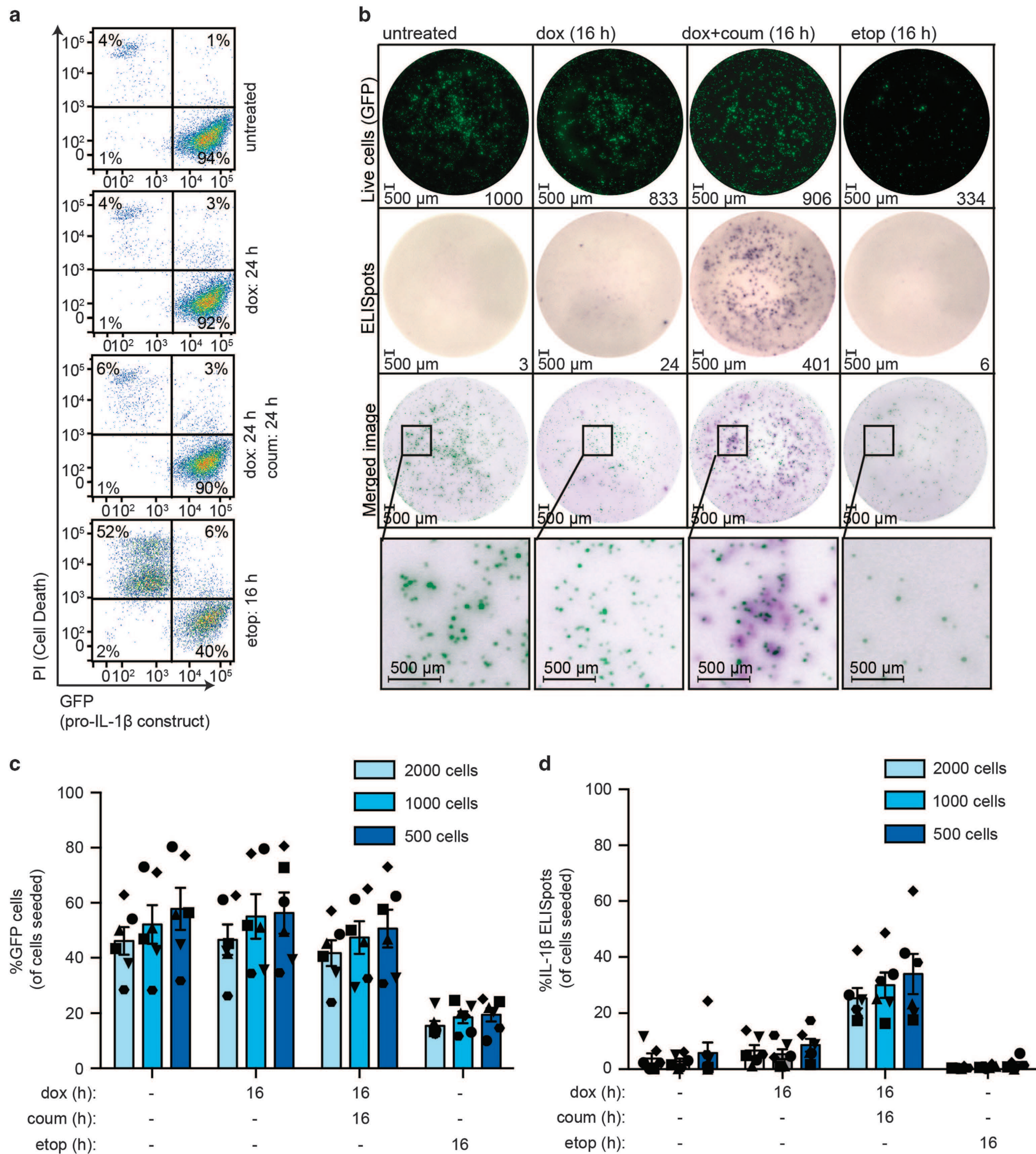

Figure 6 IL-1β is secreted from live MEFs when caspase-1-gyrase is dimerized. (**a**) GFP fluorescence is lost upon cell death. MEF cells containing pro-IL-1β and caspase-1-gyrase were treated with 1 μg/ml doxycycline and 700 nM coumermycin, or 34 μM etoposide for indicated times, and GFP fluorescence and PI uptake were analyzed by flow cytometry. (**b, c** and **d**) Caspase-1 activity causes secretion of IL-1β from live cells. MEF cells containing pro-IL-1β and caspase-1-gyrase constructs were seeded at three densities on the ELISpot plate. After 16 h treatment with 1 μg/ml doxycycline and 700 nM coumermycin, or 34 μM etoposide, GFP images were taken and then ELISPot staining was immediately performed. (**b**) Representative images of 2000 cells/well ELISpot experiment. The brightness and contrast of images were adjusted using Fiji (ImageJ) and Adobe Illustrator in a linear fashion, with settings applied equally to every image. GFP images were inverted in the merged image and pseudo-coloring was applied using Adobe Illustrator to allow overlay of ELISpots and GFP-positive cells. (**c**) Analysis of the number of GFP-positive cells as a ratio of total cells seeded. (**d**) Analysis of ELISpots represented as a percentage of cells seeded per well. Both GFP and ELISpot images were acquired and quantified using an AID ELISpot Reader. $n = 6$ independent experiments. Error bars represent the S.E.M. See also Supplementary Figure S5

The dimerization of caspase-1 in MEFs resulted in IL-1β cleavage to the mature form within 2 h – an efficiency comparable to canonical NLRP3-caspase-1 inflammasome mediated IL-1β activation in myeloid cells. This active IL-1β was secreted from living cells whose plasma membranes resisted the uptake of PI, and maintained metabolic activity.

Given that caspase-1 oligomerization is sufficient to trigger pyroptosis, it remains unclear why our dimerized caspase-1 construct was unable to induce cell death. It is possible that the fused C-terminal gyrase-GFP on caspase-1 is inhibiting its pyroptotic functions.[37] Alternatively, our immortalized MEFs may express reduced levels of important pyroptotic effector molecules required for efficient cell death downstream of inflammatory caspase activation, such as gasdermin D.[38,39] Regardless of the cause, our system has fortuitously allowed us to separate the cell death function of caspase-1 from its ability to cleave cytosolic IL-1β and cause its secretion.

Several different mechanisms for the secretion of IL-1β have been proposed, but none have been definitively proven.[4] Several groups have provided evidence that active IL-1β is released together with other inflammatory cell components when the plasma membrane ruptures.[40] Underlying this hypothesis is the observation that caspase-1 can trigger a lytic form of cell death termed pyroptosis.[29] Reports of single cell studies have concluded that once caspase-1 activation passes a threshold, cells cannot escape death.[16,19] Experimentally this means activating caspase-1 results in both cleavage of IL-1β and its release, both of which often occur almost simultaneously with the death of the cell. By bypassing the need for upstream inflammasome components and activating stimuli, which have the capacity to trigger both pyroptosis and apoptosis, we have been able to demonstrate that membrane lysis is not necessary for the release of active IL-1β from cells following caspase-1 activation.

Previous work has implicated caspase-8 in the direct processing and activation of precursor IL-1β, following induction of TLR, death receptor or dectin-1 signaling.[6,7,9,41–44] However, complicating matters are reports indicating that caspase-8 contributes to inflammasome priming,[12,45,46] may process and activate caspase-1,[10,11] or can induce NLRP3 inflammasome formation.[12,13,30] Here, we show in the absence of IL-1β-activating platforms, including ASC, caspase-1 and NLRP3, that the direct dimerization of caspase-8, or TNF and Smac-mimetic activation of endogenous caspase-8, is sufficient to cause IL-1β cleavage into a biologically active form and induce its secretion. Hence, dimerized active caspase-8 is likely to be a direct activator of IL-1β.

The literature suggests that pyroptosis has an important role in viral and bacterial responses, and sepsis.[47] However, IL-1 blockade alone suffices to treat auto-inflammatory diseases such as CAPS. Our findings show that caspase-1 activation can cause IL-1β release in the absence of cell death. Hence, caspase-1 may perform distinct functions, depending on the disease context and pathogenesis.

Materials and Methods

Cell Lines and materials. WT and *Caspase-8*$^{-/-}$ MEF cells immortalized with SV40 large T antigen were maintained and are described elsewhere.[26,48] NF-κB reporter Thp1 monocytic cells were made by infection with the lentiviral reporter vector (pTRH1-NF-κB-dscGFP, TR503PA; System Bioscience, Palo Alto, CA, USA) and sorted for GFP expression by flow cytometry. BMDMs were generated by culturing bone marrow progenitors from C57BL/6 mice (purchased from WEHI Bioservices, Kew, Australia) as described elsewhere.[12] The Walter and Eliza Hall Institute (WEHI) Animal Ethics Committee approved all animal experiments. Compounds and cytokines were obtained from the following sources: dox (Sigma-Aldrich, St. Louis, MO, USA), coumermycin (Sigma), Z-VAD-fmk (Sigma), etoposide (Clifford Hallam, Dandenong South, Victoria, Australia), human Fc-TNF (produced in house[49]) and Compound A (TetraLogic Pharmaceuticals, Malverna, PA, USA), and utilized as indicated in the figure legends.

Generation of caspase constructs. Mouse caspase-1 (wild-type, catalytically inactive (C385G), uncleavable mutant (D296N, D300N, D304N, D308N, D313N, D314N0)), caspase-8 and caspase-9 were cloned into the pFTRE 3G rtTA puro vector,[50] with N-terminal FLAG tag, C-terminal fragment of *E. coli* gyrase B (residues 2–220) that dimerizes in response to the antibiotic coumermycin and C-terminal GFP as depicted in Figure 1a. Full-length mouse IL-1β was cloned into a pMIGR retroviral vector. All constructs were verified by DNA sequencing.

BMDM cell lysates. WT BMDMs were derived, treated and lysed as described.[12] Ultrapure lipopolysaccharide from *E. coli* K12 strain (Invivogen, San Diego, CA, USA) was used as indicated in the figure legends.

Cell death analysis. MEFs were seeded at 1.5×10^5 cells per well in six-well plates and treated 24 h later as indicated in the figure legends. Adherent and non-adherent cells were harvested and incubated with 1 μg/ml PI in PBS prior to analysis by flow cytometry. Data were analyzed using FlowJo software version 7.6.5. MTS viability assays were conducted as described in.[51]

Quantification of cell viability using flow cytometry particles. Cells were seeded and treated 24 h later as indicated in the figure legends. Equal numbers of blank calibration beads (6.3×10^4 beads/well) (Spherotech, Lake Forest, IL, USA) were added to each culture well just before harvesting. Adherent and non-adherent cells were harvested and incubated with 1 μg/ml PI in PBS prior to analysis by flow cytometry. The number of PI-negative cells per 1000 beads was measured for each sample using a FACSCalibur (Becton Dickinson, Scoresby, Victoria, Australia).

Measurement of cytokines by ELISA. Cells were treated as indicated. Supernatants were removed from cultured cells and pelleted at 1 500 rpm to remove any debris. Lysates were made in DISC buffer (20 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA, 10% Glycerin, 1% Triton X-100 (Sigma-Aldrich, T9284) including protease inhibitors (Roche, Basel, Switzerland, 11697498001)) or by subjecting cells to three rounds of freeze thawing and centrifuging lysates at 13 000 rpm to remove cellular debris. IL-1β ELISA (mouse IL-1β duoSet ELISA, R&D systems, Minneapolis, MN, USA; DY401) was used to quantify cytokine levels according to the manufacturer's instructions.

NF-κB Signaling. MEF cells were treated as indicated. Supernatants were removed from cultured cells and pelleted to remove any debris. Thp1 cells bearing the NF-κB reporter construct were cultured in the MEF supernatant for 24 h prior to measuring GFP expression of Thp1 cells relative to those in media alone using a FACSCalibur (Becton Dickinson).

Immunoblotting. Reduced and denatured cell lysates (lysed in Triton X-100 based ONYX buffer (20 mM Tris, pH 7.5, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% Triton X-100 (Sigma-Aldrich, T9284) including protease inhibitors (Roche, 11697498001)) and supernatants were separated on NuPAGE Novex 4-12% Bis-Tris gels (Life Technologies, Carlsbad, CA, USA) and transferred onto nitrocellulose (Amersham, Little Chalfont, Buckinghamshire, UK) or PVDF (Millipore, Billerica, MA, USA) membranes. In total, 20 μg of protein lysate was loaded unless otherwise stated. In total, 16 μl of supernatant samples was loaded without concentration. Blots were probed with antibodies against pro- and cleaved caspase-1 (Santa Cruz Biotechnology, Dallas, TX, USA; sc-514, Adipogen, San Diego, CA, USA, AG-20B-0042-C100), IL-1β (R&D; AF-401-NA), NLRP3 (Adipogen; AG-20B-0014-C100), ASC (Santa Cruz Biotechnology; sc-22514-R), pro-caspase-8 (in house), cleaved caspase-8 (Cell Signaling Technology, Danvers, MA, USA; 8592), caspase-9 (in house), cleaved caspase-3 (Cell signaling; 9661), β-actin (Sigma; A-1978) and HSP90 (Enzo, Farmingdale, NY, USA, ADI-SPA-835). All

secondary antibodies used were conjugated to HRP and detected by ECL (Amersham or Millipore).

ELISpot. Capture antibody (mouse IL-1β duoSet ELISA, R&D systems; DY401) was incubated at 50 μg/ml in 50 μl PBS overnight on a 96-well filtration plate (Millipore, MAHAS4510). Plates were subsequently blocked with 100 μl Dulbecco's Modified Eagle's Medium supplemented with 8% FCS at 37 °C for 1 h. MEF cells bearing FLAG-caspase-1-gyrase-GFP and pro-IL-1β GFP constructs were then seeded on the plates as indicated and treated for 16 h. As a control media with recombinant IL-1β (standard from mouse IL-1β duoSet ELISA, R&D systems; DY401) at concentrations between 20 000 pg/ml to 1000 pg/ml was incubated on the plate for 16 h. An AID ELISpot Reader (Autoimmun Diagnostika GMBH, Strassberg, Germany) was used to take fluorescent images of GFP-positive live cells. Plates were immediately washed 3 × with PBS/0.05% Tween 20 then 3 × with PBS alone. Plates were incubated with biotin-conjugated detection antibody (mouse IL-1β duoSet ELISA, R&D systems; DY401) at 5 μg/ml in 50 μl PBS 1% FCS for 2 h at RT. After washing, streptavidin-ALP (Mabtech, Sweden; 3310) diluted 1/1000 in PBS/1% FCS was added to the plates for 1 h at room temperature. After washing, ELISpots were developed using 50 μl of BCIP/NBT-plus substrate (Mabtech; 3650-10). GFP-positive cells and ELISpot numbers were analyzed using the AID ELISpot software. The brightness and contrast of figure images were adjusted using Fiji (ImageJ) and Adobe Illustrator in a linear fashion, with settings applied equally to every image. Pseudo-coloring was applied to merged images using Adobe Illustrator to allow overlay of ELISpots and GFP-positive cells. Tissue culture plates were also seeded simultaneously to ELISpots to allow for microscopy imaging using the Opera Phenix, taken at 10 × with 2 × 2 binning.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Cell Death and Differentiation website (http://www.nature.com/cdd)

Figure S1.

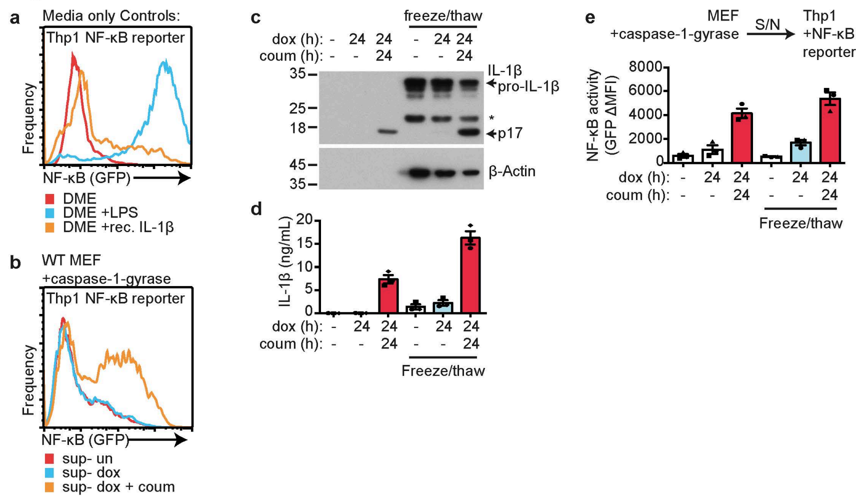
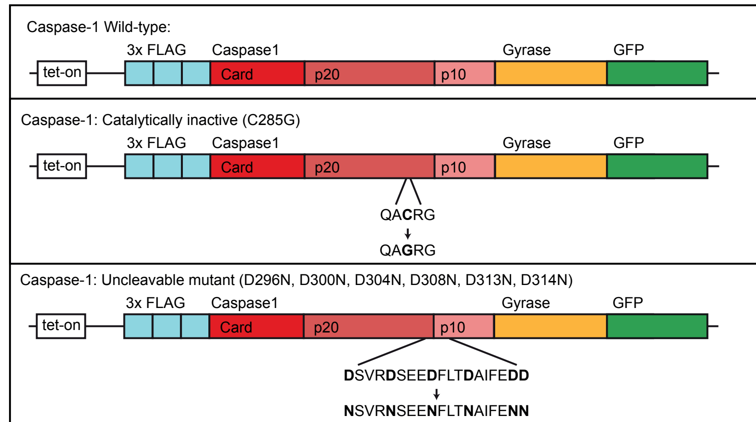


Figure S1. Dimerization of caspase-1-gyrase induces the secretion of bioactive IL-1β and activates NF-κB signalling. Related to Figure 2.

(**a and b**) IL-1β secreted from MEFs with dimerized caspase-1-gyrase is biologically active. Thp1 NF-κB reporter cells were treated with or without (a) 100 ng/mL LPS or recombinant bioactive 10 ng/mL IL-1β for 24 h. (b) Alternatively, MEFs bearing pro-IL-1β and caspase-1-gyrase constructs were treated with 1 µg/mL doxycycline and 700 nM coumermycin for 24 h, and supernatants were subsequently transferred onto Thp1 cells bearing an NF-κB GFP-reporter construct. Representative histograms of GFP expression indicating NF-κB activation. Histograms are representative of 3 independent experiments for (a) and (b), data from (b) are quantified in Figure 2c.
(**c, d and e**) NF-κB is induced in response to cleaved but not pro-IL-1β from MEFs. Cells with pro-IL-1β and caspase-1-gyrase were treated with 1 µg/mL doxycycline and 700 nM coumermycin for the indicated times in duplicate. One duplicate was frozen and thawed 3 times for efficient cellular lysis. Supernatants were analyzed by (c) Western blot for indicated proteins and (d) IL-1β ELISA. (e) Supernatant was also transferred onto Thp1 cells bearing an NF-κB GFP-reporter construct, and after a 24 h incubation GFP expression, indicating NF-κB activation, was quantified as the change in MFI relative to Thp1 cells in media alone. n = 3 independent experiments. Error bars represent the SEM in all graphs. Asterisks denote non-specific bands in Western blots.

Figure S2.

a



b

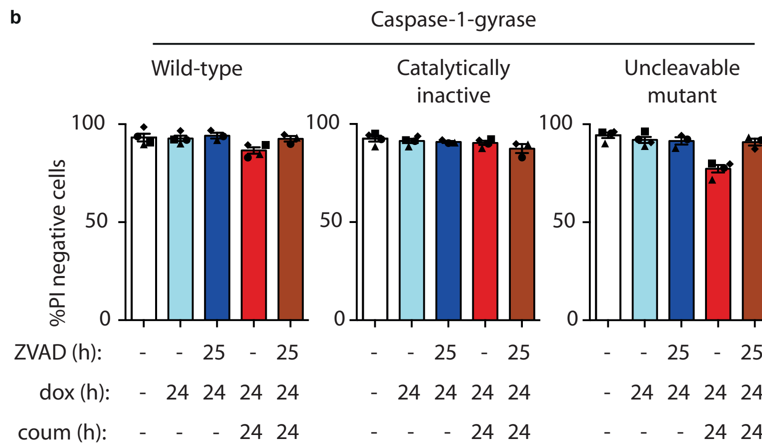


Figure S2. WT and catalytically inactive caspase-1-gyrase do not induce cell death, while the uncleavable mutant induces a small amount of cell death. Related to Figure 2.

(a) Schematic of doxycycline inducible caspase-1-gyrase fusion proteins with mutations to either the catalytic cysteine (C285), or 6 aspartic acid residues between the p10 and p20 regions of caspase-1 (D296N, D300N, D304N, D308N, D313N, D314N).
(b) Dimerization of uncleavable caspase-1-gyrase, but not the wild-type or catalytically inactive version, induces a small amount of cell death. MEFs stably infected with one of three caspase-1-gyrase vectors and pro-IL-1β were treated with 1 μg/mL doxycycline, 700 nM coumermycin and 25 μM Z-VAD-fmk. Cell viability was measured by PI uptake and assessed by flow cytometry. n = 3-4 independent experiments. Error bars represent the SEM.

Figure S3.

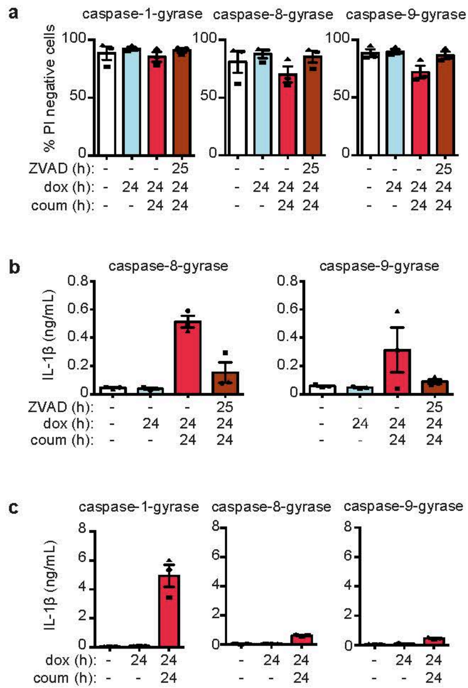


Figure S3. Caspase-1-gyrase, caspase-8-gyrase and caspase-9-gyrase activity can be inhibited by the pan-caspase inhibitor ZVAD-fmk. Related to Figure 4.

(**a and b**) Cell death and secretion of IL-1β activated by caspase dimerization can be inhibited by ZVAD-fmk. MEFs containing pro-IL-β and either caspase-1-gyrase, caspase-8-gyrase or caspase-9-gyrase constructs were treated with 1 µg/mL doxycycline, 700 nM coumermycin and 25 µM Z-VAD-FMK for the indicated times. (a) Cell viability was measured by PI uptake and flow cytometry. (b) IL-1β released into the supernatant was measured by ELISA. n = 3 independent experiments. (**c**) Supernatants used to treat NF-κB GFP-reporter Thp1 cells in Figure 4d were analyzed for IL-1β levels by ELISA. n = 3 independent experiments.
Data for caspase-1, -8 and -9 were collected in parallel but are presented in 3 separate graphs. Error bars represent the SEM in all graphs.

Figure S4.

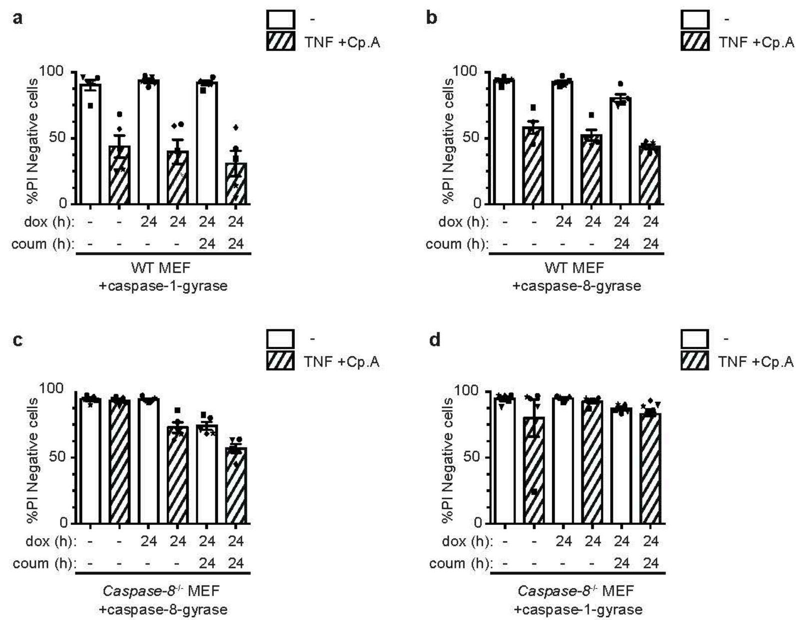


Figure S4. Caspase-8-gyrase, but not caspase-1-gyrase, can reconstitute caspase-8 mediated cell death in caspase-8-deficient MEFs. Related to Figure 5.

(a, b, c and d) MEFs containing pro-IL-1β and either (a and d) caspase-1-gyrase or (b and c) caspase-8-gyrase vectors were treated with 1 μg/mL doxycycline and 700 nM coumermycin, and 100 ng/mL TNF and 1 μM Cp.A as indicated by cross hatched columns. Loss of cell viability was measured by PI uptake and flow cytometry. n = 5 independent experiments. Error bars represent the SEM in all graphs.

Figure S5.

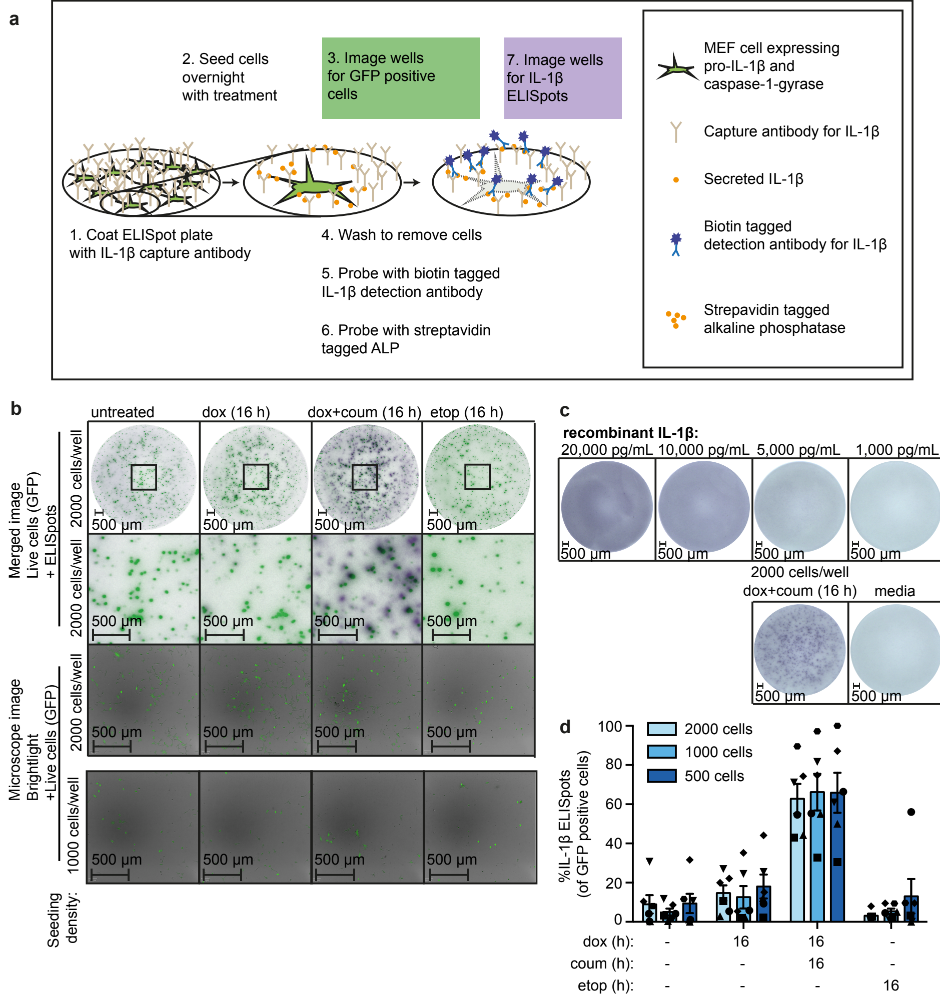


Figure S5. Caspase-1 dimerization, but not etoposide treatment or exogenous IL-1β, causes the formation of ELISpots. Related to Figure 6.

(a) Schematic of the experimental set up of ELISpot experiments presented in Figure 6b, c and d.
(b) Cells seeded at low confluency produce spatially distinct IL-1β ELISpots. Comparison of duplicate wells seeded at the same time for ELISpot or on tissue culture coated plates for microscopy. GFP positive cells and IL-1β ELISpot images were acquired and quantified using the AID ELISpot software. Microscopy images were acquired using the Opera Phenix, taken at 10x with 2x2 binning. The brightness and contrast of images were adjusted using Fiji (ImageJ) and Adobe Illustrator in a linear fashion, with settings applied equally to every image. GFP images were inverted in the merged image and pseudo-colouring applied using Adobe Illustrator to allow overlay of ELISpots and GFP positive cells.
(c) Exogenous IL-1β is detectable as diffuse staining by ELISpot. Recombinant IL-1β in media, at indicated concentrations, media alone, or MEF cells containing pro-IL-1β and caspase-1-gyrase constructs stimulated with 1 µg/mL doxycycline and 700 nM coumermycin, were visualized after 16 h for IL-1β ELISpots (see Fig S5a). Representative images of triplicate wells.
(d) Dimerized caspase-1-gyrase induces IL-1β secretion from 60-70% of GFP-positive cells. Analysis of IL-1β ELISpot results represented as a ratio to GFP positive cells, representing live cells, from the same well. GFP and ELISpot images were acquired and quantified using an AID ELISpot Reader. n = 6 independent experiments. Error bars represent the SEM.

Chapter 4: Active MLKL triggers the NLRP3 inflammasome in a cell intrinsic manner

4.1 Preface

The work within this chapter has been accepted for publication in the Proceedings of the National Academy of Sciences as the following manuscript:

Conos S A, Chen K W, De Nardo D, Hara H, Whitehead L, Núñez G, Masters S L, Murphy J M, Schroder K, Vaux D L, Lawlor K E, Lindqvist L M, Vince J E. Active MLKL triggers the NLRP3 inflammasome in a cell intrinsic manner. Proceedings of the National Academy of Sciences of the United States of America. Accepted for publication Dec 2016, published ahead of print January 17, 2017.

As the first and primary author of this paper I played a leading role in the development and realisation of the project, along with analysis of results. I completed 90% of the experiments within the manuscript and wrote the first draft of the manuscript, including compiling figures.

The remaining co-authors on this manuscript contributed to the conception, planning and design of the project and provided important input into the analysis of results, as well as contributing to editing of the manuscript and responding to reviewers. Detailed acknowledgments of their contributions of experimental data are available within the preface on pg. iv.

Active MLKL triggers the NLRP3 inflammasome in a cell-intrinsic manner

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Necroptosis is a physiological cell suicide mechanism initiated by receptor-interacting protein kinase-3 (RIPK3) phosphorylation of mixed-lineage kinase domain-like protein (MLKL), which results in disruption of the plasma membrane. Necroptotic cell lysis, and resultant release of proinflammatory mediators, is thought to cause inflammation in necroptotic disease models. However, we previously showed that MLKL signaling can also promote inflammation by activating the nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3) inflammasome to recruit the adaptor protein apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) and trigger caspase-1 processing of the proinflammatory cytokine IL-1β. Here, we provide evidence that MLKL-induced activation of NLRP3 requires (*i*) the death effector four-helical bundle of MLKL, (*ii*) oligomerization and association of MLKL with cellular membranes, and (*iii*) a reduction in intracellular potassium concentration. Although genetic or pharmacological targeting of NLRP3 or caspase-1 prevented MLKL-induced IL-1β secretion, they did not prevent necroptotic cell death. Gasdermin D (GSDMD), the pore-forming caspase-1 substrate required for efficient NLRP3-triggered pyroptosis and IL-1β release, was not essential for MLKL-dependent death or IL-1β secretion. Imaging of MLKL-dependent ASC speck formation demonstrated that necroptotic stimuli activate NLRP3 cell-intrinsically, indicating that MLKL-induced NLRP3 inflammasome formation and IL-1β cleavage occur before cell lysis. Furthermore, we show that necroptotic activation of NLRP3, but not necroptotic cell death alone, is necessary for the activation of NF-κB in healthy bystander cells. Collectively, these results demonstrate the potential importance of NLRP3 inflammasome activity as a driving force for inflammation in MLKL-dependent diseases.

MLKL | NLRP3 | necroptosis | interleukin-1β | Gasdermin D

Caspase-dependent apoptotic cell death is required for mammalian development and the prevention of autoimmune and neoplastic diseases. Programmed cell death can also act to eliminate pathogen-infected cells, with recent studies highlighting how targeted apoptosis-inducing anticancer compounds can treat viral and intracellular bacterial infections (1, 2). On the other hand, the recently characterized caspase-independent necroptotic cell death pathway is dispensable for organism development but, like apoptosis, can be triggered to kill cells harboring pathogenic microbes (3). A number of studies have also reported how pathological activation of necroptotic signaling may contribute to diverse disease states, such as ischemia–reperfusion injury, atherosclerosis, and liver disease, presumably through cell death and the release of proinflammatory mediators (4).

The execution of necroptosis is dependent on receptor interacting serine–threonine protein kinase 3 (RIPK3) phosphorylation of mixed-lineage kinase domain-like protein (MLKL), and MLKL's association with, and disruption of, plasma membrane integrity (5). In the absence of caspase activity, death receptors, such as TNF receptor 1 (TNFR1), as well as the innate immune Toll-like receptors, TLR3 and TLR4, can activate RIPK3 and MLKL to cause necroptotic cell death. Following TLR or TNFR1 signaling RIPK3 oligomerizes and becomes activated through RIP homotypic interaction motif (RHIM)–RHIM interactions with adaptor proteins, such as RIPK1 and TRIF. The resulting filamentous structure (6), termed the necrosome, triggers RIPK3 phosphorylation of the activation loop in MLKL's pseudokinase domain (7–9), which causes a redistribution of MLKL to cellular membranes, such as the plasma membrane, where it can be detected as high-molecular weight oligomers (10–13). Although MLKL lacks enzymatic activity, phosphorylation of the pseudokinase domain of MLKL is thought to cause a conformational change that exposes the killer N-terminal four-helix bundle (4HB) domain (5, 14). Recombinant MLKL (and the 4HB domain) have been shown to permeabilize artificial liposomes in vitro (12, 13, 15). However, if MLKL alone suffices to induce cell death through membrane disruption, or whether MLKL-induced killing requires other cellular factors, such as ion channel opening (10, 11), remains to be clearly defined.

The lytic nature of necroptotic killing results in the release of intracellular contents, which presumably includes damage-associated

Significance

Necroptotic cell death is mediated by activation of the mixed-lineage kinase domain-like protein (MLKL). The inflammation associated with this form of cell death is thought to be due to the release of proinflammatory cellular contents after plasma membrane rupture. In contrast to this prevailing view, we show that MLKL activates the innate immune receptor nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3) in a cell-intrinsic manner. Importantly, we show that MLKL-mediated NLRP3 and caspase-1 activation and the secretion of the proinflammatory cytokine IL-1β is a major determinant of necroptotic-derived inflammatory signals. These findings suggest that NLRP3 and IL-1β may be relevant therapeutic targets in MLKL-driven diseases.

Author contributions: S.A.C., D.D.N., J.M.M., D.L.V., K.E.L., L.M.L., and J.E.V. designed research; S.A.C., K.W.C., D.D.N., H.H., K.E.L., L.M.L., and J.E.V. performed research; S.A.C., K.W.C., L.W., and K.E.L. analyzed data; G.N., S.L.M., J.M.M., and K.S. contributed new reagents/analytic tools; and S.A.C., K.E.L., L.M.L., and J.E.V. wrote the paper.

Conflict of interest statement: D.L.V. is a consultant for Tetralogic Pharmaceuticals.

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molecular patterns (DAMPs) that can engage pattern-recognition receptors (PRRs) on bystander cells, thereby generating an inflammatory response (16). This model is widely accepted given that genetic knockout of necroptotic repressors, such as caspase-8 or RIPK1, causes inflammation in vivo through RIPK3 and MLKL activity (4). However, the specific DAMPs, or other potential processes that contribute to the inflammatory response following MLKL signaling need to be clarified.

We and others recently documented how in addition to necroptotic cell death, MLKL signaling also triggers activation of the nucleotide-binding oligomerization domain (NOD)-like receptor protein 3 (NLRP3) inflammasome complex (17–19). Inflammasome proteins, such as NLRP3, are cytosolic PRRs that can sense pathogen and host molecules, or ill-defined cellular stresses, to engage the inflammatory caspase, caspase-1. Caspase-1 subsequently cleaves and activates the potent proinflammatory cytokines IL-1β and IL-18 and induces their secretion. Although it has been suggested that cell lysis is required for IL-1β release, it has been reported by a number of groups that mature IL-1β secretion is an active cellular process and can occur in the absence of cell death (20–23).

How MLKL signaling activates NLRP3, and whether this event is separable from cell death, remains of outstanding interest. Similarly, the contribution of MLKL-induced NLRP3 inflammasome activity, as opposed to necroptotic DAMP release, on the activation of inflammatory signaling remains unknown. Here, we demonstrate that MLKL activation of NLRP3 requires a decrease in intracellular potassium levels and correlates with the translocation of MLKL to cellular membranes, but can be separated from MLKL-induced cell lysis. Using a series of inducible activated or necroptosis-defective MLKL constructs, we also show that the necroptotic activity of MLKL is required for NLRP3 inflammasome formation and IL-1β secretion. Notably, our data suggest that MLKL-induced activation of the NLRP3 inflammasome, but not death-induced release of DAMPs, is the dominant means by which necroptotic signaling activates NF-κB in healthy bystander cells. These findings suggest that in MLKL-dependent inflammatory diseases the NLRP3 inflammasome could drive pathology, and thus targeting of NLRP3 and IL-1β with available preclinical and clinical therapies may be of benefit.

Results

RIPK3–MLKL Necroptotic Signaling Activates the NLRP3 Inflammasome. Recent reports suggest that TLR and tumor necrosis factor receptor 1 (TNFR1)-induced RIPK3–MLKL signaling can trigger activation of the NLRP3 inflammasome, resulting in caspase-1 dependent IL-1β maturation and secretion (17–19). To genetically confirm that RIPK3 and MLKL can specifically activate IL-1β, we stimulated bone marrow-derived macrophages (BMDMs) from relevant gene targeted mice with the necroptosis-inducing combination of LPS, Smac-mimetic Compound A (Cp.A) [antagonizes inhibitor of apoptosis (IAP) proteins] and low-dose pan-caspase inhibitor Q-VD-OPh (QVD) [10 μM; does not inhibit caspase-1 activity (24)]. Consistent with our previous studies (17), activation of necroptosis resulted in robust cleavage (activation) and secretion of both caspase-1 and IL-1β from WT BMDMs, which was abrogated in cells lacking either RIPK3 or MLKL (Fig. 1 *A* and *B*). Importantly, RIPK3–MLKL-mediated activation of IL-1β was also dependent on NLRP3 and caspase-1, because their genetic deletion also prevented IL-1β and caspase-1 processing and secretion following LPS/QVD/Cp.A treatment (Fig. 1 *C* and *D*). Of note, inflammasome priming following LPS treatment was similar in WT and all relevant gene knockout BMDMs, as measured by TNF release (Fig. 1 *E* and *F*) and induction of pro–IL-1β expression (Fig. 1 *B* and *D*).

Potassium Efflux Links MLKL-Induced Necroptosis with NLRP3 Activation. Activated MLKL compromises plasma membrane integrity and results in ion fluxes across the plasma membrane (5).

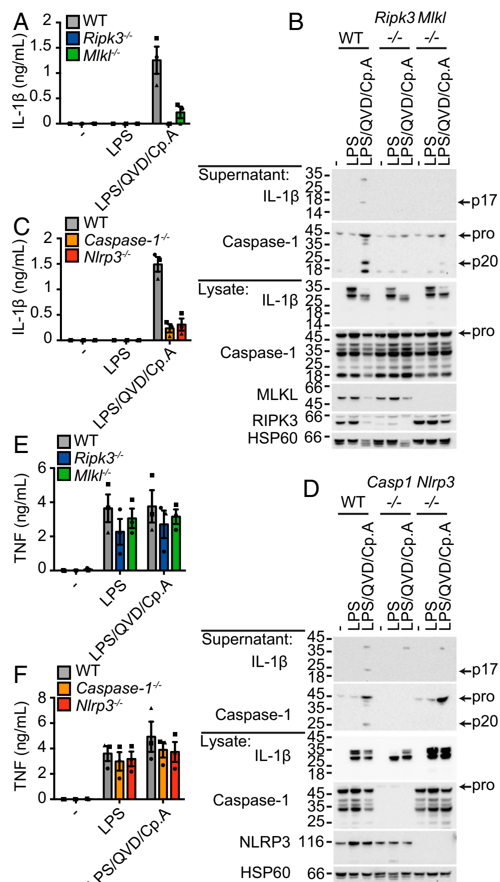

Fig. 1. MLKL activates the NLRP3 inflammasome. WT, *Ripk3*$^{-/-}$, and *Mlkl*$^{-/-}$ BMDMs (*A*, *B*, and *E*) or WT, *Caspase-1*$^{-/-}$ and *Nlrp3*$^{-/-}$ BMDMs (*C*, *D*, and *F*) were preincubated with or without LPS (100 ng/mL) for 2–3 h and were then pretreated with Q-VD-OPh (10 μM) for 30 min before stimulation with Cp.A (1 μM) for 5 h. Supernatants were assayed for IL-1β (*A* and *C*) or TNF (*E* and *F*) levels by ELISA. Data are represented as means ± SEM from three independent experiments. (*B* and *D*) Supernatants and lysates were analyzed by Western blot as indicated. Data are representative of three experiments. Triangles, circles, and squares in each graph represent independent experiments.

Given that decreased intracellular potassium levels suffice to trigger NLRP3 activity and that high levels of extracellular potassium can inhibit this process (25), we hypothesized that activated MLKL may cause potassium efflux to induce NLRP3 signaling. To test this hypothesis, we increased extracellular potassium levels to 50–150 mM before the activation of MLKL by LPS/QVD/Cp.A treatment. Consistent with our hypothesis, elevated extracellular potassium chloride blocked RIPK3–MLKL-dependent caspase-1 and IL-1β processing and secretion (Fig. 2 *A* and *B*), but did not alter TNF release (Fig. 2*C*). Importantly, necroptotic death was not affected by 50 mM potassium chloride, which maximally

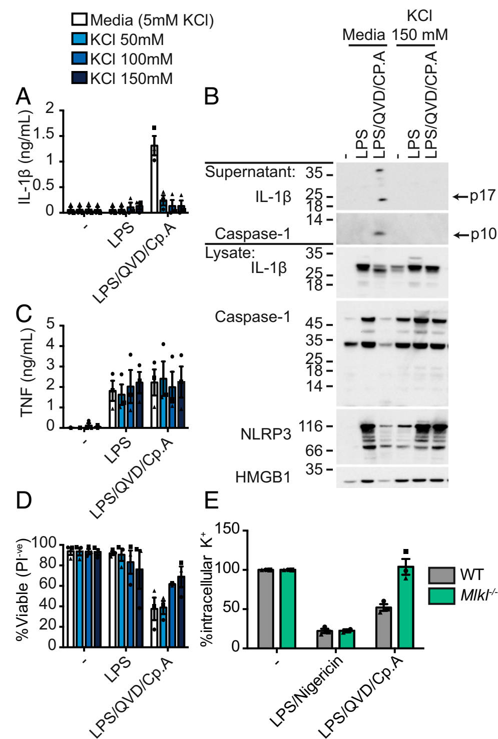

Fig. 2. MLKL induces potassium efflux to activate NLRP3. (*A–D*) WT BMDMs were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) and increasing concentrations of potassium chloride (50 mM, 100 mM, or 150 mM) 30 min before stimulation with Cp.A (1 μM) for 5 h. (*A* and *C*) Supernatants were assayed for IL-1β (*A*) or TNF (*C*) levels by ELISA. (*B*) Supernatants and lysates were analyzed by Western blot as indicated. Data are representative of three experiments. (*D*) Cell death was assessed by flow cytometric analysis of PI uptake. Data are represented as means ± SEM from three independent experiments. (*E*) WT and *Mlkl*$^{-/-}$ BMDMs were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) 30 min before stimulation with Cp.A (1 μM) for 5 h or nigericin (10 μM) for 2 h, as indicated. The intracellular levels of K^+ were quantified by inductively coupled plasma mass spectrometry. Data are represented as means ± SD from two to three independent experiments. Triangles, circles, and squares in each graph represent independent experiments.

inhibits potassium efflux and NLRP3 signaling (25), whereas higher concentrations of potassium (100–150 mM) partially rescued viability following LPS/QVD/Cp.A treatment (Fig. 2*D*). Consistent with potassium efflux being the NLRP3 trigger, the necroptotic stimuli LPS/QVD/Cp.A, like nigericin, decreased intracellular potassium levels in BMDMs in an MLKL-dependent manner, as measured by inductively coupled plasma optical emission spectrometry (Fig. 2*E*).

To investigate if NLRP3 inhibition is specific to increased extracellular potassium chloride, we examined if other alkali halides impacted NLRP3 activity. Strikingly, similar to potassium chloride, 50 mM sodium chloride, rubidium chloride and cesium chloride all markedly reduced NLRP3 activation, as measured by IL-1β secretion resulting from both apoptotic LPS/Cp.A [RIPK3–caspase-8–dependent (17)] or necroptotic LPS/QVD/Cp.A (RIPK3–MLKL-dependent) stimuli (Fig. S1*A*). Importantly, sodium chloride, rubidium chloride and cesium chloride did not impact cellular viability under apoptotic or necroptotic conditions (Fig. S1*B*). In contrast, not only did potassium chloride, rubidium chloride and cesium chloride efficiently prevent nigericin-induced canonical NLRP3 inflammasome activation and IL-1β release, they also inhibited nigericin-mediated pyroptosis (Fig. S1 *A* and *B*). Of note, although cesium chloride did reduce pro-IL-1β levels, the other alkali halides did not consistently impact NLRP3 priming by LPS (Fig. S1*C*), thus highlighting that diminished NLRP3 activation is not simply due to priming defects. These data demonstrate that a range of similarly charged higher, but not lower, atomic weight alkali metal ions can also limit canonical NLRP3 activation and pyroptotic killing.

As expected, extracellular potassium chloride, which blocks MLKL-induced NLRP3 activation, prevented LPS/QVD/Cp.A intracellular potassium levels falling below those observed in untreated macrophages (Fig. S1*D*). Unexpectedly, however, rubidium chloride and cesium chloride, which also inhibit NLRP3, resulted in a dramatic decrease in intracellular potassium even in untreated BMDMs (Fig. S1*D*). How these alkali halides prevent NLRP3 activation therefore remains unclear. Nevertheless, because these larger alkali halides only prevented NLRP3 activation of IL-1β and did not block cell death upon caspase-8 or MLKL signaling, IL-1β activation and cell death are separable events.

MLKL Oligomerization, Membrane Translocation, and Cell Death Occur Simultaneously with NLRP3 Activation. To examine if MLKL-induced IL-1β secretion correlated with MLKL-induced necroptosis, or caspase-1-induced pyroptosis, we examined cell death by propidium iodide (PI) uptake via flow cytometry. Notably, LPS/QVD/Cp.A treatment killed more than 60% of WT BMDMs within 6 h, which was prevented by RIPK3 or MLKL deficiency (Fig. 3*A*). In contrast, LPS/QVD/Cp.A killing was not affected by the absence of NLRP3 or caspase-1 (Fig. 3*B*), which suggests that despite the ability of LPS/QVD/Cp.A to activate caspase-1 in WT cells, necroptosis is the dominant mode of cell death. Consistent with this finding, LPS/QVD/Cp.A resulted in the oligomerization of MLKL and its redistribution from the cytoplasm into a cell membrane fraction within 2 h (Fig. 3*C*), which we have previously documented to be a hallmark for MLKL activation in fibroblasts treated with necroptotic stimuli TNF/QVD/Cp.A (Fig. 3*C*) (15). Indeed, accumulation of activated MLKL at membranes correlated with both macrophage cell death and the secretion of IL-1β (Fig. 3 *D* and *E*).

Activated MLKL has been postulated to directly perturb the plasma membrane, possibly via formation of pores that drive lytic cell death. Although structurally distinct, analogy can be made to the plasma membrane pores formed by the amino-terminal fragment of Gasdermin D (GSDMD). The NLRP3 inflammasome activates caspase-1, which can cleave off the suppressor C-terminal domain (CTD) of GSDMD and liberate the pore-forming N-terminal domain (NTD) to enable efficient pyroptosis and the release of mature IL-1β (26–30). We therefore tested whether GSDMD was also necessary for MLKL-induced IL-1β secretion. However, GSDMD was not essential for IL-1β activation (Fig. 3*F*) despite MLKL-induced IL-1β release requiring both NLRP3 and Caspase-1 (Figs. 1 *C* and *D* and 3*F*). Consistent with this result, GSDMD-deficient BMDMs expressed normal levels of the necroptotic machinery (RIPK3 and MLKL; Fig. S2*A*) and, similar to caspase-1, GSDMD was ultimately dispensable for MLKL killing (Fig. 3*G* and Fig. S2*B*). Hence, in BMDMs, the oligomerization and membrane association of MLKL is likely to trigger membrane damage, potassium efflux and NLRP3 activation to drive IL-1β secretion that coincides with necroptotic death.

Necroptotic Cell Death Activates NLRP3 in a Cell-Intrinsic Manner. Given that necroptotic cell death and NLRP3 activation were detected at the same time, we examined if NLRP3 activation was

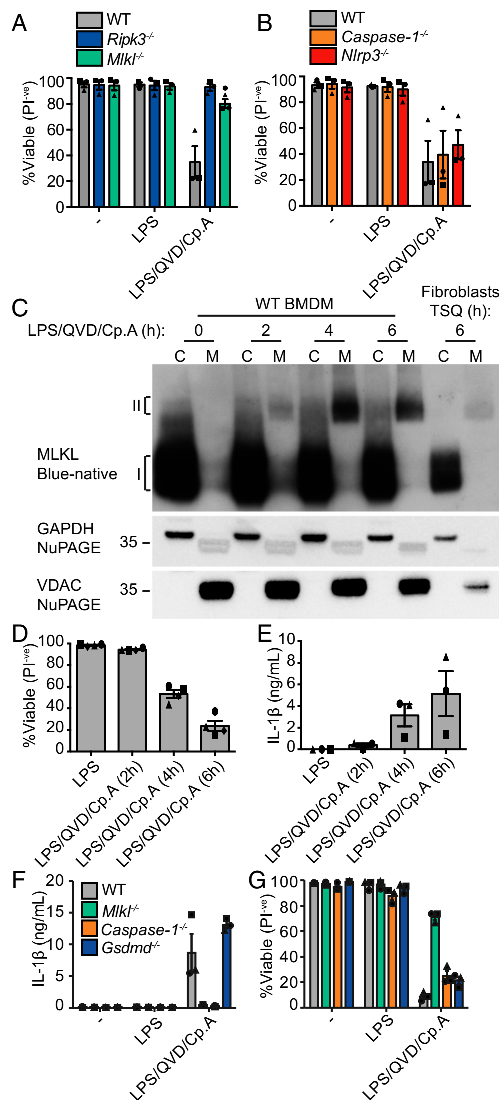

Fig. 3. MLKL oligomerization, membrane translocation and cell death correlates with MLKL-induced IL-1β secretion. (*A* and *B*) WT, *Ripk3*$^{-/-}$, and *Mlkl*$^{-/-}$ BMDMs (*A*) or WT, *Caspase-1*$^{-/-}$, and *Nlrp3*$^{-/-}$ BMDMs (*B*) were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) 30 min before stimulation with Cp.A (1 μM) for 5 h. Cell death was assessed by flow cytometric analysis of PI uptake. Data are represented as means ± SEM from three independent experiments. (*C*–*E*) WT BMDMs were preincubated with or without LPS (100 ng/mL) for 2 h and were treated with Q-VD-OPh (10 μM) 30 min before stimulation with Cp.A (1 μM) for the times indicated. MDF cells were stimulated with TNF (100 ng/mL), Q-VD-OPh (10 μM), and Cp.A (1 μM) for 6 h. (*C*) MLKL oligomerization (complex II) formation, and translocation from the cytoplasmic (C) to the membrane (M) fraction, was monitored by Blue Native PAGE. MLKL membrane complex formation was monitored by Blue Native PAGE. Membrane fractionation purity and protein abundance were assessed by immunoblotting for GAPDH and VDAC by NuPAGE gel. Data are representative of three independent repeats. (*D*) Cell death was assessed by flow cytometric analysis of PI uptake. (*E*) Supernatants were assayed for IL-1β levels by ELISA. Data are represented as means ± SEM from three independent experiments. (*F* and *G*) WT, *Mlkl*$^{-/-}$, *Gsdmd*$^{-/-}$, and *Caspase-1*$^{-/-}$ BMDMs were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) 30 min before stimulation with Cp.A (1 μM) for 24 h. (*F*) Supernatants were assayed for IL-1β levels by ELISA. (*G*) Cell death was assessed by flow cytometric analysis of PI uptake. Data are represented as means ± SEM from three independent experiments. Triangles, circles, diamonds, and squares in each graph represent independent experiments.

cell-intrinsic, or was a consequence of released cellular constituents acting on neighboring cells. The osmoprotectant glycine delays the swelling and rupture of cells during lytic cell death, thereby slowing the release of intracellular proteins, such as lactate dehydrogenase (LDH) (31). Incubating BMDMs with increasing amounts of glycine inhibited the release of LDH following either LPS/QVD/Cp.A-induced necroptotic cell death, or ATP-induced pyroptotic cell death (Fig. 4*A*), but importantly did not interfere with LDH assay readout (Fig. S3*A*). Glycine, however, did not block cell death as measured by the staining of cells with PI (Fig. 4*B*), which is consistent with glycine's ability to delay cell swelling and complete rupture, but not plasma membrane disruption (32). Notably, despite the reduction in precursor IL-1β, LDH, caspase-1 p10, and β-actin release, glycine did not block RIPK3–MLKL-induced NLRP3 activation or cytokine release, as reflected by processing and secretion of IL-1β, as well as IL-1α and TNF (Fig. 4 *C*–*E* and Fig. S3*B*). These data suggest that MLKL-induced NLRP3 inflammasome activation may be cell-intrinsic and not triggered by DAMPs released following plasma membrane rupture.

Glycine treatment only reduced, or in some cases had no effect (e.g., Bid and cytochrome *c*), on the release of cellular proteins following LPS/QVD/Cp.A-induced necroptosis (Fig. S3*B*). Therefore, to better examine if MLKL activates NLRP3 signaling to generate bioactive IL-1β in a cell-intrinsic manner, we examined the ability of necroptotic cells to induce NLRP3 inflammasome formation in neighboring cells. To do so, the formation of NLRP3-induced apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) specks was visualized following LPS/QVD/Cp.A treatment of WT and MLKL-deficient BMDMs, expressing allelic markers Ly5.1 and Ly5.2, respectively. Both genotypes responded to the canonical NLRP3 activating stimuli of LPS/nigericin by forming ASC specks (Fig. S4 *A* and *B*). In contrast, WT but not MLKL-deficient BMDMs, formed ASC specks following necroptotic LPS/QVD/Cp.A treatment (Fig. S4 *A* and *B*), and the formation of ASC specks was prevented by treatment with the NLRP3 inhibitor MCC950 (Fig. S5 *A* and *B*). When WT and MLKL-deficient cells were combined to create a mixed population, the vast majority of necroptotic LPS/QVD/Cp.A induced ASC specks were formed in WT cells and were absent from the cocultured *Mlkl*$^{-/-}$ BMDMs (Fig. 4 *F* and *G* and Fig. S5 *A* and *B*). A small number of ASC specks were observed in the Ly5.2 *Mlkl*$^{-/-}$ cells (Fig. S5*A*), however, these appeared to be due to phagocytosis of dead or dying WT cells. Therefore, necroptotic MLKL activates NLRP3-dependent ASC speck formation in a cell-intrinsic manner, and any released DAMPs have limited, if any, capacity to cause inflammasome formation in bystander cells.

Inducible Expression of Activated MLKL Is Sufficient to Activate NLRP3, Which Can Be Blocked by Increased Extracellular Potassium. We next determined whether MLKL is sufficient to activate IL-1β in the absence of other stimuli. We therefore infected the

human monocytic THP1 cell line with a range of doxycycline-inducible murine MLKL constructs (7, 14), including (*i*) a full-length WT MLKL, (*ii*) a full-length inactive MLKL mutant that cannot localize with cellular membranes (inactive; E109A, E110A), (*iii*) a CTD and brace region truncation that lacks the membrane damaging 4HB necessary to kill (CTD; residues 124–464), (*iv*) a full-length, constitutively active (CA) MLKL that mimics the activating phosphorylation of MLKL by RIPK3 (phosphomimetic mutant S345D), and (*v*) the NTD of MLKL that harbors the 4HB killing region (residues 1–180) (Fig. S6). Consistent with our previous work in fibroblasts (7, 14), upon doxycycline-induced MLKL expression in THP1 monocytic cells (Fig. 5*A*) neither the inactive or CTD MLKL proteins induced necroptosis (Fig. 5*B*). In contrast, doxycycline-induced expression of CA MLKL or the NTD MLKL efficiently killed cells (Fig. 5*B*). Remarkably, only doxycycline-induced expression of the active MLKL constructs capable of interacting with cellular

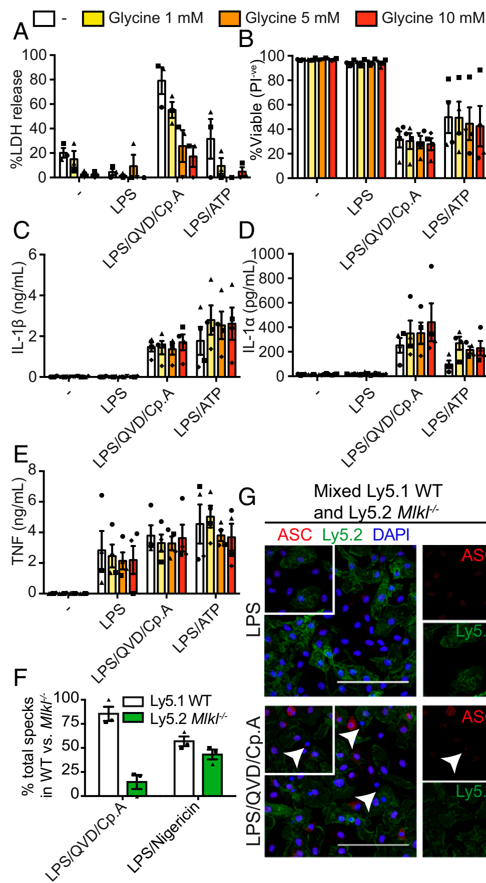

Fig. 4. MLKL activates NLRP3 in a cell-intrinsic manner. (*A*–*E*) WT BMDMs were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) and increasing concentrations of glycine (1 mM, 5 mM, or 10 mM) 30 min before stimulation with Cp.A (1 μM) for 5 h or ATP (5 mM) for 2 h. (*A*) Cytoplasmic LDH release into the supernatant was quantified compared with total intracellular LDH of untreated cells (Triton X-lysed). Data are represented as means ± SEM from three independent experiments. (*B*) Cell death was assessed by flow cytometric analysis of PI uptake. Data are represented as means ± SEM from four independent experiments. (*C*–*E*) Supernatants were assayed for IL-1β (*C*), IL-1α (*D*), or TNF (*E*) levels by ELISA. Data are represented as means ± SEM from four independent experiments. (*F* and *G*) WT Ly5.1 BMDMs and *Mlkl*$^{-/-}$ Ly5.2 BMDMs were seeded in mixed populations and stimulated with LPS (100 ng/mL) for 2–3 h and then treated with Q-VD-OPh (10 μM) 30 min before stimulation with Cp.A (1 μM) for 4 h. ASC speck formation (Alexa647, red), Ly5.2 expression (Alexa488, green), and nuclei (DAPI, blue) were assessed by confocal microscopy. (*F*) Specks were assigned to either WT or *Mlkl*$^{-/-}$ cells by automated analysis in FIJI (Fiji Is Just ImageJ) and represented as a percentage of total specks measured. Data are represented as means ± SEM from three independent experiments. (*G*) Representative images from mixed populations treated with LPS or LPS/QVD/Cp.A (necroptotic stimuli) showing ASC speck formation and are representative of five experiments. White arrows indicate ASC specks. (Scale bars: 100 μm.) A second experimental repeat is included in Fig. S5. Triangles, circles, diamonds, and squares in each graph represent independent experiments.

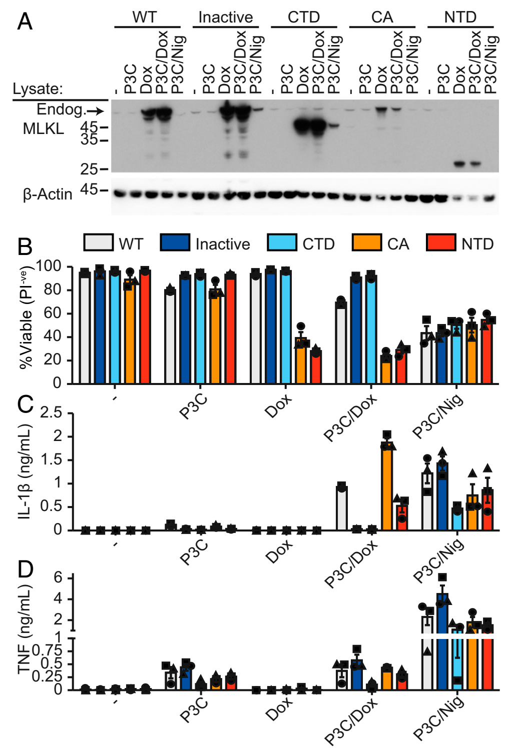

Fig. 5. Necroptosis-inducing MLKL mutants activate NLRP3 to cause IL-1β secretion. (*A*–*D*) THP1 cells infected with doxycycline inducible MLKL constructs (full-length WT, full-length inactive, CTD and brace region truncation, full-length CA, or NTD truncation) were pretreated with P3C (1 μg/mL) for 2 h and then treated with doxycycline (1 μg/mL) for 24 h or nigericin (Nig) (10 μM) for 2 h, as indicated. (*A*) Supernatants and lysates were analyzed by Western blot as indicated. Data are representative of three experiments. (*B*) Cell death was assessed by flow cytometric analysis of PI uptake. Supernatants were assayed for IL-1β (*C*) or TNF (*D*) levels by ELISA. Data are represented as means ± SEM from three independent experiments. Triangles, circles, and squares in each graph represent independent experiments.

membranes, WT MLKL, CA MLKL or the MLKL NTD, caused robust IL-1β secretion when (TLR1/2)-induced inflammasome priming was induced by Pam_3Cys (P3C) (Fig. 5*C*). In contrast, all THP1 cell lines remained responsive to nigericin-induced canonical NLRP3 inflammasome activation of IL-1β and pyroptosis (Fig. 5 *B* and *C*), as well as P3C-induced TNF secretion (Fig. 5*D*). Collectively, these data show that only necroptotic active MLKL, or the 4HB that causes membrane destabilization, are able to induce IL-1β secretion.

High levels of extracellular potassium (50 mM) efficiently prevented necroptotic (LPS/QVD/Cp.A) activation of NLRP3, but not cell death, in murine BMDMs (Fig. 2). We therefore tested whether increased levels of extracellular potassium could also block NLRP3-caspase-1 and IL-1β processing and secretion in THP1 cells following expression of the CA form of MLKL (S345D). Similar to BMDMs, high extracellular potassium blocked CA MLKL-induced processing, activation and secretion of caspase-1 and IL-1β in THP1 cells but did not prevent cell death (Fig. 6 *A–C*). Similar to potassium chloride, treatment of cells with the specific NLRP3 inhibitor MCC950 (33) abrogated processing and secretion of caspase-1 and IL-1β following CA MLKL induction, but failed to prevent MLKL-induced necroptotic killing (Fig. 6 *D–F*). Importantly, this result was not due to an inhibition of TLR-induced inflammasome priming by MCC950. Consistent with other groups (33), we observed that MCC950 treatment did not alter TLR-induced pro-IL-1β expression or TNF release from THP1 cells, or BMDMs treated with TLR-ligands and NLRP3, apoptotic or necroptotic stimuli (Fig. 6*F* and Fig. S7 *A–E*). These data demonstrate that cell death directed by MLKL, independent of caspase-1 activity, is insufficient to cause IL-1β release but that MLKL must activate the NLRP3 inflammasome to release mature IL-1β.

MLKL Activation of the NLRP3 Inflammasome, but Not Necroptotic Cell Lysis, Is Required for NF-κB Activation in Healthy Bystander Cells. We next tested whether necroptotic DAMP release, or NLRP3 activation, drives the inflammatory potential of MLKL. We examined the ability of supernatants from CA MLKL-killed cells to activate NF-κB signaling in THP1 reporter cells, in which GFP expression is driven by NF-κB signaling (Fig. 7*A*). Notably, most PRRs, including those recognizing host-derived DAMPs, are strong activators of NF-κB, making NF-κB a good readout of both DAMP signaling and NLRP3 induced IL-1β activation.

THP1 cells were differentiated into macrophages by phorbol 12-myristate 13-acetate (PMA) pulse treatment, which induces inflammasome priming and importantly bypasses the requirement for a TLR ligand priming stimulus that would otherwise contaminate the supernatant of MLKL killed cells. Similar to undifferentiated THP1 cells, doxycycline-induced expression of CA MLKL in PMA-differentiated THP1 cells resulted in the secretion of IL-1β (Fig. 7*B*) and significant cell death as measured by both LDH release and PI staining (Fig. 7 *C* and *D*). Importantly, supernatants from differentiated THP1 cells killed by CA MLKL expression activated NF-κB when added to naïve THP1 reporter cells (Fig. 7 *E* and *F*). Pretreatment of differentiated THP1 cells with the NLRP3 inhibitor MCC950 before CA MLKL killing blocked IL-1β secretion but did not prevent necroptotic killing (Fig. 7 *B–D*). Remarkably, despite not blocking necroptosis, MCC950 abrogated the ability of necroptotic supernatants to induce NF-κB (Fig. 7 *E* and *F*).

To confirm that NLRP3 activation and not cellular rupture was required to activate NF-κB signaling in bystander cells, we subjected differentiated THP1 cells to three rounds of freeze-thawing, which resulted in precursor IL-1β release detected by ELISA (Fig. 7*B*) and complete cellular rupture (Fig. 7*C*). However, like necroptotic supernatants, freeze-thawed supernatants failed to activate NF-κB when incubated with THP1 NF-κB reporter cells, unless NLRP3 was activated by CA MLKL signaling (Fig. 7*F*). Collectively, these data show that MLKL-induced activation of the NLRP3 inflammasome, but not necrotic-induced release of potential DAMPs, is the dominant means by which MLKL-mediated necroptosis signals NF-κB activity in healthy bystander cells.

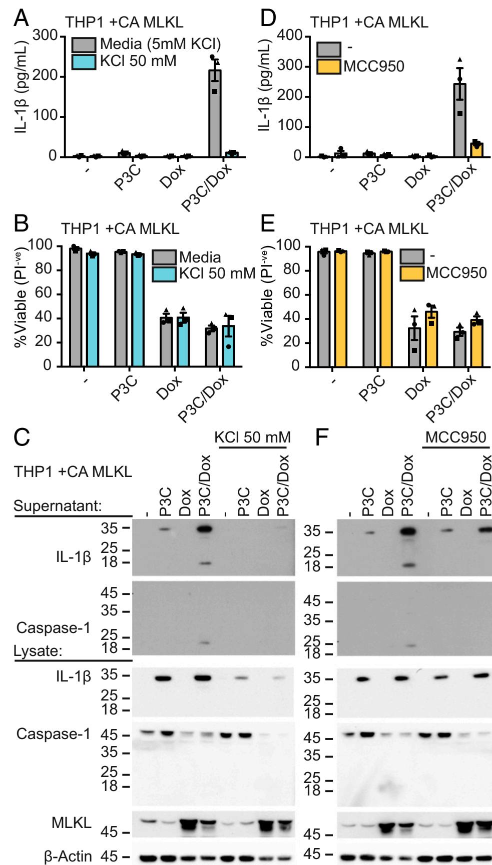

Fig. 6. MLKL-induced IL-1β secretion, but not MLKL killing, is blocked by the NLRP3 inhibitor MCC950 or high extracellular potassium. THP1 cells infected with a doxycycline-inducible CA MLKL construct were treated with P3C (1 μg/mL) and KCl (50 mM) (*A–C*) or the NLRP3 inhibitor MCC950 (1 μM) (*D–F*) before treatment with doxycycline (1 μg/mL) for 24 h. (*A* and *D*) Supernatants were assayed for IL-1β by ELISA. (*B* and *E*) Cell death was assessed by flow cytometric analysis of PI uptake. Data are represented as means ± SEM from three independent experiments. (*C* and *F*) Supernatants and lysates were analyzed by Western blot as indicated. Data are representative of three experiments. Triangles, circles, and squares in each graph represent independent experiments.

Discussion

Genetic studies have documented how excess necroptotic signaling can cause severe inflammatory disease. Necroptotic cell death is inflammatory in part due to cell lysis and the consequent release of DAMPs, which can induce or amplify inflammatory cytokine levels. We show that MLKL can also drive inflammation through activation of the NLRP3 inflammasome, which induces IL-1β activation and secretion. Our results also reveal that this pathway is responsible for the inflammatory potential of MLKL-killed cells, as measured by their ability to

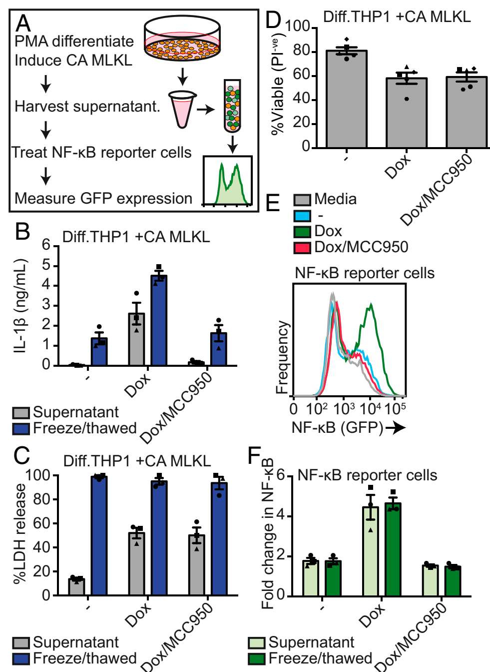

Fig. 7. MLKL-induced NLRP3 inflammasome activation, but not necroptotic cell death, induces NF-κB signaling in healthy bystander cells. (*A*) Schematic of experimental system. (*B–D*) THP1 cells infected with a doxycycline-inducible CA MLKL construct were differentiated by PMA treatment. Cells were treated with the NLRP3 inhibitor MCC950 (1 μM) and then treated with doxycycline (1 μg/mL) as indicated for 24 h. Duplicate plates were subjected to three rounds of freeze thawing to release cytosolic proteins. (*B*) Supernatants from PMA-differentiated cells were assayed for IL-1β by ELISA. (*C*) Cytoplasmic LDH release into the supernatant was quantified compared with total intracellular LDH of untreated cells. Data are represented as means ± SEM from three independent experiments. (*D*) Cell death of PMA-differentiated cells was assessed by flow cytometric analysis of PI uptake. Data are represented as means ± SEM from five independent experiments. (*E* and *F*) Cell supernatants were transferred onto THP1 cells bearing an NF-κB GFP-reporter construct. After 8 h of incubation, GFP expression was quantified by flow cytometry. (*E*) Representative histograms of GFP expression representing activation of NF-κB signaling, quantified in *F* as the fold change in mean fluorescence intensity (MFI) relative to THP1 cells in media alone. Data are represented as means ± SEM from three independent experiments. Triangles, diamonds, circles, and squares in each graph represent independent experiments.

activate NF-κB signaling in naïve bystander cells. These findings suggest that NLRP3 may represent an important inflammatory driver in MLKL-dependent diseases and hence is a prospective therapeutic target.

The NLRP3 inflammasome has been reported to be activated in healthy cells following the detection of necrotic cell debris (34). We therefore tested whether necroptotic cell death and DAMP release was the mechanism by which MLKL activated NLRP3 or whether MLKL activation of NLRP3 is a cell-intrinsic process. Glycine has previously been reported to inhibit necrotic cellular lysis, as measured by its ability to prevent LDH release (32). In our hands, the osmoprotectant glycine also inhibited the release of LDH and other, but not all, cellular proteins, and cells still became permeable to propidium iodide. Therefore, a caveat of glycine is that it delays, but does not completely prevent, plasma membrane rupture. As such, we could not conclusively determine if IL-1β secretion was an active or passive process during necroptosis. This question of whether IL-1β can be actively secreted from cells after caspase-1 activation remains controversial (20, 23, 35–37). These experiments nonetheless suggested that NLRP3 activation could be cell-intrinsic, because glycine treatment had no effect on mature IL-1β secretion following MLKL activation. In line with this idea, the assessment of intracellular ASC specks by confocal microscopy demonstrated that necroptotic cells did not activate NLRP3 and ASC speck formation in neighboring cells. Together, these experiments provide strong evidence that MLKL activation of NLRP3 is specific and cell-intrinsic and occurs as a consequence of necroptosis signaling, but before cellular disintegration.

Mechanistically, MLKL appeared to trigger NLRP3 through its necroptotic activity, where oligomerization and translocation of endogenous MLKL to membrane fractions correlated with IL-1β activation. However, external necroptotic activating stimuli can also signal through RIPK1 and RIPK3 and drive multiple inflammatory signaling pathways (38). Therefore, to confirm that NLRP3 activity was dependent on MLKL, we directly induced the expression of necroptotic-competent MLKL, which caused significant NLRP3 signaling and subsequent IL-1β secretion.

Apart from two notable exceptions (39, 40), NLRP3 activators appear to induce a loss of intracellular potassium to specifically cause inflammasome assembly (25, 41). Our data are consistent with a mechanism in which MLKL translocates to the membrane causing potassium efflux. Subsequently, the reduced intracellular potassium concentration leads to activation of NLRP3 in a cell-intrinsic manner (Fig. 8). High levels of extracellular potassium blocked potassium efflux following necroptotic stimuli, thereby preventing NLRP3 activation following both endogenous MLKL activation, or through expression of inducible necroptotically

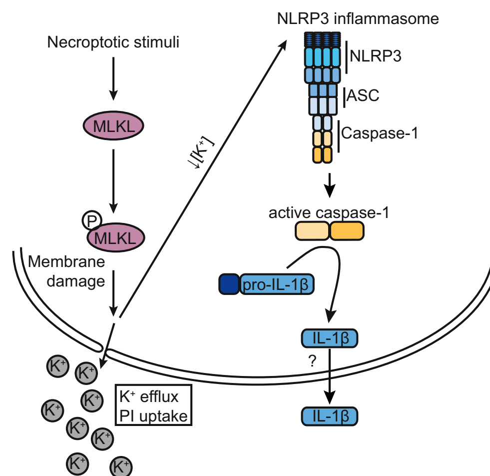

Fig. 8. Model of how MLKL activates the NLRP3 inflammasome. Necroptotic stimuli activate MLKL causing potassium efflux. Potassium efflux nucleates NLRP3 inflammasome formation, resulting in caspase-1 cleavage and activation of IL-1β. Whether IL-1β secretion subsequently occurs by an active or passive mechanism, or a combination of both, is unknown.

active MLKL. It remains unclear how rubidium chloride and cesium chloride inhibit the activation of NLRP3 given that treatment with these cations alone reduced intracellular potassium levels.

Inflammation driven by necroptotic signaling is not necessarily dependent on MLKL killing, but can instead be caused by activation of RIP kinases (42) or through MLKL-activation of NLRP3, driving cytokine secretion (17–19). Our results indicate that MLKL-induced NLRP3 activity and IL-1β secretion, not necroptotic DAMP release, is the dominant activator of NF-κB signaling in healthy bystander cells. Indeed, MLKL-mediated activation of IL-1β has been reported to cause inflammation mediated tissue damage in a model of *Staphylococcus aureus* infection (43). In addition, there are a growing number of studies that have separately implicated necroptosis or NLRP3 in driving pathology of atherosclerosis (44–46), multiple sclerosis (47–49), and ischemia–reperfusion injury of the heart (50, 51) and brain (52–54). Indeed, in models of kidney ischemia–reperfusion injury, both MLKL (42) and NLRP3 (55) deficiency are protective. Based on our findings, it is possible that MLKL-induced NLRP3 signaling is a pathological driver in these inflammatory diseases. Defining the role of MLKL-induced IL-1β signaling in these and other potential necroptotic models (56) will therefore critically inform the development and testing of new disease-specific, antiinflammatory, therapeutic strategies.

Materials and Methods

Cell Culture. BMDMs were generated from bone marrow cells harvested from femoral and tibia bones as described previously (17), except in Fig. 3 *F* and *G* and Fig. S2 which were generated as in ref. 21. BMDMs were seeded at 4×10^5 per well (24-well plate) overnight. *Ripk3*$^{-/-}$, *Nlrp3*$^{-/-}$, *Mlkl*$^{-/-}$, *Gsdmd*$^{-/-}$, and *Caspase-1*$^{-/-}$ (*ICE*$^{-/-}$, with a naturally occurring inactivating caspase-11 deletion) have been described previously (7, 27, 57–60). The Walter and Eliza Hall Institute (WEHI) and University of Queensland Animal Ethics Committee approved all animal experiments. Further experimental details are provided in *SI Materials and Methods*.

Constructs. Inducible lentiviral MLKL constructs were published previously (7, 14, 15) and used to stably infect THP1 cells. NF-κB reporter THP1 monocytic cells were made by infection with the lentiviral reporter vector (pTRH1-NF-κB–dscGFP, TR503PA; System Bioscience) and sorted by flow cytometry for GFP expression.

Cytokine ELISA. Mouse IL-1β [DY401 (R&D Systems) or 88-7013-77 (eBioscience)], human IL-1β (DY201; R&D Systems), mouse TNF (88-7324; eBioscience), human TNF (88-7346; eBioscience), and mouse IL-1α (433401; BioLegend) ELISA kits were used on supernatants according to the manufacturer's instructions.

Cell Viability. BMDMs seeded on non-tissue culture-treated 24-well plates were harvested using 5 mM EDTA/PBS. Viability of both THP1 cells and BMDMs was analyzed by propidium iodide (PI) uptake by flow cytometry on a FACSCalibur or FACSCanto (BD Immunocytometry Systems). Data were analyzed using FlowJo software version 7.6.5. LDH activity was analyzed by Cytotoxicity Detection Kit (LDH) [11644793001 (Roche) or G1780 (Promega)] as per the manufacturer's instructions.

Immunoblotting. Immunoblotting and fractionation was carried out as previously published (14, 17). Blots were probed with antibodies against pro- and cleaved mouse caspase-1 [sc-514 (Santa Cruz Biotechnology) and AG-20B-0042-C100 (Adipogen)], human caspase-1 (3866; Cell Signaling Technology), IL-1β (AF-401-NA; R&D), NLRP3 (AG-20B-0014-C100; Adipogen), ASC (sc-22514-R; Santa Cruz Biotechnology), β-actin (A-1978; Sigma), RIPK3 (PSC-2283-c100; Axxora), MLKL (3H1; in-house), VDAC1 (AB10527; EMD Millipore), GAPDH (2118; Cell Signaling Technology), HMGB1 (ab18256; Abcam), HSP60 (sc-1052; Santa Cruz Biotechnology).

Immunofluorescence of Endogenous ASC Specks by Confocal Microscopy. Immunofluorescence of ASC specks was performed similarly as described (61). Further experimental details are provided in *SI Materials and Methods*.

NF-κB Signaling. Supernatants were removed from cultured THP1 cells and pelleted to remove any debris. Duplicate seedings were subjected to three rounds of freeze thawing, supernatants were then cleared by pelleting at 13,000 rpm. THP1 cells bearing the NF-κB reporter construct were cultured in the conditioned supernatant for 8 h before measuring GFP expression of THP1 cells relative to those in media alone using a FACSCalibur (BD Immunocytometry Systems).

Intracellular Potassium Levels. Intracellular K^+ measurements were performed by inductively coupled plasma optical emission spectrometry as described previously (25).

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Supporting Information

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SI Materials and Methods

Cell Culture. THP1 cells were cultured in RPMI medium 1640 containing 8% (vol/vol) FBS [37 °C, 10% (vol/vol) CO_2] and seeded at 3 × 10^5 per mL before treatment. Differentiation of THP1 cells was achieved by incubating cells in 500 nM PMA in RPMI media containing 8% (vol/vol) FBS for 3 h (37 °C), before washing twice with RPMI media, seeding at 1.5 × 10^6 per well (6 well plate) or 3 × 10^5 per well (24 well plate) and incubating for 48 h [37 °C, 10% (vol/vol) CO_2].

Cells were primed with ultrapure LPS (100 ng/mL; Invivogen), Fc-human TNF (100 ng/mL; in-house), or Pam_3Cys (1 μg/mL; Invivogen) and stimulated as specified with Cp.A (1 μM; TetraLogic Pharmaceuticals), Q-VD-OPh (10 μM; R&D Systems), nigericin (10 μM; Sigma), ATP (5 mM; Sigma), doxycycline (1 μg/mL; D3447; Sigma-Aldrich), and MCC950 (1 μM; provided by A. Roberson and M. Cooper, University of Queensland, Brisbane, Australia) (33). Salt solutions were prepared to a concentration of 2 M in H_2O, diluted in media, and filtered. Salts were obtained from the following suppliers: lithium chloride (10374; BDH), sodium chloride (465; Univar), potassium chloride (104936; Merck), rubidium chloride (R2252; Sigma-Aldrich), cesium chloride (15507; Invitrogen). Glycine stocks (G7126; Sigma) were dissolved in media and filtered.

Immunofluorescence of Endogenous ASC Specks by Confocal Microscopy. Briefly, 8-well chamber μ-slides (Ibidi) were coated with retronectin, before WT (Ly5.1) and *Mlkl*$^{-/-}$ (Ly5.2) BMDMs were seeded in either mixed populations at 0.5 x10^5 cells per genotype, or ∼1 × 10^5 of either WT or *Mlkl*$^{-/-}$ BMDMs per well. Cells treated as described in figure legends were fixed with 4% PFA, before blocking [PBS, 10% (vol/vol) FCS] for 60 min. Cells were then stained for Ly5.2 surface expression (BD Pharmingen, 561874, 1:100) overnight at 4 °C. The following day cells were washed and permeabilized [PBS, 10% (vol/vol) FCS, 0.5% (vol/vol) Triton X-100] for 60 min, before staining for intracellular ASC (N-15; 1:500; Santa Cruz Biotechnology) overnight at 4 °C. Cells were then washed and stained with secondary antibodies staining for ASC with goat anti-rabbit–Alexa647 (A-21245; 1:1,000; Invitrogen) or Ly5.2 with goat anti-mouse-Alexa488 (A-11029; 1:300; Invitrogen) for 60 min, before nuclear staining with DAPI for 10 min. Cells were imaged using a Zeiss LSM 780 confocal microscope; 3 × 3 tile scans with Z-stacks were obtained for each experimental condition using a 40× oil objective with Immersol 518 F (Zeiss) and acquired with ZEN 2012 version 8.1 software (Zeiss). Image channels were merged and displayed as maximum projection before conversion to tagged image bitmap file (TIFF) using FIJI software. Quantification of ASC specks was performed by detecting local maxima in the red channel (ASC stain), the resulting "objects" were then determined to be either inside (*Mlkl*$^{-/-}$ cells) or outside (WT cells) the green channel (Ly5.2 stain; *Mlkl*$^{-/-}$) through morphological filtering. Total cell number (WT and *Mlkl*$^{-/-}$) per image was determined from the DAPI channel. Quantification was automated using a custom-written FIJI macro (62).

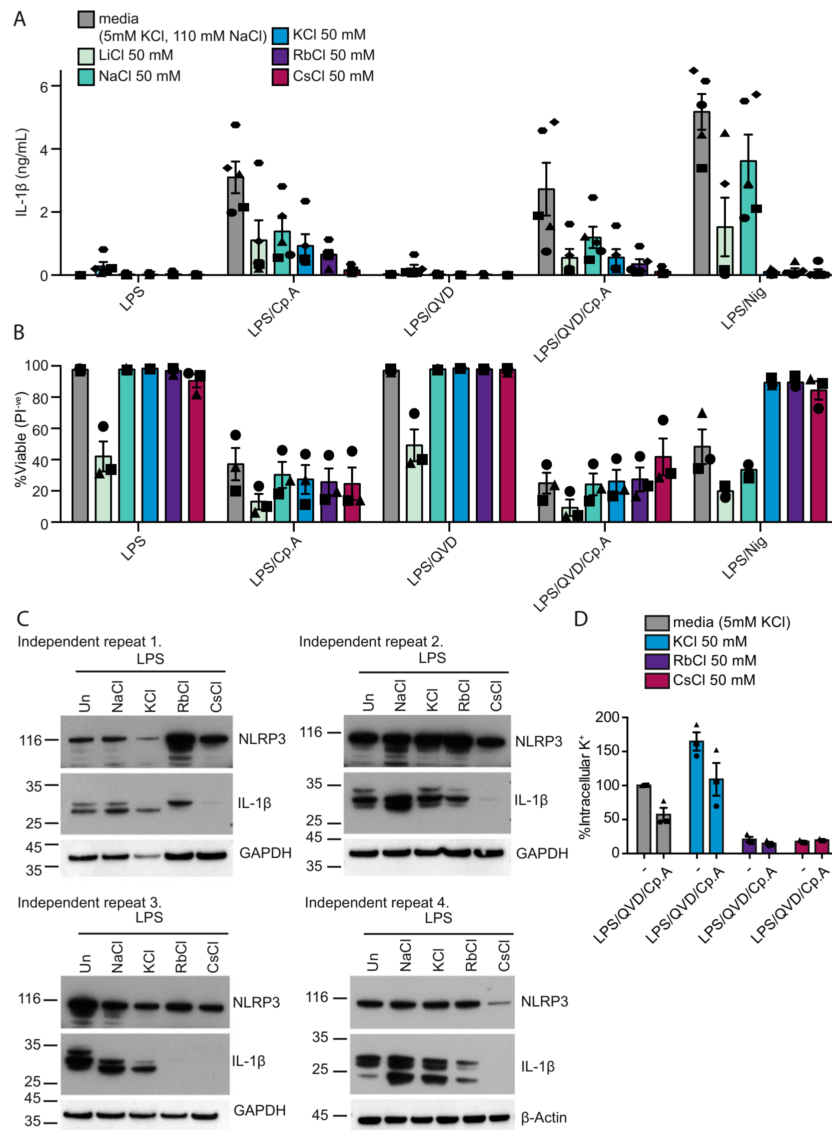


Fig. S1. Increased extracellular alkali halides prevent MLKL-induced NLRP3 activation but not cell death. (*A* and *B*) WT BMDMs were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) and one of the following salts (50 mM): lithium chloride (LiCl), sodium chloride (NaCl), potassium chloride (KCl), rubidium chloride (RbCl), or cesium chloride (CsCl) 30 min before stimulation with Cp.A (1 μM) for 5 h or nigericin (Nig) (10 μM) for 2 h, as indicated. (*A*) Supernatants were assayed for IL-1β levels by ELISA. Data are represented as means ± SEM from five independent experiments. (*B*) Cell death was assessed by flow cytometric analysis of PI uptake. Data are represented as means ± SEM from three independent experiments. (*C*) WT BMDMs were preincubated with LPS (100 ng/mL) for 2 h and then treated with one of the following salts: lithium chloride (LiCl), sodium chloride (NaCl), potassium chloride (KCl), rubidium chloride (RbCl), or cesium chloride (CsCl) (50 mM) for 5 h, as indicated. Supernatants and lysates were analyzed by Western blot as indicated (n = 4 independent experiments). (*D*) WT BMDM were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) and one of the following salts (50 mM): potassium chloride (KCl), rubidium chloride (RbCl), or cesium chloride (CsCl) 30 min before stimulation with Cp.A (1 μM) for 5 h. The intracellular levels of K^+ were quantified by inductively coupled plasma mass spectrometry. Data are represented as means ± SEM from three independent experiments. Triangles, diamonds, hexagons, circles, and squares in each graph represent independent experiments.

Figure S1C bottom right panel has been updated, corrected figure is included in appendix.

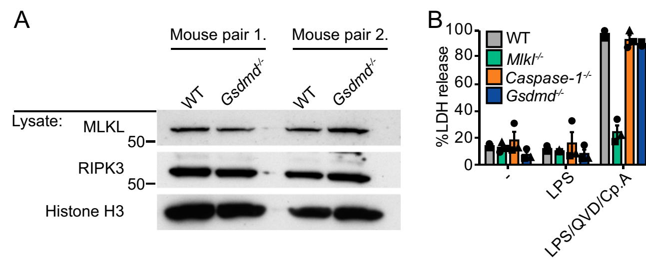


Fig. S2. GSDMD-deficient BMDMs die by necroptosis and express normal levels of RIPK3 and MLKL. (*A*) Lysates from WT and *Gsdmd*$^{-/-}$ BMDMs were analyzed by Western blot as indicated. Two mice of each genotype are shown. (*B*) WT, *Mlkl*$^{-/-}$, *Gsdmd*$^{-/-}$, and *Caspase-1*$^{-/-}$ BMDMs were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) 30 min before stimulation with Cp.A (1 μM) for 24 h. Cytoplasmic LDH release into the supernatant was quantified compared with total intracellular LDH of untreated cells (Triton X-lysed). Data are represented as means ± SEM from three independent experiments. Triangles, circles, and squares in each graph represent independent experiments.

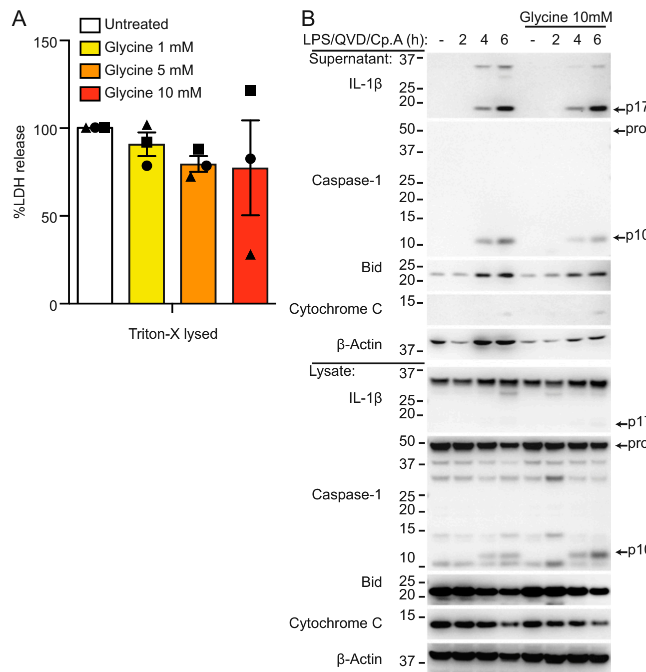


Fig. S3. Glycine does not block IL-1β cleavage and secretion. (*A*) WT BMDMs were treated with increasing amounts of glycine (1 mM, 5 mM, and 10 mM) for 8 h before addition of 1% Triton X to completely lyse cells. LDH levels were quantified. (*B*) WT BMDMs were preincubated with or without LPS (100 ng/mL) for 2–3 h and were treated with Q-VD-OPh (10 μM) and glycine (10 mM) 30 min before stimulation with Cp.A (1 μM) for the indicated times. Supernatants and lysates were analyzed by Western blot as indicated. Data are representative of two independent experiments. Triangles, circles, and squares in each graph represent independent experiments.

Fig. S4. Necroptosis induces ASC speck formation in WT but not *Mlkl*$^{-/-}$ BMDMs. WT Ly5.1 BMDMs and *Mlkl*$^{-/-}$ Ly5.2 BMDMs were seeded in separate wells and stimulated with LPS (100 ng/mL) for 2–3 h and then treated with Q-VD-OPh (40 μM) 30 min before stimulation with Cp.A (1 μM) for 5 h, or nigericin (5 μM) for 2 h as indicated. ASC speck formation (Alexa647, red), Ly5.2 expression (Alexa488, green) and nuclei (DAPI, blue) were assessed by confocal microscopy. (*A*) Representative images showing ASC speck formation, representative of three independent experiments. White arrows indicate ASC specks. Scale bars represent 100 μm. (*B*) Specks were quantified via an automated macro in FIJI and represented as percentage of cells with ASC specks. Data are represented as means ± SEM from three independent experiments. Triangles, circles, and squares in each graph represent independent experiments.

High resolution version included in appendix to assist in visualisaiton of ASC specks.

Fig. S5. Necroptosis activates NLRP3 to induce ASC speck formation. WT Ly5.1 BMDMs and *Mlkl*$^{-/-}$ Ly5.2 BMDMs were seeded in mixed populations and stimulated with LPS (100 ng/mL) for 2–3 h and then treated with Q-VD-OPh (40 μM) and MCC950 (1 μM) 30 min before stimulation with Cp.A (1 μM) for 5 h or nigericin (5 μM) for 2 h as indicate. ASC speck formation (Alexa647, red), Ly5.2 expression (Alexa488, green), and nuclei (DAPI, blue) were assessed by confocal microscopy. (*A*) Representative images showing ASC speck formation, representative of three independent experiments. White arrows indicate ASC specks within WT cells and green arrows indicate ASC specks within *Mlkl*$^{-/-}$ cells. (Scale bars: 100 μm.) (*B*) Specks were quantified via an automated macro in FIJI and represented as percentages of cells with ASC specks. Data are represented as means ± SEM from three independent experiments. Triangles, circles, and squares in each graph represent independent experiments.

High resolution version included in appendix to assist in visualisaiton of ASC specks

A Full-length wild-type MLKL (WT)

4-Helical Bundle Brace region Pseudokinase domain

(N-Term) (C-Term)

B Full-length inactive mutant MLKL (Inactive; E109A, E110A)

4-Helical Bundle Brace region Pseudokinase domain

E109A, E110A

C C-terminal domain and brace region truncation MLKL (CTD; residues 124-464))

Brace region Pseudokinase domain

D Full length constitutively active MLKL (CA; phosphomimetic mutant S345D)

4-Helical Bundle Brace region Pseudokinase domain

S345D

E MLKL N-terminal domain (NTD; residues 1-180)

4-Helical Bundle Brace region

Fig. S6. Schematic of the murine MLKL constructs used in this study. (*A*) Full-length WT MLKL. (*B*) Full-length inactive MLKL mutant (Inactive; E109A, E110A). (C) CTD and brace region truncation that lacks the membrane damaging 4HB necessary to kill (CTD; residues 124–464). (*D*) Full-length CA MLKL that recapitulates the activating phosphorylation of MLKL by RIPK3 (CA; phosphomimetic mutant S345D). (*E*) The NTD of MLKL that harbors the 4HB killing region (NTD; residues 1–180).

Fig. S7. MCC950 does not block the secretion of TNF. (*A*) THP1 cells infected with a doxycycline-inducible CA MLKL construct were treated with P3C (1 μg/mL) and the NLRP3 inhibitor MCC950 (1 μM), before treatment with doxycycline (1 μg/mL) for 24 h. Supernatants were assayed for TNF by ELISA. Data are represented as means ± SEM from three independent experiments. (*B–D*) WT BMDMs were preincubated with or without LPS (100 ng/mL) (*B* and *D*) or P3C (1 μg/mL) (*C* and *E*) for 2–3 h and were treated with Q-VD-OPh (10 μM) 30 min before stimulation with Cp.A (1 μM) for 5 h or nigericin (Nig) (5 mM) for 2 h as indicated. Supernatants were assayed for IL-1β (*B* and *C*) or TNF (*D* and *E*) by ELISA. Data are represented as means ± SEM from four independent experiments. Triangles, diamonds, circles, and squares in each graph represent independent experiments.

Chapter 5: Discussion

Programmed cell death activates inflammatory signalling through a number of interrelated pathways, including caspase-1-mediated pyroptosis, caspase-8-mediated apoptosis, or through the necroptotic activity of the RIP kinases and MLKL. Importantly these cell death pathways mediate immunity in a number of ways. Firstly they trigger the release of inflammatory cytokines which prime and activate innate and adaptive immunity. Secondly, they cause the release of intracellular pathogens from host cells, thereby mediating the detection of pathogens that would otherwise find refuge from immune surveillance in cellular niches. Finally, lytic forms of programmed cell death such as pyroptosis and necroptosis result in the release of cellular contents, including DAMPs, which can be detected by PRRs to further induce inflammatory signalling pathways and the production of inflammatory cytokines.

The results in this thesis contribute to our molecular understanding of the ways in which programmed cell death can activate inflammatory signalling. In chapter 3, I demonstrated how pyroptotic cell lysis is not required for the release of IL-1β from cells following caspase-1 activation. I further show that apoptotic caspase-8 can directly process IL-1β into an active form in the absence of caspase-1. In chapter 4, I define the mechanism by which MLKL activates the NLRP3-caspase-1 inflammasome to drive IL-1β processing. Together these results contribute to our understanding of the complex ways in which IL-1β can become activated, a process which can be therapeutically targeted to improve the inflammatory phenotype in a number of diseases.

5.1 Cell death and the secretion of IL-1β

The issue of whether IL-1β can be actively secreted has been hotly contested in recent years. A well understood feature of caspase-1 activation is that it simultaneously activates both cytokine processing and lytic cell death, making separation of the two difficult. Recent publications have demonstrated the inevitability of cell death in macrophages upon activation of the canonical NLRP3 inflammasome (Liu et al., 2014, Shirasaki et al., 2014, Cullen et al., 2015). Complicating these results is evidence that many of the commonly used experimental stimuli can trigger cell death in the absence of caspase-1 activity (Cullen et al., 2015).

Despite this, there are a growing number of reports that suggest secretion of bioactive IL-1β is able to occur in the absence of detectable LDH release (Chen et al., 2014a, Karmakar et al., 2015, Russo et al., 2016, Gaidt et al., 2016, Brough and Rothwell, 2007, Zanoni et al., 2016, Zhang et al., 2015, Martin-Sanchez et al., 2016). The results in chapter 3 contribute to this discussion, overcoming many of the experimental issues that arise when activating endogenous caspase-1 by utilizing a caspase-1-gyrase system that allowed direct activation of caspase-1. These experiments bypassed the need for external activating stimuli and provided an opportunity to study the functions of caspase-1 in isolation. This system demonstrated that IL-1β could be actively secreted from metabolically active cells, both at a population and a single cell level. Finally using ELISPOT technology, the data confirmed that this secretion was occurring from a significant proportion of the cell population. In further support of these data, I also demonstrate in chapter 4 that complete cell rupture was not required for IL-1β to be released from cells undergoing necroptosis, as pre-treatment of cells with glycine inhibited the release of cellular proteins such as LDH without blocking IL-1β release.

It remains unclear why the dimerization of caspase-1-gyrase did not kill cells. Two explanations could be that the use of a C-terminal GFP tag may have had an inhibitory effect on the activity of the caspase-1 fusion protein (Heymann et al., 2015), or alternatively that immortalized MEFs used in these experiments may lack downstream effector molecules such as GSDM-D. Regardless, the caspase-1-gyrase was enzymatically active with respect to IL-1β processing, which was inhibited by chemical caspase inhibition or catalytic inactivating point mutations. Looking forward, this experimental

system could be used to study the mechanism by which IL-1β or other cytokines such as IL-18 are secreted, as we have isolated caspase-1-dependent cytokine secretion pathways from the complication of pyroptotic cell death.

Using a similar caspase-8-gyrase system, I demonstrated that caspase-8 could process IL-1β into a biologically active form, and that it did so in the absence of caspase-1, and independent of upstream inflammatory signalling pathways. Given the controversy over the secretion of IL-1β (Monteleone et al., 2015), further research is still necessary to establish whether caspase-8 processed IL-1β can be actively secreted from cells independent of apoptosis, or whether apoptotic membrane damage is required for IL-1β release. Unlike pyroptosis, caspase-8-mediated apoptosis is a slower form of cell that that does not result in the immediate rupture of the plasma membrane and it is therefore possible that this is another circumstance where the passive release of IL-1β is not observed.

Although macrophages inevitably undergo pyroptosis following caspase-1 activation, other cell types such as neutrophils (Chen et al., 2014a, Karmakar et al., 2012), dendritic cells (Zanoni et al., 2016) and human monocytes (Gaidt et al., 2016) may not. A non-canonical pathway of NLRP3-caspase-1 induced IL-1β secretion has also been reported in BMDM (Wolf et al., 2016). It has been suggested that the lack of pyroptotic killing may allow neutrophils to continue to secrete IL-1β over a longer period of time than macrophages during *Salmonella* infection (Chen et al., 2014a). Phagocytes such as neutrophils and dendritic cells are therefore likely to drive chronic inflammation, while pyroptotic cell death may play two contrasting roles in moderating cytokine production during an innate immune response. On the one hand, pyroptosis results in the release of DAMPs and other inflammatory mediators following the activation of caspase-1, but cell rupture simultaneously limits the ability of cells such as macrophages to continue to produce inflammatory cytokines, thereby limiting chronic inflammation.

5.2 The role of cell death in driving inflammation

Inflammatory pyroptotic cell death was first described in opposition to the immunologically silent apoptotic cell death (Cookson and Brennan, 2001). The results in chapter 3 contribute to a growing understanding of the diverse ways in which the apoptotic caspase-8 can contribute to inflammation. Caspase-8 is required for the efficient priming of inflammasomes due to its ability to mediate transcriptional responses downstream of TLRs (Allam et al., 2014, Weng et al., 2014, Lawlor et al., 2015). Caspase-8 can also contribute to the processing of IL-1β either by activating the NLRP3 inflammasome or by directly activating caspase-1 (Vince and Silke, 2016). The results in chapter 3 confirm this direct processing, showing that in the absence of NLRP3 and caspase-1, caspase-8 is able to process IL-1β into a biologically active form. However, the same data showed that caspase-8 was less efficient than caspase-1 at cleaving IL-1β.

Apoptosis is considered to be a non-inflammatory form of cell death. Plasma membrane integrity is maintained throughout the apoptotic dismantling of cells *in vivo*, while apoptotic cell debris is phagocytosed quickly further inhibiting the release of cytokines and other inflammatory mediators (Fink and Cookson, 2005). In addition to this, caspase-8 activity limits the activation of RIP kinases which are capable of driving inflammation in both MLKL-dependent and independent ways (Kaiser et al., 2011, Oberst et al., 2011). The finding presented in this thesis that activation of caspase-8 can mediate processing of IL-1β in the absence of caspase-1 contradicts this idea, and suggests that apoptosis can mediate innate immune responses through pro-inflammatory cytokine production. Given that excess caspase-8 apoptosis within keratinocytes, driven by loss of upstream regulators such as Sharpin (Rickard et al., 2014, Kumari et al., 2014), TRAF2 (Etemadi et al., 2015), or cFLIP (Panayotova-Dimitrova et al., 2013), triggers pathological inflammation, it is of interest to better define what circumstances shift apoptosis to drive inflammation, and what inflammatory signalling pathways become activated, in order to better target this pathway in diseases.

Necroptosis can play a similar role to apoptosis or pyroptosis in activating innate immune responses to a range of bacteria and viruses (Guo et al., 2015). In particular viruses which inhibit caspases in an attempt to block host cell death and immune responses can inadvertently activate immune responses through necroptosis (Kaiser et al., 2013b). The

results in chapter 4 show that necroptosis additionally activates the pro-inflammatory cytokine IL-1β. Importantly the results within this thesis define a mechanism by which MLKL can trigger NLRP3-mediated IL-1β activation (Figure 8 from Chapter 4); they show that necroptosis drives plasma membrane disruption, which triggers NLRP3-caspase-1 activation through potassium efflux. Further, MLKL-induced IL-1β activation is more efficient than just necroptotic DAMP release alone at driving NF-κB-dependent inflammatory responses.

Necroptosis and IL-1β signalling have been separately implicated in a number of inflammatory diseases. In the case of kidney ischemia reperfusion injury, genetic deficiency of either MLKL (Newton et al., 2016) or NLRP3 (Bakker et al., 2014) have been effective in blocking the disease pathology. These two pathways have been further implicated to drive pathology independently of each other in inflammatory diseases such as multiple sclerosis (Ofengeim et al., 2015, Jha et al., 2010, Inoue and Shinohara, 2013) or atherosclerosis (Lin et al., 2013, Paramel Varghese et al., 2016, Sheedy and Moore, 2013), as well as in ischemia reperfusion injury of the heart (Smith et al., 2007, Toldo et al., 2012) and brain (Degterev et al., 2005, Northington et al., 2011, Brough et al., 2011).

In chapter 4, I demonstrated that inhibiting NLRP3 with MCC950 in necroptotic cells abrogated their ability to signal NF-κB activation in neighbouring healthy cells. Based on this one could hypothesise that MLKL-induced NLRP3 signalling is the critical driver of inflammatory pathology in these diseases. It would therefore be interesting to see the results from chapter 4 extended further to determine how inhibiting NLRP3 inflammasome activation would impact inflammation driven tissue damage within models of TNF-SIRS or kidney ischemia reperfusion, or other diseases where necroptosis has been reported to drive inflammatory pathologies.

5.3 Inflammation in human disease

Inflammation activated during an innate immune response is beneficial to the host as it mediates the immunological clearance of pathogens and initiates processes of wound healing; however, dysregulated and chronic inflammation is damaging to the host and can lead to the development of inflammatory diseases. IL-1 signalling has been implicated to drive pathology in a series of genetic conditions, as well as a wide range of common inflammatory diseases. For this reason a number of therapies have been developed that limit IL-1 signalling. These include the recombinant IL-1R antagonist Anakinra, soluble IL-1R Rilonacept, and IL-1β neutralizing antibody Canakinumab (Dinarello et al., 2012). The results in this thesis contribute to an improved understanding of the activation of NLRP3, as well as how it can drive the release of IL-1β downstream of a diverse range of signalling pathways, which could be used to develop better therapies for diseases such as heritable auto-inflammatory conditions, as well as other more common inflammatory diseases.

Autoinflammatory diseases are chronic diseases characterised by excess inflammatory cytokine signalling in the absence of autoantibodies or antigen-specific T-cells (Kastner, 2005). A number of genetic mutations have been found in NLRP3 that drive excess inflammation in a family of inherited autoinflammatory diseases collectively termed cryopyrin associated periodic syndromes (CAPS). These mutations all drive remarkable similar diseases with symptoms such as periodic fevers, localized inflammation in joints, and skin rashes (Table 5.1), which recur without identifiable pathogenic cause (Chitkara et al., 2007, Menu and Vince, 2011).

Table 5.1 Cyropyrin associated periodic syndromes; hereditary auto-inflammatory syndromes linked to NLRP3

Auto-inflammatory disease	Symptoms	Gene mutation	Successful treatment with anti-IL-1β therapy?
Familial cold auto-inflammatory syndrome (FCAS)	Exposure to cold causes fever, rash, joint pain	Gain of function mutation in NLRP3 (Aganna et al., 2002)	Yes, responsive to Anakinra (Ross et al., 2008)
Muckle-wells syndrome (MWS)	Exposure to cold causes fever, rash, joint pain	Gain of function mutation in NLRP3 (Aganna et al., 2002)	Yes, responsive to Anakinra (Hawkins et al., 2003)
Neonatal onset multi-systemic inflammatory disease / chronic infantile neurological cutaneous articular syndrome (NOMID/CINCA)	Exposure to cold causes fever, rash, joint pain, with neurological complications	Gain of function mutation in NLRP3 (Aksentijevich et al., 2009)	Yes, responsive to Anakinra (Goldbach-Mansky et al., 2006, Hoffman and Firestein, 2006)

In addition, mutations found in other inflammasome receptors, although driven by different genetic lesions, drive a similar range of symptoms including recurrent fever, fatigue, joint and muscle pain, skin rashes and gastro-intestinal disturbances (Dinarello et al., 2012). For example, mutations in pyrin cause two related disorders Familial Mediterranean fever (FMF) (Hoffman et al., 2001), and pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND) (McDermott et al., 1999), while a mutation in a protein upstream of pyrin also causes an inflammatory disorder termed pyoderma gangrenosum and acne (PAPA) syndrome (Wise et al., 2002). Further mutations in NLRC4, AIM2, NLRP7 and NLRP12 have all been found to drive inflammation in a range of hereditary diseases (Broderick et al., 2015). The demonstration that Il-1β is involved in driving many of these symptoms provided a basis for new clinical treatments that limit IL-1 signalling and have proved highly effective (McDermott and Tschopp, 2007). The clinical application of recombinant human IL-1R antagonist Anakinra in each of the CAPS disorders in particular has proven effective at relieving symptoms (Table 5.1).

The inhibition of IL-1 activity has also proven effective in a growing number of chronic inflammatory disorders. IL-1β blockade has even proven beneficial in disorders that are treated with therapies such as glucocorticoids, thereby enhancing disease control and reducing therapy side effects (Dinarello et al., 2012). IL-1 blocking therapies are being

tested in clinical trials in a wide range chronic inflammatory diseases such as gout, pseudogout, type-2 diabetes and atherosclerosis (Dinarello et al., 2012) (Table 5.2). In addition, IL-1β signalling has been proposed to be causative of symptoms in diseases such as multiple sclerosis, Alzheimer's disease and Parkinson's disease, although anti-IL-1 therapies have not yet been tested clinically (Table 5.2). With type 2 diabetes and gout, the application of IL-1β blocking therapies has provided confirmation that inflammasome activation of IL-1β is not just correlated with disease symptoms, but important for driving chronic inflammation.

It is therefore of great interest to further examine the ways in which IL-1β is activated within each of these diseases. In particular, an understanding of how dying cells can further activate the NLRP3 inflammasome will provide novel opportunities to limit inflammatory feed-forward loops and therapeutically target the upstream signalling pathways that activate and drive pathological inflammation.

Table 5.2 The role of NLRP3 and IL-1β in inflammatory diseases

Disease	NLRP3 activating stimulus	Role of NLRP3	Successful treatment with anti-IL-1β therapy?
Gout	Monosodium urate crystals (MSU) (Martinon et al., 2006)	Detection MSU crystals of by the NLRP3-caspase-1 inflammasome activates IL-1β driving joint inflammation	Yes (So et al., 2007)
Pseudogout	calcium pyrophosphate dihydrate (CPPD) crystals (Martinon et al., 2006)	Detection of CPPD crystals by the NLRP3-caspase-1 inflammasome activates IL-1β driving inflammatory symptoms	Yes (McGonagle et al., 2008)
Type-2 diabetes	Elevated fatty acids (Wen et al., 2011) Islet amyloid polypeptide (Masters et al., 2010)	NLRP3-caspase-1 activation of IL-1β causes the destruction of insulin producing islet beta-cells	Yes (Larsen et al., 2009, Larsen et al., 2007)
Multiple sclerosis	Uric acid (Liu et al., 2012, Amorini et al., 2009) Increased cathepsin B activity (Bever and Garver, 1995)	High levels of IL-1β and low levels of IL-1Ra (de Jong et al., 2002) enhances disease progression through demyelination and activation of T cells (Inoue and Shinohara, 2013)	No trials, but promising preclinical results (Badovinac et al., 1998)
Alzheimer's disease	Amyloid-β fibrils (Halle et al., 2008)	Activation of the NLRP3-caspase-1 inflammasome and IL-1β by amyloid depositions drive tissue damage and neuroinflammation	No trials, but promising preclinical results (Heneka et al., 2013, Kitazawa et al., 2011)
Parkinson's disease	α-synuclein (Codolo et al., 2013, Freeman et al., 2013)	Secretion of IL-1β by microglia cells drive neuroinflammation and neurodegeneration, but could also be protective under certain circumstances (Leal et al., 2013)	IL-1β plays both a protective and toxic role and may be a complex therapeutic target (Leal et al., 2013)
Atherosclerosis	Cholesterol crystals (Duewell et al., 2010, Rajamaki et al., 2010)	Detection of cholesterol crystals by the NLRP3-caspase-1 inflammasome in macrophages causes IL-1β mediated tissue destruction	Ongoing clinical trial (Ridker et al., 2011)

5.4 Conclusion

Cell death pathways and innate immunity are related through their ability to activate pro-inflammatory cytokines and drive inflammation that can either promote tissue repair, or if dysregulated cause tissue damage and cellular destruction. However, it is often unclear whether cell death drives inflammation, whether inflammation drives cell death, or whether both processes can happen at the same time in the same cell. This thesis further defines how the distinct, genetically encoded, cell death pathways of pyroptosis, apoptosis and necroptosis drive the activation and secretion of IL-1β through the NLRP3-inflammasome. We are only just beginning to fully appreciate the ways in which pathways of innate immunity, inflammation and programmed cell death work synergistically. Better defining the intersections of these pathways is of great importance, as it will inform the design of anti-inflammatory therapeutics, allowing better targeting of specific cell death and inflammasome pathways in inflammatory diseases.

Appendix

One corrected figure and two supplementary figures are provided at higher resolution here from the following publication included in chapter 4:

Conos S A, Chen K W, De Nardo D, Hara H, Whitehead L, Núñez G, Masters S L, Murphy J M, Schroder K, Vaux D L, Lawlor K E, Lindqvist L M, Vince J E. Active MLKL triggers the NLRP3 inflammasome in a cell intrinsic manner. Proceedings of the National Academy of Sciences of the United States of America. Accepted for publication Dec 2016, published ahead of print January 17, 2017.

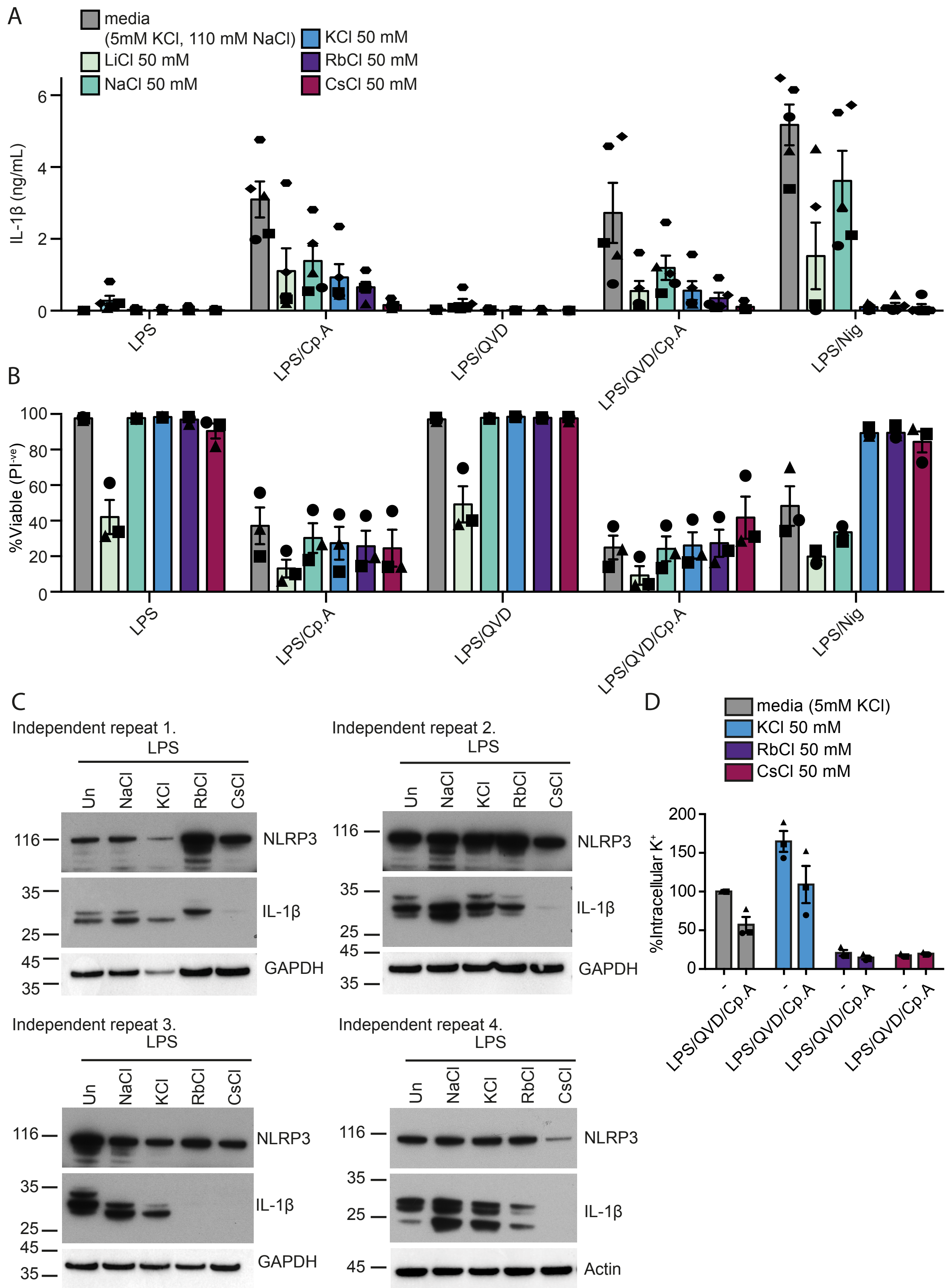


Figure S1 from Chapter 4

Figure S1C bottom right panel has been updated in a correction to the publication and is included here.

Ly5.1 WT | Ly5.2 *Mlkl*$^{-/-}$

A

LPS/QVD/Cp.A | LPS/Nigericin | LPS/QVD/Cp.A | LPS/Nigericin

Ly5.2

ASC

ASC Ly5.2 DAPI

B

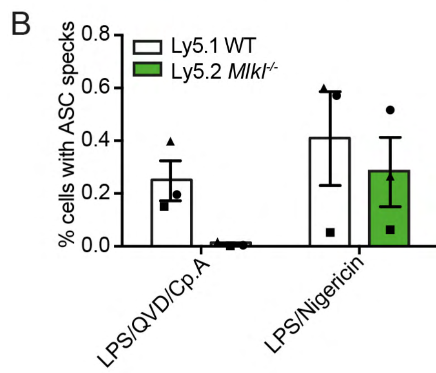
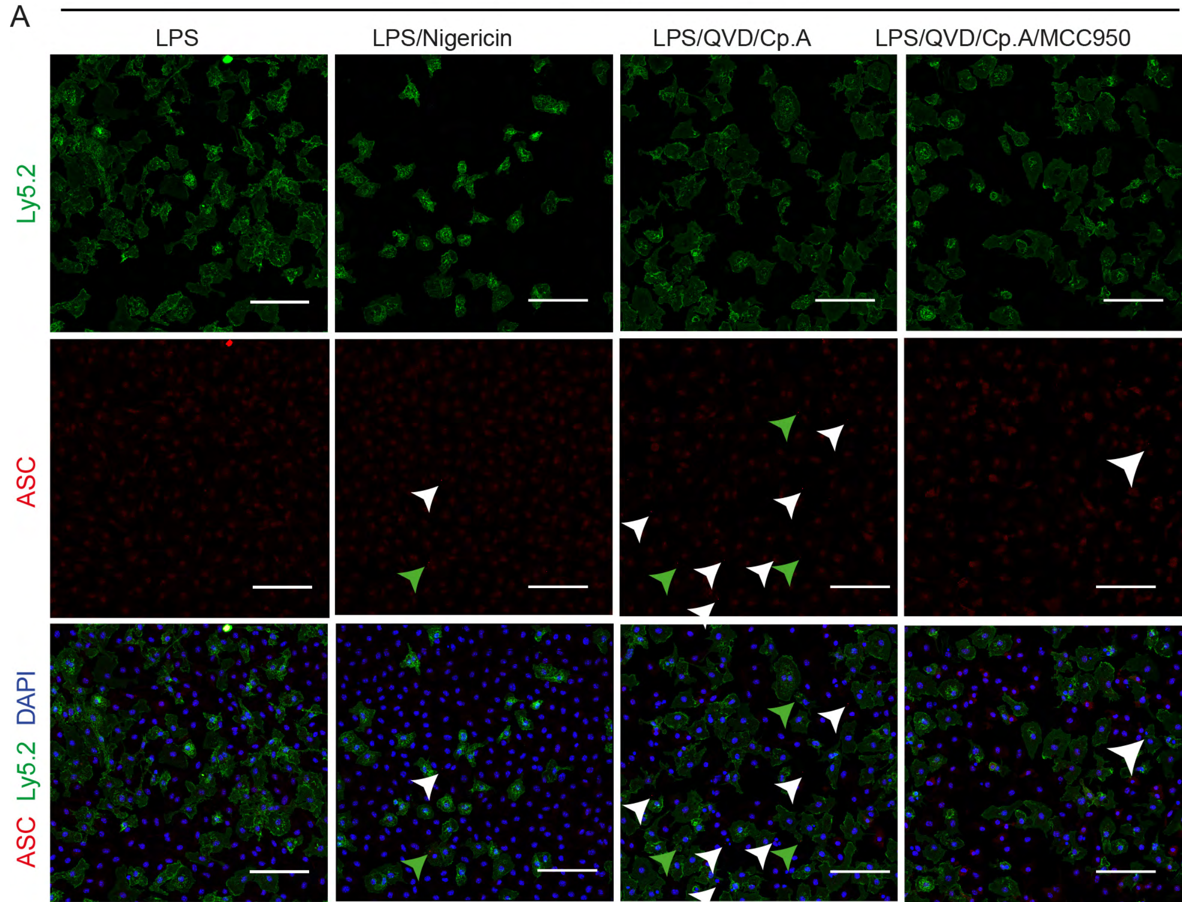


Figure S4 from Chapter 4

Necroptosis induces ASC speck formation in WT but not *Mlkl*$^{-/-}$ BMDM

Wildtype (WT) Ly5.1 BMDM and *Mlkl*$^{-/-}$ Ly5.2 BMDM were seeded in separate wells and stimulated with LPS (100 ng/mL) for 2-3 h, then treated with Q-VD-OPh (40 μM) 30 min prior to stimulation with Cp.A (1 μM) for 5 h, or Nigericin (5 μM) for 2 h as indicated. ASC speck formation (Alexa647, red), Ly5.2 expression (Alexa488, green) and nuclei (DAPI, blue) were assessed by confocal microscopy. (*A*) Representative images showing ASC speck formation, representative of 3 independent experiments. White arrows indicate ASC specks. Scale bars represent 100 μm. (*B*) Specks were quantified via an automated macro in FIJI and represented as percentage of cells with ASC specks. Data are represented as mean ± SEM from 3 independent experiments.


Mixed Ly5.1 WT and Ly5.2 Mlkl-/-
A
LPS
LPS/Nigericin
LPS/QVD/Cp.A
LPS/QVD/Cp.A/MCC950
Ly5.2
ASC
ASC Ly5.2 DAPI

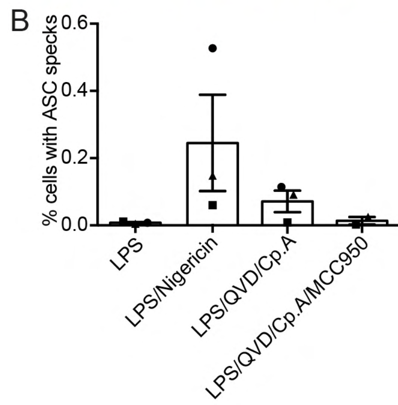

B
% cells with ASC specks
0.6
0.4
0.2
0.0
LPS
LPS/Nigericin
LPS/QVD/Cp.A
LPS/QVD/Cp.A/MCC950

Figure S5 from Chapter 4

Necroptosis activates NLRP3 to induce ASC speck formation

Wildtype (WT) Ly5.1 BMDM and *Mlkl*$^{-/-}$ Ly5.2 BMDM were seeded in mixed populations and stimulated with LPS (100 ng/mL) for 2-3 h, then treated with Q-VD-OPh (40 μM) and MCC950 (1 μM) 30 min prior to stimulation with Cp.A (1 μM) for 5 h, or Nigericin (5 μM) for 2 h as indicated in legend. ASC speck formation (Alexa647, red), Ly5.2 expression (Alexa488, green) and nuclei (DAPI, blue) were assessed by confocal microscopy. (*A*) Representative images showing ASC speck formation, representative of 3 independent experiments. White arrows indicate ASC specks within WT cells and green arrows indicate ASC specks within *Mlkl*$^{-/-}$ cells. Scale bars represent 100 μm. (*B*) Specks were quantified via an automated macro in FIJI and represented as percentage of cells with ASC specks. Data are represented as mean ± SEM from 3 independent experiments.

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