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Inflammasome function in neutrophils

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Abstract

The innate immune system protects against infection but also drives inflammatory disorders. Key molecular drivers of both processes are 'inflammasomes', multi-protein complexes that assemble in the cytosol to activate the protease, caspase-1. Active caspase-1 cleaves specific proinflammatory cytokines [e.g. interleukin (IL)-1β] into their mature, secreted forms, and initiates a form of inflammatory cell lysis called pyroptosis. Inflammasomes are assembled by select pattern recognition receptors such as NLRC4, NLRP3, AIM2, or via a non-canonical pathway involving caspase-11.

Whilst inflammasomes functions have been intensely researched, the cell types mediating inflammasome signalling in distinct *in vivo* settings were unclear. Neutrophils are one of the first cells to arrive to a site of infection or injury, and thus have the opportunity to detect inflammasome-activating molecules *in vivo*, but their ability to signal by inflammasome pathways had not been closely examined. This thesis offers a detailed investigation of NLRC4, NLRP3 and caspase-11 inflammasome signalling in neutrophils during *in vitro* or *in vivo* challenge with whole microbe, purified microbial components, or adjuvant.

This thesis demonstrates that acute *Salmonella* infection triggered NLRC4-dependent caspase-1 activation and IL-1β processing in neutrophils, and neutrophils were a major cellular compartment for IL-1β production during acute *Salmonella* challenge *in vivo*. Importantly, neutrophils did not undergo pyroptotic cell death upon NLRC4 activation, allowing these cells to sustain IL-1β production at a site of infection without compromising their crucial inflammasome-independent antimicrobial effector functions. Since IL-1β drives neutrophil recruitment and activation, the finding that neutrophils themselves produce IL-1β suggests neutrophils perform novel and important auto-regulatory functions during infection.

Neutrophil NLRP3 pathways were next examined. In macrophages, diverse molecules can trigger NLRP3 activation. Some of these agonists are insoluble molecules (particles or crystals), which activate NLRP3 through lysosomal rupture. Interestingly, the neutrophil NLRP3 inflammasome selectively responded to soluble, but not insoluble, NLRP3 agonists, and lysosomal rupture was a weak stimulus for neutrophil NLRP3 activation. This finding was replicated *in vivo*, where neutrophils did not significantly contribute to IL-1β production during alum-induced peritonitis.

Prior studies in macrophages demonstrated that NLRP3 activation triggers the oligomerisation of the inflammasome adaptor ASC, which then recruits two caspases, pro-caspase-1 and -8. Inflammasome-activated caspase-8 initiates macrophage apoptosis in parallel to caspase-1-dependent pyroptosis. This thesis shows that like macrophages, neutrophil NLRP3 activation triggered ASC oligomerisation, caspase-1 cleavage and IL-1β secretion, but unlike macrophages, caspase-1 activation did not induce cell lysis, suggesting that resistance to pyroptosis is a universal feature of neutrophil inflammasome pathways. Neutrophils also appeared unable to undergo NLRP3/ASC/caspase-8-dependent apoptosis, likely due to inefficient caspase-8 self-processing. This finding prompted examination of other caspase-8/NLRP3 pathways in neutrophils. Chemical inhibition of the inhibitor of apoptosis proteins (IAPs) in LPS-primed macrophages leads to RIPK3 activation and formation of a caspase-8 activating platform, the ripoptosome, which promotes apoptosis and can cleave pro-IL-1β into its mature form. If caspase-8 activity is suppressed, RIPK3 forms a different molecular complex, the necrosome, to induce necroptosis. In macrophages, ripoptosome and necrosome signalling induces NLRP3 inflammasome activation and downstream caspase-1 activity. Surprisingly, blockade of IAP function was a weak stimulus for cell death in LPS-stimulated neutrophils, and did not trigger caspase-8-dependent IL-1β maturation or NLRP3/caspase-1 activation, suggesting that NLRP3 regulation by RIPK3/IAPs is differentially regulated in macrophages versus neutrophils.

Caspase-11 functions are important for host defence against cytoplasmic Gram-negative bacteria, but also contribute to pathological immune responses in murine models of septic shock. Like caspase-1, caspase-11 induces pyroptosis. But caspase-11 appears unable to directly cleave cytokines, and instead triggers pro-IL-1β/18 processing through activation of the NLRP3 inflammasome. Because neutrophilia is a hallmark of sepsis, and neutrophils produce NLRP3-dependent IL-1β without concomitant cell death, the possibility that neutrophils contribute to septic shock was next investigated. Neutrophil exposure to intracellular LPS *in vitro* indeed triggered caspase-11-dependent IL-1β production but not cell death. *Nlrp3*$^{-/-}$ mice, like *Casp11*$^{-/-}$ mice, were protected from lethal endotoxin shock, suggesting that caspase-11/NLRP3-dependent cytokine production may contribute to LPS lethality. Importantly, prior neutrophil depletion protected wild type but not *Casp11*$^{-/-}$ mice from endotoxic shock. Although further investigations are required, these data suggest that

neutrophils may be a sustained cellular source of caspase-11/NLRP3-dependent, pathogenic IL-1β during murine endotoxic shock.

In summary, this thesis demonstrates that neutrophils signal via specialised inflammasome pathways to maximise host responses during pathogen challenge *in vivo.* The finding that neutrophils themselves secrete IL-1β to drive their own recruitment, activation and survival is in line with an emerging theme that neutrophils perform important immunoregulatory functions during infection. Although neutrophils are often regarded as pro-apoptotic cells, this thesis reports that, surprisingly, neutrophils resist all known forms of inflammasome-induced cell death. Presumably this allows neutrophils time to exert their important functions in host defence, but may also contribute to sustained cytokine production and thus pathology in inflammatory diseases such as endotoxic shock. The interplay between inflammation and neutrophil cell death warrants further examination to understand the underlying mechanisms of neutrophil-driven inflammatory disorders.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Original research articles

1. Sester, D.P., Thygesen, S.J., Sagulenko, V., Vajjhala, P.R., Cridland, J.A., Vitak, N., **Chen, K.W.**, Osborne, G.W., Schroder, K., and Stacey, K.J. (2015). A novel flow cytometric method to assess inflammasome formation. **Journal of immunology** *194*, 455-462.

2. **Chen, K.W.**, Gross, C.J., Sotomayor, F.V., Stacey, K.J., Tschopp, J., Sweet, M.J., and Schroder, K. (2014). The neutrophil NLRC4 inflammasome selectively promotes IL-1β maturation without pyroptosis during acute Salmonella challenge. **Cell reports** *8*, 570-582.

3. Luo, L., Wall, A.A., Yeo, J.C., Condon, N.D., Norwood, S.J., Schoenwaelder, S., **Chen, K.W.**, Jackson, S., Jenkins, B.J., Hartland, E.L.*, et al.* (2014). Rab8a interacts directly with PI3Kgamma to modulate TLR4-driven PI3K and mTOR signalling. **Nature communications** *5*, 4407.

4. Achard, M.E., **Chen, K.W.**, Sweet, M.J., Watts, R.E., Schroder, K., Schembri, M.A., and McEwan, A.G. (2013). An antioxidant role for catecholate siderophores in Salmonella. **The Biochemical journal** *454*, 543-549.

Reviews and book chapters

1. Boucher, D., **Chen, K.W.**, Schroder, K. (2015). Burn the house, save the day: pyroptosis in pathogen restriction. **Inflammasomes** *2,* 1-6.

2. **Chen, K.W.**, and Schroder, K. (2013). Antimicrobial functions of inflammasomes. **Current opinion in microbiology** *16*, 311-318.

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Conference abstracts

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2. **Chen K.W.**, Groß C.J., Sotomayor F.V., Stacey K.J., Tschopp J., Sweet M.J., Schroder K. (2014). Inflammasome-dependent antimicrobial defences *in vivo*. Lorne Infection and Immunity, Australia.

3. **Chen K.W.**, Groß C.J., Sotomayor F.V., Stacey K.J., Tschopp J., Sweet M.J., Schroder K. (2013). Inflammasome-dependent antimicrobial defences *in vivo*. Australia Society for Medical Research Conference, Brisbane.

4. **Chen K.W.**, Groß C.J., Sotomayor F.V., Stacey K.J., Tschopp J., Sweet M.J., Schroder K. (2013). Inflammasome-dependent antimicrobial defences *in vitro*. Inflammasomes in Health and Diseases Conference, Boston, USA.

5. **Chen K.W.**, Groß C.J., Sotomayor F.V., Stacey K.J., Tschopp J., Sweet M.J., Schroder K. (2013). Inflammasome-dependent antimicrobial defences *in vivo*. International Postgraduate Symposium in Biomedical Science, The University of Queensland.

6. **Chen K.W.**, Groß C.J., Sotomayor F.V., Stacey K.J., Tschopp J., Sweet M.J., Schroder K. (2012). Inflammasome activation by *Salmonella* in immune cell subsets. Brisbane Immunology Group Annual Meeting, Australia.

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Flor Sotomayor	Provided technical assistance for experiments (2%).
Katryn Stacey	Designed the study (5%). Wrote the paper (0%). Performed experiments (0%). Provided reagents (10%).
Matthew Sweet (Associate Advisor)	Designed the study (50%). Wrote the paper (0%). Performed experiments (0%). Provided reagents (10%).
Kate Schroder (Advisor)	Designed the study (50%). Wrote the paper (50%). Performed Figure 3.1A-D, S3.1A-K and S3.2 experiments (10%). Provided reagents (80%).

Contributions by others to the thesis

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Neutrophils, inflammasomes, nod-like receptors, pyroptosis, necroptosis, apoptosis, cell death, inflammation

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Abbreviations

-/-	Homozygous null genotype
APAF-1	Apoptotic protease activating factor-1
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ANOVA	Analysis of Variance
AO	Acridine orange
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BCL-2	B cell CLL/lymphoma-2
BID	BCL-2 family member BH3-interacting death domain agonist
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
BMN	Bone marrow neutrophils
BPI	Bacterial/permeability-increasing protein
BSA	Bovine serum albumin
C5a	Complement component 5a
CAPS	Cryopyrin-associated periodic fever syndromes
CARD	Caspase activation and recruitment domain
CD	Cell differentiation antigen
Cp A	Compound A
CpG	Cytosine-phosphate-guanine
CR	Complement receptor
CSF	Colony-stimulating factor
CXCL	Chemokine (C-X-C) Motif Ligand
CXCR	Chemokine (C-X-C) Motif Receptor
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DISC	Death-inducing signalling complex
DNA	Deoxyribonucleic acid
DSS	Disuccinimidyl suberate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FADD	Fas-associated death domain
FCS	Fetal calf serum
fMLP	N-Formylmethionine-leucyl-phenylalanine
G-CSF	Granulocyte-colony stimulating factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBP	Guanylate-binding protein
HEPES	N-2-Hydroxyethylpiperazine-N’2-Ethane Sulfonic Acid
HIV	Human immunodeficency virus
HMDM	Human monocyte-derived macrophages
HMGB-1	High mobility group box 1
IAP	Inhibitor of apoptosis protein
IAPP	Islet amyloid polypeptide
ICAM	Intracellular adhesion molecule

IFN	Interferon
IL	Interleukin
kDA	kilodalton
LDH	Lactate dehydrogenase
LFA-1	Leukocyte function-associated antigen 1
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
mg	Microgram
MIP-2	Macrophage inflammatory protein-2
ml	Microlitre
ml	Millilitre
MLKL	Mixed-lineage kinase domain-like
mM	Millimolar
MOI	Multiplicity of infection
MPO	Myleoperoxidase
MSU	Monosodium urate
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
MyD88	Myeloid differentiation antigen 88
NACHT	Nucleotide-binding and oligomerisation
NET	Neutrophil extracellular trap
NF-kb	Nuclear factor-kappa b
ng	nanogram
NLR	NOD-like receptor
NLRC	NOD-like receptor containing a CARD
NLRP	NOD-like receptor protein containing a PYD
PAD4	Protein arginine deiminase 4
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCV	Pathogen-containing vacuole
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-Kinase
PMN	Polymorphonuclear
poly(IC)	polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
PYD	Pyrin domain
PYHIN	Pyrin and HIN domain-containing protein
RHIM	RIP homotypic interaction motiff
RIP	Receptor interacting protein
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SCV	Salmonella-containing vacuole
SDF-1	Stromal-derived factor-1
SMAC	second mitochondria derived activator of caspase
SPI	Salmonella pathogenicity island
SPRY	splA kinase and ryanodine receptors

Stm	*Salmonella* Typhimurium
T3SS	Type 3 secretion system
T4SS	Type 4 secretion system
TNF	Tumour necrosis factor
TRADD	TNF receptor-associated death domain
TRIF	TIR domain-containing adaptor protein inducing IFN-β

1. Introduction

The innate immune system provides the first line of defence against infection and co-ordinates tissue repair during injury. Cells of the innate immune system use germ-line-encoded receptors called pattern recognition receptors (PRRs) to recognise conserved microbial structures or host-derived molecules released during cellular or tissue damage, known as pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) respectively (1) . PRRs are primarily expressed in cells at the front line of defence against infection, including macrophages, dendritic cells (DCs), neutrophils and epithelial cells. Engagement of PRRs initiates signal transduction pathways that culminate in the elimination of pathogens and/or tissue repair. A major proinflammatory pathway conferring host defence against infection is triggered by molecular complexes called 'inflammasomes' that are assembled by select members of the NOD-like receptor (NLR), pyrin and HIN domain-containing (PYHIN) protein families of 'danger' sensor proteins (2). The pyrin domain (PYD)-containing protein, PYRIN was also demonstrated to facilitate inflammasome assembly (3). Upon direct or indirect ligand sensing, inflammasome-forming NLR, PYHIN protein family members and PYRIN oligomerise and recruit partner proteins. Inflammasome complex assembly facilitates the activation of the protease, caspase-1, and downstream immune activation (2, 4).

Caspases are a family of cysteine proteases involved in cell death and inflammation. There are currently 13 caspases identified in humans and mice and their functions can be broadly divided into apoptotic or inflammatory caspases (5). The apoptotic caspases include caspase-2, -3, -6, -7, -8, -9, -10 and -14 while caspase-1, -4, -5, -11 and -12 comprise the subgroup of inflammatory caspases (5). Caspase-8, -9 and -10 are caspases that are involved in the initiation of apoptosis. These initiator caspases contain a large pro-domain that facilitate its clustering on activating complexes, driving proximity-induced auto-activation, substrate cleavage, and apoptosis. Inflammatory caspases also contain a large pro-domain for its recruitment and activation by inflammasomes (6) discussed in detail below.

1.1 Nod-like receptors and the inflammasomes

NLRs are cytosolic PRRs that detect cellular damage and cytoplasmic microbial infection. The NLR gene family is comprised of 22 human genes and 34 mouse genes, several of which assemble into inflammasome complexes (2, 6), and is defined by a common nucleotide-binding and oligomerisation (NACHT) domain. Inflammasome-forming NLRs also contain a leucine-rich repeat (LRR) domain and a caspase recruitment (CARD) or pyrin (PYD) effector domain (**Figure 1.1**) (2). The LRR domain is thought to be involved in ligand sensing, whereas the CARD or PYD domains mediate downstream signalling via homotypic interactions between protein domains.

Upon activation, NLRs oligomerise through their NACHT domain in an ATP-dependent manner (7). In the case of many inflammasome-forming NLR proteins, oligomerisation of NLRs facilitates the recruitment of apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC; **Figure 1.2**) through homotypic PYD interactions. ASC serves as an adaptor to recruit pro-caspase-1 via CARD-CARD interactions. For CARD-containing inflammasome scaffolds (i.e. NLRP1 and NLRC4), pro-caspase-1 can be directly coupled to the inflammasome complex through CARD-CARD interactions, bypassing the requirement for ASC. Clustering of pro-caspase-1 on the inflammasome complex enables its activation through homodimerisation, an essential and likely sufficient step for the acquisition of enzymatic activity that may be further influenced by self-cleavage (8-10).

Pro-IL-1β and pro-IL-18 are inactive cytokine precursors, and are key cellular substrates for active caspase-1 (2). These cytokines are critical for protective inflammatory responses during host defence; however, they can also trigger tissue pathology. IL-1β and IL-18 maturation is therefore tightly regulated, requiring at least two signals (**Figure 1.2**). Most cells do not constitutively express pro-IL-1β, and express low levels of the inflammasome sensor molecule NLRP3, thus 'Signal 1' (often referred to as a 'priming signal') induces the intracellular expression of these proteins. Priming can be achieved *in vitro* by cell stimulation with TLR agonists or proinflammatory cytokines (e.g. tumor necrosis factor, TNF) that trigger the function of the nuclear factor-κB (NF-κB) transcription factor and downstream promoter activation of the *IL1B* and *NLRP3* genes. Cytokine conversion from the inactive to the active form is regulated independently of NF-κB activity, and depends upon the inflammasome-dependent activation of caspase-1 ('Signal 2'). This second signal also regulates release of IL-1β and IL-18 via an unconventional protein secretion pathway

that is poorly understood (11). A recent study identified IL-37, a human-specific cytokine, as a substrate for caspase-1. Interestingly, cleavage of IL-37 triggers the translocation of IL-37 to the nucleus and suppresses inflammatory cytokine production (12). IL-37 also appears to possess an extracellular immunosuppressive function by engaging the IL-1 family decoy receptor IL-1R8 (13).

In addition to regulating the maturation of IL-1β and IL-18, caspase-1 activation is also associated with the initiation of pyroptosis, a form of inflammatory cell death that is distinct from apoptosis and necrosis (14). Pyroptosis occurs independently of the apoptotic caspases, and is initiated by inflammatory caspases such as caspase-1. Pyroptosis is characterised by the rapid formation of 1–2.5 nm pores, Ca^{2+} influx, DNA degradation, vacuole formation and cell swelling, leading to cell rupture and the release of cellular contents (15). This unusual form of cell death enables the release of alarmins, such as IL-1α, which is closely related to IL-1β but is not directly cleaved by caspase-1 (16).

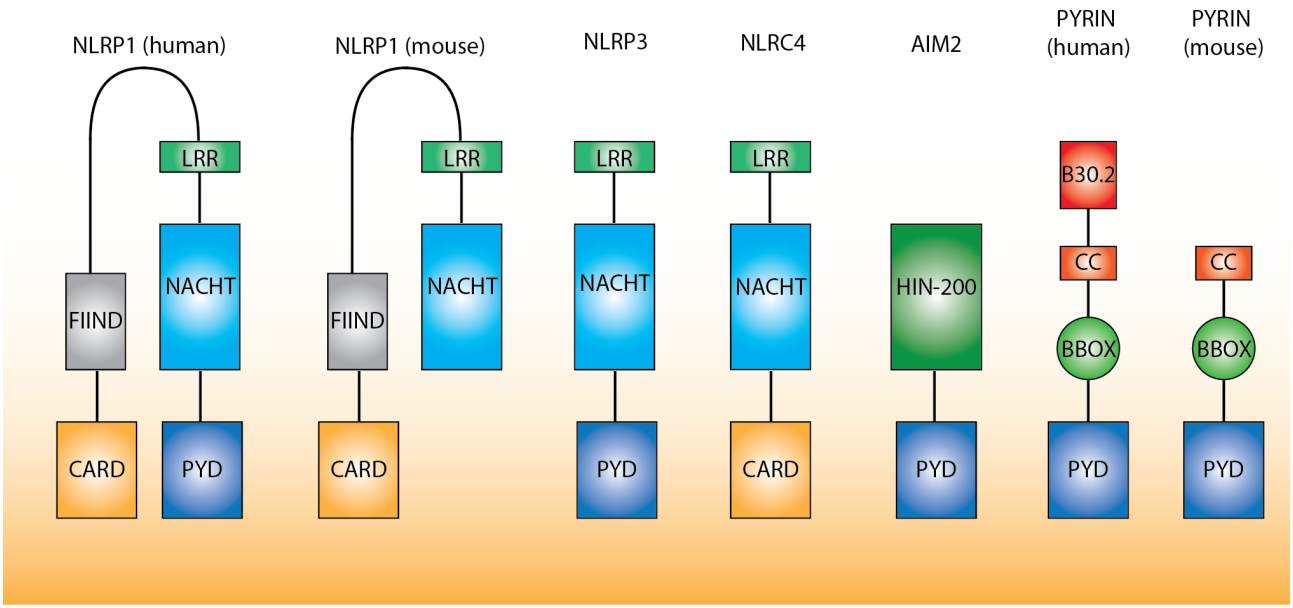


Figure 1.1 Protein domains of inflammasome scaffolds from the NLR and PYHIN families and the PYD-containing protein PYRIN.

Domains are classified according to the NCBI domain annotation tool (3). The domain structure of NLRP3, NLRC4 and AIM2 is identical between humans and mice, whereas human NLRP1, but not its murine counterparts, contains a PYD. Murine PYRIN lacks the B30.2 domain of the human PYRIN protein. Abbreviations for domain names: PYD, pyrin domain; CARD, caspase-activation and recruitment domain; NACHT, nucleotide-binding and oligomerisation domain; LRR, leucine-rich repeat; FIIND, domain with function to find; CC, coiled-coiled. Abbreviations for protein names: NLRP, Nod-like receptor protein; NLRC, NOD-like receptor containing a CARD; AIM2, absent in melanoma 2.

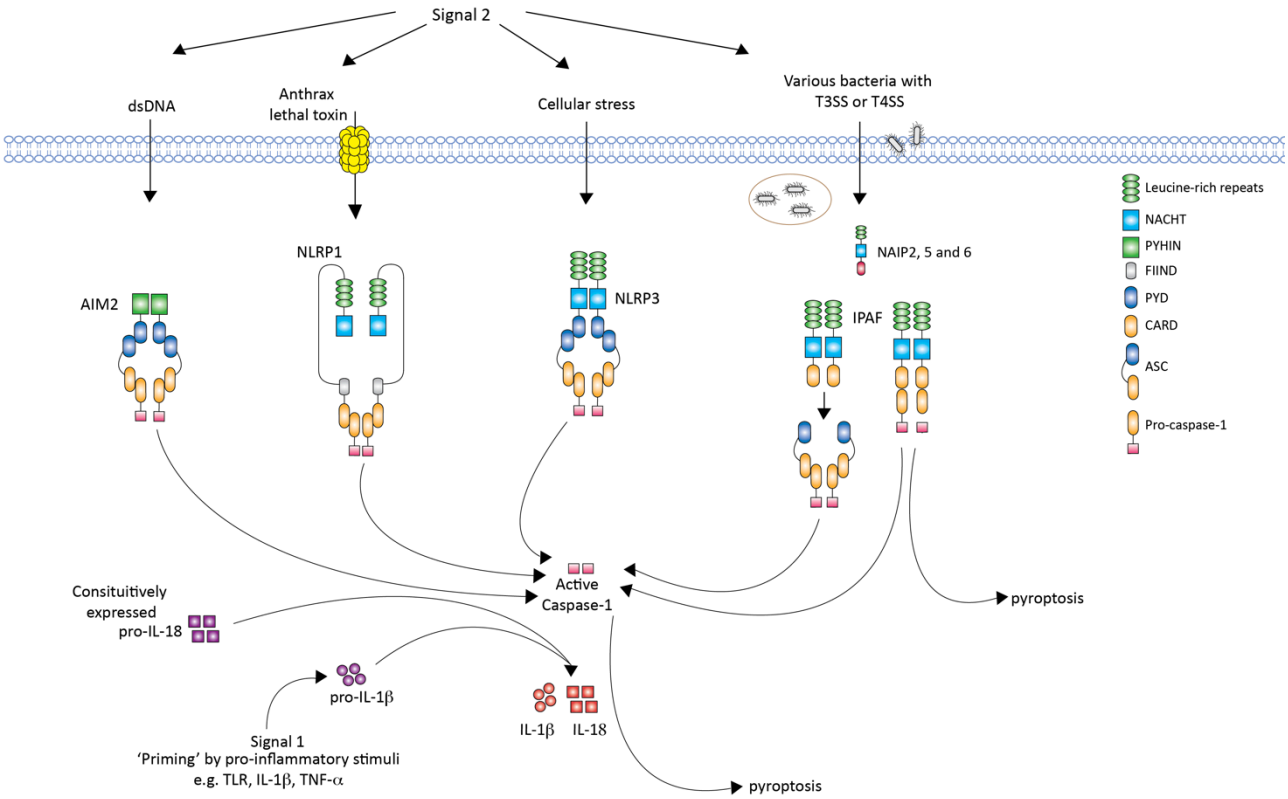


Figure 1.2 Activation pathways of AIM2, murine NLRP1, NLRP3 and NLRC4 inflammasomes.

The AIM2 inflammasome is activated by double-stranded DNA. The murine NLRP1b inflammasome is activated by anthrax lethal toxin. The NLRP3 inflammasome is triggered by various cell stress pathways triggered by infection or injury (see Table 1.1). The NLRC4 inflammasome is activated by NLRC4 interaction with other NLR family members of the neuronal apoptosis inhibitor proteins (NAIP) sub-family, upon NAIP binding to specific ligands (see Table 1.2). An ASC-containing NLRC4 inflammasome promotes cytokine processing and secretion, but ASC is dispensable for NLRC4/caspase-1-dependent cell death (pyroptosis).

1.1.1 The NLRP3 Inflammasome

The NLRP3 inflammasome is particularly interesting because it has the capacity to respond to a wide variety of unrelated activators ranging from whole pathogens, bacterial toxins, metabolic products, large insoluble particulates, environmental irritants and endogenous-derived danger signals (**Table 1.1**).

The precise molecular events governing the assembly and activation of the NLRP3 inflammasome are still unclear. The chemical diversity of NLRP3 agonists suggests these molecules elicit a common stress pathway sensed by NLRP3 that triggers NLRP3 oligomerisation and inflammasome formation. Five major models of NLRP3 inflammasome activation are proposed in the literature, and may not be mutually exclusive. The first model suggests that NLRP3 is a sensor for pathways triggering the production of reactive oxygen species (ROS) (17, 18). The second model pertains to particulate or crystalline NLRP3 agonists that are endocytosed by phagocytes and lead to lysosomal rupture. Lysosomal rupture is suggested to cause leakage of lysosomal proteases (e.g. cathepsin B) into the cytoplasm to enable NLRP3 activation by an unknown mechanism (19). The third model suggests NLRP3 is a sensor of potassium efflux, as a number of NLRP3 agonists trigger intracellular ionic flux (e.g. bacterial pore-forming toxins and extracellular ATP, which engages the P2X7 potassium efflux pump). Indeed, suppression of potassium flux (e.g. by culturing cells in high extracellular potassium) can inhibit NLRP3-dependent caspase-1 activation (20). The fourth model proposes that NLRP3 is a sensor of mitochondria perturbation (21-24). Finally, calcium mobilisation has also been implicated in triggering NLPR3 activation (21, 25). All of these models are highly controversial, and the field is yet to reach a consensus opinion.

Table 1.1 Stimuli known to activate the NLRP3 inflammasome.

Pathogens/pathogen products	References
Candida albicans	(26)
Saccharomyces cerevisiae	(26)
Staphylococcus aureus	(27)
Listeria monocytogenes	(27)
Neisseria gonorrhoeae	(28)
Salmonella Typhimurium	(29)
Group B *Streptococcus*	(30)
Burkholderia cenocepacia	(31)
Orientia tsutsugamushi	(32)
Citrobacter rodentium	(33)
Sendai virus	(34)
Influenza virus	(34)
Encephalomyocarditis virus	(35)
Measles virus	(36)
Hepatitis C virus	(37)
Pore forming toxins	(27)
Host derived molecules	
ATP	(27)
Glucose	(38)
β-amyloid	(39)
Monosodium urate	(40)
Oxidised low-density lipoprotein	(41)
Hydroxyapatite (HA) crystals	(42)
Environmental irritants	
Alum	(19)
Abestos	(17)
Silica	(19)
Others	
R837	(43)

1.1.2 The NLRC4 Inflammasome

The NLRC4 inflammasome appears to respond to a narrower spectrum of agonists compared to NLRP3. NLRC4 is primarily activated by Gram-negative bacteria such as *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium*)*, *Legionella pnuemophila* (*L. pneumophila*), *Shigella flexneri* (*S. flexneri*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) (**Table 1.2**). NLRC4 does not directly interact with its microbial agonists, instead the ligand is sensed by an NLR co-receptor called neuronal apoptosis inhibitor protein (NAIP in humans, of which there are multiple orthologs in mice) (44-49) (2). Pathogenic bacteria use a sophisticated injection apparatus, the Type 3 or 4 secretion system (T3SS or T4SS), that injects effector proteins into the host cytoplasm to mediate survival and virulence (50). Due to evolutionary conservation between the bacterial secretion system and the flagellar machinery, flagellin and components of the secretory apparatus (PrgJ and PrgI-like proteins) are 'unintentionally' translocated into the host cell cytoplasm and are detected by murine NAIP1, 5 and 6 or human NAIP (Table 2). Surprisingly, the LRR domain of NAIP is dispensable for ligand recognition. Instead, ligand specificity is mediated by the nucleotide binding domain of NAIP proteins (46).

NLRC4 contains a CARD domain and so can directly interact with pro-caspase-1 without an absolute requirement for the adapter ASC (**Figure 1.2**). Indeed, ASC-deficient macrophages are able to trigger IL-1β maturation and pyroptosis following NLRC4 activation (8, 29). However, ASC-replete macrophages show increased secretion of mature IL-1β and faster kinetics pyroptotic cell death compared to ASC-deficient macrophages following NLRC4 activation (8, 29).

Table 1.2 NLRC4 agonists.

Pathogen	Bacterial ligands	Recognition by NAIP proteins	References
Salmonella Typhimurium	Flagellin	NAIP, NAIP5 and 6	(44, 48, 49)
	PrgJ	NAIP2	(44, 48)
	PrgI	NAIP, NAIP1	(45, 47)
Pseudomonas aeruginosa	Flagellin	NAIP5	(48)
	Pscl	unknown	(51)
Legionella pnuemophila	Flagellin	NAIP5	(48)
Shigella flexneri	Mxil	unknown	(52)
	MxiH	NAIP, NAIP1	(47)
Burkholderia pseudomallei	Flagellin	unknonwn	(53)
	BsaK	NAIP2	(48)
	BsaL	NAIP, NAIP1	(47)
Chromobacterium violaceum	Cprl	NAIP, NAIP1	(47, 48)
Enteropathogenic Escherichia coli (EPEC)	Flagellin	unknown	(48, 54)
	EscL	unknown	
Enterohemorrhagic Escherichia coli (EHEC)	Eprl	NAIP, NAIP1	(47)
Listeria monocytogenes	Unknown	Unknown	(55)
Candida albicans	Unknown	Unknown	(56)

1.1.3 The NLRP1 inflammasome

The NLRP1 inflammasome was the first to be identified (57) and is activated by NLRP1 N-terminal cleavage by anthrax lethal toxin (**Figure 1.2**) (58, 59). Humans encode a single NLRP1 gene, whereas 3 orthologous genes are present in mice (*Nlrp1a-c)* (60). The *Nlrp1b* gene is highly polymorphic between inbred mice and appears to confer susceptibility to anthrax lethal toxin (60). Similar to NLRC4, human NLRP1 contains a CARD domain that can directly interact with pro-caspase-1, bypassing an absolute requirement for ASC. However, ASC can still bind to the N-terminal PYD domain of human NLRP1 (**Figure 1.2**). Unlike human NLRP1, murine NLRP1 proteins lack functional PYD domains, and so were initially believed not to interact with ASC. Surprisingly, NLRP1b activation triggered ASC polymerisation in murine macrophages, suggestive of NLRP1b-ASC CARD-CARD interactions (61). However, ASC was not required for NLRP1-dependent IL-1β maturation and pyroptosis in mouse macrophages (61, 62).

1.1.4 The AIM2 inflammasome

The cytosolic receptor AIM2 was the first non-NLR family member identified to form an inflammasome (63-66). The AIM2 inflammasome is activated by cytosolic double-stranded DNA (dsDNA), which indicates the presence of intracellular pathogens such as *Listeria monocytogenes*, *Francisella tularensis (F. tularensis)*, cytomegalovirus and vaccinia virus (67). The AIM2 inflammasome is comprised of AIM2, ASC and caspase-1 (**Figure 1.2**). Instead of clustering via an oligomerisation domain, the oligomerisation of AIM2 is likely achieved by aggregation upon the dsDNA ligand, which contains multiple AIM2 binding sites (68). AIM2 activation triggers the recruitment of ASC and pro-caspase-1 through PYD-PYD (AIM2-ASC) and CARD-CARD (ASC-procaspase-1) interactions, resulting in cytokine maturation and secretion, and lytic cell death in macrophages.

1.1.5 Non-canonical caspase-8 inflammasome

In addition to pro-caspase-1, inflammasome assembly also recruits and activates pro-caspase-8 through ASC (69-71). Caspase-8 activation downstream of AIM2, NLRP3, and NLRC4 triggers apoptosis and serves as a back up mechanism when cells fail to pyroptose or when caspase-1 expression or activity is inhibited (69, 70, 72). However, the finding that caspase-8 activation triggered apoptosis downstream of NLRC4 and ASC was recently contested by a single study. The authors of this study proposed that caspase-8 processing on the NLRC4 inflammasome did not regulate cellular viability, but the non-enzymatic function of caspase-8 was required for pro-IL-1β synthesis (71). Interestingly, a

recent study reported that *Aspergillus fumigatus* infection in mouse DCs triggers the formation of a single NLRP3-AIM2 inflammasome complex, and that caspase-8 recruitment to this complex is required for optimal caspase-1 activation (73).

Intriguingly, caspase-8 that is activated on a different intracellular platform such as the ripoptosome can process IL-1β, similar to inflammasome-activated caspase-1 (74). However, inflammasome-activated caspase-8 only triggers a small amount of IL-1β cleavage in the cell extracts and supernatant of caspase-1-deficient macrophages (69).

1.1.6 Non-canonical caspase-11 inflammasome

Caspase-11 is a murine inflammatory caspase that is required for host protection against Gram-negative cytoplasmic bacteria (75, 76) but also drives pathological inflammation in endotoxic shock (77, 78). Caspase-11 is not constitutively expressed by macrophages, but is upregulated by type I interferon signalling (79). Activation of caspase-11 during bacterial infection also requires interferon signalling, as this triggers the expression of guanylate-binding proteins (GBPs) that mediate lysis of pathogen-containing vacuoles, and ensuing release of bacteria into the cytosol (80). Cytosolic LPS is then directly sensed through the CARD domain of pro-caspase-11, triggering caspase-11 dimerisation and activation within a so-called 'non-canonical' inflammasome complex (81). Caspase-11 activity triggers the maturation of IL-1β and IL-18, but caspase-11 does not directly cleave these cytokines, rather, it triggers the assembly of the NLRP3 inflammasome, and downstream caspase-1-dependent cytokine processing (76). The mechanisms underlying caspase-11-dependent NLRP3 activation are not fully resolved, but occur via a cell-autonomous mechanism involving caspase-11-dependent K^+ efflux (82). In contrast, caspase-11 initiates pyroptosis independently of NLRP3 and caspase-1. Caspase-11 thus performs dual functions as both a ligand sensor and effector within the non-canonical inflammasome complex.

Murine caspase-11 has two human orthologs, caspase-4 and caspase-5 (83). Similar to caspase-11, caspase-4 and caspase-5 also sense LPS through their CARD domains and trigger caspase dimerisation and activation (81). Interestingly, transgenic mice that express human caspase-4 are hypersensitive to LPS challenge, suggesting that caspase-4 performs a similar biological function to caspase-11 (84). Consistent with this observation, two recent studies demonstrated that caspase-4 activation in human myeloid cells drives caspase-1-independent pyroptosis but triggers cytokine production through NLRP3/caspase-1, akin to the murine caspase-11 pathway paradigm (85, 86).

Interestingly, caspase-5 was dispensable for cell death and cytokine processing upon LPS transfection but was required during live *Salmonella* infection, suggesting that caspase-5 activation may require other bacterial products (85).

1.1.7 Cross-talk between inflammasomes and other cell death pathways

Recent studies revealed an unexpected role for inhibitor of apoptosis proteins (IAPs) as central modulators of both death receptor signalling and inflammasome activation in macrophages and DCs. IAPs were first described as negative regulators of apoptosis (87) and contain up to three copies of the baculovirus inhibitor of apoptosis protein repeat (BIR) domain. The most well studied IAPs are cellular IAP (cIAP)-1, cIAP-2 and X-linked IAP (XIAP). IAPs inhibit apoptosis by interacting with pro-apoptotic proteins, or by enhancing their degradation through the ubiquitin-proteasome pathway. IAPs also block apoptosis by inducing the transcription of pro-survival genes through the canonical and non-canonical NF-κB pathway (87). Blocking IAP function thus substantially increases the propensity of a cell to undergo apoptosis. Two recent reports revealed that genetic IAP deficiency or chemical inhibition of IAPs using second mitochondria derived activator of caspase (SMAC)-mimetic compounds drives NLRP3/caspase-1 activation in LPS-primed macrophages (74, 88). This particular NLRP3/caspase-1 activation mechanism requires signalling by the TLR3/4 adaptor, TIR domain-containing adaptor protein inducing IFN-β (TRIF), and the assembly of an intracellular multi-protein complex containing RIPK1, FADD and casapase-8 termed the 'ripoptosome'. TRIF contains a receptor-interacting protein (RIP) homotypic interactive motif (RHIM) domain and is able to recruit receptor-interacting serine-threonine kinase 3 (RIPK3) through homotypic RHIM-RHIM interactions. In the absence of IAPs, RIPK3 is not ubiquitinated and fails to deliver a pro-survival signal following LPS ligation (**Figure 1.3A**). Instead, RIPK3 promotes caspase-8 activity on the ripoptosome and triggers apoptosis and NLRP3/caspase-1 activation (88). Interestingly, caspase-8 activation by the ripoptosome also promotes IL-1β processing independently of NLRP3/caspase-1 (74, 88) (**Figure 1.3B**). When both IAPs and caspase-8 are inhibited RIPK3 phosphorylates mixed-lineage kinase domain-like (MLKL) and triggers a form of programmed cell death called 'necroptosis'; interestingly, this process also drives NLRP3 activation (**Figure 1.3B**) (88). These observations highlight the complex system of cross talk between inflammasome signalling and programmed cell death pathways (89).

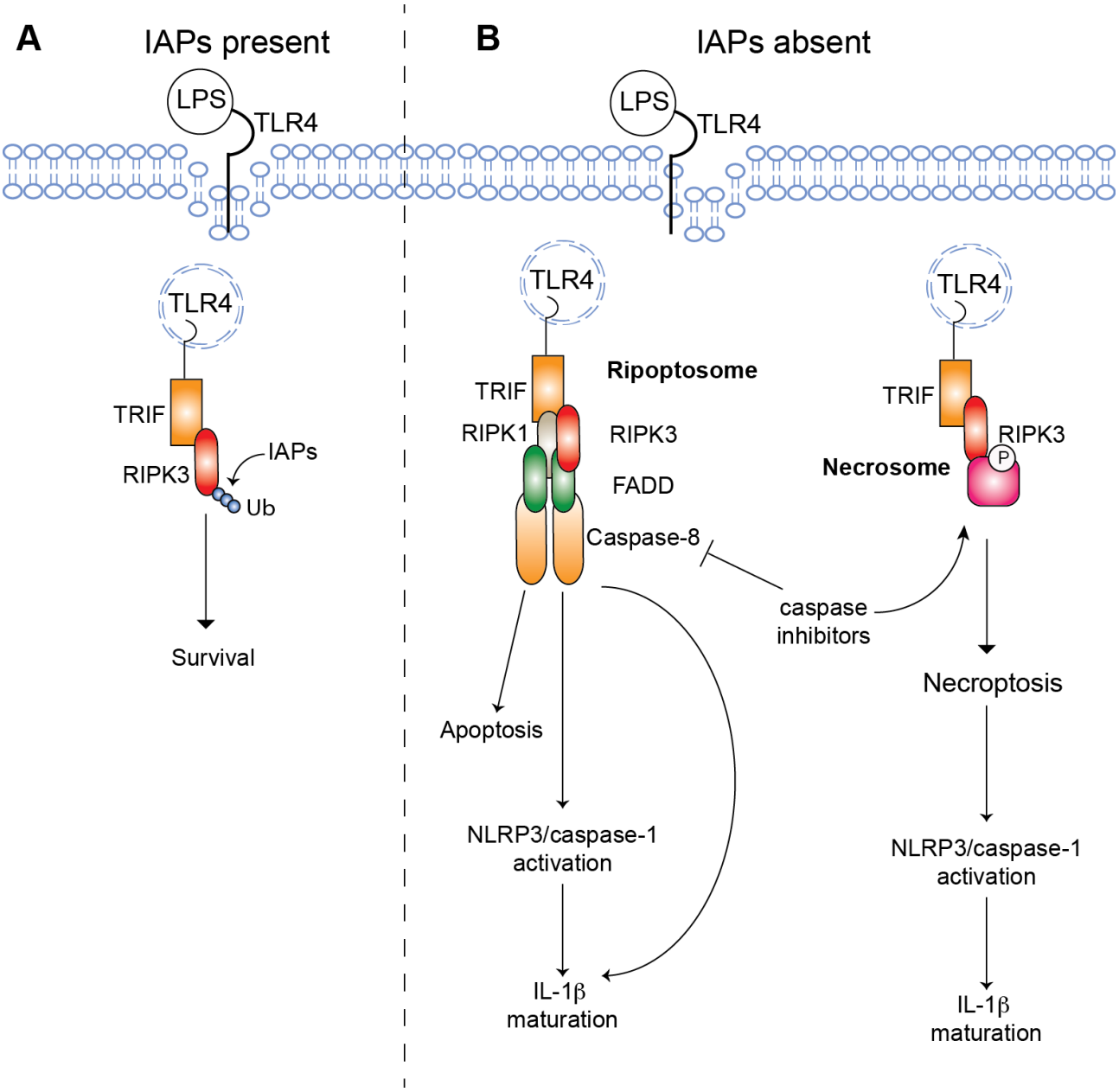


Figure 1.3 IAPs suppress apoptosis and necroptosis during TLR-TRIF-RIPK3 activation.

(A) When IAPs are present, TLR4 signalling results in IAP-mediated ubiquitination of RIPK3 to propagate pro-survival signals. (B) When IAPs are absent, LPS induces RIPK3-dependent caspase-8 activation through the ripoptosome complex and triggers apoptosis and NLRP3/caspase-1 activation. Caspase-8 activation by the ripoptosome can also directly process IL-1β. When IAPs and caspase-8 is absent, LPS induces the formation of the necrosome, which triggers necroptosis and NLRP3/caspase-1 activation.

1.2 Antimicrobial functions of the inflammasomes

Inflammasomes are critical components of the immune system, and mice that are deficient in inflammasome components are extremely susceptible to infection (**Table 1.3**). Inflammasomes critically mediate antimicrobial functions via a suite of cytokine-dependent and -independent mechanisms and will be discussed in detail below.

1.2.1 Interleukin-1β

IL-1β is one of the major cytokines secreted following inflammasome activation. IL-1β signals through the IL-1 receptor-1 (IL-1R1), which is expressed ubiquitously and therefore elicits both local and systemic responses in the host. IL-1β targets leukocytes, epithelial cells, endothelial cells, fibroblasts, as well as cells of the spleen, liver and brain. IL-1β signalling potently accentuates the antimicrobial functions of leukocytes by enhancing their phagocytic capability (90), by priming the oxidative burst (91), by regulating nutritional starvation (92) and by triggering degranulation to release antimicrobial proteins, and by forming neutrophil extracellular traps (92, 93). IL-1β also contributes to neutrophilia by enhancing neutrophil bone marrow export and granulopoiesis, by inducing the accumulation of neutrophils at inflammatory sites (94, 95) and through prolonging neutrophil lifespan (96).

In the liver IL-1β induces acute-phase responses, including hepatocyte production of complement proteins, C-reactive protein and serum amyloid A, and the co-ordinate redistribution of circulating metal ions (97). IL-1β also enhances hepatic production of C3 and Factor B, major players in the anti-microbial complement cascade. C-reactive protein recognises microbial polysaccharides and activates the complement system, promoting phagocyte opsonophagocytosis and ROS production (98). Serum amyloid A exerts various functions, including the regulation of immune cell recruitment. Iron deprivation is an established antimicrobial strategy (99) and IL-1β also triggers a reduction in the availability of free iron in the serum by enhancing hepatocyte production of the iron-sequestration protein ferritin (100, 101) and the hormone hepcidin (101), which negatively regulates iron absorption in the small intestine and iron efflux from macrophages. IL-1β also promotes lymphocyte proliferation and activation in the spleen (97). In concert with the liver-mediated acute phase response, IL-1β in the brain triggers hypothalamic prostaglandin E2 production, which raises the hypothalamic set point to induce fever (97). Although fever causes discomfort to the host, elevated body temperature augments several protective

host immune responses such as granulopoiesis, phagocytosis, T cell proliferation, antibody production and the activity of IFNs, and suppresses microbial growth (102, 103).

1.2.2 Interleukin-18

IL-18 is best known for its ability to induce the production of IFN-γ by T cells and NK cells in collaboration with IL-12 (104). IFN-γ potently affects the function of macrophages through enhancing antimicrobial pathways such as the oxidative burst, increasing the quantity and diversity of antigen presentation and by boosting cellular sensitivity to pathogen recognition receptors such as TLRs (104, 105). In addition, IFN-γ potentiates leukocyte migration and instructs the adaptive immune response. IL-18 also augments the antimicrobial functions of leukocytes more directly. For example, IL-18 signalling in neutrophils promotes the expression of formyl peptide receptors and the complement C3b receptor, allowing robust sensing and phagocytosis of pathogens (106). IL-18 also enhances microbial killing by augmenting ROS production (106) and amplifies inflammatory responses by promoting the release of proinflammatory mediators such as IL-6 (106).

Table 1.3 Examples of inflammasomes critical for *in vivo* host defence, and their microbial triggers.

Pathogen	Inflammasome	References
Aspergillus fumigatus	NLRP3, AIM2	(73)
Burkholderia pseudomallei	NLRP3, NLRC4	(53, 75)
Burkholderia thailandensis	Caspase-11	(75)
Candida albicans	NLRP3, NLRC4	(56)
Citrobacter rodentium	NLRP3, NLRC4	(33)
Francisella tularensis	AIM2	(107)
Klebsiella pneumoniae	NLRC4	(108-110)
Salmonella Typhimumrium	NLRC4, NLRP3, Caspase-11	(75, 80, 109, 111)
Pseudomonas aeruginosa	NLRC4	(110)
Staphylococcus aureus	NLRP3	(112)
Streptococcus pneumoniae	NLRP3, AIM2	(113)
Yersinia pestis	NLRP12, NLRP3	(114)

1.2.3 Pyroptotic cell death

In addition to its ability to process cytokine pro-forms as discussed above, inflammasome-activated caspase-1 also initiates a novel form of inflammatory cell death called pyroptosis. While the induction of caspase-1-dependent pyroptosis by inflammasomes has been extensively observed using *in vitro* infection models, the mechanics of caspase-1-dependent cell death remain poorly characterised. Surprisingly, pro-caspase-1 autoproteolysis appears to be dispensable for NLRP1 and NLRC4-triggered pyroptosis, while protease clustering and active site residues are required (8, 61, 62). This suggests that uncleaved, clustered pro-caspase-1 may represent an alternative activation state of the protease that elicits the pyroptotic program (8, 61, 62). Other inflammatory caspases such as caspase-4, -5 and -11 also trigger pyroptosis, however, this occurs in a caspase-1-independent manner, and the molecular mechanisms are poorly understood (76, 85, 86). The role of pyroptosis *in vivo* was only confirmed recently. Miao and co-workers elegantly demonstrated a physiological role for pyroptosis using a *Salmonella* mutant that constitutively activates the NLRC4 inflammasome (115). The authors showed that pyroptosis protects the host by releasing intracellular bacteria from their replicative niche, rendering bacteria more susceptible to destruction by neutrophils (115) and presumably other extracellular defence mechanisms which are concurrently upregulated by IL-1β and IL-18 signalling. As some pathogens exploit myeloid cells as a niche to establish systemic infection, pyroptotic cell death allows pathogen restriction to the site of infection and prevents dissemination. Various cytokines and host-derived alarmins such as IL-1α, HMGB-1 and ATP, are also released during pyroptotic cell death (76) and are likely to signal to neighbouring cells to promote their immune surveillance and antimicrobial pathways.

1.3 Pathogenic outcomes of inflammasome activation

Inflammasomes are critical for host defence, but uncontrolled or inappropriate inflammasome signalling causes significant collateral damage and can initiate or exacerbate disease. NLRP3 has been identified as a key driver of metabolic syndrome (40, 116, 117) and neurodegenerative diseases (39, 118-120). Mechanistically, aberrant production of peptides and/or metabolic by-products during these diseases is sensed by NLRP3, culminating in excessive inflammation in the affected tissue. Examples of aberrant peptides and metabolic by-products that trigger NLRP3 activation include islet amyloid polypeptide (IAPP) during T2D (116), monosodium urate (MSU) in gout (40), and β-amyloid fibrils in Alzheimer's disease (39). Genetic deficiency or pharmacological inhibition

of NLRP3, or the usage of the recombinant IL-1R antagonist, Anakinra, ameliorates several of these pathologies in humans and animal models, indicating a key role for NLRP3 in mediating these pathologies (28, 40, 119, 121-123).

Excessive inflammasome signalling during infection also mediates the inflammatory disease, endotoxic shock in murine models. While caspase-11 is important for protection against cytosolic Gram-negative bacteria, excessive caspase-11 activation triggers clinical signs of sepsis such as hypothermia, weight loss, seizure and death (77, 78).

In human immunodeficiency virus (HIV) infection, depletion of CD4 T cells is a hallmark of HIV pathophysiology and is a primary driver of acquired immunodeficiency syndrome (AIDS) (124). Two recent studies unravelled mechanisms of CD4 T cell depletion during HIV infection (125, 126). The authors demonstrated that abortive HIV infection in quiescent CD4 T cells triggered caspase-1-mediated pyroptosis. In this scenario, pyroptosis of quiescent CD4 T cells does not necessarily deprive HIV of its replicative niche, as this cell population is not permissive for viral replication. HIV instead replicates in activated CD4 T cells, which represent only a minority of the CD4 T cell population, and die by apoptosis. Pyroptosis in quiescent CD4 T cells promoted chronic CD4 T cell depletion, driving the progression of AIDS (124-126).

Another example in which caspase-1-dependent pyroptosis may be detrimental is that of NLRP1a-mediated pyroptosis in haematopoietic progenitor cells, which occurs during haematopoietic stress induced by chemotherapy or bone marrow infection (127). Although seemingly intended as a mechanism to prevent infection of daughter cells from compromised haematopoietic progenitor cells, chronic pyroptosis resulted in cytopenia and immunosuppression.

A number of mutations leading to excessive inflammasome activation have been mapped over the past decade. The most studied examples are the cryopyrin-associated periodic fever syndromes (CAPS) caused by gain-of-function mutations in *Nlrp3* (128). CAPS patients typically present with recurring episodes of fever and multiple skin rashes. Patients with mutations in NLRP12, which is the closest phylogenetic relative to NLRP3 (2), have a similar phenotypic presentation to CAPS patients (129). Whether NLRP12 forms an inflammasome is however still a subject of debate. Some studies suggested a direct role for NLRP12 in mediating IL-18 production following *Yersinia* infection (114),

while other studies claimed that NLRP12 regulates inflammasome-independent activities such as cell migration and transcription factor activity (130-133). Three independent studies recently identified single point mutations in the nucleotide-binding domain of NLRC4 in humans (134-136). These mutations drove spontaneous NLRC4 activation in the absence of a microbial trigger; resulting in excessive inflammation, cell death and neutrophil accumulation. As a result, these patients suffer from varying symptoms ranging from rashes, recurrent fever, pancytopenia, enterocolitis and intestinal tissue damage and these can lead to death.

1.4 Salmonella pathogenesis and host defence

Salmonella enterica species are Gram-negative bacteria that can infect a wide range of hosts including humans. Infection usually occurs after the ingestion of contaminated food or water and can lead to various disease manifestations. *Salmonella enterica* serovar Typhi (*S.* Typhi) is the causative agent of typhoid fever in humans. *S.* Typhimurium is a major cause of self-limiting human gastroenteritis, but can also lead to systemic disease and mortality in immunocompromised and elderly individuals. In mice, intravascular or intraperitoneal infection with *S.* Typhimurium is often used to model the pathogenesis of human systemic salmonellosis.

When ingested, *S.* Typhimurium is able to withstand the hostile environment of the gastrointestinal tract, evading digestive enzymes, acidic pH and secretory IgA to colonise the small intestine (137). A specialised secretion system termed the T3SS is essential for *S.* Typhimurium to inject virulence proteins into the host cytoplasm to successfully establish an infection. The T3SS is evolutionarily related to the flagellar export system and is present in multiple Gram-negative bacteria. At least 20 different proteins are required to assemble the secretion apparatus, which consists of a multi-ring base that spans the inner and outer membranes of the bacterial envelope, and an inner rod that joins the rings. Together, these form a supramolecular structure that is known as the needle complex (50).

S. Typhimurium expresses two virulence-associated T3SS encoded on two distinct *Salmonella* pathogenicity islands (SPI1 and SPI2). The SPI1-T3SS and SPI2-T3SS are preferentially expressed during different stages of infection (138). The SPI1-T3SS is expressed upon contact with epithelial cells and translocates effectors into the host cytoplasm and ensures the efficient uptake of the bacterium and its transcytosis to the basolateral surface of the intestinal epithelia. Once the epithelial layer is breached, *S.*

Typhimurium infects the underlying macrophages, which transport the bacteria through the reticuloendothelial system (137). Upon internalisation by phagocytes such as macrophages, *S.* Typhimurium senses the change in microenvironment and upregulates expression of the SPI2-T3SS. The SPI2-T3SS modulates cellular anti-microbial trafficking pathways including those that control the formation of an active NADPH oxidase complex (respiratory burst) and the maturation of the phagolysosome. *S.* Typhimurium subverts these trafficking pathways to form a replicative niche within a membrane-bound structure known as the *Salmonella* containing vacuole (SCV) (139, 140). The *Salmonella* SPI1-T3SS appears also to be expressed in bacteria residing in phagocytes, and may cooperate with the SPI2-T3SS to inhibit phagosome maturation and enhance intracellular replication (139, 141). This suggests that the two SPIs likely cooperate to facilitate the intracellular lifestyle of the bacterium.

1.4.1 Innate immune defence against S. Typhimurium

The successful clearance of *Salmonella* depends upon the production of cytokines and soluble factors at the early stages of infection, as well as the rapid recruitment of neutrophils and macrophages. Soluble factors such as complement bind to the bacterial membrane, and for example form a membrane attack complex that results in cell rupture. *Salmonella*-derived components such as LPS, flagellin and unmethylated CG dinucleotides (CpG DNA) can also activate Toll-like receptors (TLRs), which in turn induce potent inflammatory responses and communication between host immune cells. TLR activation results in the production of proinflammatory cytokines such as TNF, IL-6 and IL-12, which trigger the production of chemokine such KC/MIP-2 from the activated endothelium and elicit of Th1 cytokines such as IFN-γ from NK/T cells (142). These mediators in turn recruit phagocytes and induce the upregulation of multiple antimicrobial mechanisms.

1.4.2 Inflammasome-dependent defence against *S.* Typhimurium

In addition to TLRs, inflammasomes are important mediators of host defence host defence against *S.* Typhimurium. In particular, the NLRC4 and NLRP3 inflammasomes, and the non-canonical caspase-11 inflammasome, exhibit important functions (**Figure 1.4**). NLRC4-mediated cytokine secretion by murine macrophages and DCs occurs as early as 1 h after infection with *S.* Typhimurium (29, 143). Early detection of *Salmonella* by NLRC4 requires a functional SPI1-T3SS in mouse cells (29, 143, 144). The only exception to this requirement is *Salmonella* sensing by CD8+ DCs; these cells can recognise *Salmonella*-derived molecules in a SPI1-independent manner (for example, in recognising heat-killed

bacteria) by employing antigen cross-presentation pathways to transfer *Salmonella* proteins into the host cell cytoplasm for recognition by the NLRC4/NAIP system (145). At later time points, *Salmonella* switches on the SPI2-T3SS in order to stabilise its replicative niche, the SCV. Using the PhoP-PhoQ two-component system, *S.* Typhimurium downregulates flagellin expression within the SCV to evade detection by NLRC4 (143). However, low levels of bacterial flagellin are yet transferred into the cytoplasm via the SPI2-T3SS, and this can be detected by NLRC4. Interestingly, Ssal, the homolog of PrgJ encoded on the SPI2-T3SS, is not detected by NLRC4 (54). Both the NLRP3 and caspase-11 inflammasomes exert important functions in anti-*Salmonella* defence at later stages of infection (e.g. 17h). Early reports indicated a role for NLRP3 in sensing *Salmonella* infection in a SPI1/2-T3SS-independent manner, however the pathogen determinant(s), responsible for NLRP3 activation could not be identified (111). Recently, the non-canonical caspase-11 inflammasome was demonstrated to mediate antimicrobial responses to defend against *Salmonella* (75, 80). In light of this, it is possible that NLRP3 activation by *Salmonella* occurs as an indirect consequence of caspase-11 activation, rather than the previously suggested model, whereby pathogen determinant(s) from *Salmonella* are directly sensed by NLRP3. Interestingly, NLRC4 and NLRP3 can be recruited to the same molecular complex upon *S.* Typhimurium infection, which nucleated ASC into a large focus for caspase-1 processing and IL-1β/18 maturation (146).

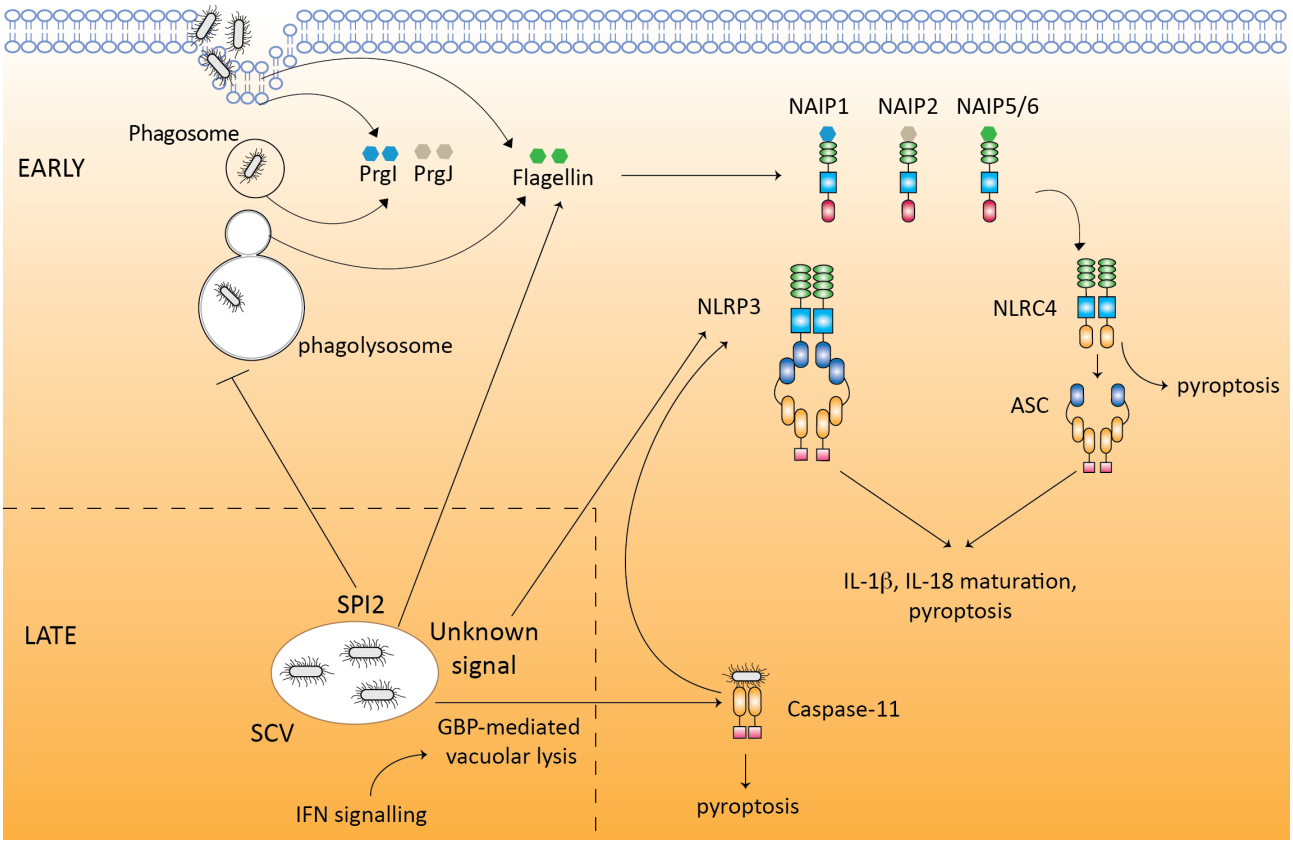


Figure 1.4 Activation of the NLRC4, NLRP3 and non-canonical caspase-11 inflammasome by *S.* Typhimurium.

Bacterial flagellin is translocated into the host cell cytosol by the SPI1-T3SS and SPI2-T3SS; PrgI/J is translocated by SPI1-T3SS only. These ligands are sensed by NAIP5/6 (flagellin), NAIP1 (PrgI) and NAIP2 (PrgJ), and NAIP proteins act as co-receptors to activate the NLRC4 inflammasome. Clustering of (uncleaved) pro-caspase-1 is sufficient to induce pyroptosis via unknown mechanisms, whereas cleaved caspase-1 promotes cytokine maturation and secretion. At later time points of infection, *S.* Typhimurium is sensed by NLRP3 through an undefined pathway, and also activates non-canonical caspase-11 inflammasome, triggering parallel caspase-11-dependent pyroptosis and NLRP3-caspase-1 activation.

1.5 Neutrophil biology

Neutrophils are the most abundant leukocytes in human blood. On average 1-2 x 10^{11} new neutrophils are formed in the bone marrow per day (147, 148). In the circulation, neutrophils constitute around 70% of the total leukocyte population (149). However, in mice, the standard model organism for *in vivo* infection studies, circulating neutrophils are significantly lower, only accounting for around 20% of blood leukocytes (150). This suggests that studies relying on mouse as a model for human immunity may underestimate the contribution of neutrophils to host defence in humans.

1.5.1 Granulopoiesis

Neutrophils are generated in the bone marrow via a process known as granulopoiesis. Myeloblasts are differentiated from pluripotent hematopoietic stem cells and are committed to develop into granulocytes (151). As neutrophils mature in the bone marrow, they undergo intensive transcription, generating a broad array of proteins including antimicrobial peptides (152), proteases (153), metal chelators (154) and cellular receptors (155, 156). These proteins are sequentially expressed during neutrophil development for storage in a series of granules (149, 157). The ability of neutrophils to deploy preformed peptides allows for rapid responses to neutralise invading pathogens. Although the arsenal of antimicrobials that neutrophils possess is usually effective in eliminating pathogens, it can also inflict cellular damage (158, 159). Thus, the deployment and activation of neutrophils requires tight regulation.

1.5.2 Release of neutrophils from bone marrow

The release of neutrophils from the bone marrow into the circulation is controlled by two chemokine receptors, CXCR2 and CXCR4 that exert opposing effects (160). Both chemokine receptors are constitutively expressed on neutrophils and bind to specific factors in the bone marrow. Stromal derived factor-1 (SDF-1; CXCL12), which is mainly produced by bone marrow osteoclasts, binds to CXCR4 and retains mature neutrophils in the bone marrow. In contrast, murine KC (CXCL1 in humans) and murine macrophage inflammatory protein -2 (MIP-2; also called CXCL2, or IL-8 in humans) are mainly synthesised by bone marrow endothelial cells, bind to CXCR2, and promote neutrophil egress from the bone marrow to the circulation. The constant tug-of-war between CXCR2 and CXCR4 ligands determines whether or not neutrophils are deployed into the circulation. Under homeostatic conditions, SDF-1 usually dominates, retaining the majority of neutrophils in the bone marrow (161). Granulocyte-colony stimulating factor (G-CSF) is

the master regulator of neutrophils and controls the generation of mature neutrophils and their release into the circulation in both the steady state and during inflammation.

1.5.3 Neutrophil recruitment

During infection, professional sentinel cells such as macrophages sense PAMPs and DAMPs, leading to the production of cytokines, chemoattractants and hematopoietic growth factors that work together to choreograph the rapid recruitment of neutrophils to a site of infection (162, 163). The production of G-CSF is greatly enhanced during infection, and increases circulating neutrophil numbers by facilitating neutrophil egress from the bone marrow. G-CSF inhibits the synthesis of SDF-1 by osteoclasts and increases the production of KC and MIP-2, causing a shift in the balance of SDF-1 versus CXCR2 ligands thereby promoting neutrophil egress into the circulation (160).

Concurrently, endothelial cells surrounding the site of infection upregulate adhesion molecules on their luminal surface to halt circulating neutrophils. The presence of bacterial products and the release of proinflammatory cytokines by resident sentinel cells induces the expression of neutrophil adhesion molecules such as P-selectin, E-selectin and several members of the intercellular adhesion molecule (ICAM) superfamily to promote neutrophil adhesion and transmigration to a site of inflammation (149, 151).

1.5.4 Neutrophil transmigration

Neutrophils constitutively express cell-surface P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin to recognise the activated endothelium (164). As neutrophils transverse the endothelium, these molecules engage P- and E-selectin, resulting in selectin-mediated tethering to the endothelium (‘rolling adhesion’). The engagement of selectin ligands activates a range of kinases, committing neutrophils to an antimicrobial state and preparing them for firm adhesion (165, 166). As loosely tethered neutrophils roll along the endothelium, they encounter further chemoattractants, cytokines and bacterial products, which cause activation and clustering of cell surface β2 integrin family members (Mac-1 and leukocyte function-associated antigen 1 [LFA-1]) (167). This triggers a second stage of neutrophil activation in which they extensively remodel their actin cytoskeleton and enhance their capacity for phagocytosis and ROS production. Neutrophils are fully activated and ready to transit into ‘firm adhesion’ at this stage. Once firmly engaged by members of the ICAM-1 superfamily on the endothelium, neutrophils exit the vasculature (‘diapedesis’).

Once they have crossed the endothelium, neutrophils travel along a chemokine gradient of either host-derived KC/MIP-2 or pathogen-derived N-Formylmethionine-leucyl-phenylalanine (fMLP). The engagement of chemoattractants onto surface receptors maintains neutrophils in an antimicrobial state (168, 169). Simultaneously, the recognition of PAMPs by TLRs enhances the phagocyte oxidative burst (169). Upon reaching the highest point of the chemokine gradient, neutrophils halt and unleash their antimicrobial arsenal.

1.5.5 Neutrophil antimicrobial mechanisms

1.5.5.1 Oxidative burst

Neutrophils employ a number of strategies to kill invading pathogens. Amongst the most potent of these is the ability of neutrophils to generate ROS via the oxidative burst. Individuals suffering from chronic granulomatous disease harbour genetic mutations in this pathway, and are unable to generate ROS; as a consequence, these individuals are more susceptible to life-threatening bacterial and fungal infections (170-172). The ROS generation via the oxidative burst requires the assembly of the multi-subunit NADPH oxidase complex, which is induced by proinflammatory cytokines such as TNF and IFN-γ in conjunction with microbial constituents such as LPS and fMLP (172). The NADPH oxidase complex is composed of several cytosolic and membrane-bound proteins. Depending on the localisation of the complex, the oxidative burst can be directed into the extracellular space or channelled towards pathogen-containing phagosomes (172). The initiation of the oxidative burst upon NADPH oxidase complex assembly results in the reduction of molecular oxygen to superoxide anion. Spontaneous reaction of the superoxide anion results in the formation of several ROS species: hydrogen peroxide, hydroxyl radical and singlet oxygen. Hydrogen peroxide also reacts with myeloperoxidase (MPO), an enzyme present in high quantities in primary granules (see 1.5.5.3), to generate hypochlorous acid (155). Because of their reactivity, ROS are able to target a wide variety of components of the invading pathogen such as nucleic acids, lipid membranes, metal centers and thiols. Taken together, these modifications impair bacterial metabolism and ultimately inhibit bacterial replication.

1.5.5.2 Phagocytosis

A hallmark of neutrophils is their ability to engulf extracellular pathogens within seconds of contact via phagocytosis (173). Once engulfted, the pathogen is contained within the membrane-bound phagosome. In contrast to macrophages, neutrophils lack a classical

endocytic pathway, and the nascent phagosome does not acidify (174, 175). Instead, the phagosomal cargo fuses with pre-formed granules, releasing potent antimicrobials that destroy the pathogen.

1.5.5.3 Granules

Neutrophils include variably sized granules in their cytoplasm that contain a variety of antimicrobial proteins, which can be either directed into the extracellular space or can fuse with pathogen-containing phagosomes. Neutrophil granules are formed sequentially during neutrophil development and can be broadly classified into three categories: azurophilic (primary), specific (secondary) and gelatinase (tertiary) granules (149, 151). Azurophilic granules are the first to be synthesised during development, are characterised by the presence of MPO, and are considered the most potent amongst the granule types. Azurophilic granules contain a variety of lytic enzymes such as serine proteases and lysozyme, as well as peptides that disrupt the bacterial cell wall such as defensins and bacterial/permeability-increasing protein (BPI) (149). Specific granules are MPO-negative and contain a number of anti-microbial compounds, including the iron chelator lactoferrin. Iron deprivation is an important antimicrobial strategy as many pathogens require iron to sustain their metabolic functions (99). Excessive iron can also dampen antimicrobial mechanisms in other myeloid cell types, such as the production of reactive nitrogen species (RNS) in macrophages (176). Gelatinase granules are the last granule type to be synthesised. Gelatinase granules contain neither MPO nor lactoferrin, but are enriched with metalloproteases. Granules are released in reverse order to their formation (177), and gelatinase granules are thought to break down the extracellular matrix and collagen to aid in neutrophil extravasation. Neutrophils also posses secretory vesicles, although their origin may differ from the three granule types mentioned above (155). These secretory vesicles are the most easily mobilised and house membrane proteins such as Mac-1 and fMLP-receptor that exert important functions during early phase of inflammation.

1.5.5.4 Neutrophil extracellular traps

In 2004, a new neutrophil antimicrobial mechanism was discovered, called neutrophil extracellular traps (NETs) (178). NET formation is associated with a form of cell death distinct from necrosis and apoptosis. ‘NETosis’ arises from the release of material such as DNA, chromatin and granular proteins into the extracellular space, to form a web that traps microbes. Antimicrobial proteins such as myeloperoxidase, lacteroferrin, defensins, elastase, proteinase-3 and calprotectin decorate NETs (179).

Although the molecular mechanisms triggering NET formation still remain elusive, it is well established that NETosis contributes to host protection against a variety of bacteria, fungus and parasites (180, 181). ROS, autophagy and IL-1 signalling have all been suggested to contribute to the induction of NETs (182), and neutrophils from chronic granulomatous disease patients less efficiently trigger NET formation (183). A single report proposed that autophagy inhibition using the chemicals 3-methyladenine (3-MA) or bafilomycin A reduced the frequency of NETosis (93). The same authors also demonstrated that gout synovial fluid triggered peripheral neutrophils from healthy donors to undergo NETosis, but this could be suppressed by co-administration of the recombinant IL-1R antagonist (Anakinra), suggesting IL-1 is required to launch NETs (93). It is generally believed that NET formation *in vivo* serves two functions; initially NETs immobilise, destroy and trap invading pathogens to prevent their dissemination, but they may additionally provide signals to orchestrate downstream inflammatory processes.

1.5.6 Neutrophil cell death pathways

Although important for antimicrobial defence, aberrant activation of neutrophils and their impaired clearance can cause collateral damage and contribute to numerous pathologies. This is exemplified in several inflammatory conditions such as gout (93), rheumatoid arthritis (184), inflammatory bowel disease (185) and sepsis (186), where neutrophilia is often observed. The clearance of neutrophils at an inflammatory focus is also critical for the resolution of inflammation (187, 188). Therefore, it is important for neutrophil lifespan to be tightly controlled. Multiple forms of programmed cell death such as apoptosis and necroptosis have been described, and will be discussed herein.

1.5.6.1 Neutrophil cell death by apoptosis

Neutrophils are relatively short-lived cells and were widely accepted to have a lifespan of less than 1 day in circulation. An increasing number of studies are now challenging this concept, and a single study demonstrated that neutrophils survive in circulation for 5 days under homeostatic conditions (189). The turn over of aged neutrophils occurs mainly through apoptosis, an immunologically silent form of cell death, and senescent neutrophils are cleared by the reticuloendothelial system and in the bone marrow (190, 191).

Apoptosis can be induced by two major mechanisms, the extrinsic and intrinsic pathways (192). The activation of the extrinsic pathway requires engagement of death receptors located on the cell surface (**Figure 1.5**). The major death receptors include the TNF receptor 1 (TNFR1), TNF-related apoptosis-inducing ligand receptor (TRAILR) receptor

and Fas (also known as CD95), and the ligands for these receptors are TNF, TRAIL and FasL, respectively. Death receptor ligation by TRAIL or FasL induces the formation of the death-inducing signalling complex (DISC), which is composed to the adaptor molecule Fas-associated death domain protein (FADD), the initiator caspase, caspase-8 and caspase-10 (in humans) (193, 194). The DISC provides a platform for pro-caspase-8 dimerisation, which is critical for its activation (195). C-FLIP, a protease-dead homolog of caspase-8, prevents apoptosis by competing with pro-caspase-8 for recruitment to the DISC and restricts its pro-apoptotic functions by forming a caspase-8/c-FLIP heterodimer (196, 197). Active caspase-8 drives apoptosis through the proteolytic activation of the executioner caspases, caspase-3 and caspase-7, which in turn execute apoptosis though the proteolytic cleavage of numerous substrates (192). TNF induces a similar DISC complex but also requires the function of TNF receptor-associated death domain (TRADD) and the kinase RIPK1 (198). Depending on the cell type involved, the sole activation of the apical caspase in this cascade (caspase-8) may or may not suffice for apoptosis induction (199). Cells that do not require an additional caspase signalling cascade are termed ‘type I’ cells. In contrast, ‘type II’ cells require caspase cascade amplification via caspase-8-mediated cleavage of pro-apoptotic B cell CLL/lymphoma-2 (BCL-2) family member BH3-interacting death domain agonist (Bid) (200). Caspase-8 cleaves Bid into its truncated form (t-Bid), which induces the depolarisation of the mitochondria, leading to the subsequent release of SMAC and cytochrome C from the mitochondria into the cytosol (201). In turn, SMAC relives XIAP-mediated inhibition of caspase-3 -7 and -9, while cytoplasmic cytochrome C is sensed by apoptotic protease activating factor 1 (APAF-1) and drives the oligomerisation of APAF-1 into a large heptameric complex that enables the recruitment of caspase-9 (202, 203). This multi-protein complex, consisting of cytochrome C, APAF-1 and caspase-9, is termed the apoptosome. Assembly of the apoptosome allows proximity-induced activation of the apical initiator caspase, caspase-9. Similar to caspase-8, activated caspase-9 triggers proteolytic activation of caspase-3 and -7 for apoptosis induction. XIAP critically discriminates whether a cell undergoes Type I or Type II apoptosis (199). Interestingly, the requirement for Bid cleavage in triggering death ligand-induced apoptosis in neutrophils is ligand-specific. While FasL-induced apoptosis was accelerated upon Bid cleavage; Bid cleavage was dispensable for TNF-induced apoptosis (204, 205).

There is no requirement for death receptor engagement for the intrinsic apoptosis pathway. The intrinsic pathway is activated following cellular stress such as DNA damage,

and growth factor withdrawal, which induces a change in ratio of BCL-2 family proteins and triggers the release of SMAC and cytochrome C (206), which promote the formation of the apoptosome to initiate the apoptotic signalling cascade (202, 203).

In neutrophils, granular proteases trigger an unusual pathway of non-canonical apoptosis. For example, the release of granular cathepsin D into the cytoplasm triggers caspase-8 activation independently of death receptor engagement and DISC complex formation (207, 208). Cathepsin D was also reported to mediate Bid cleavage independently of caspase-8 to initiate apoptosome assembly (209). Lastly, translocation of granular proteinase-3 into the cytosol allows proteolytic activation of caspase-3 independently of the canonical caspase-8 or caspase-9 pathways (210).

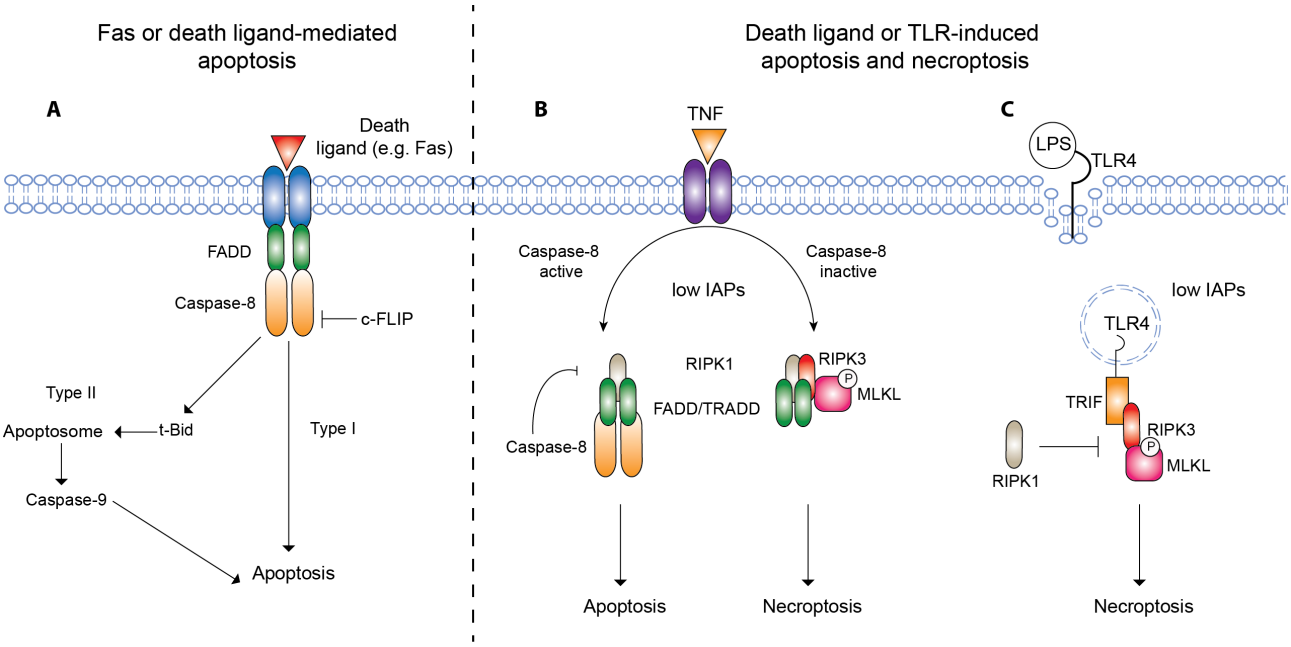


Figure 1.5 Apoptotic and non-apoptotic cell death pathways triggered by cell surface receptors.
(A) Engagement of death ligands (e.g. Fas or TNF) to death receptors triggers the formation of the death-inducing signalling complex (DISC) that activates caspase-8 to trigger apoptosis. In Type II cells, apoptosis induction requires the caspase amplification cascade through the apoptosome. (B) Death receptors trigger the assembly of a multi-protein complex containing combinations of molecules including FADD, TRADD, RIPK1, and RIPK3, and can trigger apoptosis or necroptosis. Caspase-8 cleaves and inactivates RIPK1 and RIPK3 (not shown) to inhibit necroptosis. If caspase-8 and IAPs are absent/inhibited, RIPK3 accumulates and phosphorylates MLKL to trigger necroptosis. (C) Ligation of TLR4 or TLR3 (not shown) triggers the recruitment of the signalling adaptor TRIF, which in turn recruits RIPK3 and leads to RIPK1-independent necroptosis.

1.5.6.2 Neutrophil cell death by necroptosis?

Some cell types such as macrophages trigger a form of programmed necrosis termed 'necroptosis' when the IAP and caspase-8 functions are blocked. Necroptosis is widely regarded as a proinflammatory form of cell death, as alarmins such as IL-33 and HMGB-1 are released during cell rupture (211). However, whether necroptosis is beneficial or detrimental during health and diseases is the subject of much debate, and is likely to depend on the context of necroptotic cell death.

Members of the TNF superfamily such as TNF, TRAIL and Fas can trigger necroptosis though the activity of RIPK1, RIPK3 and MLKL (212). Those TLRs that signal via the TRIF adaptor protein (i.e. TLR4, TLR3) also drive necroptosis, however, this occurs in a RIPK1-independent, RIPK3 and MLKL-dependent manner (213) (**Figure 1.5**). Ligation of other TLRs can also trigger necroptosis, however, this occurs indirectly, via autocrine TNF signalling. In many cases necroptosis occurs when cells fail to undergo apoptosis, and in keeping with this, pro-apoptotic pathways negatively regulate necroptosis. For example, caspase-8 cleaves RIPK1 to limit necroptosis (214). In contrast, a lack of caspase-8 activity (or expression) allows the accumulation of RIPK1 and RIPK3 and enables the recruitment and phosphorylation of its substrate MLKL (215). Phosphorylation induces a conformational change in MLKL (216) that allows the induction of necroptosis by either acting as a pore-forming complex or by regulating ion efflux and driving osmotic lysis (217, 218).

The physiological relevance of necroptosis is not well understood, as different studies report conflicting results when assessing necroptosis in animal disease models. Infected cells undergo apoptosis in order to limit viral replication, as viruses are obligate intracellular pathogens. In line with this, several groups have demonstrated that necroptosis serves as an alternate cell death pathway to lyse infected host cells and prevent viral replication. Indeed, *Ripk3*-deficient mice are hypersensitive to infection with cytomegalovirus (219) and herpes simplex virus-1 (220). In contrast, another study suggested that necroptotic death is not a host-driven event, and instead represents a pathogen subversion mechanism to facilitate bacterial dissemination. In that study, *Ripk3*-deficient mice harboured significantly lower bacterial burden in spleen following *S.* Typhimurium infection (220). Consistent with the notion that necroptosis is detrimental to the host, ischemic and neurodegenerative injuries are often accompanied by severe necrosis. Pharmacological inhibition of necroptosis using the RIPK1 inhibitor necrostatin-1

ameliorates ischemic brain injury (221), Huntington's disease (222) and hepatic injury (223) in animal models. However, these studies need to be carefully reassessed as necrostatin-1 was subsequently found to also target indoleamine-2,3-dioxygenase, an enzyme intimately involved in regulating both innate and adaptive immunity (224). Lastly, another study proposed that necroptosis is a mechanism to halt excessive production of proinflammatory cytokines that would otherwise be detrimental to the host if immune cells remained viable (225). The authors demonstrated that intraperitoneal administration of supernatants collected from cultured necroptotic cells (L929 cells treated with TNF and caspase inhibitor) only weakly elicited the recruitment of immune cells to the peritoneum. In contrast to cells that rapidly lysed, L929 cells there were only exposed to TNF produced sustained levels of proinflammatory cytokines, and supernatants from these cells triggered efficient recruitment of neutrophils to the peritoneum. Collectively, these data suggest that necroptosis can either be beneficial or detrimental to the host, depending on the circumstances of necroptosis induction.

1.6 Aims of this thesis

The inflammasome pathway is critical for host defence but also contributes to inflammatory diseases, so the molecular and cellular mechanisms regulating inflammasome function are of paramount medical importance. Prior to this thesis, inflammasome research was almost exclusively performed with macrophages and dendritic cells, without knowing whether these are the sole cell types that drive inflammasome-dependent responses during health and disease. Preliminary data from our laboratory demonstrated that neutrophils express a wide variety of NOD-like receptors, including NLRC4 and NLRP3, suggesting that neutrophils may signal via inflammasomes. Therefore, this thesis broadly aimed to examine the ability of neutrophils to trigger inflammasome functions upon *in vitro* and *in vivo* challenge, and investigate any possible cell type-specific adaptations to the inflammasome signalling pathway that may allow the coordination of host defence responses.

Specific project aims:

1. To determine whether neutrophils signal via NLRC4 inflammasome pathway during *in vitro* or *in vivo* challenge with *S.* Typhimurium, and examine the contribution of neutrophil inflammasome signalling to cytokine production, immune cell recruitment and activation, and pathogen control *in vivo.*

2. To determine whether the NLRP3 inflammasome pathway characterised in macrophages is conserved in neutrophils, including upstream activation mechanisms, downstream cell death pathways, and signal cross-talk from related cell death pathways (ripoptosome and necrosome).
3. To determine whether neutrophil inflammasomes contribute to pathological inflammatory responses in diseases such as endotoxin shock.

The first aim of the thesis was addressed in Chapter 3. In investigating the neutrophil NLRC4 inflammasome pathway during *in vitro* and *in vivo* infection, I made the surprising finding that neutrophils trigger cell type-specific responses upon inflammasome activation, and that modification of this signalling pathway in neutrophils is critical for host defence. The second aim was addressed in Chapters 4 and 5 of this thesis, and extended upon the data generated in Aim 1. Chapters 4 and 5 collectively demonstrate that, similar to NLRC4, the signalling pathways up and downstream of the NLRP3 inflammasome in neutrophils also appear to be distinct as compared to macrophage NLRP3 pathways. Lastly, the third aim was addressed in Chapter 6, which described the potential functions for the neutrophil caspase-11 inflammasome in driving endotoxic shock. In summary, this thesis provides detailed investigations into neutrophil NLRC4, NLRP3 and caspase-11 inflammasome signalling pathways during host defence and inflammatory disease.

2. Materials and Methods

Materials and methods used in the thesis that were not otherwise part of a publication or submitted manuscript are listed here.

2.1 Materials

2.1.1 Animals

3 week-old C57BL/6 animals were ordered from the Animal Resource Centre, WA, Australia, and aged in specific pathogen-free animal facilities within the University of Queensland Biological Resource Centre (UQBR). *Nlrc4*$^{-/-}$ (29), *Nlrp3*$^{-/-}$ (40), *Asc*$^{-/-}$ (29), *Ice*$^{-/-}$ (226), and *Casp11*$^{-/-}$ (227) mice were backcrossed at least 10 times to C57BL/6 and bred in UQBR specific pathogen-free animal facilities. Age- and sex-matched animals were used for all experiments. The University of Queensland Animal Ethics Committee approved all experimental procedures.

2.1.2 Bacterial strains

Wild type *Salmonella* enterica serovar Typhimurium SL1344 and its congenic ΔSPI1 mutant were described in Chapter 3.

2.1.3 TLR agonists

TLR ligands were prepared as follows: ultrapure LPS (TLR4 agonist; Invivogen) purified from *Escherichia coli* K12 was solubilised in sterile water at 1 mg/ml and was used at a final concentration of 100 ng/ml; LPS (TLR4 agonist; Sigma Aldrich) purified from *Escherichia coli* 0111:B4 was solubilised in sterile DPBS (Life technologies) at 1 mg/ml and used at a final dose of 10 mg/kg in a final volume of 200 μl for *in vivo* challenge; poly(IC) (TLR3 agonist; low molecular weight; Invivogen), a synthetic dsRNA analogue was solubilised in sterile water at 20 mg/ml and used at a final concentration of 50 μg/ml for cell culture and 10 mg/kg in a final volume of 200 μl for *in vivo* challenge.

2.1.4 Inflammasome agonists

Inflammasome agonists were prepared as follows: Nigericin (Sigma Aldrich) was solubilised in 100 % ethanol at 5 mM and was used at a final concentration of 5 μM; Adenosine triphosphate (ATP) (Sigma Aldrich) was solubilised in sterile water at 1 M and

used at a final concentration of 1, 1.25, 2.5 and 5 mM; alum was purchased from Pierce at 40 mg/ml and was used at a final concentration of 150, 300 or 600 μg/ml; silica (Alfa Aesar) was resuspended in PBS at a 40 mg/ml and was used at a final concentration of 150, 300 or 600 μg/ml; monosodium urate crystals were prepared from uric acid (Sigma Aldrich), resuspended in PBS at a final 50 mg/ml and were used at a final concentration of 150, 300 or 600 μg/ml; Leu-Leu-OMe (Santa Cruz) was resuspended in 100% methanol at 1 M and used at a final concentration of 0.5, 1 and 2 mM.

2.1.5 Cell death inducers

Death receptor inducers were prepared as follows: recombinant soluble Fas ligand (FasL; Enzo) was solubilised in sterile water at 100 mg/ml and used at a final concentration of 100 ng/ml; murine TNF (Peprotech) was dissolved in sterile water at 50 mg/ml and used at a final concentration of 100 ng/ml; the IAP antagonist, Compound A (Cp A, a kind gift from the Walter and Eliza Hall Institute of Medical Research, Australia), was diluted in Opti-MEM (Life Technologies) at 100 mg/ml and used at a final concentration of 500 ng/ml.

2.1.6 Caspase inhibitors

Caspase inhibitors were prepared as follows: z-VAD-fmk was solubilised in DMSO at 50 mM and used at a final concentration of 50 μM; biotin-VAD-fmk was solubilised in DMSO at 10 mM and used at a final concentration of 10 μM.

2.1.7 Antibodies for *in vivo* injection

Antibodies used for *in vivo* injections were described in Chapters 3 and 4.

2.1.8 Antibodies for flow cytometry

Antibodies used for flow cytometry were described in Chapters 3 and 4.

2.1.9 Antibodies for western blot

Antibodies that were used in Chapter 5 that were not otherwise described in Chapters 3 and 4 are listed in Table 2.1.

Table 2.1 Antibodies used for western blot.

Antibody	Specificity	Antibody type	Dilution	Expected molecular weight (kDa)	Source
Primary antibodies					
α-c-IAP1	Human, mouse	Rat monoclonal	1:1000	62	Enzo
α-c-FLIP	Human, mouse	Rat monoclonal	1:500	55 (c-FLIP$_L$), 26 (c-FLIP$_s$)	Adipogen
α-Bid	Mouse	Rat monoclonal	1:1000	23	eBiosciences
α-caspase-8	Mouse	Rat monoclonal	1:1000	55 (Full length); 46, 28, 18 (cleavage)	Enzo
α-TRIF	Human, mouse, rat	Rabbit polyclonal	1:1000	76	Novus Biological
α-ASC	Human, mouse	Rabbit polyclonal	1:1000	20	Santa Cruz
Secondary antibodies					
Goat α-rabbit IgG HRP	Rabbit IgG	-	1:5000	-	Cell signaling
Goat α-rat IgG HRP	Rat IgG	-	1:5000	-	Cell signaling

2.2 Methods

2.2.1 Bacterial preparation

S. Typhimurium strains for *in vitro* and *in vivo* infection were prepared as stated in Chapter 3.

2.2.2 Bone marrow-derived macrophage cell culture

Bone marrow-derived macrophages were prepared and cultured as described in Chapter 3.

2.2.3 Bone marrow neutrophil purification

Bone marrow neutrophils were prepared and cultured as described in Chapter 3.

2.2.4 Peritoneal-elicited neutrophil purification

Neutrophils were purified from the peritoneal exudate of thioglycollate-challenged mice as described in Chapter 3.

2.2.5 Human cell preparation

Immune cells were fractionated from human blood as described in Chapter 3.

2.2.6 Inflammasome activation assay

Inflammasome activation assays were performed as described in Chapters 3 and 4.

2.2.7 LPS transfection

Cells were transfected with LPS in order to activate the cytosolic LPS receptor, caspase-11. Cells were primed with 100 ng/ml ultrapure *Escherichia coli* K12 LPS for 6 h in Opti-MEM. Cells were then washed once with Opti-MEM and the culture media replaced to remove residual LPS. 2 μg/ml LPS was transfected into the cytoplasm by application of 0.5 μl Fugene (Promega) in a final volume of 200 μl Opti-MEM. Cells were incubated a further 8 h before cell harvest.

2.2.8 Gentamicin protection assay

Intracellular bacterial loads in neutrophils and macrophages were quantified using gentamicin protect assay as described in Chapter 3.

2.2.9 Enzyme-linked immunosorbent assay (ELISA)

The production of inflammatory cytokines IL-1α, IL-1β, IL-18 and TNF following cell stimulation was quantified using enzyme-linked immunosorbent assay (ELISA) as described in Chapters 3 and 4. IL-6 concentrations were quantified using matched-pair antibodies (BD Bioscience). Nunc MaxiSorp 96-well plates were coated with capture antibody (50 µL/well; diluted in 0.1 M NaHCO3) and incubated overnight at 4 ^{o}C. Plates were washed twice with PBST (PBS/0.05% Tween-20) to remove unbound antibody and blocked with 200 µL/well 10% FCS/PBS for 1 hour at 37 ^{o}C. Plates were washed twice with PBST, dried and 50 µL/well of cytokine standards or sample were added and incubated overnight at 4 ^{o}C. Unbound sample was removed from the plate by four washes in PBST. Detection antibody (100 µL/well; diluted in 10% FCS/PBS) was then added and incubated for 1 hour at 37 ^{o}C. Unbound detection antibody was removed by washing the plate six times in PBST. Extra-avidin peroxidase (Sigma Aldrich) was diluted 1:1000 in 10% FCS/PBS, and 50 µL was added to each well and incubated at 37℃ for 30 mins. Plates were washed eight times in PBST before addition of 100 µL/well TMB substrate (Sigma Aldrich). The reaction was stopped when lower standards had developed using 100 µL/well of 2 N H_2SO_4. Absorbance at 450 nm was measured using a Powerwave XS spectrophotometer (Bio Tek) and cytokine concentration in samples was calculated using data points generated from the standard curve.

2.2.10 MTT reduction assay

The colorimetric MTT assay measures cellular metabolic activity by monitoring cleavage of the tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) by the mitochondrial enzyme succinate dehydrogenase, forming an insoluble blue formazan product that indirectly indicates cellular viability. Neutrophils and macrophages were plated in Opti-MEM at 0.4 x 10^6 or 0.1 x 10^6 cells per well, respectively, in a final volume of 200 µl in a 96-well plate. Cell culture supernatant was removed after cell stimulation and replaced with 50 µl of MTT reagent at a final concentration of 1 mg/ml (diluted in Opti-MEM), and incubated for 1-2 h at 37 ^{o}C (the extent of formazan production can be visualised under a light microscope). MTT reagent was then replaced and with 200 µl isopropanol and incubated in the dark with gentle shaking in room temperature to dissolve the formazan. The absorbance of the solubilised formazan was measured at 570 nm using the Powerwave XS spectrophotometer (Bio Tek) 96-well plate reader.

2.2.11 Lactate Dehydrogenase (LDH) release assay

Lactate dehyrodgenase (LDH) is a cytosolic enzyme that is released during cellular lysis and can be measured using a colorimetric assay that produces a red formazan product. The intensity of the final red formazan product can be used as an indirect indicator of cellular toxicity. LDH toxicity assays were performed as described in Chapter 3.

2.2.12 Live cell imaging

The ability of neutrophils to phagocytose silica particles was investigated by live cell imaging as described in Chapter 4.

2.2.13 Flow cytometric analysis of propidium iodide uptake

Propidium iodide is a cell-impermeable DNA-intercalating agent excluded from viable cells. Propidium iodide can be detected at 617 nm by flow cytometry, and is used to label dying cells. Neutrophils were plated on non-adherent 24-well tissue culture plastic (Nunc) and stimulated. Semi-adherent and floating cells were then harvested in ice-cold DPBS supplemented with 1% FCS and 5 mM EDTA, and collected by centrifugation at 500 g for 5 min at 4^0C. Cells were resuspended in 100 μl ice-cold DPBS supplemented with 1% FCS, 5 mM EDTA and 1 μg/ml propidium iodide, and samples were acquired on a FACS Canto II (BD Bioscience) cytometer and analysed using FlowJo 9.4.4 (Tree Star).

2.2.14 Gene profiling analysis

NLRs were profiled for mRNA expression in human and murine immune cell fractions as described in Chapter 3.

2.2.15 SDS-PAGE and immunoblotting

Proteins from whole cell lysate and cell culture supernatant were separated using pre-cast 4-12% or 12% polyacrylamide gels (Bio-rad) and analysed by immunoblotting as described in Chapters 3 and 4.

2.2.16 ASC crosslinking

Cell extracts were crosslinked in order to monitor ASC polymerisation following inflammasome stimulation. Neutrophils and macrophages were plated at 3 x 10^6/well and 1.65 x 10^5/well, respectively, in order for ASC expression levels to be equivalent in untreated cell extracts (macrophages express ~18-fold more ASC protein on a per-cell basis, which approximately scales with cell volume). Following stimulation, cells were washed twice with 250 μl of ice-cold 50 mM HEPES and lysed in 250 μl ice-cold buffer (0.5

mM HEPES, 0.5% Triton X-100, 0.3% Benzonase, Roche general protease inhibitor) for 10 min on ice. Cell lysates were centrifuged at 6000 *g* for 15 min at 4 ^{o}C, and the soluble fraction removed. The Triton X-100 insoluble cell pellet enriched with ASC polymers was resuspended in 200 μl 50 mM HEPES supplemented with 50 mM NaCl and 2 mM disuccinimidyl suberate (DSS; Thermo Scientific) and incubated for 45 min at 37 ^{o}C to cross-link ASC oligomers. The ASC polymer-enriched insoluble fraction was then isolated by centrifugation at 6000 *g* for 15 mins at 4 ^{o}C, and resuspended in 20 μl of 1x LDS loading buffer (Invitrogen). Samples were separated by gel electrophoresis (precast 4-12% polyacrylamide gel; Bio-rad), transferred to nitrocellulose membrane, and analysed by immunoblotting.

2.2.17 Active caspase pulldown

Cells were treated with biotin-VAD-fmk in order to label active caspases following inflammasome activation. 10 μM biotin-VAD-fmk (Santa Cruz) was added to 4 x 10^6 neutrophils 30 min prior to cell stimulation. Cell extracts were then lysed in 200 μl ice-cold buffer (1% IPEGAL, 50 mM HEPES, 50 mM NaCl, 0.3% benzonase) for 10 min on ice. Paramagnetic streptavidin beads (Promega) were added to the cell culture supernatant or cell extract, and incubated overnight with gentle rotation at 4 ^{o}C. Beads were washed thrice in 500 μl buffer with increasing NaCl concentration (1% IPEGAL, 50mM HEPES plus 150 mM, 300 mM, or 500 mM NaCl). Pellets were then resuspended in 20 μl 1x LDS buffer. Beads were boiled for 95 ^{0}C for 10 min and separated by centrifugation at 6000 *g* for 1 min and the soluble fraction containing the biotin-VAD-fmk-trapped proteins was separated by gel electrophoresis (precast 12% polyacrylamide gel; Bio-rad) before protein transfer to nitrocellulose, and immunoblot analysis.

2.2.18 *In vivo* Salmonella challenge

Mice were challenged with *Salmonella* as described in Chapter 3.

2.2.19 Alum-induced peritonitis

Alum was administered by intraperitoneal injection, and immune cell recruitment to the peritoneum was quantified as described in Chapter 3.

2.2.20 Murine endotoxic shock

Mice were challenged with 10 mg/kg poly(IC) i.p. in a total volume of 200 μl DPBS at 0700 h. 6h later, mice were re-challenged, this time by intraperitoneal injection with 10 mg/kg *Escherichia coli* 0111:B4 LPS (Sigma Aldrich) in a final volume of 200 μl DPBS. Poly(IC)

and LPS injection times were kept constant between experiments to account for murine circadian rhythm. Rectal temperature was monitored using a BART 12R-220 thermometer (World Precision Instruments) every 2 h for the first 10 h post-LPS challenge, and were monitored every 4-6 h thereafter. Mice were considered moribund and sacrificed immediately when their rectal temperature dropped below 24^0C or when they were unable to regain posture.

2.2.21 Collection of mouse serum

Serum was collected and analysed by ELISA to assess the production of proinflammatory cytokines in control and infected animals. Post-euthanasia, whole blood was collected by heart puncture using a 27-gauge needle. Whole blood was left to clot in room temperature for 45 min-1 h. The serum was separated by centrifugation at 1000 g for 10 mins at room temperature.

2.2.22 Analysis of immune infiltrate by flow cytometry

Immune cell recruitment to the peritoneum following intraperitoneal *Salmonella* challenge was analysed as described in Chapter 3.

2.2.23 Analysis of cellular toxicity *in vivo* by flow cytometry

Cellular toxicity in the immune cell infiltrate following *Salmonella* challenge was quantified as described in Chapter 3.

2.2.24 Statistical analysis

Statistically significant differences were calculated using Prism 6 (GraphPad software). For comparisons between two normally distributed data sets, an unpaired t-test with two-tailed distribution (Mann-Whitney t-test) was used. For comparisons between 3 or more samples across different time points, a two-way ANOVA was used. For all data contained in this thesis, statistically significant data is represented as *$p<0.05$, **$p< 0.01$, ***$p<0.005$.

3. The neutrophil NLRC4 inflammasome selectively promotes IL-1β maturation without pyroptosis during acute *Salmonella* challenge

The data presented here in Chapter 3 were previously published and were unformatted to meet The University of Queensland's thesis requirement.

Chen, K.W., Gross, C.J., Sotomayor, F.V., Stacey, K.J., Tschopp, J., Sweet, M.J., and Schroder, K. (2014). The neutrophil NLRC4 inflammasome selectively promotes IL-1β maturation without pyroptosis during acute Salmonella challenge. **Cell reports** *8*, 570-582.

Contributor	Statement of contribution
Kaiwen Chen (Candidate)	Designed the study and wrote the paper. Performed all experiments except Figure 3.1, S3.1 and S3.2 (40%).
Christina Groß	Performed Figure 3.1E-F, and S3.1L-Q experiments (5%).
Flor Sotomayor	Provided technical assistance for cell culture (2%).
Katryn Stacey	Provided reagents and intellectual input (8%).
Matthew Sweet (Associate Advisor)	Provided reagents and intellectual input (10%).
Kate Schroder (Advisor)	Performed Figure 3.1A-D, S3.1A-K and S3.2 experiments (35%). Designed the study and wrote the paper.

3.1 Summary

The NLRC4 inflammasome drives potent innate immune responses against *Salmonella*, by eliciting caspase-1-dependent pro-inflammatory cytokine production (e.g. interleukin-1β, IL-1β) and pyroptotic cell death. Macrophages are well established to trigger inflammasome-mediated host defense during acute *Salmonella*-induced peritonitis, however the potential contribution of other cell types was unclear. Here we demonstrate that neutrophils, typically viewed as cellular targets of IL-1β, themselves activate the NLRC4 inflammasome during acute *Salmonella* infection, and are a major cell compartment for IL-1β production during acute peritoneal challenge *in vivo* (10^6 CFU/mouse). Importantly, we also find that neutrophils are resistant to pyroptosis, a unique specialization of the inflammasome pathway amongst cells so far described. NLRC4 pathway specialization allows neutrophils to sustain IL-1β production at a site of infection, without compromising the crucial inflammasome-independent antimicrobial effector functions that would be lost if neutrophils rapidly lysed upon caspase-1 activation. Inflammasome pathway specialization in neutrophils thus maximizes host pro-inflammatory and antimicrobial responses during pathogen challenge.

3.2 Introduction

The innate immune system engages an array of pattern recognition receptors (PRR) to detect signals of host infection, tissue injury and cellular stress. Among these receptors are the Toll-like receptors, C-type lectins, cytoplasmic nucleic acid receptors (e.g. RIG-like helicases and HIN-200 proteins) and Nod-like receptors (NLRs). NLRs are activated by pathogen-associated molecules and host-derived alarmins indicating cellular injury or stress, and are potent mediators of inflammation. The human NLR family has 22 members, and is defined by the presence of a central NACHT domain that triggers self-oligomerization. A functional subgroup of the NLR family form high molecular weight complexes known as inflammasomes (e.g. NLRP1, NLRP3, NLRP6, NLRP12 and NLRC4) (2).

Inflammasomes are ‘danger’ sensor complexes that trigger immune system activation via cytokine processing. Inflammasome activation involves oligomerization of the NLR scaffold, followed by recruitment, clustering and auto-activation of a pro-inflammatory caspase, caspase-1, usually via a protein adaptor ASC (2). Upon activation, caspase-1 mediates the maturation and secretion of pro-inflammatory cytokines such as interleukin (IL)-1β and IL-18, and rapidly induces a lytic form of inflammatory cell death (pyroptosis).

These caspase-1 functions together mediate host-protective inflammatory and antimicrobial responses. IL-1β and IL-18 drive pro-inflammatory responses, including the recruitment and activation of phagocytes (4), whilst pyroptotic cell death releases intracellular pathogens into the extracellular environment, rendering them susceptible to neutrophil-mediated destruction (115). Specific Gram-negative bacteria encoding type 3 or 4 secretion systems, such as *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium, *Stm*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) trigger activation of the NLRC4 inflammasome (29, 51). NLRC4 activation by *S.* Typhimurium occurs upon cytosolic recognition of bacterial ligands (needle protein, PrgJ rod protein, bacterial flagellin) following their translocation into the host cell cytosol by the *Salmonella* pathogenicity island-1 type 3 secretion system (SPI1-T3SS) (45, 47, 54). NLRC4 activation by these ligands is mediated by NAIP co-receptors, also encoded within the NLR family (44, 45, 47, 48). NLRC4 has a CARD domain and so can interact directly with pro-caspase-1 through homotypic CARD-CARD interactions, bypassing an absolute requirement for the ASC inflammasome adaptor. This ASC-independent NLRC4 inflammasome was reported to form a death complex that drives caspase-1-dependent macrophage lysis (8). However, maximal cytokine processing by the NLRC4 inflammasome in macrophages requires ASC (8).

Although recent years have seen great progress in our understanding of the molecular mechanisms underlying NLRC4 inflammasome activation, several key questions regarding NLRC4 biology remain outstanding. One such question is the identity of the cell types responsible for initiating NLRC4 pathways during *in vivo* infection; most NLRC4 research to date has concentrated on macrophages and dendritic cells without clear data indicating these are the dominant cell types triggering NLRC4 activation, IL-1β/IL-18 production and pyroptosis *in vivo*. Another key question is the extent to which inflammasome-dependent processes (cytokine production versus cell death) are inter-regulated. Production of active IL-1β/IL-18 is controlled by caspase-1 at two levels: cytokine processing from the inactive precursor to the mature form, and cytokine release from the cytosol to the extracellular space. It is currently unclear whether the release of mature cytokine occurs solely via an active caspase-1-dependent unconventional secretion pathway (11) or whether passive release during caspase-1-dependent cell death contributes to cytokine export. It is thus presently unresolved whether pyroptotic cell death is actually necessary for full inflammasome-dependent cytokine production (through contributing to cytokine release) or alternatively, whether pyroptosis serves to limit cytokine production by preventing the

further processing of pro-caspase-1, pro-IL-1 and pro-IL-18 cytosolic pools. If the latter, this raises the further intriguing question of how inflammasome pathways trigger sustained cytokine production during *in vivo* infection and inflammatory disease, when inflammasome-activated cells would be expected to rapidly lyse, limiting their ability to produce further cytokines.

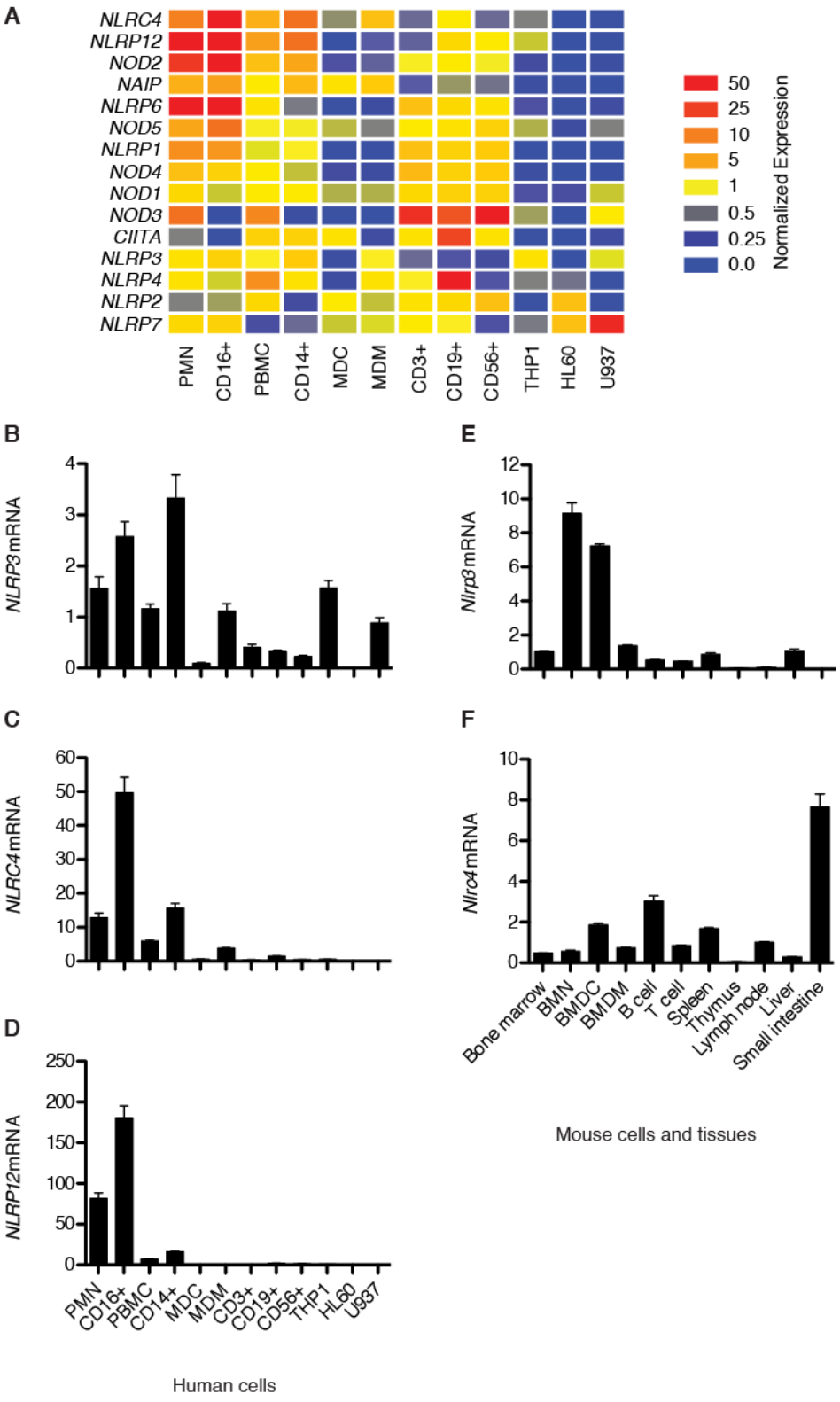
Neutrophils are the first cells recruited in large quantities to a site of infection or injury, and inflammasome activity itself induces IL-1-dependent neutrophil influx *in vivo*. The possibility that neutrophils themselves are competent for inflammasome function and thereby contribute to IL-1-dependent amplification of neutrophil recruitment/activation and pathogen clearance has not been carefully investigated. Herein, we report that the NLRC4 inflammasome is expressed by both human and mouse neutrophils, and that neutrophil NLRC4 selectively promotes *S.* Typhimurium-dependent cytokine processing, but not pyroptosis, *in vitro* and *in vivo*. This ability of neutrophils to produce caspase-1-dependent IL-1β without concomitant cell death is unique amongst NLRC4-signaling cells so far described, and for the first time explains the extended kinetics of inflammasome-dependent IL-1β production during acute infection. Furthermore, our finding that neutrophils trigger caspase-1-dependent IL-1β without concomitant pyroptosis challenges the dogma that these inflammasome-dependent processes are always initiated together. Taken together, our results indicate that inflammasome pathway specialization in neutrophils allows their prolonged release of IL-1β, allowing a positive amplification loop of neutrophil recruitment and activation, without compromising the crucial inflammasome-independent antimicrobial effector functions of neutrophils that would be lost if neutrophils were to rapidly lyse by pyroptotic cell death. Inflammasome pathway specialization in neutrophils thus maximizes host pro-inflammatory and antimicrobial responses during *Salmonella* challenge.

3.3 Results

3.3.1 Neutrophils express multiple NLRs

Neutrophils express a wide variety of PRR (228) and are likely candidates for NLR function. However, the expression and potential function of most NLRs in neutrophils is unclear (228). To date, only one recent study has specifically shown a function for a neutrophil inflammasome, that of NLRP3, in mediating host inflammatory responses during *in vivo* infection (229). In order to define the neutrophil NLR repertoire, we examined the

mRNA expression of all 22 NLR family members amongst purified immune cell fractions from human blood. Human blood was separated into polymorphonuclear cell (PMN) and peripheral blood mononuclear cell (PBMC) fractions by discontinuous density sedimentation. The PMN and PBMC fractions were then further purified on the basis of cell surface markers. 15 of the 22 human NLRs are reliably detectable in such cells (**Figure 3.1A**). Human neutrophils purified from the PMN fraction by CD16 positive selection express numerous NLRs, including the inflammasome scaffolds *NLRP1, NLRP3*, *NLRP6, NLRP12* and *NLRC4* (**Figure 3.1A, Supplementary Figure 3.1**). For example, *NLRP3* and *NLRC4* mRNAs were expressed in purified human neutrophils at similar or greater levels than other cell types for which NLRP3 and NLRC4 function is well characterized, such as $CD14^+$ monocytes, monocyte-derived macrophages (MDM) and monocyte-derived DCs (MDC) (**Figure 3.1B, C**), and *NLRP12* mRNA showed a striking enrichment in neutrophils versus other white blood cell populations within the panel (**Figure 3.1D**). Mouse bone marrow neutrophils (BMN) also expressed *Nlrp3*, *Nlrc4*, and *Nlrp1a* at similar or greater levels to bone marrow macrophages (BMDM) and/or bone marrow dendritic cells (BMDC) (**Figure 3.1E, F, Supplementary Figure 3.1**). In human and murine macrophages, cell stimulation with the TLR4 agonist, lipopolysaccharide (LPS) upregulates the expression of *Nlrp3* and *Nlrc4* (230). In keeping with this, we observed LPS-dependent upregulation of *Nlrp3* and *Nlrc4* expression in murine neutrophils (**Supplementary Figure 3.2**). Constitutive and inducible expression of inflammasome-forming NLRs in neutrophils suggests that like other PRR, inflammasomes may modulate neutrophil responses during inflammation.


A
NLRC4
NLRP12
NOD2
NAIP
NLRP6
NOD5
NLRP1
NOD4
NOD1
NOD3
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NLRP3
NLRP4
NLRP2
NLRP7
PMN
CD16+
PBMC
CD14+
MDC
MDM
CD3+
CD19+
CD56+
THP1
HL60
U937
50
25
10
5
1
0.5
0.25
0.0
Normalized Expression
B
NLRP3 mRNA
C
NLRC4 mRNA
D
NLRP12 mRNA
Human cells
E
Nlrp3 mRNA
F
Nlrc4 mRNA
Bone marrow
BMN
BMDC
BMDM
B cell
T cell
Spleen
Thymus
Lymph node
Liver
Small intestine
Mouse cells and tissues

Figure 3.1 Human and mouse neutrophils express multiple NLRs.

(A) Heat map showing median-normalized qPCR profiles of all NLRs reliably detected in human blood cell populations or myeloid cell lines. Expression profile detail for **(B)** *NLRP3*, **(C)** *NLRC4* and **(D)** *NLRP12* in purified human leukocyte populations and cell lines. Neutrophil expression of **(E)** *Nlrp3* and **(F)** *Nlrc4* was confirmed in a panel of mouse tissues and purified cell populations. Data are mean + SD of technical replicates and are representative of three independent experiments. Human cells: polymorphonuclear fraction (PMN; containing neutrophils and eosinophils), neutrophils ($CD16^+$ cells from PMN fraction, which excludes monocytes), bulk peripheral blood mononuclear cell fraction (PBMC), monocytes ($CD14^+$ cells from PBMC fraction), monocyte-derived dendritic cells (MDC), monocyte-derived macrophages (MDM), T cells ($CD3^+$), B cells ($CD19^+$), NK cells ($CD56^+$), and the monocytic cell lines THP-1, HL60 and U937. Mouse cells: bone marrow neutrophils (BMN), bone marrow-derived dendritic cells (BMDC), bone marrow-derived macrophages (BMDM), and splenic B and T lymphocytes. See also Figure S3.1 and S3.2.

3.3.2 The neutrophil NLRC4 inflammasome drives caspase-1 and IL-1β activation

Neutrophil expression of *NLRC4* mRNA suggested that neutrophils may participate in inflammasome-dependent antimicrobial responses against *Salmonella*. We investigated this possibility by infecting mature bone marrow neutrophils purified from wild-type (WT) and caspase-1/11 doubly-deficient (*Ice*$^{-/-}$) mice. Neutrophils were primed with LPS to induce pro-IL-1β expression, prior to infection with varying doses of log-phase *S.* Typhimurium SL1344. BMDMs, which are well established to activate NLRC4-dependent pathways (8, 54), were prepared and treated in parallel for comparison. Treatment with LPS alone did not stimulate IL-1β secretion in either neutrophils or macrophages, but 5 h *S.* Typhimurium infection triggered robust IL-1β secretion in LPS-primed neutrophils (**Figure 3.2A**), similar to infected WT macrophages (**Figure 3.2B**). *Salmonella*-induced IL-1β secretion in both neutrophils and macrophages was ablated in *Ice*$^{-/-}$ cells, indicating that IL-1β production was mediated by an inflammasome. Of note, the optimal dose of *S.* Typhimurium, and the timing of IL-1β secretion varied between neutrophils and macrophages.

Maximal *Salmonella*-dependent cytokine production by the NLRC4 inflammasome in macrophages requires the ASC inflammasome adaptor (8). We found that ASC was also required for optimal *Salmonella*-dependent IL-1β production from neutrophils (**Figure 3.2C**), similar to macrophages (**Figure 3.2D**). At higher multiplicities of infection, moderate ASC-independent IL-1β production was apparent for both neutrophils and macrophages, and we confirmed by immunoblotting that ASC-independent IL-1β was indeed the mature form (**Figure 3.2E**). As *Salmonella*-dependent IL-1β production was maximal in neutrophils and macrophages at distinct multiplicities of infection, for subsequent *in vitro* experiments we infected neutrophils and macrophages with *Salmonella* doses that yield robust IL-1β production (MOIs of 25 and 5, respectively).

In macrophages, IL-1β production during acute *Salmonella* infection relies upon the *Salmonella* SPI1-T3SS needle (54), which translocates bacterial proteins into the macrophage cytosol for recognition by the NAIP co-receptors of the NLRC4 inflammasome (44, 45, 47, 48). NLRP3 is also reported to contribute to *Salmonella* sensing in unprimed macrophages at later time points of infection (111, 146); the late time course of NLRP3 function in unprimed cells presumably reflects the functional requirement for infection-induced NLRP3 upregulation. We thus examined whether similar host and pathogen

determinants underlie IL-1β production from neutrophils. We infected WT or inflammasome-deficient neutrophils and macrophages with *S.* Typhimurium or its isogenic mutant (ΔSPI1) and assayed IL-1β production at 5 h post-infection. The *Salmonella* SPI1-T3SS translocon was required for IL-1β secretion from both macrophages and neutrophils (**Figure 3.2E, Supplementary Figure 3.3A-B**). As for macrophages, NLRC4 was essential for neutrophil IL-1β secretion at 5h post infection (**Figures 3.2E, Supplementary Figure 3.3A-B**). Western blot confirmed that IL-1β and caspase-1 are both efficiently processed, and their mature forms (IL-1β p17, caspase-1 p20) were released from WT but not *Nlrc4*$^{-/-}$ or caspase1/11-deficient *Ice*$^{-/-}$ neutrophils infected with WT *S.* Typhimurium (**Figure 3.2E**). We next examined whether neutrophils secrete other caspase-1-dependent cytokines upon NLRC4 activation. IL-18 secretion by *Salmonella*-infected neutrophils and macrophages followed a similar pattern to IL-1β production by these cells (**Supplementary Figure 3.3B-C**); however, LPS-primed neutrophils were poorer producers of IL-18, as compared to IL-1β. Consistent with previous reports (111, 146), we observed that *Nlrp3* deficiency led to a modest but reproducible suppression of IL-1β and IL-18 production in LPS-primed neutrophils and macrophages at 5 h post-infection (**Supplementary Figure 3.3A-D**). In examining this further, we observed that NLRP3 appeared to provide a supportive function to the NLRC4 inflammasome at 1 h post-infection, but the contribution of NLRP3 to NLRC4-dependent IL-1β production diminished as the time course progressed (**Supplementary Figure 3.3E**). We confirmed that caspase-1l was dispensable for neutrophil IL-1β production (**Supplementary Figure 3.3F**) under our experimental conditions. Together, these data indicate neutrophils can trigger NLRC4 function, wherein NLRC4-dependent caspase-1 drives the production of inflammasome target cytokines. IL-1β was the dominant cytokine secreted by this pathway in neutrophils, perhaps due to stronger intracellular expression of this cytokine precursor as compared to pro-IL-18 in LPS-primed cells.

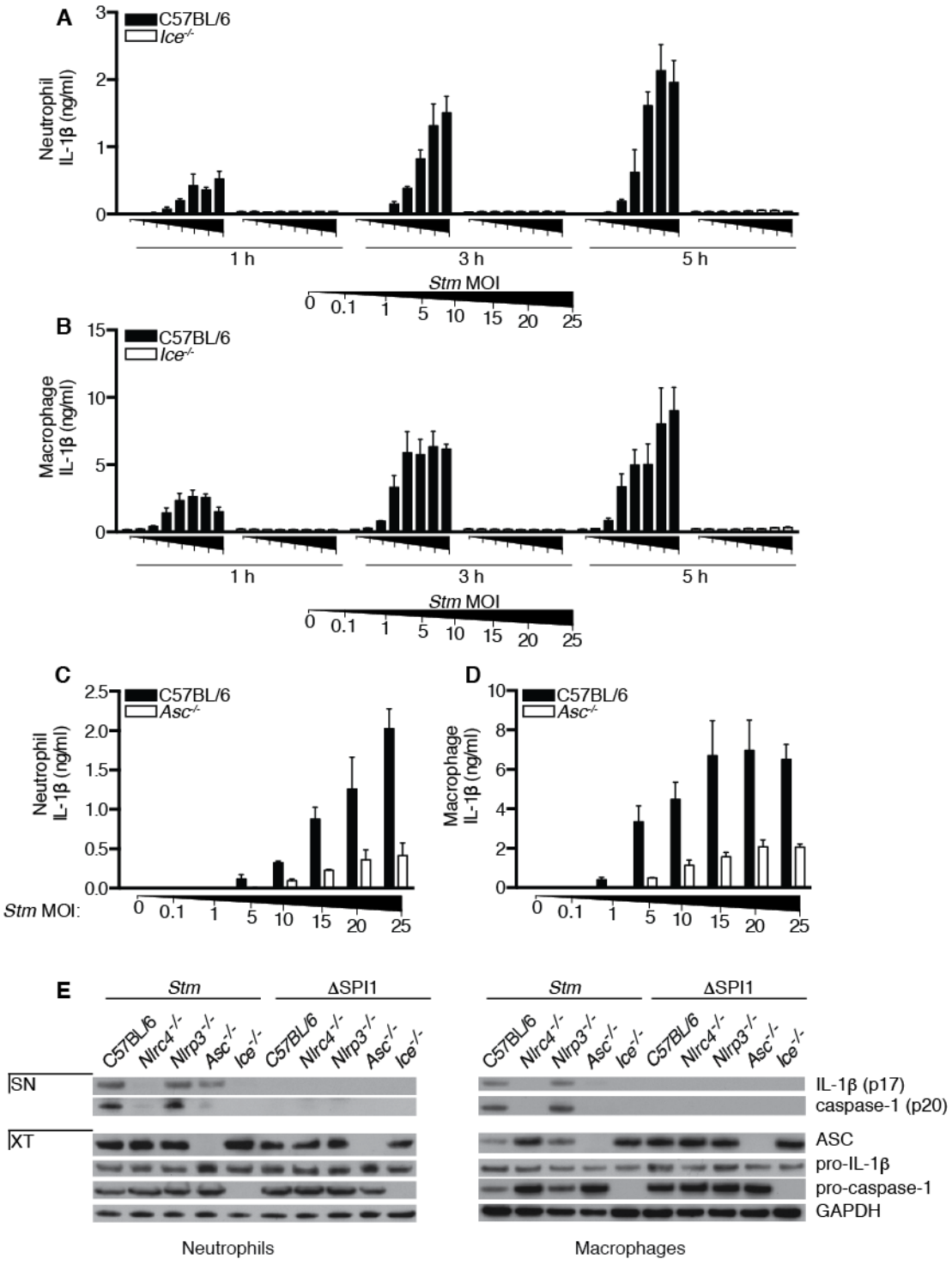

A
C57BL/6
Ice-/-
Neutrophil IL-1β (ng/ml)
1 h
3 h
5 h
Stm MOI
0 0.1 1 5 10 15 20 25
B
C57BL/6
Ice-/-
Macrophage IL-1β (ng/ml)
1 h
3 h
5 h
Stm MOI
0 0.1 1 5 10 15 20 25
C
C57BL/6
Asc-/-
Neutrophil IL-1β (ng/ml)
Stm MOI:
0 0.1 1 5 10 15 20 25
D
C57BL/6
Asc-/-
Macrophage IL-1β (ng/ml)
0 0.1 1 5 10 15 20 25
E
Stm
ΔSPI1
C57BL/6
Nlrc4-/-
Nlrp3-/-
Asc-/-
Ice-/-
SN
XT
IL-1β (p17)
caspase-1 (p20)
ASC
pro-IL-1β
pro-caspase-1
GAPDH
Neutrophils
Macrophages

Figure 3.2 Neutrophil NLRC4 activation triggers caspase-1 and IL-1β cleavage and secretion.
(A, C, E left panel) bone marrow neutrophils or **(B, D, E right panel)** bone marrow-derived macrophages were primed with 100 ng/ml LPS for 4 h before infection with *S.* Typhimurium (*Stm*). Cells were infected with increasing doses of *Stm* and IL-1β secretion was measured by ELISA at **(A, B)** 1, 3 and 5h post-infection, or **(C, D)** 5h post-infection. **(E)** Cells were infected with *Stm* or its isogenic ΔSPI1 mutant (MOI of 25 or 5 for neutrophils and macrophages, respectively) and cell extracts and supernatants were harvested at 5 h post-infection. Cleaved caspase-1 and IL-1β in cell supernatants (SN) and expression of pro-caspase-1, pro-IL-1β, ASC and GAPDH (loading control) in cell extracts (XT) were detected by western blot. All ELISA data are mean + SD of technical triplicate cell stimulations. All data are representative of 3 independent experiments, except B-D data, which are representative of 2 independent experiments. See also Figure S3.3.

3.3.3 The neutrophil inflammasome is activated during *in vivo* infection

We next examined whether, like bone marrow neutrophils challenged *in vitro*, neutrophils elicited and infected by *Salmonella in vivo* can produce inflammasome-dependent IL-1β. WT and *Ice*$^{-/-}$ mice were infected (i.p.) for 6 h with 10^6 CFU *S.* Typhimurium, after which elicited neutrophils were purified and cultured *ex vivo* in the presence of the membrane-impermeable antibiotic, gentamicin, to eliminate extracellular bacteria. Despite only low levels of active cell infection at these time points (**Figure 3.3A**), WT neutrophils efficiently secreted IL-1β *ex vivo* (**Figure 3.3B**), and IL-1β secretion was abrogated in *Ice*$^{-/-}$ mice, indicating that neutrophil IL-1β secretion is inflammasome-dependent. Consistent with *in vitro* data (**Supplementary Figure 3.3C**), purified neutrophils infected *in vivo* produced minimal caspase-dependent IL-18 (**Figure 3.3C**).

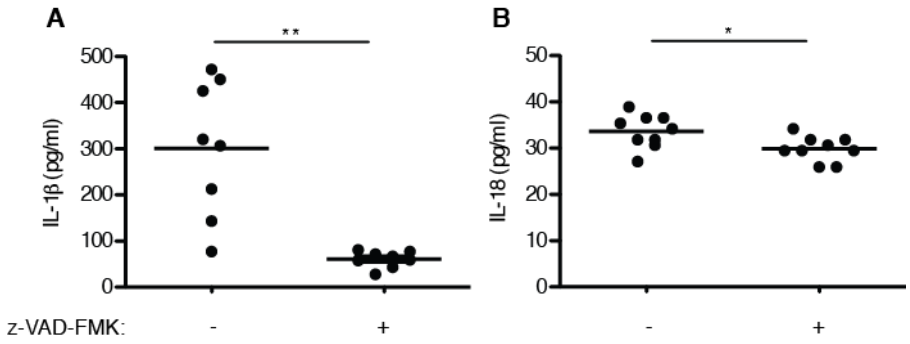


Figure 3.3 Neutrophils infected with *S.* Typhimurium *in vivo* trigger inflammasome-dependent IL-1β secretion.

Mice were challenged with *S.* Typhimurium (*Stm*) and sacrificed at 6 h post-infection. Ly6G$^+$ neutrophils in the peritoneal exudate were purified by positive selection. **(A)** Neutrophil intracellular bacterial loads were analyzed at 30 mins post-purification. **(B)** *In vivo* elicited neutrophils were cultured *ex vivo* for 8 h, and supernatants were collected for **(A)** IL-1β and **(B)** IL-18 quantification by ELISA. Data are mean of neutrophils elicited from individual mice (8-10 mice per genotype) combined from two independent experiments.

3.3.4 Neutrophils are a major source of IL-1β during acute Salmonella infection

Given that neutrophils possess a functional NLRC4 inflammasome (**Figure 3.2**), and neutrophils comprise a large component of the cellular infiltrate in many inflammatory settings, we hypothesized that the neutrophil compartment may be a significant contributor to IL-1β production during acute infection. We first measured the kinetics of immune cell recruitment during *Salmonella*-induced peritonitis. In uninfected mice, neutrophils ($CD11b^+Ly6C^+Ly6G^+$) and monocytes ($CD11b^+Ly6C^+Ly6G^-$) are barely detectable, and resident macrophages ($CD11b^+F4/80^+$) are the major $CD11b^+$ myeloid cells in the peritoneal cavity (**Figures 3.4A-B**). Neutrophils were recruited as early as 1 h post infection and neutrophil numbers increased by 165-fold compared to uninfected mice at 6h (**Figure 3.4B**). Monocytes were recruited more slowly and weakly as compared neutrophils, showing significant recruitment at 4 h (11-fold) and 6 h (26-fold) as compared to uninfected mice (**Figure 3.4B**). In contrast, macrophage abundance in the peritoneal exudate declined throughout the infection time course, showing significantly decreased abundance at 4 and 6 h (**Figures 3.4A-B**), consistent with previous reports (231, 232). We also profiled the kinetics of intracellular pro-IL-1β expression in myeloid cell subsets over the same infection time course. Macrophages, monocytes and neutrophils all showed a rapid induction in intracellular pro-IL-1β expression in the first 2 h of *S.* Typhimurium challenge (**Supplementary Figure 3.4A**).

To quantify the contribution of the neutrophil compartment to inflammasome-dependent IL-1β production *in vivo*, we examined the effect of neutrophil depletion. 24 h after α-Ly6G antibody injection, mice were challenged with *S.* Typhimurium over 6 h. α-Ly6G administration efficiently depletes neutrophils (**Figure 3.4C)** without affecting the abundance of other myeloid cells (**Supplementary Figures 3.4B-C**), as shown previously (233, 234). Consistent with the strong expression of intracellular pro-IL-1β in macrophages ($CD11b^+F4/80^+$) 1 h post *Salmonella* challenge (**Supplementary Figure 3.4A**), IL-1β levels in the peritoneal lavage fluid dramatically increased at 1 h post-infection in both undepleted and neutrophil-depleted mice (**Figure 3.4D**), suggesting that resident peritoneal macrophages provide the initial wave of IL-1β during *Salmonella* challenge. However, IL-1β levels continued to increase by 2-fold between 1 h and 2 h in undepleted mice (coinciding with neutrophil recruitment, **Figure 3.4B**) whilst IL-1β levels remained unchanged in depleted mice (**Figure 3.4D**). IL-1β levels remained higher (3-fold) in undepleted mice as compared neutrophil-depleted mice at 6 h post-infection (**Figure**

3.4D). Importantly, neutrophil depletion did not affect monocyte/macrophage intracellular pro-IL-1β (**Supplementary Figures 3.4D-E**), indicating that neutrophil depletion did not indirectly influence IL-1β expression or secretion from monocyte/macrophages. Collectively, these data suggest that resident macrophages provide the first wave of IL-1β production within the first hour of infection, whereas newly-recruited neutrophils continue to produce IL-1β thereafter. Similar results were obtained 12h post-infection, where neutrophil depletion reduced IL-1β levels in the peritoneum by 60% (**Supplementary Figures 3.4F-G**) and in the serum by 50% (**Figure 3.4E**). These results, coupled with our earlier data showing that *in vivo*-challenged neutrophils efficiently produce IL-1β *ex vivo*, suggest that neutrophils are a major cellular compartment for IL-1β production from 1 to 12 h post-infection. As expected given the known anti-microbial functions of neutrophils during *Salmonella* infection, neutrophil-depleted mice exhibited significantly higher bacterial burdens in both liver and spleen (**Figures 3.4F-*G***). Neutrophil-derived IL-1β likely contributes to host defense, as IL-1β neutralization increased bacterial burden in the liver and spleen (**Figure 3.4H-I**).

Consistent with our earlier observations that primed neutrophils are poor producers of IL-18 (**Figure 3.3B, Supplementary Figure 3.3**), acute (1-6 h) infection did not induce IL-18 levels in the peritoneum (**Supplementary Figure 3.5A**), and neutrophil depletion did not significantly affect IL-18 production after 12 h *Salmonella* challenge *in vivo* (**Supplementary Figure 3.5B**), despite modest IL-18 induction at this time point (**Supplementary Figure 3.5C**). Thus non-neutrophilic cells are the primary producers of IL-18 during acute *Salmonella* challenge.

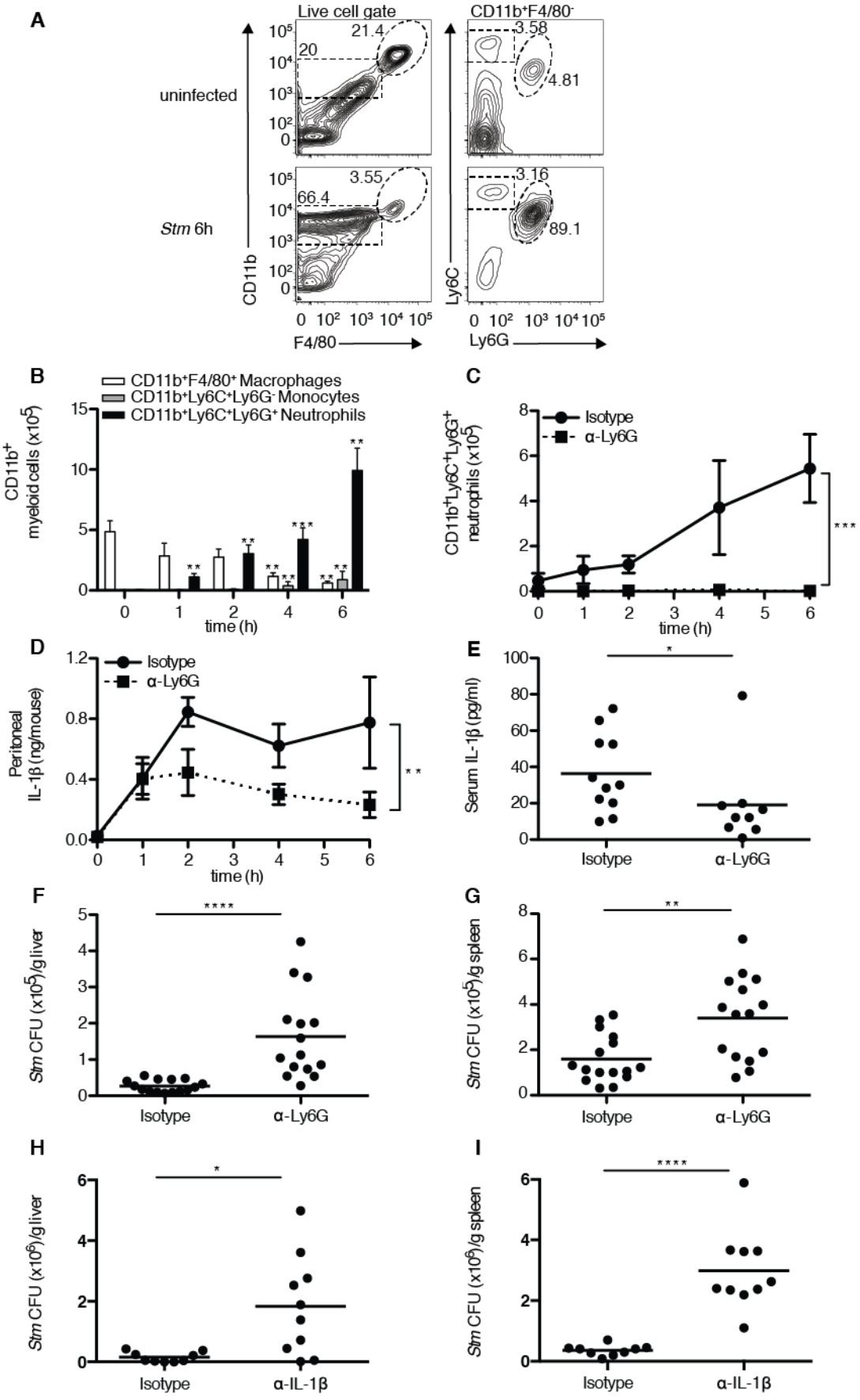

A
Live cell gate
CD11b+F4/80-
uninfected
Stm 6h
21.4
20
3.58
4.81
3.55
66.4
3.16
89.1
CD11b
Ly6C
F4/80
Ly6G
B
CD11b+F4/80+ Macrophages
CD11b+Ly6C+Ly6G- Monocytes
CD11b+Ly6C+Ly6G+ Neutrophils
CD11b+ myeloid cells (x10^5)
time (h)
C
Isotype
α-Ly6G
CD11b+Ly6C+Ly6G+ neutrophils (x10^5)
time (h)
D
Isotype
α-Ly6G
Peritoneal IL-1β (ng/mouse)
time (h)
E
Serum IL-1β (pg/ml)
Isotype
α-Ly6G
F
Stm CFU (x10^5)/g liver
Isotype
α-Ly6G
G
Stm CFU (x10^5)/g spleen
Isotype
α-Ly6G
H
Stm CFU (x10^6)/g liver
Isotype
α-IL-1β
I
Stm CFU (x10^6)/g spleen
Isotype
α-IL-1β

Figure 3.4 Neutrophils are major producers of IL-1β during acute *Salmonella* infection.

(A) Flow cytometry plots and **(B)** absolute abundance of macrophages ($CD11b^+F4/80^+$), neutrophils ($CD11b^+Ly6C^+Ly6G^+$) and monocytes ($CD11b^+Ly6C^+Ly6G^-$) following infection with *Stm.* The statistical significance for each cell type at each time point was calculated relative to uninfected. **(C-G)** Mice were administered with isotype control (mock-depleted) or α-Ly6G antibody (neutrophil-depleted) for 24 h before infection with *Stm*. **(C)** Neutrophil abundance in the peritoneum and **(D)** IL-1β levels in the peritoneal lavage fluid up to 6 h post-infection. At 12 h post-infection **(E)** serum IL-1β was measured by ELISA, and pathogen loads in the **(F)** liver and **(G)** spleen were quantified by serial dilution. **(H-I)** Mice were administered with isotype control or α-IL-1β neutralizing antibody for 16 h prior to infection with *Stm*. At 24 h post-infection, pathogen loads in the **(H)** liver and **(I)** spleen were quantified by serial dilution. Flow cytometry plot in **(A)** is representative of at least 4 independent experiments. Data are mean of **(B-D)** 3-8 individual mice per time point combined from 2-3 independent experiments, **(E-G)** 14-16 mice pooled from 3 independent experiments, or **(H-I)** 9-10 mice pooled from 2 independent experiments. See also Figures S3.4 and S3.5.

3.3.5 Neutrophils are resistant to pyroptosis *in vitro* and *in vivo*

In addition to the regulation of inflammasome-dependent cytokines, a hallmark of NLRC4 activation in macrophages and dendritic cells is the rapid induction of caspase-1-dependent but cytokine-independent pyroptotic cell death (29). We thus examined whether *Salmonella* infection also triggered NLRC4-dependent neutrophil death in concert with cytokine regulation. As for previous experiments, we primed neutrophils and macrophages with LPS and then infected cells *in vitro* with increasing doses of *S.* Typhimurium. Surprisingly, NLRC4-dependent caspase-1 activation (**Figure 3.2**) did not trigger concomitant neutrophil death, as assessed by light microscopy (not shown) or by quantifying the release of intracellular lactate dehydrogenase (LDH) into the supernatant (**Figure 3.5A**). By contrast, macrophages underwent rapid caspase-1-dependent cell death upon *S.* Typhimurium infection (**Figure 3.5B**). Macrophage pyroptosis was evident as early as 1 h post-infection, whereas neutrophils showed no evidence of caspase-1-dependent cell death as late as 5h (**Figures 3.5A-B**) or even 16h (**Supplementary Figure 3.6A**) post-infection, despite clear neutrophil death when treated with an apoptotic trigger, staurosporine, at these time points (**Supplementary Figure 3.6B**). In keeping with the lack of inflammasome-dependent cell death in neutrophils, and the reported ASC-independence of NLRC4/caspase-1-triggered pyroptosis in macrophages (8), *Asc*-deficiency did not affect LDH release in neutrophils or macrophages at 5 h post-infection (**Figures 3.5C-D**). While NLRC4 and the *Salmonella* SPI1-T3SS were absolutely required for *Salmonella*-dependent cell death in macrophages, neither WT *Salmonella* nor its isogenic ΔSPI1 mutant triggered neutrophil cell death, and this was unaffected by knockout of inflammasome components (**Supplementary Figure 3.6C-D**). To test whether the ability of neutrophils to resist caspase-1-dependent cell death was a general feature of inflammasome pathways in this cell type, we examined pyroptotic cell death downstream of a non-NLR inflammasome. AIM2 is a cytosolic dsDNA sensor of the HIN200 family, and a potent driver of pyroptotic cell death in macrophages. While AIM2 activation by transfected DNA transfection enabled caspase-1 processing, it did not trigger caspase-1-dependent neutrophil death (**Figure 3.5E**). Together, these *in vitro* data indicate that while NLRC4 activation in macrophages triggers rapid caspase-1-dependent cell death, neutrophils are resistant to this arm of caspase-1 signaling, despite the concomitant activity of other caspase-1 functions (e.g. IL-1β processing, **Figure 3.2**).

Pyroptotic death is a host defense mechanism that restricts the ability of *Salmonella* to replicate intracellularly within macrophages (115). As neutrophils were resistant to

pyroptotic cell death, we hypothesized that the beneficial function of caspase-1 in suppressing intracellular pathogen loads in macrophages may be absent in neutrophils. We thus infected neutrophils and macrophages with increasing doses of *Salmonella in vitro* as for previous experiments (in the presence of gentamicin to neutralize extracellular bacteria), and monitored intracellular bacterial loads over a time course of *in vitro* infection (**Figure 3.5F-G**). In line with previous studies (54, 115), caspase-1deficiency dramatically increased the intracellular bacterial burden of macrophages (**Figure 3.5G**), coinciding with their inability to undergo cell death, and both caspase-1-dependent and -independent mechanisms contributed to suppressing intracellular pathogen loads by 16 h post-infection. By contrast, the intracellular bacterial burden of neutrophils was not strongly affected by caspase-1 deficiency (**Figure 3.5F**). The intracellular loads of live bacteria were lower in neutrophils versus macrophages, likely reflecting their superior anti-microbial defense mechanisms, and we observed the presence of *Salmonella* in most neutrophils by microscopy (not shown). Surprisingly, both WT and *Ice*$^{-/-}$ neutrophils failed to suppress intracellular bacterial burden by 16 h post-infection. The inability of neutrophils to undergo pyroptosis may thus dramatically compromise their ability to defend against intracellular infection.

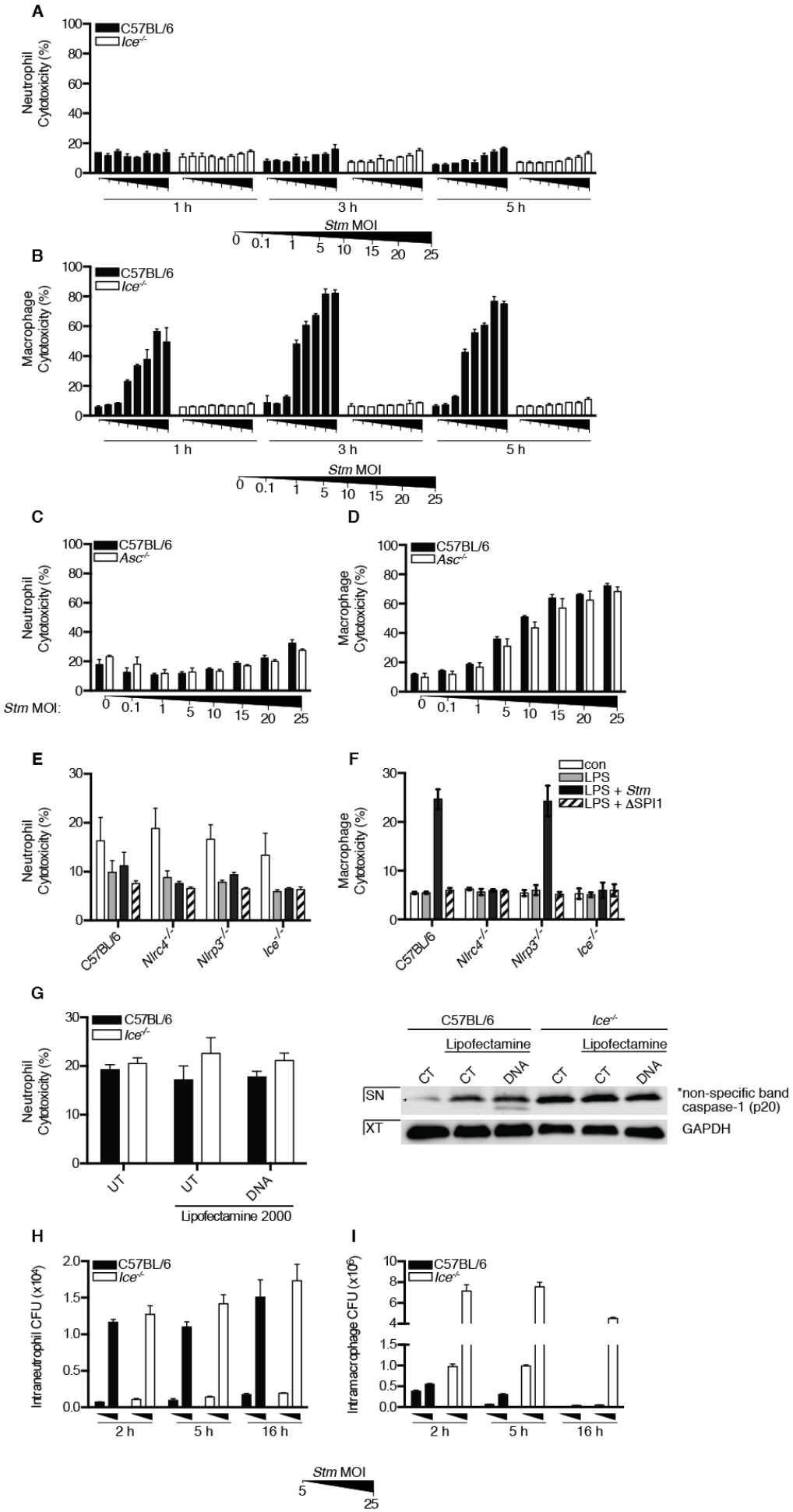

A
Neutrophil Cytotoxicity (%)
C57BL/6
Ice-/-
1 h
3 h
5 h
Stm MOI
0 0.1 1 5 10 15 20 25
B
Macrophage Cytotoxicity (%)
C57BL/6
Ice-/-
1 h
3 h
5 h
Stm MOI
0 0.1 1 5 10 15 20 25
C
Neutrophil Cytotoxicity (%)
C57BL/6
Asc-/-
Stm MOI:
0 0.1 1 5 10 15 20 25
D
Macrophage Cytotoxicity (%)
C57BL/6
Asc-/-
0 0.1 1 5 10 15 20 25
E
Neutrophil Cytotoxicity (%)
C57BL/6
Nlrc4-/-
Nlrp3-/-
Ice-/-
F
Macrophage Cytotoxicity (%)
con
LPS
LPS + Stm
LPS + ΔSPI1
C57BL/6
Nlrc4-/-
Nlrp3-/-
Ice-/-
G
Neutrophil Cytotoxicity (%)
C57BL/6
Ice-/-
UT
UT
DNA
Lipofectamine 2000
C57BL/6
Ice-/-
Lipofectamine
Lipofectamine
CT
CT
DNA
CT
CT
DNA
SN
XT
*non-specific band caspase-1 (p20)
GAPDH
H
Intraneutrophil CFU (x10⁴)
C57BL/6
Ice-/-
2 h
5 h
16 h
I
Intramacrophage CFU (x10⁶)
C57BL/6
Ice-/-
2 h
5 h
16 h
Stm MOI
5
25

Figure 3.5 Neutrophils do not undergo *Salmonella*-dependent pyroptosis *in vitro*.
Cytoplasmic LDH release into the supernatant, as compared to total intracellular LDH of uninfected cells was quantified as a measure of cell death. **(A, C, F)** bone marrow neutrophils and **(B, D, G)** bone marrow-derived macrophages were all primed for 4 h with LPS before infection with *Stm*. Cells were infected with increasing *Stm* doses and LDH release was measured at **(A, B)** 1, 3 and 5 h, or **(C, D)** 5 h post-infection. **(E)** Cells were LPS-primed for 3 h before lipofectamine transfection of calf thymus DNA. LDH release was measured at 6 h post transfection. Caspase-1 cleavage was measured in cell supernatants (SN) by immunoblotting, relative to expression of the GAPDH loading control in cell extracts (XT). **(F-G)** Cells were LPS-primed for 4 h and infected with *Stm* (MOI 5 or 25). Intracellular bacterial CFU of bone marrow neutrophils **(F)** and bone marrow-derived macrophages **(G)** were enumerated. Data are mean + SD of technical triplicate cell stimulations and are representative of **(A-B)** 3 or **(C-G)** 2 independent experiments.

To investigate whether *in vivo* challenged neutrophils are also resistant to pyroptosis, we infected WT and *Ice*$^{-/-}$ mice with *S.* Typhimurium i.p for 6 h, purified the *in vivo*-infected neutrophils and cultured them *ex vivo* for 8 h. Despite inflammasome-dependent IL-1β release (**Figure 3.3A**), caspase-1/11 deficiency did not affect the release of intracellular LDH (**Figure 3.6A**). To further investigate this phenomenon in a fully *in vivo* setting, we challenged mice with *S.* Typhimurium i.p. for 1.5 h. As inflammasome-dependent cell death triggers a rapid loss in plasma membrane integrity, pyroptotic cells can be identified with the membrane impermeable dye, 7-AAD (115). 18% of resident peritoneal macrophages were no longer viable at 1.5 h post-*Salmonella* challenge, but macrophage death was two-fold reduced in *Ice*$^{-/-}$ animals (**Figure 3.6B**). In contrast, 7-AAD uptake by neutrophils at the same time point was low, and slight differences in neutrophil death in WT versus *Ice*$^{-/-}$ animals were not statistically significant (**Figure 3.6B**). To ensure sufficient time for the activation of caspase-1-dependent programs in newly-recruited neutrophils, the experiment was repeated using an extended infection time course of 6 h. As for 1.5 h (**Figure 3.6B**), 7-AAD uptake remained low amongst neutrophils at 6 h post-infection, and showed no significant difference between WT and *Ice*$^{-/-}$ neutrophils (**Figure 3.6C**). In all, our *in vitro* and *in vivo* data indicate that while neutrophils drive substantial inflammasome-dependent IL-1β release, they possess a specialized inflammasome pathway that renders them resistant to pyroptotic cell death during challenge with *Salmonella.*

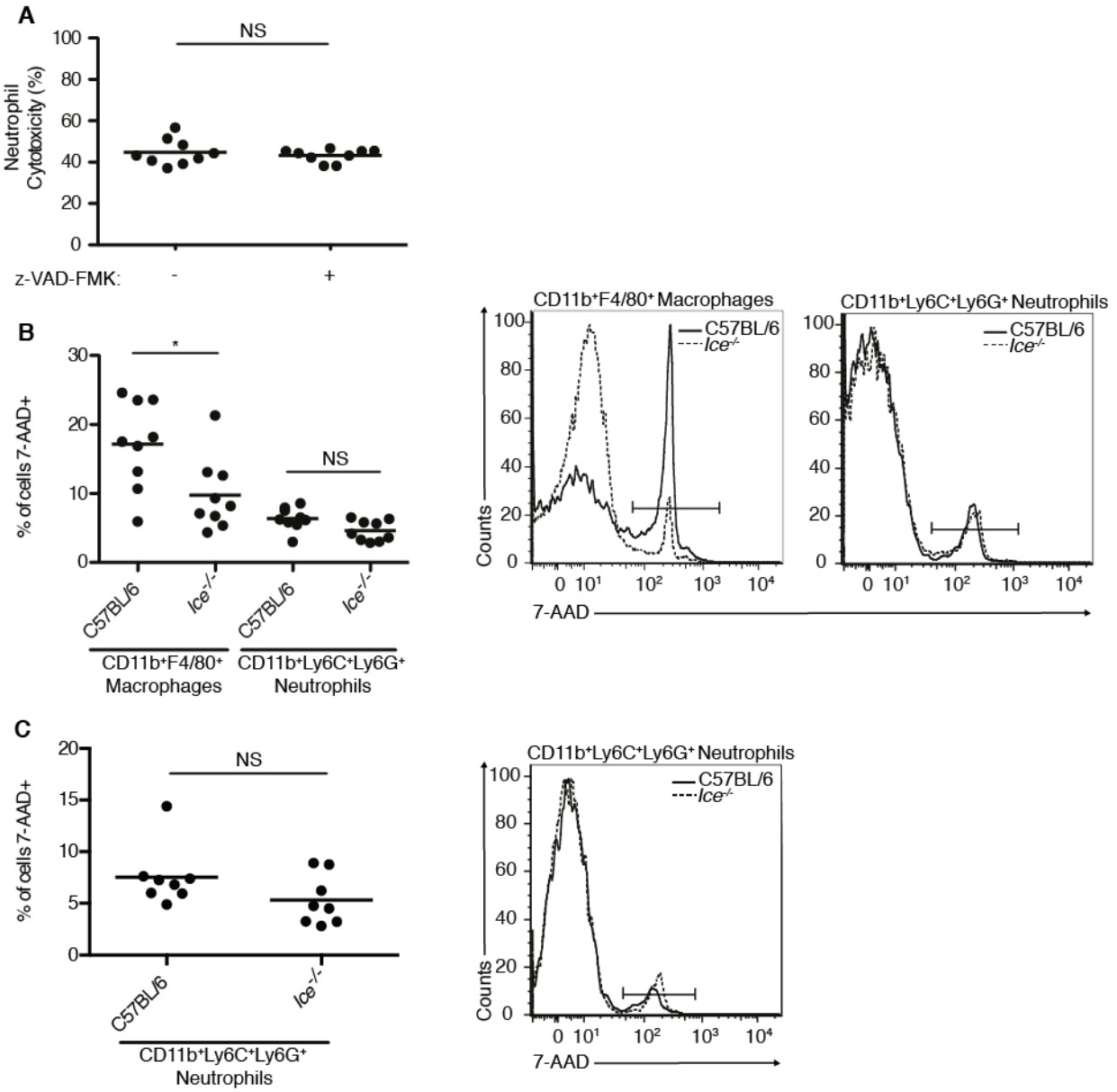


Figure 3.6 Neutrophils infected with *Salmonella in vivo* resist pyroptotic cell death.

(A) Mice were challenged with *Stm* and Ly6G$^+$ neutrophils in the peritoneal exudate at 5.5 h post-infection were purified by positive selection. *In vivo* elicited neutrophils were cultured *ex vivo* for 5 h in the presence or absence of 50 μM z-VAD-FMK, and assayed for LDH release. Data are mean of neutrophils elicited from 8 individual mice combined from two independent experiments. **(B, C)** Percentage of 7-AAD$^+$ cells amongst macrophages or neutrophils in the peritoneal exudate following *Stm* challenge for **(B)** 1.5 h or **(C)** 6 h, and representative flow cytometry plots. Data are mean of 8-9 individual mice combined from two independent experiments.

3.4 Discussion

The NLRC4 inflammasome pathway is now well established to mediate host inflammatory and antimicrobial responses, leading to pathogen control and clearance. However, the key cell types responsible for initiating NLRC4 pathways during *in vivo* infection with *Salmonella* were unclear. During *Salmonella* intra-peritoneal challenge, peritoneal macrophages quickly respond to *Salmonella* challenge with NLRC4 inflammasome formation and caspase-1 activation, culminating in rapid caspase-1-dependent IL-1β production and pyroptotic cell death. However, despite rapid macrophage lysis (apparent as early as 1.5 h), peritoneal IL-1β levels continue to rise past 24 h post-infection, suggesting the possibility that other cells may be able to signal via inflammasomes at a site of infection. Neutrophils predominate the early inflammatory infiltrate, presenting these cells as excellent candidates for driving NLRC4-dependent IL-1β production. Neutrophils were previously reported not to trigger NLRC4 inflammasome-dependent pathways, because they did not undergo pyroptotic cell death when infected with typical NLRC4 activators such as *S.* Typhimurium (53, 115), however, such studies did not assess the capacity for neutrophils to cleave caspase-1 or produce inflammasome-target cytokines.

Here we provide the first demonstration that *Salmonella* infection indeed triggers neutrophil NLRC4-dependent caspase-1 activation and IL-1β production *in vitro* and that neutrophils are a major compartment for inflammasome-dependent IL-1β production *in vivo*. By specifically depleting neutrophils *in vivo* prior to infection with *S.* Typhimurium, we quantified the contribution of neutrophils to both the IL-1β response and pathogen burden during acute infection. Neutrophil depletion dramatically increased pathogen loads in liver and spleen, consistent with the important function of these cells in controlling murine *Salmonella* infection as is well established in intravenous, intraperitoneal and oral challenge models (115, 234-240). Our data suggest that after an initial burst of IL-1β release from resident macrophages, recruited neutrophils become the predominant source of secreted IL-1β from 1 to 12 h post-infection. Neutrophil-derived IL-1β is thus likely to mediate a positive amplification loop driving further neutrophil recruitment and activation, presenting a new auto-regulatory paradigm for these cells during pathogen control. Of note, similar pathways may control neutrophil function downstream of other neutrophil-expressed inflammasomes, such as AIM2 and NLRP3. Three recent reports suggest that the NLRP3 inflammasome enables IL-1β processing in neutrophils, *in vitro* (241, 242) and *in vivo* (229), while pyroptosis was not examined. We confirmed herein that, as for

macrophages as was previously published (111, 146), NLRP3 appears to collaborate with the NLRC4 inflammasome in neutrophils. The published literature for NLRP3 function in neutrophils, and our studies presented here characterizing neutrophil NLRC4, NLRP3 and AIM2 inflammasome function, collectively overturn the general assumption that neutrophils can only trigger IL-1β production via inflammasome-independent mechanisms such as Fas receptor ligation (243) or cleavage via granular proteases (244). Indeed, in the *Salmonella* model studied here, neutrophil-derived IL-1β was entirely dependent on inflammasome-activated caspase-1. The inflammasome dependency of neutrophil IL-1β may depend on the microbial trigger, as neutrophils produced IL-1β independently of inflammasomes during *P. aeruginosa* infection (244).

The contribution of IL-1β to host defense in murine *Salmonella* challenge is somewhat controversial. In oral infection models, deficiency in *Il-1b* or its receptor was associated with moderately increased susceptibility to *Salmonella* infection in BALB/c (234) or C57BL/6 (245) mice, although the latter is controversial (234). For intraperitoneal infection, IL-1β/IL-1R appears dispensable for pathogen control and mouse survival at 3 days post-infection, while the important protective functions of IL-18 in host defense are evident at this time point (234, 245). Since IL-1β exerts a range of activities to promote neutrophil-mediated pathogen clearance in a number of other bacterial infection models (4), and our data indicate that IL-18 levels are unchanged in the acute (0-6 h) phase of intraperitoneal challenge when neutrophils predominate, we investigated whether IL-1β contributes to pathogen control during acute infection. Indeed, significantly increased bacterial burdens were evident in the organs of IL-1β-neutralized mice at 12 h post-infection. In light of previous studies, our data suggest a model in which macrophage- and neutrophil-derived IL-1β supports host defense in the acute phase of infection, while IL-18 production becomes important for microbial control in the latter stages of infection.

The neutrophil NLRC4 pathway is unique amongst cells described to date. Despite clear NLRC4-dependent caspase-1 activation, neutrophils did not undergo pyroptotic cell death, *in vitro* or *in vivo.* The resistance of neutrophils to pyroptotic cell death appears to allow sustained IL-1β release, as compared to macrophages where cytokine production plateaued within 3 h, coinciding with cell lysis. While our observation of caspase-1-dependent cytokine processing uncoupled from pyroptotic death is the first report of neutrophil signal specialization downstream of NLRs, specialization in other PRR signaling pathways (e.g. TLRs, CLRs) is already well documented (228). For example, while TLR4 in macrophages elicits dual signaling pathways via MyD88 and TRIF, TLR4 solely signals through MyD88 in human neutrophils (246).

Our *in vivo* data suggest a model in which resident macrophages and recruited neutrophils collaborate, together coordinating caspase-1-dependent inflammation and pathogen elimination. Resident peritoneal macrophages respond to infection by secreting a rapid burst of IL-1β within the first hour to initiate inflammation and neutrophil influx, while neutrophils are the dominant IL-1β-secreting cells from 1 to 12 h post *Salmonella* challenge. A recent study from Miao and co-workers posits that macrophage pyroptosis is an innate defense mechanism that prevents *Salmonella* replication within macrophages, and allows bacterial release for more effective killing by neutrophils (115). Since neutrophils are well established as the primary cells mediating *Salmonella* elimination, the lack of neutrophil pyroptosis we observe makes intuitive sense, as microbial destruction rather than cell lysis is the appropriate response for a neutrophil encountering *Salmonella.* If neutrophils could not resist pyroptosis, their ability to eliminate *Salmonella* through classic antimicrobial mechanisms (reactive oxygen species, degranulation, phagocytosis, etc.) would be severely compromised, leading to pathogen dissemination. While neutrophil NLRC4 pathway specialization is probably necessary to ensure pathogen clearance in the short term, the lack of a functional pyroptotic pathway in neutrophils, coupled with the extended lifespan of neutrophils in inflammatory microenvironments (147, 228), may ultimately render these cells susceptible to intracellular pathogens. Indeed, several reports suggest that *Salmonella* (247, 248) as well as other pathogens such as *Neisseria gonorrhoeae* (249)*, Staphylococcus aureus* (250), *Chlamydia pneumoniae* (251), *Burkholderia pseudomallei* (53) and *Anaplasma phagocytophilum* (252) reside and replicate efficiently within neutrophils *in vivo,* and may use neutrophils to acquire nutrients, evade the immune system and disseminate to other tissues. Indeed, our observations from neutrophil infection *in vitro* suggest that their resistance to pyroptosis may contribute to the

inability of neutrophils to restrict intracellular *Salmonella* survival, similar to caspase-1-deficient macrophages.

The surprising lack of neutrophil pyroptosis has fundamental implications for our understanding of inflammasome pathways. Firstly, it suggests that the caspase-1-dependent pathways mediating cytokine processing versus death are mechanistically separable, in support of an earlier proposal that two distinct caspase-1 activation states drive cytokine processing versus lytic death (8). Until caspase-1-dependent cell death programs are elucidated, the mechanism by which neutrophils resist this process will remain elusive, but a likely scenario is that a caspase-1 proteolytic substrate required for the initiation of lytic cell death is not expressed in neutrophils. The finding that neutrophils have uncoupled IL-1β release from pyroptotic cell death also unequivocally confirms the existence of caspase-1-dependent unconventional protein secretion pathways, refuting the alternative possibility that IL-1β is passively released during caspase-1-dependent cell death (253). Furthermore, the extended kinetics of IL-1β production in neutrophils as compared to macrophages suggests that pyroptotic cell death actually functions to curtail macrophage IL-1β processing and release.

In summary, our data are the first to indicate that neutrophils posses a unique inflammasome pathway that resists caspase-1-directed lytic cell death, thereby permitting extended cytokine production. Our finding that neutrophils are major contributors to inflammasome-dependent IL-1β during acute *Salmonella* infection may actually underestimate the importance of neutrophil inflammasomes in human infection and disease, as neutrophils are grossly under-represented in murine as compared to human blood (254). NLRP3/12 dysfunction is associated with genetic and acquired human inflammatory diseases, including hereditary fever syndromes and gout (2), and these diseases are currently attributed to inflammasome dysfunction in monocytes/macrophages, in which cytokine production is intrinsically linked to cell death. This raises the intriguing question of why IL-1β production and inflammation are not self-limiting in these diseases, as one might expect IL-1β release to be transient if IL-1β-producing cells rapidly lyse. Our demonstration that human neutrophils express a range of inflammasome scaffolds, and that neutrophil IL-1β production proceeds without restriction by inflammasome-dependent cell death pathways, suggests that neutrophils may be critical cellular drivers of pathogenic IL-1β in human inflammatory disease.

3.5 Experimental Procedures

3.5.1 Bacterial strains

S. Typhimurium SL1344 strains were grown at 37°C in Luria-Bertani medium at 200 rpm. The SL1344 ΔSPI1 (Δ*InvA*) mutant was previously described (145). Overnight bacteria were diluted 1:40 and grown for 3 h to induce expression of the SPI1-T3SS. Antibiotics used were 20 μg/ml streptomycin and 50 μg/ml gentamicin.

3.5.2 Mice

Mice were backcrossed at least 10 times to C57BL/6 and all experiments were conducted with age- and sex-matched mouse cohorts. C57BL/6, *Nlrc4*$^{-/-}$ (29), *Nlrp3*$^{-/-}$ (40), *Asc*$^{-/-}$ (29), *Ice*$^{-/-}$ (226) and *Casp11*$^{-/-}$ (227) mice were housed in specific pathogen-free facilities at the University of Queensland. All protocols were approved by the Animal Ethics Committee at the University of Queensland.

3.5.3 Preparation of human and mouse cells

Fresh human peripheral blood was subject to discontinuous density sedimentation (Histopaque 1119 and 1077, Sigma) to separate neutrophils and eosinophils (densities >1.077 g/ml; polymorphonuclear PMN fraction) from cells with lower densities (peripheral blood mononuclear cells, PBMC). CD16$^+$ neutrophils were further purified from the polymorphonuclear fraction, and CD14$^+$ monocytes, CD4$^+$ T cells, CD19$^+$ B cells and CD56$^+$ NK cells were purified from the mononuclear fraction by magnetic-assisted cell sorting (MACS), according to standard protocols (Miltenyi Biotec). All MACS-purified cell preparations were assayed for purity by flow cytometry. Monocyte-derived DCs and monocyte-derived macrophages were differentiated for 7 days with GM-CSF (100 ng/ml) plus IL-4 (25 ng/ml), or M-CSF (100 ng/ml), respectively (all Immunotools). Preparations of CD16$^+$, CD14$^+$ and CD3$^+$ cells achieved 97-99% purity, while less abundant cell types were enriched (55% CD19$^+$ B cells; 70% CD56$^+$ NK cells). All mouse tissues for mRNA profiling were prepared as single cell suspensions. Bone marrow cells and splenocytes were subject to erythrocyte lysis prior to fractionation. Splenocytes were stained with B220 and CD3, and B and T lymphocytes were enriched using standard MACS techniques with anti-fluorochrome beads (Miltenyi Biotec). Bone marrow and elicited neutrophils were purified by surface labeling using α-Ly6G-FITC (NIMP-R14 or 1A8), and purified by MACS with α-FITC beads. MACS-purified fractions were assayed for cell purity by flow cytometry. Bone marrow and elicited neutrophil populations achieved >98% purity. Splenic B and T

cells were typically enriched to 88% and 64% purity, respectively. Bone marrow-derived macrophages and dendritic cells were differentiated as previously described (255, 256).

3.5.4 mRNA expression profiling

Human and mouse mRNA expression profiling by quantitative PCR was performed normalized to a human and mouse reference gene (HPRT) as previously described (255). All primer sequences are available on request. Primer pairs were designed to flank exon-exon junctions to avoid amplification of contaminating genomic DNA, and primer pair efficiencies were quantified and used in calculations to generate cDNA profiles.

3.5.5 Neutrophil and macrophage in vitro infection assays

BMN and BMDM were prepared as described above. BMNs were used for experiments on the day of purification and were plated in RPMI-1640 supplemented with 10% FCS and 10 mM HEPES (all Life Technologies), and 0.4 μg/mL aprotinin (Sigma) at a density of 3.3 x10^6 cells/ml, except experiments with ΔSPI1 bacteria that were plated at 2 x 10^7 neutrophils/ml. Differentiated BMDM were cultured at a density of 0.8 x 10^6 cells/ml in RPMI-1640 supplemented with 10% FCS, 1x Glutamax (Life Technologies), and 10^4 units/ml recombinant human M-CSF (a gift from Chiron). BMN or BMDM were primed with 100 ng/ml ultrapure *E. coli* K12 LPS (Invivogen) for 4 h to induce the expression of pro-IL-1β. BMN and BMDM were infected at the indicated MOI and centrifuged immediately at 700 g for 10 min at room temperature. Cells were incubated at 37 ^oC for 25 min to allow phagocytosis of extracellular bacteria. Complete media was then replaced with Opti-MEM (Life Technologies) supplemented with 50 μg/ml gentamicin (Life Technologies) for the remainder of the assay to kill extracellular bacteria. Cells were then further incubated for the indicated times before harvesting cell-free supernatants and cell extracts for cytokine production. Cell-free supernatants were analyzed at the indicated time points post-infection for LDH release.

3.5.6 Intracellular bacterial survival

BMN and BMDM were infected as described above with the following modifications. 25 min after centrifugation, cells were washed thrice with complete media containing 200 μg/ml gentamicin and cultured for a further 30 min to kill extracellular bacteria. Subsequently, cells were cultured in complete media containing 20 μg/ml gentamicin for up to 16 h.

3.5.7 *In vivo* infection and neutrophil depletion

Neutrophils were depleted by i.p. administration of 0.1 mg α-Ly6G antibody (endotoxin-free 1A8 clone, BioXcell), or mock-depleted by i.p. administration of 0.1 mg isotype control antibody (endotoxin-free 2A3 clone, BioXcell). 24 h after antibody injection, mice were challenged with i.p. 1 x 10^6 CFU log-phase *S.* Typhimurium SL1344. For IL-1β neutralization experiments, mice were administered 0.1 mg of IL-1β neutralizing antibody (endotoxin-free B122 clone, BioXcell) or isotype antibody (endotoxin-free, polyclonal hamster IgG, BioXcell) i.p. 16 h prior to *Salmonella* challenge. Mice were sacrificed at 1-24 h post-infection as indicated and the peritoneal cavity was flushed with 10 ml of DPBS. Cytokine levels and cellularity in the peritoneal lavage fluid were analyzed by ELISA and flow cytometry, respectively. Liver and spleen were homogenized and bacterial loads were determined using serial dilution. To culture *in vivo* challenged neutrophils *ex vivo*, neutrophils were purified from the peritoneal exudate using α-Ly6G MACS and cultured at 2 x 10^5 cells/well for a further 8 h in 125 μl Opti-MEM supplemented with 50 μg/ml gentamicin. Bacterial loads were quantified by serial dilution following 0.5 h incubation with 50 μg/ml gentamicin.

3.5.8 Flow cytometry

Myeloid cells were identified using α-CD11b (Pac Blue-conjugated, M1/70, BioLegend), macrophages were labeled with α-F4/80 (APC-conjugated, BM8, BioLegend), and neutrophils and monocytes were discriminated by α-Ly6G (FITC, PeCy7 or PE-conjugated 1A8, BioLegend) and α-Ly6C (PE-conjugated, HK1.4, BioLegend) staining. T cells were labeled with α-CD3 (APC/Cy7-conjugated, 17A2, BioLegend) and B cells were labeled with α-B220 (FITC-conjugated, RA3-6B2). Intracellular pro-IL-1β was detected using the NJTEN3 antibody clone (eBioscience). Dead cells were labeled with 7-AAD (Becton Dickinson). Cell profiles were acquired using a Canto II (Becton Dickinson) and analyzed using FlowJo software (Tree Star).

3.5.9 Inflammasome and pyroptosis assays

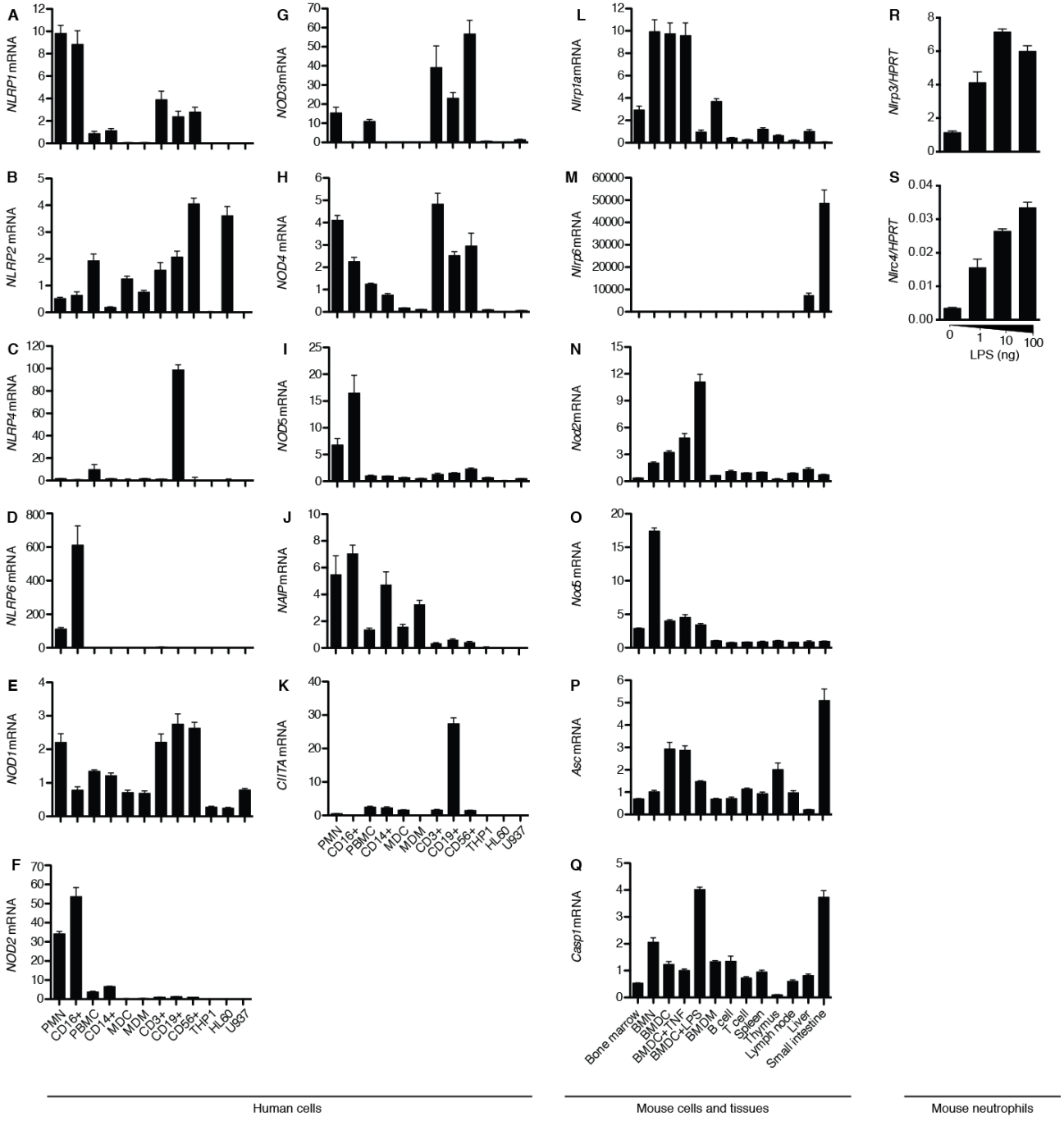
IL-1β (R&D Systems) and IL-18 (MBL, eBioscience) levels in cell-free supernatants and serum were analyzed by ELISA. For AIM2 inflammasome activation studies, neutrophils (10^6) were transfected with 0.125 μg calf thymus DNA using Lipofectamine 2000. Western blots were performed as previously described (256). In brief, cell-free supernatants were precipitated with chloroform and methanol, subject to SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted using standard methods. Antibodies included

IL-1β (polyclonal goat, R&D Systems), caspase-1 (Casper-1, Adipogen), ASC (polyclonal rabbit, Santa Cruz), and GADPH (polyclonal mouse, BioScientific). Cytotoxicity was analyzed by lactate dehydrogenase release (TOX7-1KT, Sigma).

3.5.10 Statistical analysis

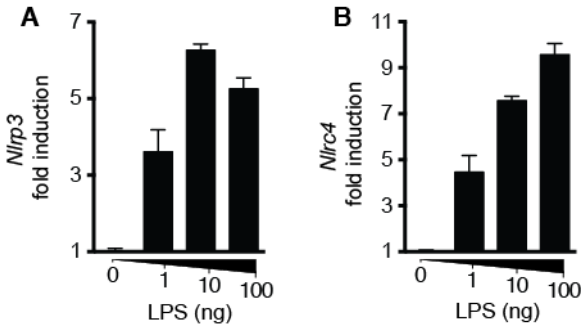
Statistical analyses were performed using non-parametric Mann-Whitney t-test or a two-way ANOVA using Prism Graphpad software. Data were considered significant when $p \leq 0.05$ (*), 0.005 (**), 0.001(***) and 0.0001(****).

3.6 Supplementary figures



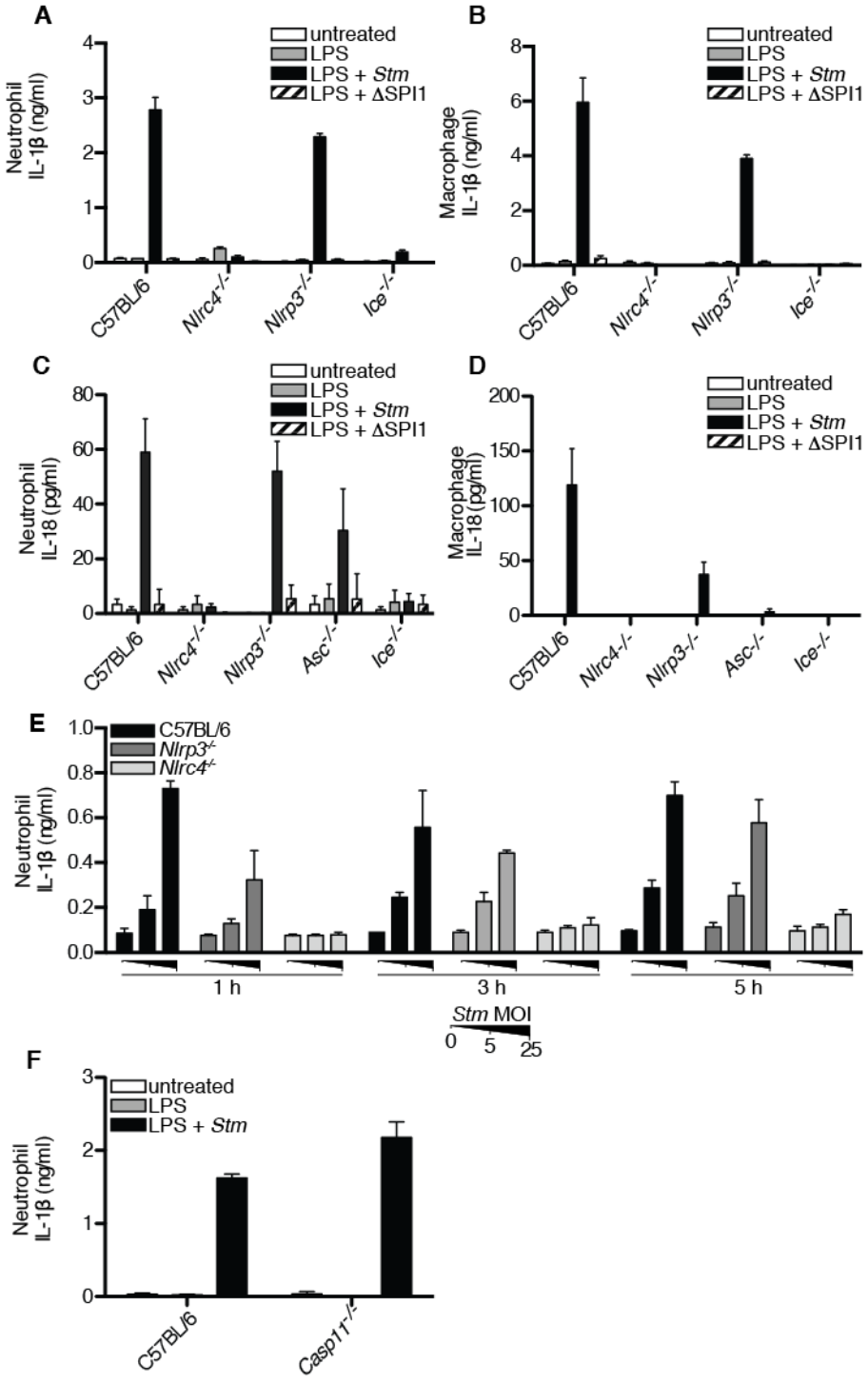
Supplementary Figure 3.1 Expression profile detail for NLRs expressed in purified human and mouse leukocyte populations, cell lines and tissues, related to Figure 3.1.

mRNA expression of **(A)** *NLRP1*, **(B)** *NLRP2,* **(C)** *NLRP4*, **(D)** *NLRP6*, **(E)** *NOD1*, **(F)** *NOD2*, **(G)** *NOD3*, **(H)** *NOD4*, **(I)** *NOD5*, **(J)** *NAIP*, and **(K)** *CIITA* was quantitated by qPCR in primary human blood populations: polymorphonuclear fraction (PMN; containing neutrophils and eosinophils), neutrophils ($CD16^+$ cells from PMN fraction, which excludes monocytes), bulk peripheral blood mononuclear cell fraction (PBMC), monocytes ($CD14^+$ cells from PBMC fraction), $CD14^+$ monocyte-derived dendritic cells (MDC), $CD14^+$ monocyte-derived macrophages (MDM), $CD3^+$ T cells, $CD19^+$ B cells and $CD56^+$ NK cells. The myeloid cell lines, THP-1, HL60 and U937 were also included on the panel. Data are mean + SD of technical triplicates and is representative of three experiments with independent blood donors. NLRs that were highly expressed in human neutrophils were examined for expression in mouse immune cell subsets and tissues by qPCR: **(L)** *Nlrp1a,* **(M)** *Nlrp6*, **(N)** *Nod2,* and **(O)** *Nod5*. mRNAs for the inflammasome components **(P)** *Asc* and **(Q)** *Casp1* were also measured in parallel. Cell populations are: BMN, bone marrow neutrophils; BMDC, bone marrow dendritic cells left untreated or treated overnight with TNF (25 ng/ml) or LPS (50 ng/ml); BMDM, bone marrow derived macrophages; B cells, $B220^+$ splenocytes; T cells, $CD3^+$ splenocytes. Data are mean + SD of technical triplicates and are representative of three independent experiments.


A
Nlrp3
fold induction
7
5
3
1
0
1
10
100
LPS (ng)
B
Nlrc4
fold induction
11
9
7
5
3
1
0
1
10
100
LPS (ng)

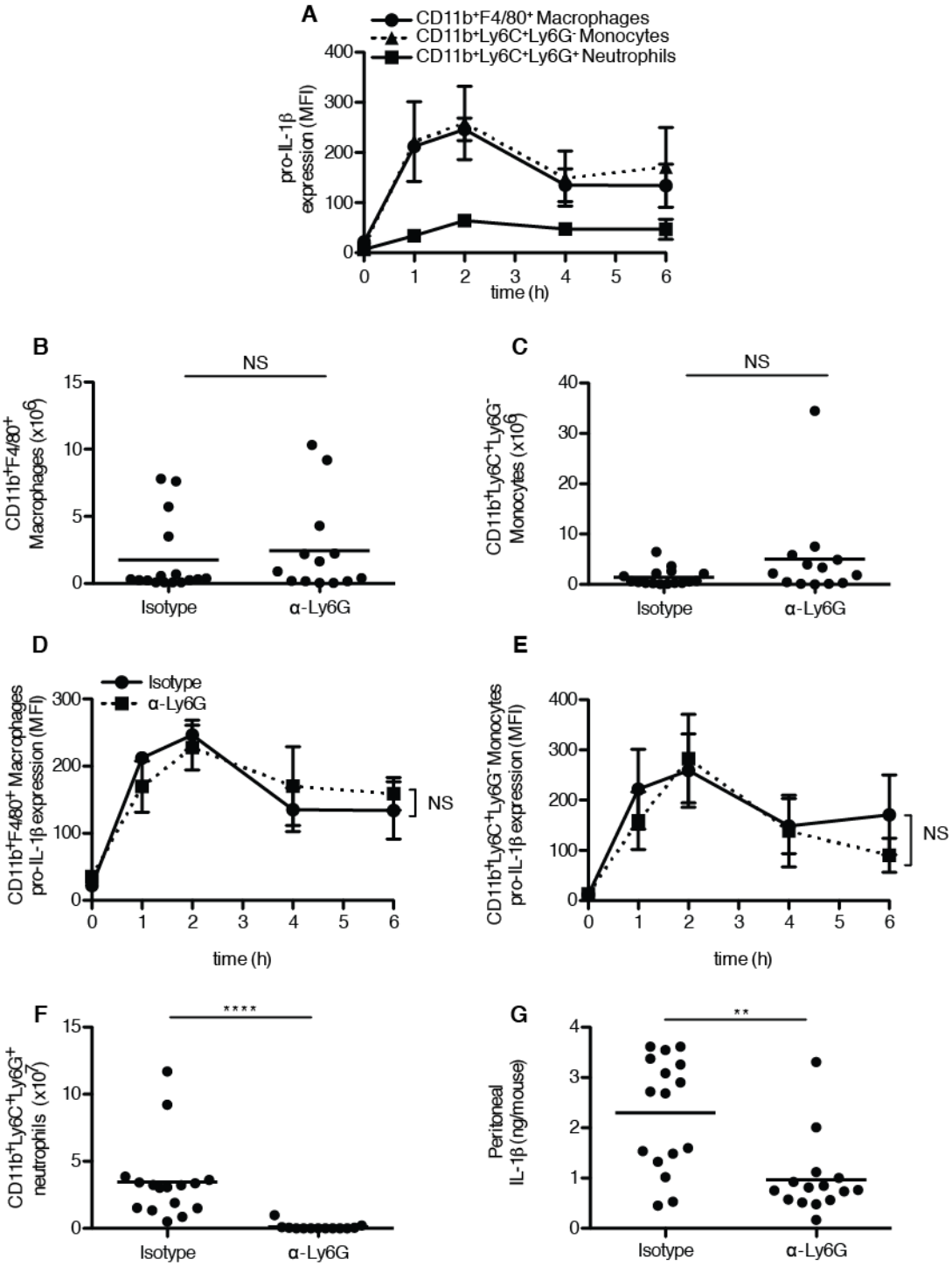
Supplementary Figure 3.2 Neutrophil *Nlrp3* and *Nlrc4* expression is induced by LPS, related to Figure 3.1.

BMN were treated with an increasing dose of LPS for 4 h and mRNA expression was quantified by qPCR. Data are mean + SD of technical triplicates.


A
Neutrophil IL-1β (ng/ml)
untreated
LPS
LPS + Stm
LPS + ΔSPI1
C57BL/6
Nlrc4-/-
Nlrp3-/-
Ice-/-
B
Macrophage IL-1β (ng/ml)
C
Neutrophil IL-18 (pg/ml)
Asc-/-
D
Macrophage IL-18 (pg/ml)
E
C57BL/6
Nlrp3-/-
Nlrc4-/-
Neutrophil IL-1β (ng/ml)
1 h
3 h
5 h
Stm MOI
0
5
25
F
Neutrophil IL-1β (ng/ml)
Casp11-/-

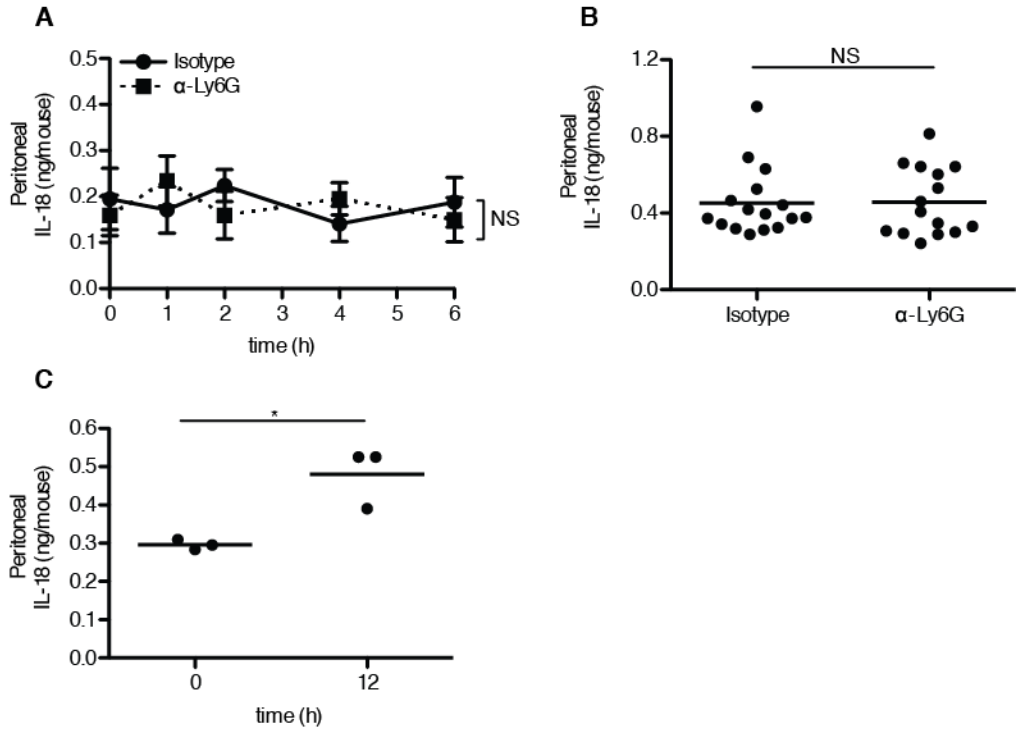
Supplementary Figure 3.3 NLRP3 collaborates with the NLRC4 inflammasome during *Salmonella* infection, related to Figure 3.2.

BMNs or BMDMs were treated with 100 ng/ml LPS before infection with *S.* Typhimurium (*Stm*) or its isogenic ΔSPI1 mutant. Neutrophils (**A, C, F**) and macrophages (**B, D**) were infected (MOI of 25 or 5 for neutrophils and macrophages, respectively) for 5 h and IL-1β (**A-B, F**) and IL-18 (**C-D**) release were measured by ELISA. (**E**) Neutrophils were infected with increasing doses of *Stm* and IL-1β secretion was measured by ELISA at 1, 3 and 5 h post-infection. Data are (**A-B**) mean + SD of technical triplicate cell stimulations and are representative of (**A**) 3 or (**B**) 2 individual experiments, **(C)** mean + SEM of 3 independent experiments, (**D**) mean + SD of technical triplicate cell stimulations and are representative of 3 independent experiments, **(E)** mean + SD of technical triplicate cell stimulations and are representative of 2 independent experiments, (**F**) mean + SD of technical triplicate cell stimulations.


A
CD11b+F4/80+ Macrophages
CD11b+Ly6C+Ly6G- Monocytes
CD11b+Ly6C+Ly6G+ Neutrophils
pro-IL-1β expression (MFI)
time (h)
B
NS
CD11b+F4/80+ Macrophages (x10^6)
Isotype
α-Ly6G
C
NS
CD11b+Ly6C+Ly6G- Monocytes (x10^6)
Isotype
α-Ly6G
D
Isotype
α-Ly6G
CD11b+F4/80+ Macrophages pro-IL-1β expression (MFI)
NS
time (h)
E
CD11b+Ly6C+Ly6G- Monocytes pro-IL-1β expression (MFI)
NS
time (h)
F
****
CD11b+Ly6C+Ly6G+ neutrophils (x10^7)
Isotype
α-Ly6G
G
**
Peritoneal IL-1β (ng/mouse)
Isotype
α-Ly6G

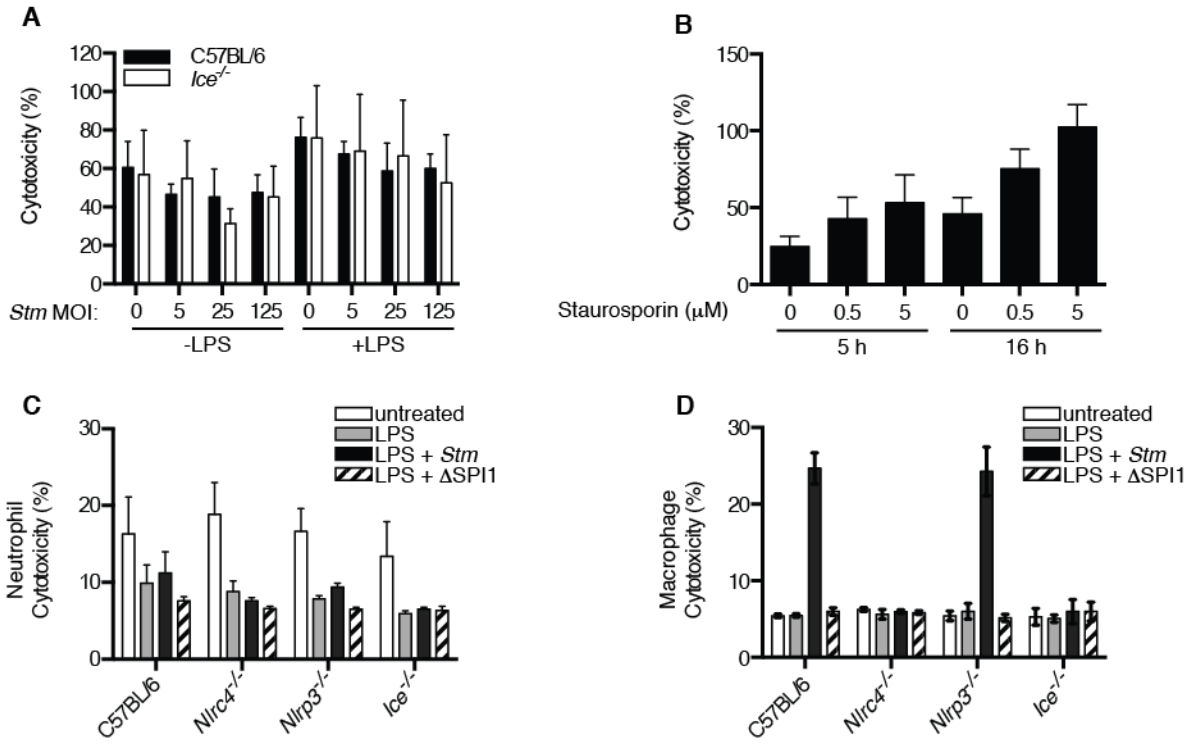
Supplementary Figure 3.4 Neutrophil depletion does not affect the abundance or intracellular pro-IL-1β expression of macrophages or monocytes, related to Figure 3.4.

(A) Mice were challenged with i.p. *Stm* and the peritoneal exudate was collected up to 6 h post-infection. Expression of intracellular pro-IL-1β in myeloid cells was measured by flow cytometry (mean fluorescence intensity). Data are mean ± SEM of 3-4 individual mice per time point combined from 2 independent experiments. **(B-G)** Mice were administered with isotype control (mock-depleted) or α-Ly6G antibody (neutrophil-depleted) for 24 h before infection with *Stm*. The abundance of (**B**) macrophages and (**C**) monocytes in the peritoneal exudate at 12 h post-infection was assessed by cell counting and flow cytometry. Data are mean of 14-16 mice pooled from 3 independent experiments. The intracellular expression of pro-IL-1β in peritoneal exudate (**D**) macrophages and (**E**) monocytes over a time course of infection was assessed by flow cytometry. Data are mean ± SEM of 3-5 individual mice per time point combined from 2 independent experiments. **(F)** Neutrophil abundance and **(G)** IL-1β levels in the peritoneal lavage fluid were also measured at 12 h post-infection. Data are mean of 14-16 mice pooled from 3 independent experiments.



Supplementary Figure 3.5 IL-18 is produced from 6-12 h post infection by non-neutrophilic cells, related to Figure 3.4.

Peritoneal IL-18 levels in mice **(A-B)** administered isotype versus α-Ly6G antibody for 24 h or **(C)** not administered antibody before *Stm* challenge for **(A)** 0-6 or **(B, C)** 12 h before quantitation of peritoneal IL-18 by ELISA. Data are mean ± SEM of (**A**) 3-5 individual mice per time point, combined from 2 independent experiments, mean of **(B)** 14-16 mice pooled from 3 independent experiments, or **(C)** 3 individual mice.



Supplementary Figure 3.6 Neutrophils are resistant to pyroptotic cell death, related to Figure 3.5.

(A) BMNs were plated in complete media and primed for 4 h with LPS or left untreated before infection with increasing doses of *Stm* for 16 h. **(B)** BMNs were treated with increasing concentrations of staurosporin to trigger apoptotic cell death, for 5 h and 16 h. (**C-D**) Cells were primed for 4 h with LPS and infected with *Stm* or its isogenic mutant (MOI of 25 or 5 for neutrophils and macrophages, respectively) and LDH release was measured at 1 h post-infection. Cytoplasmic LDH release into the supernatant, as compared to total intracellular LDH, was quantified as a measure of cell death. (**A-B**) Data are mean + SD of quadruplicate cell stimulations or (**C-D**) mean + SD of technical triplicate cell stimulations and are representative of 3 independent experiments.

4. The neutrophil NLRP3 inflammasome is activated by soluble but not particulate or crystalline agonists

The data presented here in Chapter 4 were submitted for consideration as a short communication to the European Journal of Immunology on 22 July 2015.

Contributor	Statement of contribution
Kaiwen Chen (Candidate)	Designed the study (45%). Wrote the paper (40%). Performed Figure 4.1C, 4.2B-E and 4.3 (40%).
Christina Groß	Designed the study (0%). Wrote the paper (5%). Provided initial observations investigated in this study, performed experiments (10%).
Adam Wall	Designed the study (0%). Wrote the paper (0%). Performed Figure 4.2A and Supplementary Figure 4.2 (10%).
Jennifer Stow	Designed the study (5%). Wrote the paper (0%). Provided reagents (5%). Performed experiments (0%).
Matthew Sweet (Associate Advisor)	Designed the study (0%). Wrote the paper (5%). Provided reagents (0%). Performed experiments (0%).
Kate Schroder (Advisor)	Designed the study (50%). Wrote the paper (50%). Provided reagents (95%). Performed Figure 4.1A-D, S4.1A-K and Supplementary Figure 3.4 (40%).

4.1 Abstract

Neutrophils express pattern recognition receptors (PRR), and regulate immune responses via PRR-dependent cytokine production. An emerging theme is that neutrophil PRRs often exhibit cell type-specific adaptations in their signalling pathways, which prompted us to examine NLRP3 inflammasome signalling in neutrophils, as compared to macrophages where NLRP3 function is well established. Here, we demonstrate that while neutrophils can indeed signal via the NLRP3 inflammasome, neutrophil NLRP3 selectively responds to soluble agonists but not particulate/crystalline agonists that trigger macrophage phagolysosomal rupture. In keeping with this, the lysosomotropic peptide Leu-Leu-OMe stimulated only weak neutrophil NLRP3-dependent IL-1β production, suggesting that lysosomal rupture is not a strong stimulus for NLRP3 activation in neutrophils. We validated our *in vitro* findings for poor neutrophil NLRP3 responses to particles *in vivo*, where we demonstrated that neutrophils do not significantly contribute to alum-induced IL-1β production *in vivo*. In all, our studies highlight that myeloid cell identity and the nature of the danger signal can strongly influence signalling by a single PRR, thus shaping the nature of the resultant immune response.

4.2 Introduction

Inflammasomes are cytoplasmic multi-protein complexes that drive the maturation of specific interleukin-1 (IL-1) family cytokines and induce an inflammatory form of cell death called pyroptosis. Inflammasomes consist of a sensor protein (e.g. a Nod-like receptor (NLR) protein), the caspase-1 protease, and often contain the common inflammasome adaptor, ASC (2). The NLRP3 inflammasome is of particular importance because it has the capacity to sense a wide variety of structurally unrelated molecules, including whole pathogens, bacterial toxins, metabolic products, insoluble molecules (particles, crystals and protein aggregates), and alarmins released from damaged tissues. Consequently, the NLRP3 inflammasome is central to host defence but also mediates pathological immune responses in numerous inflammatory and metabolic disorders such as Alzheimer's Disease, silicosis, asbestosis and gout (2). How a single protein responds to such a diverse range of agonists remains an enigma. Many studies posit that these distinct agonists elicit convergent cell stress signals that trigger NLRP3 activation and inflammasome assembly. Proposed mechanisms include K^+ efflux (20), lysosomal rupture by crystals, particles and protein aggregates (19), reactive oxygen species production (18) and calcium mobilisation (25). However, the precise nature of the NLRP3-activating stress

signal, and the mechanism by which NLRP3 senses such a signal, remains obscure. Also unclear is how a single receptor signalling module can tailor an *in vivo* immune response that is appropriate to such diverse cues such as tissue injury, infection, metabolic stress or environmental irritants; one possibility is that distinct NLRP3 agonists trigger NLRP3 signalling in different immune cell suites.

While the biology of NLRP3 has been widely studied in macrophages and dendritic cells, increasing evidence suggests that these cells are not the sole cell types driving NLRP3-dependent responses *in vivo.* Two recent studies demonstrated that neutrophils are major contributors to NLRP3-dependent IL-1β production during murine infection with *Staphylococcus aureus* (229) and *Streptococcus pneumoniae* (257). NLRP3 signalling pathways are poorly characterised in neutrophils, and it is increasingly appreciated that immune signalling pathways often display cell type-specific effects. For example, we recently reported that neutrophils selectively trigger caspase-1-dependent cytokine processing but not pyroptosis following NLRC4 activation by *Salmonella* (**Chapter 3**), while in macrophages, the NLRC4 inflammasome drives both cytokine processing and pyroptosis. Here, we investigated NLRP3 signalling in neutrophils, with the hypothesis that like NLRC4, the NLRP3 pathway may exhibit neutrophil-specific adaptations. In doing so, we demonstrate that soluble but not particulate or crystalline NLRP3 agonists trigger NLRP3-dependent responses in neutrophils. Our findings highlight cell type specificity in the ability to respond to individual NLRP3 agonists, and provide a possible mechanism by which NLRP3-dependent immune responses are tailored to the specific danger encountered.

4.3 Results and discussion

4.3.1 The neutrophil NLRP3 inflammasome selectively responds to soluble agonists

The neutrophil NLRP3 inflammasome is reported to trigger caspase-1-dependent IL-1β cleavage and release following infection with whole pathogen (*S. aureus* and *S. pneumoniae*) or bacterial pore-forming toxins (229, 241, 257). However, it was unclear whether other compounds established to activate NLRP3 in macrophages, such as insoluble compounds that cause phagolysosomal rupture, also activate NLRP3 in neutrophils. We investigated this possibility by stimulating purified bone marrow neutrophils and bone marrow-derived macrophages (BMDM) with a range of NLRP3 agonists. Cells were stimulated with lipopolysaccharide (LPS) to upregulate NLRP3 and pro-IL-1β prior to challenge with NLRP3 agonists (230, 258). The bacterial pore-forming

toxin and potassium ionophore, nigericin, triggered IL-1β secretion from both wild type (WT) neutrophils and macrophages (**Figure 4.1A-C**). IL-1β production by neutrophils required an inflammasome, as IL-1β secretion was significantly reduced in caspase-1/11-deficient *Ice*$^{-/-}$ neutrophils (**Figure 4.1A**), similar to *Ice*$^{-/-}$ macrophages (**Figure 4.1B**). Moreover, caspase-1/11 deficiency ablated IL-1β processing by neutrophils (**Figure 4.1C**). Other soluble activators of the NLRP3 inflammasome (e.g. extracellular ATP, R837) also triggered NLRP3/caspase-1-dependent IL-1β release in neutrophils (**Supplementary Figure 4.1A-B**), as for macrophages (27, 259). In macrophages and dendritic cells, a range of insoluble compounds can also trigger NLRP3 activation by rupturing phagolysosomes (16, 19). We next investigated if this pathway is also active in neutrophils. Surprisingly, neither alum nor silica particles, nor MSU crystals, triggered caspase-1-dependent IL-1β secretion from neutrophils (**Figure 4.1D-F**), while all elicited strong caspase-1-dependent IL-1β secretion from macrophages as anticipated (**Figure 4.1G-J**). Thus, neutrophils can mount robust NLRP3 inflammasome responses to soluble but not insoluble NLRP3 agonists.

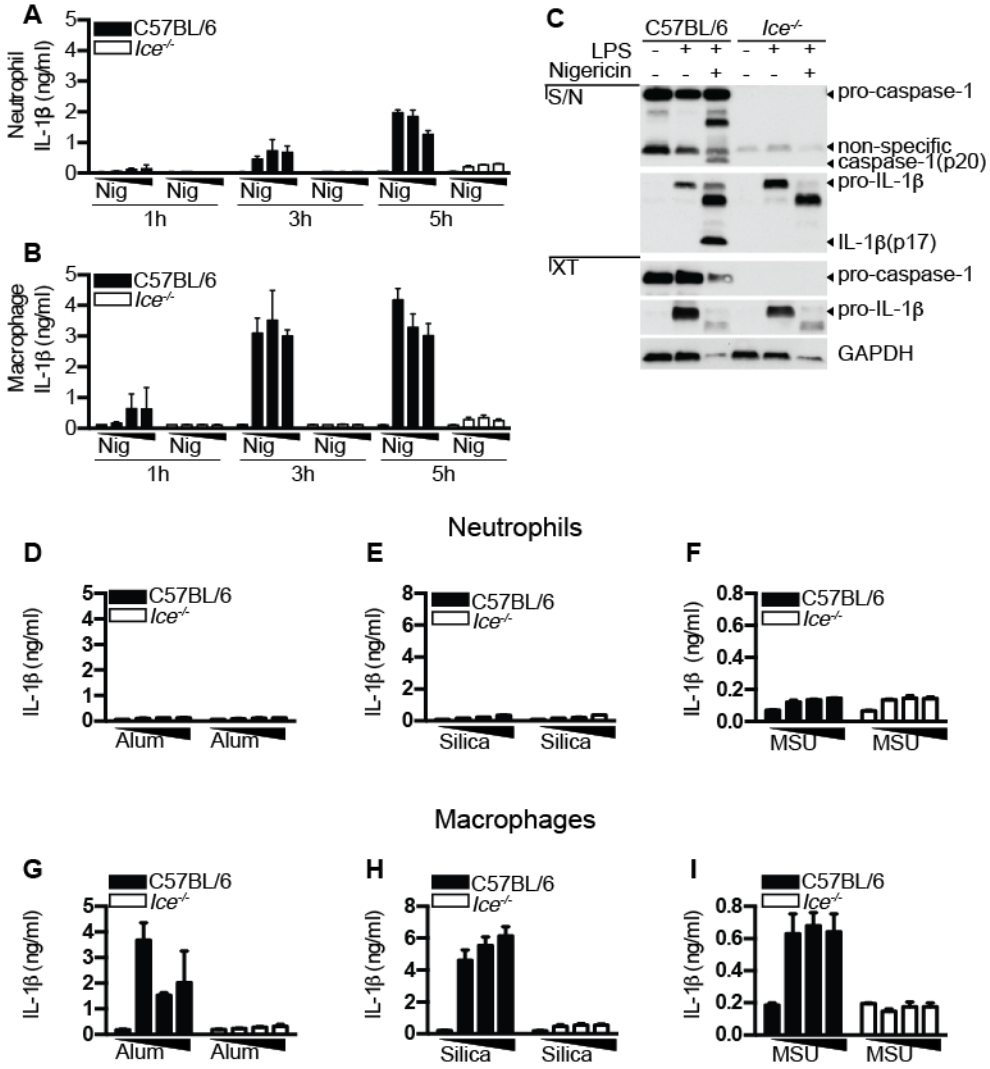


Figure 4.1 Neutrophils produce mature IL-1β in response to soluble but not particulate/crystalline agonists of the NLRP3 inflammasome.

Bone marrow neutrophils (**A**, **C**, **D-F**) or bone marrow-derived macrophages (**B**, **G-I**) were primed with 100 ng/ml LPS for 4 h before challenge with nigericin (Nig) (**A-B**: 0, 1.25, 2.5, 5 μM; C: 5 μM), alum (**D**, **G**: 0, 150, 300, 600 μg/ml), silica (**E**, **H**: 0, 150, 300, 600 μg/ml) or MSU (**F**, I: 0, 150, 300, 600 μg/ml). IL-1β secretion was measured by ELISA at 1, 3, 5 h (**A-B**) or 7 h post challenge (**D-I**). (**C**) Neutrophil supernatants (SN) and cell extracts (XT) were harvested at 5 h post-nigericin treatment, and examined by western blot. All ELISA data are mean + SD of technical triplicate cell stimulations, and are representative of 3 independent experiments.

4.3.2 The phagolysosomal rupture pathway is a weak stimulus for neutrophil NLRP3

Insoluble NLRP3 agonists trigger NLRP3 activation in macrophages by destabilising phagolysosomes (19). The observed lack of inflammasome activation in neutrophils stimulated with insoluble NLRP3 agonists could either reflect ineffective phagocytosis of these stimuli, or a deficiency in the NLRP3-activating phagolysosomal rupture pathway. We first investigated the cellular uptake of silica particles by live cell imaging. Neutrophils were incubated with silica in the presence of the fluid phase marker, Dextran, over 2 h. Silica particles were detected in Dextran-positive compartments (**Figure 4.2A, Supplementary Figure 4.2**), indicating that neutrophils phagocytose these particles, as for MSU crystals (260). We next examined the lysosomal rupture pathway in neutrophils versus macrophages by treating the cells with the small lysosomotropic peptide L-leucyl-L-leucine methyl ester (Leu-Leu-OMe) to induce phagolysosomal damage (19). Phagolysosomal integrity can be measured using acridine orange (AO), which fluoresces red in the low pH of lysosomes (19). AO fluorescence was 3-fold higher in bone marrow macrophages compared to neutrophils (**Figure 4.2B-C**), suggesting that macrophages harbour a larger, or more acidic, lysosomal compartment than neutrophils. Leu-Leu-OMe treatment triggered a loss of AO fluorescence in both cell types (**Figure 4.2B-C**), although the effect was much more prominent in macrophages. The loss of AO fluorescence correlated inversely with IL-1β secretion for both neutrophils and macrophages (**Figure 4.2D-E**). However, IL-1β production in response to Leu-Leu-OMe was weak in neutrophils, while producing a robust IL-1β response in macrophages. This indicates that while Leu-Leu-OMe can elicit phagolysosome rupture and resultant IL-1β production in neutrophils, this pathway is a very weak stimulus for neutrophil NLRP3 activation as compared to macrophages.

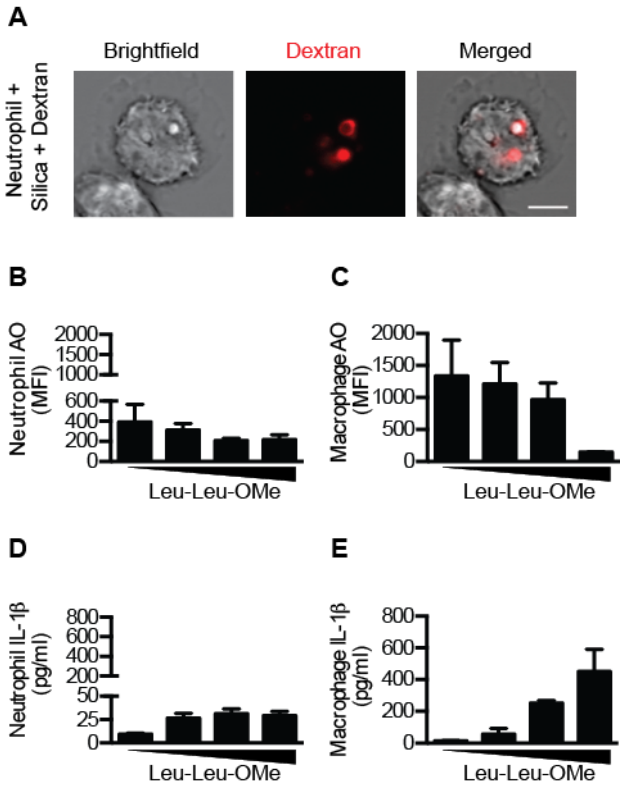


Figure 4.2 The phagolysosomal rupture pathway is a weak stimulus for neutrophil IL-1β production.

(**A**) Bone marrow neutrophils were exposed to silica (15 μg/ml) for 2 h in the presence of Dextran555 (200 μg/ml) and silica internalisation was analysed by confocal microscopy. Brightfield image shows refractive silica particles with one particle (arrow) co-localising with the fluid phase marker, dextran. Scale bar = 5 μM. (**B**, **D**) Bone marrow neutrophils or (**C**, **E**) bone marrow-derived macrophages were primed with 100 ng/ml LPS for 4 h before exposure to increasing doses of Leu-Leu-OMe (0, 0.5, 1, 2 mM). Acridine orange (AO) staining and IL-1β secretion was measured at 7 h by flow cytometry and ELISA, respectively. (**B-E**) Data are mean + SD of technical triplicate cell stimulations, and are representative of (**A**) 2 or (**B-E**) 3 independent experiments.

4.3.3 Neutrophil depletion does not alter IL-1β production in alum-induced peritonitis

We, and others, demonstrated that neutrophils are a major cellular compartment for caspase-1-dependent IL-1β production during *in vivo* bacterial infection (229, 257). Given our surprising observation that alum did not trigger IL-1β production by the neutrophil NLRP3 inflammasome *in vitro* (**Figure 4.1D**), we anticipated that, unlike the case for *in vivo* bacterial challenge (229, 257), neutrophils would not be a significant cellular source of IL-1β during alum-induced peritonitis. To examine this hypothesis, we administered C57BL/6 mice with an isotype control antibody or an α-Ly6G (1A8) antibody to specifically deplete neutrophils (**Chapter 3**). At 16 - 24 h post-depletion, mice were challenged with 350 μg alum for 6 h and peritoneal IL-1β levels were quantified. Alum triggered neutrophil recruitment and IL-1β production in mock-depleted mice (**Figure 4.3A**), but neutrophil depletion did not affect peritoneal IL-1β levels (**Figure 4.3B**). This indicates that, as anticipated, neutrophils are not a significant cellular source of IL-1β in this setting.

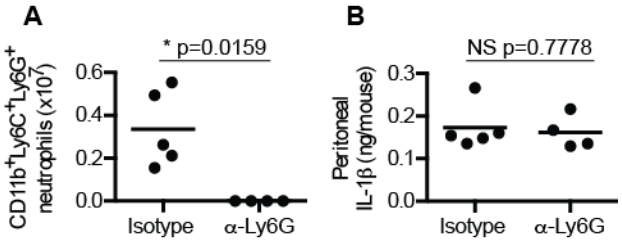


Figure 4.3 Neutrophils do not significantly contribute to IL-1β production during alum challenge *in vivo*.

Mice were i.p injected with 100 μg of α-Ly6G (1A8) or isotype antibody. After 16-24 h, mice were challenged i.p with 350 μg alum for 6 h. (**A**) Absolute abundance of $CD11b^{+}Ly6C^{+}Ly6G^{+}$ neutrophils, and (**B**) peritoneal IL-1β levels in the peritoneal exudate. Data are 4-5 mice per condition, combined from 2 independent experiments. Statistical analyses were performed using the non-parametric Mann-Whitney t-test. Data were considered significant when $p \leq 0.05$ (*).

4.4 Concluding remarks

Neutrophils have been traditionally viewed as anti-microbial effector cells, but an emerging literature documents the wide diversity of neutrophil functions, including their role as important modulators of inflammation (261). The NLRP3 inflammasome is a key pathway for eliciting inflammatory responses, but the circumstances in which neutrophils contribute to NLRP3-driven inflammation were poorly characterised. Here we challenged neutrophils with a panel of 7 different NLRP3 agonists that trigger NLRP3 activation by phagolysosomal rupture (MSU, Alum, Silica, Leu-Leu-OMe) or by mechanisms independent of phagolysosomal rupture (nigericin, ATP, R837). Human and mouse neutrophils express NLRP3 (**Chapter 3**), and in keeping with this, we and others (241) observe robust NLRP3-dependent responses to soluble agonists (e.g. nigericin, ATP, R837) that signal the presence of infection or tissue damage. Surprisingly, we discovered that the neutrophil NLRP3 inflammasome was poorly responsive to insoluble NLRP3 agonists such as alum, silica and MSU that trigger macrophage NLRP3 activation via phagolysosomal rupture. Neutrophils were likewise poorly responsive to the lysosomotropic peptide Leu-Leu-OMe, suggesting that lysosomal rupture is not a prominent pathway for NLRP3 activation in neutrophils. Accordingly, we found that neutrophils did not significantly contribute to IL-1β production during alum-induced peritonitis. This is in sharp contrast with *in vivo* bacterial challenge models, where neutrophils were major producers of IL-1β (229, 257) (**Chapter 3**) and thereby drove an amplification loop to ensure large numbers of activated neutrophils were recruited to resolve the infection (**Chapter 3**).

Given the diversity of NLRP3 activating signals, a key outstanding question is how the immune system tailors an *in vivo* response that is appropriate to each stimulus. For example, in scenarios where neutrophil-mediated clean up is important (e.g. infection, injury), one might expect that the immune response will be dominated by neutrophils, while this may be less important in other scenarios of NLRP3 activation, such as metabolic stress (e.g. MSU) or exposure to environmental irritants (e.g. alum, silica). Our data broadly support such a model, as intraperitoneal *Salmonella* infection was a more potent stimulus for neutrophil recruitment than alum injection (**Figure 4.3** and **Figure 3.4B**). The finding that neutrophils produce IL-1β in response to NLRP3 agonists indicative of infection or injury, but not those signalling metabolic stress or irritant exposure, suggests a possible mechanism by which immune responses are tailored according to stimulus; neutrophil-derived IL-1β and the ensuing positive feedback loop of neutrophil recruitment and

activation likely allows for a strong neutrophil response when it is appropriate, but this positive feedback loop is not engaged to prevent collateral damage in circumstances where neutrophil function is less important. Our proposed model is noteworthy from a clinical perspective, as the NLRP3 pathway contributes to the adjuvant activity of alum (262), and is central to a number of inflammatory disorders triggered by insoluble aggregates, such as gout and silicosis. Our data suggest that other cell types (e.g. macrophages, dendritic cells) are responsible for driving inflammasome-mediated inflammatory responses in these settings.

Our finding that the signalling pathways eliciting NLRP3 activation in macrophages are not always conserved in neutrophils emphasises the strong influence of cell identity on innate immune signalling pathways. Instances of such cell type-specificity in signalling pathways are already appreciated within the myeloid compartment; for example, neutrophils exhibit various cell type-specific adaptations to the Toll-like receptor signalling pathways described for macrophages (228). We speculate that cell type-specific tailoring of immune signalling networks may be common, and warrants further investigation to gain a more nuanced mechanistic understanding of immune response sculpting in health and disease.

4.5 Materials and methods

4.5.1 Mice

C57BL/6 and *Ice*$^{-/-}$ (226) mice were housed in specific-pathogen-free facilities at the University of Queensland. *Ice*$^{-/-}$ mice were backcrossed at least ten times to C57BL/6, and all experiments were conducted with age- and sex-matched mouse cohorts. The University of Queensland's animal ethics committee approved all experimental protocols.

4.5.2 Primary cell culture and inflammasome assays

Bone marrow neutrophils were surface labelled using α-Ly6G-FITC (1A8, Biolegend), and purified by MACS with α-FITC beads. MACS-purified fractions were assayed for cell purity (>98%) by flow cytometry. Macrophages were differentiated from bone marrow as previously described (255). Bone marrow neutrophils were always used for experiments on the day of purification and were plated in OPTI-MEM (Life Technologies) supplemented with 0.4 μg/mL aprotinin (Sigma) at a density of 3.3 x10^6 cells/ml, except in Figure S2B where 5 x10^6 cells/ml were used. Bone marrow-derived macrophages were cultured at a density of 1 x 10^6 cells/ml in OPTI-MEM (Life Technologies) supplemented with 100 ng/ml units/ml recombinant human M-CSF (ImmunoTools). To activate the NLRP3

inflammasome, neutrophils and macrophages were primed with 100 ng/ml ultrapure *E. coli* K12 LPS (Invivogen) for 4 h to induce the expression of pro-IL-1β before stimulation with NLRP3 agonists. IL-1β levels in cell-free supernatants were analysed by ELISA (eBioscience). Cytotoxicity was measured by intracellular LDH release (Promega). Cell culture supernatants were precipitated with chloroform and methanol as previously described (256), and western blots on cell extracts and precipitated supernatants were performed using standard procedures (256). Antibodies included IL-1β (polyclonal goat, R&D Systems), caspase-1 (Casper-1, Adipogen), ASC (polyclonal rabbit, Santa Cruz), and tubulin (B-5-1-2, Sigma).

4.5.3 Live cell imaging of neutrophil uptake of silica

Purified neutrophils were plated on Poly-L-Lysine (Sigma) treated Mat-tek dishes for live cell imaging experiments. Cells were incubated with silica (15 μg/ml) particles and Dextran (200 μg/ml; 10,000 MW, Alexa Fluor® 555). After 2 hours incubation, cells were washed three times with warm OPTI-MEM (Life Technologies) and imaged live using a Zeiss 710 confocal microscope.

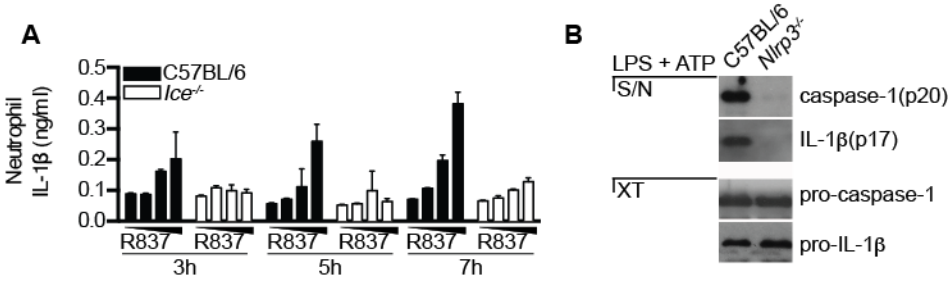
4.5.4 Acridine orange labelling and flow cytometry

Lysosomes were loaded with acridine orange as previously described (19). In brief, cells were incubated with acridine orange (Santa Cruz, 1 μg/ml) for 15 min and washed thrice prior to stimulation with Leu-Leu-OMe. Lysosomal rupture was quantified using flow cytometry at a loss of emission at 600 – 650 nm. Data were acquired on BD Canto II and were analysed with FlowJo software (Tree Star).

4.5.5 Neutrophil depletion and *in vivo* alum challenge

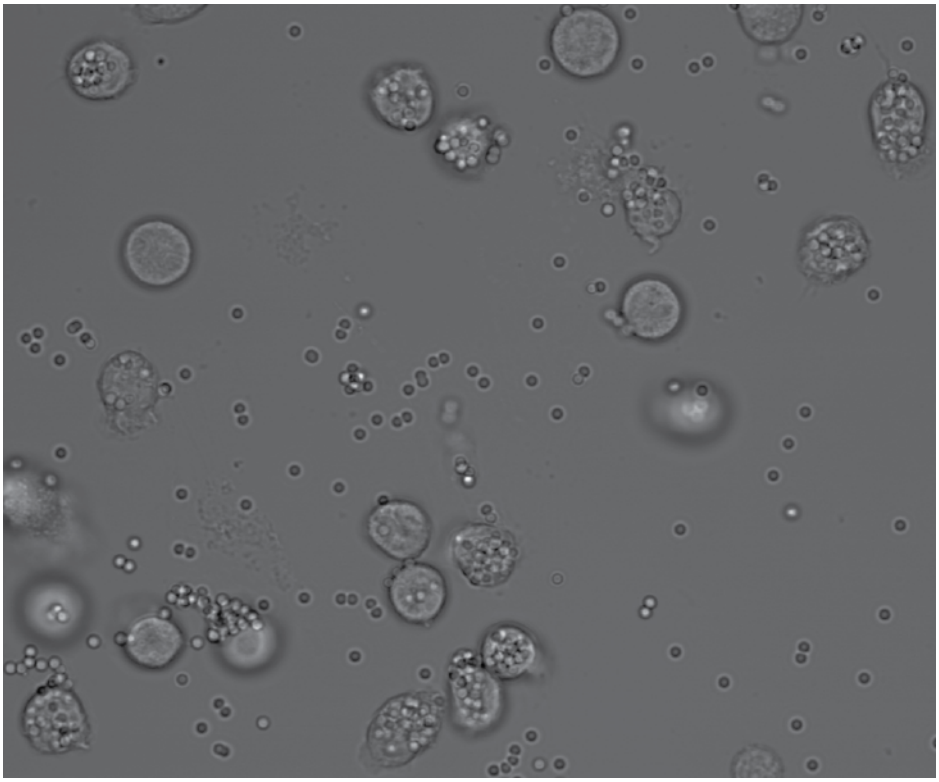
Neutrophils were depleted or mock-depleted by i.p. administration of 100 μg α-Ly6G antibody (endotoxin-free 1A8 clone, BioXcell) or isotype control anti- body (endotoxin-free 2A3 clone, BioXcell). 16-24 h post depletion, mice were challenged with i.p with 350 μg alum (Pierce) and sacrificed after 6 h. The peritoneal cavity was flushed with 10 ml of ice-cold DPBS. Collected cells were analysed for neutrophil abundance by flow cytometry, and cell-free supernatants were subject to IL-1β ELISA.

4.6 Supplementary figures



Supplementary Figure 4.1 Neutrophils produce mature IL-1β in response to soluble agonists of the NLRP3 inflammasome.

(**A**) Bone marrow neutrophils were primed with 100 ng/ml LPS for 4 h before challenge with R837 (0, 5, 10, 20 μg/ml) and IL-1β secretion was measured by ELISA at 3, 5, and 7 h post-challenge. Data are mean + SD of technical triplicate cell stimulations, and are representative of 3 independent experiments. (**B**) Wild type versus *Nlrp3*$^{-/-}$ neutrophils were primed with 100 ng/ml LPS for 3 h and challenged with 5 mM ATP for 1 h. Supernatants (SN) and cell extracts (XT) were harvested and examined for pro-IL-1β and pro-caspase-1 cleavage by western blot.



Supplementary Figure 4.2 Neutrophils internalise silica particles.

Neutrophils were incubated with silica (15 μg/ml) for 2 h. Uncropped brightfield image shows refractive silica particles within neutrophils.

5. Neutrophils resist multiple caspase-8-dependent cell death pathways

5.1 Introduction

The NLRP3 inflammasome is of particular medical importance because it triggers inflammatory responses to a wide variety of structurally unrelated molecules, including whole pathogens, bacterial toxins, protein aggregates, metabolites, environmental irritants and cellular danger signals released as a consequence of infection or tissue damage (2). A key molecular function of the NLRP3 inflammasome is to recruit pro-caspase-1 to the ASC polymer for cluster-induced auto-activation, and thereby facilitate caspase-1-dependent programs (e.g. cytokine processing, pyroptosis). Recent studies have revealed an additional function for the NLRP3 inflammasome; the ASC polymer also recruits pro-caspase-8, driving caspase-8 activation and apoptotic cell death in macrophages (69, 70). NLRP3/caspase-8-dependent macrophage apoptosis occurs more slowly than caspase-1-dependent pyroptosis, and is posited to be a backup mechanism to ensure the suicide of infected cells if caspase-1 function is absent (e.g. in cells that do not express caspase-1, or if caspase-1 function inhibited by pathogen virulence factors) (69, 70).

Inhibitor of apoptosis proteins (IAPs) are potent inhibitors of pro-apoptotic proteins, and also regulate the transcription of pro-survival proteins. Blocking or depleting IAPs thus significantly increases the propensity of a cell to undergo cell death. IAP depletion triggers multiple intracellular signalling complexes that culminate in caspase-8-dependent apoptosis (**Figure 5.1**). In some cells, IAP antagonism triggers NF-κB activation, leading to autocrine TNF signalling (263). TNF signalling in IAP-depleted cells triggers the formation of an intracellular signalling complex consisting of RIPK1, TRADD, FADD and caspase-8. This caspase-8 activating platform is termed 'complex II' and triggers caspase-8 dependent apoptosis (**Figure 5.1A**) (198). In other cell types, IAP depletion may also trigger caspase-8-dependent apoptosis that is independent of TNF signalling. Caspase-8-mediated apoptosis in this case requires the formation of a complex II-like platform called the 'ripoptosome', consisting of RIPK1, FADD and caspase-8, which triggers caspase-8-dependent apoptosis (**Figure 5.1B**) (264, 265). In cells such as macrophages, IAP depletion in conjunction with TLR4 signalling leads to TRIF-dependent activation of RIPK3 to trigger ripoptosome-mediated apoptosis (**Figure 5.1C**) (88). Interestingly, cell death is

not prevented if caspase function is blocked in IAP-depleted cells. Instead, cells switch to another form of lytic cell death that is dependent on RIPK3 and MLKL, and is termed 'necroptosis' (**Figure 5.1A-C**) (266).

Intriguingly, recent reports have revealed that ripoptosome and necrosome signalling in macrophages triggers NLRP3 inflammasome activation and IL-1β maturation, and that IAPs suppress this process (74, 88, 267). Genetic ablation or pharmacological inhibition of IAPs in LPS-primed macrophages triggered RIPK3-dependent caspase-8 activation via the ripoptosome (74, 264), and ensuing caspase-1-independent IL-1β maturation (74) and NLRP3 function (88). If caspase-8 function was suppressed in addition to IAP ablation, RIPK3 instead triggered MLKL-dependent necroptosis and NLRP3 signalling (88). It seems likely that in both RIPK3-mediated pathways, the NLRP3 inflammasome is assembled as a consequence of falling intracellular K^+ levels during apoptotic or necroptotic cell death.

We have previously demonstrated that neutrophils do not undergo caspase-1-dependent pyroptosis downstream of NLRC4 inflammasome activation by *Salmonella* (Chapter 3). The data presented in this chapter aimed to investigate possible cross-talk between RIPK3 cell death and NLRP3 inflammatory pathways in neutrophils, with the hypothesis that, similar to previous chapters, innate immune signalling pathways may be specialised in neutrophils. Here, we demonstrate that neutrophils resist all known forms of NLRP3-dependent cell death. In addition, our data suggest that differential TLR signalling in neutrophils allows neutrophils to resist RIPK3-dependent cell death and inflammatory responses.

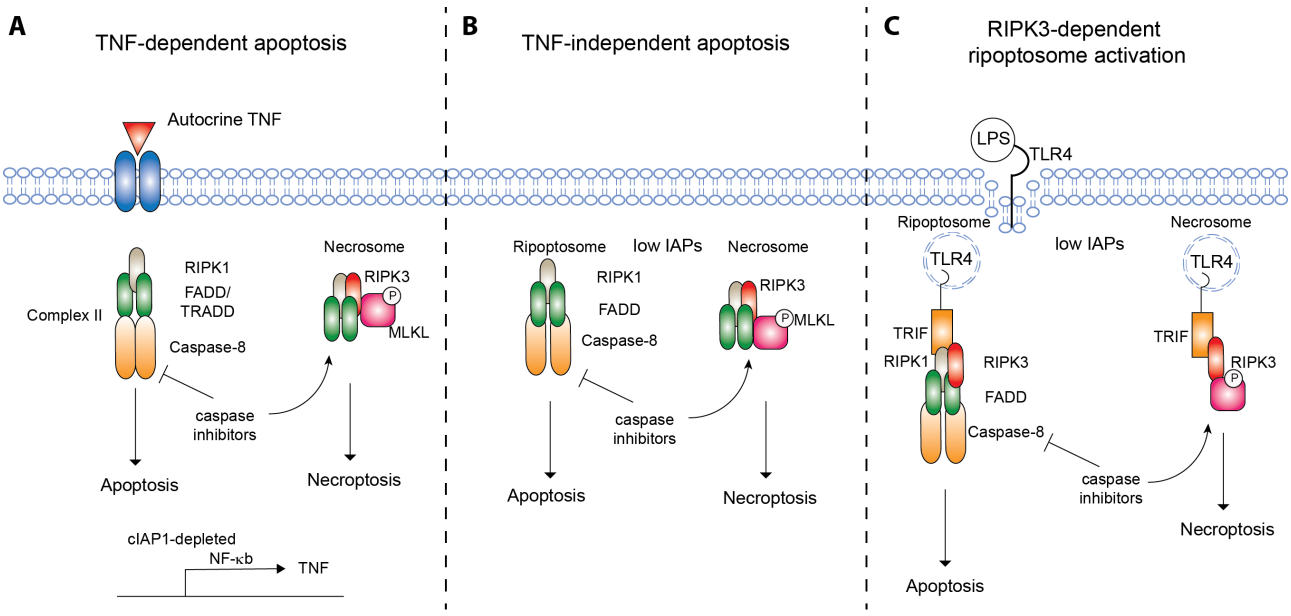


Figure 5.1 Different cell death pathways engaged in IAP-depleted cells.

(**A**) IAP depletion can trigger autocrine TNF production and complex II-dependent caspase-8 activation. (**B**) IAP depletion can trigger caspase-8 activation and apoptosis through the ripoptosome, with apoptosis occurring independently of TNF signalling. (**C**) TLR-TRIF signalling can trigger RIPK3-dependent caspase-8 activation via the ripoptosome. In all cases, caspase-8 inhibition leads to the assembly of the necrosome, resulting in RIPK3-dependent MLKL phosphorylation and necroptosis.

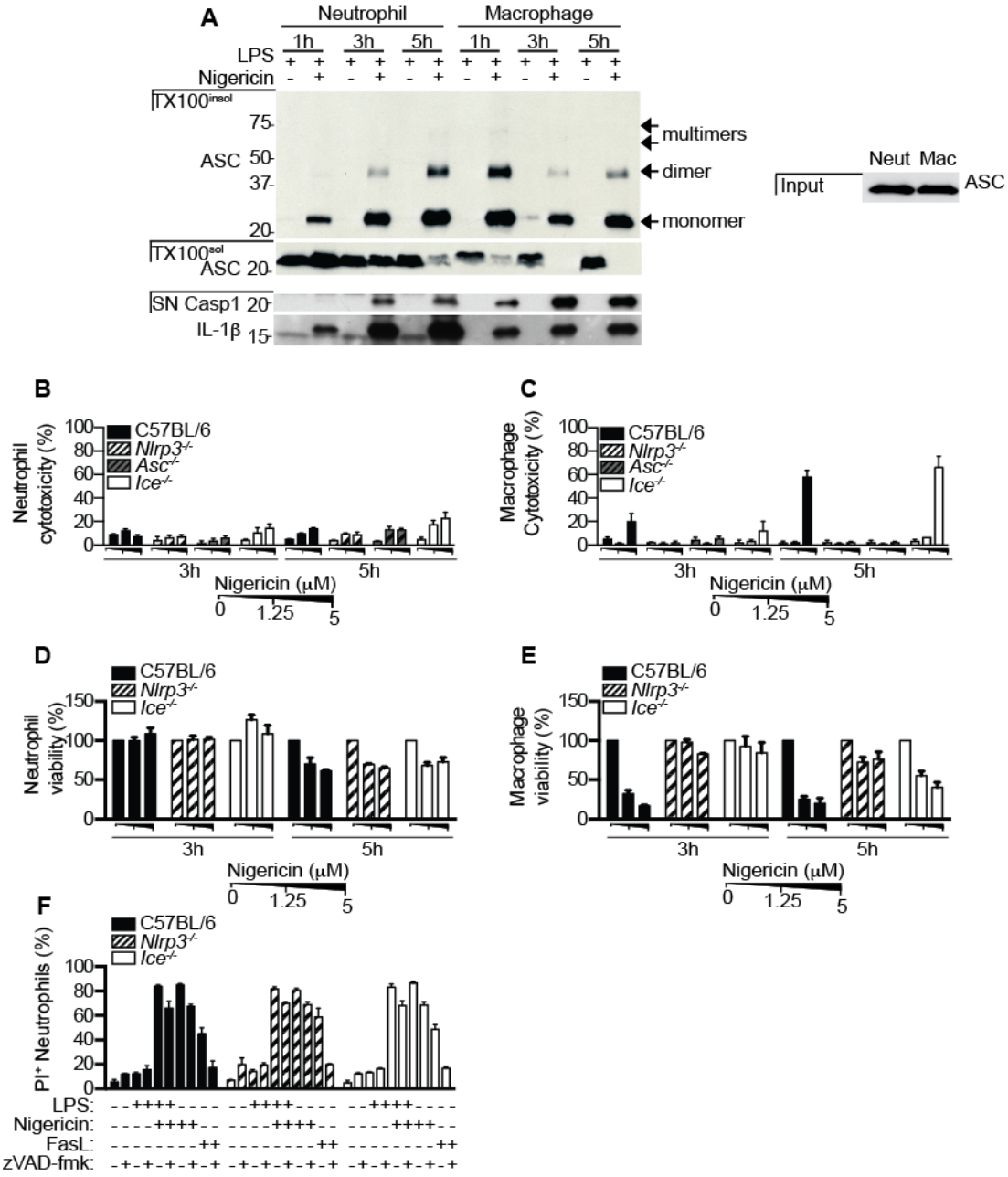
5.2 Results

5.2.2 NLRP3 activation triggers ASC oligomerisation, caspase-1 processing and IL-1β maturation but not pyroptosis or apoptosis in neutrophils

We and others reported that the NLRP3 agonist, nigericin, stimulates NLRP3-dependent IL-1β production in neutrophils (**Chapter 4** and (241)), but the NLRP3 signalling pathway and its cellular outcomes have not been closely examined in neutrophils. In macrophages, NLRP3 signals by triggering the polymerisation of the inflammasome adaptor protein, ASC. We first examined whether nigericin elicits ASC polymerisation in neutrophils, as for macrophages. LPS-primed bone marrow neutrophils (BMN) and bone marrow-derived macrophages (BMDM) were stimulated with nigericin, and cellular extracts were fractionated into Triton X100 soluble and insoluble fractions (containing monomeric and polymeric ASC, respectively). The Triton X100 insoluble fractions were then subject to disuccinimidyl suberate (DSS) to crosslink ASC oligomers (268, 269). Treatment with LPS alone triggered minimal ASC polymerisation in both neutrophils and macrophages (**Figure 5.2A**). Nigericin treatment of LPS-primed cells triggered ASC polymerisation in both cell types, although ASC polymerisation occurred more slowly in neutrophils compared to macrophages, as ASC remained largely in the Triton X-100 soluble fraction in neutrophils up to 3 h post-nigericin treatment, when it underwent a major redistribution to the Triton X-100 insoluble, ASC polymer fraction (**Figure 5.2A**). In contrast, in macrophages much of the cellular ASC redistributed from the cytoplasmic Triton X-100 soluble to the insoluble fraction within 1 h of nigericin treatment, and ASC redistribution appeared complete by 3 h post-nigericin stimulation (**Figure 5.2A**). Consistent with the delayed polymerisation of ASC, nigericin-stimulated caspase-1 processing and IL-1β maturation occurred more slowly in neutrophils compared to macrophages (**Figure 5.2A**).

Having established that neutrophil NLRP3, like macrophage NLRP3, triggers the polymerisation of ASC and downstream caspase-1 and IL-1β processing, the cellular outcomes of neutrophil NLRP3 inflammasome activation were next examined. In macrophages, the ASC polymer facilitates caspase-1 and caspase-8 activation for the initiation of pyroptosis and apoptosis, respectively. LPS-primed wild type (WT), *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ and *Ice*$^{-/-}$ neutrophils and macrophages were stimulated with nigericin over 5 h, and cell death and viability were measured by intracellular LDH release, MTT reduction and propidium iodide (PI) uptake. Substantial macrophage cell death was apparent at high doses of nigericin as early as 3 h in WT macrophages, reflecting caspase-1-dependent

pyroptosis, while WT neutrophil viability remained relatively high even at 5 h post-nigericin (**Figure 5.2B-E**). *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ macrophages were protected from nigericin-induced cell death at all time points, while caspase-1-deficient *Ice*$^{-/-}$ macrophages lost viability at 5 h (**Figure 5.2C, E**), reflecting NLRP3/ASC/caspase-8-dependent apoptotic death (69, 70). Importantly, genotype had no apparent effect on neutrophil cytotoxicity or viability following nigericin treatment even after 5 h (**Figure 5.2B, D**), indicating that neutrophils did not undergo any form of NLRP3 inflammasome-dependent cell death. To ensure that the lack of NLRP3-dependent cell death in neutrophils was not a consequence of delayed signalling kinetics, WT, *Nlrp3*$^{-/-}$ and *Ice*$^{-/-}$ neutrophils were stimulated with nigericin in the presence or absence of the pan-caspase inhibitor z-VAD-fmk for 7 h and the loss of membrane integrity was measured by PI uptake in these cells. At this time point, a high proportion (~80%) of nigericin-stimulated neutrophils were positive for PI, but this proportion was not affected by LPS pre-treatment or neutrophil genotype (**Figure 5.2F**). Caspase inhibition by z-VAD-fmk modestly reduced PI uptake in nigericin-treated neutrophils of all genotypes, but greatly reduced PI uptake in Fas-treated neutrophils (**Figure 5.2F**), indicating that while Fas and nigericin do trigger neutrophil cell death, and nigericin-stimulated cell death is partially dependent on apoptotic caspases, nigericin-induced cell death occurs via mechanisms independent of the NLRP3 inflammasome. In these experiments, z-VAD-fmk was added to the cell culture media at the same time as LPS and FasL to prevent FasL-induced caspase activation, however, z-VAD-fmk may alter LPS-priming of NLRP3 responses. To circumvent this possibility, z-VAD-fmk will be added to the cells 30 min prior to nigericin stimulation and not throughout the LPS priming process in future experiments. Taken together, these data indicate that while part of the macrophage NLRP3 signalling pathway is mirrored in neutrophils (i.e. ASC polymerisation, caspase-1 processing and IL-1β maturation), neutrophils are resistant to all forms of NLRP3-mediated cell death.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1h
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TX100sol
ASC 20
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IL-1β 15
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ASC
B
Neutrophil cytotoxicity (%)
C57BL/6
Nlrp3-/-
Asc-/-
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F
PI+ Neutrophils (%)
LPS:
Nigericin:
FasL:
zVAD-fmk:

Figure 5.2 NLRP3 triggers ASC polymerisation, and caspase-1 and IL-1β processing but not cell death in neutrophils.

(**A**) BMN and BMDM were primed with 100 ng/ml LPS for 4 h and then stimulated with 5 μM nigericin for 1, 3, or 5 h. At the indicated time points, cells were lysed and the Triton X-100 insoluble fraction ($TX100^{insol}$) was crosslinked with DSS. Soluble and insoluble cell fractions ($TX100^{sol}$, $TX100^{insol}$), and cell supernatants (SN) were subject to western blot for ASC, cleaved caspase-1 (p20) and mature IL-1β (p17). Cell numbers for neutrophils (3×10^6) and macrophages (1.65×10^5) were optimised such that total ASC abundance was equivalent between samples (right: input). (**B**, **D**) BMN or (**C**, **E**) BMDM were primed with 100 ng/ml LPS and treated with a dose range of nigericin for 3 or 5 h. (**B-C**) Cytoplasmic LDH release into the supernatant or (**D-E**) MTT reduction was quantified as a measure of cell death/viability. (**F**) BMN were left untreated or primed with 100 ng/ml LPS in the presence or absence of 50 μm z-VAD-fmk. PI uptake at 7 h post-nigericin was quantified by flow cytometry. Data are mean + SD of technical triplicate cell stimulations and are representative of (**A-C**) four or (**D-F**) two independent experiments.

5.2.3 The neutrophil NLRP3 inflammasome recruits and activates caspase-8, but does not facilitate caspase-8 self-processing

In macrophages, NLRP3 drives apoptotic cell death by recruiting caspase-8 to the ASC polymer for caspase-8 activation (69, 70). Hypothesising that neutrophil protection from NLRP3/ASC/caspase-8-dependent cell death may be due to inefficient caspase-8 activation by neutrophil NLRP3/ASC, pro-caspase-8 recruitment to the ASC speck was next examined. LPS-primed neutrophils were stimulated with nigericin over 5 h in the presence of z-VAD-fmk to prevent caspase-8 proteolysis and release from the ASC polymer. The Triton X-100 insoluble fraction was separated from the soluble fraction by centrifugation, to enrich for polymeric ASC. Caspase-8 was not present in the Triton X-100 insoluble fraction in untreated and LPS-primed neutrophils. Nigericin treatment triggered caspase-8 redistribution into the ASC polymer-containing insoluble fraction (**Figure 5.3A**). As caspase-8 appeared to be recruited to the neutrophil NLRP3 inflammasome, possible mechanisms suppressing caspase-8 activity were next investigated. c-FLIP is a caspase-8 homologue that lacks the caspase catalytic cysteine, and heterodimerises with pro-caspase-8 to inhibit specific caspase-8 functions (270). c-FLIP exists as long and short splicoforms (c-$FLIP_L$ and c-$FLIP_s$). At high c-FLIP concentrations, both c-FLIP isoforms outcompete pro-caspase-8 for binding to its signalling complex and thereby limit caspase-8 auto-proteolysis. However, at low cellular concentrations, c-$FLIP_L$ can enhance specific pro-caspase-8 activities, as the protease-dead domain of c-$FLIP_L$ is able to allosterically activate pro-caspase-8 (197). However, the c-$FLIP_L$/caspase-8 heterodimer cleaves a narrower range of substrates compared to a caspase-8 homodimer (197). c-FLIP localisation was next examined, with the hypothesis that c-FLIP may be recruited to the neutrophil NLRP3 inflammasome alongside caspase-8 to suppress caspase-8-dependent apoptosis. Interestingly, nigericin triggered the redistribution of both c-FLIP isoforms, c-$FLIP_L$ and c-$FLIP_s$, to the Triton X-100 insoluble fraction (**Figure 5.3A**). These data broadly suggest that both caspase-8 and c-FLIP are recruited to the inflammasome upon neutrophil nigericin treatment, and caspase-8 auto-proteolysis on the neutrophil NLRP3 inflammasome may be suppressed by c-FLIP. Further experiments using *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ neutrophils will confirm that pro-caspase-8 and c-FLIP recruitment to the Triton X-100 insoluble fraction indeed reflects recruitment to the inflammasome, and c-FLIP silencing experiments will reveal possible functions for c-FLIP in inhibiting NLRP3/caspase-8-dependent apoptosis.

A caspase activity probe was next employed to clarify whether inflammasome-recruited caspase-8 gains enzymatic activity in neutrophils. Biotin-VAD-fmk is a biotinylated substrate mimic that binds irreversibly to the active site of caspases, and can thereby be used in conjunction with streptavidin-coupled beads to isolate activated caspases for identification by western blot. Caspase-1-deficient *Ice*$^{-/-}$ neutrophils were used for these studies in order to more clearly observe caspase-8-dependent processes, which occur more slowly than pyroptosis (69, 70). Surprisingly, given that neutrophils did not undergo inflammasome-mediated apoptosis (**Figure 5.2B-F**), pro-caspase-8 was indeed detected in the pulldown fraction upon neutrophil nigericin stimulation. Intriguingly, active caspase-8 was detected in the cell supernatant, but not in the ASC polymer-containing cell extracts. The active species pulled down by this approach were full-length (55 kDa) pro-caspase-8 and a larger caspase-8 species of approximately 75 kDa (**Figure 5.3B**). The latter may reflect caspase-8 post-translational modification, which can reportedly positively or negatively regulate caspase-8 activity (271-273). In this experiment, biotin-VAD-fmk was applied 30 min prior to cell stimulation with nigericin, and so it remains possible that biotin-VAD-fmk prevented pro-caspase-8 self-processing upon the ASC speck. To test whether inflammasome-recruited caspase-8 undergoes auto-processing or supports substrate cleavage in neutrophils, LPS-primed neutrophils were exposed to nigericin for 5 h without the addition of a caspase inhibitor, and cell extracts and supernatants were analysed by immunoblot. Nigericin triggered the release of full-length caspase-8 into the cell supernatant, but caspase-8 cleavage was not apparent in any neutrophil fraction (**Figure 5.3C**). In contrast, nigericin triggered robust caspase-8 processing in *Ice*$^{-/-}$ macrophages (**Figure 5.3C**). Under certain conditions, c-FLIP$_L$ can stabilise the pro-caspase-8 active site loop to form an enzymatically-active heterodimer with limited substrate repertoire (197, 215, 270, 274). Because both c-FLIP isoforms were enriched in the Triton X-100-insoluble ASC polymer fraction following nigericin treatment (**Figure 5.3A**), possible correlations between c-FLIP expression levels and caspase-8 processing were next investigated, as high c-FLIP levels might explain altered caspase-8 processing in neutrophils. Nigericin stimulation did not influence c-FLIP$_L$ expression in neutrophils, and c-FLIP$_s$ was barely detected in cell extracts under all tested conditions. In contrast, nigericin induced a marked reduction of c-FLIP$_L$ in macrophages, while c-FLIP$_s$ was not detected (**Figure 5.3C**). In all, these data suggest a likely role for c-FLIP in limiting caspase-8 cleavage upon the NLRP3 inflammasome in neutrophils, thereby suppressing inflammasome-mediated apoptotic cell death. Future experiments will knock down c-FLIP expression in neutrophils using siRNA, in order to assess whether this increases neutrophil susceptibility to NLRP3-dependent

apoptosis. Alternatively, c-FLIP$_L$ will be overexpressed in *Ice*$^{-/-}$ macrophages in order to block NLRP3/caspase-8 dependent apoptosis.

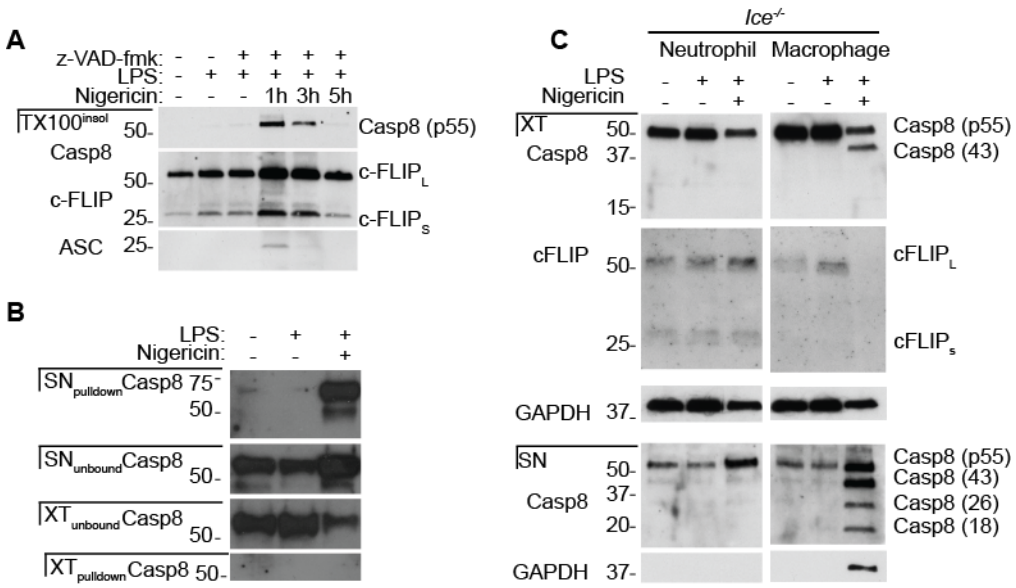


Figure 5.3 Nigericin triggers pro-caspase-8 recruitment to the ASC speck, pro-caspase-8 activity, but not pro-caspase-8 processing.

(**A**) BMN were primed with 100 ng/ml LPS for 4 h in the presence or absence of 50 μM zVAD-fmk co-administration, before stimulation with 5 μM nigericin for 1, 3, 5 h. At the indicated time points, cells were lysed and the Triton X-100 insoluble ($TX100^{insol}$) and soluble fractions were analysed by western blot. (**B**) LPS-primed *Ice*$^{-/-}$ neutrophils were stimulated with 5 μM nigericin for 5 h in the presence of 10 μM biotin-VAD-fmk (added 30 min prior to nigericin). Western blot detected biotin-VAD-trapped and untrapped caspase-8 in the supernatant (SN) and cell extracts (XT). (C) LPS-primed *Ice*$^{-/-}$ BMN and BMDM were treated with 5 μM nigericin for 5 h and the cell extracts (XT) were analysed by western blotting. Blots are representative of (**A**) a single experiment or (**B-C**) 3 independent experiments.

5.2.4 LPS/Cp A does not trigger RIPK3/caspase-8-dependent apoptosis or NLRP3 signalling in neutrophils

The surprising finding that neutrophils resist NLRP3/caspase-8-dependent cell death prompted us to investigate whether other caspase-8 signalling platforms are also deficient in neutrophils. The ripoptosome consists of RIPK1, FADD, caspase-8, and under some conditions, the caspase-8 inhibitor, c-FLIP (264, 265). Both c-FLIP isoforms were reported to limit caspase-8 auto-processing upon the ripoptosome, and thereby inhibit caspase-8-dependent cleavage of apoptotic substrates (264, 265). If c-FLIP is absent, ripoptosome-recruited pro-caspase-8 undergoes complete auto-proteolysis and is able to cleave its full range of substrates to drive the apoptotic program. Caspase-8 function in the context of the ripoptosome was next examined in neutrophils, as ripoptosome assembly in macrophages triggers apoptosis and NLRP3 signalling (74, 275). Neutrophils were treated with the IAP antagonist, Compound A (Cp A), to induce ripoptosome assembly (74, 264, 265). Surprisingly, Cp A treatment was a weak stimulus for neutrophil death (**Figure 5.4A**), but elicited robust macrophage death (**Figure 5.4B**). LPS pre-treatment did not enhance cell death levels in Cp A-treated neutrophils (**Figure 5.4A**), but accentuated Cp A-mediated macrophage cytotoxicity (**Figure 5.4B**). Enhanced cell death in LPS-treated macrophages is due to TLR4-mediated RIPK3 activation, which enhances ripoptosome-dependent apoptosis (88). The lack of Cp A-induced cell death in IAP-inhibited neutrophils suggested the possibility that in these cells, Cp A may not induce autocrine TNF signalling. Indeed, exogenous TNF enhanced Cp A-mediated death in neutrophils but LPS pre-treatment had no effect on Cp A-induced neutrophil cytotoxicity (**Figure 5.4A**). In all this suggests that IAP-depletion licenses neutrophils to undergo TNF-dependent apoptosis, demonstrating that the ripoptosome can indeed be formed and trigger apoptosis in neutrophils. However, IAP depletion did not license neutrophils for LPS-mediated RIPK3/ripoptosome-driven cell death. By contrast, macrophages were susceptible to both cell death pathways.

Since Cp A did not elicit cell death in LPS-primed neutrophils (**Figure 5.4A**), we hypothesised that IAP inhibition would be similarly unable to drive caspase-8- and NLRP3/caspase-1-dependent pro-IL-1β processing in neutrophils. Indeed, Cp A did not trigger IL-1β secretion from either WT or caspase-1-deficient *Ice*$^{-/-}$ LPS-primed neutrophils, while nigericin induced IL-1β secretion from LPS-primed WT neutrophils (**Figure 5.4B**). In contrast, and as expected (74), Cp A triggered robust IL-1β secretion from LPS-primed WT macrophages (**Figure 5.4C**), reflecting RIPK3/caspase-8-dependent IL-1β processing and

RIPK3/caspase-8-dependent NLRP3 activation (88). IAP inhibition triggers ASC polymerisation in LPS-stimulated macrophages and dendritic cells (267), however, it was unclear whether IAPs directly regulate ASC polymerisation, or this occurs as a secondary outcome of ripoptosome/caspase-8-driven NLRP3 activation. To investigate this, ASC polymerisation in LPS/Cp A-stimulated neutrophils and macrophages was monitored using the DSS crosslinking protocol. Nigericin stimulated ASC polymerisation in LPS-primed neutrophils, while Cp A did not trigger neutrophil ASC polymerisation regardless of LPS priming (**Figure 5.4E**). In contrast, Cp A triggered ASC polymerisation in macrophages, and LPS pre-treatment boosted this effect (**Figure 5.4E**). These data are consistent with NLRP3 activation following LPS/Cp A treatment in macrophages but not neutrophils (**Figure 5.4C-E**), and suggest that IAPs do not directly regulate ASC polymerisation, but instead, the reported ASC polymerisation in LPS-treated IAP-deficient macrophages and dendritic cells occurs as a consequence of ripoptosome/caspase-8-dependent NLRP3 signalling. If this is the case, the NLRP3 inhibitor, MCC950, should block ASC oligomerisation in LPS/Cp A-treated macrophages, and future experiments will be carried out to verify this. As anticipated given the dual pathways (caspase-8 versus caspase-8/NLRP3/caspase-1) mediating IL-1β production downstream of the ripoptosome (88), caspase-1 deficiency only partially suppressed Cp A-induced IL-1β secretion from LPS-primed macrophages (**Figure 5.4D-E**). The lack of cell death and mature IL-1β release in LPS/Cp A-stimulated neutrophils suggests that this treatment regimen may fail to trigger pro-caspase-8 processing in neutrophils.

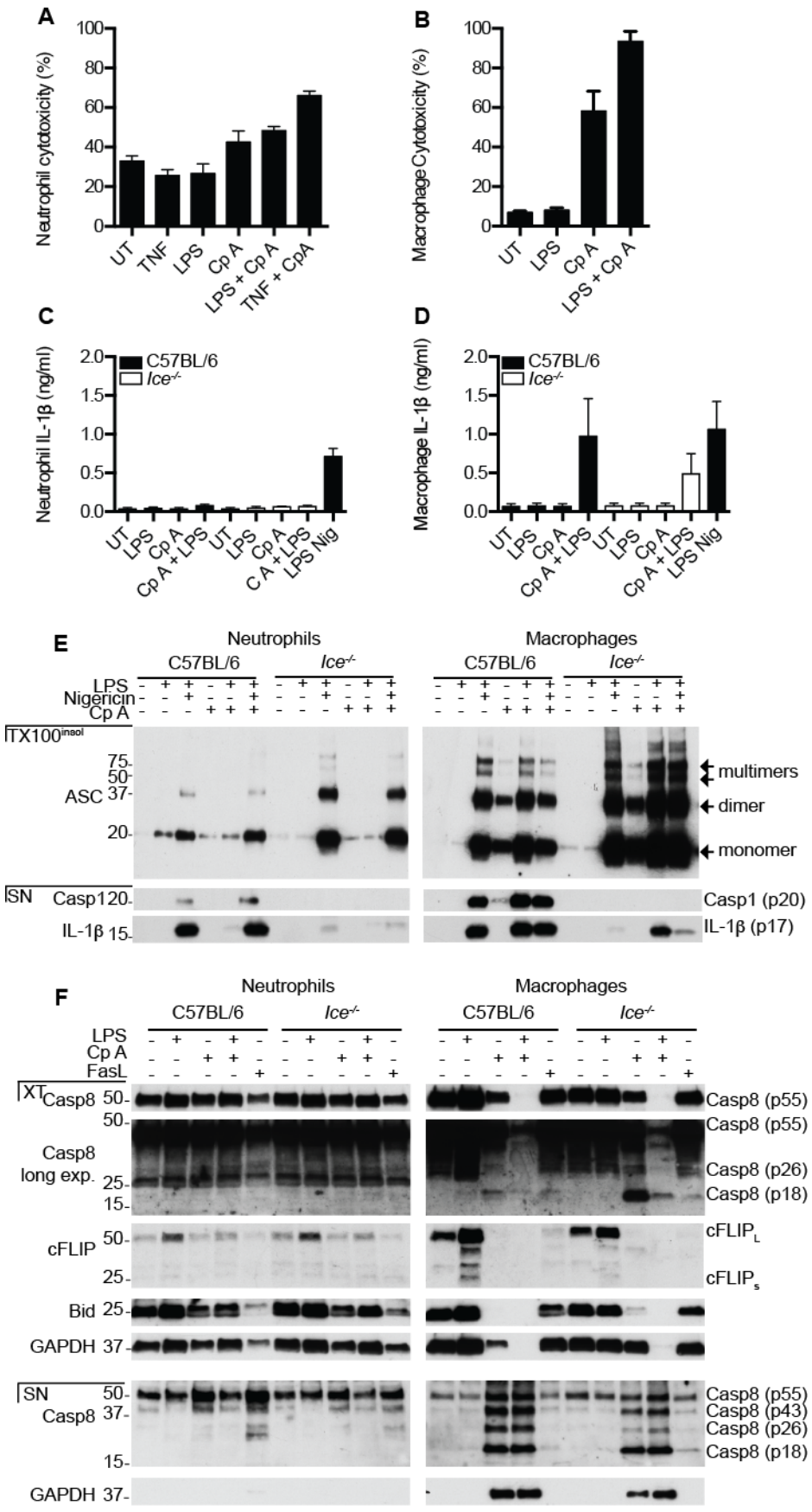
We therefore next investigated the cleavage status of pro-caspase-8 in neutrophils and macrophages exposed to LPS/Cp A versus a positive control for caspase-8 processing, FasL. Expression of full-length caspase-8 was unaffected by Cp A treatment in neutrophils (**Figure 5.4F**). Cp A did not elicit caspase-8 cleavage, while FasL triggered robust caspase-8 processing as observed by the loss of full-length pro-caspase-8 in the neutrophil cell extracts and the appearance of caspase-8 cleavage fragments in the supernatant (**Figure 5.4F**). In line with the lack of Cp A-induced caspase-8 proteolysis, expression of the caspase-8 substrate, Bid, was unchanged following Cp A treatment, while FasL triggered a loss of full-length Bid in the neutrophil cell extracts (**Figure 5.4F**). In contrast in LPS-primed macrophages, Cp A triggered a loss of full-length pro-caspase-8 and appearance of processed caspase-8 in the cell extracts and supernatants (**Figure 5.4F**), as well as IL-1β processing (**Figure 5.4E**). Similarly, Bid cleavage was readily observed in both Cp A and FasL-treated macrophages (**Figure 5.4F**).

c-FLIP hemizygosity enhances ripoptosome/caspase-8-mediated IL-1β processing in macrophages (276), so high c-FLIP expression may underlie the lack of pro-caspase-8 cleavage in neutrophils exposed to LPS/Cp A. LPS treatment induced c-$FLIP_L$ expression both cell types (**Figure 5.4F**), consistent with the view that LPS is a pro-survival signal (Chapter 3). c-$FLIP_s$ was barely detected in all treatments in either cell type. FasL induced c-$FLIP_L$ degradation in both neutrophils and macrophages, and accordingly, both cell types underwent caspase-8 cleavage and death (**Figure 5.4F**). Importantly, Cp A treatment did not affect c-$FLIP_L$ expression in neutrophils, but led to a complete loss of cellular c-$FLIP_L$ in macrophages, suggesting a possible role for c-$FLIP_L$ in regulating caspase-8 auto-processing and substrate repertoire in these cells. In all, the lack of cell death and mature IL-1β release in LPS/Cp A-stimulated neutrophils suggested at least three possible explanations: (1) the ripoptosome complex failed to be assembled in these cells; (2) caspase-8 was not activated because the ripoptosome was not recruited by TRIF to the TLR signalling complex; or (3) c-$FLIP_L$ was recruited to the TRIF-associated ripoptosome, and suppressed caspase-8 pro-apoptotic functions. Preliminary experiments to investigate the latter two possibilities were commenced, but full investigations were not possible due to time constraints. Future experiments will investigate each other these possible scenarios in detail.

5.2.5 Neutrophils appear unable to signal via TRIF

In macrophages, LPS treatment in IAP-depleted cells triggers ripoptosome-dependent cell death, IL-1β processing and NLRP3 activation (74, 88). This process requires signalling through the TLR3 and TLR4 adaptor TRIF, which recruits and activates RIPK3 kinase through homotypic RHIM-RHIM interactions (88). Interestingly, human neutrophils weakly express TRIF, and were unable to trigger TRIF-dependent signalling pathways such as the production of type I interferon following LPS engagement (246). TRIF signalling in murine neutrophils was therefore next investigated, as absent or altered TRIF signalling may provide a mechanism for the absence of caspase-8-dependent outputs (cell death, IL-1β maturation, NLRP3 activation) following LPS/Cp A treatment in neutrophils (**Figure 5.4**). TRIF was readily detected in neutrophil cell lysates (**Figure 5.5A**). Interestingly, the synthetic TLR3 agonist, poly(IC), appeared to provided a survival signal to neutrophils, similar to LPS (Chapter 3), as indicated by an increase in the level of tubulin loading control (**Figure 5.5A**), which may indicate that murine neutrophils can signal through TLR3, although this remains to be formally investigated. Having established that murine

neutrophils express TRIF, we next investigated whether LPS- or poly(IC)-treated neutrophils could signal via TRIF, by measuring proinflammatory cytokine production. TNF production can occur via the TRIF-dependent or -independent pathways downstream of TLR4 (277), but all cytokine production downstream of TLR3 depends upon TRIF. LPS triggered TNF (**Figure 5.5B**) but not IL-6 (not detected, data not shown) production in murine neutrophils, while poly(IC) did not trigger detectable levels of secreted TNF (**Figure 5.5B**) or IL-6 (not detected, data not shown). In all, these data suggest that murine neutrophils may trigger only a subset of the full spectrum of macrophage TLR/TRIF signals, as previously suggested for human neutrophils (246). Future experiments will assess the TRIF-dependent signalling events (e.g. IFN-β gene expression) following stimulation with various TLR ligands to confirm if LPS-dependent TRIF signalling is indeed deficient or altered in neutrophils.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Casp8 long exp.
Casp8 (p26)
Casp8 (p18)
cFLIP
cFLIPL
cFLIPs
Bid
GAPDH
SN
Casp8
Casp8 (p43)

Figure 5.4 LPS-primed neutrophils do not trigger RIPK3-dependent inflammatory responses upon functional blockade of IAPs.

(**A**) BMN or (**B**) BMDM were left untreated or treated with 500 nM Cp A in the presence or absence of 100 ng/ml LPS or 100 νg/ml TNF for 6 h. Cytoplasmic LDH release into the supernatant, as compared to total intracellular LDH of untreated cells was quantified as a measure of cell death. (**C-F**) BMN or BMDM were primed for 3 h with 100 ng/ml LPS before stimulation with 500 nM Cp A for 6 h. Cells were treated with 100 ng/ml FasL for 9 h. (C-D) IL-1β secretion was quantified by ELISA. (**E**) ASC oligomers in the Triton X-100 insoluble ($TX100^{insol}$) fraction and cleaved caspase-1 (p20) and mature IL-1β (p17) in the cell supernatant were detected by western blotting. (F) Caspase-8, c-FLIP and Bid in the cell extracts (XT) and supernatants (SN) were detected by western blotting. GAPDH was used as loading control. (**A**, **C**, **D**) Data are mean + S.E.M of three independent experiments, performed each in triplicate cell stimulations. (**B**) Data are mean + SD of technical triplicates, and representative of two experiments. (**E-F**) Western blots are representative of three independent experiments.

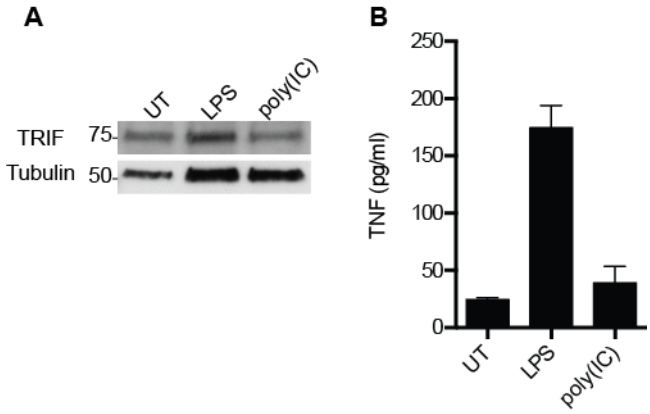

Figure 5.5 Murine neutrophils express TRIF, but may not signal via TRIF-dependent pathways.
Neutrophils were left untreated or stimulated with 100 ng/ml LPS or 50 μg/ml poly(IC) for 10 h. (**A**) TRIF expression in the whole cell lysate was detected by western blotting, versus a tubulin loading control. (**B**) TNF production was measured by ELISA. Data are mean SD of technical triplicate from a single experiment.

5.2.5 TNF but not LPS triggers RIPK3-dependent necroptosis in neutrophils

The earlier observation that TNF plus Cp A triggered neutrophil death, but not Cp A alone or in combination with LPS (**Figure 5.4A**), suggested that TNF/Cp A-induced cell death in neutrophils most likely occurred through Complex II, while Cp A plus LPS was not a strong stimulus for neutrophil ripoptosome signalling. To examine whether neutrophils can undergo necroptotic cell death, neutrophils were treated with TNF in the presence of Cp A and the pan-caspase inhibitor Q-VD-OPh (QVD), and cellular toxicity was measured by PI uptake after 24 h. 30% of untreated neutrophils were PI positive after culture for 24 h, reflecting basal apoptosis. In line with earlier observations (**Figure 5.4A**), Cp A or TNF alone had little effect on cell viability but in combination synergised to induce cell death in both the presence and absence of caspase inhibition (**Figure 5.6**). TNF/Cp A-induced cytotoxicity was unaffected by *Ripk3* deficiency, suggesting that in the absence of caspase inhibition, neutrophils underwent Complex II-dependent apoptosis. However, *Ripk3* deficiency protected neutrophils during TNF/Cp A/QVD-induced cell death (**Figure 5.6**), indicating that neutrophils underwent TNF-dependent necroptosis when caspase-8 function was inhibited. When a similar experiment was repeated in which TNF was substituted for LPS, neutrophils did not undergo cell death after any combination of Cp A, LPS and QVD, while Cp A/LPS and CpA/LPS/QVD did kill macrophages (88). Taken together, these data suggest that TNF but not LPS can trigger cell death pathways in IAP-depleted neutrophils.

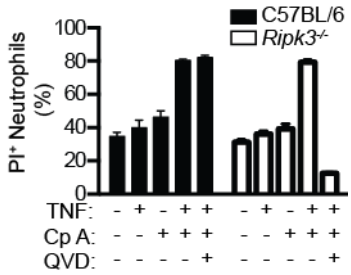

Figure 5.6 Cp A sensitises neutrophils to TNF-induced, RIPK3-independent death, while caspase inhibition triggers RIPK3-dependent necroptosis.

Bone marrow neutrophils were purified by cell sorting, and PI uptake was measured 24 h after cell exposure to TNF (100 ng/ml), Cp A (500 nM), QVD (20 μM) or combinations therein. Data are mean + SD of technical triplicate cell stimulations from a single experiment. This experiment was performed by Motti Gerlic (Walter and Eliza Hall Institute), who kindly granted permission for this data to be presented in this thesis.

5.3 Discussion

Previous data presented in this thesis demonstrated that NLRC4/caspase-1 activation in neutrophils selectively drives cytokine processing but not pyroptosis in neutrophils, and that this pathway specialisation is critical for *in vivo* defence against *Salmonella* (**Chapter 3**). The data presented in the current chapter extends these findings, demonstrating that neutrophil NLRP3 activation also does not elicit caspase-1-dependent pyroptosis, or indeed caspase-8-dependent apoptosis, in line with emerging data that this myeloid subset often exhibits specialised inflammatory signalling pathways. Neutrophil resistance to NLRP3/caspase-8-dependent cell death correlated with a lack of nigericin-stimulated pro-caspase-8 processing, and may reflect inhibition of pro-caspase-8 processing and apoptosis-initiating function by c-FLIP isoforms. Future experiments will use siRNA to silence c-FLIP expression in neutrophils, or use myeloid-specific conditional c-FLIP knockout neutrophils, to determine whether c-FLIP inhibition of pro-caspase-8 underlies the inability of neutrophils to undergo NLRP3-dependent apoptosis. The mechanism by which neutrophils avoid NLRP3/caspase-1-directed pyroptosis remain unclear, but may reflect reduced or absent expression of the unknown cell machinery required for pyroptosis execution.

As for previous results for neutrophil NLRC4, the inability of neutrophils to undergo NLRP3 inflammasome-dependent pyroptotic and apoptotic cell death likely represents an advantage to the host in specific circumstances. A key *in vivo* function of the NLRP3 inflammasome is to recruit neutrophils to a site of infection or injury, in order to clear infection or engulf cell debris. In these settings, neutrophils sensing NLRP3-activating stimuli are likely also exposed to inflammatory mediators such as IL-1β that prolong neutrophil lifespan (228). The inability of neutrophils to undergo NLRP3-dependent cell death, coupled with increased lifespan at sites of inflammation, may represent a mechanism by which neutrophil function is ensured until the cause of inflammation is resolved, at which time neutrophils will be allowed to undergo their underlying apoptotic program. If NLRP3 agonists could induce neutrophil death, this would compromise neutrophil antimicrobial mechanisms (e.g. ROS, neutrophil extracellular traps, granules) and phagocytic function at sites of injury or infection. The lack of inflammasome-mediated cell death in neutrophils may however pose a disadvantage under some circumstances; for example, it may result in increased susceptibility to specific neutrophil-trophic intracellular pathogens. For example, *Anaplasma phagocytophilum* and *Leishmania major* avoid immune surveillance by replicating within neutrophils, and conversely, neutrophil

depletion protected animals from *Anaplasma phagocytophilum* (278) and *Leishmania major* (279) infection. Interestingly, *Leishmania major* activates the inflammasome (280), and so one might predict that infected mice would show improved survival if neutrophils were able to trigger inflammasome-dependent cell death, similar to the improved survival of neutrophil-depleted as compared control mice (279, 281). Another setting in which the inability of neutrophils to undergo inflammasome-mediated cell death might contribute to pathology is in inflammatory disease, as the sustained viability of inflammasome-activated neutrophils may result in prolonged neutrophil cytokine production. NLRP3 dysregulation is associated with hereditary fever syndromes and acquired pathologies such as arthritis (2). As neutrophils make up more than 70% of white blood cells in the human circulation (228), and NLRP3-dependent inflammatory responses from neutrophils are not restricted by inflammasome-dependent cell death, neutrophils may be a key cell type for driving NLRP3-dependent pathologies through sustained IL-1β production in humans. The next chapter in this thesis investigates potential roles for neutrophils in mediating pathological inflammasome responses to bacterial LPS in mice, in order to provide proof-of-concept that neutrophil inflammasomes can drive human inflammatory diseases.

The surprising inability of neutrophils to undergo inflammasome-mediated apoptotic and pyroptotic pathways led us to investigate whether neutrophils could die via other inflammatory cell death pathways elicited during infection, such as ripoptosome- or necrosome-induced cell death downstream of LPS/TLR4 or TNF/TNFR signalling. TNF alone did not induce neutrophil cell death, but IAP inhibition sensitised the cells to RIPK3-independent cell death, likely mediated by TNF-induced complex II signalling to the caspase-8-driven apoptotic program. If caspase-8 function was inhibited by QVD, neutrophils instead underwent RIPK3-depedent necroptotic cell death. Intriguingly, LPS did not appear to induce any form of cell death in IAP-inhibited neutrophils, and correspondingly, Cp A/LPS did not trigger neutrophil NLRP3 signalling (**Figure 5.4C** and (88)). At least three possible reasons may account for the lack of ripoptosome- and necrosome-mediated cell death in LPS-stimulated neutrophils: (1) IAP depletion may not induce ripoptosome complex assembly in neutrophils, similar to observations in some cancer cells, such as chronic lymphocytic leukaemia (282); (2) High expression of c-FLIP isoforms in neutrophils may block these cell death modalities; or (3) TLR4 signalling in neutrophils may be unable to engage ripoptosome-mediated responses, due to deficient or altered TRIF signalling. To investigate if IAP depletion triggers ripoptosome formation in neutrophils, reciprocal co-immunoprecipitation experiments for components of the

ripoptosome (e.g. RIPK1, FADD and caspase-8) will be performed in Cp A-treated neutrophils. Such experiments will be performed using macrophages as a positive control, as macrophages are well established to signal via the ripoptosome in response to Cp A/LPS exposure. If the ripoptosome is indeed assembled in neutrophils, the role of c-FLIP in suppressing apoptosis and necroptosis will then be investigated. Gene silencing (siRNA) approaches will knock down the expression of both c-$FLIP_L$ and c-$FLIP_s$ isoforms, as both isoforms can block RIPK1/3-mediated cell death (264, 265). These experiments may be technically challenging, as neutrophils are highly apoptotic cells, and because c-FLIP suppresses caspase-8-mediated apoptosis. To circumvent these potential issues, neutrophils will be cultured in the presence of growth factors (e.g. G-CSF) during gene silencing and stimulation. Alternatively, experiments may be conducted using myeloid-specific c-FLIP-deficient cells. In macrophages, TLR4 activates RIPK3 via TRIF-RIPK1 interaction, leading to ripoptosome-mediated cell death and NLRP3 signalling (88). The possibility that TLR4 signalling does not activate the ripoptosome in IAP-inhibited neutrophils because of altered TRIF signalling in these cells will be a key focus of future investigations. TLR4-stimulated human neutrophils are unable to induce expression of type I IFN, possibly due to an inability to engage the TRIF signalling pathway (246). TRIF is a signalling adaptor that is recruited to TLR4 in the endosome following TLR4 internalisation. Importantly, internalisation of the TLR4:LPS complex into endosomes requires the TLR4 co-receptor, CD14 (277). Human neutrophils display ~20-fold fewer CD14 molecules on their cell surface compared to blood monocytes (283, 284); so decreased neutrophil CD14 expression may block neutrophil TRIF signalling and consequent RIPK1/3-mediated cell death pathways. We will investigate this possibility by profiling the expression of TRIF-dependent target genes such as IFN-β downstream of LPS stimulation in murine neutrophils. If indeed neutrophils do not trigger TRIF-dependent target genes, expression of cell surface CD14 will be investigated and LPS-mediated internalisation of TLR4 will be monitored. We anticipate that TLR4 will remain on the cell surface following LPS engagement cells such as neutrophils that weakly express CD14, while cells such as macrophages that express high levels of CD14 will internalise LPS-TLR4 as previously shown (277).

Apoptosis is traditionally regarded as an immunologically silent form of cell death; so it is intriguing that ripoptosome-dependent apoptosis triggers proinflammatory NLRP3 signalling in macrophages. It remains unclear what advantage this gives to the host, and likewise, what advantage is offered from neutrophil protection from such pathways. These

observations for macrophages contribute to an emerging literature suggesting that apoptosis induced by cellular stress or microbial infection can trigger pathways that propagate inflammation through the production of proinflammatory cytokines. For example, in the presence of LPS, FasL triggered apoptosis and concomitant IL-1β release in specific cell types, including neutrophils (243, 285, 286). Similarly, endoplasmic reticulum stress can also trigger apoptosis with accompanied IL-1β release (287). Apoptosis is a key innate immune mechanism to protect against viral infection, and is launched in infected cells via depletion of endogenous IAPs. Since viral infection often triggers IAP depletion and TLR3-TRIF-dependent signalling, RIPK3-mediated cell death and NLRP3 activation in this scenario likely provides important antiviral innate immune responses in macrophages. While neutrophils are traditionally viewed as mediators of anti-bacterial defences, emerging studies suggest that neutrophils can enhance antiviral responses as they express a number of intracellular nucleic acid sensors that can signal independently of TRIF such as AIM2 (**Chapter 3**), RIG-1 and MDA5 (288), and neutrophils can cast NETs to entrap human immunodeficiency virus-1 (HIV-1) (289). Therefore, it seems likely that RIPK3-dependent cell death pathways are switched off in neutrophils in order to prolong neutrophil viability at a site of infection.

In conclusion, this chapter demonstrates that NLRP3 activation in neutrophils triggers ASC polymerisation, caspase-1 cleavage and secretion of mature IL-1β. Unlike macrophages, however, neutrophils do not die via NLRP3/caspase-1 and NLRP3/caspase-8 pathways. Neutrophils also appeared to be protected from other cell death pathways induced by inflammatory stimuli in macrophages. Unlike macrophages, IAP depletion did not trigger neutrophil death, but IAP depletion did sensitise neutrophils to TNF-mediated apoptosis. Intriguingly, LPS exposure did not trigger death in IAP-depleted neutrophils, regardless of the presence or absence of caspase inhibition. In all, these data highlight the important influence of cell identity on immune signalling pathways, even within closely related myeloid cells. These cell type-specific signalling adaptations have likely evolved to provide a coordinated inflammatory response *in vivo.*

6. The neutrophil caspase-11 inflammasome in endotoxin shock

The PhD candidate led the experimental design and interpretation of data. However, due to the laborious nature of the experiments, all data presented in this chapter were acquired in conjunction with Dr Dave Boucher (50%).

6.1 Introduction

Cells of the innate immune system express a wide range of PRRs to detect the presence of microbial infection (1). One mechanism by which immune cells sense Gram-negative bacteria is by detecting LPS (also known as endotoxin), a major constituent of the Gram-negative bacterial cell wall. TLR4 is a well-characterised cell surface receptor that recognises extracellular LPS (290-292). TLR4 ligation triggers the induction of proinflammatory gene expression, including proinflammatory cytokines such as IL-6 and TNF (293), and components of the inflammasome signalling pathway such as NLRP3, IL-1β and caspase-11 (227, 230, 258). A recent report identified a second LPS receptor, caspase-11 (81). Unlike TLR4, caspase-11 is located in the cell cytoplasm, and so detects cytosolic LPS from intracellular bacteria. LPS binding appears to cluster caspase-11, triggering proximity-induced caspase-11 autoactivation (81). In macrophages, active caspase-11 drives pyroptotic cell death, leading to the passive release of alarmins such as IL-1α and HMGB1, and also directs NLRP3/caspase-1-dependent IL-1β secretion through an ill-defined mechanism termed “non-canonical NLRP3 activation” (76) that involves caspase-11-dependent K^+ efflux (82, 86, 294).

Sepsis is life-threating condition characterised by a complex systemic hyperinflammatory response that can lead to multi-organ failure if the host fails to control infection (295). Sepsis-induced sustained inflammatory dysregulation can progress to septic shock upon circulatory decompensation (i.e. hypotension) (296). While both TLR4 and caspase-11 pathways contribute to microbial clearance during infection, excessive activation of either pathway during murine experimental LPS challenge drives endotoxic shock, a systemic hyperinflammatory syndrome resembling clinical signs of septic shock in humans (77, 78, 291, 292, 296). Indeed, *Tlr4*-deficient and *Casp11*-deficient mice were both protected during challenge with a single high dose of LPS (up to 54 mg/kg) (77, 78, 292), but the precise pathways controlling pathogenic inflammation in this model are still incompletely understood. A 1995 study demonstrated that *Ice*$^{-/-}$ mice were resistant to challenge with a single high dose of LPS, similar to *Tlr4*$^{-/-}$ mice (226). Until recently, these mice were considered to be *Casp1* knockouts. Given that *Ice*$^{-/-}$ mice secreted significantly lower amount of IL-1β following LPS challenge, it was concluded that *Ice*$^{-/-}$ mice were protected from LPS lethality due to their inability to trigger caspase-1-dependent IL-1β secretion (226). Later studies showed that that caspase-1 activation required the assembly of an inflammasome complex (57), which suggested the existence of an unknown

inflammasome that assembles upon cytosolic LPS recognition. In 2011, Kayagaki and co-workers demonstrated that the commonly used caspase1-deficient *Ice*$^{-/-}$ mouse strain carry a passenger mutation in the *Casp11* locus, suggesting possible roles for caspase-11 in many of the phenotypes previously attributed to caspase-1, including endotoxin sensitivity (76). Subsequent studies used *Tlr4*$^{-/-}$ mice to investigate the role of caspase-11 in endotoxin sensitivity, in order to examine caspase-11 functions in isolation to LPS/TLR4-dependent signalling. Because caspase-11 is not constitutively expressed in most cell types but can be induced by TLR ligation, *Tlr4*$^{-/-}$ mice were first pre-administered the TLR3 ligand, poly(IC), to induce the expression of caspase-11. Remarkably, subsequent LPS challenge in poly(IC)-primed *Tlr4*$^{-/-}$ mice caused 100% lethality within 60 h, while more than 60% of *Casp11*-deficient mice were protected for 4 days or more during poly(IC)/LPS challenge. However, these studies did not investigate LPS sensitivity in the caspase-1/11-double deficient *Ice*$^{-/-}$ mice, and so it remained unclear whether there was an additional protection from the loss of caspase-1 (77, 78).

In addition to the poly(IC)/LPS model, mounting evidence also supports a pathological role for caspase-11 in the single high dose LPS challenge model. Kayagaki and co-workers demonstrated that *Ice*$^{-/-}$ mice reconstituted for *Casp11* function by BAC transgenesis (*Casp1*$^{-/-}$*Casp11*^{Tg}) became susceptible to single dose LPS challenge (76). Given that LPS-TLR4 signalling upregulates not only *Casp11* expression, but also expression of NLRP3 and pro-IL-1β (227, 258), this raises the question of whether caspase-11-driven lethality in this study is mediated by caspase-11-dependent pyroptosis and subsequent alarmin release, or by non-canonical NLRP3 activation and ensuing cytokine release. It also questions to what extent the resistance conferred by *Tlr4* knockout is attributable to deficient caspase-11 pathways during LPS challenge. Kayagaki *et al.* posited that caspase-11-dependent pyroptosis, and not non-canonical NLRP3 pathways, were likely responsible for LPS-induced lethality (76, 77). However, one of these studies showed that *Casp1*$^{-/-}$*Casp11*^{Tg}, *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice all showed significant protection during LPS challenge relative to WT mice (76), suggesting the possibility that the non-canonical NLRP3/ASC/caspase-1 pathway may also contribute to LPS-induced lethality via IL-1β or IL-18 production. In line with this, other studies supported a pathogenic role for IL-1 signalling in LPS-induced lethality as IL-1 receptor 1 (IL-1R1) deficiency or treatment with exogenous IL-1 receptor antagonist (IL-1Ra) protected animals from LPS-induced lethality (297-299). Subsequent studies using *Il1β*$^{-/-}$ mice revealed that IL-1β deficiency only partially protected mice during LPS challenge, while neutralisation of IL-18 in *Il1β*$^{-/-}$ mice

provided additional resistance (300). This suggests that IL-1β and IL-18 synergise to contribute to endotoxic shock. IL-1α is an alarmin released by pyroptotic cells that also signals through IL-1R1, although unlike IL-1β, it is not cleaved by caspase-1. Whether IL-1α also contributes to shock is currently unclear.

To my knowledge, no studies have yet directly assessed the impact of non-canonical NLRP3/caspase-1 activity in the poly(IC)/LPS challenge model. If cytokines indeed mediate pathogenic caspase-11 responses during endotoxic shock, then it is yet unclear which cytokines are responsible for driving lethality, and which cell types produce these cytokines. As outlined above, potential culprits are IL-1β and IL-18 released via non-canonical NLRP3 function, and/or IL-1α released upon NLRP3-independent cell lysis. This thesis previously demonstrated that neutrophils do not undergo NLRC4, NLRP3 or AIM2-dependent pyroptosis (**Chapters 3** and **5**) or inflammasome-mediated apoptosis (**Chapter 5**) but are a major cellular source of IL-1β during acute *Salmonella* infection (**Chapter 3**). Given that a key feature in LPS shock is neutrophilia (301), and neutrophils can produce substantial and sustained IL-1β, we hypothesised that caspase-11-activated neutrophils may present a sustained cellular source of pathogenic IL-1β that contributes to caspase-11-dependent LPS lethality. Thus, this chapter presents preliminary investigations into the potential contributions of the neutrophil caspase-11 inflammasome, and neutrophil-derived IL-1β, to endotoxin sensitivity.

6.2 Results

6.2.1 LPS transfection triggers caspase-11 activation in neutrophils

Previous data from the Schroder laboratory demonstrated that caspase-11 is constitutively expressed in bone marrow neutrophils (data not shown), suggesting the potential for neutrophils to contribute to caspase-11-driven inflammation. To examine whether neutrophils can signal via the caspase-11 inflammasome, we first purified bone marrow neutrophils from wild type (WT), *Casp11*$^{-/-}$, *Ice*$^{-/-}$ (*Casp1/11*$^{-/-}$) and *Nlrp3*$^{-/-}$ mice. These cells were primed with LPS for 4 h to upregulate the expression of NLRP3 and the caspase-1 substrate pro-IL-1β (230, 258). Subsequently, the cell culture media was replaced to remove residual LPS, and cells were transfected with ultrapure *E. coli* K12 LPS to the cytosol by chemical transfection to activate caspase-11. Macrophages, which are well established to activate caspase-11 upon LPS transfection (76), were prepared and stimulated in parallel as positive controls. The level of IL-1β secreted into the cell culture

supernatant was quantified by ELISA at 8h post-transfection as an output for caspase-11 activation. LPS priming alone was ineffective for triggering IL-1β secretion from neutrophils and macrophages (**Figure 6.1A, B**), but LPS transfection for 8 h triggered robust IL-1β secretion in neutrophils (**Figure 6.1A**), similar to macrophages (**Figure 6.1B**). Mechanisms underlying IL-1β production from neutrophils were the same as macrophages, as IL-1β secretion from both cell types was suppressed in *Casp11*$^{-/-}$, *Nlrp3*$^{-/-}$ and *Ice*$^{-/-}$ cells (**Figure 6.1A, B**). These data for neutrophils and macrophages are consistent with the prevailing paradigm that intracellular LPS activates the caspase-11 inflammasome, and triggers IL-1β production via non-canoncial NLRP3/caspase-1 activity. We presume that IL-1β production in WT neutrophils stimulated with intracellular LPS was accompanied by IL-1β maturation and caspase-1 processing, but this will need to be formally examined in future investigations.

6.2.2 Neutrophils are resistant to caspase-11-mediated pyroptosis

Another hallmark of caspase-11 activation is the initiation of the caspase-11-dependent, caspase-1-independent pyroptosis (76). Given that LPS transfection triggered robust caspase-11-dependent IL-1β secretion from neutrophils (**Figure 6.1A**), their potential to undergo caspase-11-mediated pyroptosis was next investigated. Neutrophils and macrophages of different genotypes were prepared as above, and the release of intracellular LDH was measured as an indicator of lytic cell death. LPS priming delivered a survival signal to neutrophils (**Figure 6.2A**) as previously reported (**Chapter 3** and **5**). 8 h LPS transfection did not induce LDH release from wild type neutrophils, and LDH release was not clearly altered by *Casp1/Casp11*, *Casp11* or *Nlrp3* deficiency in these cells (**Figure 6.2A**), despite clear caspase-11-dependent IL-1β secretion at this time point (**Figure 6.1A**). This was in striking contrast to WT macrophages, which underwent caspase-11-dependent cell death (**Figure 6.2B**) concomitant to IL-1β secretion (**Figure 6.1B**) upon intracellular LPS delivery. Thus, LPS transfection in neutrophils selectively triggers non-canonical NLRP3/caspase-1-dependent IL-1β production but not caspase-11-dependent pyroptosis.

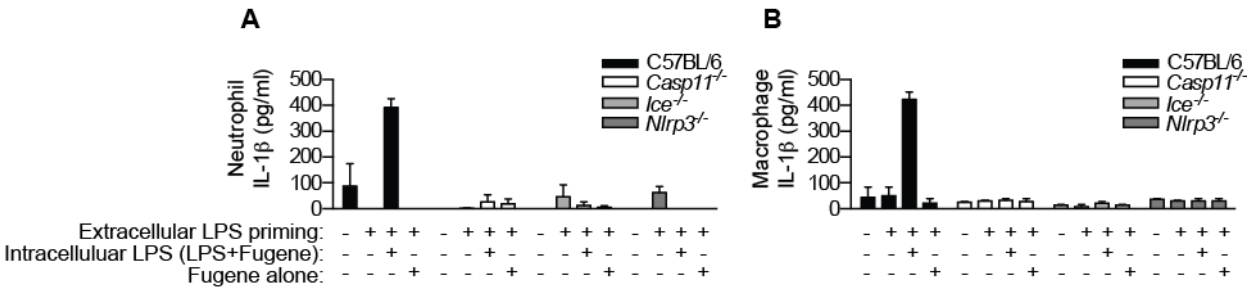

Figure 6.1 LPS transfection triggers caspase-11 activation in neutrophils.

(**A**) Bone marrow neutrophils or (**B**) bone marrow-derived macrophages were primed with 100 ng/ml LPS for 4 h and washed. 2 μg/ml ultrapure LPS *E.coli* K12 was then transfected using Fugene. IL-1β secretion was quantified by ELISA at 8 h post-transfection. Data are mean + range of duplicate cell stimulations and are representative of 3 independent experiments.

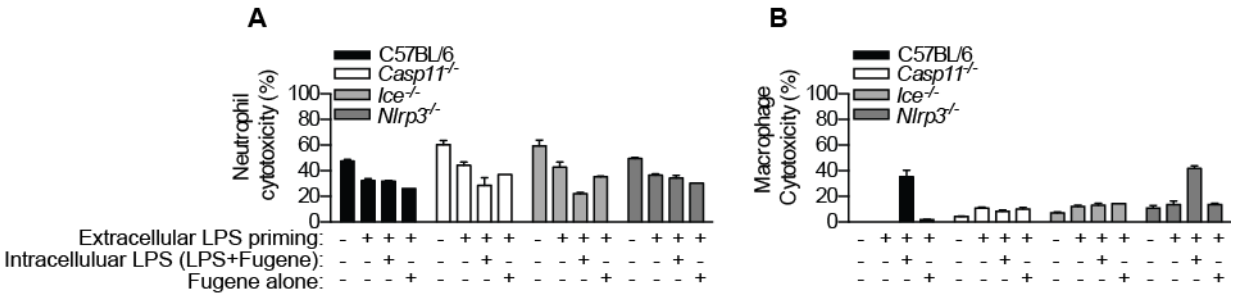

Figure 6.2 Neutrophils do not undergo caspase-11-mediated pyroptosis.

(**A**) Bone marrow neutrophils or (**B**) bone marrow-derived macrophages were primed with 100 ng/ml LPS for 4 h and washed. 2 μg/ml ultrapure LPS *E.coli* K12 was then transfected using Fugene. Intracellular LDH release was quantified as a measurement of cell death at 8 h post transfection, and expressed as a percentage of the total intracellular LDH of untreated cells. Data are mean + range of duplicate cell stimulations and are representative of 3 independent experiments.

6.2.3 NLRP3 contributes to lethality during *in vivo* poly(IC)/LPS challenge

The possibility that NLRP3 signalling contributes to LPS lethality in the poly(IC)/LPS challenge model was next examined by challenging WT and *Nlrp3*$^{-/-}$ mice with a non-lethal dose of poly(IC) (10 mg/kg) followed by 10 mg/kg of *E. coli* 0111:B4 LPS. Mouse rectal temperature and survival was monitored over 72 h. Consistent with previous reports, low dose of poly(IC) or LPS treatment alone did not induce hypothermia or septic shock-like symptoms (**Figure 6.3A, B**) (77, 78). However, LPS challenge in poly(IC)-primed mice triggered hypothermia in both WT and *Nlrp3*$^{-/-}$ animals up to 10 h post LPS challenge (**Figure 6.3C**). Interestingly, NLRP3 deficiency protected mice from further hypothermia, and rectal temperature recovered progressively towards 72 h (**Figure 6.3C**). In keeping with this, *Nlrp3*$^{-/-}$ mice displayed a significantly smaller change in body temperature compared to wild type animals (**Figure 6.3D**). Importantly, 100% of *Nlrp3*$^{-/-}$ mice were protected from poly(IC)/LPS-induced lethality, while all WT mice succumbed, by 24 h (**Figure 6.3E**), suggesting that NLRP3 is an important driver of caspase-11-mediated endotoxin shock.

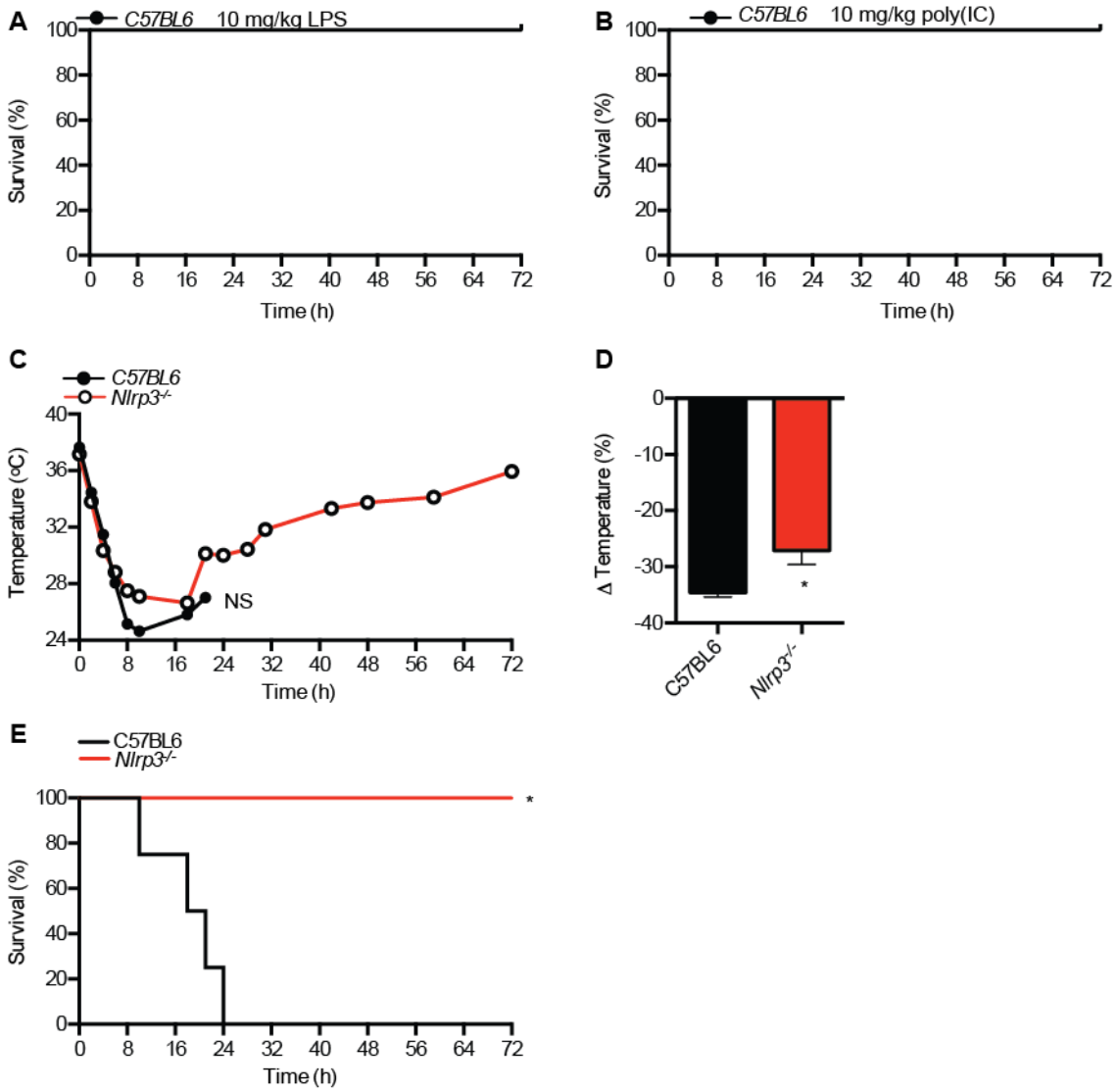

Figure 6.3 NLRP3 deficiency protects mice from caspase-11-mediated endotoxin shock.

Mice were injected with (**A**) 10 mg/kg of *E. coli* 0111:B4 LPS or (**B**) 10 mg/kg of poly(IC) and monitored for signs of endotoxemia up to 24 h. (**C-E**) Mice were injected i.p with 10 mg/kg of poly(IC) for 6 h followed by a subsequent i.p injection with 10 mg/kg *E. coli* 0111:B4 LPS, and monitored for signs of endotoxemia. Mice were considered moribund and sacrificed immediately when rectal temperature fell below 24 ^{o}C or when mice were unable to regain their posture. (**C**) Rectal temperature of poly(IC)-primed mice following LPS challenge. The statistical significance for *Nlrp3*$^{-/-}$ was calculated relative to WT mice up to 20 h post-LPS challenge. (**D**) Percentage change in rectal temperature in panel (**A**) at 10 h post-LPS challenge. (E) Kaplan-Meier survival curve for LPS challenge in poly(IC)-primed mice. (**A-B**) 2 mice or (**C-E**) 4 mice were used per group, and data are representative of a single experiment.

6.2.4 Neutrophil depletion reduces susceptibility to caspase-11-dependent endotoxemia *in vivo*

The contribution of neutrophil caspase-11 function to endotoxic shock *in vivo* was next investigated. Mice were administered a neutrophil-specific α-Ly6G antibody to transiently deplete neutrophils *in vivo,* or alternatively, an isotype control antibody (as for **Chapter 2**). 16 to 20 h after antibody injection, mice were challenged as before with poly(IC) and LPS to activate caspase-11. LPS challenge in poly(IC)-primed mice triggered hypothermia in both WT and *Casp11*$^{-/-}$ mice, regardless of neutrophil depletion (**Figure 6.4A**). Although *Casp11*$^{-/-}$ mice were as susceptible as wild type mice to hypothermia at early stages (**Figure 6.4A, B**), *Casp11*$^{-/-}$ mice did not succumb to LPS lethality (**Figure 6.4C**) and rectal temperature recovered over the course of 72 h (**Figure 6.4A**). Similar to caspase-11 deficiency, neutrophil depletion protected WT mice from hypothermia (**Figure 6.4B**), with mice recovering quickly from 8 h post-LPS challenge, while neutrophil-sufficient WT mice remained hypothermic until they became moribund and were sacrificed at 33 h post-LPS (**Figure 6.4A**). Moreover, neutrophil depletion conferred protection from LPS lethality, with 100% of neutrophil-sufficient WT mice becoming moribund within 32 h, while only 34% of neutrophil-depleted animals succumbed by 72 h post-LPS challenge (**Figure 6.4C**). Poly(IC)/LPS-induced death in this setting is mediated by caspase-11-dependent responses, as *Casp11*$^{-/-}$ mice were protected during poly(IC)/LPS challenge (**Figure 6.4C**). Importantly, neutrophil depletion had no effect on hypothermia or survival rates in *Casp11*$^{-/-}$ mice, suggesting that neutrophils contribute to these responses via caspase-11 function (**Figure 6.4A-C**). To investigate mechanisms underlying the protective effect of neutrophil depletion, cytokine levels in serum were quantified at 4 h post LPS challenge in poly(IC)-treated mice, with and without prior neutrophil depletion. TNF production is elicited by TLRs independently of inflammasomes, and TNF levels were unaffected by neutrophil depletion (**Figure 6.4D**), suggesting that both groups of mice responded equally to acute poly(IC)/LPS challenge. Because neutrophils contributed to pathological inflammation in poly(IC)/LPS-challenged mice, and caspase-11 activation in neutrophils triggers NLRP3-dependent IL-1β but not cell lysis and ensuing alarmin (e.g. IL-1α) release (**Figure 6.1A and 6.2A**); we hypothesised that neutrophils contribute to septic shock via production of IL-1β but not IL-1α. We thereby predicted that neutrophil depletion would specifically reduce serum IL-1β levels, however, we observed no significant effect of neutrophil depletion on either serum IL-1α (**Figure 6.4E**) or IL-1β (**Figure 6.4F**) at 4 h post LPS challenge in poly(IC) primed mice. This was very surprising as IL-1β drives hypothermic

responses (302), and neutrophil depletion protected 66% of wild type animals from poly(IC)/LPS-induced lethality (**Figure 6.4C**), and markedly protected against hypothermia (**Figure 6.4A**). The protective effect of caspase-11 deficiency or neutrophil depletion on body temperature was most evident at later stages of challenge (e.g. 18-31 h), thus, it is likely that neutrophil caspase-11-dependent IL-1β secretion becomes prevalent at later stages of challenge. Additional kinetic experiments to address this were not performed as part of this thesis, due to time constraints. Taken together, these results suggest that pathological immune responses in poly(IC)/LPS challenge model are mediated in three distinct phases: (1) in the acute phase (0-4 h post-challenge), caspase-11-independent mechanisms, possibly involving TNF, initiate hypothermic responses; (2) in the intermediate phase (4-8 h post-challenge) caspase-11-dependent pyroptotic cell death releases alarmins, thereby exacerbating hypothermia and septic shock-like symptoms; and (3) in the late phase (>8 h), non-canonical NLRP3 dependent pathways (e.g. neutrophil-derived IL-1β secretion) further amplify inflammation and ultimately lead to mortality. Although neutrophil depletion conferred protection in the caspase-11-mediated endotoxemia model used here, the mechanisms responsible remain to be definitively established. Neutrophil-mediated cytokine production at later time points of challenge will thus be a key focus of future investigations.

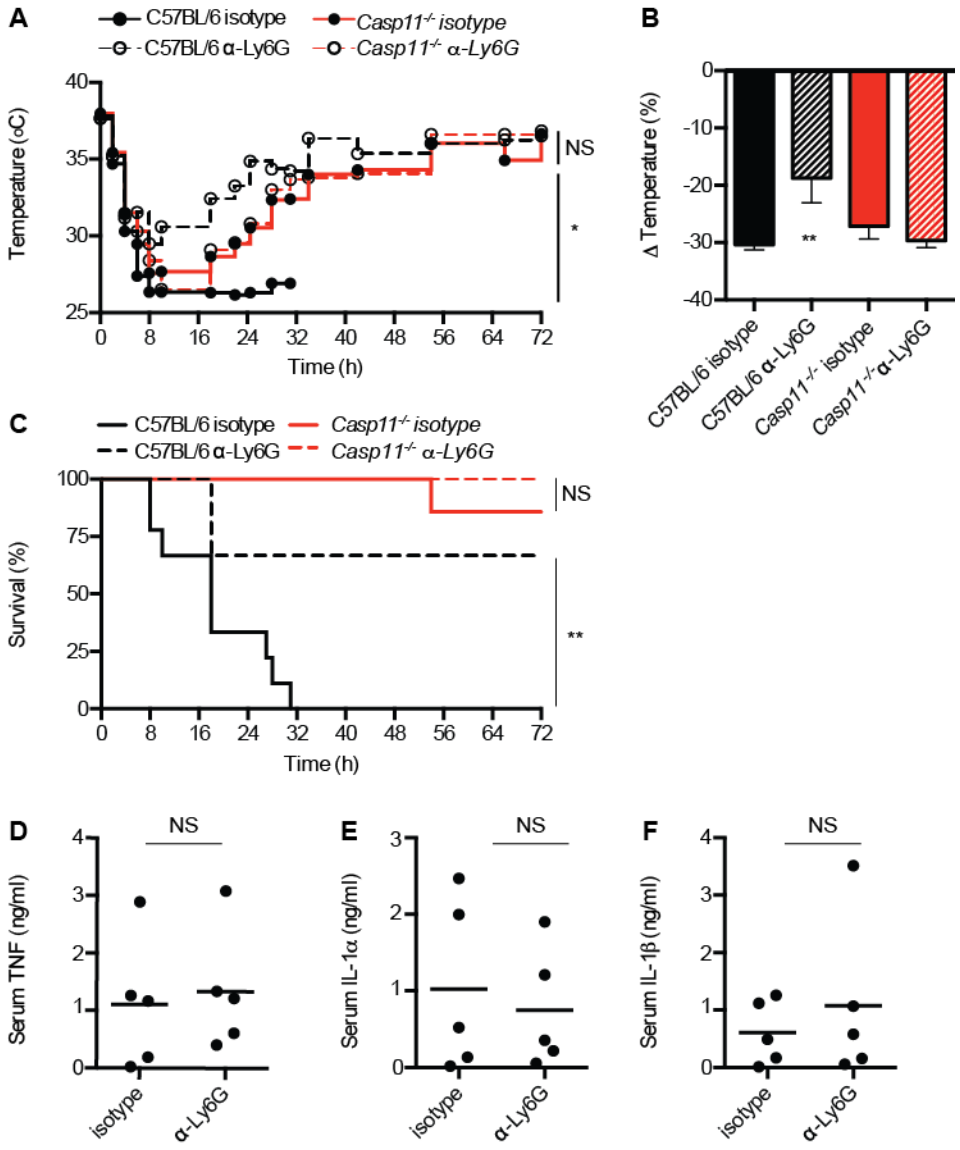

Figure 6.4. Neutrophil depletion protects mice from caspase-11-mediated endotoxic shock.

Mice were administered a single injection of 100 μg α-Ly6G or isotype antibody. 16 to 20 h later, mice were administered 10 mg/kg of poly(IC) for 6 h, followed by 10 mg/kg *E. coli* 0111:B4 LPS, and monitored for signs of endotoxemia. Mice were considered moribund and sacrificed immediately when rectal temperature dropped below 24°C or when mice were unable to retain their posture. (**A**) Rectal temperature of poly(IC)-primed mice following LPS challenge. The statistical significance for WT mice was calculated relative to neutrophil-depleted WT mice up to 32 h post-LPS challenge. (**B**) Percentage change in rectal temperature of panel (**A**) at 10 h post-LPS challenge. (**C**) Kaplan-Meier survival curve for LPS challenge in poly(IC)-primed mice. (**D-F**) Serum cytokine levels of wild type mice with and without neutrophil depletion. (**A-B**) Data are from 5 mice per group, and are representative of 3 independent experiments. (**C**) Data are pooled from 2 independent experiments and contain 7 to 10 mice per group. (**D-F**) Data are means from serum collected from individual mice (5 mice per group).

6.2.5 Adoptive transfer of B220$^-$ bone marrow cells

In order to formally establish that caspase-11 function in neutrophils drives endotoxic shock responses *in vivo*, future experiments will adoptively transfer wild type versus *Casp11*$^{-/-}$ (CD45.2) donor neutrophils into congenic CD45.1 wild type hosts, or congenic CD45.1 wild type donor neutrophils into (CD45.2) wild type versus *Casp11*$^{-/-}$ hosts, prior to poly(IC)/LPS challenge. Preliminary experiments attempting systemic neutrophil adoptive transfer using tail vein injection were unsuccessful; and colleagues from the University of Queensland observed that donor neutrophils were cleared in the lung at 2-4 h post-transfer (unpublished data; personal communications with Marion Brunk). To circumvent this issue, future experiments will deliver donor neutrophils by intraperitoneal injection. In an initial trial experiment, B220$^+$ B cells were depleted from total CD45.2 donor bone marrow cells. 1 x 10^7 B220-depleted, unlabelled donor bone marrow cells were transferred via i.p. injection into CD45.1 recipient mice that were previously administered with an α-Ly6G antibody to deplete endogenous neutrophils. Flow cytometry was used to examine whether donor cells were recoverable from the recipient animal 6 h after poly(IC) priming, before proceeding with LPS challenge. Although CD45.2$^+$ donor cells were indeed recovered from poly(IC) challenged mice (**Figure 6.5A**), the majority of CD45.2$^+$ cells were Ly6C$^+$Ly6G$^-$ monocytes rather than CD11b$^+$Ly6G$^+$ neutrophils (**Figure 6.5A-B**). Apoptotic neutrophils can home to the bone marrow (303), but in this case donor CD45.2$^+$Ly6G$^+$ neutrophils were not present in the bone marrow (**Figure 6.5B**). The possibility that the unlabelled α-Ly6G antibody used for neutrophil depletion may have masked the α-Ly6G antibody epitope was investigated, but this possibility was excluded by alternative gating methods (CD11b$^+$SSC^{hi}, data not shown). It is likely that under this experimental regimen, the α-Ly6G antibody used to deplete recipient mice of neutrophils was still circulating at the time of neutrophils transfer, leading to donor neutrophil lysis. Future experiments will either optimise the timing or dose of antibody administration, or will utilise neutrophil-sufficient recipient mice.

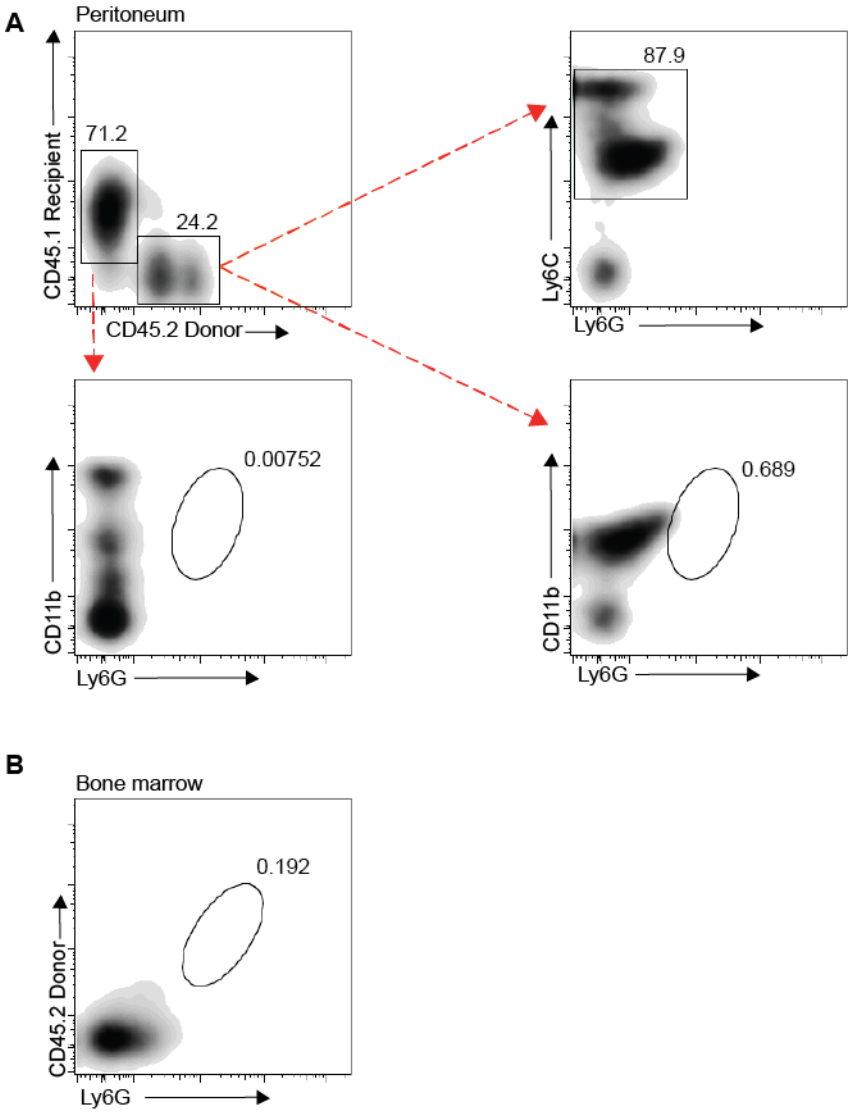

Figure 6.5 Adoptive transfer of B220- bone marrow cells.

Recipient CD45.1 mice were given a single i.p. injection of α-Ly6G (100 μg) 72 h prior to adoptive transfer. 1 x 10^7 $B220^-$ CD45.2 donor bone marrow cells were injected i.p. into CD45.1 recipient mice. Mice were challenged 1 h later with 10 mg/kg of poly(IC) and sacrificed at 6 h post-challenge. Flow cytometry analysis of the cellularity of the (**A**) peritoneal exudate cells and (**B**) bone marrow from the CD45.1 recipient mouse. Profiles are representative of data from 3 individual mice.

6.3 Discussion

The caspase-11 inflammasome is an important mediator of antimicrobial responses to cytoplasmic bacteria (75); however, excessive signalling through this pathway drives lethal endotoxemia (77, 78). A previous study demonstrated that *Casp1/11*-deficient *Ice*$^{-/-}$ mice reconstituted with a *Casp11* transgene using a bacterial artificial chromosome (*Casp1*$^{-/-}$ *Casp11*^{Tg}) were substantially more susceptible to LPS-induced lethality than *Ice*$^{-/-}$ mice, while mortality in *Casp11*$^{-/-}$ mice was significantly delayed relative to WT (76). On the basis of these observations, it was suggested that caspase-1-dependent cytokines (IL-1β and IL-18) are not important mediators of caspase-11-dependent septic shock (77). However, this conclusion needs to be revisited, as transcriptional regulation and expression of the *Casp11* transgene may not reflect endogenous *Casp11* in wild type mice, because the gene context (e.g. chromatin structure, enhancer environment, etc) is fundamentally changed, despite retaining the natural *Casp11* proximal promoter. In support of this, the same study demonstrated that *Casp1*$^{-/-}$*Casp11*^{Tg}*, Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice all showed significant protection during LPS challenge relative to WT mice (76), suggesting that the non-canonical NLRP3/ASC/caspase-1 pathway may also contribute to LPS-induced lethality. It is also important to highlight that the role of poly(IC) priming was not investigated in the previous study, and was only regarded as a means to induce caspase-11 expression. But poly(IC) stimulation also induces the expression of NLRP3, and the caspase-1 substrate pro-IL-1β in immune cells (304, 305). In support of poly(IC) upregulating the NLRP3/caspase-1/IL-1β signalling axis, our data demonstrate that *Nlrp3* deficiency protected poly(IC)-primed mice during LPS lethality, suggesting that the NLRP3-dependent cytokines, IL-1β and IL-18, are likely candidates for driving endotoxin shock following poly(IC)/LPS challenge. At this stage, we are unable to speculate whether IL-1β or IL-18, or the combination of both cytokines, are likely to drive septic shock-like symptoms, as both cytokines were previously implicated in LPS shock models (297-300). If further experiments suggest that excessive cytokine production through the NLRP3/caspase-1 axis indeed contributes to caspase-11-mediated endotoxic shock, this will open up new therapeutic avenues for the treatment of endotoxin shock, for example, the small molecule NLRP3 inhibitor, MCC950 (123).

If NLRP3-dependent cytokines drive lethal septic shock responses *in vivo*, then the cellular source of such cytokines needs to be formally established. Monocytes/macrophages are clear candidates, but other cell types should also be considered. Because neutrophilia is a hallmark of sepsis (301), and neutrophils secrete IL-1β in a manner not curtailed by

inflammasome-mediated cell death, this presents neutrophils as a likely and perhaps major cellular source of IL-1β during *in vivo* lethal endotoxemia. Neutrophil-derived IL-18 will also be investigated, however, neutrophils were weak producers of IL-18 downstream of NLRC4 (**Chapter 3**), suggesting that if IL-18 indeed contributes to endotoxic shock, it is likely derived from other cellular sources. Supporting our hypothesis for pathogenic neutrophil caspase-11 function in endotoxic shock, neutrophil depletion strongly protected wild type mice during poly(IC)/LPS challenge, while neutrophil depletion did not significant impact *Casp11*$^{-/-}$ mice; this indicates that the protective effect of neutrophil depletion lies within the caspase-11 pathway. Surprisingly, while neutrophil depletion protected 66% of mice from lethality, it did not significantly alter serum IL-1β levels at 4 h post-LPS challenge. Since IL-1β is a key regulator of the hypothermic response (302), and that neutrophil depletion protected mice from hypothermia at 8 h post-LPS challenge and later, this suggests that neutrophil-derived IL-1β levels may peak at a later stage of LPS challenge. Future experiments will investigate the time course of neutrophilia and serum IL-1β profiles in the poly(IC)/LPS challenge model, and assess serum IL-1β levels and cleavage status in control and neutrophil depleted mice at later stages of poly(IC)/LPS challenge. We will also purify and culture *in vivo*-challenged neutrophils *ex vivo*, to demonstrate that these neutrophils secrete IL-1β via NLRP3-dependent means. As a complementary approach, we will quantify the expression of intracellular pro-IL-1β in the inflammatory infiltrate of WT and caspase-11-deficient animals by flow cytometry. If neutrophil caspase-11 function indeed contributes to IL-1β production during LPS challenge, we expect a concomitant loss of intracellular pro-IL-1β expression *in vivo*, and a corresponding release of IL-1β *ex vivo,* in WT but not *Casp11*-deficient neutrophils following poly(IC)/LPS challenge.

The protective effect of neutrophil depletion during poly(IC)/LPS challenge suggests two possible scenarios by which neutrophils may contribute to LPS-induced lethality: (1) caspase-11 function in neutrophils may drive pathogenic IL-1β production through non-canonical NLRP3 activation, or (2) caspase-11-independent neutrophil functions such as NET formation may promote caspase-11 signalling in other cells (306-308). Therefore, additional experiments are required to definitively establish whether neutrophils influence caspase-11 pathways in a cell-autonomous fashion (i.e. caspase-11 signalling *in neutrophils* drives septic shock). An elegant approach for delineating *in vivo* caspase-11 functions in neutrophils would be to use a neutrophil-specific conditional *Casp11* knockout.

However, genetic approaches for neutrophil-specific gene knockout are not currently available (233); the widely used 'neutrophil-specific' *LysMCre* line for Cre-mediated gene deletion also targets macrophages and other myeloid cells, as the *LysM* promoter is active throughout the myeloid compartment (309). An alternative method for defining the role of caspase-11 in neutrophils is to perform adoptive transfer experiments with wild type versus *Casp11*$^{-/-}$ donor neutrophils and recipient mice, and assessing their responses upon LPS challenge. This thesis presents data from preliminary experiments aimed at establishing such a protocol for neutrophil adoptive transfer, but this trial unfortunately failed to recover any donor neutrophils from the recipient animal following poly(IC) challenge. At least three possible explanations may explain the lack of donor CD45.2^{+}CD11b^{+}Ly6G^{+} neutrophils recovered from recipient mice. Firstly, and perhaps most likely, residual circulating α-Ly6G antibodies in recipient mice may have caused the transferred neutrophils to lyse. Future experiments will use recipient mice that have not been exposed to the α-Ly6G antibody, or will optimise the time between application of the α-Ly6G antibody and injection of CD45.2^{+} cells, to allow sufficient time for the α-Ly6G antibody to be cleared from the circulation. Secondly, the transferred neutrophils may have been phagocytosed by resident peritoneal macrophages upon injection, as the presence of a large number of neutrophils is uncommon in tissues during the steady state. This is however unlikely, as phagocytosis of neutrophils generally occurs after the exposure of 'eat-me' signals that are usually only expressed on apopototic neutrophils (310). Thirdly, the process of neutrophil enrichment and adoptive transfer in this experiment may have activated the neutrophils, triggering neutrophil cell death. Future experiments will examine the expression of cell surface activation and apoptotic markers (e.g. CD62L, annexin V) and plasma membrane integrity (e.g. propidium iodide staining) on purified neutrophils prior to adoptive transfer. Additional experiments will be performed to optimise the neutrophil adoptive transfer protocol and to assess the contribution of neutrophil-intrinsic caspase-11, caspase-1 and NLRP3 pathways, and the cytokines IL-1α, IL-1β and IL-18 in driving lethal endotoxin shock. We will also monitor the fitness of poly(IC)/LPS-challenged wild type, *IL-1*$\alpha^{-/-}$, *IL-1*$\beta^{-/-}$ and *IL-1R*$^{-/-}$ animals to assess possible IL-1 synergies in septic shock.

Caspase-11 is not constitutively expressed in most cell types *in vivo*. *Casp11* expression is upregulated following interferon (IFN) signalling (79, 227), which occurs downstream of TLR engagement by LPS or poly(IC). Since caspase-11 activation during bacterial infection requires type I IFN signalling (80), this suggests that individuals with pre-existing infections or autoimmune diseases are likely to exhibit sensitised caspase-11 activation

pathways, and perhaps be more susceptible to caspase-11-mediated pathologies during bacterial infection, compared to otherwise healthy individuals. In line with this, individuals with autoimmune diseases such as systemic lupus erythematosus (311) or viral infection are often predisposed bacterial infection and its associated septic shock (312). In fact, 38% of sepsis cases in the United States were caused by viral infection followed by secondary Gram-negative bacterial superinfection (312) and recent autopsy investigations revealed that the leading cause of death in the 1918 'Spanish flu' pandemic was due to influenza A virus plus bacterial co-infection (313). Since poly(IC) is a synthetic RNA analogue that mimics viral infection, and that it triggers TLR signalling and induces autocrine and paracrine IFN signalling, the poly(IC)/LPS challenge model provides a system to study functional links between IFN and septic shock during human diseases such as bacterial superinfection.

Because TLR4-deficient mice were resistant to high dose LPS challenge, and LPS administration as low as 4 ng/kg in healthy human subjects triggered cardiovascular dysfunction similar to that observed during septic shock (314), various approaches to neutralise extracellular LPS or TLR4 were designed as potential therapies to ameliorate septic shock. However, these neutralising antibodies lacked efficacy in numerous large-scale clinical trials (315). It is not entirely clear how to interpret this lack of efficacy, as it raises three distinct possibilities: (1) LPS-independent mechanisms drive septic shock in human patients (e.g. TLR signalling by other microbial structures); (2) LPS signalling is not neutralised by anti-LPS or anti-TLR4 antibodies. For example, such antibodies may not access intracellular compartments to neutralise LPS signalling via endosomal (e.g. TLR4) or cytosolic (e.g. caspase-4/5) receptors; and/or (3) Neutralising antibodies were not administered early enough in disease progression to effectively block the clinical signs of septic shock.

It should be noted that the murine models of endotoxic shock presented in this chapter hold some limitations for understanding the corresponding human pathology. First, there is no direct one-to-one ortholog of *Casp11* in humans. Humans instead encode two orthologs, *CASP4* and *CASP5* (83). Caspase-4 and -5 do however share the same LPS-binding properties of caspase-11, and cytosolic bacterial infection in human myeloid cells triggers caspase-4/5-dependent cell death and caspase-4/NLRP3-dependent cytokine secretion (85, 86). Importantly, transgenic animals encoding human caspase-4 were hypersensitive to LPS challenge, implying that caspase-4, like caspase-11, may also be

involved in endotoxin sensitivity in humans. Second, the reliance of murine models to understand human diseases, in particular, endotoxin shock, has been frequently questioned because mice are significantly more resistant to LPS challenge than humans (316), and the LPS concentration in human plasma does not necessarily correlate with mortality in human septic shock patients (317, 318). In addition, transcriptional analysis of murine and human samples during endotoxin shock revealed poor correlation between the two species, further questioning the relevance of modelling human endotoxin shock using mice challenged with LPS (319). However, subsequent re-analysis of the same gene expression data set by two independent groups using different methodologies revealed contrary results, that is, that gene expression during inflammatory diseases such as endotoxin shock were indeed correlated between human patients and mouse models (320, 321). It should also be highlighted that septic shock patients are frequently cytopenic or pan-cytopenic (322). The use of unfractionated leukocytes for the aforementioned transcriptional analyses thus confounds data interpretation, as leukocyte composition is likely to be significantly altered between sepsis patients and healthy controls. The profiling of highly purified immune cell subsets would be a more elegant approach for interrogating the transcriptional changes associated with human septic shock. While we acknowledge that murine models of endotoxin shock may not completely represent mirror human sepsis, they do provide a tractable system for examining inflammatory signalling pathways, their cellular players, and downstream pathology.

In summary, the work presented in this chapter demonstrates that neutrophil LPS transfection triggers caspase-11-dependent IL-1β secretion but not pyroptosis *in vitro*. It also demonstrates that NLRP3 and caspase-11 functions are important drivers of LPS-induced lethality in poly(IC)-primed mice. Lastly, this chapter demonstrates a novel role for neutrophils in poly(IC)/LPS-induced lethality in WT but not *Casp11*-deficient mice. These data support a model in which neutrophils drive pathological immune responses during endotoxin shock via caspase-11/NLRP3-driven cytokine production. This hypothesis will be the focus of future studies that will define the precise role of neutrophils during murine septic shock.

7. Final discussion

Inflammasome activation in all cell types studied prior to this thesis triggers a burst of IL 1β/18 production that is accompanied by rapid cellular lysis. Given this literature, one might expect that inflammasome-dependent cytokines would only be produced transiently *in vivo*. However, IL-1β levels remain elevated over extended periods during infection and inflammatory disease (323, 324). This suggests that cytokine production and cell death occur in repeated waves of inflammasome signalling by incoming cells, or alternatively, that some cell types are protected from inflammasome-mediated death, allowing them to sustain inflammasome-dependent cytokine production within inflammatory foci. A major biological function of IL-1β is to recruit neutrophils to a site of inflammation and activate them to engulf invading pathogens or remove cellular debris (4). Human neutrophils were reported to regulate their own recruitment and activation by secreting IL-8 upon pathogen recognition (325), and so this thesis investigated whether neutrophils utilise the inflammasome-IL-1β signalling axis as a similar auto-regulatory strategy. Previous studies had discounted possible functions for neutrophil inflammasomes, after observing that human and murine neutrophils did not undergo pyroptosis when infected with NLRC4 activators such as *S.* Typhimurium and *Burkholderia pseudomallei* (53, 115), and reporting that neutrophils contributed to IL-1β processing through caspase-1-independent mechanisms such as Fas ligation (243) or pro-IL-1β cleavage by neutrophil granular proteases (324, 326-328). Collectively, these reports led to an assumption that neutrophils do not themselves signal via inflammasomes and are thus merely cellular targets of inflammasome-derived IL-1β. We and others observed that neutrophils express multiple NLRs including NLRC4 and NLRP3, and other critical components of the inflammasome signal transduction pathway such as the ASC inflammasome adaptor, and the zymogen pro-caspase-1, indicating that neutrophils may be able to sense inflammasome-activating stimuli (241, 242, 257). Indeed, this thesis demonstrates that neutrophils can signal via NLRC4, NLRP3, AIM2 and caspase-11 inflammasomes, although the cellular outcomes of signalling are distinct as compared macrophages.

A surprising but major finding of this thesis is that inflammasome activation in neutrophils selectively triggered caspase-1-dependent cytokine maturation without concomitant pyroptotic death. Neutrophil protection from caspase-1-driven pyroptosis appears to be a universal mechanism, as activation of the four major inflammasomes (NLRC4, NLRP3, AIM2 and caspase-11) failed to trigger neutrophil lysis. Macrophage inflammasomes also

recruit and activate caspase-8, and trigger apoptotic cell death if pyroptosis is blocked by caspase-1 deficiency or inhibition (69, 70). Interestingly, neutrophils also resisted inflammasome-dependent apoptosis following NLRP3 activation. Although it was not formally tested in this thesis, the failure of neutrophils to undergo inflammasome-dependent apoptosis is anticipated to also apply to other inflammasomes (e.g. AIM2, NLRC4), and provides a likely explanation for why neutrophils but not *Ice*$^{-/-}$ macrophages remained viable after prolonged (16 h) *Salmonella* infection (**Chapter 3**, **Supplementary Figure 3.6A**). The primary known function of pyroptosis is to prevent intracellular pathogens from establishing a replicative niche, and to expose these microbes to neutrophil-mediated destruction (115). So the unique ability of neutrophils to resist inflammasome-mediated cell death is likely critical for enabling these cells to perform their classical antimicrobial functions (e.g. phagocytosis, production of ROS, NETs and antimicrobial peptides), while simultaneously driving an IL-1β-mediated auto-regulatory loop to ensure further neutrophil recruitment and activation. In addition to inflammasome-dependent cell death, neutrophil protection from TLR4/RIPK3-dependent apoptosis and necroptosis also likely represents a mechanism to ensure neutrophil viability for IL-1β production and antimicrobial defence during *in vivo* microbial infection. While the inability of neutrophils to undergo inflammasome-dependent cell death may facilitate host defence in the short term, it may ultimately render these cells susceptible to infection by specific intracellular microbes. Indeed, neutrophils are a replicative niche for several intracellular pathogens such as *Salmonella, Anaplasma phagocytophilum*, *Leishmania* and *Neisseria* (248, 249, 279, 329, 330). In fact, a key virulence strategy of *Anaplasma phagocytophilum* is to induce the production of the neutrophil chemoattractant IL-8 (KC in mice) to recruit further neutrophils for infection (331). It will be of great interest in future investigations to determine whether professional intra-neutrophil pathogens such as *Anaplasma phagocytophilum* and *Neisseria* trigger inflammasome functions in neutrophils, and if so, whether the auto-regulatory IL-1β feedback loop provided by neutrophils increases host susceptibility to these microbes.

An emerging concept in the disciplines of inflammation and regulated cell death is the striking similarities in receptor domain structure, organisation and activation mechanisms between the two pathways (89). For example, the organisation and activation mechanism of the NLRC4 inflammasome is highly reminiscent of the APAF-1 apoptosome (332). Both NLRC4 and APAF-1 contain N-terminal CARD domains and central nucleotide-binding domains. Following activation, both APAF-1 and NLRC4 oligomerise through their

nucleotide-binding domain, which directs the recruitment of caspases through CARD-CARD interactions to drive proximity-induced caspase activation (333). Another similarity between some forms of regulated cell death signalling and inflammasomes is the requirement for a signalling adaptor (89). Signalling via the extrinsic apoptosis pathway (e.g. by Fas/FasL), and some inflammasomes requires a protein adaptor (FADD and ASC, respectively) to bridge receptor-caspase interactions. Perhaps a more important function of such signalling adaptors is to present clustered sites for caspase binding, thereby increasing the local caspase concentration to force caspase self-activation (334).

C-FLIP$_L$ is a protease-dead homolog of caspase-8 and is a key regulator of caspase-8-dependent cell death (196). At high cellular concentrations, c-FLIP$_L$ competes with pro-caspase-8 for recruitment to signalling complexes, and consequently limits caspase-8 auto-processing and activity. At low cellular concentrations however, the protease-dead domain of c-FLIP$_L$ can allosterically activate pro-caspase-8, although the c-FLIP$_L$/pro-caspase-8 heterodimer cleaves a narrower range of substrates compared to caspase-8 homodimers (197, 274). Importantly, the c-FLIP$_L$/caspase-8 heterodimer cleaves RIPK3 to prevent necroptosis (215). A straightforward explanation for why neutrophils do not undergo pyroptosis may be that the caspase-1-regulated pyroptotic effector is not expressed in these cells. However, it is also possible that neutrophils express a c-FLIP$_L$-like molecule to regulate the substrate repertoire of caspase-1 in neutrophils, akin to the c-FLIP$_L$/caspase-8 model for suppressing apoptosis and necroptosis. Work from others in our laboratory indicated that caspase-12 is highly expressed in murine neutrophils but only weakly expressed in murine macrophages (data not shown). Caspase-12 is highly homologous to caspase-1 (335), and caspase-12 overexpression suppresses caspase-1-dependent IL-1β processing (336), but pyroptosis was not examined in that study (336). One possibility is that a caspase-12/caspase-1 heterodimer can support IL-1β processing (albeit less efficiently than a caspase-1 homodimer) but cannot cleave the substrate that executes pyroptosis. If indeed caspase-12 blocks the induction of neutrophil death, one would expect inflammasome activation to trigger pyroptosis in caspase-12-deficient neutrophils. Thus, future experiments will investigate whether overexpression of caspase-12 in macrophages supresses pyroptosis induction whilst supporting sustained IL-1β production.

Prior studies revealed that pro-caspase-1 self-cleavage was not required for the induction of pyroptosis while caspase-1 processing in the interdomain linker (p20↓p10) was

necessary for caspase-1-dependent cytokine processing in CARD-containing inflammasomes (e.g. NLRC4). On the basis of this observation, it was proposed CARD-containing inflammasome scaffolds can form two distinct caspase-1-activing platforms in macrophages, one that does not contain ASC and one that does, and these mediate pyroptosis or cytokine processing, respectively (8). However, an inability of neutrophils to form the death-inducing inflammasome complex cannot explain why neutrophils do not undergo pyroptosis, as similar observations were made for the NLRP3 inflammasome that is believed to support cytokine processing and cell death from a single complex. It is possible, however, that the caspase-1 species that triggers macrophage lysis is not generated in stimulated neutrophils, for example, due to differences in ASC speck structure in neutrophils.

The surprising lack of inflammasome/caspase-8-dependent apoptosis in neutrophils was likely due to inefficient caspase-8 processing. High expression of the caspase-8 inhibitor, c-FLIP$_L$ or c-FLIP$_s$, in neutrophils compared to macrophages may provide a simple explanation for why caspase-8 was not processed in neutrophils. Another possible explanation is that the physical properties of ASC polymers may be different in neutrophils as compared macrophages, and this influences the ability of inflammasome-recruited pro-caspase-8 (or indeed pro-caspase-1) to self-process. Whilst NLRP3 activation triggered rapid redistribution of soluble ASC into a single large ASC focus in macrophages, ASC polymerisation in neutrophils appeared delayed and less efficient. Importantly, NLRP3 was reported to trigger multiple ASC clusters in neutrophils (257), while ASC collapses into a single focus in macrophages. Coupled with the fact that neutrophils express approximately 18-fold less ASC on a per cell basis (**Chapter 5**), this raises the likelihood that ASC foci are smaller in neutrophils relative to macrophages, and so likely possess different physical characteristics. This may influence the ability of neutrophil ASC foci to provide binding sites with optimal orientation for caspase-8 clustering and allosteric activation.

Interestingly, neutrophils also appear to be protected from LPS-mediated necroptosis, possibly as a mechanism to sustain neutrophil viability for antimicrobial defence. Our preliminary data suggest that altered or deficient TLR4 signalling via the TRIF-dependent pathway may allow neutrophils to avoid LPS-mediated necroptosis. If this is the case, it indicates that the advantages conferred to the host immune response by resisting neutrophil cell death outweigh the possible benefits of neutrophil production of TRIF-dependent cytokines (e.g. type I IFN). Interestingly, neutrophils appear to express high

levels of specific interferon-inducible genes, such as pro-caspase-11, under homeostatic conditions; this suggests that perhaps these cells have evolved mechanisms independent of autocrine type I IFN signalling to upregulate key host defence genes. The interferon-inducible guanylate-binding proteins (GBPs) are other important players in the caspase-11 pathway. In macrophages, GBPs mediate the lysis of the pathogen-containing vacuole (PCV), exposing bacteria to the cytosol for recognition by caspase-11 (80). It will be of great interest in future investigations to examine whether exposure of phagosomal bacteria into the cytoplasm similarly requires GBP functions in neutrophils.

Bacterial infection induces a form of cell death in neutrophils called 'NETosis' (178). Neutrophils undergoing NETosis release various cellular contents, including DNA, histones, proteases and antimicrobial peptides. These molecules form a web to entrap extracellular pathogens and are collectively termed 'Neutrophil Extracellular Traps' (NETs). A single study demonstrated that cells undergoing NETosis remain viable up to 24 h (337) and others suggested that neutrophils undergoing NETosis retain their ability to migrate and phagocytose pathogens (338), and that the process of NETosis does not release intracellular LDH (178, 339). Interestingly, many processes that trigger inflammasome function also are also inducers of NETosis, such as TLR engagement, induction of ionic efflux and ROS production (178, 337). Therefore, the lack of inflammasome activation by insoluble and particulate NLRP3 agonists may be a consequence of neutrophils undergoing NETosis. In fact, a single study suggested that MSU-induced NETosis serves an important mechanism to supress inflammation by entrapping and digesting cytokines and chemokines (340). The calcium-dependent enzyme, protein arginine deiminase 4 (PAD4), is a key regulator of NETosis (341). Interestingly, PAD4 contains a putative caspase-1 cleavage site (personal communication with Dr Dave Boucher), suggesting a possible mechanism where by caspase-1 activation might actually suppresses NETosis via PAD4 proteolytic inactivation.

Alternatively, it is possible that inflammasome-dependent IL-1β triggers NET formation at a site of infection as an additional antimicrobial defence strategy to classic neutrophil functions. IL-1β signalling enhances NETosis (93) and patients with Familial Mediterranean Fever often release IL-1β-decorated NETs during disease attacks (342). Interestingly, NETs were recently shown to serve as an endogenous danger signal that primes the expression of NLRP3 and pro-IL-1β in macrophages during atherosclerosis (343). Increasing studies are now suggesting that neutrophils are a heterogeneous

population and various subtypes exist *in vivo* (344). It may be possible that specific neutrophil subsets avoid cell death to sustain viability during infection, while others undergo NETosis to entrap pathogens that are too large to be engulfed (345), or to signal to other immune cells such as macrophages. Collectively, these data suggest the possibility that in some circumstances, inflammasome-dependent IL-1β may trigger neutrophil recruitment, classic neutrophil antimicrobial functions, but also NET release. In these settings, NETs would serve as an additive antimicrobial mechanism, and also as an inflammasome-priming signal for newly recruited monoctyes and macrophages.

An emerging concept from this thesis is that pattern recognition receptor signalling pathways are not always conserved between cell types, including between closely-related subsets of the myeloid lineage. We propose that cell type-specific specialisation of inflammatory signalling pathways allows the immune system to tailor an *in vivo* response so that it is appropriate to each stimulus. In support of this, neutrophil inflammasome signalling and the ensuing amplification loop of neutrophil recruitment and activation was only triggered under scenarios where neutrophil-mediated clean up are important (infection and tissue injury; *Salmonella*, LPS, nigericin, ATP), while exposure to metabolic stress signals (MSU), or irritants (alum, silica) did not trigger neutrophil inflammasome function. This indicates that neutrophils are unlikely to be a source of pathogenic IL-1β during inflammatory diseases such as silicosis and gout, but may contribute via sustained IL-1β production to pathology in inherited diseases such as those driven by gain-of-function mutations in NLRP3 (128) or NLRC4 (134-136), or heritable diseases of NLRP12 dysfunction (129). Another scenario where neutrophil inflammasome function may be pathogenic is during endotoxic shock, a condition that is often accompanied by neutrophilia (301). This thesis reported that neutrophil LPS transfection triggered caspase-11-dependent IL-1β secretion without concomitant cell death *in vitro*, and *in vivo* neutrophil depletion was protective for caspase-11-driven endotoxic shock. Interestingly, neutrophil depletion was recently proposed as a possible treatment for human septic shock (346). Further experiments will be required to establish whether the protective effect of neutrophil depletion on LPS-induced lethality occurred as a direct consequence of blocking cell-autonomous neutrophil caspase-11 signalling in mice.

Two recent studies demonstrated that ASC polymers released during macrophage pyroptosis further propagate inflammation by triggering inflammasome activation in neighbouring cells. These studies showed that phagocytosis of extracellular ASC polymers

triggered lysosomal rupture and NLRP3-independent ASC polymerisation and caspase-1 activation in recipient macrophages (347, 348). It is unclear how such processes will be halted *in vivo*, if engulfment of extracellular ASC polymers is always destined to trigger inflammasome signalling in a recipient cell, leading to release of further ASC polymers into the extracellular space, in a vicious inflammatory cycle. This thesis demonstrated that in neutrophils (1) ASC polymerisation occurs relatively slowly, (2) caspase-1 does not trigger pyroptosis, and (2) the lysosomal rupture pathway is a poor stimulus for NLRP3 activation; in all, this presents neutrophils as possible cellular candidates for suppressing inflammation driven by extracellular ASC polymers. However, it is unclear at present whether neutrophils are able to digest these protein aggregates. Future experiments will examine whether extracellular ASC polymers trigger caspase-1 activation and IL-1β release in neutrophils, and if indeed ASC polymers do not trigger neutrophil caspase-1 activation, ASC-deficient neutrophils will be exposed to purified ASC polymers to reveal whether neutrophils can degrade exogenous ASC, by western blotting and microscopy.

IL-1β, -18 and -37 are unusual cytokines that lack signal peptides and are secreted in an unconventional, ER/Golgi-independent manner (349). As inflammasome activation in macrophages elicits near-concurrent secretion of mature IL-1β and cell lysis, it is often proposed that inflammasome-dependent cytokines are passively released during pyroptotic cell death (253, 350). The discovery that caspase-1 activation downstream of NLRC4, NLRP3, AIM2 and caspase-11 in neutrophils selectively triggers cytokine maturation but not pyroptotic death refutes this possibility, and unequivocally confirms that cytokine secretion and pyroptotic cell death can be uncoupled. In line with this, ripoptosome-dependent release of caspase-8-processed IL-1β occurs before the loss of membrane integrity (74), indicating that cell lysis is not required for unconventional cytokine export.

In summary, this thesis demonstrates that neutrophils exhibit specialised inflammasome signalling pathways, and these signalling modifications are likely to allow sculpting of the resultant immune response *in vivo.* Interestingly, neutrophils only produced inflammasome-dependent IL-1β after exposure to stimuli that mimic infection or injury, and this provided a feed-forward loop of neutrophil recruitment and activation; this is likely to represent a mechanism by which specific inflammasome agonists can elicit a neutrophil-dominated response. Neutrophils appear to be resistant to multiple forms of microbe-induced cell death, and IL-1β provides a neutrophil survival signal (96). Such mechanisms

likely provide these cells with sufficient time to perform their important phagocytic and/or antimicrobial functions at a site of injury or infection, after which neutrophil pro-survival factors decline, and the recruited neutrophils can undergo their underlying apoptotic program during the resolution phase of inflammation. However, prolonged neutrophil presence and inflammatory function may also drive pathologies such as endotoxin shock. So detailed knowledge of neutrophil inflammatory responses and cell death pathways are required in order to better understand neutrophil-driven inflammatory disease, and will be a focus of future investigations. The findings presented in this thesis add to an emerging literature that documents the important immuno-modulatory functions of neutrophils during infection and inflammation, and also emphasises the importance of cell identity in tailoring an appropriate immune response.

8. References

1. Medzhitov R (2009) Approaching the asymptote: 20 years later. *Immunity* 30(6):766-775.
2. Schroder K & Tschopp J (2010) The inflammasomes. *Cell* 140(6):821-832.
3. Yu JW*, et al.* (2006) Cryopyrin and pyrin activate caspase-1, but not NF-kappaB, via ASC oligomerization. *Cell Death Differ* 13(2):236-249.
4. Chen KW & Schroder K (2013) Antimicrobial functions of inflammasomes. *Curr Opin Microbiol* 16(3):311-318.
5. Martinon F & Tschopp J (2004) Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* 117(5):561-574.
6. Lamkanfi M & Dixit VM (2014) Mechanisms and functions of inflammasomes. *Cell* 157(5):1013-1022.
7. Zurek B, Proell M, Wagner RN, Schwarzenbacher R, & Kufer TA (2012) Mutational analysis of human NOD1 and NOD2 NACHT domains reveals different modes of activation. *Innate Immun* 18(1):100-111.
8. Broz P, von Moltke J, Jones JW, Vance RE, & Monack DM (2010) Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. *Cell Host Microbe* 8(6):471-483.
9. Fuentes-Prior P & Salvesen GS (2004) The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J* 384(Pt 2):201-232.
10. MacCorkle RA, Freeman KW, & Spencer DM (1998) Synthetic activation of caspases: artificial death switches. *Proc Natl Acad Sci U S A* 95(7):3655-3660.
11. Keller M, Ruegg A, Werner S, & Beer HD (2008) Active caspase-1 is a regulator of unconventional protein secretion. *Cell* 132(5):818-831.
12. Bulau AM*, et al.* (2014) Role of caspase-1 in nuclear translocation of IL-37, release of the cytokine, and IL-37 inhibition of innate immune responses. *Proc Natl Acad Sci U S A* 111(7):2650-2655.
13. Li S*, et al.* (2015) Extracellular forms of IL-37 inhibit innate inflammation in vitro and in vivo but require the IL-1 family decoy receptor IL-1R8. *Proc Natl Acad Sci U S A* 112(8):2497-2502.
14. Cookson BT & Brennan MA (2001) Pro-inflammatory programmed cell death. *Trends Microbiol* 9(3):113-114.
15. Brennan MA & Cookson BT (2000) Salmonella induces macrophage death by caspase-1-dependent necrosis. *Mol Microbiol* 38(1):31-40.

16. Gross O*, et al.* (2012) Inflammasome activators induce interleukin-1alpha secretion via distinct pathways with differential requirement for the protease function of caspase-1. *Immunity* 36(3):388-400.
17. Cassel SL*, et al.* (2008) The Nalp3 inflammasome is essential for the development of silicosis. *Proc Natl Acad Sci U S A* 105(26):9035-9040.
18. Cruz CM*, et al.* (2007) ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages. *J Biol Chem* 282(5):2871-2879.
19. Hornung V*, et al.* (2008) Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol* 9(8):847-856.
20. Munoz-Planillo R*, et al.* (2013) K(+) efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter. *Immunity* 38(6):1142-1153.
21. Horng T (2014) Calcium signaling and mitochondrial destabilization in the triggering of the NLRP3 inflammasome. *Trends Immunol* 35(6):253-261.
22. Iyer SS*, et al.* (2013) Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation. *Immunity* 39(2):311-323.
23. Nakahira K*, et al.* (2011) Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol* 12(3):222-230.
24. Shimada K*, et al.* (2012) Oxidized mitochondrial DNA activates the NLRP3 inflammasome during apoptosis. *Immunity* 36(3):401-414.
25. Murakami T*, et al.* (2012) Critical role for calcium mobilization in activation of the NLRP3 inflammasome. *Proc Natl Acad Sci U S A* 109(28):11282-11287.
26. Gross O*, et al.* (2009) Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* 459(7245):433-436.
27. Mariathasan S*, et al.* (2006) Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature* 440(7081):228-232.
28. Duncan JA*, et al.* (2009) Neisseria gonorrhoeae activates the proteinase cathepsin B to mediate the signaling activities of the NLRP3 and ASC-containing inflammasome. *J Immunol* 182(10):6460-6469.
29. Mariathasan S*, et al.* (2004) Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature* 430(6996):213-218.
30. Costa A*, et al.* (2012) Activation of the NLRP3 inflammasome by group B streptococci. *J Immunol* 188(4):1953-1960.

31. Rosales-Reyes R, Aubert DF, Tolman JS, Amer AO, & Valvano MA (2012) Burkholderia cenocepacia type VI secretion system mediates escape of type II secreted proteins into the cytoplasm of infected macrophages. *PLoS One* 7(7):e41726.
32. Koo JE, Hong HJ, Dearth A, Kobayashi KS, & Koh YS (2012) Intracellular invasion of Orientia tsutsugamushi activates inflammasome in asc-dependent manner. *PLoS One* 7(6):e39042.
33. Liu Z*, et al.* (2012) Role of inflammasomes in host defense against Citrobacter rodentium infection. *J Biol Chem* 287(20):16955-16964.
34. Kanneganti TD*, et al.* (2006) Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. *J Biol Chem* 281(48):36560-36568.
35. Ito M, Yanagi Y, & Ichinohe T (2012) Encephalomyocarditis virus viroporin 2B activates NLRP3 inflammasome. *PLoS Pathog* 8(8):e1002857.
36. Komune N, Ichinohe T, Ito M, & Yanagi Y (2011) Measles virus V protein inhibits NLRP3 inflammasome-mediated interleukin-1beta secretion. *J Virol* 85(24):13019-13026.
37. Burdette D*, et al.* (2012) Hepatitis C virus activates interleukin-1beta via caspase-1-inflammasome complex. *J Gen Virol* 93(Pt 2):235-246.
38. Zhou R, Tardivel A, Thorens B, Choi I, & Tschopp J (2010) Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat Immunol* 11(2):136-140.
39. Halle A*, et al.* (2008) The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol* 9(8):857-865.
40. Martinon F, Petrilli V, Mayor A, Tardivel A, & Tschopp J (2006) Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* 440(7081):237-241.
41. Jiang Y*, et al.* (2012) Oxidized low-density lipoprotein induces secretion of interleukin-1beta by macrophages via reactive oxygen species-dependent NLRP3 inflammasome activation. *Biochem Biophys Res Commun* 425(2):121-126.
42. Jin C*, et al.* (2011) NLRP3 inflammasome plays a critical role in the pathogenesis of hydroxyapatite-associated arthropathy. *Proc Natl Acad Sci U S A* 108(36):14867-14872.
43. Fujisawa A*, et al.* (2007) Disease-associated mutations in CIAS1 induce cathepsin B-dependent rapid cell death of human THP-1 monocytic cells. *Blood* 109(7):2903-2911.

44. Kofoed EM & Vance RE (2011) Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* 477(7366):592-595.
45. Rayamajhi M, Zak DE, Chavarria-Smith J, Vance RE, & Miao EA (2013) Cutting edge: Mouse NAIP1 detects the type III secretion system needle protein. *J Immunol* 191(8):3986-3989.
46. Tenthorey JL, Kofoed EM, Daugherty MD, Malik HS, & Vance RE (2014) Molecular basis for specific recognition of bacterial ligands by NAIP/NLRC4 inflammasomes. *Mol Cell* 54(1):17-29.
47. Yang J, Zhao Y, Shi J, & Shao F (2013) Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation. *Proc Natl Acad Sci U S A* 110(35):14408-14413.
48. Zhao Y*, et al.* (2011) The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus. *Nature* 477(7366):596-600.
49. Kortmann J, Brubaker SW, & Monack DM (2015) Cutting Edge: Inflammasome Activation in Primary Human Macrophages Is Dependent on Flagellin. *J Immunol* 195(3):815-819.
50. Cornelis GR (2006) The type III secretion injectisome. *Nat Rev Microbiol* 4(11):811-825.
51. Miao EA, Ernst RK, Dors M, Mao DP, & Aderem A (2008) Pseudomonas aeruginosa activates caspase 1 through Ipaf. *Proc Natl Acad Sci U S A* 105(7):2562-2567.
52. Suzuki T*, et al.* (2007) Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages. *PLoS Pathog* 3(8):e111.
53. Ceballos-Olvera I, Sahoo M, Miller MA, Del Barrio L, & Re F (2011) Inflammasome-dependent pyroptosis and IL-18 protect against Burkholderia pseudomallei lung infection while IL-1beta is deleterious. *PLoS Pathog* 7(12):e1002452.
54. Miao EA*, et al.* (2010) Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. *Proc Natl Acad Sci U S A* 107(7):3076-3080.
55. Wu J, Fernandes-Alnemri T, & Alnemri ES (2010) Involvement of the AIM2, NLRC4, and NLRP3 inflammasomes in caspase-1 activation by Listeria monocytogenes. *J Clin Immunol* 30(5):693-702.
56. Tomalka J*, et al.* (2011) A novel role for the NLRC4 inflammasome in mucosal defenses against the fungal pathogen Candida albicans. *PLoS Pathog* 7(12):e1002379.

57. Martinon F, Burns K, & Tschopp J (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. *Mol Cell* 10(2):417-426.
58. Chavarria-Smith J & Vance RE (2013) Direct proteolytic cleavage of NLRP1B is necessary and sufficient for inflammasome activation by anthrax lethal factor. *PLoS Pathog* 9(6):e1003452.
59. Levinsohn JL*, et al.* (2012) Anthrax lethal factor cleavage of Nlrp1 is required for activation of the inflammasome. *PLoS Pathog* 8(3):e1002638.
60. Boyden ED & Dietrich WF (2006) Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin. *Nat Genet* 38(2):240-244.
61. Van Opdenbosch N*, et al.* (2014) Activation of the NLRP1b inflammasome independently of ASC-mediated caspase-1 autoproteolysis and speck formation. *Nat Commun* 5:3209.
62. Guey B, Bodnar M, Manie SN, Tardivel A, & Petrilli V (2014) Caspase-1 autoproteolysis is differentially required for NLRP1b and NLRP3 inflammasome function. *Proc Natl Acad Sci U S A* 111(48):17254-17259.
63. Burckstummer T*, et al.* (2009) An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. *Nat Immunol* 10(3):266-272.
64. Fernandes-Alnemri T, Yu JW, Datta P, Wu J, & Alnemri ES (2009) AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature* 458(7237):509-513.
65. Hornung V*, et al.* (2009) AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 458(7237):514-518.
66. Roberts TL*, et al.* (2009) HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. *Science* 323(5917):1057-1060.
67. Rathinam VA*, et al.* (2010) The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat Immunol* 11(5):395-402.
68. Rathinam VA, Vanaja SK, & Fitzgerald KA (2012) Regulation of inflammasome signaling. *Nat Immunol* 13(4):333-342.
69. Sagulenko V*, et al.* (2013) AIM2 and NLRP3 inflammasomes activate both apoptotic and pyroptotic death pathways via ASC. *Cell Death Differ* 20(9):1149-1160.

70. Pierini R*, et al.* (2012) AIM2/ASC triggers caspase-8-dependent apoptosis in Francisella-infected caspase-1-deficient macrophages. *Cell Death Differ* 19(10):1709-1721.
71. Man SM*, et al.* (2013) Salmonella infection induces recruitment of Caspase-8 to the inflammasome to modulate IL-1beta production. *J Immunol* 191(10):5239-5246.
72. Puri AW, Broz P, Shen A, Monack DM, & Bogyo M (2012) Caspase-1 activity is required to bypass macrophage apoptosis upon Salmonella infection. *Nat Chem Biol* 8(9):745-747.
73. Karki R*, et al.* (2015) Concerted activation of the AIM2 and NLRP3 inflammasomes orchestrates host protection against Aspergillus infection. *Cell Host Microbe* 17(3):357-368.
74. Vince JE*, et al.* (2012) Inhibitor of apoptosis proteins limit RIP3 kinase-dependent interleukin-1 activation. *Immunity* 36(2):215-227.
75. Aachoui Y*, et al.* (2013) Caspase-11 protects against bacteria that escape the vacuole. *Science* 339(6122):975-978.
76. Kayagaki N*, et al.* (2011) Non-canonical inflammasome activation targets caspase-11. *Nature* 479(7371):117-121.
77. Kayagaki N*, et al.* (2013) Noncanonical inflammasome activation by intracellular LPS independent of TLR4. *Science* 341(6151):1246-1249.
78. Hagar JA, Powell DA, Aachoui Y, Ernst RK, & Miao EA (2013) Cytoplasmic LPS activates caspase-11: implications in TLR4-independent endotoxic shock. *Science* 341(6151):1250-1253.
79. Rathinam VA*, et al.* (2012) TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria. *Cell* 150(3):606-619.
80. Meunier E*, et al.* (2014) Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced GTPases. *Nature* 509(7500):366-370.
81. Shi J*, et al.* (2014) Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* 514(7521):187-192.
82. Ruhl S & Broz P (2015) Caspase-11 activates a canonical NLRP3 inflammasome by promoting K+ efflux. *Eur J Immunol*.
83. Wang S*, et al.* (1996) Identification and characterization of Ich-3, a member of the interleukin-1beta converting enzyme (ICE)/Ced-3 family and an upstream regulator of ICE. *J Biol Chem* 271(34):20580-20587.
84. Kajiwara Y*, et al.* (2014) A critical role for human caspase-4 in endotoxin sensitivity. *J Immunol* 193(1):335-343.

85. Baker PJ*, et al.* (2015) NLRP3 inflammasome activation downstream of cytoplasmic LPS recognition by both caspase-4 and caspase-5. *Eur J Immunol*.
86. Schmid-Burgk JL*, et al.* (2015) Caspase-4 mediates non-canonical activation of the NLRP3 inflammasome in human myeloid cells. *Eur J Immunol*.
87. Silke J & Meier P (2013) Inhibitor of apoptosis (IAP) proteins-modulators of cell death and inflammation. *Cold Spring Harb Perspect Biol* 5(2).
88. Lawlor KE*, et al.* (2015) RIPK3 promotes cell death and NLRP3 inflammasome activation in the absence of MLKL. *Nat Commun* 6:6282.
89. Blander JM (2014) A long-awaited merger of the pathways mediating host defence and programmed cell death. *Nat Rev Immunol* 14(9):601-618.
90. Ogle JD*, et al.* (1990) Comparison of abilities of recombinant interleukin-1 alpha and -beta and noninflammatory IL-1 beta fragment 163-171 to upregulate C3b receptors (CR1) on human neutrophils and to enhance their phagocytic capacity. *Inflammation* 14(2):185-194.
91. Ferrante A, Nandoskar M, Walz A, Goh DH, & Kowanko IC (1988) Effects of tumour necrosis factor alpha and interleukin-1 alpha and beta on human neutrophil migration, respiratory burst and degranulation. *Int Arch Allergy Appl Immunol* 86(1):82-91.
92. Arena A*, et al.* (2010) Both IL-1beta and TNF-alpha regulate NGAL expression in polymorphonuclear granulocytes of chronic hemodialysis patients. *Mediators Inflamm* 2010:613937.
93. Mitroulis I*, et al.* (2011) Neutrophil extracellular trap formation is associated with IL-1beta and autophagy-related signaling in gout. *PLoS One* 6(12):e29318.
94. Kaushansky K, Lin N, & Adamson JW (1988) Interleukin 1 stimulates fibroblasts to synthesize granulocyte-macrophage and granulocyte colony-stimulating factors. Mechanism for the hematopoietic response to inflammation. *J Clin Invest* 81(1):92-97.
95. van der Velden VH*, et al.* (1998) Interleukin-1beta and interferon-gamma differentially regulate release of monocyte chemotactic protein-1 and interleukin-8 by human bronchial epithelial cells. *Eur Cytokine Netw* 9(3):269-277.
96. Hsu LC*, et al.* (2011) IL-1beta-driven neutrophilia preserves antibacterial defense in the absence of the kinase IKKbeta. *Nat Immunol* 12(2):144-150.
97. Dinarello CA (1984) Interleukin-1 and the pathogenesis of the acute-phase response. *N Engl J Med* 311(22):1413-1418.

98. Prasad K (2004) C-reactive protein increases oxygen radical generation by neutrophils. *J Cardiovasc Pharmacol Ther* 9(3):203-209.
99. Nairz M, Schroll A, Sonnweber T, & Weiss G (2010) The struggle for iron - a metal at the host-pathogen interface. *Cell Microbiol* 12(12):1691-1702.
100. Rogers JT (1996) Ferritin translation by interleukin-1and interleukin-6: the role of sequences upstream of the start codons of the heavy and light subunit genes. *Blood* 87(6):2525-2537.
101. Lee P, Peng H, Gelbart T, Wang L, & Beutler E (2005) Regulation of hepcidin transcription by interleukin-1 and interleukin-6. *Proc Natl Acad Sci U S A* 102(6):1906-1910.
102. Blatteis CM (1986) Fever: is it beneficial? *Yale J Biol Med* 59(2):107-116.
103. Capitano ML*, et al.* (2012) Elevating body temperature enhances hematopoiesis and neutrophil recovery after total body irradiation in an IL-1-, IL-17-, and G-CSF-dependent manner. *Blood* 120(13):2600-2609.
104. Schroder K, Hertzog PJ, Ravasi T, & Hume DA (2004) Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 75(2):163-189.
105. Schroder K*, et al.* (2007) PU.1 and ICSBP control constitutive and IFN-gamma-regulated Tlr9 gene expression in mouse macrophages. *J Leukoc Biol* 81(6):1577-1590.
106. Wyman TH*, et al.* (2002) Physiological levels of interleukin-18 stimulate multiple neutrophil functions through p38 MAP kinase activation. *J Leukoc Biol* 72(2):401-409.
107. Fernandes-Alnemri T*, et al.* (2010) The AIM2 inflammasome is critical for innate immunity to Francisella tularensis. *Nat Immunol* 11(5):385-393.
108. Cai S, Batra S, Wakamatsu N, Pacher P, & Jeyaseelan S (2012) NLRC4 inflammasome-mediated production of IL-1beta modulates mucosal immunity in the lung against gram-negative bacterial infection. *J Immunol* 188(11):5623-5635.
109. Lara-Tejero M*, et al.* (2006) Role of the caspase-1 inflammasome in Salmonella typhimurium pathogenesis. *J Exp Med* 203(6):1407-1412.
110. Sutterwala FS*, et al.* (2007) Immune recognition of Pseudomonas aeruginosa mediated by the IPAF/NLRC4 inflammasome. *J Exp Med* 204(13):3235-3245.
111. Broz P*, et al.* (2010) Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. *J Exp Med* 207(8):1745-1755.

112. Kebaier C*, et al.* (2012) Staphylococcus aureus alpha-hemolysin mediates virulence in a murine model of severe pneumonia through activation of the NLRP3 inflammasome. *J Infect Dis* 205(5):807-817.
113. Fang R*, et al.* (2014) Type I interferon signaling regulates activation of the absent in melanoma 2 inflammasome during Streptococcus pneumoniae infection. *Infect Immun* 82(6):2310-2317.
114. Vladimer GI*, et al.* (2012) The NLRP12 inflammasome recognizes Yersinia pestis. *Immunity* 37(1):96-107.
115. Miao EA*, et al.* (2010) Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria. *Nat Immunol* 11(12):1136-1142.
116. Masters SL*, et al.* (2010) Activation of the NLRP3 inflammasome by islet amyloid polypeptide provides a mechanism for enhanced IL-1beta in type 2 diabetes. *Nat Immunol* 11(10):897-904.
117. Vandanmagsar B*, et al.* (2011) The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nat Med* 17(2):179-188.
118. Inoue M, Williams KL, Gunn MD, & Shinohara ML (2012) NLRP3 inflammasome induces chemotactic immune cell migration to the CNS in experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 109(26):10480-10485.
119. Heneka MT*, et al.* (2013) NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice. *Nature* 493(7434):674-678.
120. Codolo G*, et al.* (2013) Triggering of inflammasome by aggregated alpha-synuclein, an inflammatory response in synucleinopathies. *PLoS One* 8(1):e55375.
121. Stienstra R*, et al.* (2011) Inflammasome is a central player in the induction of obesity and insulin resistance. *Proc Natl Acad Sci U S A* 108(37):15324-15329.
122. Larsen CM*, et al.* (2007) Interleukin-1-receptor antagonist in type 2 diabetes mellitus. *N Engl J Med* 356(15):1517-1526.
123. Coll RC*, et al.* (2015) A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nat Med* 21(3):248-255.
124. Okoye AA & Picker LJ (2013) CD4(+) T-cell depletion in HIV infection: mechanisms of immunological failure. *Immunol Rev* 254(1):54-64.
125. Doitsh G*, et al.* (2014) Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection. *Nature* 505(7484):509-514.
126. Monroe KM*, et al.* (2014) IFI16 DNA sensor is required for death of lymphoid CD4 T cells abortively infected with HIV. *Science* 343(6169):428-432.

127. Masters SL*, et al.* (2012) NLRP1 inflammasome activation induces pyroptosis of hematopoietic progenitor cells. *Immunity* 37(6):1009-1023.
128. Agostini L*, et al.* (2004) NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity* 20(3):319-325.
129. Jeru I*, et al.* (2008) Mutations in NALP12 cause hereditary periodic fever syndromes. *Proc Natl Acad Sci U S A* 105(5):1614-1619.
130. Allen IC*, et al.* (2012) NLRP12 suppresses colon inflammation and tumorigenesis through the negative regulation of noncanonical NF-kappaB signaling. *Immunity* 36(5):742-754.
131. Arthur JC*, et al.* (2010) Cutting edge: NLRP12 controls dendritic and myeloid cell migration to affect contact hypersensitivity. *J Immunol* 185(8):4515-4519.
132. Zaki MH*, et al.* (2011) The NOD-like receptor NLRP12 attenuates colon inflammation and tumorigenesis. *Cancer Cell* 20(5):649-660.
133. Zaki MH, Man SM, Vogel P, Lamkanfi M, & Kanneganti TD (2014) Salmonella exploits NLRP12-dependent innate immune signaling to suppress host defenses during infection. *Proc Natl Acad Sci U S A* 111(1):385-390.
134. Canna SW*, et al.* (2014) An activating NLRC4 inflammasome mutation causes autoinflammation with recurrent macrophage activation syndrome. *Nat Genet* 46(10):1140-1146.
135. Kitamura A, Sasaki Y, Abe T, Kano H, & Yasutomo K (2014) An inherited mutation in NLRC4 causes autoinflammation in human and mice. *J Exp Med* 211(12):2385-2396.
136. Romberg N*, et al.* (2014) Mutation of NLRC4 causes a syndrome of enterocolitis and autoinflammation. *Nat Genet* 46(10):1135-1139.
137. Haraga A, Ohlson MB, & Miller SI (2008) Salmonellae interplay with host cells. *Nat Rev Microbiol* 6(1):53-66.
138. Hansen-Wester I & Hensel M (2001) Salmonella pathogenicity islands encoding type III secretion systems. *Microbes Infect* 3(7):549-559.
139. Kuhle V, Abrahams GL, & Hensel M (2006) Intracellular Salmonella enterica redirect exocytic transport processes in a Salmonella pathogenicity island 2-dependent manner. *Traffic* 7(6):716-730.
140. Vazquez-Torres A*, et al.* (2000) Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. *Science* 287(5458):1655-1658.

141. Brawn LC, Hayward RD, & Koronakis V (2007) Salmonella SPI1 effector SipA persists after entry and cooperates with a SPI2 effector to regulate phagosome maturation and intracellular replication. *Cell Host Microbe* 1(1):63-75.
142. Mittrucker HW & Kaufmann SH (2000) Immune response to infection with Salmonella typhimurium in mice. *J Leukoc Biol* 67(4):457-463.
143. Miao EA*, et al.* (2006) Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat Immunol* 7(6):569-575.
144. Franchi L*, et al.* (2006) Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat Immunol* 7(6):576-582.
145. Kupz A*, et al.* (2012) NLRC4 inflammasomes in dendritic cells regulate noncognate effector function by memory CD8(+) T cells. *Nat Immunol* 13(2):162-169.
146. Man SM*, et al.* (2014) Inflammasome activation causes dual recruitment of NLRC4 and NLRP3 to the same macromolecular complex. *Proc Natl Acad Sci U S A* 111(20):7403-7408.
147. Croker BA, Roberts AW, & Nicola NA (2012) Towards a four-dimensional view of neutrophils. *Methods Mol Biol* 844:87-99.
148. Summers C*, et al.* (2010) Neutrophil kinetics in health and disease. *Trends Immunol* 31(8):318-324.
149. Amulic B, Cazalet C, Hayes GL, Metzler KD, & Zychlinsky A (2012) Neutrophil function: from mechanisms to disease. *Annu Rev Immunol* 30:459-489.
150. Lieschke GJ*, et al.* (1994) Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood* 84(6):1737-1746.
151. Borregaard N (2010) Neutrophils, from marrow to microbes. *Immunity* 33(5):657-670.
152. Ganz T*, et al.* (1985) Defensins. Natural peptide antibiotics of human neutrophils. *J Clin Invest* 76(4):1427-1435.
153. Kang T*, et al.* (2001) Subcellular distribution and cytokine- and chemokine-regulated secretion of leukolysin/MT6-MMP/MMP-25 in neutrophils. *J Biol Chem* 276(24):21960-21968.
154. Cramer E, Pryzwansky KB, Villeval JL, Testa U, & Breton-Gorius J (1985) Ultrastructural localization of lactoferrin and myeloperoxidase in human neutrophils by immunogold. *Blood* 65(2):423-432.

155. Borregaard N, Sorensen OE, & Theilgaard-Monch K (2007) Neutrophil granules: a library of innate immunity proteins. *Trends Immunol* 28(8):340-345.

156. Calafat J*, et al.* (1993) Evidence for small intracellular vesicles in human blood phagocytes containing cytochrome b558 and the adhesion molecule CD11b/CD18. *Blood* 81(11):3122-3129.

157. Faurschou M & Borregaard N (2003) Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect* 5(14):1317-1327.

158. Chen M*, et al.* (2006) Neutrophil-derived leukotriene B4 is required for inflammatory arthritis. *J Exp Med* 203(4):837-842.

159. Leffler J*, et al.* (2012) Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. *J Immunol* 188(7):3522-3531.

160. Semerad CL, Liu F, Gregory AD, Stumpf K, & Link DC (2002) G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the blood. *Immunity* 17(4):413-423.

161. Eash KJ, Greenbaum AM, Gopalan PK, & Link DC (2010) CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest* 120(7):2423-2431.

162. Jagels MA & Hugli TE (1992) Neutrophil chemotactic factors promote leukocytosis. A common mechanism for cellular recruitment from bone marrow. *J Immunol* 148(4):1119-1128.

163. Ley K, Laudanna C, Cybulsky MI, & Nourshargh S (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 7(9):678-689.

164. Kansas GS (1996) Selectins and their ligands: current concepts and controversies. *Blood* 88(9):3259-3287.

165. Mueller H*, et al.* (2010) Tyrosine kinase Btk regulates E-selectin-mediated integrin activation and neutrophil recruitment by controlling phospholipase C (PLC) gamma2 and PI3Kgamma pathways. *Blood* 115(15):3118-3127.

166. Yago T*, et al.* (2010) E-selectin engages PSGL-1 and CD44 through a common signaling pathway to induce integrin alphaLbeta2-mediated slow leukocyte rolling. *Blood* 116(3):485-494.

167. Constantin G*, et al.* (2000) Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* 13(6):759-769.

168. Parker LC, Whyte MK, Dower SK, & Sabroe I (2005) The expression and roles of Toll-like receptors in the biology of the human neutrophil. *J Leukoc Biol* 77(6):886-892.
169. Sabroe I, Dower SK, & Whyte MK (2005) The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis. *Clin Infect Dis* 41 Suppl 7:S421-426.
170. Blumental S*, et al.* (2011) Invasive mold infections in chronic granulomatous disease: a 25-year retrospective survey. *Clin Infect Dis* 53(12):e159-169.
171. Gordon MA (2008) Salmonella infections in immunocompromised adults. *J Infect* 56(6):413-422.
172. Fang FC (2004) Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat Rev Microbiol* 2(10):820-832.
173. Segal AW, Dorling J, & Coade S (1980) Kinetics of fusion of the cytoplasmic granules with phagocytic vacuoles in human polymorphonuclear leukocytes. Biochemical and morphological studies. *J Cell Biol* 85(1):42-59.
174. Henry RM, Hoppe AD, Joshi N, & Swanson JA (2004) The uniformity of phagosome maturation in macrophages. *J Cell Biol* 164(2):185-194.
175. Nordenfelt P & Tapper H (2011) Phagosome dynamics during phagocytosis by neutrophils. *J Leukoc Biol* 90(2):271-284.
176. Weiss G*, et al.* (1994) Iron regulates nitric oxide synthase activity by controlling nuclear transcription. *J Exp Med* 180(3):969-976.
177. Sengelov H, Kjeldsen L, & Borregaard N (1993) Control of exocytosis in early neutrophil activation. *J Immunol* 150(4):1535-1543.
178. Brinkmann V*, et al.* (2004) Neutrophil extracellular traps kill bacteria. *Science* 303(5663):1532-1535.
179. Urban CF*, et al.* (2009) Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. *PLoS Pathog* 5(10):e1000639.
180. Meng W*, et al.* (2012) Depletion of neutrophil extracellular traps in vivo results in hypersusceptibility to polymicrobial sepsis in mice. *Crit Care* 16(4):R137.
181. Riyapa D*, et al.* (2012) Neutrophil extracellular traps exhibit antibacterial activity against burkholderia pseudomallei and are influenced by bacterial and host factors. *Infect Immun* 80(11):3921-3929.
182. Remijsen Q*, et al.* (2011) Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Res* 21(2):290-304.

183. Bianchi M*, et al.* (2009) Restoration of NET formation by gene therapy in CGD controls aspergillosis. *Blood* 114(13):2619-2622.
184. Sur Chowdhury C*, et al.* (2014) Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility. *Arthritis Res Ther* 16(3):R122.
185. Sandborn WJ*, et al.* (2002) A review of activity indices and efficacy endpoints for clinical trials of medical therapy in adults with Crohn's disease. *Gastroenterology* 122(2):512-530.
186. Ueda T*, et al.* (2014) A simple scoring system based on neutrophil count in sepsis patients. *Med Hypotheses* 82(3):382-386.
187. Haslett C (1999) Granulocyte apoptosis and its role in the resolution and control of lung inflammation. *Am J Respir Crit Care Med* 160(5 Pt 2):S5-11.
188. Stark MA*, et al.* (2005) Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity* 22(3):285-294.
189. Pillay J*, et al.* (2010) In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood* 116(4):625-627.
190. Furze RC & Rankin SM (2008) The role of the bone marrow in neutrophil clearance under homeostatic conditions in the mouse. *FASEB J* 22(9):3111-3119.
191. Saverymuttu SH, Peters AM, Keshavarzian A, Reavy HJ, & Lavender JP (1985) The kinetics of 111indium distribution following injection of 111indium labelled autologous granulocytes in man. *Br J Haematol* 61(4):675-685.
192. Ashkenazi A & Dixit VM (1998) Death receptors: signaling and modulation. *Science* 281(5381):1305-1308.
193. Kischkel FC*, et al.* (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J* 14(22):5579-5588.
194. Chinnaiyan AM, O'Rourke K, Tewari M, & Dixit VM (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell* 81(4):505-512.
195. Muzio M*, et al.* (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. *Cell* 85(6):817-827.
196. Irmler M*, et al.* (1997) Inhibition of death receptor signals by cellular FLIP. *Nature* 388(6638):190-195.

197. Pop C*, et al.* (2011) FLIP(L) induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity. *Biochem J* 433(3):447-457.
198. Micheau O & Tschopp J (2003) Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* 114(2):181-190.
199. Jost PJ*, et al.* (2009) XIAP discriminates between type I and type II FAS-induced apoptosis. *Nature* 460(7258):1035-1039.
200. Yin XM*, et al.* (1999) Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. *Nature* 400(6747):886-891.
201. Li H, Zhu H, Xu CJ, & Yuan J (1998) Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell* 94(4):491-501.
202. Zou H, Henzel WJ, Liu X, Lutschg A, & Wang X (1997) Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell* 90(3):405-413.
203. Li P*, et al.* (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 91(4):479-489.
204. Geering B, Gurzeler U, Federzoni E, Kaufmann T, & Simon HU (2011) A novel TNFR1-triggered apoptosis pathway mediated by class IA PI3Ks in neutrophils. *Blood* 117(22):5953-5962.
205. Croker BA*, et al.* (2011) Fas-mediated neutrophil apoptosis is accelerated by Bid, Bak, and Bax and inhibited by Bcl-2 and Mcl-1. *Proc Natl Acad Sci U S A* 108(32):13135-13140.
206. Chipuk JE, Moldoveanu T, Llambi F, Parsons MJ, & Green DR (2010) The BCL-2 family reunion. *Mol Cell* 37(3):299-310.
207. Conus S*, et al.* (2008) Caspase-8 is activated by cathepsin D initiating neutrophil apoptosis during the resolution of inflammation. *J Exp Med* 205(3):685-698.
208. Conus S, Pop C, Snipas SJ, Salvesen GS, & Simon HU (2012) Cathepsin D primes caspase-8 activation by multiple intra-chain proteolysis. *J Biol Chem* 287(25):21142-21151.
209. Blomgran R, Zheng L, & Stendahl O (2007) Cathepsin-cleaved Bid promotes apoptosis in human neutrophils via oxidative stress-induced lysosomal membrane permeabilization. *J Leukoc Biol* 81(5):1213-1223.
210. Loison F*, et al.* (2014) Proteinase 3-dependent caspase-3 cleavage modulates neutrophil death and inflammation. *J Clin Invest* 124(10):4445-4458.

211. Kaczmarek A, Vandenabeele P, & Krysko DV (2013) Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. *Immunity* 38(2):209-223.
212. Vandenabeele P, Galluzzi L, Vanden Berghe T, & Kroemer G (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nat Rev Mol Cell Biol* 11(10):700-714.
213. He S, Liang Y, Shao F, & Wang X (2011) Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. *Proc Natl Acad Sci U S A* 108(50):20054-20059.
214. Lin Y, Devin A, Rodriguez Y, & Liu ZG (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev* 13(19):2514-2526.
215. Oberst A*, et al.* (2011) Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature* 471(7338):363-367.
216. Sun L*, et al.* (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* 148(1-2):213-227.
217. Hildebrand JM*, et al.* (2014) Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and necroptotic cell death. *Proc Natl Acad Sci U S A* 111(42):15072-15077.
218. Cai Z*, et al.* (2014) Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. *Nat Cell Biol* 16(1):55-65.
219. Cho YS*, et al.* (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* 137(6):1112-1123.
220. Robinson N*, et al.* (2012) Type I interferon induces necroptosis in macrophages during infection with Salmonella enterica serovar Typhimurium. *Nat Immunol* 13(10):954-962.
221. Degterev A*, et al.* (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol* 1(2):112-119.
222. Zhu S, Zhang Y, Bai G, & Li H (2011) Necrostatin-1 ameliorates symptoms in R6/2 transgenic mouse model of Huntington's disease. *Cell Death Dis* 2:e115.
223. Zhou Y*, et al.* (2013) Protective effects of necrostatin-1 against concanavalin A-induced acute hepatic injury in mice. *Mediators Inflamm* 2013:706156.
224. Takahashi N*, et al.* (2012) Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models. *Cell Death Dis* 3:e437.

225. Kearney CJ*, et al.* (2015) Necroptosis suppresses inflammation via termination of TNF- or LPS-induced cytokine and chemokine production. *Cell Death Differ* 22(8):1313-1327.
226. Kuida K*, et al.* (1995) Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme. *Science* 267(5206):2000-2003.
227. Wang S*, et al.* (1998) Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. *Cell* 92(4):501-509.
228. Thomas CJ & Schroder K (2013) Pattern recognition receptor function in neutrophils. *Trends Immunol* 34(7):317-328.
229. Cho JS*, et al.* (2012) Neutrophil-derived IL-1beta is sufficient for abscess formation in immunity against Staphylococcus aureus in mice. *PLoS Pathog* 8(11):e1003047.
230. Schroder K*, et al.* (2012) Acute lipopolysaccharide priming boosts inflammasome activation independently of inflammasome sensor induction. *Immunobiology* 217(12):1325-1329.
231. Gomez IG*, et al.* (2012) Metalloproteinase-mediated Shedding of Integrin beta2 promotes macrophage efflux from inflammatory sites. *J Biol Chem* 287(7):4581-4589.
232. Xu T, Maloy S, & McGuire KL (2009) Macrophages influence Salmonella host-specificity in vivo. *Microb Pathog* 47(4):212-222.
233. Daley JM, Thomay AA, Connolly MD, Reichner JS, & Albina JE (2008) Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol* 83(1):64-70.
234. Franchi L*, et al.* (2012) NLRC4-driven production of IL-1beta discriminates between pathogenic and commensal bacteria and promotes host intestinal defense. *Nat Immunol* 13(5):449-456.
235. Cheminay C, Chakravortty D, & Hensel M (2004) Role of neutrophils in murine salmonellosis. *Infect Immun* 72(1):468-477.
236. Dejager L, Pinheiro I, Bogaert P, Huys L, & Libert C (2010) Role for neutrophils in host immune responses and genetic factors that modulate resistance to Salmonella enterica serovar typhimurium in the inbred mouse strain SPRET/Ei. *Infect Immun* 78(9):3848-3860.
237. Seiler P*, et al.* (2000) Rapid neutrophil response controls fast-replicating intracellular bacteria but not slow-replicating Mycobacterium tuberculosis. *J Infect Dis* 181(2):671-680.

238. Vassiloyanakopoulos AP, Okamoto S, & Fierer J (1998) The crucial role of polymorphonuclear leukocytes in resistance to Salmonella dublin infections in genetically susceptible and resistant mice. *Proc Natl Acad Sci U S A* 95(13):7676-7681.
239. Conlan JW (1997) Critical roles of neutrophils in host defense against experimental systemic infections of mice by Listeria monocytogenes, Salmonella typhimurium, and Yersinia enterocolitica. *Infect Immun* 65(2):630-635.
240. Conlan JW (1996) Neutrophils prevent extracellular colonization of the liver microvasculature by Salmonella typhimurium. *Infect Immun* 64(3):1043-1047.
241. Mankan AK, Dau T, Jenne D, & Hornung V (2012) The NLRP3/ASC/Caspase-1 axis regulates IL-1beta processing in neutrophils. *Eur J Immunol* 42(3):710-715.
242. Bakele M*, et al.* (2014) Localization and functionality of the inflammasome in neutrophils. *J Biol Chem* 289(8):5320-5329.
243. Miwa K*, et al.* (1998) Caspase 1-independent IL-1beta release and inflammation induced by the apoptosis inducer Fas ligand. *Nat Med* 4(11):1287-1292.
244. Karmakar M, Sun Y, Hise AG, Rietsch A, & Pearlman E (2012) Cutting edge: IL-1beta processing during Pseudomonas aeruginosa infection is mediated by neutrophil serine proteases and is independent of NLRC4 and caspase-1. *J Immunol* 189(9):4231-4235.
245. Raupach B, Peuschel SK, Monack DM, & Zychlinsky A (2006) Caspase-1-mediated activation of interleukin-1beta (IL-1beta) and IL-18 contributes to innate immune defenses against Salmonella enterica serovar Typhimurium infection. *Infect Immun* 74(8):4922-4926.
246. Tamassia N*, et al.* (2007) The MyD88-independent pathway is not mobilized in human neutrophils stimulated via TLR4. *J Immunol* 178(11):7344-7356.
247. Dunlap NE, Benjamin WH, Berry AK, Eldridge JH, & Briles DE (1992) A Safe-Site for Salmonella-Typhimurium Is within Splenic Polymorphonuclear Cells. *Microbial Pathogenesis* 13(3):181-190.
248. Geddes K, Cruz F, & Heffron F (2007) Analysis of cells targeted by Salmonella type III secretion in vivo. *PLoS Pathog* 3(12):e196.
249. Casey SG, Shafer WM, & Spitznagel JK (1986) Neisseria gonorrhoeae survive intraleukocytic oxygen-independent antimicrobial capacities of anaerobic and aerobic granulocytes in the presence of pyocin lethal for extracellular gonococci. *Infect Immun* 52(2):384-389.

250. Rogers DE & Tompsett R (1952) The survival of staphylococci within human leukocytes. *J Exp Med* 95(2):209-230.
251. van Zandbergen G*, et al.* (2004) Chlamydia pneumoniae multiply in neutrophil granulocytes and delay their spontaneous apoptosis. *J Immunol* 172(3):1768-1776.
252. Chen SM, Dumler JS, Bakken JS, & Walker DH (1994) Identification of a granulocytotropic Ehrlichia species as the etiologic agent of human disease. *J Clin Microbiol* 32(3):589-595.
253. Bortoluci KR & Medzhitov R (2010) Control of infection by pyroptosis and autophagy: role of TLR and NLR. *Cell Mol Life Sci* 67(10):1643-1651.
254. Mestas J & Hughes CC (2004) Of mice and not men: differences between mouse and human immunology. *J Immunol* 172(5):2731-2738.
255. Schroder K*, et al.* (2012) Conservation and divergence in Toll-like receptor 4-regulated gene expression in primary human versus mouse macrophages. *Proc Natl Acad Sci U S A* 109(16):E944-953.
256. Gross O (2012) Measuring the inflammasome. *Methods Mol Biol* 844:199-222.
257. Karmakar M*, et al.* (2015) Neutrophil IL-1beta processing induced by pneumolysin is mediated by the NLRP3/ASC inflammasome and caspase-1 activation and is dependent on K+ efflux. *J Immunol* 194(4):1763-1775.
258. Bauernfeind FG*, et al.* (2009) Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *J Immunol* 183(2):787-791.
259. Kanneganti TD*, et al.* (2006) Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature* 440(7081):233-236.
260. Schorn C*, et al.* (2012) Monosodium urate crystals induce extracellular DNA traps in neutrophils, eosinophils, and basophils but not in mononuclear cells. *Front Immunol* 3:277.
261. Mocsai A (2013) Diverse novel functions of neutrophils in immunity, inflammation, and beyond. *J Exp Med* 210(7):1283-1299.
262. Eisenbarth SC, Colegio OR, O'Connor W, Sutterwala FS, & Flavell RA (2008) Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. *Nature* 453(7198):1122-1126.
263. Vince JE*, et al.* (2007) IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. *Cell* 131(4):682-693.
264. Tenev T*, et al.* (2011) The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. *Mol Cell* 43(3):432-448.

265. Feoktistova M*, et al.* (2011) cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. *Mol Cell* 43(3):449-463.
266. He S*, et al.* (2009) Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell* 137(6):1100-1111.
267. Yabal M*, et al.* (2014) XIAP restricts TNF- and RIP3-dependent cell death and inflammasome activation. *Cell Rep* 7(6):1796-1808.
268. Coll RC*, et al.* (2015) A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases. *Nat Med*.
269. Yin Q*, et al.* (2013) Molecular mechanism for p202-mediated specific inhibition of AIM2 inflammasome activation. *Cell Rep* 4(2):327-339.
270. Micheau O*, et al.* (2002) The long form of FLIP is an activator of caspase-8 at the Fas death-inducing signaling complex. *J Biol Chem* 277(47):45162-45171.
271. Gonzalvez F*, et al.* (2012) TRAF2 Sets a threshold for extrinsic apoptosis by tagging caspase-8 with a ubiquitin shutoff timer. *Mol Cell* 48(6):888-899.
272. Jin Z*, et al.* (2009) Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. *Cell* 137(4):721-735.
273. Li Y*, et al.* (2013) The HECTD3 E3 ubiquitin ligase facilitates cancer cell survival by promoting K63-linked polyubiquitination of caspase-8. *Cell Death Dis* 4:e935.
274. Hughes MA*, et al.* (2009) Reconstitution of the death-inducing signaling complex reveals a substrate switch that determines CD95-mediated death or survival. *Mol Cell* 35(3):265-279.
275. Allam R*, et al.* (2014) Mitochondrial apoptosis is dispensable for NLRP3 inflammasome activation but non-apoptotic caspase-8 is required for inflammasome priming. *EMBO Rep* 15(9):982-990.
276. Wu YH*, et al.* (2014) Participation of c-FLIP in NLRP3 and AIM2 inflammasome activation. *Cell Death Differ* 21(3):451-461.
277. Zanoni I*, et al.* (2011) CD14 controls the LPS-induced endocytosis of Toll-like receptor 4. *Cell* 147(4):868-880.
278. Birkner K*, et al.* (2008) The elimination of Anaplasma phagocytophilum requires CD4+ T cells, but is independent of Th1 cytokines and a wide spectrum of effector mechanisms. *Eur J Immunol* 38(12):3395-3410.
279. Peters NC*, et al.* (2008) In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science* 321(5891):970-974.

280. Gurung P*, et al.* (2015) An NLRP3 inflammasome-triggered Th2-biased adaptive immune response promotes leishmaniasis. *J Clin Invest* 125(3):1329-1338.
281. Ingersoll MA, Kline KA, Nielsen HV, & Hultgren SJ (2008) G-CSF induction early in uropathogenic Escherichia coli infection of the urinary tract modulates host immunity. *Cell Microbiol* 10(12):2568-2578.
282. Maas C*, et al.* (2013) CLL cells are resistant to smac mimetics because of an inability to form a ripoptosome complex. *Cell Death Dis* 4:e782.
283. Jersmann HP (2005) Time to abandon dogma: CD14 is expressed by non-myeloid lineage cells. *Immunol Cell Biol* 83(5):462-467.
284. Antal-Szalmas P, Strijp JA, Weersink AJ, Verhoef J, & Van Kessel KP (1997) Quantitation of surface CD14 on human monocytes and neutrophils. *J Leukoc Biol* 61(6):721-728.
285. Bossaller L*, et al.* (2012) Cutting edge: FAS (CD95) mediates noncanonical IL-1beta and IL-18 maturation via caspase-8 in an RIP3-independent manner. *J Immunol* 189(12):5508-5512.
286. Croker BA*, et al.* (2011) Neutrophils require SHP1 to regulate IL-1beta production and prevent inflammatory skin disease. *J Immunol* 186(2):1131-1139.
287. Shenderov K*, et al.* (2014) Cutting edge: Endoplasmic reticulum stress licenses macrophages to produce mature IL-1beta in response to TLR4 stimulation through a caspase-8- and TRIF-dependent pathway. *J Immunol* 192(5):2029-2033.
288. Berger M*, et al.* (2012) Neutrophils express distinct RNA receptors in a non-canonical way. *J Biol Chem* 287(23):19409-19417.
289. Saitoh T*, et al.* (2012) Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. *Cell Host Microbe* 12(1):109-116.
290. Poltorak A*, et al.* (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282(5396):2085-2088.
291. Hoshino K*, et al.* (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* 162(7):3749-3752.
292. Takeuchi O*, et al.* (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* 11(4):443-451.
293. Takeuchi O & Akira S (2010) Pattern recognition receptors and inflammation. *Cell* 140(6):805-820.

294. Case CL*, et al.* (2013) Caspase-11 stimulates rapid flagellin-independent pyroptosis in response to Legionella pneumophila. *Proc Natl Acad Sci U S A* 110(5):1851-1856.
295. Buras JA, Holzmann B, & Sitkovsky M (2005) Animal models of sepsis: setting the stage. *Nat Rev Drug Discov* 4(10):854-865.
296. Fink MP & Heard SO (1990) Laboratory models of sepsis and septic shock. *J Surg Res* 49(2):186-196.
297. Ohlsson K, Bjork P, Bergenfeldt M, Hageman R, & Thompson RC (1990) Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. *Nature* 348(6301):550-552.
298. Fischer E*, et al.* (1992) Interleukin-1 receptor blockade improves survival and hemodynamic performance in Escherichia coli septic shock, but fails to alter host responses to sublethal endotoxemia. *J Clin Invest* 89(5):1551-1557.
299. Joosten LA*, et al.* (2010) Differential susceptibility to lethal endotoxaemia in mice deficient in IL-1alpha, IL-1beta or IL-1 receptor type I. *APMIS* 118(12):1000-1007.
300. Netea MG*, et al.* (2000) Neutralization of IL-18 reduces neutrophil tissue accumulation and protects mice against lethal Escherichia coli and Salmonella typhimurium endotoxemia. *J Immunol* 164(5):2644-2649.
301. Kovach MA & Standiford TJ (2012) The function of neutrophils in sepsis. *Curr Opin Infect Dis* 25(3):321-327.
302. Alheim K*, et al.* (1997) Hyperresponsive febrile reactions to interleukin (IL) 1alpha and IL-1beta, and altered brain cytokine mRNA and serum cytokine levels, in IL-1beta-deficient mice. *Proc Natl Acad Sci U S A* 94(6):2681-2686.
303. Furze RC & Rankin SM (2008) Neutrophil mobilization and clearance in the bone marrow. *Immunology* 125(3):281-288.
304. Guarda G*, et al.* (2011) Type I interferon inhibits interleukin-1 production and inflammasome activation. *Immunity* 34(2):213-223.
305. Guarda G*, et al.* (2011) Differential expression of NLRP3 among hematopoietic cells. *J Immunol* 186(4):2529-2534.
306. Martinod K*, et al.* (2015) PAD4-deficiency does not affect bacteremia in polymicrobial sepsis and ameliorates endotoxemic shock. *Blood* 125(12):1948-1956.
307. Fuchs TA*, et al.* (2010) Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci U S A* 107(36):15880-15885.

308. Xu J*, et al.* (2009) Extracellular histones are major mediators of death in sepsis. *Nat Med* 15(11):1318-1321.
309. Clausen BE, Burkhardt C, Reith W, Renkawitz R, & Forster I (1999) Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res* 8(4):265-277.
310. Silva MT (2011) Macrophage phagocytosis of neutrophils at inflammatory/infectious foci: a cooperative mechanism in the control of infection and infectious inflammation. *J Leukoc Biol* 89(5):675-683.
311. Naveau C & Houssiau FA (2005) Pneumococcal sepsis in patients with systemic lupus erythematosus. *Lupus* 14(11):903-906.
312. Beadling C & Slifka MK (2004) How do viral infections predispose patients to bacterial infections? *Curr Opin Infect Dis* 17(3):185-191.
313. Morens DM, Taubenberger JK, & Fauci AS (2008) Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. *J Infect Dis* 198(7):962-970.
314. Suffredini AF*, et al.* (1989) The cardiovascular response of normal humans to the administration of endotoxin. *N Engl J Med* 321(5):280-287.
315. Freeman BD & Natanson C (2000) Anti-inflammatory therapies in sepsis and septic shock. *Expert Opin Investig Drugs* 9(7):1651-1663.
316. Warren HS (2009) Editorial: Mouse models to study sepsis syndrome in humans. *J Leukoc Biol* 86(2):199-201.
317. Antonelli M (1999) Sepsis and septic shock: pro-inflammatory or anti-inflammatory state? *J Chemother* 11(6):536-540.
318. Opal SM*, et al.* (1999) Relationship between plasma levels of lipopolysaccharide (LPS) and LPS-binding protein in patients with severe sepsis and septic shock. *J Infect Dis* 180(5):1584-1589.
319. Seok J*, et al.* (2013) Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A* 110(9):3507-3512.
320. Takao K & Miyakawa T (2015) Genomic responses in mouse models greatly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A* 112(4):1167-1172.
321. Shay T, Lederer JA, & Benoist C (2015) Genomic responses to inflammation in mouse models mimic humans: we concur, apples to oranges comparisons won't do. *Proc Natl Acad Sci U S A* 112(4):E346.
322. Croker BA, O'Donnell JA, & Gerlic M (2014) Pyroptotic death storms and cytopenia. *Curr Opin Immunol* 26:128-137.

323. Lachmann HJ*, et al.* (2009) In vivo regulation of interleukin 1beta in patients with cryopyrin-associated periodic syndromes. *J Exp Med* 206(5):1029-1036.
324. Greten FR*, et al.* (2007) NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta. *Cell* 130(5):918-931.
325. Bazzoni F*, et al.* (1991) Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. *J Exp Med* 173(3):771-774.
326. Coeshott C*, et al.* (1999) Converting enzyme-independent release of tumor necrosis factor alpha and IL-1beta from a stimulated human monocytic cell line in the presence of activated neutrophils or purified proteinase 3. *Proc Natl Acad Sci U S A* 96(11):6261-6266.
327. Guma M*, et al.* (2009) Caspase 1-independent activation of interleukin-1beta in neutrophil-predominant inflammation. *Arthritis Rheum* 60(12):3642-3650.
328. Joosten LA*, et al.* (2009) Inflammatory arthritis in caspase 1 gene-deficient mice: contribution of proteinase 3 to caspase 1-independent production of bioactive interleukin-1beta. *Arthritis Rheum* 60(12):3651-3662.
329. Carlyon JA, Abdel-Latif D, Pypaert M, Lacy P, & Fikrig E (2004) Anaplasma phagocytophilum utilizes multiple host evasion mechanisms to thwart NADPH oxidase-mediated killing during neutrophil infection. *Infect Immun* 72(8):4772-4783.
330. Dunlap NE, Benjamin WH, Jr., Berry AK, Eldridge JH, & Briles DE (1992) A 'safe-site' for Salmonella typhimurium is within splenic polymorphonuclear cells. *Microb Pathog* 13(3):181-190.
331. Akkoyunlu M, Malawista SE, Anguita J, & Fikrig E (2001) Exploitation of interleukin-8-induced neutrophil chemotaxis by the agent of human granulocytic ehrlichiosis. *Infect Immun* 69(9):5577-5588.
332. Martinon F & Tschopp J (2007) Inflammatory caspases and inflammasomes: master switches of inflammation. *Cell Death Differ* 14(1):10-22.
333. Poyet JL*, et al.* (2001) Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1. *J Biol Chem* 276(30):28309-28313.
334. Kagan JC, Magupalli VG, & Wu H (2014) SMOCs: supramolecular organizing centres that control innate immunity. *Nat Rev Immunol* 14(12):821-826.
335. Nakagawa T*, et al.* (2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 403(6765):98-103.
336. Saleh M*, et al.* (2006) Enhanced bacterial clearance and sepsis resistance in caspase-12-deficient mice. *Nature* 440(7087):1064-1068.

337. Yousefi S, Mihalache C, Kozlowski E, Schmid I, & Simon HU (2009) Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ* 16(11):1438-1444.

338. Yipp BG*, et al.* (2012) Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med* 18(9):1386-1393.

339. Pilsczek FH*, et al.* (2010) A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. *J Immunol* 185(12):7413-7425.

340. Schauer C*, et al.* (2014) Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. *Nat Med* 20(5):511-517.

341. Li P*, et al.* (2010) PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med* 207(9):1853-1862.

342. Apostolidou E*, et al.* (2014) Neutrophil extracellular traps regulate IL-1beta-mediated inflammation in familial Mediterranean fever. *Ann Rheum Dis*.

343. Warnatsch A, Ioannou M, Wang Q, & Papayannopoulos V (2015) Inflammation. Neutrophil extracellular traps license macrophages for cytokine production in atherosclerosis. *Science* 349(6245):316-320.

344. Scapini P & Cassatella MA (2014) Social networking of human neutrophils within the immune system. *Blood* 124(5):710-719.

345. Branzk N*, et al.* (2014) Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat Immunol* 15(11):1017-1025.

346. Lewis SM, Khan N, Beale R, Treacher DF, & Brown KA (2013) Depletion of blood neutrophils from patients with sepsis: treatment for the future? *Int Immunopharmacol* 17(4):1226-1232.

347. Baroja-Mazo A*, et al.* (2014) The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. *Nat Immunol* 15(8):738-748.

348. Franklin BS*, et al.* (2014) The adaptor ASC has extracellular and 'prionoid' activities that propagate inflammation. *Nat Immunol* 15(8):727-737.

349. Monteleone M, Stow JL, & Schroder K (2015) Mechanisms of unconventional secretion of IL-1 family cytokines. *Cytokine* 74(2):213-218.

350. Cullen SP, Kearney CJ, Clancy DM, & Martin SJ (2015) Diverse Activators of the NLRP3 Inflammasome Promote IL-1beta Secretion by Triggering Necrosis. *Cell Rep* 11(10):1535-1548.