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Regulation of the pro-inflammatory cytokine IL-1β by ubiquitination and its role in modulating IL-1β activity

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Regulation of the pro-inflammatory cytokine IL-1β by ubiquitination and its role in modulating IL-1β activity

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By

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Abstract

Interleukin-1β (IL-1β) is a potent proinflammatory cytokine that requires inflammasome-associated caspase-1, a cysteine protease, to cleave inactive precursor IL-1β, to its active secreted p17 fragment. Excessive IL-1β activation and release promotes autoinflammatory diseases, such as Cryopyrin associated periodic syndromes (CAPS), gout and cancers. Despite this role in inflammatory disease, the post-translational regulation of IL-1β is poorly understood. This thesis studies the post-translational modifications (PTMs) of IL-1β, with a particular focus on the ubiquitination of precursor IL-1β and its role in modulating IL-1β activity.

Ubiquitination is one of the most common regulatory PTMs, that occurs by the covalent addition of ubiquitin moieties onto a target protein, and acts to maintain protein homeostasis and co-ordinate cellular signalling cascades, including innate immunity. In this thesis, *in vitro* ubiquitination assays using Tandem Ubiquitin Binding Entities (TUBEs) and Mass spectrometric analysis define novel precursor IL-1β PTMs. Experiments show that Toll-like Receptor (TLR)-mediated expression of endogenous precursor IL-1β (inflammasome priming) causes its polyubiquitination. In particular, IL-1β lysine 133 (K133) is identified as a target residue for ubiquitination, while serine 134 (S134) is shown to be phosphorylated. Structural modelling of IL-1β also demonstrated the presence of an electrostatic interaction between IL-1β K133 and D129. In contrast to loss of IL-1β S134 phosphorylation, disruption of the IL-1β K133 and D129 electrostatic interaction, using K133A IL-1β and D129A mutants, abrogated IL-1β processing and secretion. Therefore, it is likely that IL-1β K133 is not just ubiquitinated, but its electrostatic interaction with D129 is critical for caspase-1 recognition and cleavage of precursor IL-1β.

Interestingly, expression studies in cell lines suggested that IL-1β K133R acts to stabilise precursor IL-1β and prevent it from being efficiently degraded. In line with this idea, experiments showed that IL-1β is a very short-lived protein relative to other inflammasome components and associated cytokines, and that IL-1β is targeted for K48- and K63-linked polyubiquitylation, which correlates with its targeting for proteasomal degradation.

To study the physiological role of IL-1β K133 ubiquitylation in primary cells and *in vivo,* an IL-1β K133R Knockin (KI) mouse was generated (*IL-1β*$^{K133R/K133R}$) using Clustered, regularly, interspersed, short, palindromic repeats (CRISPR)/Cas9 gene editing technology. Consistent with cell line-based assays, IL-1β from macrophages and neutrophils isolated from *IL-1β*$^{K133R/K133R}$ mice displayed increased levels and enhanced stability upon TLR-mediated inflammasome priming and, consequently, increased IL-1β secretion upon inflammasome activation. *In vivo* experiments mirrored these findings, as following endotoxic shock, *IL-1β*$^{K133R/K133R}$ mice responded by producing more precursor and more bioactive IL-1β relative to wildtype control animals. Thus, IL-1β K133 ubiquitination of precursor IL-1β limits inflammation by inducing IL-1β turnover, thereby reducing its availability to be activated by inflammasomes. These findings thereby delineate an additional post-translational mechanism to try and safeguard against excessive, and potentially damaging, IL-1β-driven inflammatory responses.

Declaration

This is to certify that:

i) This thesis comprises only my original work towards the degree of the PhD except where indicated below

ii) Due acknowledgement has been given to all other information used; and

iii) This thesis is lesser than 100,000 words in length, exclusive of tables, figures and references;

The following authors contributed data in Chapter 3 and 4: Dr. James Vince contributed data presented in Figures 3.2.2, 3.2.5, 3.2.11, 4.2.5, 4.2.8 and 4.2.11D. Dr. Kate Lawlor contributed data to Figure 4.2.11A, B and C. Dr. Rebecca Feltham contributed data to Figure 3.2.4B and Dr. Maryam Rashidi contributed data to Figure 4.2.3D. Generation of genetically modified mice in Chapter 4 was performed by the Melbourne Advanced Genomic Editing Centre (MAGEC) and basic animal husbandry was performed by Bioservices department at the Walter and Eliza Hall Institute of Medical Research. Mass spectrometry runs were performed by Dr. Laura Dagley from Proteomics lab at the Walter and Eliza Hall Institute of Medical Research.

My overall contribution to the results described in thesis is 90%.

Swarna Lekha Vijayaraj

Preface

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Abbreviations

α	alpha
β	beta
κ	kappa
μ	micro
AD	alzheimer's disease
AGE	advanced glycation end products
AIM2	absent in melanoma 2
AOSD	adult-onset still's disease
AP-1	activator protein-1
APAF	apoptotic protease activating factor 1
AQUA	absolute quantification
ARIH	ariadne ring-binding-ring domain e3 ubiquitin protein ligase 1
ATP	adenosine triphosphate
BafA1	bafilomycin A1
BAK	B-cell lymphoma 2 (BCL2) - antagonist/ killer1
BAX	Bcl-2-associated X protein
BMDC	bone marrow-derived dendritic cell
BMDM	bone marrow-derived macrophage
BRCC3	Breast cancer gene 1/2 (BRACA1/2)- containing complex 3
CANTOS	canakinumab anti-inflammatory thrombosis outcomes study
CAPS	cryopyrin-associated periodic syndrome
CARD	caspase activation and recruitment domain
cGAS	cyclic (guanine monophosphate) GMP- (adenine monophosphate) AMP synthase
CHX	cyclohexamide
cIAP	cellular inhibitor of apoptosis protein
CINCA	chronic infantile neurological cutaneous and articular syndrome
CLR	C-type lectin receptor
Cp.A.	Smac mimetic compound A
CRISPR	clustered, regularly, interspersed, short palindromic repeats
CRP	C-reactive protein

CUL1	E3 ligase cullin1
DAMP	damage-associated molecular pattern
DISC	death-inducing signalling complex
DRD	dopamine D1 receptor
DTT	dithiothreitol
DUB	deubiquitinase
EDTA	ethylene-diamine-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FACS	fluorescence-activated single cell sorting
FCAS	familial cold autoinflammatory syndrome
FINND	function-to-find domain
FMF	familial mediterranean fever
GFP	green fluorescent protein
GSDMD	gasdermin-D
HECT	homologous to E6AP C-terminus
HEK	human embryonic kidney
HCMV	human cytomegalovirus
HSP	heat shock protein
HSV	herpes simplex virus
IAV	influenza A virus
IFN	interferon
IKK	IκB kinase
IL	interleukin
IL-1R1	interleukin-1 receptor type 1
IL-1RAcP	interleukin-1 receptor accessory protein as a co-receptor
IMM	inner mitochondrial membrane
IRAK	IL-1 receptor-associated kinase 4
IRES	internal ribosome entry site
IRF	interferon regulatory factor
ISG15	interferon stimulated gene 15
JNK	c-Jun N-terminal kinase
K	lysine

KCL	potassium chloride
KI	knockin
LDH	lactate dehydrogenase
LeTx	lethal toxin
LGP2	laboratory of genetics and physiology 2
LMP	lysosomal membrane permeabilization
LPS	lipopolysaccharide
LRR	leucine rich repeats
LRRK2	leucine rich repeat containing kinase-2
M1	methionine1
MAPK	mitogen-activated protein kinase
MARCH	membrane-associated RING-CH-type finger protein
MAS	macrophage activation syndrome
MAVS	mitochondrial anti-viral signalling protein
MCL	myeloid leukemia cell differentiation protein
MDA5	melanoma differentiation-associated protein 5
MDP	muramyl dipeptide
MEFV	mediterranean fever
MLKL	mixed lineage kinase domain-like protein
MMP	mitochondrial membrane permeabilization
MS	mass spectrometric
MSU	monosodium urate
MWS	muckle-wells syndrome
NACHT	nucleotide-binding-and-oligomerization domain
NAIP	NLR family member apoptosis inhibitor protein
NEK	never in mitosis gene-A
NEM	N-ethylmaleimide
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells protein
NLR	nucleotide-binding domain, leucine rich containing protein
NLRP3	NLR family pyrin domain containing 3 protein
NOMID	neonatal-onset multisystem inflammatory disease
OMM	outer mitochondrial membrane
PAAND	Pyrin-associated autoinflammation with neutrophilic dermatitis

PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PYHIN	PYD and HIN domain containing proteins
PI	propidium iodide
PKD	protein kinase D
PRR	pattern recognition receptor
PTM	post-translational modification
PYD	pyrin
RBR	RING-between RING-RING domain families
RING	really interesting new gene
RIP2	receptor-interacting serine/threonine-protein kinase 2
RIPK	receptor interacting protein kinase
RLR	retinoic acid-inducible gene 1-like receptor
ROS	reactive oxygen species
SCF	Skp, Cullin, F-box containing complex
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIJA	systemic onset juvenile idiopathic arthritis
SILAC	stable isotopes labeling by amino acids in cell culture
STING	stimulator of interferon genes
SUG	suppressor of gal1
T4SS	type 4 secretion system
TAB	TGF β-activated kinase (TAK) binding protein
TGN	trans golgi network
TIR	toll/interleukin-1 receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR1	tumor necrosis factor receptor 1
TOLLIP	toll-interacting protein
TRAF	TNF receptor-associated factor
TRIM	tripartite motif-11
TUBE	tandem ubiquitin binding entity
Ub	ubiquitin
UBE3A	ubiquitin-protein ligase E3A /E6AP

UBR	ubiquitin-protein ligase E3 component N-recognin
UPS	ubiquitin-proteasome system
USP	ubiquitin-specific protease
VACV	vaccinia virus
WDR1	WD repeat-containing protein1
WT	Wildtype

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Chapter 1: Literature review

1.1 Innate Immunity and Pattern Recognition Receptors (PRRs)

1.1.1 Toll-like receptors (TLRs)

Pattern recognition receptors (PRRs) are sensors that specialise in detecting pathogenic molecules and host "danger" signals (that are released by damaged or dead cells), playing a crucial part in innate immunity. Activation of PRRs leads to signalling cascades that eventually culminate in pro-inflammatory responses and inflammation. The first identified and best characterised PRRs are Toll-like receptors (TLRs). TLRs were first reported in *Drosophila melanogaster* (Anderson KV. 1985). Currently, 10 TLRs are identified in humans (TLR1-TLR10) and 13 TLRs in mice (TLR1-TLR13) (Akira 2006). **Figure 1.1** depicts different classes of PRRs identified in the innate immune system.

TLRs are widely expressed in immune cells such as monocytes, macrophages and dendritic cells (DCs), as well as non-immune cells such as fibroblasts, endothelial and epithelial cells (Akira 2006). Depending on their cellular localisation, TLRs are classified as cell surface or intracellular endosomal TLRs. Cell surface TLRs include TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10. Bacterial components such as lipopolysaccharide (LPS), flagellin and a wide variety of "pattern associated molecular patterns" (PAMPs) are recognised by cell surface TLRs (Akira 2006, Kawai 2010, Regan 2013). Apart from PAMPs, cell surface TLRs also recognise "damage associated molecular patterns" (DAMPs), such as HMGB1, DNA and RNA released from damaged cells (Park 2004, Wagner 2006, Vidya 2018). Intracellular TLRs are mostly located in endosomes and includes TLR3, TLR7, TLR8, TLR9, TLR11, TLR12 and TLR13. Primarily, TLR3 binds to double stranded RNAs (dsRNAs) (Alexopoulou 2001), TLR7 and TLR8 binds to single stranded RNA (ssRNAs) (Diebold 2004, Heil 2004), TLR9 detects unmethylated CpG-DNA motifs (Hochrein 2004, Kumar 2009), TLR11 and TLR12 recognises prolifin-like molecules derived from *Toxoplasma gondii* (Yarovinsky 2005, Koblansky 2013) and TLR13 recognises bacterial 23S rRNA and unknown components of vesicular stomatitis virus (Vidya 2018).

TLRs are type 1 transmembrane glycoproteins and their general structure consist of three domains. The extracellular domain consists of repeated (22-29) Leucine-Rich Repeats (LRRs)

motif assembled in a horseshoe-like structure that facilitates the binding of PAMPs or DAMPs (Botos 2011). Upon ligand recognition, TLRs recruit adaptor proteins to the intracellular TIR (Toll/interleukin-1 receptor) domain that initiate the signalling cascade that results in the activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cell protein (NF-κB), interferon (IFN) regulatory factors (IRFs) or mitogen-activated protein kinases (MAPKs) to regulate the expression of cytokines, chemokines and type 1 IFNs that ultimately protect the host from infection or cellular stress (Akira 2004).

Depending on adaptor proteins that are recruited to the TIR domain, two distinct signalling pathways are classified; namely, the myeloid differentiation primary response protein 88 (MyD88)-pathway and the TIR domain-containing adaptor-inducting IFNβ (TRIF)-pathway (Akira 2004). With the exception of TLR3, all TLRs utilise the MyD88 pathway (Kumar 2009). The binding of MyD88 to the TIR domain through homotypic/heterotypic interaction leads to the recruitment of IL-1 Receptor-Associated Kinase 4 (IRAK4) resulting in the formation of the Myddosome complex and phosphorylation of IL-1 Receptor-Associated Kinase 1 (IRAK1)(Lin 2010). Subsequently, Tumor Necrosis Factor (TNF) Receptor-Associated Factor 6 (TRAF6) is activated and leads to the K63-linked polyubiquitination of TAK1 and TRAF6 activating TAK1/TGF-β activated kinase (TAB) complex (Gorjestani 2012). This ultimately, culminates in the nuclear translocation of previously inactive NF-κB to its active form as the inhibitor IκBα is degraded. The active NF-κB allows the transcription of various genes inducing the expression of inflammatory cytokines (Wang 2001).

The TLR-TRIF-dependent pathway is specific to TLR3 and TLR4 and induces the transcription factor NF-κB, Activating Protein-1 (AP-1) and IRF family members. The TRIF pathway leads to the upregulation of IFN-inducible genes including type-1 IFNs (Hoebe 2003). TLR4 is the only TLR, that can activate both MyD88 and TRIF signalling pathways (Lu 2008). In the context of inflammasomes, TLR4 activation leads to NF-κB activation and results in the transcriptional upregulation of the inflammasome sensor protein, NOD-,LRR- and pyrin domain containing protein 3 (NLRP3) and IL-1β genes (Hiscott 1993, Bauernfeind 2009). This step is termed as “inflammasome priming”.

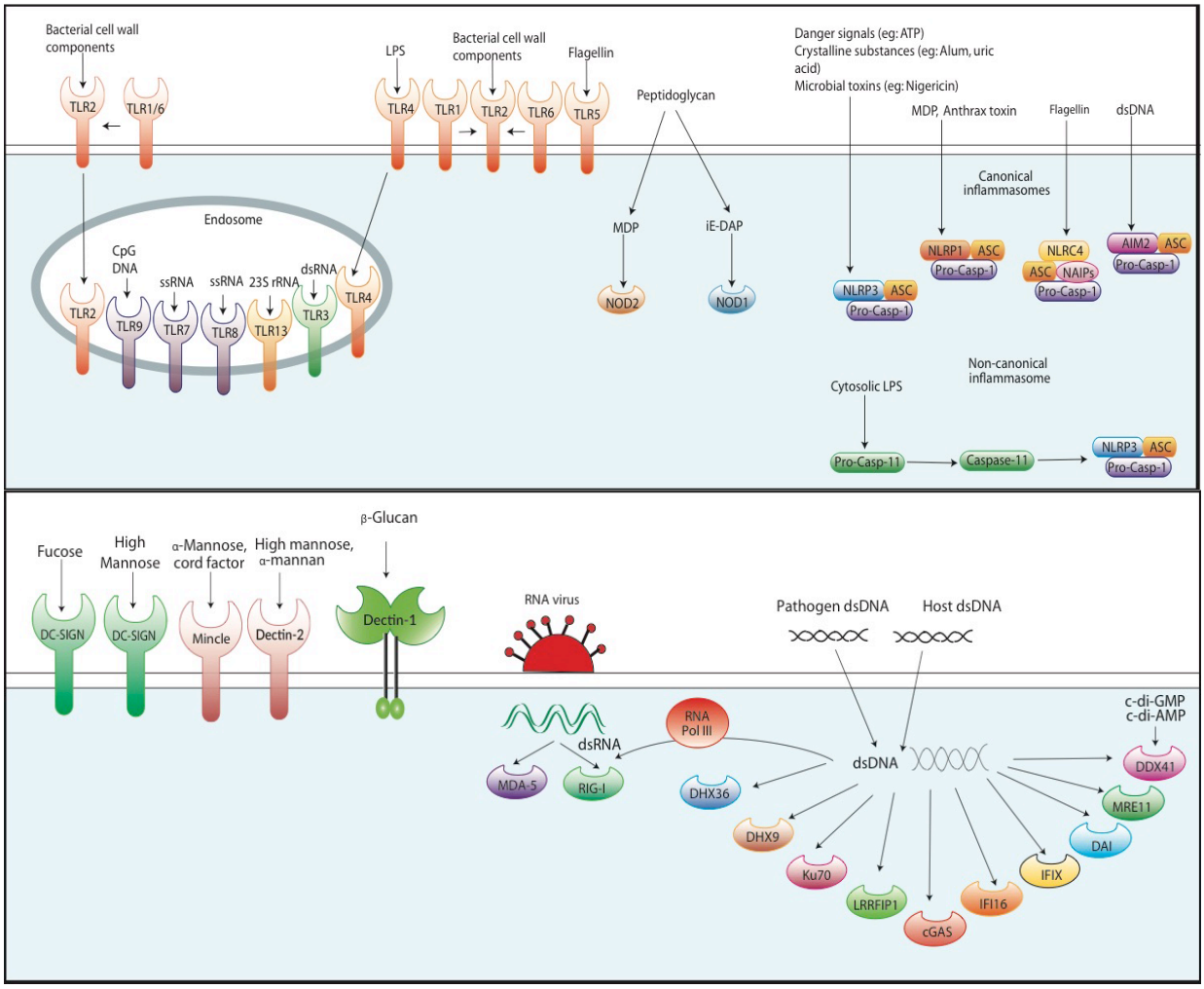


Figure 1.1 PRRs of innate immunity.

PRRs that serve as sensors to screen intracellular and extracellular pathogenic molecules, named pathogen-associated molecular patterns (PAMPs) and host cellular stressors, named damage-associated molecular patterns (DAMPs). Upon recognition of various ligands by PRRs, signalling cascades are activated to evoke a pro-inflammatory response to eliminate pathogens.

1.1.2 NOD like receptors (NLRs)

Nucleotide binding Oligomerization Domain (NOD)- like receptors are cytoplasmic receptors that recognise PAMPs (e.g. peptidoglycan and viral RNA) and DAMPs (e.g. UV radiation and ATP). Upon recognition and binding to various PAMPs and DAMPs, an inflammatory response is activated (Kim, Shin et al. 2016). A huge variability in the presence of NLR genes is witnessed among various species. In humans, more than 22 NLR genes have been identified (Schroder and Tschopp 2010). In mouse, 34 NLR genes are discovered and a staggering number of 200 NLR receptors are identified in sea urchin (Motta, Soares et al. 2015).

The structure of NLRs are similar to plant disease-resistance (R) genes that are involved in responses against plant pathogens (Bent and Mackey 2007). There are three characteristic domains present in each NLR: 1) A N-terminal caspase activation and recruitment domain (CARD) or pyrin (PYD) domain that is responsible for signal transduction by mediating homotypic protein-protein interactions. The NLR family member apoptosis inhibitor protein (NAIP5) is an exception in that it contains a baculovirus inhibitor of apoptosis repeat (BIR) domain (Ting, Lovering et al. 2008) in addition to a central nucleotide-binding and oligomerization domain (NACHT) that mediates self-oligomerization and C-terminal leucine-rich repeats (LRR) that are responsible for ligand sensing and autoregulation. Interestingly, the NACHT domain has similarities to the NB-ARC motif of the apoptotic mediator APAF1 that forms the apoptosome platform to activate caspase-9 (Proell, Riedl et al. 2008).

The first NLRs described as PRRs were NOD1 and NOD2. The peptidoglycan component of Gram-negative bacteria, γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), activates NOD1 signalling (Girardin, Boneca et al. 2003), whereas Muramyl DiPeptide (MDP), a peptidoglycan component of both Gram-positive and Gram-negative bacteria, activates NOD2 signalling (Girardin, Boneca et al. 2003). NOD1/NOD2 recognition of peptidoglycan components leads to its interaction with Receptor Interacting Protein 2 (RIP2) and subsequent activation of NF-κB and MAPK signalling causing transcriptional upregulation of inflammatory genes (Girardin, Tournebize et al. 2001, Ogura, Bonen et al. 2001, Kobayashi, Inohara et al. 2002).

1.1.3 RIG like receptors (RLRs)

RIG-I like receptors (RLRs) are cytoplasmic viral RNA sensors that belong to the family of DExD/H box RNA helicases (Onoguchi, Yoneyama et al. 2011). Upon recognition of viral RNA ligands in the cytoplasm, RLRs trigger inflammation to control viral infection. The most well characterised member of RLRs is RIG-1 (Retinoic acid-inducible gene-1). Other members of RLRs identified include MDA5 (Melanoma Differentiation Associated Factor 5) and LGP2 (Laboratory of Genetics and Physiology 2) (Yoneyama and Fujita 2009).

Both RIG-1 and MDA-5 share structural similarity and contain three domains. i) Tandem Caspase Activation and Recruitment Domains (CARD) located at N-terminus, ii) a central DExD/H box RNA helicase domain with the capability to bind and hydrolyse ATP to unwind RNA and iii) a C-terminal Repressor Domain (RD) inserted within the C-terminal Domain

(CTD). LGP2, though structurally similar to RIG-1 and MDA5, lacks the tandem CARD domain at the N-terminus (Yoneyama, Kikuchi et al. 2005).

RLRs are widely expressed in many tissues including myeloid cells, epithelial cells and plasmacytoid dendritic cells. Typically, RLR expression is low. However, upon IFN exposure and viral infection, RLR expression is significantly increased implying that regulation of RLR is partly controlled by its expression (Kang, Gopalkrishnan et al. 2004). RIG-1 can detect members of viral genera Paramyxoviridae, Rhabdoviridae Orthomyxoviridae and DNA viruses (Epstein-Barr virus EBER). Similarly, MDA5 recognises members of viral genera Picornaviridae and DNA viruses (vaccinia virus) (Kato, Takeuchi et al. 2006). Electron microscopy studies revealed recognition of dsRNA resulted in MDA5 forming fibre-like polymers (Peisley, Lin et al. 2011, Berke, Yu et al. 2012, Wu, Peisley et al. 2013). Recently, it was reported that LGP2 acted as a positive regulator in viral RNA recognition by aiding MDA5 fibre formation and elongating the fibre length (Duic, Tadakuma et al. 2020). Atomic Force Microscopy (AFN) imaging showed upon viral RNA recognition, LGP2 incorporated into MDA5 fibres and induced structural changes to MDA5, exposing MDA-5's CARD domains, allowing it to participate in downstream signalling, eliciting an antiviral response (Duic, Tadakuma et al. 2020).

RIG-1 preferentially binds to RNA sequences marked with 5' phosphorylated (5'ppp) ends, presumably to differentiate between self and pathogenic RNA sequences (Hornung, Ellegast et al. 2006). Structural analysis revealed that MDA5 CTD preferentially binds to dsRNA with blunt ends (Murali, Li et al. 2008). Upon viral infection and recognition of viral PAMPs, RIG-1 and MDA5 binds to an adaptor protein MAVS (Mitochondrial Anti-Viral Signalling protein, also known as IPS-1, VISA or Cardiff, located on the outer mitochondrial membrane) through caspase recruitment domain (CARD-CARD) interactions (Seth, Sun et al. 2005). Relocation of RLRs to MAVS-associated membranes leads to formation of the MAVs signalosome which includes TRAF3 and subsequently the IKKε/TBK1 complex. Following this, latent transcription factors IRF3 (Interferon Regulatory Factor 3), IRF7 and NF-κB are activated leading to the production of type 1 (including IFNα and IFNβ) and type III interferons (IFN-γ and IL-10 related cytokine) (Dixit, Boulant et al. 2010, Poeck, Bscheider et al. 2010).

RIG-1 signalling can also be activated independently of MAVS as studies have shown RIG-1 directly associates with ASC protein to drive caspase-1 dependent inflammasome activation, leading to the processing of pro-inflammatory cytokines IL-1β and IL-18 into their mature forms (Poeck, Bscheider et al. 2010, Loo and Gale 2011).

1.1.4 C-type lectin receptors (CLRs)

CLRs are a heterogenous family of PRRs containing both soluble and transmembrane proteins that can specifically bind to carbohydrates (beta-glucan and mannan) present on fungal cell walls (Netea, Brown et al. 2008). CLRs are expressed in most cell types including macrophages and dendritic cells, that play an important role in anti-fungal immunity. Studies have also shown that CLRs are involved in eliciting anti-mycobacterial immune responses (Kerrigan and Brown 2011). Transmembrane CLRs can be divided in to two sub groups: Type-1 receptors that belong to the mannose receptor family (e.g. CD206-macrophage mannose receptor) and Type-II receptors that belong to the asialoglycoprotein receptor family (e.g. Dectin-1, Dectin-2 and Mincle) (Geijtenbeek and Gringhuis 2009).

Upon recognition of fungal pathogens by CLRs and their phagocytosis, an anti-fungal CARD9 signalling pathway is activated, inducing NF-κB activation, leading to the production of cytokines and chemokines. In addition to inducing innate immune responses, CLRs also induce adaptive immune responses by promoting development of Th1 and Th17 responses (LeibundGut-Landmann, Gross et al. 2007).

In order to induce optimal anti-fungal responses, the host immune system engages “crosstalk” pathways between multiple TLRs that can recognise fungal PAMPs and CLRs (Kawai and Akira 2011). Such collaborative “crosstalk” pathways are well described in Dectin-1 signalling pathway where anti-inflammatory cytokines such as TNF-α, IL-1β and IL-10 are synergistically induced by Dectin-1 and multiple TLRs (Dennehy, Ferwerda et al. 2008) (Huang, Ostroff et al. 2009).

1.1.5 Cytosolic DNA sensors and STING

The cGAS-STING (Cyclin GMP-AMP synthase- Stimulator of Interferon Genes) pathway has been shown to be essential for DNA-mediated immune responses. Cyclic GMP-AMP synthase (cGAS) is a PRR that senses cytosolic cyclic-dinucleotides (CDNs) and triggers innate immune responses (Li, Shu et al. 2013, Zhang, Wu et al. 2014). Upon recognition of CDNs, cGAS is activated to form a cGAS-DNA dimeric complex. cGAS-DNA dimeric complex triggers a reaction from ATP and GTP and produces 2'3'-cyclic GMP-AMP (cGAMP) (Kranzusch, Lee et al. 2014). Through high affinity ligand binding, cGAMP binds to adaptor protein STING (Wu, Sun et al. 2013). Subsequently, STING is activated through various structural changes and recruits and phosphorylates IRF3 via TBK1. Phosphorylation of IRF3 leads to phosphorylation of IκB-α and its degradation. IRF3 then translocates to the nucleus and induces the transcription of type I interferons and other cytokines (Cai, Chiu et al. 2014).

Apart from sensing unsolicited, self-DNA released from damaged cells, cGAS plays an important role in antiviral immunity. Infectious studies in mice have shown DNA viruses such as Herpes Simplex Virus 1 (HSV1) and vaccinia virus (VACV) to activate the cGAS-STING pathway and is essential to contribute host defense responses (Li, Wu et al. 2013). In addition to DNA viruses, the cGAS-STING pathway plays a critical role in conferring host immune defence responses against retroviruses such as HIV. HIV contains single-stranded RNA which requires a reverse transcriptase to convert RNA to DNA in order to propagate infection. Studies with inhibitors of retroviral reverse transcription abrogated IFN responses suggesting that viral cDNA could be activating the cGAS-STING pathway (Gao, Wu et al. 2013).

Contrary to host defence responses, chronic activation of the cGAS-STING pathway leads to autoimmune disease. In Aicardi-Goutieres syndrome (AGS), an autoimmune condition, due to mutations in 3'repair exonuclease TREX1, an overaccumulation of cytosolic DNA occurs (Crow, Hayward et al. 2006). Consistently, mice deficient in TREX1 develop severe autoimmunity. Deletion of STING rescues this phenotype, suggesting the cGAS-STING pathway is involved in this condition (Gall, Treuting et al. 2012). In addition to host defense mechanisms and autoimmune disease, the cGAS STING pathway is critical for cellular senescence and antitumor immunity; it can detect accumulated cytoplasmic DNA in tumour cells or DNA released from cancer cells that is phagocytosed by immune cells such as, dendritic

cells and macrophages to trigger anti-tumour immune responses (Wang, Hu et al. 2017, Yang, Wang et al. 2017).

1.2 Inflammasome sensor proteins

Innate immune sensor proteins that can form inflammasomes belong to three different families: Nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), the PYHIN family (PYD and HIN domain containing proteins) and the tripartite motif family (TRIM). In humans, more than 22 NLR genes have been identified and the most well characterised NLR proteins are NLRP3, NLRC4 and NLRP1b. Apart from NLRs, the other well studied inflammasome sensor proteins include: Absent in Melanoma 2 (AIM2) which belongs to the HIN-200 family and Pyrin (also known as TRIM20) which belongs to the TRIM family, as the name suggests.

1.2.1 NLRP1

The expression of NLRP1 is highest in the thymus and spleen and therefore was suspected to play a functional role in the immune system. In 2002, NLRP1 was the first NLR protein reported to form an inflammasome (Martinon, Burns et al. 2002). Due to gene duplication events, NLRP1 exists as three paralogs (NLRP1a, NLRP1b and NLRP1c) in mouse (Boyden and Dietrich 2006). The structure of human NLRP1 is unique in that it contains a N-terminal PYD domain, C-terminal (function-to-find) FINND domain followed by CARD domain and was shown to bind and activate caspase-1 to trigger inflammasome signalling (Hiller, Kohl et al. 2003, D'Osualdo, Weichenberger et al. 2011, Jin, Curry et al. 2013).

The NLRP1 CARD domain encompasses six anti-parallel α helices in a greek key fold model and has very similar structure and charge surface patches to the other members of the death domain-fold family; NOD1, pro-caspase-9 and Apoptotic protease activating factor 1 (Apaf-1) (Jin, Curry et al. 2013). Recently crystal structures of the NLRP1 LRR domain were described, however the PAMP, muramyl dipeptide (MDP) believed to activate NRLP1, failed to co-crystallize with the NLRP1 LRR domain (Reubold, Hahne et al. 2014). Indeed, bone marrow-derived macrophages (BMDMs) that lack NLRP1b in mice on a 129 background showed unaltered IL-1β secretion in response to MDP, TiO_2 (titanium dioxide) or a combination of both MDP and TiO_2, (Kovarova, Hesker et al. 2012). Also, it is well known nano-TiO_2 used to package MDP can alone trigger NLRP3 inflammasome activation (Yazdi, Guarda et al. 2010). These studies strongly suggest that MDP is not the ligand that triggers the NLRP1

inflammasome. Recently, human NLRP1 but not murine NLRP1 is reported as a direct sensor of double stranded RNA (dsRNA) via its LRR and NACHT domain resulting in its activation (Bauernfried, Scherr et al. 2020).

Murine NLRP1b does not contain a PYD domain and the presence of C-terminal CARD domain indicates that the NLRP1b inflammasome activation does not require ASC and may directly interact with caspase-1. This is true in some conditions when BMDMs or bone marrow-derived dendritic cells (BMDCs) stimulated with *Bacillus anthracis* lethal toxin (LeTx) showed IL-1β cleavage and secretion in the absence of ASC (Guey, Bodnar et al. Dec 2014). Lethal factor (LF) of the *Bacillus anthracis* LeTx is reported to cleave mouse Nlrp1b in N-terminal region between K44 and L45 site to trigger its activation (Hellmich, Levinsohn et al. 2012).

NLRP1 activity is also reported to be dependent on the autolytic proteolysis in the FINND domain and single nucleotide polymorphisms or alternative mRNA near the highly conserved cleavage site (H1186) could impact NLRP1 inflammasome activity (Finger, Lich et al. 2012). Polymorphisms of NLRP1 are involved in many diseases. 19 genetic variants of NLRP1 are associated with vitiligo (Jin, Birlea et al. 2007). Other diseases associated with NLRP1 polymorphisms include systemic lupus erythematosus, inflammatory bowel disease, rheumatoid arthritis, psoriasis, Alzheimer's disease and malignant melanoma (Yu, Moecking et al. 2018).

1.2.2 NAIP-Nlrc4

Poyet and colleagues were the first to describe IPAF-ICE-protease Activating Factor (now called NLRC4) as an Apaf-1 like molecule that could activate caspase-1 (Poyet, Srinivasula et al. 2001). Due to its high structural similarity to other NLR proteins and presence of a CARD domain analogous to NLRP1, IPAF was then renamed as NLRC4. However, NLRC4 is unique as it does not sense pathogens directly. NLRC4 requires specific cytosolic co-receptor NAIPs (NLR family of apoptosis inhibitory proteins) for sensing various bacterial ligands (Kofoed and Vance 2011). Cytoplasmic flagellin and basal body rod and needle proteins of the secretion systems present in many Gram-negative bacteria, including the type III secretion system of *Salmonella typhimurium* (T3SS) and *Legionella pneumophila* type IV secretion system (T4SS) can be sensed by specific NAIPs, which then activate the NLRC4 inflammasome (Galan and

Wolf-Watz 2006, Miao, Mao et al. 2010, Zhao, Yang et al. 2011). Though NLRC4 has a CARD domain that and can interact with caspase-1 directly, like other inflammasome forming proteins described in this section, ASC recruitment to the NLRC4 inflammasome complex augments caspase-1 activation in response to *Salmonella* (Mariathasan, Newton et al. July 2004). Cells lacking caspase-1 cannot die by pyroptosis, however, NLRC4 activation may still trigger caspase-8 dependent apoptosis (Mascarenhas, Cerqueira et al. 2017).

From detailed structural studies done on NLRC4 it has been reported that a single NAIP can induce the NLRC4 inflammasome complex formation (Zhang, Chen et al. 2015). Recently, NLRC4 and NLRP3 were reported to be part of the same inflammasome complex in infected cells (Man, Hopkins et al. 2014). Previous co-Immunoprecipitation studies have also shown heterotypic interactions between NLRC4 and other NLR proteins (Damiano, Oliveira et al. 2004). A protein kinase, Leucine Rich Repeat containing Kinase-2 (LRRK2) was recently shown to interact with NLRC4 along with phosphorylation of NLRC4 at S533. This post-translational modification at S533 seems to be crucial for holding NLRC4 in an inactive state (Liu, Liu et al. 2017). Taken together, the NLRC4 activation mechanism still remain unclear, particularly as inflammasome platforms are shown to heteroligomerize and/or be activated in parallel, vary in function in different cell types, and have been implicated in the pathogenesis of non-infectious diseases.

Several gain-of-function missense mutations in NLRC4 have been recently been discovered that cause infantile enterocolitis and recurrent Macrophage Activation Syndrome (MAS) that is often featured in common autoinflammatory diseases, such as Systemic Juvenile Idiopathic Arthritis (sJIA) and Adult-Onset Still's Disease (AOSD) (Canna, de Jesus et al. 2014, Romberg, Al Moussawi et al. 2014). MAS is a severe syndrome that includes hectic fever, coagulopathy, hemophagocytosis and dramatically elevated serum ferritin and is a similar to familial Heamophagocytic Lymphohistiocytosis (fHLH) a disease categorized as immunodeficiency (Weaver and Behrens 2014). Also, patients with MAS have dramatic elevated levels of IL-18 and respond acutely to IL-18 blockade therapies (de Torre-Minguela, Mesa Del Castillo et al. 2017). However, not all patients with NLRC4 mutations develop MAS. Recently a patient with a NLRC4 mutation developed CAPS-like symptoms (explained below) and responded to IL-1β inhibitors (Kawasaki, Oda et al. 2017). Interestingly, in human lung epithelial cell lines a NLRC4 H433P mutation increased its interaction with ubiquitinated proteins and the proteasomal component, SUG1, and resulted in caspase-8 dependent apoptosis

(Raghawan, Sripada et al. 2017). These findings highlight the importance of mechanistically deciphering inflammasome regulation to best predict therapeutic intervention to allow a personalized approach for the treatment of autoinflammatory diseases.

1.2.3 AIM2

The AIM2 inflammasome detects cytosolic dsDNA and provides immuno-surveillance for the host against pathogenic infections. AIM2 contains a c-terminal HIN-200 domain and PYD domain at the N-terminus. The HIN-200 domain senses double stranded (dsDNA) and triggers inflammasome assembly by releasing the PYD domain and allowing specific homotypic interactions with the PYD domain from ASC. During pathogenic infections one of the key requirements for AIM2 inflammasome activation is the release of bacterial/viral DNA (Man, Karki et al. 2016). Upon sensing dsDNA in the cytoplasm, from host or pathogens, AIM2 recruits the adaptor protein commonly involved in inflammasome formation, ASC (abbreviated as Apoptosis-associated Speck-like protein containing a CARD domain) via PYD-PYD interaction and initiates inflammasome activation (Lugrin and Martinon 2018).

As a defense mechanism, pathogens, such as *Legionella pneumophilia,* are reported to inhibit bacterial DNA release into cytosol of infected host macrophages and therefore block AIM2 inflammasome activation via its secretory effector protein, SdhA (Ge, Gong et al. 2012). Recently, several other molecules including, viral proteins pul83 and IE86 released during human cytomegalovirus (HCMV) infection, Hepatitis B e-antigen (HbeAG), Interferon-inducible protein 16β (IFI16β) (a newly identified human IFI16β isoform), mouse p202 (a disease locus for lupus) are all reported to inhibit AIM2 inflammasome as well at various stages of the activation pathway (Huang, Ma et al. 2017, Chen, He et al. 2018, Wang, Ye et al. 2018, Botto, Abraham et al. 2019). Thus, the tight control of the AIM2 inflammasome is important for host immune responses.

Conversely, unnecessary AIM2 inflammasome activation is implicated in the development of several inflammatory diseases and cancer (Sharma, Karki et al. 2019). For instance, upregulated AIM2 expression is associated with skin inflammatory diseases such as psoriasis and atopic dermatitis (de Koning, Bergboer et al. 2012). In a mouse model of unilateral urethral obstruction (UUO), AIM2 deficiency resulted in reduced inflammation compared to wildtype animals, suggesting the involvement of the AIM2 inflammasome in recognising necrotic DNA

released in chronic kidney disease (CKD) (Komada, Chung et al. 2018). Similarly, in atherosclerosis prone *ApoE* knockout model, loss of the AIM2 inflammasome showed improved histopathological features in high-fat diet-fed $ApoE^{-/-}$ mice compared to littermate control $ApoE^{-/-}$ mice (Paulin, Viola et al. 2018). The source of cytosolic dsDNA in all these diseases remains unknown, though it could be the extracellular DNA shed from dying cells, and its uptake by bystander cells, that causes AIM2 inflammasome activation.

1.2.4 Pyrin

Pyrin is encoded by the MEFV (Mediterranean Fever) gene (Seshadri, Duncan et al. 2007) and belongs to the TRIM family proteins that play an important role in innate immunity to viruses (Nisole, Stoye et al. 2005). Pyrin contains a N-terminal PYD domain, a basic leucine zipper domain (b-ZIP), a B-box domain, a central coiled-coiled domain and C-terminal B30.2 (PRY/SPRY) domain (Arakelov, Arakelov et al. 2018), which is notably absent in the mouse. Pyrin is activated by bacterial toxins, such as TcdB (a virulence factor of *Clostridium Difficile*), C3 toxin (*Clostridium botulinum*) and IbpA (*Histophilus somni*), which inactivate RhoA at the GTPase switch-1 region leading to dephosphorylation and activation of pyrin (Xu, Yang et al. 2014). RhoA blocks pyrin by activating serine-threonine kinases PKN1 and PKN2 to phosphorylate pyrin at Ser208 and Ser242 thereby allowing 14-3-3 binding (Park, Wood et al. 2016).

Familial Mediterranean Fever (FMF) is the most common monogenic autoinflammatory disease caused by autosomal recessive gain-of-function mutations in both alleles of MEFV locus that encode pyrin. The onset of FMF manifests with periodic fever, synovitis and amyloidosis (Moradian, Babikyan et al. 2017). The majority of FMF mutations are harboured within the B320.2/SPRY domain, although how they trigger pyrin activation remains unclear. Perturbed actin depolymerization has been implicated in pyrin activation to bacterial toxins. In fact, mice lacking the actin depolymerizing cofactor WD Repeat-containing protein 1 (WDR1) exhibit spontaneous autoinflammation associated with pyrin inflammasome activation and excessive IL-18 levels (Kile, Panopoulos et al. 2007). Moreover, drugs that inhibit microtubule polymerization, such as colchicine, inhibit pyrin activation to *C.difficile* toxins TcdA and TcdB (Gao, Yang et al. 2016). However, colchicine failed to block dephosphorylation and loss of 14-3-3 binding to pyrin, suggesting that colchicine acts downstream of these activation events to limit pyrin activity. Unexpectedly, FMF patient monocytes were also recently shown to secrete

IL-1β and IL-18 in responses to TcdA, indicating inflammasome activation can occur independent of microtubule function (Van Gorp, Saavedra et al. 2016).

Recently, Masters and colleagues documented a clinically distinguishable autoinflammatory disease, PANND (Pyrin-Associated Autoinflammation with Neutrophilic Dermatitis), caused by S242R mutation that is located in the +2site of the 14-3-3 binding motif of pyrin (Moghaddas, Llamas et al. 2017). PANND patients also exhibit a differential cytokine response, with spontaneous IL-1β release, and have therefore benefited from IL-1β blockade with the biologic, Anakinra.

1.2.5 NLRP3 inflammasome

NLRP3 is the most well characterized inflammasome due to the diverse range of PAMPS (eg. Influenza A virus) host-derived DAMPs (eg. ATP) and environmental particles (eg, asbestos fibres) that trigger its assembly. These diverse triggers have implicated NLRP3 in the pathogenesis of a variety of infectious diseases and common inflammatory diseases (He, Hara et al. 2016). The importance of tight regulation of NLRP3 is best highlighted by the potentially life threatening autoinflammatory diseases, called Cyropyrin Associated Periodic Syndrome (CAPS), that are caused in humans by autoactivating mutations in NLRP3 (See Chapter 1.4) (Kuemmerle-Deschner, Ozen et al. 2017). Canonical and non-canonical NLRP3 inflammasome activation are described below and illustrated in **Figure 1.2**.

1.2.5a Canonical NLRP3 inflammasome activation

NLRP3 contains a typical tripartite structure with a N-terminal PYD domain, a central NACHT domain and a C-terminal LRR domain (Schroder and Tschopp 2010). The NLRP3 LRR domain is thought to sense activating stimuli to promote NLRP3 oligomerization via the NACHT domain, which allows the PYD domain to recruit the adaptor ASC, thereby triggering homotypic CARD-CARD interactions with caspase-1 (Franchi, Warner et al. 2009). Like other inflammasome sensor proteins, upon caspase-1 activation and proteolysis the active dimers p20 and p10 cleave the pro-inflammatory proteins pro-IL-1β and pro-IL-18 in to their active forms (Walker, Talanian et al. 1994, Boatright, Renatus et al. 2003). Simultaneously, caspase-1 cleaves and activates gasdermin-D (GSDMD), a protein with pore-forming activity that causes cell membrane lysis and a cell death known as pyroptosis (Kayagaki, Stowe et al. 2015,

Liu, Zhang et al. 2016). Gasdermin-D cleavage by caspase-1 results in the GSDMD pyroptotic N-terminal fragment being released from the inhibitory C-terminal domain. Due to affinity for phospholipids, the N-terminal fragment of gasdermin-D targets the cytoplasmic face of the plasma membrane (Liu, Zhang et al. 2016). Accumulation and oligomerisation of N-terminal fragments of gasdermin-D results in pore-formation (Aglietti, Estevez et al. 2016, Ding, Wang et al. 2016), resulting in cell death and the release of intracellular, inflammatory components, that can help clear pathogens (Lamkanfi, Vande Walle et al. 2011, Aachoui, Sagulenko et al. 2013).

1.2.5b Non-canonical inflammasome activation

The non-canonical caspae-11 inflammasome was identified following the pivotal discovery by Kayagaki et al. that loss of caspase-11, rather than caspase-1, protected mice from a lethal dose of LPS (Kayagaki, Warming et al. 2011). Subsequently, caspase-11 was revealed to be activated by Gram-negative bacteria, including *Citrobacter rodentium*, *Escherichia coli* and *Salmonella enterica serovar Typhimurium*, to promote host pathogen clearance. Bacterial-derived LPS, specifically the lipid A moiety, binds caspase-11, as well as human orthologues caspase-4 and -5, leading to their activation and the cleavage of the pore-forming effector gasdermin D to induce pyroptotic cell death, independent of caspase-1 (Shi, Zhao et al. 2014). Caspase-11-GSDMD activation also allows potassium ion efflux to trigger NLRP3 inflammasome activation to generate bioactive IL-1β. Hence, like caspase-1, caspase-11 (and caspase-4 and caspase-5) cleave GSDMD and cause pyroptosis in innate immune cells such as macrophages and monocytes and non-myeloid cells such as endothelial, epithelial cells and keratinocytes (Knodler, Crowley et al. 2014).

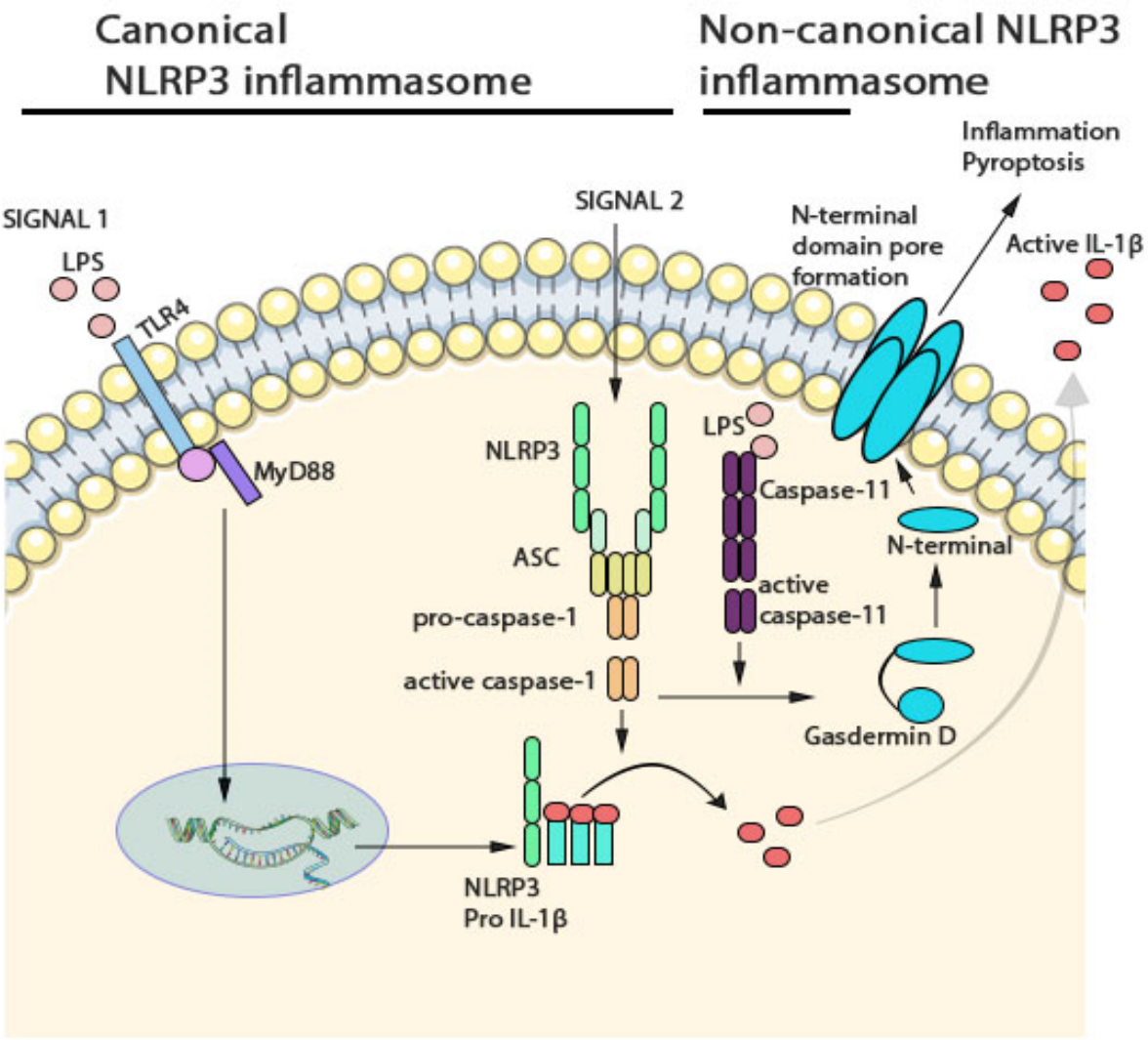


Figure 1.2 Activation of canonical and non-canonical NLRP3 inflammasome.

Canonical NLRP3 inflammasome requires signal 1 for TLR activation (shown LPS and TLR4 as an example), leading to NLRP3 and precursor IL-1β transcription and signal 2 (provided by DAMPs) to form inflammasome complex leading to caspase-1 activation and IL-1β secretion. Gram-negative bacteria (cytosolic LPS shown here) is detected by caspase-11 and leads to caspase-11 activation. Both active caspase-1 and caspase-11 can process gasdermin-D and the N-terminal fragment of gasdermin-D is released to cause pore formation, leading to pyroptosis.

1.2.5c Alternative inflammasome activation via TLR/TNFR1 signalling

Caspase-8 is an initiator caspase known to play an important role in orchestrating death-receptor superfamily-mediated extrinsic apoptosis. Death receptor activation leads to recruitment of caspase-8 into the Death-Inducing Signalling Complex (DISC). Caspase-8 homodimerisation in this complex then results in its activation and cleavage of the executioner caspase 3 and 7 leading to apoptosis (Feltham, Vince et al. 2017). The structure of caspase-8

contains two death effector domains (DEDs) at the amino terminal followed by p18 and p10 protease subunits at the carboxyl end (Gurung and Kanneganti 2015). Apart from activation of death receptors, pro-caspase-8 mediated apoptosis can also be initiated by inflammasomes. When BMDMs/BMDCs that lack caspase-1 were infected with "cytosolic" bacteria *Francisella novicida* upon DNA sensing, AIM2/ASC inflammasome complex activated caspase-8 and induced apoptosis (Pierini, Juruj et al. 2012, Pierini, Perret et al. 2013). Subsequently, it was also shown that *Francisella novicida* infection of *caspase-1*$^{-/-}$ BMDMs resulted in the activation of AIM2/ASC/caspase-8 non-canonical pathway that triggered cleavage and release of bioactive IL-18 (Pierini, Perret et al. 2013). This was significant, as although many groups had previously reported direct cleavage of IL-1β by caspase-8 (Maelfait, Vercammen et al. 2008, Vince, Wong et al. 2012), IL-18 cleavage by caspase-8 had yet to be documented (Pierini, Perret et al. 2013). Moreover, the IL-18 secretion mediated by caspase-8 was shown to be physiologically important as it was reported to drive the production of IFN-ϒ by *caspase-1*$^{-/-}$ NK cells to clear bacterial infection.

Apart from AIM2, other inflammasomes (NLRC4, NLRP1b and NLRP3) can also activate the caspase-8 non-canonical inflammasome pathway (Sagulenko, Thygesen et al. 2013, Chung, Vilaysane et al. 2016, Schneider, Gross et al. 2017, Van Opdenbosch, Van Gorp et al. 2017, Lee, Mirrashidi et al. 2018). BMDMs lacking caspase-1 electroporated with ultra-purified flagellin to induce NLRC4/NAIP5 activation died similar to wildtype cells though with slower kinetics and displayed chromatin condensation and an intact plasma membrane; hallmark features of apoptosis. Indeed, a genome-wide CRISPR/Cas9 screen in caspase-1$^{-/-}$ immortalized BMDMs (iBMDMs) infected with a lentivirus-based, single-guide RNA (sgRNA) expression library and then electroporated with flagellin revealed that *ASC* and *caspase-8* were the highest scoring genes in surviving cells supporting the hypothesis of an alternative apoptotic pathway downstream of NLRC4, in the absence of caspase-1.

The role of caspase-8 in caspase-1 independent NLRC4 activated BMDMs was confirmed genetically by using caspase-8$^{-/-}$RIPK3$^{-/-}$ cells (as caspase-8$^{-/-}$ animals are embryonic lethal). Moreover, this study also showed that caspase-1 does not compete with caspase-8 to bind to ASC, and NLRC4/caspase-8 apoptotic BMDMs undergo plasma membrane damage ultimately leading to secondary necrosis (Lee, Mirrashidi et al. 2018). Other independent reports published recently also support a caspase-1 independent ASC/caspase-8 mediated apoptosis non-canonical pathway upon NLRC4 activation (Van Opdenbosch, Van Gorp et al. 2017, Lee,

Mirrashidi et al. 2018). Interestingly, the other NLR, which like NLRC4 that can directly recruit caspase-1, NLRP1b, was also reported to induce an ASC/caspase-8 non-canonical pathway in response to Anthrax Lethal Toxin (LeTx) independent of caspase-1 (Van Opdenbosch, Van Gorp et al. 2017). In the same study, NLRP1b and NLRC4 induced caspase-8 apoptosis was inhibited when cells were TLR primed due to de novo synthesis of the known caspase-8 modulator, c-FLIP (Van Opdenbosch, Van Gorp et al. 2017). Similarly, ASC-dependent non-canonical NLRP3 inflammasome activation of caspase-8 was also reported when BMDMs were treated with bacterial pore-forming toxin nigericin in the absence of caspase-1 (Sagulenko, Thygesen et al. 2013, Schneider, Gross et al. 2017). Recently, NLRP3 non-canonical inflammasome caspase-8 activation was also reported in tubular epithelial cells (TECs) in response to non-canonical NLRP3 inflammasome stimuli (TNF-α /Cyclohexamide) and this may be due to lack of caspase-1 expression in TECs compared to BMDMs (Chung, Vilaysane et al. 2016). Interestingly, dectin-1 signalling also can activate the non-canonical caspase-8 inflammasome in addition to triggering canonical NLRP3 formation (Gringhuis, Kaptein et al. 2012, Ganesan, Rathinam et al. 2014). Thus, the interaction of inflammasome-associated ASC pyrin domains with both DED domains of caspase-8 can facilitate inflammasome responses in the absence of caspase-1 (Sagulenko, Thygesen et al. 2013, Vajjhala, Lu et al. 2015).

1.2.6. Mechanism of NLRP3 inflammasome activation

NLRP3 inflammasome activation requires two steps: Step 1 is called the priming step that usually occurs through a PAMP like LPS binding to its receptor (in this case Toll Like Receptor 4, TLR4) followed by activation of NF-κB transcription factor and transcription of NLRP3 and inactive precursor IL-1β and IL-18 (Hornung and Latz 2010, Guarda, Zenger et al. 2011). Step 2 is called the activation step that can occur through PAMPs or DAMPs (Mariathasan, Weiss et al. 2006, Hornung, Bauernfeind et al. 2008) which trigger inflammasome sensor protein activation, allowing formation of the inflammasome complex and proximity-induced activation of caspase-1 (Martinon and Tschopp 2004). Precursor IL-1β and IL-18 are cleaved by caspase-1 and to their bioactive fragments which are released from cells (Thornberry, Bull et al. 1992). Given the structural diversity of all the various stimuli involved in activating NLRP3 inflammasome (described in NLRP3 activation in health and disease), it's unlikely all these stimuli can directly bind to NLRP3 in order to activate it. Thus, it is hypothesized that there is a common mechanism underlying all these stimuli that is sensed by NLRP3. Some of

the well-studied mechanisms of NLRP3 activation and their recent updates are discussed in this section.

Cells under steady-state conditions have a high intracellular potassium (K+) concentration. However, upon loss of cell membrane integrity and K+ efflux, the NLRP3 inflammasome is activated (Petrilli, Papin et al. 2007). It is unclear if NLRP3 is a direct sensor of K+ efflux. Canonical stimuli of NLRP3 activation commonly used in lab experiments, ATP and nigericin, both involve K+ efflux to trigger NLRP3 signalling (Perregaux and Gabel 1994). In fact, Munoz-Planillo et al showed that K+ efflux is the common trigger involved in NLRP3 activation upon phagocytosis of particulate matter, ATP and bacterial toxins. Further, K+ efflux was reported as a specific trigger only for NLRP3 and not for AIM2 or NLRC4 inflammasome activation (Munoz-Planillo, Kuffa et al. 2013). NLRP3 inflammasome activation triggered by murine caspase-11 upon LPS transfection is also dependent on K+ efflux. Moreover, potassium efflux triggered by potassium-free medium is adequate to activate NLRP3 (Rivers-Auty and Brough 2015, Ruhl and Broz 2015, Schmid-Burgk, Gaidt et al. 2015).

Recently NEK7, a serine/threonine kinase that belongs to the Never in Mitosis gene-A (NIMA)-related kinase family and a regulator of microtubule dynamics was shown to interact with NLRP3. NEK7 interaction with NLRP3 was shown as a global requirement for NLRP3 activation in response to multiple key stimuli (including particulate matter, lysosome membrane damaging agents and pore-forming toxins) but was disposable for AIM2 and NLRC4 activation (Schmid-Burgk, Chauhan et al. 2016). Potassium efflux was not affected upon NEK7 deletion, however high extracellular KCl in the medium blocked the NEK7-NLRP3 interaction indicating that NEK7-NLRP3 interaction occurs downstream of potassium efflux. Interestingly, NLRP3 activation was weakened in NEK7-depleted macrophages containing a NLRP3 R258W mutation associated with Muckle wells syndrome, which was shown previously to activate NLRP3 in a potassium efflux independent manner (He, Zeng et al. 2016). Macrophages treated with ROS scavengers also reduced the NEK7-NLRP3 interaction and phosphorylation of NEK7 was required for its interaction with NLRP3 (Shi, Wang et al. 2016). MCC950, a NLRP3 inhibitor, was also suggested to reduce the NEK7 and NLRP3 interaction (Zhang, Lv et al. 2017). Thus, phosphorylated NEK7 interaction with NLRP3 is a key requirement for NLRP3 activation in potassium efflux dependent and independent pathways.

Dysfunctional mitochondria and Reactive Oxygen Species (ROS) generated from damaged mitochondria with reduced membrane potential are reported as upstream activators of the NLRP3 inflammasome. ROS scavengers are commonly utilized in most studies to study ROS involvement in NLRP3 activation, however ROS scavengers are chemical inhibitors that can be toxic and may have off target effects; therefore, it is quite controversial and not clear if ROS are a direct NLRP3 trigger (Juliana 2012, Munoz-Planillo, Kuffa et al. 2013, Won, Park et al. 2015).

Various mitochondrial factors such as cardiolipin, cytochrome C and mitochondrial DNA (mt DNA) are also implicated in NLRP3 inflammasome activation. Cardiolipin, located in the inner mitochondrial membrane (IMM) was shown to interact directly with the LRR domain of NLRP3 (Iyer, He et al. 2013). A recent study utilized the unique property of cardiolipin binding to annexin V and reported that upon LPS priming, cardiolipin translocates from the IMM to Outer Mitochondrial Membrane (OMM) in a ROS dependent manner (Elliott, Miller et al. 2018). Further, cardiolipin binding to NLRP3 was reported to result in higher molecular weight NLPR3 complex formation (Elliott, Miller et al. 2018).

Cytochrome C, an enzyme associated with Inner Mitochondrial Membrane (IMM) was also reported to interact with NLRP3 and the interaction of NLRP3 with cytochrome C interfered its binding with cardiolipin. Further cytochrome C treatment weakened NLRP3 activation and reduced NEK7 interaction with NLRP3 (Shi and Kehrl 2016). This suggests that cytochrome C release, which occurs during apoptotic cell death, may act to dampen NLRP3 signalling to reduce a cells inflammatory potential.

Shimada et al have suggested that oxidized mitochondrial DNA (mt DNA) released into the cytosol bound and activated NLRP3 preferentially upon ATP or nigericin treatment (Shimada, Crother et al. 2012). However, other mitochondrial proteins MAVS, Parkin and mitochondrial apoptosis mediated by BAK and BAX, are dispensable for NLRP3 activation (Allam, Lawlor et al. 2014).

NLRP3 is also reported to translocate to mitochondria in order to be activated (Subramanian, Natarajan et al. 2013). Consistent with previous studies, recently it was shown that upon LPS priming NLRP3 associated with outer mitochondrial membranes, with nigericin allowing ASC

to be recruited to NLRP3 at the OMM (Elliott, Miller et al. 2018). ROS inhibitor pre-treatment before priming reduced the recruitment of caspase-1 and NLRP3 to the mitochondrial fraction, suggesting ROS is required to trigger NLRP3 inflammasome activation. The role of potassium efflux in the translocation of NLRP3, caspase-1 and ASC to mitochondrial membranes has not been tested and therefore it is unclear if NLRP3 inflammasome protein association with the mitochondria is a K+ efflux dependent or independent process. However, other groups have challenged the translocation of NLRP3 to mitochondria (Bauernfeind, Bartok et al. 2011, Juliana 2012). These studies reported that upon NLRP3 activation stimuli, Mitochondria-associated endoplasmic reticulum membranes (MAMs) localised adjacent to Golgi membranes and that NLRP3 was recruited to these MAMs (Zhang, Meszaros et al. 2017). Golgi localised protein kinase D (PKD) was reported to phosphorylate NLRP3, releasing NLRP3 from MAMs and promoting NLRP3 inflammasome activation (Zhang, Meszaros et al. 2017). More recently, it was reported that NLRP3 stimuli induced the disassembly of the *trans*-Golgi network (TGN) into vesicles described as a dispersed TGN (dTGN) (Chen and Chen 2018). Phosphatidylinositol-4-phosphate (PtdIns4P) on dTGN was showed to recruit NLRP3 and dTGN thus was suggested to serve as a scaffold for NLRP3 aggregation, leading to ASC polymerisation and downstream signalling (Chen and Chen 2018). Recent co-localisation studies revealed that centrosome, an organelle that acts as microtubule-organizing center (MTOC) is the activation site of NLRP3. Targeted deletion of dynein adaptor, histone deacetylase-6 (HDAC6) *in vitro* and *in vivo* further revealed that HDAC6 was indispensable for NLRP3 inflammasome activation and may be involved in the microtubule transport of NLRP3 to the MTOC (Magupalli, Negro et al. 2020).

Apart from mitochondria, lysosomes destabilization is reported to trigger NLRP3 inflammasome (Hornung, Bauernfeind et al. 2008). Multiple studies showed, CA-074 Me commonly believed to be a specific inhibitor of cathepsin B, (a lysosomal enzyme) blocked IL-1beta secretion (Halle, Hornung et al. 2008, Dostert, Guarda et al. 2009). However, it was later showed CA-074 blocked multiple cathepsins and activity of cathepsin D to cleave cathepsin C is required for IL-1 beta secretion (Orlowski, Colbert et al. 2015). However, these studies have been called into question with data showing that cathepsin inhibitors, such as CA-074, are potent blockers of inflammasome priming and may therefore prevent NLRP3 signalling indirectly (Rashidi, Simpson et al. 2019).

Moreover, lysosomal destabilization along with mitochondrial damage, dependent on

mitochondrial ROS was shown to induce lysosomal membrane permeabilization (LMP) and NLRP3 activation (Heid, Keyel et al. 2013). However, high concentrations of KCl blocked both LMP and Mitochondrial Membrane Permeabilization (MMP), suggesting K+ efflux is the convergence point for NLRP3 inflammasome activation. Moreover, compromised cell membranes due to NLRP3 activation can increase cell permeability and lead to multiple ion fluxes. Indeed, studies reported Ca^{2+} influx may be involved in NLRP3 activation (Brough, Le Feuvre et al. 2003, Chu, Thomas et al. 2009, Murakami, Ockinger et al. 2012, Rossol, Pierer et al. 2012) although this has also been disputed (Munoz-Planillo, Kuffa et al. 2013). Thus K+ efflux seems to be the only unified, common mechanism of NLRP3 activation, triggered by various stimuli that researchers can agree on, although potassium efflux-independent NLRP3 triggers have been suggested (Gross, Mishra et al. 2016, Sanman, Qian et al. 2016).

1.3 The NLRP3 inflammasome in health

NLRP3 inflammasome activity is required for host-defense against pathogenic infections. Pathogens such as bacteria, virus and fungi and host-derived and environmental danger molecules can all cause NLRP3 activation. In this section I will discuss in detail the role of NLRP3 activation in protecting against these microbial pathogens and its activation by environmental stressors.

1.3.1 Bacterial activation of NLRP3

Both Gram-positive (such as *Staphylococcus aureus, Listeria monocytogenes*) and Gram-negative bacteria (enterohemorrhagic *Escherichia coli, Citrobacter rodentium*) can activate the NLRP3 inflammasome (Kim and Jo 2013). NLRP3 usually senses the fluctuations in the host environment caused by pathogens and is not activated by direct contact with microbial pathogens. For example, in response to the bacterial pore-forming toxin, nigericin, NLRP3 senses ion fluxes in order to get activated (Latz, Xiao et al. Jun 2013). A virulence factor utilized by *S. aureus* called Alpha Toxin (AT), which acts as a pore forming toxin at low concentrations also activate NLRP3 inflammasome via potassium flux (Craven, Gao et al. 2009). Typically, NLRP3 confers protection against pathogens and in some cases works in conjunction with other inflammasomes to clear pathogens. In a recent report, NLRP3 along with AIM2 expression was upregulated in dual influenza and Methicillin-Sensitive *S. aureus* (MSSA) infections and inhibition of NLRP3 by MCC950 in later stage (days 4 and 6 after influenza) of super infection reduced bacterial burden and inflammation (Robinson, Ramanan

et al. 2018). However, in *S. aureus* infection induced by AT, NLRP3 inflammasome activation fosters infection. In another study, it was demonstrated that the mechanism underlying the promotion of *S. aureus* infection was not dependent on IL-1β or IL-18 but through mitochondrial engagement and activation of caspase-1 and killing bacteria by phagosomal acidification (Cohen, Boland et al. 2018).

NLRP3 also plays an important role in *Mycobacterium tuberculosis* (*M.tb*)-induced immunity (Wawrocki and Druszczynska 2017). Both NLRP3 and ASC knockout mice are reported to be highly susceptible to *M.tb* infection (van de Veerdonk, Netea et al. 2011, Eklund, Welin et al. 2014, Lai, Meintjes et al. 2015). Virulent factors of *M.tb*, such as Zn-MetalloProtease (ZMP-1) were reported to inhibit NLRP3 inflammasome activation (Master, Rampini et al. 2008). Mice infected with ZMP1 deleted *M.tb* was reported to enhance IL-1β secretion and *M.tb* clearance in the lung. Other bacteria, such as such as *Streptococcus pneumoniae, Chlamycia pneumoniae, Salmonella typhimurium* are all recognized by NLRP3 and genetic studies have been implicated it in conferring host resistance to numerous bacterial infections (Menu and Vince 2011).

1.3.2 Viral activation of NLRP3

Sendai virus and Influenza A Virus (IAV) were the first two viruses shown to activate the NLRP3 inflammasome to cause IL-1β and IL-18 activation (Kanneganti, Body-Malapel et al. 2006, Ichinohe, Pang et al. 2010). Subsequently, a number of both RNA viruses (such as Encephalomyocarditis virus- ECMV, Vesicular Stomatitis virus – VSV, Measles virus – MV and Hepatitis C virus – HCV) and DNA viruses (such as herpesviruses, Modified vaccinia virus Ankara – MVA and Varicella- Zoster virus – VZV) were all shown to activate NLRP3 (Lupfer, Malik et al. 2015). Zika virus infection was also reported to activate NLRP3 inflammasome in human PBMCs and BMDCs (Wang, Li et al. 2018). Previously, SARS (severe acute respiratory syndrome) outbreak caused by coronavirus (CoV), named as SARS-CoV, was shown to target innate immune responses. The SARS-CoV open reading frame (ORF)8b was suggested to interact with LRR domain of NLRP3 to cause its activation (Shi, Nabar et al. 2019). However, ORF8b from the current pandemic caused by SARS-CoV-2 is highly divergent from previous SARS-CoV and activates NLRP3 inflammasome (Ratajczak, Bujko et al. 2020, Yuen, Ye et al. 2020). Further studies are required to understand the different

proinflammatory responses between SARS-CoV and SARS-CoV2 to understand its pathogenicity, and if NLRP3 signaling plays are role in disease.

In Influenza A Virus (IAV) infection, it was shown that Interferon regulatory factor 1 (IRF)-1 promoted NLRP3 inflammasome activity and cell death through transcriptional regulation of Z-DNA Binding Protein-1 (ZBP-1) (Kuriakose, Zheng et al. 2018). ZBP-1 was also previously reported as a regulator of inflammasome activation during IAV infection (Kesavardhana, Kuriakose et al. 2017). Though early NLPR3 activation has protective responses, excessive NLRP3 activation during IAV infection leads to severe lung injury and mortality. This phenomenon is well exhibited where juvenile mice (4-week old) in response to IAV infection with persistent NLRP3 activation (characterised with increased caspase-1 and IL-18 secretion) had severe disease compared to adult mice (8-10 weeks old). Further, it was reported administration of anti-CCR2 antibodies and depletion of monocytes thus decreasing interferon induced NLRP3 activation led to increased survival in juvenile mice in response to IAV infection (Coates, Staricha et al. 2018).

Neutrophils also play an important role, as in a recent report it was shown that following neutrophil depletion in mice infected with influenza X31 strain, IL-1β levels were reduced to control levels 24 hours post infection (Peiro, Patel et al. 2017). Further, a mouse homolog of neutrophil-derived antimicrobial peptide, mCRAMP that was present in the Bronchoalveolar lavage fluid (BALF) of mice after 24 hours of influenza infection was significantly reduced with neutrophil depletion. Analysis of all influenza-infected mice revealed that there was strong correlation between mCRAMP and neutrophil numbers as well as mCRAMP and IL-1β levels. Thus, mCRAMP from neutrophils can amplify macrophage NLRP3 activation and crosstalk between neutrophils and macrophages are important to confer protective immunity.

1.3.3 Fungal activation of NLRP3

Apart from bacterial and viral infections, NLRP3 inflammasome activation is also associated upon fungal pathogen recognition. *Microsporium canis* (*M.canis*), a fungus that causes tinea capitis, a cutaneous infection of the scalp, was reported to activate the NLRP3 inflammasome in human monocytes and murine DCs (Mao, Zhang et al. 2014). In the same study, Dectin-1, a C-type lectin receptor, was reported to regulate pro IL-1β expression and therefore IL-1β secretion in cells infected with *M.canis*. Also, Dectin-1/Syk- mediated release of ROS was

reported to be important for the secondary NLRP3 activation step. Thus dectin-1/Syk signalling pathway is involved in both the priming and activation step of the NLRP3 inflammasome. Consistently, humans with polymorphisms in dectin-1 resulting in defective surface expression of dectin-1 were reported to be more susceptible to *Candida albicans* (*C. albicans*) infection (Ferwerda, Ferwerda et al. 2009) and, interestingly, polymorphisms in NLRP3 are also associated with *C. albicans* infection in women (Lev-Sagie, Prus et al. 2009).

Recently, it was reported NLRP3 activation in B-lymphocytes stimulated with fungal antigens (CpG) secreted IgM antibodies via a mTOR pathway, but did not activate IL-1β (Ali, Dasari et al. 2017). Thus, NLRP3 has been demonstrated to regulate both innate and adaptive immunity in response to fungal infections. Fungal glucan, curdlan and Candida Secreted aspartic protease 2 and 6 (Sap 2 and 6) have both been demonstrated to activate the NLRP3 inflammasome (Pietrella, Pandey et al. 2013). *In vivo* studies have also shown both the IL-1 receptor and NLRP3 inflammasome components are important for clearing fungal pathogens (Hise, Tomalka et al. 2009).

1.3.4 Host derived molecules that can trigger NLRP3 activation

Damaged cells or dying cells release molecules that can trigger sterile NLRP3 inflammasome activation. These molecules, called DAMPs include extracellular ATP, monosodium urate (MSU) crystals and amyloid-β peptide (Mariathasan, Weiss et al. 2006, Martinon, Petrilli et al. 2006, Halle, Hornung et al. 2008, Zhou, Tardivel et al. 2010). In the case of sterile inflammation that results following tissue damage, host cytokines such as TNF, have been reported to sensitize cells (macrophages and DCs) for ATP-induced NLRP3 inflammasome activation (Franchi, Eigenbrod et al. 2009).

The canonical DAMP, ATP, induces NLRP3 inflammasome formation via the purinergic receptor P2X7 and decreasing intracellular K+. The detailed mechanisms of NLRP3 activation are discussed in the chapter 1.2.6. In liver transplant patients undergoing acute rejection, elevated levels of the P2X7 receptor were reported (Amores-Iniesta, Barbera-Cremades et al. 2017). *In vivo* studies have reported that the NLRP3 inflammasome is activated in allografts and NLRP3 is upregulated during graft rejection (Amores-Iniesta, Barbera-Cremades et al. 2017). Thus, NLRP3 or P2X7 inhibitors may reduce inflammation and aid transplant tolerance.

Advanced Glycation End products (AGEs) that are glycated proteins, nucleic acids and lipids formed due to elevated glucose levels in the case of chronic diseases such as diabetes, atherosclerosis and chronic kidney disease can lead to elevated NLRP3 and IL-1β expression and induce both pancreatic β cell death and renal injury *in vivo*, possibly suggesting NLRP3 inflammasome activation (Kong, Lu et al. 2017, Yeh, Yang et al. 2017). However, another recent report, on the contrary, showed AGEs dampen NLRP3 inflammasome activation specifically in BMDMs and THP-1 cells (Son, Hwang et al. 2017). These differences could be due to the difference in cell types or in the preparation of AGEs. Further studies are required to understand the role of AGEs in NLRP3 inflammasome activation.

Dietary metabolites that accumulate due to poor lifestyle choices of high fat or sugar diet leads to chronic inflammation and activation of NLRP3 inflammasome and IL-1β secretion (Camell, Goldberg et al. 2015). Chronic diseases such as atherosclerosis and gout involve NLRP3 inflammasome activation via fatty acids, cholesterol crystals and MSU crystals respectively (addressed in detail in NLRP3 inflammasome disease related section, Chapter 1.4). Recently, it was reported that a ketone body, β-hydroxybutyrate, usually produced as an ATP source during starvation conditions can inhibit NLRP3 inflammasome activation (Youm, Nguyen et al. 2015).

1.3.5 Environmental stimuli causing NLRP3 activation

Environmental stimuli such as crystals of silica, aluminum, asbestos, nano-titanium dioxide (TiO_2) and silica dioxide (SiO_2) are reported to activate the NLRP3 inflammasome (Hornung, Bauernfeind et al. 2008, Baron, Gombault et al. 2015, Dostert, Pétrilli et al. May 2008). Both Alum formulation and nanoparticles are commonly used as vaccine adjuvants. However, not all vaccine delivery systems, only positively charged particles, activate NLRP3 (Neumann, Burkert et al. Jul 2014). In human keratinocytes, ultraviolet radiation induces NLRP3 inflammasome activation by Ca^{2+} mobilization (Awad, Assrawi et al. 2018). Also, NLRP3 inflammasome activation is reported to be triggered by air pollutants from diesel exhaust, cigarette smoke, volcanic ash and fine particulate matter extracts from water (Bauer, Diaz-Sanchez et al. 2012, Damby, Horwell et al. 2017). These particulate matters are all likely to be taking up into cells by phagocytosis where they accumulate in lysosomes and are unable to be broken down efficiently. Consequently, this results in lysosome membrane damage and potassium ion efflux to induce NLRP3 signaling (Rashidi, Wicks et al. 2020).

1.4 The NLRP3 inflammasome in Disease

Cyropyrin- Associate Periodic Syndromes (CAPSs, also known as cryopyrinopathies) are a group of rare autoinflammatory disorders that occur due to gain-of-function mutations in NLRP3 (also known as cryopyrin, PYPAF1, PYRIN-containing APAF1-like protein or CIAS1) (Kuemmerle-Deschner 2015). In Australia, the prevalence of CAPS is estimated to be 1 case in a million adults (Mehr, Allen et al. 2016). CAPS represent a continuum of three inflammatory disease conditions; namely Familial Cold Autoinflammatory Syndrome (FCAS), Muckle-Wells Syndrome (MWS) and Neonatal-Onset Multisystem Inflammatory Disease (NOMID) or Chronic Infantile Neurological Cutaneous and Articular Syndrome (CINCA) (Ozkurede and Franchi 2012). While FCAS and MWS (reported first in 1940 and 1962 respectively) are the mild-moderate disease phenotype that occurs commonly due to hereditary autosomal dominant mutations in familial cases, NOMID described first in 1981 is a moderate-severe phenotype that is usually observed due to *de novo* NLRP3 mutations (Hoffman, Mueller et al. 2001, Aksentijevich, Nowak et al. 2002). Gain-of-function mutations mostly observed in the NACHT domain of NLRP3 drive aberrant inflammasome activation and therefore results in spontaneous, excessive, unregulated release of IL-1β resulting in raised inflammatory markers (namely C-reactive protein; CRP and serum amyloid A; SAA) in blood serum of CAPS patients (Carta, Penco et al. 2015). Other key clinical features of CAPS patients range from mild symptoms like urticarial-like rash, fever, joint pain to arthritis, sensorineural hearing loss, chronic aseptic meningitis and skeletal abnormalities (epiphyseal overgrowth/frontal bossing) in the most severe cases (Hawkins, Lachmann et al. 2004).

Since excessive IL-1β secretion is the major contributor for CAPS pathogenesis, blocking IL-1β secretion is the current therapy for CAPS (Eskola, Pohjankoski et al. 2018). IL-1β blocking agents used in the treatment of CAPS include Anakinra, recombinant IL-1 receptor antagonist, canakinumab, a monoclonal antibody against IL-1β and Rilonacept, engineered IL-1β trap (Landmann and Walker 2017).

Though IL-1β is currently targeted, the ideal aim is to target the NLRP3 inflammasome directly for CAPS treatment so that the immunosuppressive effects of inhibiting IL-1 produced from other inflammasome sensor proteins can be avoided. Pfizer recently developed MCC950 (formerly known as CP-456773 or CRID3), a NLRP3 inflammasome inhibitor. MCC950 was shown to improve the survival of NLRP3-mutant mice and when PBMCs from patients with

MWS were pretreated with MCC950, processing of both IL-1β and caspase-1 was inhibited (Coll, Robertson et al. 2015). Apart from treatment of CAPS, MCC950 has been recently reported to have beneficial effects in animal models of diabetic encephalopathy and traumatic brain injury (Ismael, Nasoohi et al. 2018, Zhai, Meng et al. 2018). Recently, another β-sulfonyl nitrile compound known as OLT1177 was shown to selectively inhibit NLRP3 inflammasome, reduce IL-1β levels compared to MCC950 and safe to be used in treating humans (Marchetti, Swartzwelter et al. Jan 2018). Thus, both MCC950 and OLT1177 seem to be promising drugs to treat autoinflammatory diseases.

1.4.1 Gout

Gout is a common inflammatory arthropathy and is mostly present in men and menopausal women (Doherty 2009). Currently, the region with highest prevalence of gout in the world is Taiwan (9.5% men and 3% female) followed by New Zealand including Pacific islands and Australia (Pascart and Liote 2018). Hyperuricemia defined as serum urate concentration of equal to or higher than 6.8mg/dl (416 μmol/l) leads to monosodium urate (MSU) crystal formation and causing gout (Sattui and Gaffo 2016). MSU crystals are demonstrated to activate the NLRP3 inflammasome in macrophages and neutrophils (Martinon, Petrilli et al. 2006). However, neutrophils recruited to the site of inflammation are reported to form neutrophil extracellular traps (NETs) that can degrade pro- inflammatory cytokines (Schauer, Janko et al. 2014). Thus, neutrophils are important for both the initial inflammatory phase and the resolution phase of gout disease.

MSU crystals have been proposed to trigger necroptosis in some cell types (Mulay, Desai et al. 2016), although not in macrophages (Rashidi, Simpson et al. 2019). It could be the DAMPS from necroptosis can activate the NLRP3 inflammasome. However, it's been reported that active-MLKL, independent of DAMP release, can trigger the NLRP3 inflammasome (Conos, Chen et al. 2017). Further investigations are required to understand the combined contribution of these pathways in releasing inflammatory mediators in gout patients. Genetic studies showed associations between gout and functional variants in *CARD8* gene, a potential negative regulator of the NLRP3 inflammasome and TLR4 (Qing, Zhou et al. 2013, McKinney, Stamp et al. 2015), reinstating the importance of NLRP3 inflammasome role in the development of gout. Thus, targeting NLRP3 inflammasome activity is one potential new strategy in the treatment of gout.

Colchicine that is currently administered in low dose to manage active gout has been demonstrated to block MSU crystal-mediated NLRP3 activation (Martinon, Petrilli et al. 2006, Graf, Whittle et al. 2015). IL-1 inhibitors canakinumab and anakinra are also used in the treatment of acute gout symptoms commonly in patients who are non-respondents to conventional treatments such as NSAIDs or colchicine. Canakinumab treatment has been demonstrated to reduce gout flares and lowering serum urate levels in clinical trials, however efficacy of canakinumab in gout patients with cardiovascular or renal diseases is yet to investigated (Dumusc and So 2015).

1.4.2 Alzheimer's disease (AD)

Alzheimer's disease (AD) is a common form of dementia and is the major contributing factor for disability in Australian aged 65 years or over (Annear 2018). Accumulation of amyloid-beta (Aβ) plaques and hyperphosphorylated microtubule-associated protein tau in the brain is the hallmark of AD disease. Recently, neuroinflammation has been associated with several neurodegenerative diseases including AD (Cribbs, Berchtold et al. 2012).

In vitro studies have revealed NLRP3 inflammasome activation by Aβ peptides (Halle, Hornung et al. 2008). Also, NLRP3 knockout mice with familial AD associated mutations showed less deposition of Aβ plaques and protection from spatial memory impairment. NLRP3 inflammasome activation by Aβ plaques in microglial cells (the tissue resident macrophages of brain) leads to secretion of IL-1β and IL-18, neuronal cell death, release of DAMPs and further activation of the NLRP3 inflammasome (Heneka, Kummer et al. 2013). These results are consistent with increased expression of IL-1β reported in the brains of patients with chronic neurodegenerative diseases (Shaftel, Griffin et al. 2008). Interestingly, overexpression of antioxidant defense enzyme peroxiredoxin 3 reduced the levels of both caspase-1 and IL-1β suggesting an important role for NLRP3 inflammasome in AD disease pathogenesis (Chen, Na et al. Sep 2015). Recent studies have also implicated dysfunctional autophagy and lysosomal pathways along with NLRP3 inflammasome activation implicated in hippocampal neuronal cell death and impairment in learning and memory (Ahmed, Iyer et al. 2017, Wang, Zhang et al. 2017).

HT (3,4-dihydroxyphenylethanol), an anti-oxidant and natural ingredient of olive oil administered to AD transgenic mice for six months showed reduced neuroinflammation and increased cognitive function. Interestingly, NLRP3 inflammasome activation was significantly decreased upon treatment with HT (Peng, Hou et al. 2016). In humans, study of immune related gene expression study in healthy aging and AD patients revealed upregulated caspase-1 expression in both cases, implicating the NLRP3 inflammasome activation with aging in general (Heneka, Kummer et al. 2013). Interestingly, caspase-1 and NLRP3 inhibition or loss has been reported to provide therapeutic benefits effects in mouse AD models (Rashidi, Wicks et al. 2020). Therefore, a further understanding of the activation and inhibitory pathways of the NLRP3 inflammasome in the brain and determining key targets could unravel new therapeutic approaches for AD.

1.4.3 Atherosclerosis

Atherosclerosis is chronic vascular inflammatory condition, characterised by the formation of plaques (composed of lipids and inflammatory cells) on arteries. Atherosclerosis is the underlying condition of cardiovascular diseases and a common complication seen in diabetic patients. Cholesterol crystals, a common component of atherosclerotic lesions, were reported to activate NLRP3 inflammasome *in vitro* and *in vivo* (Duewell, Kono et al. 2010). This study also utilized lethally irradiated Low-Density Lipoprotein Receptor $(LDLR)^{-/-}$ mice reconstituted with bone marrow lacking NLRP3 inflammasome components fed with high fat diet to study the effect of NLRP3 inflammasome in chronic inflammation. All mice reconstituted with bone marrow lacking NLRP3 inflammasome components reported reduced early atherosclerosis. In particular $LDLR^{-/-}$ mice with reconstituted bone marrow that lack IL-1β were protected from high fat diet induced atherosclerosis. Caspase-1 was also reported to be important for the development of atherosclerosis in mice by different groups (Hendrikx, Jeurissen et al. 2015, Usui, Shirasuna et al. Oct 2014). Thus, pre-clinical data of the role of IL-1β in atherosclerosis led to the development of a large clinical trial using IL-1β blockade in cardiovascular disease patients, which will be discussed later in a separate section (See CANTOS trial, section 1.4.6).

Apart from macrophages, vascular endothelial cells play an important role in the pathogenesis of atherosclerosis. Indeed, NLRP3 inflammasome activation was reported in response to oxidative stress induction by atheroprone oscillatory shear flow in endothelial cells (Xiao, Lu

et al. Aug 2013). NLRP3, IL-1β mRNA expression and related inflammasome machinery were reported to be higher in plaques of myocardial patients (Paramel Varghese, Folkersen et al. 2016). This study also showed human carotid plaques stimulated with cholesterol crystals even in the absence of TLR4 priming secreted significant levels of IL-1β. This could be due to the increased mRNA expression of NLRP3 and IL-1β in the plaques at steady state conditions. Thus, NLRP3 inflammasome components could serve as potential biomarkers for early atherosclerosis and further investigations are required to understand the exact mechanism of NLRP3 activation in this disease for designing new therapeutic interventions.

1.4.4 Obesity

Obesity affects about 1.2 million people including children in Australia according to the Australian Bureau of Statistics (ABS). Three major factors, namely; high calorie food intake, lack of physical activity and environmental factors (such as unhealthy microbiome, stress, etc.) attribute to the rising level of obesity in today's society (Ravussin and Ryan 2018). Obesity is the major risk factor of many diseases including cardiovascular diseases, type II diabetes and stroke (Rovella, Anemona et al. 2018). Chronic low-grade inflammation is one of the hallmarks of obesity and macrophage infiltration and activation in white adipose tissue (WAT) are positively correlated with adipocyte tissue expansion in humans and mice (Xu, Barnes et al. 2003, Cinti, Mitchell et al. 2005). Saturated fatty acids (SFA) including palmitate and stearate were reported to activate the NLRP3 inflammasome, while the converse effect of NLRP3 inactivation was reported with unsaturated fatty acids (UFA) oleate and linoleate (L'Homme, Esser et al. 2013). Ceramide, a lipid metabolite deposited in tissues of obese patients, has also been shown to activate NLRP3 (Vandanmagsar, Youm et al. 2011). Indeed, reduction of ceramide in skeletal muscle is reported to improve insulin sensitivity and may help obese Type II diabetic patients in controlling their blood glucose levels (Aburasayn, Al Batran et al. Jul 2016). Whether targeting IL-1 and/or NLRP3 might decrease disease-risk in obese patients remains to be determined.

1.4.5 Cancer

The NLRP3 inflammasome activation has been implicated with various inflammation-induced cancers. *Helicobacter pylori* (H. *pylori*), a Gram-negative bacterial infection, causes chronic inflammation and is a major risk factor for gastric cancer (Salama, Hartung et al. 2013). NLRP3

inflammasome activation was reported in response to H. *pylori* in with spontaneous IL-1β secretion in human PBMCs and conventional IL-1β secretion dependent on LPS priming (Semper, Mejias-Luque et al. 2014). H. *pylori* was reported to regulate NLRP3 expression through a NLRP3- targeting 3' arm miRNA hsa-miR-223-3p. However, the same study reported that unlike human PBMCs, THP-1 monocytes do not secrete IL-1β in response to H. *pylori* indicating cell-specific inflammasome regulatory mechanisms (Pachathundikandi and Backert 2018). In a gene expression study NLRP3 was not significantly regulated in H. *pylori* infected cells, though there were strong associations between NLRP3 polymorphisms and H. *pylori* infection (Castano-Rodriguez, Kaakoush et al. 2014). Polymorphisms of IL-1β gene is associated with increased risk of gastric carcinoma in H. *pylori* infected patients and IL-1β is involved in the pathogenesis of the disease (Khazim, Azulay et al. 2018). Antagonism of IL-1 receptor signaling in IL-1β transgenic mouse model infected with *Helicobacter* infection resulted in improved gastric pathology (Tu, Bhagat et al. 2008).

Epstein-Barr virus (EBV) infection in a risk factor associated with cancers that include Hodgkin's lymphoma, Burkitt's lymphoma and nasopharyngeal carcinoma (NPC). Both NLRP3 and IL-1β expression were upregulated in NPC tumor tissues. This study also showed in NPC patients, up regulation of NLRP3 and AIM2 was strongly correlated with local recurrence-free survival, with no tumor recurrence for 5 years after treatment (Chen, Wang et al. 2012), providing anti-tumor effects. Like most viruses that inhibit inflammasome activation, EBV was reported to inhibit NLRP3 inflammasome activation via a miRNA that binds to the 3'-untranslated region of NLRP3 (Haneklaus, Gerlic et al. 2012). However, In a recent study, EBV-infected cells did not show upregulation of NLRP3, and the AIM2 inflammasome was reported to the key inflammasome activated (Torii, Kawada et al. 2017).

1.4.5a Colitis and colon cancer

Ulcerative colitis (UC), a chronic inflammatory condition that is commonly classified as Inflammatory Bowel Disease (IBD) is a major risk factor for developing colorectal cancer, with 18% of UC patients estimated to develop cancer in 30 years (Lakatos and Lakatos 2008). NLRP3, ASC and caspase-1 deficient mice are reported to be highly susceptible to colitis-associated cancer (CAC) induced by the azoxymethane, a mutagenic agent/dextran sulfate sodium, a proinflammatory chemical (AOM/DSS) mice model (Allen, TeKippe et al. 2010). However, the role of NLRP3 in the AOM/DSS model is controversial with some studies

reporting that a loss of NLRP3 results in mice resistant, or sensitive, to colitis upon the induction of AOM/DSS (Bauer, Duewell et al. 2010, Kantono and Guo 2017). These differences could be attributed because of the distinct microbiota of mice maintained in different animal facilities. Further studies are required to understand the role of NLRP3 in CAC.

1.4.5b Melanoma

Melanoma is a type of skin cancer with the highest incidences reported in Australia and New Zealand in the world (Sneyd and Cox Aug 2013). In late-stage human melanoma cells, there is spontaneous NLRP3 inflammasome activation and IL-1β secretion without a clear activation signal, which is usually required for NLRP3 triggering in human monocytes. On the contrary, in the early stage of disease, both IL-1 receptor and co-stimulation with Muramyl Dipeptide (MDP) was required for NLRP3 activation and IL-1β secretion (Okamoto, Liu et al. 2010). Genetic studies showed NLRP3 polymorphisms are positively correlated with risk of developing melanoma. The SNP rs35829419 (Q705K) in particular was shown to be a major risk factor for developing sporadic Malignant Melanoma (MM) in Swedish males (Verma, Sarndahl et al. Apr 2012). In *in vitro* studies, SNP rs35829419 of NLRP3 was reported to provide gain-of-function response including spontaneous IL-1β secretion (Verma, Bivik et al. 2012).

1.4.5c Leukemia

Apart from solid tumors, NLRP3 is inferred to play a role in various types of leukemia. Adult T-cell leukemia (ATL) and chronic inflammatory disorders like uveitis is caused by Human T-cell Lymphotropic Virus type-1 (HTLV-1) (Arisawa, Soda et al. 2006). NLRP3 polymorphisms with the presence of SNP rs10754558 G allele, previously reported to increase NLRP3 mRNA expression, conferred protection against HTLV-1 infection (Kamada, Pontillo et al. 2014). P2X7, a receptor that induces NLRP3 inflammasome activation in response to ATP stimulation, is overexpressed in CLL lymphocytes (Cabrini, Falzoni et al. 2005). However, NLRP3 is down regulated in mRNA and at the protein level and promotes cell growth in CLL lymphocytes (Salaro, Rambaldi et al. 2016). Reducing NLRP3 expression could be one of the strategies that tumor cells use to make cells more susceptible to malignant transformation, as cells with lower NLRP3 expression will not be able to sense danger signals

in their environment. This is consistent with independent reports that showed both NLRP3 and IL-1β mRNA was decreased in CML patients and human hepatocellular carcinoma (Wei, Mu et al. 2014, Salaro, Rambaldi et al. 2016).

1.4.6 CANTOS trial

Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) is a large clinical study involving 10,061 patients with history of myocardial infarction and C-reactive protein (hsCRP) with equal to or more than 2mg/L (Ridker, Everett et al. 2017). As the name of the study suggests, Canakinumab, a fully human IL-1β monoclonal antibody was tested in cardiovascular patients and its anti-inflammatory responses were evaluated. Three doses (50mg, 150mg and 300 mg) of canakinumab were administered subcutaneously every 3 months. The results of this study recently published showed that 150mg dose every 3 months significantly reduced the recurrent cardiovascular in patients independent of their cholesterol levels. Anti-inflammatory effects evaluated after 3.7 years showed lowering pro-inflammatory mediators such as CRP and IL-6 concentrations of 26-41% and 25-43 % respectively. Though no effect of IL-1β blockade therapy was reported on lowering patient glucose levels. Despite exclusions of patients with chronic infections, canakinumab treatment was associated with higher incidences of fatal infection and sepsis. Additionally, IL-1β blockade was reported to reduce adverse cardiovascular event rates in patients with chronic kidney disease (CKD) and categorised as high-risk for atherosclerosis with no adverse renal effects (Ridker, Macfadyen et al. 2018).

Further analysis conducted from CANTOS trial revealed that canakinumab treatment significantly reduced the incidences of lung cancer and related mortality (about 77%). The lower incidence of lung cancer was reported in both 150mg and 300 mg dose treatment groups (Baylis, Gomez et al. 2017). These anti-tumor effects of canakinumab could be due to lowering the inflammatory environment associated with the development of new cancers. Canakinumab treatment therefore may not be effective to treat late-stage cancers or metastatic cases. A formal new clinical trial is required to address the effectiveness of canakinumab in lung cancer.

1.5 Proinflammatory cytokines

1.5.1 Interleukin-1β

The IL-1 family contains both pro-inflammatory and anti-inflammatory cytokines and comprises of 11 members (Dunn, Sims et al. 2001, Sims, Nicklin et al. 2001). Since the focus of this thesis is the pro-inflammatory cytokine IL-1β, the following section provides a brief overview of some of the key pro-inflammatory cytokines involved in the innate immune system, starting with IL-1β in this section.

Activated IL-1β binds the ubiquitously expressed IL-1 receptor type 1 (IL-1R1) (Vigers, Anderson et al. 1997). Apart from IL-1β, IL-1α can also independently bind to IL-1R1 (Schreuder, Tardif et al. 1997). Hence, the term IL-1 signalling refers to both IL-1α and IL-1β. As for most IL-1 superfamily receptors, IL-1R1 includes three immunoglobin like domains in their ectodomains and an intracellular domain with similar homology to members of the TLR family, known as the Toll-like/IL-1R (TIR) domain (O'Neill 2008). Upon IL-1β binding to IL-1R1, a ligand-induced conformational change induces the recruitment of the IL-1 receptor accessory protein (IL-1RAcP) as a co-receptor, which results in the dimerization of the TIR domains from both IL-1RAcP and IL-1R (Schreuder, Tardif et al. 1997). The IL-1-IL-1R1-IL-1RAcP ternary complex initiates intracellular signalling by recruitment of adaptor molecules, including myeloid differentiation factor 88 (MYD88) and IL-1R-associated kinases (IRAK)(Dunne and O'Neill 2003). Autophosphorylation of IRAK-4 leads to subsequent phosphorylation of IRAK1 and IRAK2 and recruitment of Tumor Necrosis Factor-associated factor 6 (TRAF 6)(Kawagoe, Sato et al. 2008) (Cao, Xiong et al. 1996). IRAK1, IRAK2 and TRAF6 functions as adaptor molecules to activate NF-kB, as well as mitogen-activated protein kinases (MAPKs) (Walsh, Kim et al. 2008). This ultimately results in an inflammatory response by increasing gene expression of IL-1 responsive genes (such as IL-6, IL-8, IL-1α, IL-1β)(Weber, Wasiliew et al. 2010). Both in mice and human studies, the absence of MyD88 or IRAK4 and mutations in IRAK4 alone leads to defective IL-1 signalling implying an essential role of these adaptor proteins in the IL-1 signalling pathway (Adachi, Kawai et al. 1998, Picard, Puel et al. 2003). Termination of IL-1 signalling is transient and conserved. IL-1R interacts with Toll-interacting protein (TOLLIP), which inhibits IRAK1, and targets IL-1R1 to endosomes for its degradation (Burns, Clatworthy et al. 2000). Also, activation of

several negative-feedback inhibitors results in the termination of the IL-1 signalling pathway (Kobayashi, Hernandez et al. 2002, Burns, Janssens et al. 2003, Rao, Nguyen et al. 2005).

1.5.2 interleukin-18

IL-18 was firstly termed as "IFN-γ inducing factor" in 1989 when isolated from mouse serum following intraperitoneal injection of endotoxin (Nakamura, Okamura et al. 1989). Later, upon purification and sequencing, it was defined as a proinflammatory cytokine belonging to the IL-1 family (Okamura, Nagata et al. 1995). Similar to IL-1β, IL-18 is synthesised as an inactive protein form of 24 KDa (Dinarello 2018). However, unlike IL-1β, it is reported that the IL-18 precursor form is constitutively expressed in disease free conditions in most cells including macrophages and dendritic cells in humans and mice (Chen, Li et al. 2013).

IL-18 signalling is initiated by active IL-18 binding to its primary receptor, IL-18 receptor α chain (IL-18Rα). This complex then binds to the co-receptor, IL-18 receptor β chain (IL-18Rβ) to induce downstream signalling (Kim, Reznikov et al. 2001). X-ray crystallography studies have shown structural similarities between IL-1R1 complex and IL-18 receptor complex bound to its co-receptor (Tsutsumi, Kimura et al. 2014). Downstream signalling of IL-18 is also identical to IL-1 signalling and involves the dimerization of TIR domains, recruitment of adaptor proteins MyD88, IRAKs and TRAF6, leading to activation of NFkB and MAPK signalling (Weber, Wasiliew et al. 2010).

The IL-18 receptor is mainly expressed in haematopoietic cells such as T cells and mast cells, although the highest expression of IL-18 receptor is found in NK cells (Nakamura, Otani et al. 2000). In the presence of IL-12 or IL-15, IL-18 was reported to activate NK cells and induce the production of IFNγ resulting in a Th1 response and host defense against intracellular pathogens (Chaix, Tessmer et al. 2008). Recently, non-immune cells such as nerve cells or epithelial cells have also been shown to express the IL-18 receptor (Nakanishi 2018). Independently, IL-18 is reported to induce IL-4 and IL-13, driving a Th2 immune response (Yoshimoto, Tsutsui et al. 1999). Also, IL-18 has been shown to induce the death ligands, TNF-α and FasL, which can cause apoptotic cell death (Tsutsui, Kayagaki et al. 1999, Zhang, Hile et al. 2011). Regulation of IL-18 is dependent on levels of IL-18 binding protein (IL-18BP), which binds to IL-18 and inhibits its ability to signal (Kim, Eisenstein et al. 2000) (Dinarello,

Novick et al. 2013). Recent studies have reported IL-37, an anti-inflammatory cytokine also acts as a negative regulator that binds to IL-18Ra and inhibits downstream IL-18 signalling (Nold-Petry, Lo et al. 2015).

1.5.3 IL-1β blocking therapies

In alignment with the focus of this thesis on IL-1β, the following section illustrates the significance of IL-1β activity, by describing current treatments and clinical trials involving blocking IL-1β. Readers are referred to other literature highlighting distinct conditions alleviated by blocking other cytokines such as TNF-α (Rider, Carmi et al. 2016, Choy, De Benedetti et al. 2020).

Currently, IL-1β blocking is used in the treatment of CAPS. CAPS are a group of rare auto-inflammatory disorders that occur due to gain-of-function mutations in NLRP3 (also known as cryopyrin, PYPAF1, PYRIN-containing APAF1-like protein or CIAS1), the gene that codes for the cyropyrin protein (Cuisset, Jeru et al. 2011). Gain-of-function mutations mostly observed in the NACHT domain of NLRP3 drive aberrant inflammasome activation and therefore results in spontaneous, excessive, unregulated release of IL-1β resulting in raised inflammatory markers (namely C-reactive protein; CRP and serum amyloid A; SAA) in blood serum of CAPS patients (Lachmann, Lowe et al. 2009). Other key clinical features of CAPS patients range from mild symptoms like urticarial-like rash, fever, joint pain to arthritis, sensorineural hearing loss, chronic aseptic meningitis, skeletal abnormalities (epiphyseal overgrowth/frontal bossing) in the most severe cases (Neven, Prieur et al. 2008).

In Australia, the prevalence of CAPS is estimated to be 1 case in a million adults (Mehr, Allen et al. 2016). CAPS represent a continuum of three inflammatory disease conditions; namely Familial Cold Autoinflammatory Syndrome (FCAS), Muckle-Wells Syndrome (MWS) and Neonatal-Onset Multisystem Inflammatory Disease (NOMID) or Chronic Infantile Neurological Cutaneous and Articular Syndrome (CINCA) (Aksentijevich 2007, Levy, Gerard et al. 2015). FCAS and MWS (reported first in 1940 and 1962 respectively) are mild-moderate disease phenotype that occurs commonly due to hereditary autosomal dominant mutations. NOMID, described first in 1981, results in a more severe phenotype that is usually observed due to *de novo* NLRP3 mutations (Muckle and Wellsm 1962, Prieur and Griscelli 1981,

Hoffman, Wanderer et al. 2001). Recently (from 1st June 2015), Anakinra, recombinant IL-1 receptor antagonist was included on Pharmaceutical Benefits Scheme (PBS) making it widely available for the treatment of CAPS (Mehr, Allen et al. 2016).

Another chronic inflammatory disease, Systemic onset Juvenile Idiopathic Arthritis (SJIA), a severe form of juvenile idiopathic arthritis involves significant inflammation. Several reports showed that Anakinra treatment of SIJA patients refractory to steroid and TNF blockers resulted in increased efficacy (Pascual, Allantaz et al. 2005). Subsequently, Canakinumab was approved for treatment in SIJA patients (Ruperto, Quartier et al. 2012). Similarly, Canakinumab is also used in the treatment of Rheumatoid arthritis and gout patients who are non-respondents to TNF-α blockers, NonSteroidal Anti-Inflammatory Drugs (NSAIDs) and colchicine (Alten, Gomez-Reino et al. 2011, So and Martinon 2017).

The therapeutic efficacy of Canakinumab has been investigated in clinical trials for various other conditions. The largest reported clinical study of blocking IL-1β is Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) involving 10,061 patients with a history of myocardial infarction. Results showed that a 150 mg dose of Canakinumab every 3 months significantly reduced the recurrent cardiovascular in patients independent of their cholesterol levels. Anti-inflammatory effects evaluated after 3.7 years showed a lowering of pro-inflammatory mediators such as CRP and IL-6 concentrations of 26-41% and 25-43% respectively. Though no effect of IL-1β blockade therapy was reported on lowering patient glucose levels, consistent with reductions in CRP levels IL-1β blockade was effective in chronic kidney disease. Notably, despite the exclusion of patients with chronic infections, canakinumab treatment was associated with higher incidences of fatal infection and sepsis (Ridker, Everett et al. 2017).

Interestingly, this study also reported cancer mortality was significantly lower in patients treated with canakinumab. In the additional analysis conducted from this study revealed that canakinumab treatment significantly reduced the incidences of lung cancer and related mortality (about 77%) (Ridker, MacFadyen et al. Oct 2017). These anti-tumor effects of canakinumab could be due to lowering the inflammatory environment associated with the development of new cancers. Canakinumab treatment therefore may not be effective to treat

late-stage cancers or metastasis cases. A formal new clinical trial is therefore required to address the effectiveness and safety of canakinumab in lung cancer.

1.6 Molecular mechanism of IL-1β secretion

1.6.1 Passive secretion of IL-1β secretion in macrophages

IL-1β release is described as a passive event and a consequence of pyroptosis at the site of inflammation in the literature (Brough and Rothwell 2007, Liu, Yamaguchi et al. 2014, Shirasaki, Yamagishi et al. 2014).

Recently, it was reported Gasdermin-D cleaved by caspase-11, -4, -5 and -1 causes pyroptosis by forming pores in the plasma membrane, and that this also enables IL-1β release (Kayagaki, Stowe et al. 2015). Proteolytic cleavage of Gasdermin-D by caspases results in the N-terminal fragment binding to inner membrane lipids and forming pores in the range of 10-15nm in diameter. These pores facilitate the release of mature IL-1β (of 4.5nm in diameter), as well as allowing water and ions to enter the cells, resulting in increased cell volume and pore size. Eventually, most cytoplasmic contents are released into the extracellular environment (Shi, Zhao et al. 2015, Aglietti, Estevez et al. 2016, Ding, Wang et al. 2016). Similar to gasdermin-D, the death effector MLKL can damage the plasma membrane resulting in altered ion homeostasis and leading to another form of cell death termed necroptosis (Chen, He et al. 2016). MLKL activation was shown to result in potassium ion efflux and NLRP3 activation leading to IL-1β release. Further, it was reported MLKL-dependent IL-1β release could occur independent of gasdermin-D, suggesting plasma membrane damage alone suffices to release bioactive IL-1 (Conos, Chen et al. 2017, Gutierrez, Davis et al. 2017). Interestingly, however, a polybasic motif within mature IL-1β upon proteolytic cleavage has been shown to promote its association with phospholipids on the plasma membrane; facilitating its externalisation upon gasdermin-D activation. Pro IL-1β (prior to cleavage) is predominantly negatively charged and therefore avoids recruitment to the plasma membrane and possible IL-1β secretion (Monteleone, Stanley et al. 2018). Therefore, IL-1β secretion cannot be wholly termed as passive secretion.

1.6.2 Active secretion of IL-1β secretion in neutrophils

Pyroptosis may allow the passive release of IL-1β. However, recent reports indicate that the events of pyroptosis and IL-1β release can be uncoupled. By culturing cells in the presence of the osmoprotectant glycine that inhibits cell lysis, IL-1β release was unaffected (Evavold, Ruan et al. 2018). In cell lines containing an inducible and dimerizable caspase-1 construct to induce cleavage and secretion of IL-1β, it was shown at the single cell level that, following caspase-1 activation, IL-1β secretion could occur in living cells (Conos, Lawlor et al. 2016). Although not reflecting a true physiological response, this study provided important proof-of-principle evidence that IL-1 release may not always depend on a loss of cell viability. Consistent with this idea, an intracellular Lactate Dehydrogenase (LDH) release assay to measure cell death in neutrophils following Salmonella infection and NLRC4 inflammasome activation was negative post 5 hours of infection, compared to macrophages that underwent pyroptosis as early as 1-hour post infection, despite considerable levels of neutrophil-derived IL-1 being secreted. (Chen 2014). Similarly, NLRP3-dependent IL-1β release in human monocytes challenged with Salmonella typhimurium is reported to occur independent of pyroptosis (Diamond, Leong et al. 2017). In agreement with Salmonella infection studies, human peripheral neutrophils infected with Streptococcus pneumoniae resulted in IL-1β secretion independent of pyroptosis (Karmakar 2015). Thus, the resistance of neutrophils to pyroptosis suggests the presence of a caspase-1-dependent alternative secretion pathway for IL-1β release.

1.6.3 Other theories of IL-1β secretion

IL-1β must be able to cross the plasma membrane of a viable cell in order to be secreted. Indeed, pro IL-1β is found exclusively inside cells while, upon activation, mature IL-1β is rapidly released into the extracellular environment (Singer, Scott et al. 1995). Various mechanisms to facilitate IL-1β translocation have been hypothesised including type I unconventional protein secretion, and intracellular vesicles or organelles (Type III secretion) that fuse with the plasma membrane to deliver mature IL-1β to the extracellular environment (Piccioli and Rubartelli 2013). In primary human monocytes activated by LPS, it was shown that pro-IL-1β and caspase-1 were located in vesicles containing the endolysosomal hydrolase, cathepsin-D and lysosomal marker, Lamp-1 (Singer, Scott et al. 1995). Conventionally, lysosomal proteins intended for secretion are produced in the Endoplasmic Reticulum (ER) and are sorted to lysosomes via the trans golgi network. IL-1β is reported not to be produced

in the ER, therefore it is unlikely lysosomal vesicles are utilised for IL-1β secretion (Rubartelli, Cozzolino et al. 1990). Other vesicular secretion mechanisms, such as extracellular vesicles and exosomes, are also reported to facilitate IL-1β secretion. In Thp-1 cells stimulated with LPS, after only two minutes of adding the inflammasome activator signal ATP, microvesicles containing IL-1β have been reported to be released (MacKenzie, Wilson et al. 2001). Other cell types such as microglial and dendritic cells have also been documented to mediate IL-1β secretion via microvesicles (Bianco, Pravettoni et al. 2005, Pizzirani, Ferrari et al. 2007). In mouse macrophages, upon ATP stimulation, exosomes that fuse with the plasma membrane to deliver their contents are reported to contain IL-1β, caspase-1 and other inflammasome components (Qu, Franchi et al. 2007).

Autophagy is another mechanism that can mediate leaderless protein secretion (Curwin, Brouwers et al. 2016). In regard to IL-1β secretion, autophagy is reported to both promote (Dupont, Jiang et al. 2011, Zhang, Kenny et al. 2015) and inhibit inflammasome activation (Saitoh, Fujita et al. 2008, Crisan, Plantinga et al. 2011, Harris, Hartman et al. 2011, Zhou, Yazdi et al. 2011). Recently, two KEFRQ-like motifs were identified in IL-1β and mutations of these resulted in defective IL-1β secretion, suggesting IL-1β secretion could occur through a Chaperone-Mediated Autophagy (CMA) mechanism (Zhang, Kenny et al. 2015). The cytosolic chaperone, Hsc70, recognises KEFRQ-motifs and recruits proteins to the lysosomal membrane (Zhang, Kenny et al. 2015). The Heat Shock Protein 90 (HSP90) and lysosomal membrane LAMP2A provide further assistance to facilitate translocation of target proteins (Zhou, Li et al. 2005). Interestingly, in addition to IL-1β, a KEFRQ-motif is also present in caspase-1. Further investigation into secretory lysosomal vesicles and CMA-mediated IL-1β translocation is required.

1.7 Post- translational modifications (PTMs)

PTMs play a critical role in regulating inflammasome signalling and ubiquitin system, the focus of this thesis, orchestrates one of the most complex, multifaceted, post-translational modifications of proteins in eukaryotes. This section is a brief background of the ubiquitination process and their role in inflammasome signalling.

1.7.1 The Ubiquitin Proteasome System (UPS)

Protein ubiquitination occurs in an enzymatic three-step process governed by E1 (activating), E2 (conjugating) and E3 (ligating) enzymes (Hershko and Ciechanover 1998) **(Figure 1.3)**. E1 enzymes comprises of 2 family members (UBA1 and UBA6 in human), E2 enzymes includes approximately 40 members and there are more than 600 E3 ligases belonging to three families; Homologous to E6AP C-terminus (HECT), Really interesting new gene (RING) and RING-between RING-RING (RBR) families (Schulman and Harper 2009, Komander and Rape 2012, Zheng and Shabek 2017).

In the first step, the E1 enzyme establishes a thioester bond between carboxyl terminal of ubiquitin and cysteine at the active site of the E1 by utilising ATP. Structural conformational changes lead to increased affinity for E2 enzymes and through a trans-thioesterification reaction, the ubiquitin molecule is transferred from E1 to an E2 cysteine at its active site. E3 ligases recruit E2 enzymes laden with ubiquitin molecule and either directly catalyses the ubiquitylation of target protein or enable the transfer of ubiquitin to the target protein from the E2 enzyme via its HECT, RING or RBR domains (Pickart 2001, Bernassola, Karin et al. 2008, Dove, Stieglitz et al. 2016). Specificity of ubiquitination is primarily dependent on E3 ligases (Hershko and Ciechanover 1998). However, given the sheer number of more than 600 E3 ligases identified in the human genome (mostly uncharacterised) (Bernassola, Karin et al. 2008), the redundancy of E3 ligases and the dynamic nature of ubiquitination (Nalepa, Rolfe et al. 2006), the identification of E3 ligase substrates is enormously challenging. Nonetheless, in some well-studied E3 ligases, conserved targeting sequences in substrates have been identified. For example, *N*-degrons sequences are recognised by UBR (Ubiquitin-Protein ligase E3 component N-recognin) E3 ligases (Tasaki, Mulder et al. 2005). PTMs also play a key role in substrate recognition by E3 ligases; such as phosphorylated tyrosine motifs in substrates are targeted by c-Cbl (named after Casitas B-lineage lymphoma encoding gene) E3 ligase (Deckert, Elly et al. 1998).

Ubiquitination of a target protein can occur in multiple ways **(Figure 1.3)**. Modification involving a single ubiquitin molecule on a single lysine residue of a target protein is termed as monoubiquitination, while modification of multiple lysine residues of a target protein by the addition of single ubiquitin molecule is referred as multiubiquitination (Sasaki, Carracedo et al. 2011, Su, Gao et al. 2013). Polyubiquitination is referred to the sequential rounds of

ubiquitination that occurs on a ubiquitin molecule of a lysine initially linked to a target protein. Ubiquitin linkages can occur on the amine group of one of the seven different lysines of ubiquitin and the amino-terminal of methionine, termed as K6, K11, K27, K29, K33, K48, K63 and M1 respectively. The detailed architecture of ubiquitin linkages are collectively termed as the ubiquitin code. The ubiquitin code dictates the fate and regulation of target proteins (Komander and Rape 2012) **(Figure 1.4)**.

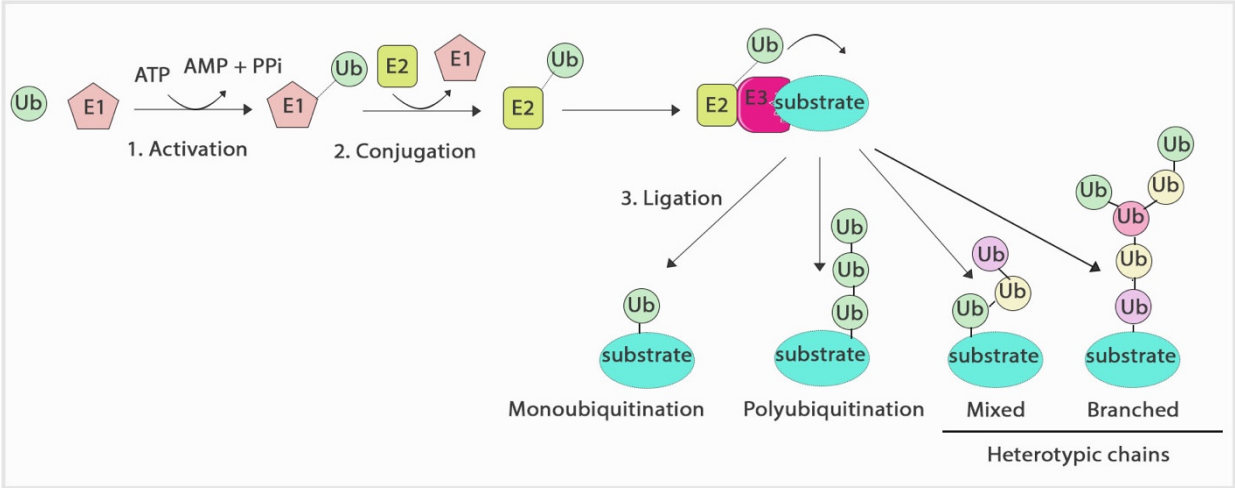


Figure 1.3 Schematic representation of ubiquitination mechanism.

Ubiquitination occurs in three steps. E1 activating enzyme activates ubiquitin in step 1, followed by the ubiquitin molecule passed on to E2, conjugating enzyme and in the third step, E3 ligase enzyme transfers ubiquitin to the target substrate on to the target's lysine residue. Protein substrates can be modified in multiple ways as shown. Monoubiquitination occurs when a ubiquitin molecule is accepted at one lysine residue of substrate. Alternatively, polyubiquitination occurs when the same acceptor site receives multiple ubiquitin molecules. In mixed chains, only one acceptor site of substrate is modified, however each ubiquitin molecule is modified by an acceptor site. Branched ubiquitin chains contain at least one ubiquitin molecule that is modified at multiple acceptor sites, hence forming a branch.

K48 and K63-linked ubiquitin chains are the two-most well characterised ubiquitin linkages (Komander and Rape 2012). K-48 ubiquitin linkages target proteins for 26S proteosomal degradation; one of the main degradative apparatuses in cells (Hershko and Ciechanover 1998) and K-63 ubiquitin linkages function in non-degradative roles (opposed to K-48 linkages), such as protein trafficking and signalling (Spence, Sadis et al. 1995). The rest of the ubiquitin linkages; K6, K11, K27, K29, K33 and M1 are termed as 'atypical' ubiquitin modifications due to the lack of tools to study these linkages until recently. Development of innovative technologies have provided more data on 'atypical' ubiquitin linkage functions as follows: K6-linked ubiquitin chains are reported to be associated with DNA damage and maintaining

mitochondria homeostasis (Morris and Solomon 2004, Nishikawa, Ooka et al. 2004, Ordureau, Sarraf et al. 2014); K11-linked ubiquitin chains are stated to drive additional proteosomal degradation and regulate cell cycle (Bremm and Komander 2011, Wickliffe, Williamson et al. 2011); K27 linked ubiquitination are described to recruit mediators during DNA damage response and stimulator of interferon genes "STING" activation in response to exogenous DNA (Wang, Liu et al. 2014, Gatti, Pinato et al. 2015); K29 ubiquitination functions to cause proteosomal degradation (Kim, Bennett et al. 2011), although additional non-degradative roles in inhibition of Wnt signalling, that plays important role in embryogenesis (Clevers and Nusse 2012) and epigenetic regulation is also reported (Tran, Hamada et al. 2008, Jin, Xie et al. 2016); K33 ubiquitination regulates T-cell receptor signalling and protein trafficking (Huang, Jeon et al. 2010, Yuan, Lee et al. 2014) and M1 linkages play as key positive regulators of NF-κB signalling (Kirisako, Kamei et al. 2006, Rahighi, Ikeda et al. 2009, Emmerich, Ordureau et al. 2013). In addition to the homotypic and heterotypic linkages, branched ubiquitin linkages (where, a ubiquitin molecule in a ubiquitin chain is modified at one or more lysine residues forming a "branch" structure) are recently identified (Meyer and Rape 2014), representing increasing layers of ubiquitin diversification.

Another key player in the ubiquitin system are deubiquitinases (DUBs). DUBs remove ubiquitin modifications from proteins (Husnjak and Dikic 2012). There are approximately 100 of these enzymes discovered in humans, being classified into 5 families; namely, ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), ubiquitin C-terminal hydrolases (UCH), Josephin family and motif interacting with ubiquitin (MIU)- containing novel DUBs family (MINDY) (Mevissen and Komander 2017). Recent discoveries of other post-translational modifications such as phosphorylation or acetylation of ubiquitin and DUBs has revealed further complexity of the ubiquitin system (Ohtake, Saeki et al. 2015, Swaney, Rodriguez-Mias et al. 2015, Song and Luo 2019)

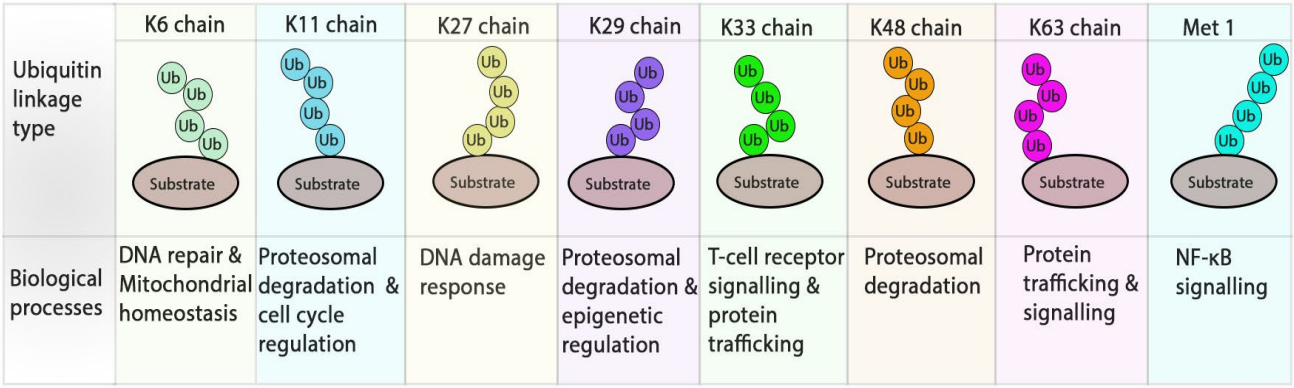


Figure 1.4 Diversity of ubiquitin linkages.

Various homotypic polyubiquitination of substrate can lead to different fate and regulation of the target substrate resulting in different biological outcomes as shown.

1.7.2 Ubiquitination and inflammasome signalling

NLRP1B inflammasome activation in response to *Bacillus anthracis* Lethal Toxin (LT) and subsequent caspase-1 activation is well characterised. Recently, a study utilising genome-wide CRISPR-Cas9 screening approach in RAW264.7 cells identified UBR2, an E3 ligase of the N-end rule protein degradation pathway, which states that N-terminal amino acid dictates the half-life of a protein (Varshavsky 2011) was required for NLRP1B activation (Chui, Okondo et al. 2019). Another separate study also identified that the *Shigella* ubiquitin E3 ligase IpaH7.8 directly ubiquitinates and promotes proteosomal degradation of the N-terminus, leading to NLRP1B inflammasome activation (Sandstrom, Mitchell et al. 2019). Recently, it was reported upon N-end rule mediated degradation of the N-terminus, the released C-terminal fragment containing CARD domain recruits CARD domain of ASC to activate NLRP1B inflammasome (Xu, Zhou et al. 2021).

The AIM2 inflammasome is activated in response to double stranded DNA. Tripartite motif-11 (TRIM 11) was identified as a negative regulator of the AIM2 inflammasome by targeting it for polyubiquitination at K458 to promote its interaction with the cargo receptor p62, leading to autophagic degradation of AIM2 (Liu, Tang et al. 2016).

Regulation of NLRP7 through the endolysosomal pathway was recently identified in which a deubiquitinase, STAM (signal transducing adaptor molecule or AMSH), recruited ubiquitinated NLRP7 (on K288 and K290) to rescue it from endolysosomal degradation and

thereby allow inflammasome activation upon LPS or Pam3cys stimulation (Bednash, Weathington et al. 2017).

A K48-linked ubiquitination was identified on transiently expressed and immunoprecipitated NLRC4 (also known as Ipaf), and its ubiquitination was increased in the presence of MG132 (a peptide aldehyde that inhibits the proteolytic activity of 26S proteasome complex), consistent with ubiquitination targeting NLRC4 for degradation. Notably, SUG1 (suppressor of gal1), a component of 26S proteasome, was shown to interact and enhance NLRC4 ubiquitination. Interestingly, tagging ubiquitin molecules to NLRC4 led to NLRC4 complex formation with enhanced caspase-8 activation and cell death (Kumar, Radha et al. 2010). Recently, the autoinflammatory syndrome caused by a H443P mutant of NLRC4 was reported to undergo increased polyubiquitination and interaction with SUG1 compared to wildtype NLRC4 in an overexpression system (Raghawan, Sripada et al. 2017), suggesting that altered interaction of H433P NLRC4 mutant with SUG1 and increased polyubiquitination is sufficient to trigger constitutive caspase-8 mediated NLRC4 activation and autoinflammation.

1.7.3 Regulation of the NLRP3 inflammasome by ubiquitination

Several E3 ligases are reported to negatively regulate the NLRP3 inflammasome. TRIM31 was suggested to promote K48-linked NLRP3 ubiquitination leading to NLRP3 proteosomal degradation (Song, Liu et al. 2016). An E3 ligase belonging to the Skip-Cullin-F-box (SCF) family, FBXL2, was also reported to ubiquitinate NLRP3 on lysine 689 and cause NLRP3 proteosomal degradation (Han, Lear et al. 2015). Interestingly, another component of the SCF family, the ubiquitin E3 ligase Cullin1 (CUL1), was suggested to negatively regulate NLRP3 inflammasome activation by ubiquitination, not by proteasomal targeting, but by keeping NLRP3 in an inactive state. Upon inflammasome activation, CUL1 was suggested to dissociate and release NLRP3 to interact with ASC and form an active inflammasome complex (Wan, Zhang et al. 2019). Similarly, NLRP3 ubiquitination is promoted by the E3 ligase Ariadne homolog 2 (ARIH2) without causing NLRP3 degradation and loss of ARIH2 is reported in increased NLRP3 signalling (Kawashima, Karasawa et al. 2017).

NLRP3 has been implicated in neurodegenerative conditions. In this regard, the neurotransmitter dopamine signals via activation of dopamine D1 receptor (DRD1), and it was reported that dopamine binding to DRD1 caused increased cAMP production, resulting in the

ubiquitination of NLRP3 and activate autophagy for its degradation by the ubiquitin E3 ligase MARCH7 (Yan, Jiang et al. 2015). The site of NLRP3 ubiquitination by MARCH7 is yet to be reported. Another key player in neuroinflammation, the E3 ligase PARKIN, was also shown to inhibit NLRP3 inflammasome activation (Mouton-Liger, Rosazza et al. 2018). However, in this case PARKIN appears to act indirectly, as it was suggested that a lack of A20 gene induction, a ubiquitin-modifying enzyme and NFκB inhibitor, was responsible for excessive NLRP3 activation upon PARKIN loss (Mouton-Liger, Rosazza et al. 2018). A20, a ubiquitin-modifying enzyme belonging to the otubain protease family is reported to negatively regulate NLRP3 inflammasome activation. Upon LPS priming, spontaneous NLRP3 activation dependent on RIPK3 occurred in macrophages lacking A20 (Duong, Onizawa et al. 2015). However, the role of A20 DUB activity in inflammatory signalling seems ambiguous and further investigations are required (De, Dainichi et al. 2014).

Some ubiquitin E3 ligases such as Pellino2 and TRAF6 have been reported to positively regulate the NLRP3 inflammasome. In response to inflammasome priming, Pellino2 deficiency reduced NLRP3 ubiquitination and activation. It was reported that Pellino2 ubiquitinates IRAK1, and that IRAK1-mediated regulation acts as counter mechanism to reduce NLRP3 ubiquitination and supress its activation in cells lacking Pellino2 (Humphries, Bergin et al. 2018). TRAF6 signalling downstream of IRAK activation also regulate NLRP3 activation. Macrophages lacking TRAF6 resulted in similar NLRP3 expression to WT cells but reduced its activation. Though NLRP3 is not the target of ubiquitination by TRAF6, TRAF6 was required for ASC oligomerisation and the NLRP3-ASC interaction (Xing, Yao et al. 2017).

Deubiquitinases (DUBs) are also involved in both the positive and negative regulation of the NLRP3 inflammasome. BRCC3, a zinc metalloprotease DUB, was reported to interact with the LRR domain of NLRP3, causing NLRP3 deubiquitination and promoting its activation (Py, Kim et al. 2013). Positive and negative regulation of NLRP3 inflammasome by ubiquitination is illustrated in **Figure 1.5**.

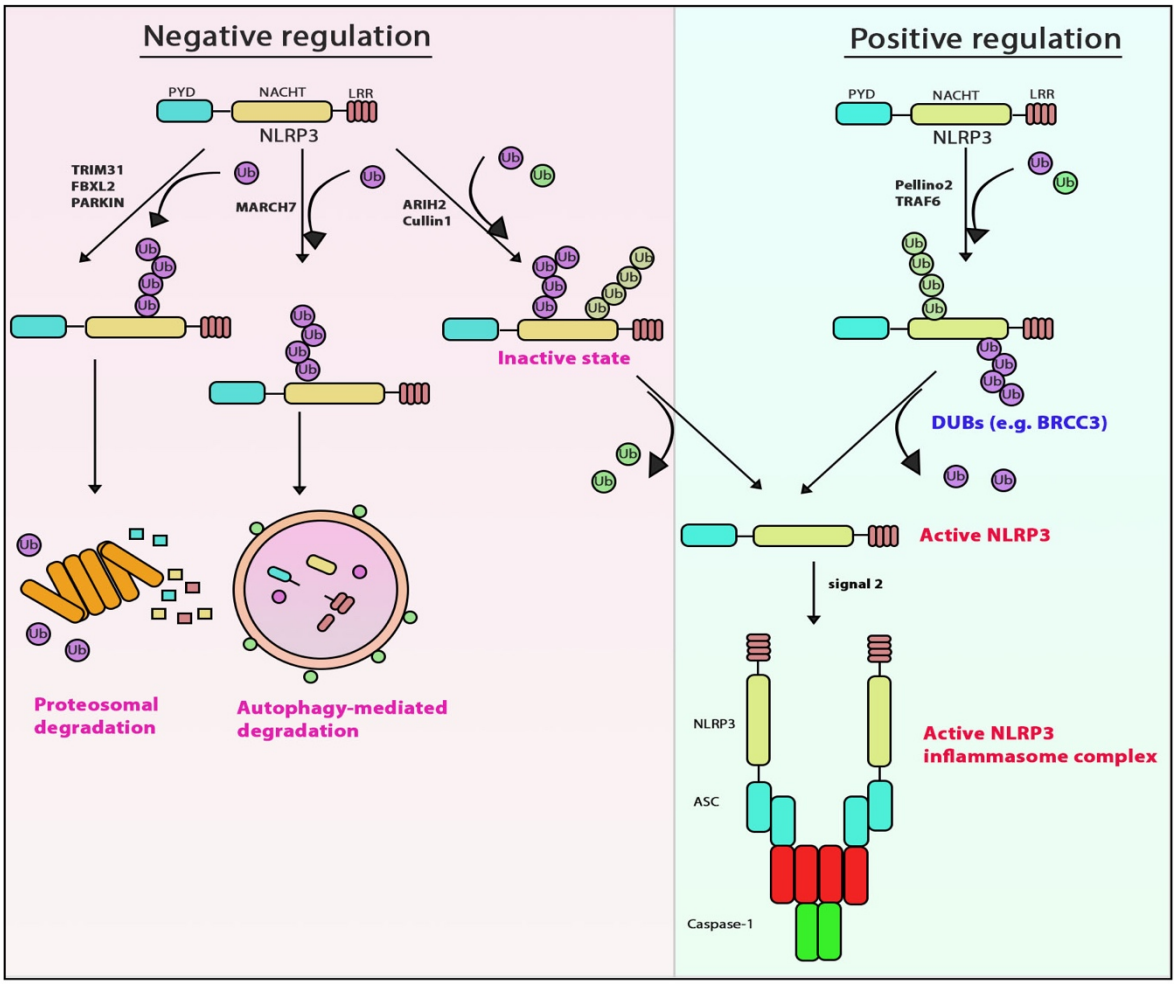


Figure 1.5 Regulation of NLRP3 inflammasome by ubiquitination.

Negative regulation is provided by identified E3 ligases TRIM31, FBXL2 and PARKIN leading to proteosomal degradation of NLRP3. E3 ligase MARCH7 targets NLRP3 for autophagy-mediated ubiquitination, whereas E3 ligases ARIH2 and Cullin1 holds NLRP3 inflammasome in an inactive state. Upon sensing signal 2 (activation signal), E3 ligases release NLRP3 from inactive to active state to form the inflammasome complex. On the contrary, E3 ligases Pellion2, TRAF6 leads to positive regulation of NLRP3 inflammasome. DUBs such as BRCC3 also promote NLRP3 inflammasome activation by removing its ubiquitination.

Collectively, these results suggest that regulation by ubiquitination play a key role in the activation and regulation of NLRP3 inflammasome sensor. Targeting identified E3 ligases and DUBs that regulate NLRP3 inflammasome activation present viable, potential therapeutic options to treat aberrant inflammatory signalling. However, a clear understanding of the complex, ubiquitin biology in inflammasome signalling is first required and this area of research is rapidly evolving with exciting times ahead for novel discoveries.

1.8 Hypothesis and Aims

IL-1β is a potent proinflammatory cytokine and plays a critical role in the clearance of pathogens. However, excess IL-1β secretion could lead to devastating autoinflammatory diseases. Therefore, tight regulation of IL-1β is required to avoid host damaging responses. Upon NLRP3 inflammasome activation, IL-1β requires caspase-1, a cysteine protease to cleave its precursor form in an inactive state to its active secreted p17 fragment. The secretory mechanism of IL-1β is not completely understood as it lacks the signal sequence required for conventional secretory pathway and therefore referred to as a leaderless protein (Monteleone, 2015). Since precursor IL-1β exists in an inactive state, it is hypothesised that precursor IL-1β undergoes post-translational modification (PTM) to modulate its activity.

The following are the aims of the thesis:

- To define and characterise the PTMs that occur on precursor IL-1β upon inflammasome priming.

- To understand the functional consequences of the IL-1β PTMs; and

- To provide a detailed mechanism of PTM-related regulation of inflammasome-driven IL-1β responses.

Chapter 2: Materials and Methods

2.1. Cell culture

2.1.1 Bone marrow derived macrophages (BMDMs)

Primary BMDMs were derived from C57BL/6J (WEHI Bioservices, *IL-1β* $^{K133R/K133R}$ (IL-1β K133R knockin; MAGEC facility, WEHI), *IL-1β*$^{-/-}$ (Horai 1998), *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ and *Caspase-1*$^{-/-}$ (Seth Masters, WEHI) mice. Bone marrow was flushed from femoral and tibial bones of mice and plated in a final concentration of 20% (v/v) L929 cell-conditioned medium diluted in DMEM medium (Gibco, Thermo Fisher Scientific #11885084) (containing 10% (v/v) foetal bovine serum (FBS, Sigma Aldrich #12003C), 100 U/mL penicillin, 100 U/mL streptomycin and 2 mg/mL L-Glutamine). Cells were grown on 15 cm bacterial petri plates (37^{O}C, 10% CO_2) and supplemented with fresh medium on day 4. Macrophages were harvested after 6-8 days of culture and plated for stimulations at 4 x 10^5 cells per well and 2 x 10^5 cells per well of a 24-well plate and 96-well plate, respectively. For tandem ubiquitin binding entity (TUBE) and immunoprecipitation assays and mass spectrometry analysis macrophages were seeded in 10 cm^2 and 20 cm^2 bacterial petri plates at 10 x 10^6 and 20 x 10^6 cells per plate, respectively. BMDMs were allowed to settle for 16-24 hours prior to receiving treatments. The Walter and Eliza Hall Institute (WEHI) Animal Ethics Committee approved all animal experiments.

2.1.2 Human Embryonic Kidney (HEK) 293T cells

HEK 293T cells were cultured in DMEM medium containing 10% FBS supplemented with 100 U/mL penicillin, 100 U/mL streptomycin and 2 mg/mL L-Glutamine (referred to henceforth as complete DMEM) at 37^{O}C, 10% CO_2 atmosphere and passaged by trypsinization every 4-5 days to maintain subconfluence.

2.1.3 Protein expression in HEK293T cells

293T cells were grown to ~80% confluence in complete media. Transfections were carried out using Lipofectamine (Thermo Fisher Scientific) as mentioned below.

For mass spectrometry analysis and immunoprecipitation assays, transfections of constructs were carried out in 100 mm dishes. Prior to transfection, cells at 70-80% confluency in

complete DMEM were replaced with 7 ml of Opti-MEM^{TM} (serum-free) medium (Thermo Fisher Scientific) for 12-18 hours. For each transfection sample, 20 µg DNA and 50 µl lipofectamine was combined in total of 3 ml Opti-MEM^{TM} and incubated for 20 minutes at room temperature. After incubation, the combined DNA and lipofectamine complexes were added dropwise to each plate. Cells in Opti-MEM^{TM} were replaced with complete DMEM after 4-5 hours and incubated at 37^{O}C for 24-48 hours.

For caspase-1 cleavage and IL-1β stability assays, transfections of constructs were carried out in 24-well tissue culture plates. One day prior to transfection, 2 x10^{5} cells were plated in 400 µl of DMEM medium containing no antibiotics and serum. Each transfection sample was prepared with a total of 800 ng DNA or lower as indicated in the relevant results section (Chapters 3.2 and 4.2) and 2 µl of lipofectamine in total of 100 µl Opti-MEM^{TM} and incubated for 20 minutes at room temperature. After incubation, 100 µl of complexes was added to each well. Medium was replaced with complete DMEM after 4-5 hours and incubated at 37^{O}C for 24-48 hours.

IL-1β plasmids harbor internal ribosome entry site (IRES) driven GFP expression, therefore transfection efficiency was routinely assessed for GFP^{+} HEK 293T cells by inverted fluorescent microscopy.

2.1.4 Neutrophil isolation from mouse bone marrow using Fluorescence-activated cell sorting (FACS)

WT littermate and *IL-1β*$^{K133R/K133R}$ KI mouse bone marrow cells were harvested from femurs and tibias. All mouse genotypes were confirmed (as described in section 2.8). Cells were pelleted (1500 revolutions per minute (rpm), 5 min) and resuspended for 30 seconds in red cell removal buffer (RCRB, 0.168 M ammonium chloride; NH4CL, pH7.3) to remove red blood cells, then washed and resuspended in FACS buffer (2 mM Ethylenediaminetetraacetic acid; EDTA, 0.1% Bovine serum albumin; BSA in phosphate-buffered saline, PBS). Phycoerythrin; PE- conjugated anti-Ly6G (clone 1A8) and Allophycocyanin; APC-Cy-7 conjugated anti-CD11b (Mac1) FACS antibodies (BD biosciences) were incubated (1:400 dilution in FACs buffer) with bone marrow cells for 30 mins at 4^{O}C in the dark. Excess antibodies were removed by adding 1mL of FACS buffer followed by centrifugation at 1500 rpm for 5 mins at 4^{O}C (repeated twice). Cells were subsequently resuspended in complete DMEM and sorted using

flow cytometry by gating on live $CD11b^+$ $Ly6G^+$ cells. Sorted neutrophils were then seeded in a 24-well plate at 1-2x 10^5 cells per well for inflammasome assays (as described in Chapter 4.2).

Table 2.1 Reagents

The following list of reagents were used in experiments as indicated

Reagent	**Catalogue number**	**Manufactured Company**
ATP	A2383	Sigma- Aldrich
MG132	tlrl-mg132	Invivogen
Nigericin	N1743	Sigma- Aldrich
Alum	PI-77161	Thermo Scientific
Ultrapure lipopolysaccharide from E.coli.K	tlrl-3pelps	Invivogen
Doxycycline	D3447	Sigma- Aldrich
Cyclohexamide	C7698	Sigma- Aldrich
Compound A	N/A	TetraLogic Pharmaceuticals
Bafilomycin A-1	B1793	Sigma- Aldrich
Mouse Recombinant Interferon-γ	485-MI	R&D
Cyclosporin A	tlrl-cyca	Invivogen
FK506	tlrl-fk5	Invivogen
BAPTA-AM	A1076	Sigma- Aldrich
Pam3Csk4	tlrl-pms	Invivogen
Ubiquitin Isopeptidase inhibitor G5	662125	Merck-millipore
Deubiquitinase inhibitor WP1130	681685	Merck-millipore
Polybrene	107689	Sigma- Aldrich
Propidium iodide	81845	Sigma- Aldrich
Q-VD-OPh	1170-5	Biovision (Biocore)
Go Taq green	M7123	Promega
L-Broth	N/A	WEHI
Mini/Maxi/gel purification kits	A1330/ 12163/ 28704	Promega/Qiagen
Phusion High-Fidelity Polymerase	F530S	Thermo Fisher Scientific
Restriction enzymes: Mfe1-HF & Not1-HF	R3589S & R3189S	New England Biolabs
Sepharose Protein G beads	N/A	WEHI
T4 DNA Ligase	M1801	Promega
Triton-X100	T9284	Sigma- Aldrich
Trypsin	15090-046	Life Technologies
Urea	17-1319-01	GE Healthcare Life Sciences

Agarose TUBE (Tandem Ubiquitin Binding Entities)	UM401	Life Sensors
Agarose control-TUBE	UM400	Life Sensors
PR619	SML0430	Sigma- Aldrich
N-ethylmaleimide (NEM)	E3876	Sigma- Aldrich
Ampicillin	11593-027	Life technologies
Fetal bovine serum	12003c	Sigma- Aldrich
Mouse IL-1β/IF2 ELISA kit	DY401	R&D Systems
Mouse TNF-α ELISA kit	88-7324	eBioscience
Hydrogen peroxide	TA-060-HP	Thermo Scientific
DAB substrate	K3467	Dako
Mini-EDTA-free protease inhibitor	11836170001	Roche
Enhanced chemiluminescence (ECL) reagent	WBKLS0050	Millipore
NuPAGE gel (4-12%)	WG1402A	Invitrogen
Nitrocellulose membranes (0.45μM)	10600003	GE Healthcare Life Sciences
Precision Plus pre-stained protein standards	1610375	BIO-RAD
Competent E.coli	C4040-03	Invitrogen

2.2 Cloning of IL-1β DNA constructs

Generation of various murine IL-1β constructs were cloned into a retroviral pMIGRMCS vector containing IRES driven GFP expression previously gifted by Dr. Kate Schroder. All point mutations were introduced through overlapping PCR (detailed below in section 2.2.1). N' terminal FLAG epitope tagged IL-1β was cloned by primers containing nucleotides encoding the FLAG fragment (described below in section 2.2.2).

2.2.1 Site-directed mutagenesis PCR

For IL-1β site-directed mutagenesis PCR, three sets of PCRs were performed. Forward and reverse primers were designed for introducing point mutations at a specific site (Refer Figure 2.1).

First and second PCRs were performed with the forward point mutation primers (Primer Nos. #3/5/7/9 in Table 2.2) and overall reverse primer of IL-1β sequence (Primer No. #2 in Table 2.2), and the reverse point mutation primers (Primer Nos. #4/6/8/10 in Table 2.2) and overall forward primer of IL-1β sequence (Primer No. #1 in Table 2.2), respectively (Figure 2.1). The third PCR were performed using first and second PCR products (2 µl each) and overall forward and reverse primers of IL-1β sequence (Primer No. #1 and #2 in Table 2.2) to amplify full-length point-mutant IL-1β cDNA (PCR conditions are outlined in 2.2.2). The resulting product was agarose gel purified and cloned into the pMIGRMCS vector (**Figure 2.1**; conditions outlined in 2.2.3-2.2.5).

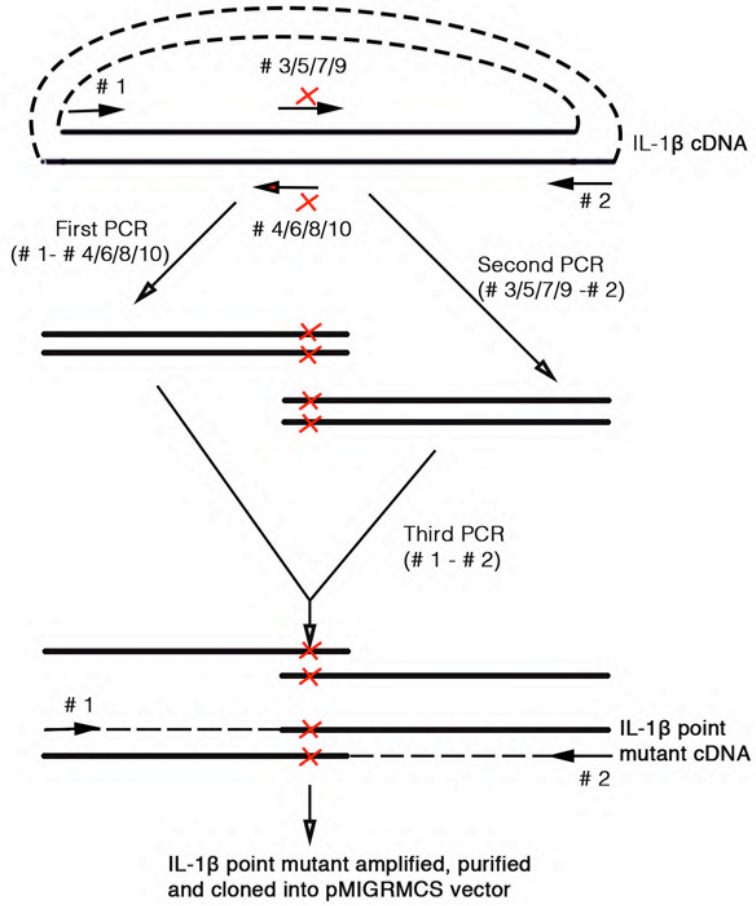


Figure 2.1 Schematic representation of site-directed mutagenesis PCR.

Double stranded cDNA and the primers (numbers according to table 2.2) are represented by with arrows indicating 5'- 3' orientation. Site of point mutation is represented by a red cross. First and second PCRs produced fragments of IL-1β cDNA both containing the point mutation. In the third PCR step, denatured fragments were annealed at the overlap and extended by DNA polymerase. Primers #1 & 2 further amplified the mutant IL-1β cDNA, which was then purified and cloned into pMIGRMCS vector.

Table 2.2 Primers used in overlapping PCR for IL-1β site directed mutagenesis2

Primer No.	Primer name	Primer sequence
#1	IL-1β F'primer	5' AGTCCAATTGATGGCAACTGTTCCTGAAC 3'
#2	IL-1β R'primer	5' AGTCGCGGCCGCTTAGGAAGACACAGATTCC 3'
#3	IL-1β K133R F'primer	5'AGGCTCCGAGATGAACAACAAAGAAGCCTCGTGCTGTCG 3'
#4	IL-1β K133R R'primer	5' CGACAGCACGAGGCTTCTTTGTTGTTCATCTCGGAGCCT 3'
#5	IL-1β K133A F'primer	5' AGGCTCCGAGATGAACAACAAGCAAGCCTCGTGCTGTCG 3'
#6	IL-1β K133A R'primer	5' CGACAGCACGAGGCTTGCTTGTTGTTCATCTCGGAGCCT 3'
#7	IL-1β S134A F'primer	5' CGAGATGAACAACAAAAAGCCCTCGTGCTGTCGGACCCA 3'
#8	IL-1β S134A R'primer	5' TGGGTCCGACAGCACGAGGGCTTTTTGTTGTTCATCTCG 3'
#9	IL-1β S134E F'primer	5' CGAGATGAACAACAAAAAGAGCTCGTGCTGTCGGACCCA 3'
#10	IL-1β S134E R'primer	5' TGGGTCCGACAGCACGAGCTCTTTTTGTTGTTCATCTCG 3'

2 2* In table 2.2, IL-1β K133R refers to a lysine to arginine point mutation at residue 133 of murine IL-1β, IL-1β K133A refers to a lysine to alanine mutation at residue 133, IL-1β S134A refers to serine to alanine mutation (phospho-mutant) at residue 134 and IL-1β S134E refers to serine to glutamine mutation (phospho-mimetic) at residue 134. Residues in red represent restriction sites of Mfe1 in #1 and Not1 in #2 primers respectively. Residues in green represent amino acid point mutations in the primers. F'and R' primers denotes forward and reverse primers respectively.

2.2.2 Polymerase Chain Reaction (PCR)

Traditional cloning approach was used to generate N' terminal flag of IL-1β cDNA construct. PCR was performed with forward primer containing the flag fragment (Primer No. #11, flag sequence in blue; Table 2.3) and the overall reverse primer of IL-1β sequence (Primer No. #2 in Table 2.2 & 2.3). PCR conditions are described below. PCR products were agarose gel purified and cloned into the pMIGRMCS vector (conditions described in 2.2.3-2.2.5).

Table 2.3 Primers used for cloning IL-1β cDNA construct[2]*

Primer No.	Primer name	Primer sequence
#11	IL-1β N-FLAG forward primer	5' AGTCCAATTGATGGACTACAAGGACGACGATGACAAGGCAACTGTTCCTGAACTCAAC 3'
#2	IL-1β reverse primer	5' AGTCGCGGCCGCTTAGGAAGACACAGATTCC 3' 3

PCR Reaction Mix

10X Pfu DNA buffer	-	5 µl
Pfu DNA polymerase	-	0.5 µl
dNTPs (10 mM)	-	1 µl
DNA (50-500 ng)	-	1 µl
5' primer (0-.1-1 µM)	-	1 µl
3' primer (0-.1-1 µM)	-	1 µl

Nuclease-Free water filled to final volume of 50 µl

Thermocycling conditions

1. 98 °C - 5 minutes
2. 98 °C - 15 seconds
3. 55 °C - 15 seconds
4. 72 °C - 15 seconds

Steps 2-4 cycled 30 times

5. 72 °C - 30 seconds

*Annealing temperature was optimised for different primer pairs

3 *In table 2.3, residues in red represent restriction sites of Mfe1 in #11 and Not1 in #2 primers respectively.

Agarose gel electrophoresis was used to separate all PCR products on a 1% Agarose Tris-acetate gel with gel red. All gels were visualized using a UV trans-illuminator and DNA was extracted using a gel purification kit (Qiagen) according to the manufacturers protocol.

2.2.3 Restriction enzyme digestion and ligation

Mfe1-HF and Not1-HF were used to digest various full-length IL-1β constructs and pMIGRMCS vector according to manufacturer's instructions. The digested vectors and inserts were separated by agarose gel electrophoresis and purified (2.2.1). Ligations were carried out at 4^{O}C overnight.

Ligation Reaction Mix

10X T4 DNA ligase buffer - 1 µl

T4 DNA ligase - 1 µl

Vector (100-200ng)

Insert [calculated according to vector to insert ratio of 1:3 (w/w) calculated by the NEBioCalculator tool (www.nebiocalculator.neb.com)]

Nuclease-Free water to a final volume of 15 µl

2.2.4 Transformation

Chemically competent *E. coli* DH10β cells were transformed with the ligation mix. 50 µl of competent cells were combined with 10 µl of ligation reaction. Competent cells containing the ligation mixture were incubated at 42 ^{O}C for ~40 seconds, then on ice for a further 10 minutes. Bacteria were then cultured in 250 µl Luria broth (LB) for 20 minutes at 37 ^{O}C, pelleted by centrifugation at 5000 rpm for 1 minute, and 200 µl of supernatant discarded. Pellets were resuspended in the remaining 100 µl of supernatant and this was spread on LB Agar plates containing 100 µg/ml ampicillin. Plates were incubated at 37 °C overnight.

2.2.5 Miniprep and Maxiprep of plasmid DNA

Single colonies were picked at random from agar plates and cultured in 5 ml LB medium (tryptone 1% (v/v), yeast extract 0.5% (v/v), sodium chloride 1% (v/v) and sodium hydroxide 5M, final pH 7.3-7.4). Ampicillin (100 µg/ml) was added to the LB medium for selection of positive colonies containing plasmid DNA. Bacterial cultures were incubated at 37^{O}C overnight with agitation. Cells were pelleted, and plasmid DNA was isolated according to the

manufacturer's protocol (Promega). Isolated plasmid DNA was sent to the AGRF facility, Melbourne for Sanger sequencing. Sequencing primers used were primer nos.#1 and #2 in table 2.2. For further sequencing confirmation, pMIGRMCS vector sequencing primers (listed in table 2.4) were also used for sequencing. Bacterial colonies harbouring the correct plasmids were then cultured at 37^{O}C overnight with agitation in 100 ml LB medium containing ampicillin (100 μg/ml), and large-scale plasmid DNA purification was performed according to the manufacturers protocol (Qiagen Maxi prep). Purified plasmid was stored at -20 ^{O}C.

Table 2.4 pMIGRMCS vector sequencing primers

Primer No.	Primer name	Primer sequence
#12	Sequencing forward primer	5' GATCTGATATCGGATCCCAATTGACGC GTGCGGCCGCCTCGAGGTTAACG 3'
#13	Sequencing reverse primer	5'AATTCGTTAACCTCGAGGCGGCCGCACGCGTCAATTG GGATCCGATATCA 3'

Table 2.5 DNA constructs gifted

The following constructs were provided as shown below

DNA constructs	Vector	Selection	Provided by
IL-1β wildtype	pMIGRMCS	Ampicillin	Dr James Vince
C-IL-1βK8A	pMIGRMCS	Ampicillin	Dr James Vince
IL-1βK17A	pMIGRMCS	Ampicillin	Bioneer
Caspase-1	pcDNA3.1	Ampicillin	Dr Seth Masters
pcDNA3.1	-	Ampicillin	Dr Seth Masters
Caspase-8	pFTre	Ampicillin	Dr Stephanie Conos

In table 2.5, the DNA construct containing 8 lysine to alanine mutations within residues 205-211 (residues K205, K209-211, K214, K220, K224 and K226) of murine IL-1β is represented as C-IL-1βK8A mutant. All 17 conserved lysines (K30, K32, K58, K72, K133, K144, K172, K180, K182, K192, K205, K209-211, K214, K220 and K226) between the human and mouse were mutated to alanine in murine IL-1β and this IL-1β construct is denoted as IL-1β K17A mutant (Figure 3.2.12 provides schematic representation of all lysine residues and mutations of IL-1β DNA constructs described here).

2.3 Ubiquitination analysis

2.3.1 Preparation of samples for Mass Spectrometry (MS) analysis

293T cells were plated in 100 mm dishes at cell density of 5 X 10^6. IL-1β constructs tagged at the N' terminus with FLAG tag were transfected using lipofectamine 2000 (Thermo Fisher Scientific). After 48 hours, cells were washed with ice-cold PBS and lysed with 700 µl of non-denaturing DISC lysis buffer (30 mM Tris-HCl (pH 7.4), 120 mM NaCl, 2mM EDTA, 2mM KCL, 1% Triton X-100, Roche complete protease inhibitor cocktail, 10 µM PR619 and 1 mM NEM; N-ethylmaleimide) on ice for 30 minutes. Cell lysates were spun at ~13000x g (4^OC) and the supernatant collected. A 50 µl slurry of M2 agarose anti-FLAG conjugated beads (WEHI proteomics lab) was added to the clarified cell lysate and the mix rotated overnight at 4^OC. Subsequently, the M2 agarose anti-FLAG beads were washed 4 times with DISC lysis buffer and bound FLAG-IL-1β eluted 3 times with 50µl of elution buffer (0.5% SDS and 5mM DTT) at 65^OC. Eluted proteins were run on NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen). SYPRO ruby staining was used to view the purified proteins and bands of interest were cut out. In-gel trypsin digestion was performed, and digested peptides analysed by MS at the proteomics facility, WEHI (kindly performed by Laura Dagley).

2.3.2 Tandem Ubiquitin Binding Entities (TUBEs) purification of ubiquitinated proteins

BMDMs were stimulated with treatments, as specified in the results section. All mouse genotypes were confirmed (as described in Section 2.8). Macrophages were washed with ice-cold PBS and lysed in 500 µl of DISC lysis buffer [30 mM Tris-HCl (pH 7.4), 120 mM NaCl, 2 mM EDTA, 2 mM KCL, 1% Triton X-100 containing Roche complete protease inhibitor cocktail (Roche, 11697498001) and DUB inhibitor 10 µM PR619)] on ice for 25 minutes. Cell lysates were clarified by centrifugation for 15 minutes at ~13000x g (4^OC). Equilibrated Agarose-TUBE (TUBE1, Life Sensors; 40 µl slurry per condition) was added to the clarified lysate and samples rotated for 20 hours at 4^OC. Bound proteins were eluted using Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol, 0.01% (v/v) bromophenol blue, 0.05% (v/v) 2-mercaptoethanol) after 4 x washes with DISC lysis buffer.

2.4 Immunoprecipitation (IP)

2.4.1 IPs from 293T cells

293T cells at cell density of 5 X 10^6 were transfected in 100 mm petri plates using lipofectamine 2000 (Thermo Fisher Scientific). After 48 hours, cells were washed with ice-cold PBS and lysed with 500 µl of denaturing DISC lysis buffer (30 mM Tris-HCl (pH 7.4), 120 mM NaCl, 2 mM EDTA, 2 mM KCL, 1% Triton X-100 containing Roche complete protease inhibitor cocktail and DUB inhibitors10 µM PR619 and 1 mM NEM (N-ethylmaleimide). Lysates were pre-cleared with 40 µl of Protein G 50% bead slurry (provided by WEHI proteomics lab) and incubated on a rotator at 4^OC for 60 minutes. Pre-cleared lysates were collected after centrifugation for 10 minutes at ~13000x g (4^OC). Myc antibody (2 µg) was added to each pre-cleared lysate sample along with 40 µl of Protein G 50% bead slurry and rotated overnight at 4^OC. Protein G and anti-Myc protein complexes were collected after 3-4 washes with DISC lysis buffer containing DUB inhibitor 10 µM PR619 and 1 mM NEM and eluted in SDS-PAGE sample buffer.

2.4.2 IPs from BMDMs

BMDMs at cell density of 2 X 10^7 treated with LPS (500 ng/ml, 6 hours) to induce IL-1β expression were lysed on ice with DISC buffer supplemented with protease inhibitors, followed by centrifugation at 13000rpm for 15 mins at 4ºC. Lysates were then pre-cleared with protein G beads at 4ºC and centrifuged at 13000rpm for 20 mins. Protein G beads were cross linked with 7µg IL-1β antibody in the presence of crosslinking agent, 5mM BS3 at room temperature for 30 mins on rotator. Cross linking reactions were quenched by adding 1MTris HCL (pH7.5) and rotated at room temperature for 15 mins. Crosslinked protein G beads with IL-1β antibody were washed thrice in DISC lysis buffer and eluted in 0.1M glycine-HCL (pH 2.5). Elutions were spun to remove any unbound antibody that is not crosslinked. Pre-cleared lysates were then rotated with IL-1β antibody crosslinked protein G beads overnight at 4ºC. Four washes were performed in lysis buffer containing protease and phosphatase inhibitors and eluted in 0.5%SDS and 5mM DTT. MS analysis of the eluted peptides were carried out at the proteomics facility, WEHI (kindly performed by Laura Dagley).

2.4.3 IPs using ubiquitin linkage-specific antibodies

BMDMs from WT, *IL-1β*$^{K133R/K133R}$ and *IL-1β*$^{-/-}$ mice were seeded in 15 cm dish at cell density of 2 X 10^7. All mice genotypes were confirmed (as described in Section 2.8). Cells were primed with LPS (100 ng/ml for 5 hours) and treated with the proteasomal inhibitor, MG132 (20 μM in the last 2 hours of priming). Cells were washed with ice-cold PBS and lysed with 500 μl of denaturing DISC lysis buffer (30 mM Tris-HCl (pH 7.4), 120 mM Nacl, 2 mM EDTA, 2 mM KCl, 1% Triton X-100, Roche complete protease inhibitor cocktail, 10 μM PR619 supplemented with 4 M urea). Lysates were pre-cleared with 100 μl of Protein G-sepharose slurry. Immunoprecipitation was carried out by adding 4 μg of K48 and K63-linkage specific polyubiquitin antibody (Newton, Matsumoto et al. 2008), or human anti-E25 (IgG isotype-matched control) to cleared cell lysates. Samples were rotated for 20 hours at 4^{O}C followed by a 1-hour incubation with 40 μl of protein G-sepharose slurry to capture antigen-antibody complexes. The protein G beads were washed twice with DISC lysis buffer (without urea) and once with PBS. Each wash was carried out for 10 minutes while rotating at 4^{O}C. Protein complexes bound to the beads were eluted with 25 μl of 2 x SDS sample buffer (50 mM Tris pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol, 0.01% (v/v) bromophenol blue, 0.05% (v/v) 2-mercaptoethanol). Input and IP samples were interrogated by immunoblot (as described in section 2.7).

2.5 Propidium Iodide (PI) uptake by flow cytometry

293T cells were seeded on a 24-well plate at cell density of 2 X 10^5 cells per well and transfected using lipofectamine 2000 (Thermo Fisher Scientific) with IL-1β and caspase-1 constructs at various concentrations, as indicated in the results (Results 3.2.9 and 3.2.10). After 48 hours of transfection, cells were harvested using trypsin (Life Technologies). Cells were washed and suspended in 200-500μl DMEM + 10% FCS medium containing 1 μg/ml of PI. Uptake of PI was measured on a FACSCanto (BD Immunocytometry Systems) and data analysed using FlowJo software version 7.6.5.

2.6 Enzyme linked Immunosorbent assay (ELISA)

Cell supernatants, serum and peritoneal fluid samples were collected after specified treatments. Mouse IL-1β (R&D Systems; DY401), mouse TNF (Ebioscience; 88-7324) and IL-6 (Thermo Fisher Scientific; BMS603-2) were performed according to manufacturer's instructions.

2.7 Immunoblot analysis

2.7.1 Preparation of cell lysates and supernatants for Immunoblot analysis

After centrifugation (10,000 g for 5 minutes) of samples, cell supernatants were collected and diluted with 2x SDS-PAGE sample buffer (50mM Tris pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol, 0.01% (v/v) bromophenol blue, 0.05% (v/v) 2-mercaptoethanol). Adherent cells were lysed and harvested directly in 1x SDS-PAGE buffer.

2.7.2 Immunoblotting

All lysates and supernatant samples were boiled for ~10 minutes prior to immunoblotting. NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen) were used to separate the samples, and proteins subsequently transferred onto nitrocellulose (Amersham) membranes. Membranes were blocked in 5% skim milk/0.1% Tween/PBS for 1 hour at room temperature. Ponceau S staining [0.2% (w/v) Ponceau S, Sigma Aldrich; P3584; 5% acetic acid) was performed to assess successful transfer of proteins to membranes for 1 minute, followed with a couple of washes with reverse osmosis water to remove excess staining. Membranes were photographed and destained with a couple of washes in PBS/0.1% Tween for 5 mins each. Primary antibodies diluted in 5% skim milk/0.1% Tween/PBS were incubated on a shaker overnight at $4^{O}C$. Incubations of membranes with secondary antibodies conjugated to Horse radish peroxidase (HRP) diluted in 5% skim milk/0.1% Tween/PBS were performed at room temperature for 1-2 hours. Membranes were subsequently washed 3-4 times between antibody incubations (0.1% Tween/PBS), with each wash carried out for 10 minutes at room temperature. Enhanced chemiluminescence (ECL) reagent (Amersham) was used to develop all membranes. Imaging was done using both X-ray film (Cytvia; formerly GE Healthcare Life Sciences) and ChemiDoc^{TM} Touch imaging system (BIO-RAD).

Table 2.6 List of antibodies

Antibody	Dilutions	Species reactivity	Source	Catalogue No	Company
IL-1β (full length and p17 fragment)	1/1000	Mouse	Goat	AF-401-NA	R&D
NLRP3	1/1000	Human, Mouse	Mouse	AG-20B-0014	Adipogen
Caspase-1 (full length and p20 fragment)	1/1000	Mouse	Mouse	AG-20B-0042	Adipogen
Caspase-1 (full length and p10 fragment)	1/500	Mouse	Rabbit	Sc-514	Santa Cruz Biotechnology
ASC	1/1000	Human, Mouse, Rat	Rabbit	Sc-22514-R	Santa Cruz Biotechnology
Ubiquitin	1/1000	All	Rabbit	3933S	Cell Signalling
K48-linked Polyubiquitin, clone Apu 2.07	1/1000	Human, Mouse, Others	*E. coli*	gift	Genentech
K63-linked Polyubiquitin, Apu 3.A8	1/1000	Human, Mouse, Others	*E. coli*	gift	Genentech
Anti-E25 Isotype	1/1000	Human	*E. coli*	gift	Genentech
β-Actin	1/10,000	Human, Mouse, others	Mouse	A-1978	Sigma
Goat Anti-Mouse Ig-Horseradish peroxidase	1/10,000	Mouse	Goat	1010-05	Southern Biotech
Goat Anti-Rabbit Ig- Horseradish peroxidase	1/10,000	Rabbit	Goat	4010-05	Southern Biotech
Horseradish peroxidase conjugated anti-goat IgG	1/10,000	Goat	Rabbit	HAF017	R&D
Mcl-1 (D35A5)	1/1000	Human, Mouse, Monkey	Rabbit	5453S	Cell Signalling

2.8 Generation of IL-1β K133R CRISPR KI mutant mice

Knock-in mutant mice harbouring a lysine to arginine mutation at amino acid position 133 of IL-1β (IL-1β K133R**)** was created by the MAGEC facility, WEHI. To generate *IL-1β*$^{K133R/K133R}$ CRISPR mice, sgRNA targeting oligonucleotide donor sequence (as shown in Results chapter-4.2.1) was injected into the cytoplasm of fertilized one-cell stage embryos of WT C57BL/6 breeders. Viable founder mice were identified by next-generation sequencing. To remove potential sgRNA off-target hits, IL-1β K133R CRISPR KI founder mice were backcrossed onto C57BL/6 animals for 2 generations. Since F0 mice are often mosaic, next-generation sequencing was performed on F1&F2 offspring, to confirm the transmission of the targeted allele (as described in 2.8.1). Heterozygous *IL-1β*^{K133R} mice were then inter-crossed to generate WT littermates and homozygous *IL-1β*$^{K133R/K133R}$ knockin mouse lines.

For routine genotyping, mouse tail clips were digested in tail lysis buffer (Viagen, #102-T) and 5 mg/ml proteinase K (Worthington) at 55^{O}C overnight in a shaker. Tail samples were then heated at 80^{O}C for an hour to inactivate the proteinase K and subsequently extracted DNA used for PCR genotyping following the conditions stated below. Genotyping PCRs were run for each tail sample with 2 sets of primers (in Table 2.7). Wildtype allele PCRs were run with primers #14 (recognising the WT IL-1β sequence) and #16 (in Table 2.7). KI allele PCRs were run with primers #15 (recognising the KI IL-1β sequence, KI mutation in red) and #16 (in Table 2.7).

Table 2.7 List of genotyping primers

Primer No.	Primer name	Primer sequence
#14	IL-1β Wildtype forward	5’ TCCGAGATGAACAACAAAAAAGC 3’
#15	IL-1β K133R forward	5’ TCCGAGATGAACAACAAAGAAGT 3’
#16	IL-1β Wildtype reverse	5’ GTTATCCCTATATGACTGGGCTGG 3’

Genotyping PCR Reaction Mix

2X GoTaq - 10 µl

Forward primer (10 µM) - 1 µl

Reverse primer (10 µM) - 1 µl

DNA - 2 µl

Nuclease-Free water to final volume of 20 µl

Genotyping thermocycling conditions were the same as described in section 2.2.1.

2.8.1. Next-generation sequencing

Amplification of gDNA was performed with primers (#17&18 in table 2.8) carrying specific indexing overhang (OH) sequences at their 5' end (in blue in table 2.8). PCR conditions for gDNA amplification are described below. PCR products were purified from the reaction mix using 20 µl Agencourt AMPure XP beads (Beckman Coulter, A63880) on a magnetic rack, followed by two washes with 70% ethanol and resuspended in 30 µl nuclease-free water. Purified PCR products were then indexed using an indexing PCR in a 96-well format with 8 forward and 12 reverse primers (#19 &20 in table 2.8, provided by the WEHI Genomics facility). Indexing PCR conditions are also described below. A 2200 TapeStation was used to assess the quality of all PCR products as per manufacturers instructions (D1000 protocol, Agilent Technologies). 10 µl of indexing PCR products were pooled together and purified again using 35 µl Agencourt AMPure XP beads and eluted into 30 µl nuclease-free water. Success of the purification was assessed again using the 2200 TapeStation and the pool was diluted to 12 pM for sequencing on the MiSeq platform (Illumina). To perform single reads, a 300-cycle kit was used for 281 cycles, followed by second index read for 44 cycles. Sequencing was performed by the WEHI Genomics facility.

Table 2.8 List of next-generation sequencing primers[4]*

Primer No.	Primer name	Primer sequence
#17	IL-1β K133R OH forward	5' **GTGACCTATGAACTCAGGAGTC** gAGCCCATCCTCTGTGACTC 3'
#18	IL-1β K133R OH reverse	5' **CTGAGACTTGCACATCGCAGC** aTGACTGGGCTGGAAAAATG 3'
#19	Indexing forward primer targeting OH primer above	5'CAAGCAGAAGACGGCATACGAGATCCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT*NNNNNNNN*GTGACCTATGAACTCAGGAGTC 3'
#20	Indexing reverse primer targeting OH primer above	5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT*NNNNNNNN*CTGAGACTTGCACA TCGCAGC 3'

gDNA PCR Reaction Mix

2X GoTaq - 10 µl

Forward primer (10 µM) - 0.5 µl

Reverse primer (10 µM) - 0.5 µl

DNA - 1 µl

Nuclease-Free water to final volume of 20 µl

gDNA Thermocycling conditions

1. 95 °C - 3 minutes
2. 95 °C - 30 seconds
3. 60 °C - 30 seconds
4. 68 °C - 30 seconds

Steps 2-4 cycled 18 times

5. 68 °C - 7 minutes

[4] * In primers #19 & #20 *NNNNNNNN* denotes unique Index sequences, 12 sequences used for #19 and 8 sequences for #20.

Indexing PCR Reaction Mix

2X GoTaq - 10 µl
Forward primer (10 µM) - 0.5 µl
Reverse primer (10 µM) - 0.5 µl
Purified gDNA PCR product - 10 µl
Nuclease-Free water to final volume of 21 µl

Indexing Thermocycling conditions

1. 95 °C - 3 minutes
2. 95 °C - 15 seconds
3. 60 °C - 30 seconds
4. 72 °C - 30 seconds

Steps 2-4 cycled 25 times

5. 72 °C - 7 minutes

2.9 *In vivo* endotoxin model

Female IL-1β K133R CRISPR KI mice and littermate controls at 6-8 weeks of age were injected intraperitoneally with 100 µg of LPS (Ultra-pure, Invivogen). Blood samples were collected at baseline via retro-orbital venous plexus and at 2 hours via a cardiac bleed (after CO_2-induced asphyxia). Peritoneal lavages were collected by injection of 1.5 ml of 5 mM EDTA diluted in PBS into the peritoneal cavity. Serum and peritoneal fluid cytokine levels were measured by ELISA (as described in 2.6). Peritoneal fluid was analysed by immunoblot (as described in 2.7).

2.10 Statistical analyses

The Mann-Whitney U-test was used to compare *in vivo* cytokine levels between IL-1β K133R CRISPR KI mice and C57BL/6 mice. For each test, p values <0.05 were considered statistically significant.

Chapter 3: IL-1β is ubiquitinated on Lysine 133 and phosphorylated on Serine 134

3.1 Introduction

Inflammasome activation and subsequent IL-1β activity is required for the clearance of various pathogens, whereas over activation of inflammasomes and excess IL-1β secretion can promote cancer, and inflammatory driven diseases, such as atherosclerosis (Menu and Vince 2011, Strowig 2012). Therefore, the regulation of inflammasome signalling and IL-1β secretion is key for maintaining good health.

Post-translational protein modification can exquisitely control the activity of innate immune cellular signalling pathways, including the inflammasomes. In particular, as covered in Chapter 1.7, NLRP3 inflammasome components are regulated by various post-translational modifications, including ubiquitination and phosphorylation. However, there are inconsistencies in the literature about the function of NLRP3 ubiquitination. For example, ubiquitination of NLRP3 has recently been suggested to target it for proteasomal degradation, contrasting to previous studies suggesting that NLRP3 ubiquitination does not target it to the proteasome (Juliana 2012, Py 2013, Song, Liu et al. 2016). Given the large number of lysines present in the NLPR3 sequence that may be targeted for ubiquitination, the number of ubiquitin E3 ligases implicated in regulating NLRP3 function (Han, Lear et al. 2015, Yan 2015, Song, Liu et al. 2016, Kawashima, Karasawa et al. 2017, Humphries, Bergin et al. 2018), and the diversity of ubiquitin chains (both K63- and K48-linked) detected on NLRP3 (Py, Kim et al. 2013, Kawashima, Karasawa et al. 2017), it is likely that the decoration of NLRP3 by both degradative and non-degradative ubiquitin chains plays important roles in regulating NLRP3 signalling.

Similar to NLRP3, other inflammasome components, including ASC (Hara 2013, Martin 2014, Rodgers 2014, Guan 2015) and caspase-1 (Labbe, McIntire et al. 2011, Van Opdenbosch 2014), have been reported to undergo post-translational modification. Upon NLRP3 activation, ASC was reported to be ubiquitinated by the Linear Ubiquitination Assembly Complex (LUBAC), and this was required for NLRP3 dependent IL-1β secretion (Rodgers 2014). Moreover, TNFR-associated factor 3 (TRAF3), an ubiquitin E3 ligase, has been suggested to

promote NLRP3 activity by ubiquitinating ASC on Lysine 174 (K174) with predominantly K63-linked ubiquitin chains (Guan 2015). Phosphorylation of ASC by C-Jun N-terminal kinases (JNKs) and Spleen tyrosine kinases (Syk) are reportedly also required for ASC speck formation and subsequent caspase-1 activation (Hara 2013). On the other hand, IκB kinase α (IKKα) kinase was reported to phosphorylate ASC on Serine 193 (S193) and Serine 16 (S16), and this event was critical for the interaction of ASC with IKKα kinase in the nucleus to prevent ASC translocation into the cytoplasm and NLRP3 inflammasome assembly (Martin 2014).

Thus, post-translational modifications are critical in regulating the inflammasome machinery, including NLRP3, ASC, and possibly caspase-1. However, the targeting of cytokines themselves, including IL-1β, and the impact on their function and secretion is less clear, and is the major aim of this chapter.

3.1.1 Chapter overview

To define whether IL-1β is targeted for post-translational modification, Mass spectrometry analysis of purified IL-1β was carried out. Results demonstrated that IL-1β can be both ubiquitinated on lysine 133 (K133) and phosphorylated on serine 134 (S134). The direct targeting of IL-1β for ubiquitination was validated using an *in vitro* assay using recombinant ubiquitinating enzymes (E1/E2 and E3), and the endogenous ubiquitination of IL-1β documented to occur in macrophages via purification of the ubiquitinated proteome. Further assays demonstrated that both TLR4 and TLR2 mediated inflammasome priming of macrophages suffice to trigger the ubiquitination of IL-1β.

Several IL-1β point mutants were created to investigate the functional impact of ubiquitination and phosphorylation on IL-1β cleavage and secretion. The mutation of IL-1β Lys 133 to Alanine (K133A), obliterated its ability to be secreted following caspase-1 activation, whereas phospho-mimetic or phospho-dead IL-1β Ser 134 mutants had no impact on IL-1β secretion.

Structural modelling of IL-1β demonstrated an electrostatic interaction between K133, the site targeted for ubiquitination, and aspartic acid 129 (D129) to form a salt bridge. Therefore, the effect of ubiquitination of K133 on electrostatic interactions with aspartic D129, and on

caspase-1-mediated proteolysis of IL-1β were examined. Interestingly, mutation of IL-1β Lysine 133 to Arginine (K133R), to generate a ubiquitination defective mutant at this site, did not have any impact on caspase-1 cleavage or IL-1β release. However, abrogation of the salt bridge between K133 and D129 of IL-1β curtailed caspase-1 processing.

Finally, additional Mass spectrometry experiments on endogenous purified IL-1β identified interacting ubiquitin E3 ligases that might target IL-1β for modification. Future studies will include creating gene knock out cell lines of the IL-1β interacting ubiquitin E3 ligases to define how their loss impacts IL-1β ubiquitination and its ability to be activated and secreted.

Chapter 3.2 Results:

3.2.1: TLR4-mediated inflammasome priming with LPS induces IL-1β and NLRP3 expression and their ubiquitination

BMDMs were treated with the TLR4 activator LPS, which induces transcriptional upregulation of NLRP3 and precursor IL-1β, but does not cause inflammasome activation (termed inflammasome priming) (Hiscott 1993, Bauernfeind 2009). Subsequently, the ubiquitination status of NLRP3 inflammasome components was examined using TUBEs. TUBEs are agarose beads coupled with tandem Ubiquitin Binding Domains (UBDs) that purify polyubiquitinated proteins of all chain linkages. Purified polyubiquitinated proteins, and cell lysate input, can then be analysed by immunoblotting to discern potentially important modifications on isolated protein complex components. As expected, upon LPS treatment, control input cell lysate demonstrated robust induction of NLRP3 and precursor IL-1β expression, while there was no change in the expression of ASC or caspase-1 (Fig. 3.2.1). Notably, TUBE purification resulted in the efficient purification of ubiquitinated proteins (left panel in Fig. 3.2.1). The detection of high molecular weight laddering of NLRP3 and IL-1β in the TUBE purified samples demonstrated that endogenous precursor IL-1β and NLRP3 were ubiquitinated in response to 3 hours of LPS stimulation, which increased further after 6-8 hours. In contrast, the ubiquitination of caspase-1 and ASC was not detected (Fig. 3.2.1). It should be noted that unmodified IL-1β and NLRP3 were co-purified with their poly-ubiquitinated forms (Fig. 3.2.1), which likely represents complexing of unmodified IL-1β and NLRP3 with their ubiquitylated species. Use of an agarose bead-only control (lacking UBDs) demonstrated the specificity of the TUBE approach, as no purified ubiquitin signal of either NLRP3 inflammasome components or the ubiquitinated proteome was detected when cell lysates were incubated with agarose control beads (agarose control lane in Fig.3.2.1). Overall, these results suggest that priming BMDMs with LPS leads to the expression of endogenous precursor IL-1β and NLRP3 as well as their ubiquitination.

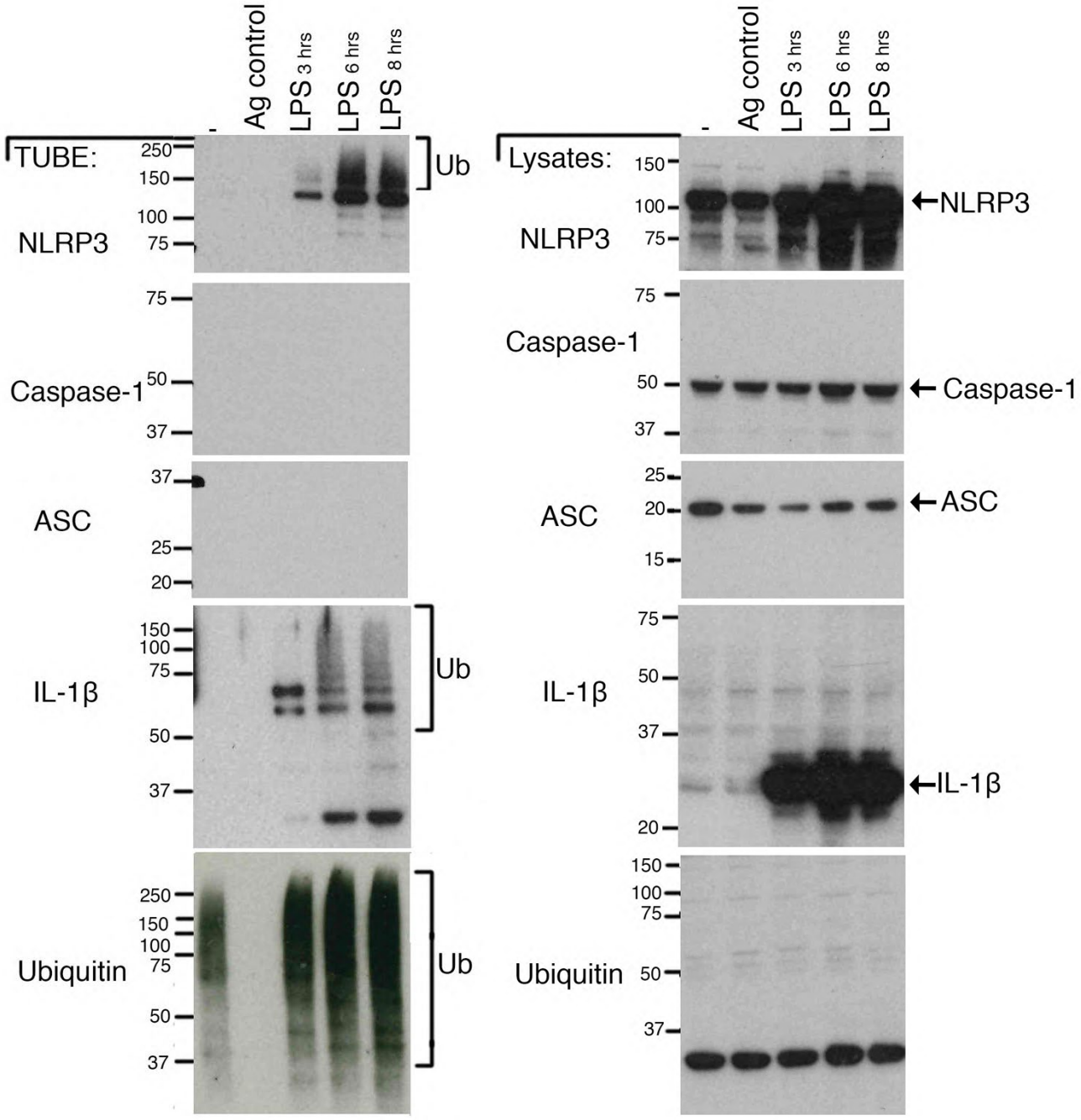


Figure 3.2.1 TLR4-mediated inflammasome priming with LPS induces precursor IL-1β and NLRP3 expression and their ubiquitination.

WT BMDMs were treated with 100 ng/ml LPS for the indicated times and ubiquitinated proteins purified by TUBE and analysed by immunoblot. Data is representative of three independent experiments. Ag-Agarose control; Ub- Ubiquitinated laddering of indicated protein; LPS-Lipopolysaccharide.

3.2.2: TLR2-mediated inflammasome priming with Pam3Cys induces precursor IL-1β and NLRP3 expression and their ubiquitination

To understand if the ubiquitination of endogenous IL-1β and NLRP3 was specific to TLR4 signalling, or if other TLR ligands could also induce ubiquitination of these proteins, BMDMs were treated with the TLR2 ligand, Pam_3Cys, over time. Subsequently, the ubiquitination of precursor IL-1β and NLRP3 was examined by TUBE purification of the ubiquitinated proteome and immunoblot. Notably, modification of precursor IL-1β and NLRP3, but not ASC or caspase-1, with ubiquitin chains was detected after 8 hours of Pam3Cys treatment (Fig. 3.2.2), which was similar to the IL-1β and NLRP3 ubiquitination pattern observed in response LPS stimulation (Fig. 3.2.1). Thus, ubiquitination of endogenous precursor IL-1β and NLRP3 can be induced with different inflammasome-priming TLR ligands.

Next, the ubiquitination status of NLRP3 and IL-1β was examined upon NLRP3 inflammasome activation induced by ATP or nigericin treatment (Figure 3.2.2). Intriguingly, NLRP3 ubiquitination decreased following both ATP or nigericin treatment (Fig. 3.2.2), consistent with reports suggesting that the deubiquitination of NLRP3 promotes its activation (Py 2013). In contrast, precursor IL-1β ubiquitination increased following 20 minutes of ATP treatment, while at later time points both ATP and nigericin decreased IL-1β ubiquitination. Similar to TLR priming, ATP or nigericin stimulation did not result in any detectable caspase-1 or ASC ubiquitination (Fig. 3.2.2). Of note, decreased ubiquitination of NLRP3 and precursor IL-1β at later time points (40 mins) with ATP or nigericin stimulation is likely due to cell death. However, it is possible NLRP3 and precursor IL-1β are also actively targeted for deubiquitination at later times.

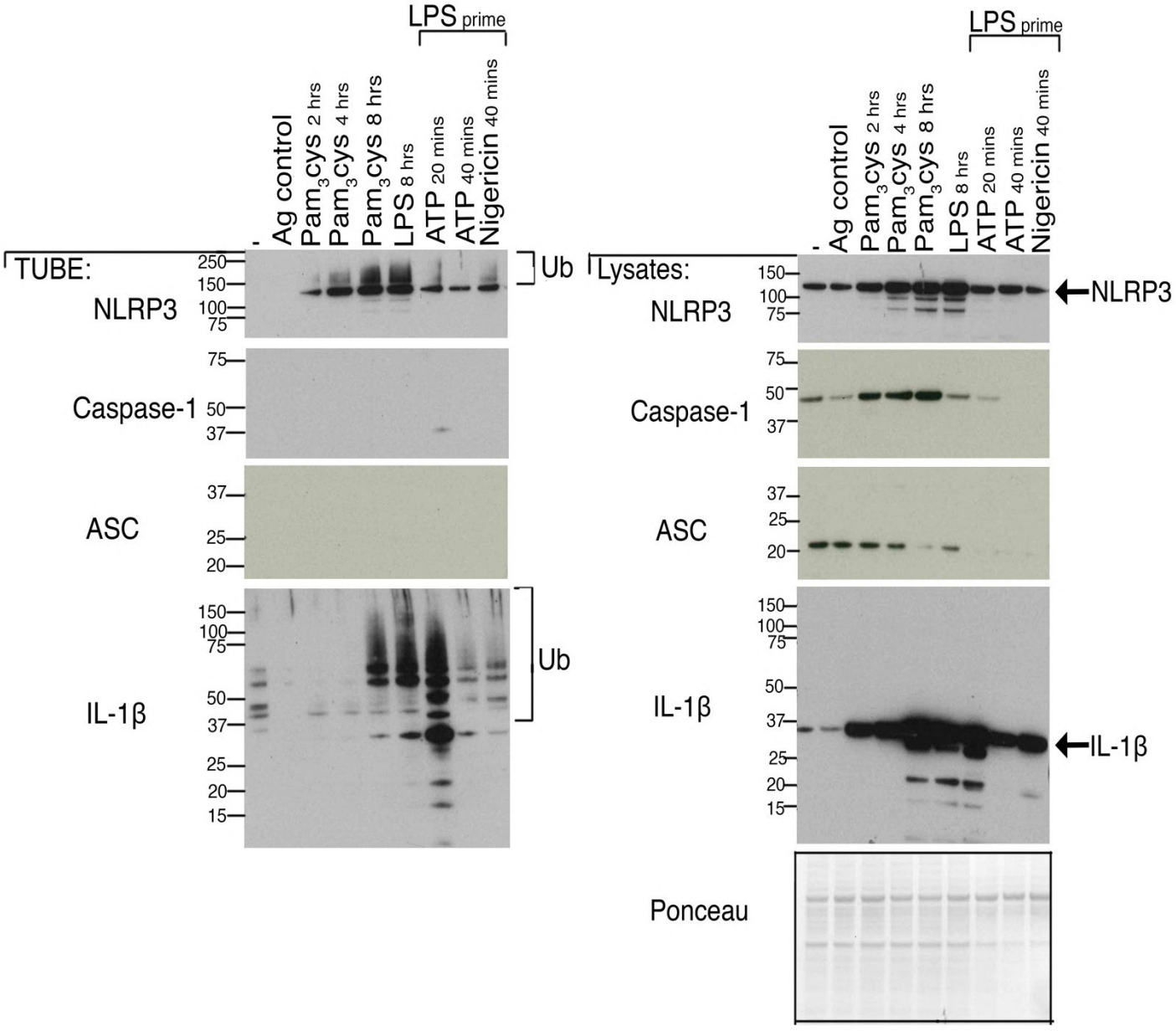


Figure 3.2.2 TLR2-mediated inflammasome priming with Pam3Cys induces precursor IL-1β and NLRP3 expression and their ubiquitination.

Wildtype **(**WT) BMDMs were treated with either 1 μg/ml of Pam3cys or 100 ng/ml LPS for the indicated times. After LPS priming (100 ng/ml, 7 hours), NLRP3 inflammasome activation was also induced by ATP (5 mM) or nigericin (10 μM) treatment, as indicated. Ubiquitinated proteins were purified by TUBE and analysed by immunoblot. Ponceau staining shows protein input loading control. Results represent one of three independent experiments.

3.2.3: TLR and ATP stimulation induced IL-1β ubiquitination independent of NLRP3 inflammasome components

To examine if the observed increased ubiquitination of IL-1β with TLR and ATP treatment (Figure. 3.2.2) is due to inflammasome signalling, or relates to ubiquitin-mediated proteasomal degradation, TUBE experiments were carried out in BMDMs that lack inflammasome components. BMDMs from wildtype (WT), *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ and *Caspase-1*$^{-/-}$ mice were primed with LPS for 3 hours and treated with canonical inflammasome stimuli ATP for 20 mins. Ubiquitinated proteins were purified from cell lysates by TUBE. Notably, BMDMs lacking NLRP3, ASC and Caspase-1 showed increased IL-1β ubiquitination with LPS and ATP treatment similar to WT cells (Figure 3.2.3), suggesting that the NLRP3 activator ATP, can induce IL-1β ubiquitination independent of inflammasome activity. Caspase-1 modification is noted in TUBE samples across all conditions in WT, *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ BMDMs, suggesting possible mono-ubiquitination (Figure 3.2.3) and is inconsistent with previously shown data (Figure 3.2.1). This inconsistency could be due to the lysis of BMDMs in non-denaturing conditions and therefore unable to dissociate any rapidly forming complexes of non-specific ubiquitinated proteins that bind with caspase-1.

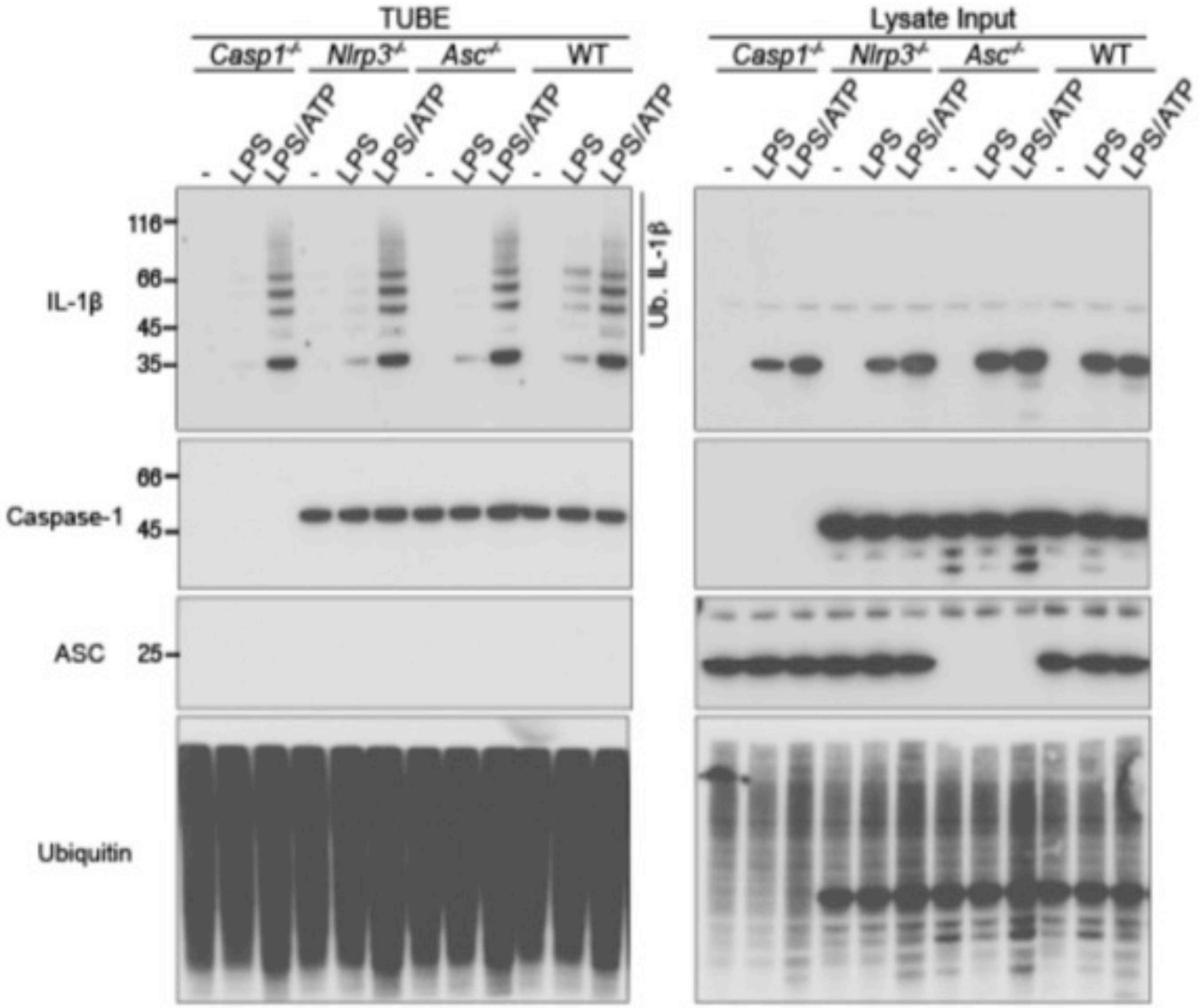


Figure 3.2.3 LPS and ATP induce IL-1β ubiquitination independent of NLRP3 inflammasome components.

BMDMs from WT, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$ and *Caspase-1*$^{-/-}$ were primed with LPS (50 ng/ml) for 3 hours and stimulated with ATP for 20 minutes. TUBEs were used to purify ubiquitinated proteins. Immunoblots were performed on cell lysates and TUBE isolated ubiquitylated proteins for the indicated proteins. One of 2 experiments.

3.2.4: *In vitro* ubiquitination of IL-1β

LPS induces IL-1β expression but also dramatically alters macrophage signalling, metabolism and transcriptional networks. Therefore, to test if expression of IL-1β alone is sufficient to cause its ubiquitination, 293T cells were transfected with N-terminal FLAG epitope tagged IL-1β with or without a myc tagged ubiquitin construct and subjected to immunoprecipitation with a myc antibody to purify the ubiquitinated proteome. Immunoblot analysis showed that IL-1β is modified with myc tagged ubiquitin (Figure 3.2.4a), and thereby suggests that the expression of IL-1β alone, in the absence of any TLR stimulus, can be targeted for ubiquitination.

Next, an *in vitro* ubiquitination assay was performed to further validate that IL-1β is indeed modified by ubiquitin chains. N-terminal FLAG epitope tagged IL-1β was expressed in 293T cells and purified with FLAG beads. Subsequently, purified FLAG-IL-1β was incubated with recombinant E1 enzyme preloaded with Ubiquitin, UbcH5a E2, E3 enzymes (cIAP1/UBE3A) and ATP in a ubiquitin assay buffer (40 mM Tris-HCL pH7.5, 10 mM $MgCl_{2,}$, 0.6 mM DTT (Dithiothreitol)) at 37ºC for 90 mins and the reaction stopped by adding SDS-PAGE loading dye. Immunoblotting demonstrated that, as expected, in the absence of an E3 ligase IL-1β ubiquitination was not detected (Lane 2, Figure 3.2.4b). However, IL-1β was strongly modified by ubiquitin chains in the presence of the E3 ligase cIAP1, thereby proving that IL-1β can be directly targeted for ubiquitin modification (Lane 4, Figure 3.2.4b). A different E3 ligase, UBE3A, failed to ubiquitinate IL-1β (lane 3, Figure 3.2.4b), which likely reflects that UBE3A either cannot recognise the E2 enzyme UbcH5a, or IL-1β itself.

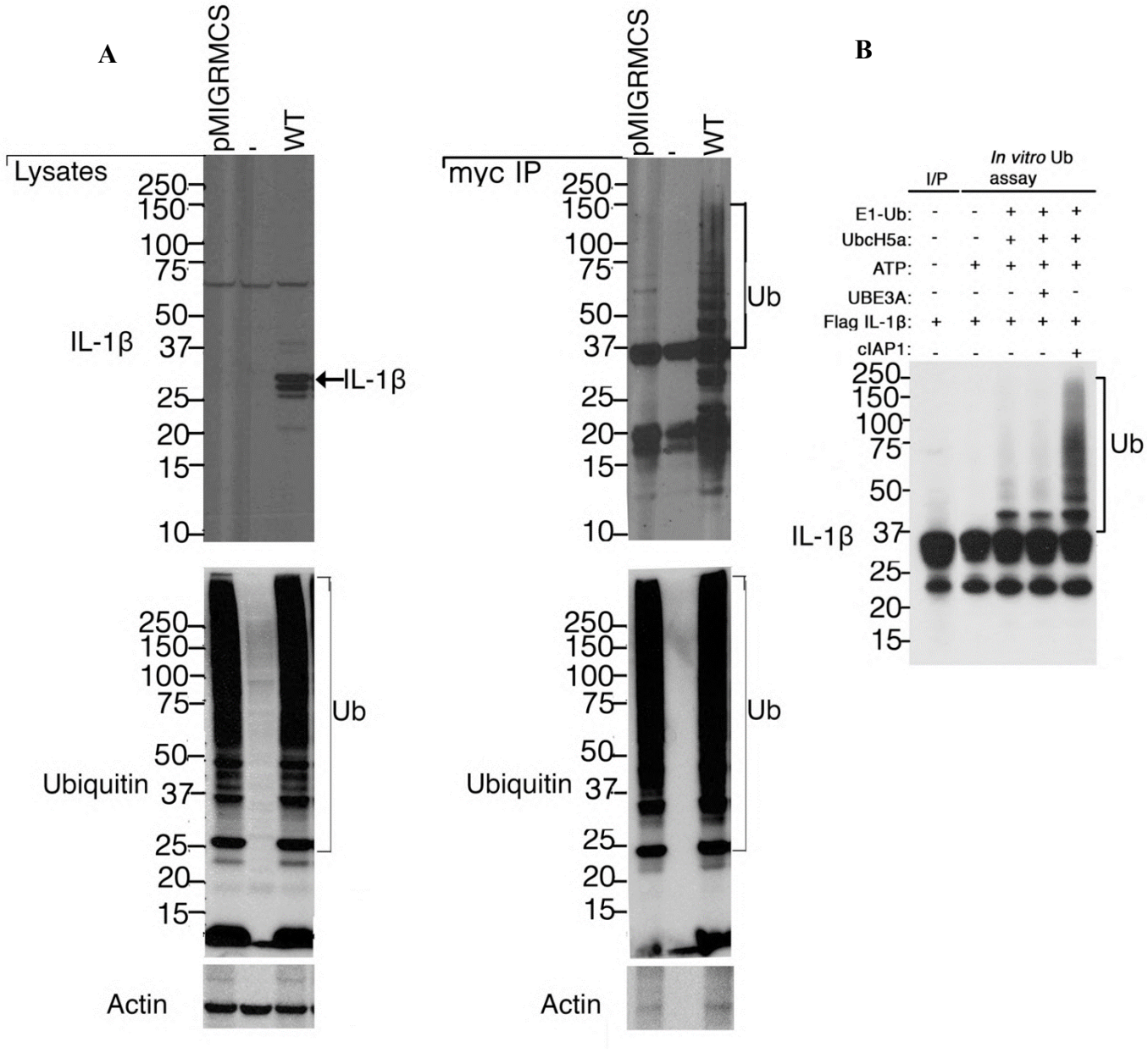


Figure 3.2.4 IL-1β can be ubiquitinated in the absence of TLR priming.

A) Immunoblot analysis of 293T cells transfected for 24 hours with N-terminal flag tagged IL-1β and myc-tagged ubiquitin or empty vector control, pMIGRMCS and subjected to immunoprecipitation with myc antibody. B) Lane 1 indicates Flag-IL-1β resuspended in ubiquitination buffer alone. Second and third lane is ubiquitination of IL-1β with E1 conjugated with Ubiquitin and UbcH5a, E2 enzymes combined and UBE3A E3 enzymes respectively. Lane 4 is ubiquitination reaction mix containing E1 conjugated with Ubiquitin, UbcH5a E2 enzyme and E3 ligase cIAP1.

3.2.5: Mature IL-1β is unlikely to be ubiquitinated in response to NLRP3 activation.

To examine if the secreted bioactive IL-1β p17 fragment is targeted for ubiquitination, the ubiquitinated proteome was purified using TUBEs from BMDM lysate and supernatant following nigericin-induced NLRP3 activation. BMDMs were treated with LPS or with LPS and nigericin, to induce either precursor IL-1β expression, or NLRP3 activation and IL-1β cleavage and secretion, respectively. Nigericin treatment was performed using serum free media (OptiMEM) to remove contaminating ubiquitinated proteins likely to be present in L929 conditioned media containing FBS. As expected, LPS resulted in precursor IL-1β expression, while nigericin treatment triggered IL-1β cleavage and secretion of the p17 bioactive fragment (Figure 3.2.5; left panel). The significant loss of anti-ubiquitin signal in the cell lysates and supernatants following TUBE purification (post-TUBE samples, Fig. 3.2.5, right panel), demonstrated that the majority of ubiquitinated proteins were successfully captured.

The concentrated (~200 fold) TUBE purified ubiquitin proteome supernatant, relative to supernatant input and post-TUBE supernatant, showed that ubiquitinated precursor IL-1β was released into the supernatant upon nigericin treatment, and is likely to reflect nigericin-induced pyroptotic cell death. The concentrated TUBE purified ubiquitin proteome supernatant also contained a small amount of IL-1β p17 following nigericin treatment. However, no modifications, indicative of ubiquitination, were detected directly above the IL-1β p17 fragment, unlike prominent precursor IL-1β modification. Moreover, the majority of nigericin-induced IL-1β p17 fragment was present in the input cell lysates/supernatant and post-TUBE lysates/supernatant sample in equal amounts and, importantly, was not depleted by removal of the ubiquitinated proteome by TUBE (compare Fig. 3.2.5 left panel vs right panel). Therefore, this data suggests that the mature IL-1 β fragment is not ubiquitinated, and that ubiquitination of IL-1β is restricted to precursor IL-1β.

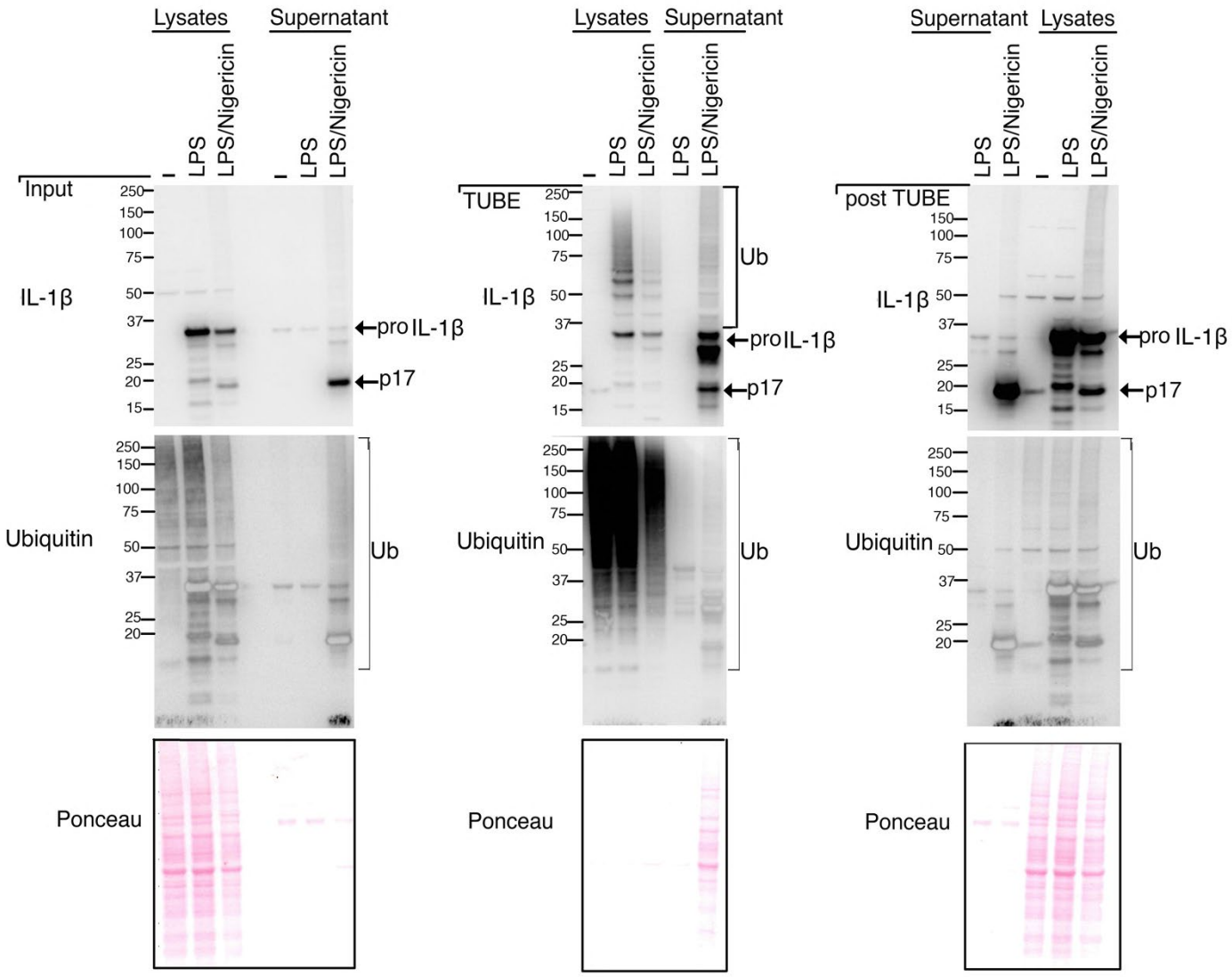


Figure 3.2.5 Mature bioactive IL-1β is unlikely to be ubiquitinated.

Wild type (WT) BMDMs were treated with 50 ng/ml of LPS for 3 hours alone in L929 conditioned media containing FBS or followed by nigericin (10 μM) treatment for 40 mins in 6 ml of OptiMEM media. Ubiquitinated proteins were purified by TUBE from cell lysates and 6 ml of supernatant. Input of the supernatant and post-TUBE samples in the immunoblot analysis shown here contains only 25 μl of the 6 ml supernatant sample. Left panel shows analysis of IL-1β and ubiquitin in cell lysates and supernatants pre-TUBE. Middle panel shows analysis of IL-1β and ubiquitin bound to the TUBE and the right panel shows the analysis of IL-1β and ubiquitin in cell lysates and supernatants post-TUBE. One of two independent experiments. Ponceau staining shows protein loading.

3.2.6: Precursor IL-1β is ubiquitylated on lysine 133 and phosphorylated on serine 134

To elucidate the function of IL-1β ubiquitination, and other potential post-translational modifications (PTM), I sought to identify the specific IL-1β residues targeted by Mass Spectrometry (MS) analysis. N-terminal FLAG-epitope tagged IL-1β cDNA was transfected into 293T cells and cells treated with proteasome inhibitor MG132 to inhibit IL-1β proteasomal degradation (Lane 2 in left panel, Fig 3.2.6a). Immunoprecipitation using a FLAG antibody was performed to purify IL-1β. Elutions were performed in 0.5% SDS with 5mM DTT and run on an SDS-PAGE gel (Right panel, Fig 3.2.6a). For in-gel digestion, the SDS-PAGE gel was stained with SYPRO ruby stain to visualise the proteins (Lower panel, Fig 3.2.6a). Protein bands corresponding to the molecular weight of precursor IL-1β (34 kDa) along with other visible protein bands were cut from the middle lane of the right, lower panel (indicated in red box, Fig 3.2.6a), and after trypsin digestion, samples were subjected to MS analysis.

Conjugation of mature ubiquitin to the target protein occurs between the last glycine (Gly) residue at the C-terminus of mature ubiquitin containing terminal residues Arg-Gly-Gly and the lysine residue of the target protein. Upon trypsinisation of ubiquitinated proteins, arginine is cleaved leaving the Gly-Gly dipeptide remaining on the lysine residue of the target protein. Trypsin digests of purified precursor IL-1β revealed a diglycine modified lysine (Lys-ε-Gly-Gly) signature on lysine 133 (K133) (Figure 3.2.6b). Further analysis revealed that precursor IL-1β serine 134 (S134), immediately adjacent to K133, was phosphorylated (Figure 3.2.6c). No other post-translational modifications were detected.

A comparison of precursor IL-1β sequences across different animal species, showed that IL-1β K133 is evolutionarily conserved among mouse, rat, human and rhesus IL-1β (Figure 3.2.6d). However, IL-1β S134 is conserved in mouse, human and rhesus, but not rat, IL-1β (Figure 3.2.6d).

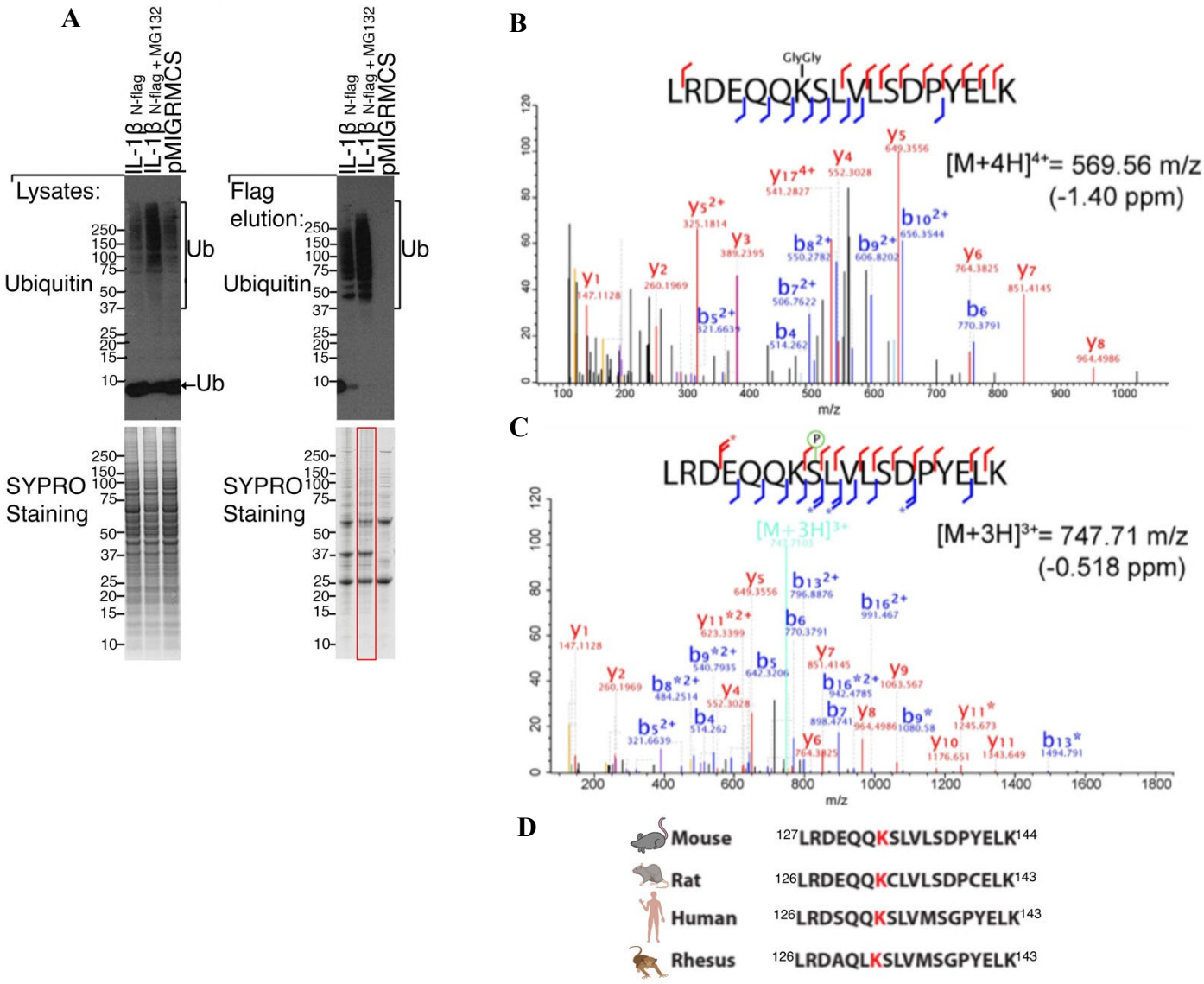


Figure 3.2.6 Precursor IL-1β is ubiquitinated on lysine 133 and phosphorylated on serine 134.

A) 293T cells grown in a 100 mm dish (at cell density of 5 x 10^6) were transfected with N-terminal FLAG tagged IL-1β for 48 hours followed by treatment with or without MG132 (20 μM) in the last 6 hours of the transfection. Immunoprecipitations were carried out using the FLAG antibody and FLAG elutions were performed in 0.5% SDS with 5 mM DTT, followed by immunoblot analysis and SYPRO ruby staining. B) Mass Spectrometry analysis of IL-1β showing diglycine remnant on K133. C) Mass Spectrometry analysis of IL-1β showing phosphorylation on S134. d) Amino acid sequence alignment of precursor IL-1β showing the conversation of the region containing K133 and S134 in different animals, as indicated.

3.2.7: Structural modelling of IL-1β K133 and S134

Posttranslational modifications (PTMs) can affect protein stability, interactions and signalling. Therefore, structural modelling of IL-1β was done to understand how the residues that are targeted for the identified PTMs might impact its structure, and consequently its function. Both PTM targeted residues of IL-1β, K133 and S134 are located downstream of the caspase-1 cleavage site within the p17 mature IL-1β fragment (Figure 3.2.7a and 3.2.7.b). In a secondary structure model of IL-1β, K133 and S134 are located at the linker region just before the start of the second beta strand of p17 fragment protein structure (Figure 3.2.7b). Remarkably, analysis of the solved structure of IL-1β (PDB: 2MIB), identified that IL-1β K133 forms a salt bridge with aspartate 129 (D129) (Figure 3.2.7c). Therefore, ubiquitination of K133 residue may impact the electrostatic interaction between IL-1β K133 and D129.

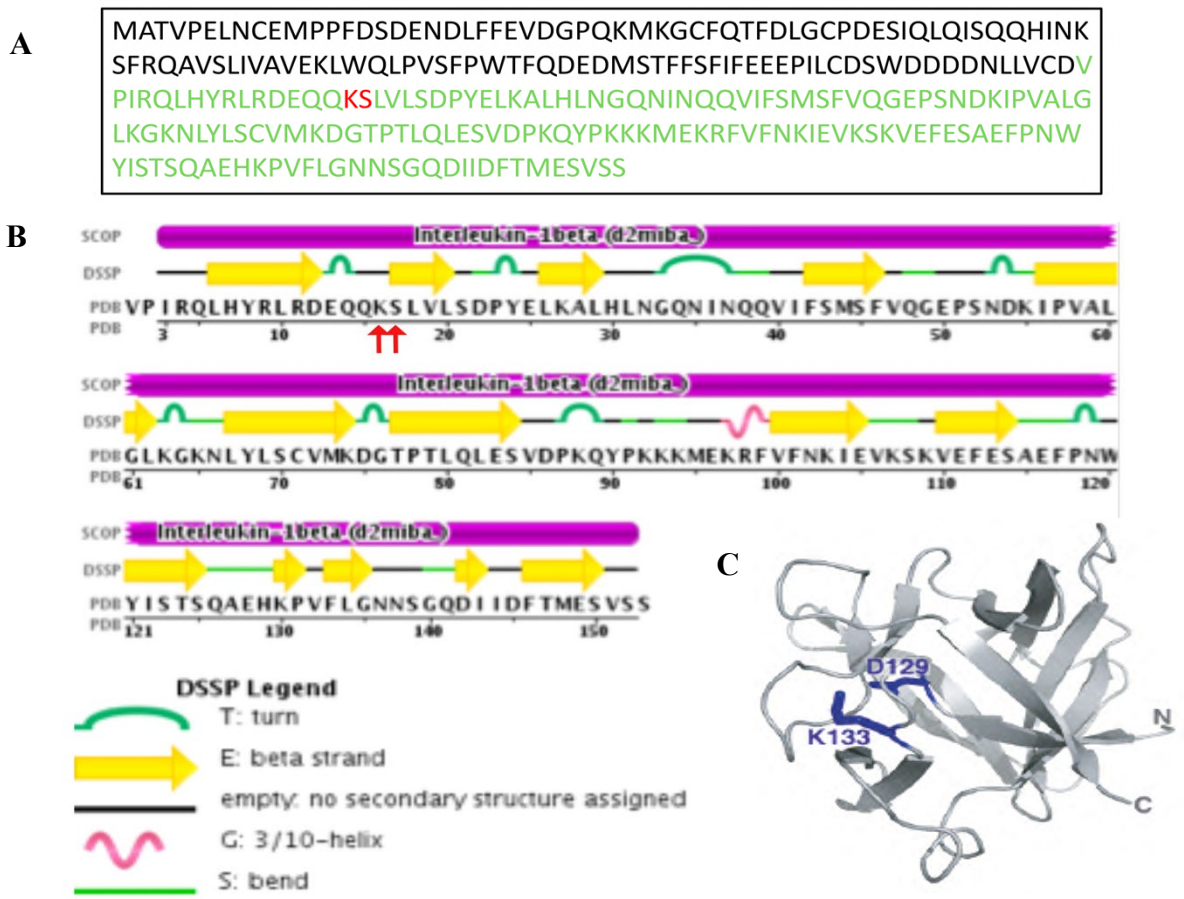


Figure 3.2.7 Structure modelling of IL-1β K133 and S134.

A) Complete mouse IL-1β sequence with p17 fragment highlighted in green and PTM sites identified highlighted in red. Caspase-1/8 cleavage site is aspartate 117 (D117) B) p17 fragment of mouse IL-1β protein secondary structure (www.rcsb.org) with PTM sites identified here highlighted by red arrows and C) Structure of mouse IL-1β (PDB: 2MIB) showing the electrostatic interaction between K133 and D129.

3.2.8: Analysis of IL-1β K133 and S134 ubiquitination in 293T cells.

To investigate the impact of post-translational IL-1β modifications identified using Mass Spectrometry (MS) analysis *in vitro*, IL-1β K133 and S134 mutants were generated. 293T cells were transfected with wildtype (WT) IL-1β, IL-1β K133A (unable to be ubiquitinated on K133), IL-1β S134A (phospho-dead) and IL-1β S134E (phospho-mimetic) cDNAs along with a myc-tagged ubiquitin construct. Ubiquitinated proteins from cell lysates were immuno-precipitated with a myc antibody and analysed by immunoblot. IL-1β K133A showed reduced ubiquitination when compared to WT IL-1β (Figure 3.2.8). In comparison, the IL-1β S134E mutant displayed reduced ubiquitination akin to the IL-1β K133A mutant (Figure 3.2.8), while the IL-1β S134A ubiquitination was similar to WT IL-1β (Figure 3.2.8). This suggests that S134 phosphorylation may limit IL-1β K133 ubiquitination.

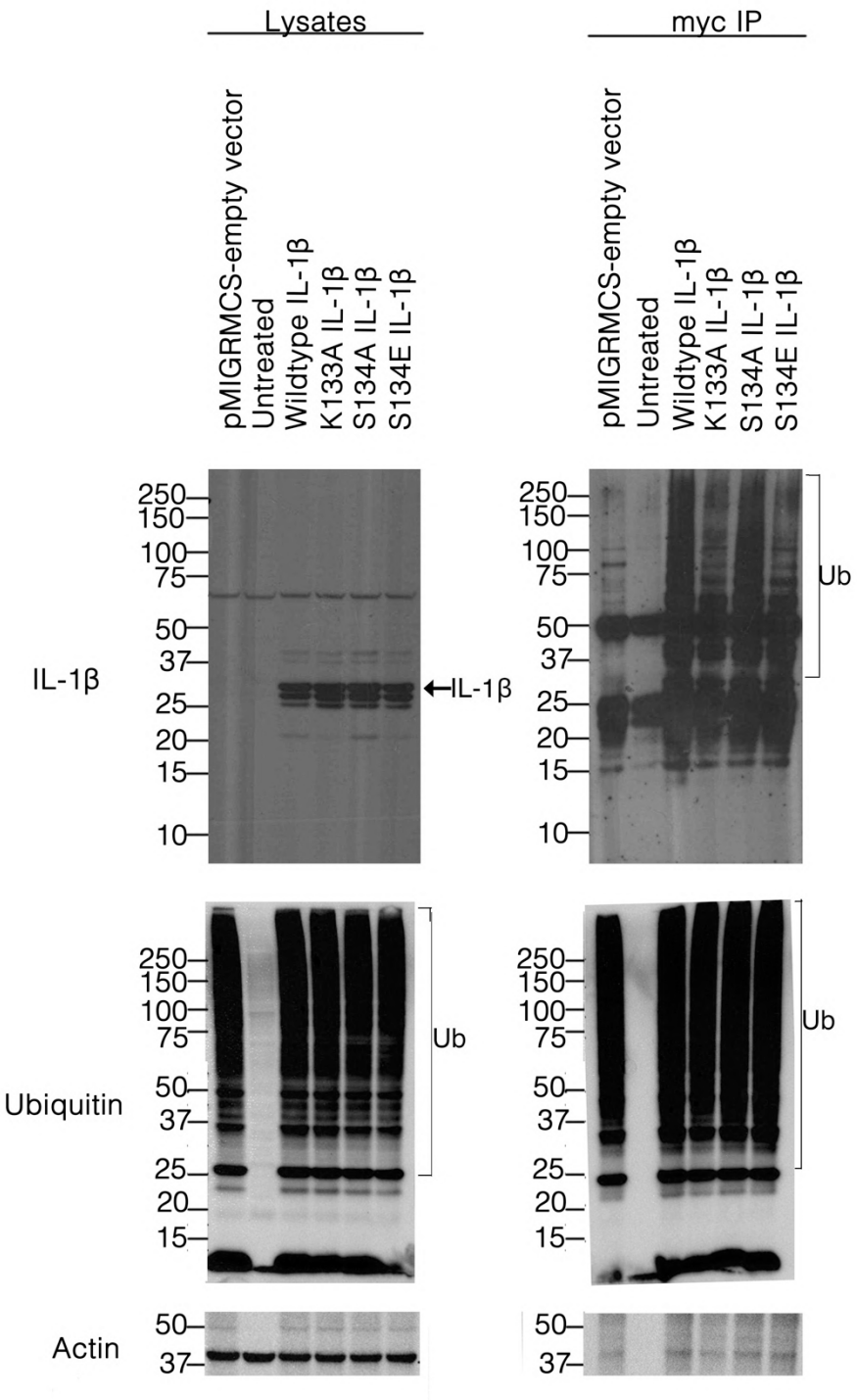


Figure 3.2.8 Analysis of K133 and S134 ubiquitination in 293T cells.

Immunoblot analysis of 293T cells transfected for 24 hours with N-terminal flag tagged WT IL-1β or the IL-1β mutants, as indicated, along with myc-tagged ubiquitin. Empty vector control was pMIGRMCS. Lysates were subjected to immunoprecipitation with myc antibody. One of two independent experiments. Actin depicts protein loading.

3.2.9: Expression of IL-1β K133A prevents caspase-1-mediated IL-1β cleavage, while IL-1β S134A has no impact on the proteolytic activity of caspase-1.

To examine the functional consequences resulting from K133 ubiquitination and S134 phosphorylation of IL-1β, 293T cells were transfected with wildtype (WT) IL-1β, IL-1β K133A, IL-1β S134A and IL-1β S134E. In parallel, caspase-1 was co-transfected (100-400 ng) into cells and the ability caspase-1 dependent IL-1β secretion quantified by ELISA. Co-transfection with caspase-1 and WT IL-1β resulted in robust IL-1β secretion (Figure 3.2.9a and b), which was comparable with IL-1β S134A (phospho-mutant) and IL-1β S134E (phospho-mimetic) release (Figure 3.2.9a and b). In contrast, caspase-1 failed to induce significant secretion of IL-1β K133A (Figure 3.2.9a and b). As expected, transfection of IL-1β or caspase-1 alone with the appropriate empty vector controls (pMIGRMCS and pcDNA3.1) did not result in IL-1β secretion.

To examine if caspase-1-mediated cell death might impact IL-1β release, 293T cells expressing IL-1β and caspase-1 were examined for propidium iodide (PI) uptake by flow cytometry. PI is a cell membrane impermeable stain which, upon loss of plasma membrane integrity and cell viability, intercalates with double stranded DNA. Notably, no significant increase in cell death was detected upon caspase-1 transfection. This is consistent with previous studies indicating that in certain cell types, including 293Ts, IL-1β secretion can occur independent of cell-death (Chen 2014, Conos, Lawlor et al. 2016, Evavold, Ruan et al. 2018).

Next, immunoblots were performed to test whether impaired IL-1β K133A secretion was due to defective caspase-1 cleavage into the bioactive p17 IL-1β fragment. This analysis revealed that both IL-1β S134A (phospho-mutant) and IL-1β S134E (phospho-mimetic) can be cleaved by caspase-1 and consequently the mature IL-1β p17 fragmented is secreted and detected in the supernatant (Figure 3.2.9b). In contrast, IL-1β K133A was resistant to cleavage by caspase-1 and, consistent with ELISA results (Figure 3.2.9a), mature IL-1β was not detected in the supernatant (Figure 3.2.9b). Collectively, these results show that IL-1β K133A prevents caspase-1 cleavage which therefore limits IL-1β secretion, and this may result from disrupted ubiquitination and/or the electrostatic interaction with D129 when lysine 133 is mutated to alanine. On the other hand, the phosphorylation of IL-1β on S134 is unlikely to impact its cleavage by caspase-1.

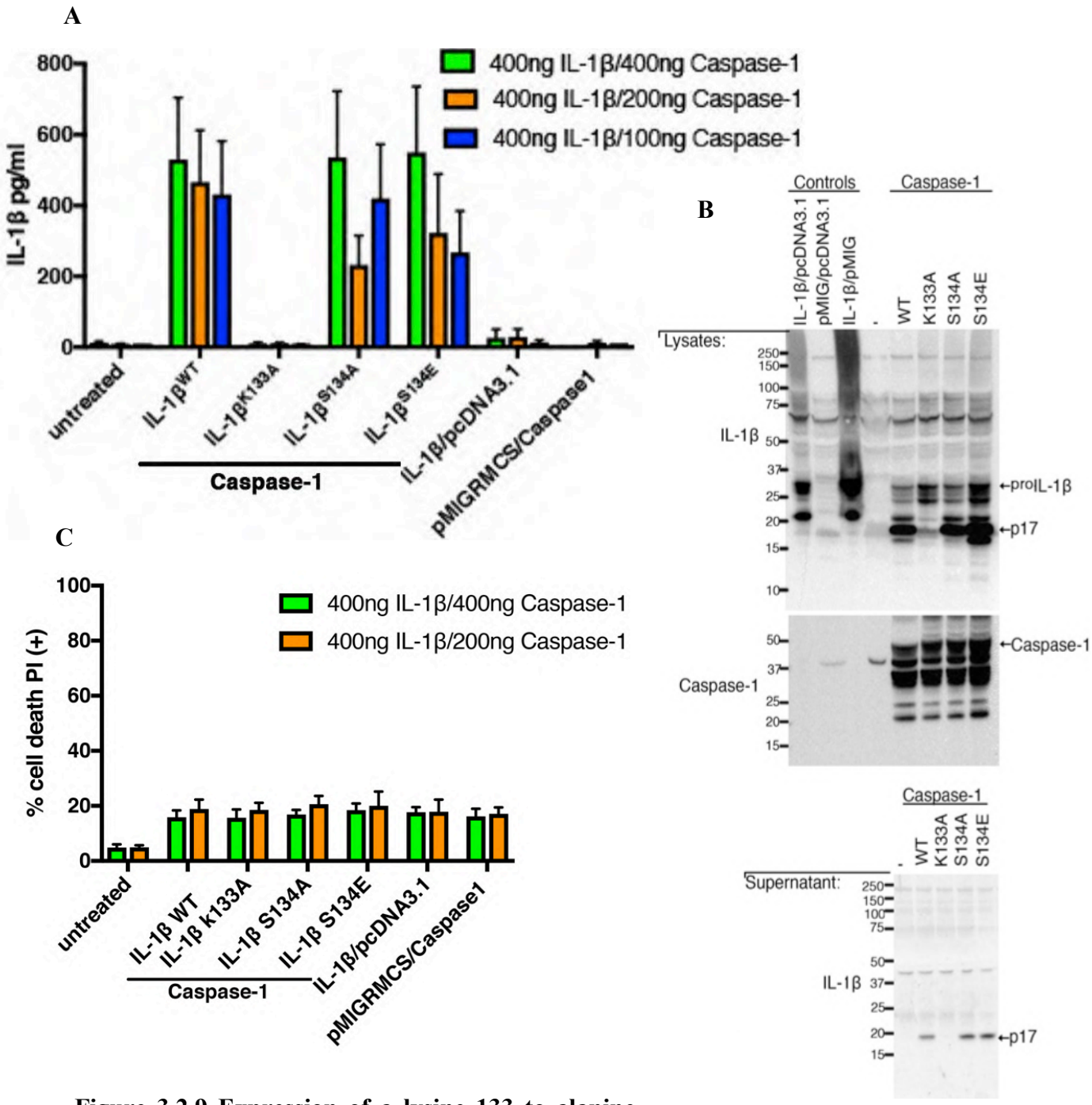


Figure 3.2.9 Expression of a lysine 133 to alanine (K133A) IL-1β point mutant prevents caspase-1 cleavage, while a serine 134 alanine (S134A) IL-1β mutation has no impact.

A) IL-1β in the supernatant of 293T cells (seeded in a 24-well plate) transfected with IL-1β and caspase-1, as indicated, for 24-48 hours and analysed by ELISA. n=3 independent experiments. Error bars represent Mean + S.E.M. B) 293T cells transfected with IL-1β (400 ng) along with caspase-1 (400 ng), as indicated, for 48 hours were analysed by immunoblot. Representative of three independent experiments. C) 293T cells transfected with IL-1β and caspase-1 constructs at various concentrations, as indicated, were stained with PI and its uptake quantified by flow cytometry. Data are representative of three independent experiments. Error bars represent Mean + S.E.M of 3 independent experiments. (A-C) pMIGRMCS and pcDNA3.1 are empty vector controls for IL-1β and caspase-1, respectively.

3.2.10: Expression of IL-1β K133A prevents caspase-8 cleavage, while S134A IL-1β has no impact.

The death receptor apoptotic caspase, caspase-8, has been reported to cleave and activate IL-1β (Maelfait, Vercammen et al. 2008, Bossaller, Chiang et al. 2012, Vince, Wong et al. 2012, Lawlor, Khan et al. 2015) . Therefore, to define if the inability of caspase-1 to process IL-1β K133A is specific, or impairs IL-1β maturation by other proteases, IL-1β K133A (lysine mutant), IL-1β S134A (phospho mutant), IL-1β S134E (phospho mimetic) were transfected into 293T cells with caspase-8. While caspase-8 expression induced IL-1β S134A and IL-1β S134E secretion comparable to WT IL-1β, IL-1β K133A release into the supernatant was not detected (Figure 3.2.10 a and b). PI staining and FACS analysis also revealed that these cells retained cellular viability, similar to caspase-1 transfection experiments (Figure 3.2.10c).

Capsase-8 is reported to cleave precursor IL-1β directly at same site, Asp117, as caspase-1 (Maelfait, Vercammen et al. 2008, Vince 2012). To examine if caspase-8 cleavage was defective in IL-1β K133A mutant, immunoblot analysis was performed. The analysis of cell supernatants revealed mature IL-1β secretion after cleavage of IL-1β S134A and IL-1β S134E by caspase-8 (Figure 3.2.10b). In contrast, lysine mutant IL-1β K133A was resistant to caspase-8 cleavage, and consistent with ELISA results (Figure 3.2.10a), mature IL-1β was not detected in the cell supernatant (Figure 3.2.10b). Therefore, IL-1β K133A is resistant to both caspase-1 and caspase-8 mediated cleavage and secretion.

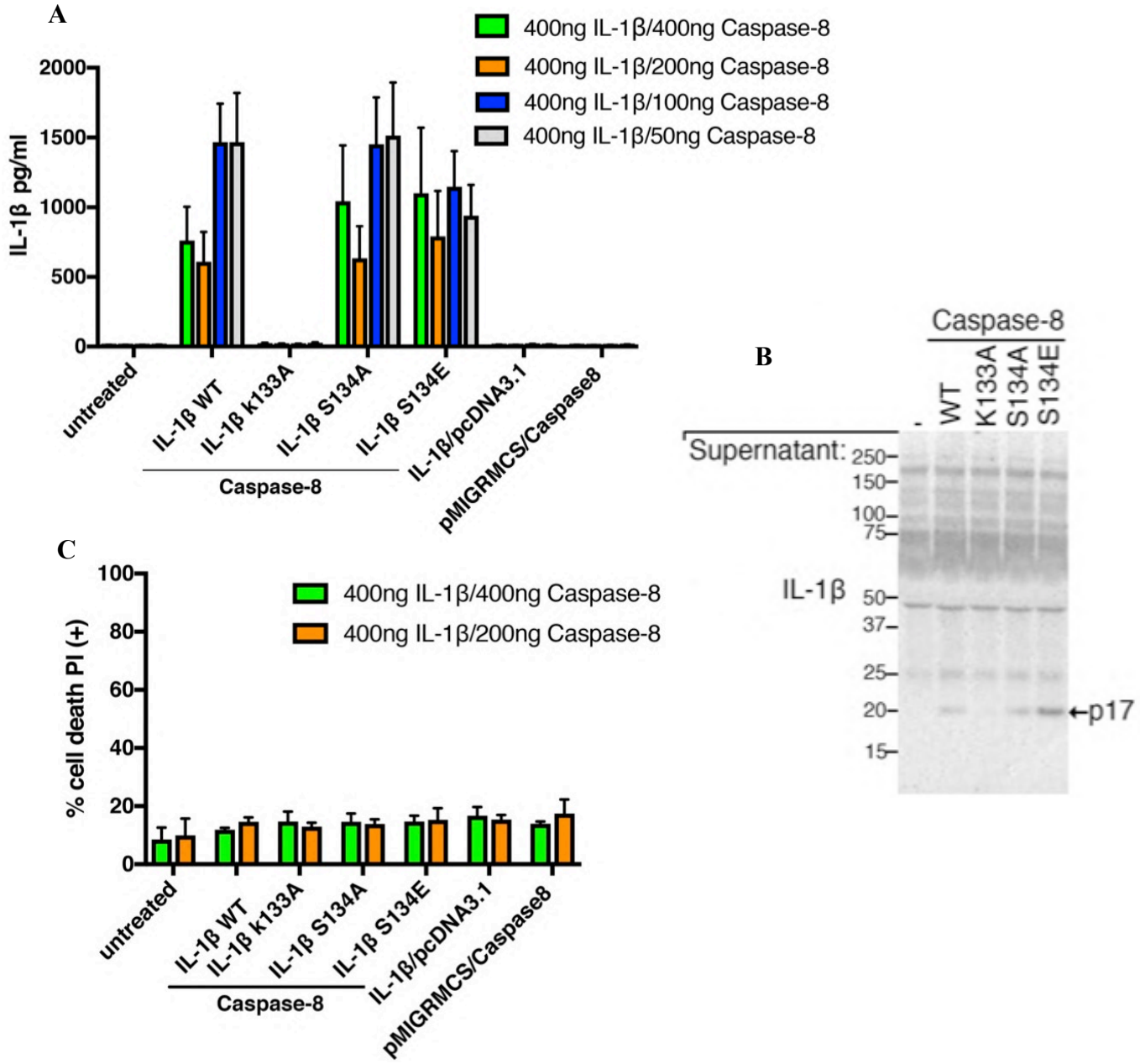


Figure 3.2.10 Expression of IL-1β K133A prevents its cleavage by caspase-8, while IL-1β S134A has no impact.

A) IL-1β in the medium of 293T cells (seeded in a 24-well plate) transfected with caspase-8 (24-48hours) at various concentrations as indicated and analysed by ELISA. n=3 independent experiments. Error bars represent Mean + S.E.M. B) 293T cells transfected with IL-1β (400 ng) constructs along with caspase-8 (400 ng) for 48 hours were analysed by immunoblot. Representative of three independent experiments C) 293T cells transfected with IL-1β and caspase-8 constructs at various concentrations, as indicated, were quantified for PI uptake by flow cytometry. Error bars represent Mean + S.E.M of 3 independent experiments. (A-C) pMIGRMCS and pcDNA3.1 are empty vector controls.

3.2.11: Loss of the IL-1β K133:D129 salt bridge prevents caspase-1 cleavage.

To examine if the ubiquitination of IL-1β K133 and/or the electrostatic interaction between IL-1β K133 and D129 impacted caspase-1 cleavage and IL-1β secretion, several new IL-1β mutations were generated and analysed. Specifically, comparisons were made between WT IL-1β , IL-1β K133A (abrogates both K133 ubiquitination and the D129 electrostatic interaction), IL-1β D129A (unable to form the electrostatic interaction with K133), IL-1β K133R (abrogates K133 ubiquitination but not the salt bridge with D129) and IL-1β D117A (caspase-1 cleavage site mutant). Western blot analysis of 293T cells co-transfected with caspase-1 and IL-1β constructs showed that WT IL-1β was cleaved by caspase-1 and secreted as p17 mature fragment of IL-1β was identified in both lysates and supernatants (Figure 3.2.11), consistent with the above findings. In the presence of the chemical pan-caspase inhibitor, ZVAD-fmk, or mutation of the caspase-1 cleavage site, IL-1β D117A, no cleavage and secretion of IL-1β was detected, as expected (Figure 3.2.11). Interestingly, the loss of the IL-1β K133:D129 salt bridge (IL-1β K133A and IL-1β D129A) resulted in defective caspase-1 processing and thus reduced IL-1β secretion. In contrast, IL-1β K133R, which cannot be ubiquitinated on K133 but retains an electrostatic interaction with D129, did not perturb caspase-1 processing and mature IL-1β secretion (Figure 3.2.11). Thus, the electrostatic interaction of IL-1β K133 and D129 is required for efficient caspase-1 processing of IL-1β.

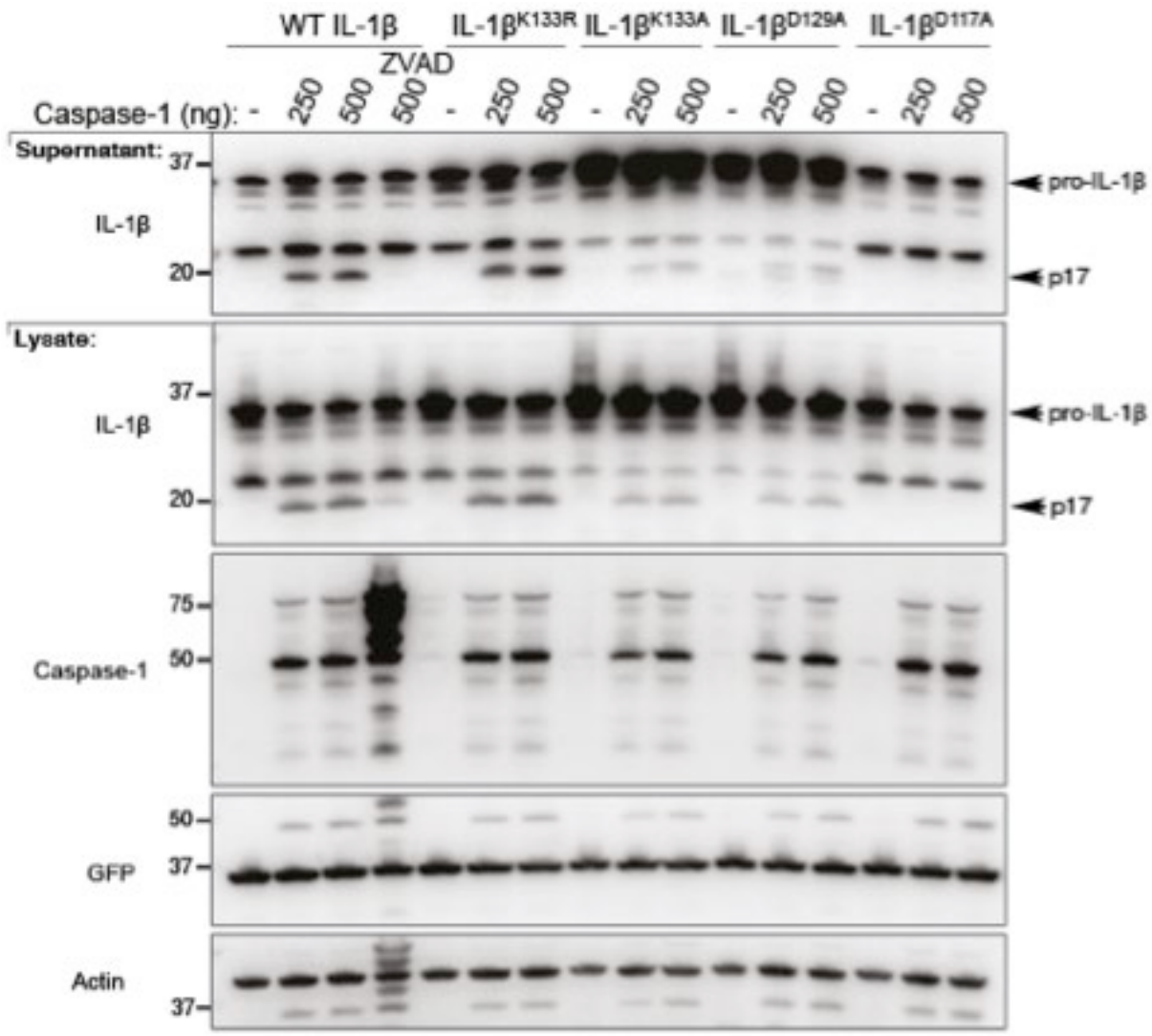


Figure 3.2.11 Loss of the IL-1β K133:D129 salt bridge prevents caspase-1 cleavage of IL-1β.

293T cells co-transfected with IL-1β (400 ng) constructs along with caspase-1 (200 or 500 ng) for 24 hours in the presence or absence of the pan-caspase inhibitor Z-VAD-fmk (50 μM). Cell lysates and supernatant were analysed for the indicated proteins by immunoblot. GFP was blotted for to confirm IL-1β plasmid expression levels and actin incorporated as a loading control. Representative of three independent experiments.

3.2.12: Precursor IL-1β ubiquitination is not restricted to K133.

Precursor IL-1β contains 19 lysine residues that may act as ubiquitination sites (Figure 3.2.12a). To examine if there are other ubiquitination sites apart from K133, 293T cells were transfected with WT IL-1β (WT), a C-terminal lysine cluster mutant (C-IL-1βK8A) where 8 IL-1β lysines were mutated to alanine (residues K205, K209-211, K214, K220, K224 and K226) and an IL-1β mutant where all 17 conserved lysines (IL-1β K17A) between mouse and human IL-1β were mutated to alanine (Figure 3.2.12a). After 48 hours post-transfection, polyubiquitinated proteins were purified by TUBEs. Western blotting of TUBE purified samples revealed reduced ubiquitination of C-IL-1β K8A compared to WT IL-1β (Figure 3.2.12b). However, the expression of C-IL-1β K8A was lower compared to WT IL-1β (Figure 3.2.12b), indicating the C-terminal lysine residues may play an important role in the stability of IL-1β. Interestingly, IL-1β K17A, where all conserved lysines are mutated including K133, showed increased ubiquitin modification compared to WT IL-1β (Figure 3.2.12b). This result indicates that IL-1β ubiquitination may occur on the non-conserved lysine residues not mutated in IL-1β K17A (K224, K247), and/or other residues (e.g. cysteine, serine, threonine) reported to undergo non-canonical ubiquitination (McDowell & Philpott, 2013) are preferentially targeted for modification. Importantly, the parental empty vector plasmid, pMIGRMCS (empty vector control; EVC), and agarose control beads, documented the specificity of IL-1β and ubiquitination (Figure 3.2.12b). Thus, IL-1β likely contains multiple residues that can be targeted for ubiquitination in the 293T cell expression system.

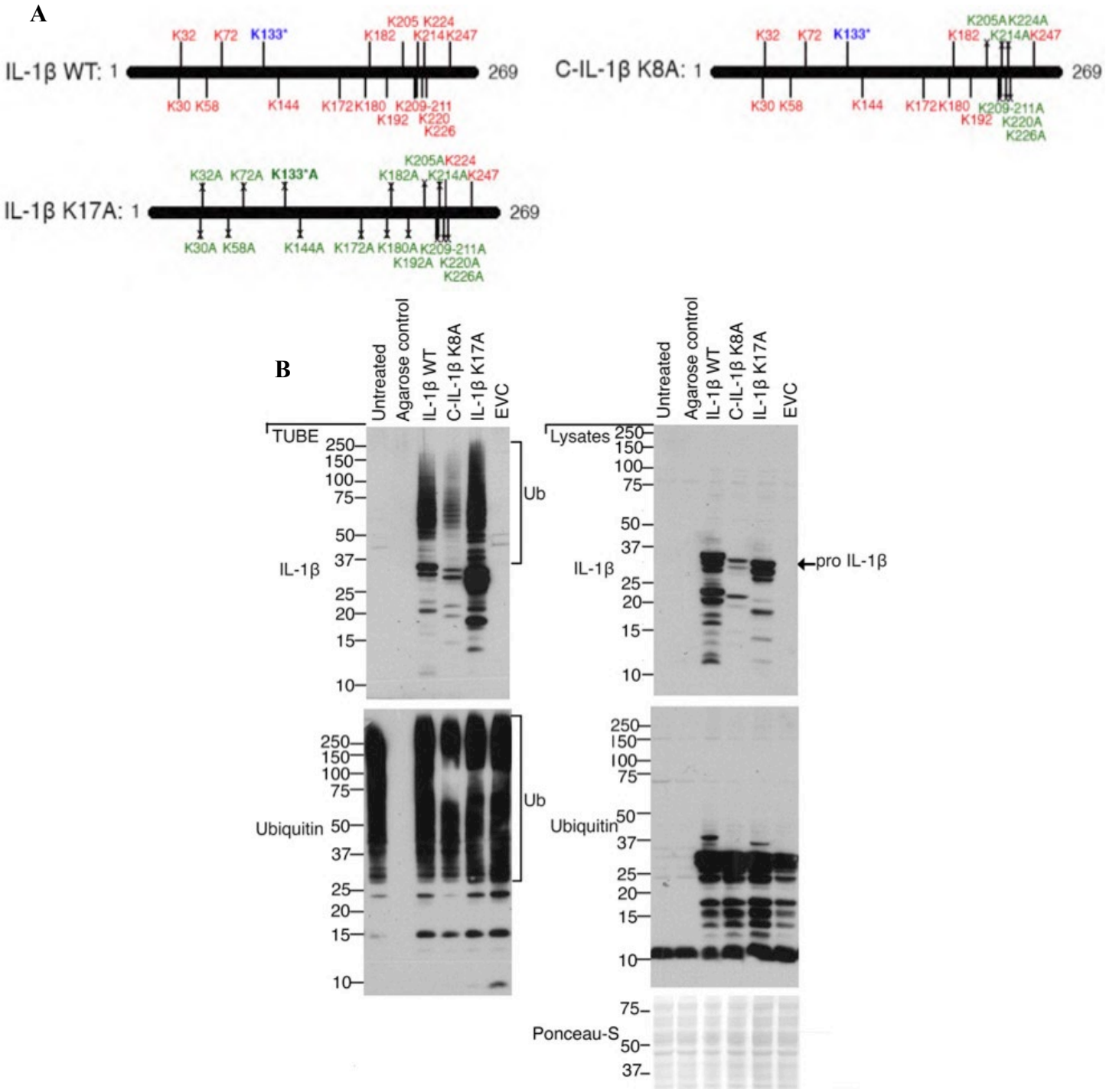
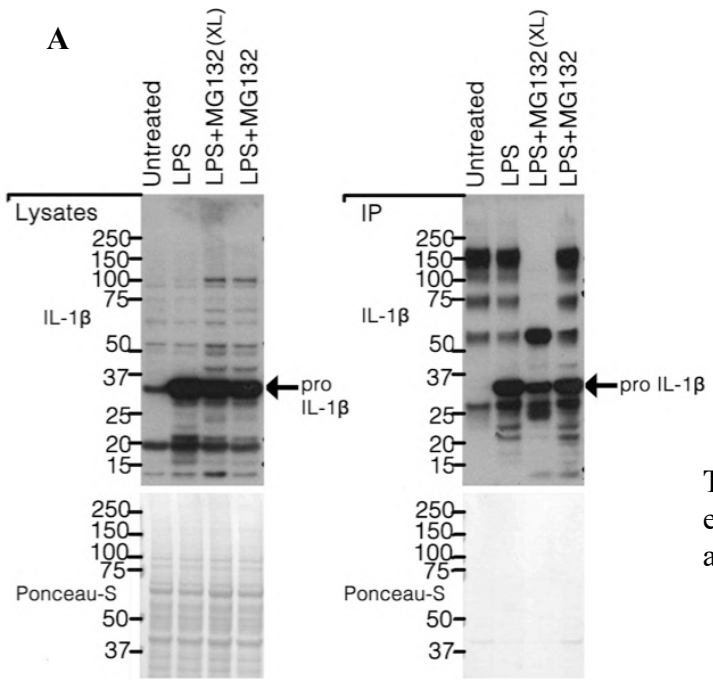


Figure 3.2.12 Precursor IL-1β ubiquitination is not restricted to K133.

A) Schematic representation of all 19 lysine residues (in red) of pro IL-1β full length (IL-1β WT), the C-terminal lysine IL-1β mutant (C-IL-1β K8A) with 8 lysines mutated to alanine (in green), and complete conserved lysine IL-1β mutant (IL-1β K17A) containing 17 lysines mutated to alanine (in green). Mass spectrometry identified K133 is marked in blue and highlighted by an asterisk. B) 293T cells were transfected with IL-1β WT and mutant constructs: C-IL-1β K8A and for IL-1β K17A for 48 hours. Polyubiquitinated proteins were purified by TUBE and analysed by immunoblot for the indicated proteins. Ponceau-S blot shows loading control. Representative of two experiments.

3.2.13: Mass spectrometry analysis of IL-1β interacting proteins

To identify the E3 ligases that might ubiquitinate IL-1β, MS analysis of endogenous IL-1β was conducted. BMDMs were treated with LPS to induce IL-1β expression or with LPS and the proteasome inhibitor, MG132, and endogenous IL-1β purified using IL-1β antibody cross-linked to protein G beads. Immunoblot analysis of IL-1β demonstrated that efficient IL-1β purification was achieved (Figure 3.2.13a). Subsequent MS analysis identified several candidate IL-1β interacting proteins in samples treated with LPS that were not enriched without LPS (Figure 3.2.13b). Candidates with Log2 protein ratios above 3 indicates highly significant enrichment in the IL-1β purification compared to WT samples with no LPS treatment and are represented by green dots (Figure 3.2.13b). Candidates with Log2 protein ratios above 1 and less than 3, represented by red dots, indicate significant enrichment and a possible interaction with IL-1β (Figure 3.2.13b). As expected, highly significant levels of IL-1β were detected, with a Log2 protein ratio of 5.097372581 (Figure 3.2.13b). As previously reported RIPK3 (Receptor-interacting serine/threonine-protein kinase 3) was found to significantly interact with IL-1β (Duong, Onizawa et al. 2015) (Figure 3.2.13b, Log2 Protein ratio 2.030986588). Interestingly, the E3 ligases Arih1, Dtx3l, Rnf213 and Trim21 were also detected at significant levels within IL-1β complexes (Table 3.2.1). Further optimisation of IL-1β enrichment from BMDMs is required to confirm the interactions with the proteins identified, and gene targeting studies of the potential IL-1β ubiquitin E3 ligases will be carried out.



Gene Name	Accession Number	Log 2 Protein Ratio
IL-1β	P10749	5.097372581
Arih1	Q9Z1K5	3.101094068
Dtx3l	Q3UIR3-2	1.125588232
Rnf213	F7A6H4	1.600062586
Trim21	Q3U7K7	1.148967802

Table 3.2.1 E3 ligases pulled down with endogenous mouse IL-1β from BMDMs and identified by MS.

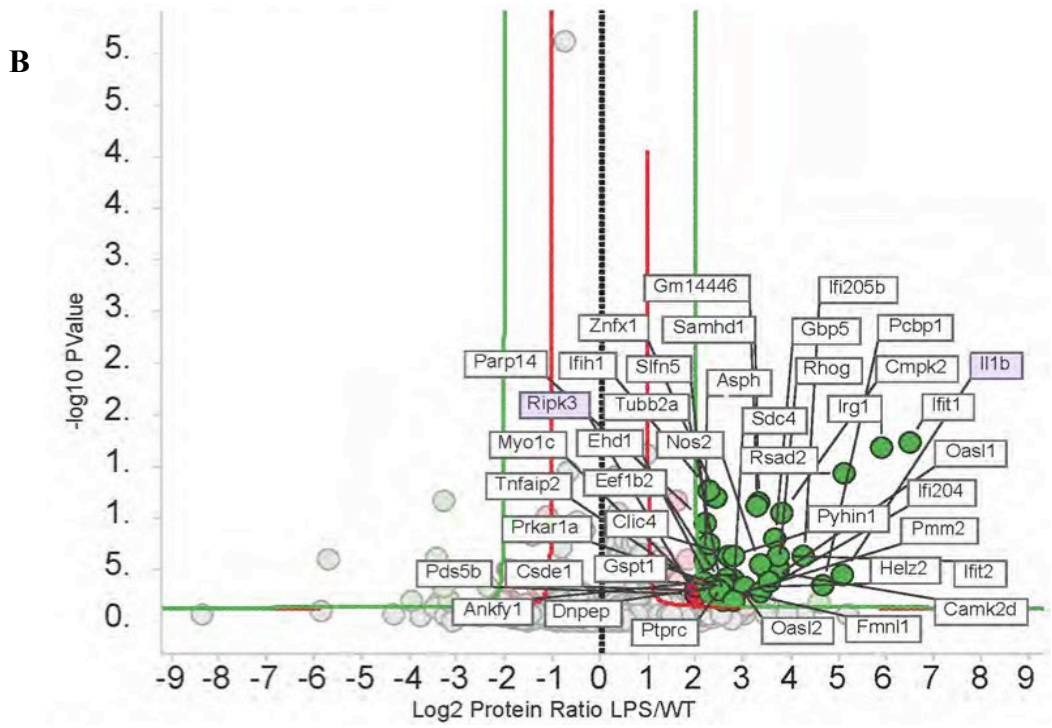


Figure 3.2.13 1 Mass spectrometry analysis of IL-1β interacting proteins.

A) BMDMs (2 X 10^7) were treated with LPS (500 ng/ml) for 6 hours followed with or without MG132 treatment (20 μM) in last 2 hours. Immunoprecipitations were carried out with an IL-1β antibody (with or without crosslinking-XL as indicated) and elutions were performed in 0.5% SDS with 5 mM DTT followed by MS analysis B) Volcano plot of MS analysis of samples treated with or without LPS as indicated in the x-axis showing mouse IL-1β pulled down and possible RIPK3 interaction (shaded in purple) along with other possible candidates that are likely to interact with IL-1β (shaded in purple). Experiment conducted in BMDMs collected from three different mice.

3.3 Discussion

Innate immune signalling is complex and subject to various evolutionary pressures. Posttranslational modifications (PTMs) provide regulation of innate immune signalling and can be important for both activating innate immunity, or preventing detrimental, excessive, signalling. This is highlighted by the fact that the mutation of PTM sites are associated with monogenic autoinflammatory diseases (Harapas 2018). For example, genetic studies in mice have documented that mutation of the NLRP3 phosphorylation site, Serine 295 (S295), causes spontaneous NLRP3 inflammasome activity that phenocopies human autoactivating NLRP3 mutations (CAPS disease). Moreover, CAPS can result from mutations in the immediate surrounding region of NLRP3 S295, suggesting these mutations may perturb inhibitory NLRP3 S295 phosphorylation and, consequently, drive disease-causing IL-1β activation (Mortimer 2016). While anti-IL-1β therapies are currently used in the clinic to treat CAPS patients (Neven 2008, Sibley 2012), little is known about IL-1β PTMs and how they might impact IL-1β signalling. Therefore, the key aim of this chapter was to define PTMs of IL-1β and study their impact on IL-1β secretion.

The findings reported in this chapter show that i) inflammasome priming induces both IL-1β expression and its ubiquitination, ii) inflammasome priming and ATP-mediated activation enhances IL-1β ubiquitination further independent of NLRP3 inflammasome components, iii) mature bioactive IL-1β is unlikely to be ubiquitinated, iv) IL-1β undergoes ubiquitination on K133 and phosphorylation on S134, v) IL-1β K133 ubiquitination is not required for caspase-1 processing of IL-1β, vi) IL-1β contains an electrostatic interaction between K133 and D129 which is required for efficient caspase-1 processing, and vi) four ubiquitin E3 ligases (Ari1, Dtx31, Rnf213 and Trim 21) potentially interact with endogenous IL-1β complexes.

Both NLRP3 and IL-1β were ubiquitinated in response to the stimulation of BMDMs with TLR4 and TLR2 ligands. Ubiquitination of NLRP3 inflammasome components were examined by Tandem Ubiquitin Binding Entities (TUBEs). While TUBEs are excellent approach to isolate endogenous ubiquitinated proteins, it is theoretically possible to co-purify IL-1β along with ubiquitin-like proteins, including SUMO, NEDD8 and ISG15 as ubiquitin-like proteins can covalently attach to IL-1β similar to ubiquitin. Therefore, it was necessary to perform in

vitro ubiquitination assays to confirm ubiquitination of IL-1β. Indeed, immunoprecipitation and *in vitro* ubiquitination assays validated ubiquitination of precursor IL-1β.

Notably, unlike their triggering of IL-1β ubiquitination, LPS and Pam3Cys treatment failed to induce the ubiquitination of caspase-1 and ASC. This demonstrates that inflammasome priming signals alone that induce the upregulation of IL-1β and NLRP3 are sufficient to trigger their ubiquitination, while inflammasome components that are constitutively expressed, such as caspase-1 and ASC, are not modified by inflammasome priming stimuli. Consistent with these findings, other studies failed to observe caspase-1 ubiquitination upon the treatment of BMDMs with LPS (Van Opdenbosch 2014), although caspase-1 ubiquitination has been reported following the activation of several inflammasome sensor proteins (Van Opdenbosch 2014). In a recent study performed in mouse primary peritoneal macrophages (PMs), ASC ubiquitination was reported after both LPS priming and nigericin stimulation (Chiu, Chen et al. 2016). Consistent with my data, LPS priming alone did not result in ASC ubiquitination (Chiu, Chen et al. 2016). ASC is also reported to undergo linear ubiquitination and linear ubiquitination of ASC was shown to promote NLRP3 inflammasome activation (Rodgers 2014). It is possible to have missed linear ubiquitination of ASC in my experiments as TUBE utilised were of higher affinity to K63-linked poly ubiquitination. Experiment using anti-M1 (linear) TUBE with a higher selectivity for M1-linked polyubiquitin may be able to capture linear ubiquitination of endogenous ASC.

Interestingly, my results demonstrated that the ubiquitination of IL-1β in response to the TLR2 ligand Pam3Cys was only detected after 4-8 hours of stimulation, which was delayed compared to the detection of LPS-induced IL-1β ubiquitination within 3-6 hours. Both TLR2 and TLR4 signalling trigger NFκB to induce precursor IL-1β. Therefore, the difference in IL-1β ubiquitination kinetics resulting from TLR2 and TLR4 stimulation may reflect the increased efficiency of LPS-induced levels of IL-1β expression with the doses of TLR ligands used, as suggested by previous work (Lawlor, Feltham et al. 2017). This would be consistent with TLR2 and TLR4-induced mRNA analysis, which showed that TLR4 stimulation increases the expression of cytokines to a higher magnitude (Blankley 2014). It is therefore possible that the ubiquitination of precursor IL-1β is triggered once its expression reaches a specific threshold, which may act to ensure its levels and/or activity are tightly controlled to limit damaging inflammatory responses, as the results in Chapter 4 demonstrate. Indeed, this would be

consistent with NLRP3 ubiquitination promoted by E3 ligases such as MARCH7 or TRIM31, targeting NLRP3 for its degradation to limit excessive inflammasome activation (Yan, Jiang et al. 2015, Song, Liu et al. 2016).

Priming and activation signal provided by canonical NLRP3 agonist ATP enhanced IL-1β ubiquitination independent of inflammasome components. Thus, activation signal alone can induce IL-1β regulation and act as additional molecular switch to keep the levels of IL-1β in check to limit any deleterious inflammatory response under cell stress.

Mass Spectrometric analysis detected ubiquitination of precursor IL-1β on lysine 133 (K133) and phosphorylation on serine 134 (S134) residue. Both these residues are located within the C-terminal bioactive region of IL-1β, suggesting these residues may play a role in IL-1β secretion. *In vitro* analysis showed that IL-1β K133A was ubiquitinated less when compared to WT IL-1β. Remarkably, IL-1β S134E, a phospho mimetic mutant, also displayed reduced ubiquitin modification, suggesting that phosphorylation of IL-1β S134 could act to limit IL-1β K133 modification. Creation of phospho-knockin $IL\text{-}1\beta^{S134E/S134E}$ mouse model and ubiquitination analysis of precursor IL-1β in this model may reveal if combined regulation of phosphorylation and ubiquitination occurs on IL-1β and if it does exist, then how combined regulation may affect IL-1β activity and secretion can also be studied. Indeed, cross-talk between PTM mechanisms has been reported in particular between phosphorylation and ubiquitination (Hunter 2007, Nguyen, Kolch et al. 2013). For example, NLRP3 inflammasome inhibition via the Bile acids (BAs)- Transmembrane G protein coupled receptor (TGR5) – Cyclic AMP (cAMP) – Protein Kinase A (PKA) axis, promoted PKA-dependent phosphorylation on S291 residue and ubiquitination of NLRP3 (Guo 2016). In another study, C-Jun N-terminal kinase (JNK1) mediated phosphorylation on S194 residue of NLRP3 during priming was reported to promote deubiquitination of NLRP3 (Song, Liu et al. 2017). Therefore, similar to NLRP3, it is plausible that combined PTM regulation of IL-1β may occur and will be examined in the future.

My findings also indicated that bioactive IL-1β is unlikely to be targeted for ubiquitination. This suggests that mature IL-1β is not modified with ubiquitin and therefore active IL-1β may be derived from non-ubiquitinated fraction of precursor IL-1β, although the K133 ubiquitination site is present in mature IL-1β fragment. Further fractionation studies of ubiquitinated and non-ubiquitinated pools of IL-1β are required to test this hypothesis.

Experiments to generate pure fractions of ubiquitinated and non-ubiquitinated IL-1β species and subsequent recombinant caspase-1 cleavage assays on the collected IL-1β fractions have been difficult as ubiquitination could occur any lysine residue and obtaining pure fractions of non-ubiquitinated IL-1β is challenging.

Interestingly, although the MS analysis only detected IL-1β K133 ubiquitination, the expression of an IL-1β mutant lacking all 17 conserved lysine residues in 293T cells still resulted in prominent IL-1β ubiquitination. This may reflect the artificial induction of IL-1β ubiquitination that results from its overexpression in 293T cells, although results from IL-1β K133R mutant mice argue that this is unlikely (see Chapter 4). Alternatively, redundancy might exist in IL-1β ubiquitination whereby the loss of one, or more, target lysines simply results in the transfer of ubiquitin onto other possible target lysines (two non-conserved lysine residues remained in the IL-1β mutant lacking the 17 conserved lysines). The other possible explanation is the occurrence of non-canonical ubiquitination that is reported to occur on non-lysine residues; such as the N-terminal amine group, hydroxyl group of serine and threonine residues, or the thiol group of cysteines residues(McDowell 2013). Further detailed biochemical studies analysing endogenous IL-1β are required to determine if additional PTM sites are present, or if non-canonical ubiquitination might occur on IL-1β.

Four E3 ligases, namely, Ari1, Dtx31, Rnf213 and Trim 21 were identified to possibly interact with IL-1β by MS analysis in this study. The only known E3 ligase to ubiquitinate IL-1β until now is the human papillomavirus type 16 associated E6-oncoprotein associated protein (E6-AP) (Niebler, Qian et al. 2013). E6-AP mediated IL-1β ubiquitination targets IL-1β for proteosomal degradation, thus allowing virus to escape host immune surveillance (Niebler, Qian et al. 2013). Future studies are required to validate the identifies putative four E3 ligases by co-immunoprecipitation studies. Knock out studies of the potential E3 ligase will be performed to validate ubiquitination of IL-1β *in vitro* and *in vivo*. As discussed in chapter 1.7.3, several E3 ligases are reported to positively and negatively regulate NLRP3. Therefore, it will be interesting to examine if such similar nuanced regulation also occurs on IL-1β as keeping levels of IL-1β in check is critical for the health of the host.

IL-1β K133A (lysine mutant), IL-1β S134A (phospho-dead) and IL-1β S134E (phospho-mimetic) were generated to determine if these residues and their modifications had any effect on IL-1β cleavage and secretion. While the IL-1β S134 phospho mutants did not have a

significant impact on IL-1β secretion, IL-1β K133A significantly reduced caspase-1 cleavage and IL-1β secretion. Notably, this mutation will abrogate of the IL-1β K133:D129 salt bridge, and in agreeance with this idea, an IL-1β D129A mutant also resulted in defective caspase-1 processing and reduced IL-1β secretion. On the other hand, IL-1β K133R mutant which cannot be ubiquitinated but retains the positive charge required to form salt bridge with D129, did not hinder caspase-1 processing and IL-1β secretion. Therefore, an intact K133:D129 electrostatic interaction is key for caspase-1 cleavage of IL-1β. It can be hypothesised that K133 modification of IL-1β could hinder the salt bridge between K133 and D129 and possibly hinder IL-1β recognition and cleavage by caspase-1.

Of note, despite robust secretion of cleaved activated IL-1β when co-expressed with caspase-1 or caspase-8 in 293T cells, cell death, was not detected. IL-1β is a leaderless secretory protein that lacks the N-terminal signal sequence and able to bypass the classical endoplasmic reticulum ER-Golgi trafficking pathway (Monteleone 2015). Therefore, the central dogma in the field of cell death and inflammation is that inflammasome activators causes pyroptosis and allow the passive secretion of IL-1β (Liu 2014, Shirasaki 2014, Cullen 2015). However, our results of caspase-1 or caspase-8 mediated IL-1β secretion without increases in cell death in 293T cells, which lack pore-forming gasdermin D (GSDMD) expression, are consistent and adds to a growing evidence of research suggesting that IL-1β secretion can occur independent of cell death in certain cell types and conditions, such as 293T cells, neutrophils and DCs (Brough 2007, Kang 2013, Chen 2014, Conos 2016). Recently, the absence of an innate immune protein, SARM (Sterile and α HEAT Armadillo motif-containing protein), which contains a Toll-IL-1R (TIR) domain was reported to increase IL-1β secretion and associated with decreased pyroptosis (Carty, Kearney et al. 2019). Therefore, further investigation of the role of SARM in 293T cells could reveal new regulated pathways of IL-1β secretion.

Contrary to my findings, expression of IL-1β K133R in 293T cells was previously reported to undergo moderately reduced caspase-1 processing and less secretion compared to WT IL-1β (Duong, Onizawa et al. 2015, Zhang, Liu et al. 2018). The main difference between my experiments and these published studies is that they transfected ASC along with caspase-1 and IL-1β, while I transfected caspase-1 and IL-1β constructs without including ASC. Further studies are required to understand if ubiquitination of IL-1β on K133 residue could impact the interaction between ASC and caspase-1 and therefore hinder caspase-1 processing of IL-1β.

Regardless, my findings highlight that ubiquitination of IL-1β K133 may have an alternative impact on IL-1β signalling, independent of caspase-1, such as IL-1β turnover, and this possibility is explored further in the following results chapter.

Chapter 4: Ubiquitination of IL-1β on K133 reduces IL-1β stability to impact its activation by caspase-1

4.1 Introduction:

The effect of IL-1β ubiquitination on IL-1β secretion in macrophages and neutrophils:

Inflammasome machinery, including NLRP3 and IL-1β, are highly expressed or inducible in macrophages and have been extensively studied. Upon NLRP3 inflammasome activation, pro-caspase-1 is cleaved into its active form which then cleaves both precursor IL-1β and IL-18 into their mature forms. Additionally, active caspase-1 can activate pore-forming Gasdermin D to cause a proinflammatory cell death termed pyroptosis (He 2016). Thus, the central dogma in the field of cell death is that in macrophages both pyroptosis and IL-1β secretion occur simultaneously. However, in neutrophils, another major source of IL-1β during inflammation and infection, IL-1β secretion can occur independently of pyroptosis in response to canonical NLRP3 stimuli, ATP or nigericin, or following various infections (Chen 2014, Karmakar 2015, Karmakar 2016). The resistance to pyroptosis in neutrophils could be attributed to the lesser abundance of caspase-1 on a single cell basis and lack of gasdermins (GSDMs) expression when compared to macrophages (Boucher, Monteleone et al. 2018, Monteleone, Stanley et al. 2018). Moreover, in contrast to macrophages, where single ASC specks are formed in a cell indicating NLRP3/ASC/caspase-1 oligomerization, multiple ASC specks are formed in neutrophils upon inflammasome activation (like gain of function in inflammasome genes) (Karmakar 2015). Thus, the exact mechanism by which neutrophils avoid pyroptosis is not yet clear. One of the hypotheses for the absence of pyroptosis in neutrophils is that during infection, prolonged survival of neutrophils is required for killing of intracellular bacteria (Chen 2014). Thus, both macrophages and neutrophils operate synergistically to drive caspase-1 mediated IL-1β secretion during infection, and these are the two cell types that have been analysed in the context of IL-1β ubiquitination and in *IL-1β*$^{K133R/K133R}$ mice in this chapter.

4.1.1 Chapter overview

In Chapter 3, I documented the ubiquitination of IL-1β and identified lysine 133 as being an IL-1β residue that is targeted for ubiquitination. To examine the regulatory effects of ubiquitination of IL-1β in a more physiological system, a Clustered Regular Inter Spaced Palindromic Repeats (CRISPR)/Cas9 gene targeted Knock In (KI) point mutant mice harboring a lysine (K) to arginine (R) mutation on lysine 133 of IL-1β (*IL-1β*$^{K133R/K133R}$) was successfully generated, and the study of this mouse forms a primary focus of this chapter.

Initial experiments examined the ubiquitination pattern of precursor IL-1β in WT and *IL-1β*$^{K133R/K133R}$ mice and documented the presence of degrative K48, activating K63-ubiquitin chains. Moreover, studies in both 293T cells and macrophages showed that IL-1β was rapidly turned over via proteasomal degradation, and that the loss of IL-1β ubiquitination on K133 increased precursor IL-1β stability, and therefore increased levels of bioactive IL-1β released upon NLRP3 inflammasome activation. Finally, upon LPS injection *in vivo*, precursor *IL-1β*$^{K133R/K133R}$ was stabilised, which resulted in increased IL-1β levels in serum and peritoneal lavage fluids. Therefore, ubiquitination on K133 of IL-1β targets IL-1β for proteosomal degradation, thereby limiting inflammation by diminishing its ability to be triggered by inflammasomes.

Chapter 4.2 Results

4.2.1. Loss of IL-1β K133 ubiquitination stabilises precursor IL-1β in 293T cells

Ubiquitination of target proteins can alter their stability (Varshavsky 1992, Johnson, Ma et al. 1995). Preliminary studies were conducted in 293T cells to examine stability of wildtype IL-1β along with the identified post-translational site mutants; IL-1β K133 and S134 mutants (Chapter 3, Figure 3.2.6). WT IL-1β , IL-1β K133A, IL-1β S134A and IL-1β S134E were expressed in 293T cells and cell then treated with the protein synthesis inhibitor cycloheximide (CHX) to allow IL-1β turnover to be examined. Notably, WT IL-1β degradation was detected after 3 hours of CHX treatment, and IL-1β was almost undetectable after 6-9 hours (Figure 4.2.1A). However, IL-1β K133A was moderately protected from degradation following CHX treatment, particularly at early (3 hr) time points (Figure 4.2.1A). On the other hand, both IL-1β S134A and IL-1β S134E mutants behaved similar to WT IL-1β and were rapidly degraded (Figure 4.2.1A). Negative controls of empty vector, (pMIGRMCS) transfections showed no precursor IL-1β expression (Figure 4.2.1A), as expected.

Treatment of 293T cells with the proteasome inhibitor MG132 limited IL-1β degradation following CHX addition (third row panel, Figure 4.2.1A). However, even in the presence of MG132, IL-1β K133A was destabilised at later time points (6 and 9 hours) (third row panel, Figure 4.2.1A). This could be due to a loss of MG132 efficacy at these later time points.

Next, K-to-A mutants of lysine-rich mutants of IL-1β were also tested in CHX chase assays. These included alanine mutants of all the four lysines present between residues 205-211 of IL-1β, namely K(205-211)A mutant, alanine mutants of all the eight lysines present between residues 205-211 and 214-226 of IL-1β, namely C-IL-1β K8A mutant, and all the seventeen conserved lysines between human and mouse species of IL-1β, namely IL-1β K17A mutant (as shown in Figure 4.2.1C). The C- IL-1β K8A mutant resulted in very low expression levels (Figure 4.2.1B). The other two IL-1β mutants, K(205-211)A and IL-1β K17A, could be expressed but were rapidly degraded (Figure 4.2.1B).

MG132 treatment rescued the degradation of IL-1β K17A and also IL-1β K(205-211)A (Figure 4.2.1B). As expected, C-IL-1β K8A could not be rescued with MG132 as IL-1β expression of the mutant was extremely weak to start with (Figure 4.2.1B). Therefore, the IL-1β lysines between residues 214-226 of IL-1β could be important for stabilizing the protein. However, it is hard to make solid conclusions of which particular lysines are actually important for IL-1β stabilization from these cycloheximide chase assays alone and further protein studies are required. Collectively, these results show that the K133 residue of IL-1β stabilises IL-1β in 293T cell assays and the lysine rich region of IL-1β between 214 – 226 may play a role in IL-1β stability.

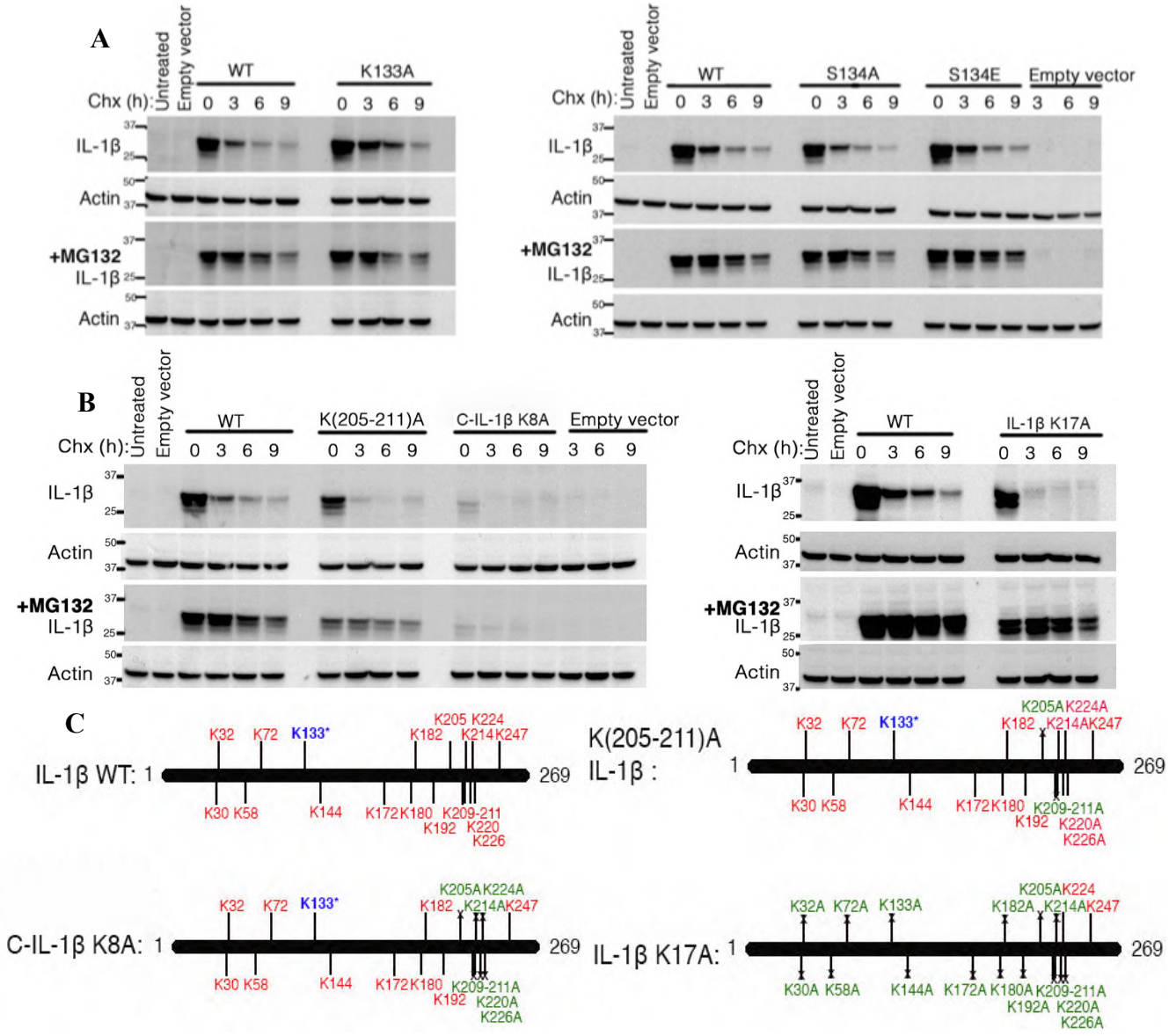


Figure 4.2.1 Loss of IL-1β K133 ubiquitination stabilizes precursor IL-1β when expressed in 293T cells.

A) 293T cells were transfected with IL-1β K133A and WT IL-1β constructs (on left panel), IL-1β S134A, IL-1β S134E and WT IL-1β constructs (on right panel), B) IL-1β K(205-211)A, C-IL-1β K8A constructs (on left panel) and IL-1β K17A and WT IL-1β constructs (on right panel) in a 24-well plate at a cell density of 2 x 10^5 cells per well for 48 hours followed by cycloheximide (CHX) treatment (20 µg/ml) at indicated time points. MG132 (20 µM for 2 hours) treatment added to cells during the CHX chase assay is indicated as + MG132 (in A & B). Subsequent to chase assay, cells were lysed and analysed by immunoblot. Empty vector serves as negative control with no IL-1β expression and actin blots show protein loading. Data representative of three independent experiments. C) Schematic representation of all 19 lysine residues (in red) of IL-1β full length (IL-1β WT), K(205-211)A IL-1β mutant with 4 lysines mutated to alanine, C-IL-1β K8A mutant with 8 lysines mutated to alanine and complete conserved lysine IL-1βK17A mutant containing 17 lysines mutated to alanine. Mass spectrometry identified K133 is marked in blue and asterisk (when not mutated to alanine). All mutated lysine residues to alanine are in green and represented by a cross; x.

4.2.2. Precursor IL-1β ubiquitination is increased following inhibition of the proteasome or deubiquitinating enzyme activity.

To examine if the degradation of endogenous precursor IL-1β was mediated by the proteasome pathway, wildtype BMDMs were treated with LPS followed by treatment with MG132 followed by TUBE purification of ubiquitinated proteins. Western blot analysis showed that IL-1β ubiquitination was increased upon MG132 treatment (Figure 4.2.2A), indicating that IL-1β ubiquitylation likely targets it for proteasomal degradation, consistent with the presence of K48-linked ubiquitin chains (Figure 4.2.4B).

Small molecule inhibitors of deubiquitinases (DUBs), G5 and WP1130, block the removal of ubiquitin chains from proteins and can therefore increase their polyubiquitination (Aleo, Henderson et al. 2006, Kapuria, Peterson et al. 2010). G5 (3,5-bis [(4-Nitrophenyl) methylene]-1,1-dioxide, tetrahydro-4H-thiopyran- 4-one) is a broad-spectrum Isopeptidase inhibitor, whereas WP1130 selectively inhibits DUBs; USP9x, USP5, USP14, UCH37 and USP24 (Aleo, Henderson et al. 2006, Kapuria, Peterson et al. 2010, Luo, Jing et al. 2019). Both these DUB inhibitors were used in previous studies to examine the impact of NLRP3 ubiquitination on inflammasome activity (Juliana 2012, Lopez-Castejon, Luheshi et al. 2013, Py, Kim et al. 2013, Ghonime, Shamaa et al. 2014). To examine if G5 and WP1130 promoted the ubiquitination of precursor IL-1β, similar to MG132 treatment, BMDMs from wild type mice were treated with LPS in the presence or absence of G5, WP1130, or MG132. TUBEs were utilized to purify ubiquitinated proteins. Immunoblot of IL-1β showed that precursor IL-1β was ubiquitinated with LPS treatment, and in the presence of both DUB inhibitor treatments ubiquitination of IL-1β was increased, similar to MG132 treatment (Figure 4.2.2B). Lysates showed equal expression of precursor-IL-1β across all conditions except for untreated cells; precursor IL-1β expression requires LPS treatment (Figure 4.2.2B). DMSO, the vehicle control, did not have any effect on IL-1β ubiquitylation (Figure 4.2.2B). Agarose control showed specificity of TUBE reagents with no non-specific, background ubiquitination detected (Figure 4.2.2B). These results suggest that ubiquitination regulates precursor IL-1β and provides further validation of the MS analysis (Chapter 3, Figure 3.2.6B). In a previous study, WP1130 induced the ubiquitination of proteins containing both K48- and K63-linked ubiquitin chains (Kapuria, Peterson et al. 2010). Thus, this work provides further evidence that polyubiquitination of precursor IL-1β contains both these ubiquitin linkage types (Figure 4.2.4B).

To examine the regulation of ubiquitination upon NLRP3 inflammasome activation, BMDMs from WT mice were subjected to inflammasome assays in the presence or absence of DUB inhibitors. The NLRP3 activators ATP and Alum were used to trigger the NLRP3 inflammasome. Following priming, both ATP and Alum induced the secretion of IL-1β into the cell supernatants, as measured by ELISA. NLRP3 activation and subsequent IL-1β activation was reduced in a dose dependent manner by the presence of either G5 or WP1130 (Figure 4.2.2C). LPS treatment alone did not result in any IL-1β secretion, as expected (Figure 4.2.2C). Thus, de-ubiquitination may allow for NLPR3 activity, consistent with previous reports (Juliana 2012, Py 2013), although DUB inhibition also induces polyubiquitination of precursor IL-1β (as shown in Fig 4.2.2B), which may target precursor IL-1β for proteasomal degradation, thereby potentially limiting IL-1β release.

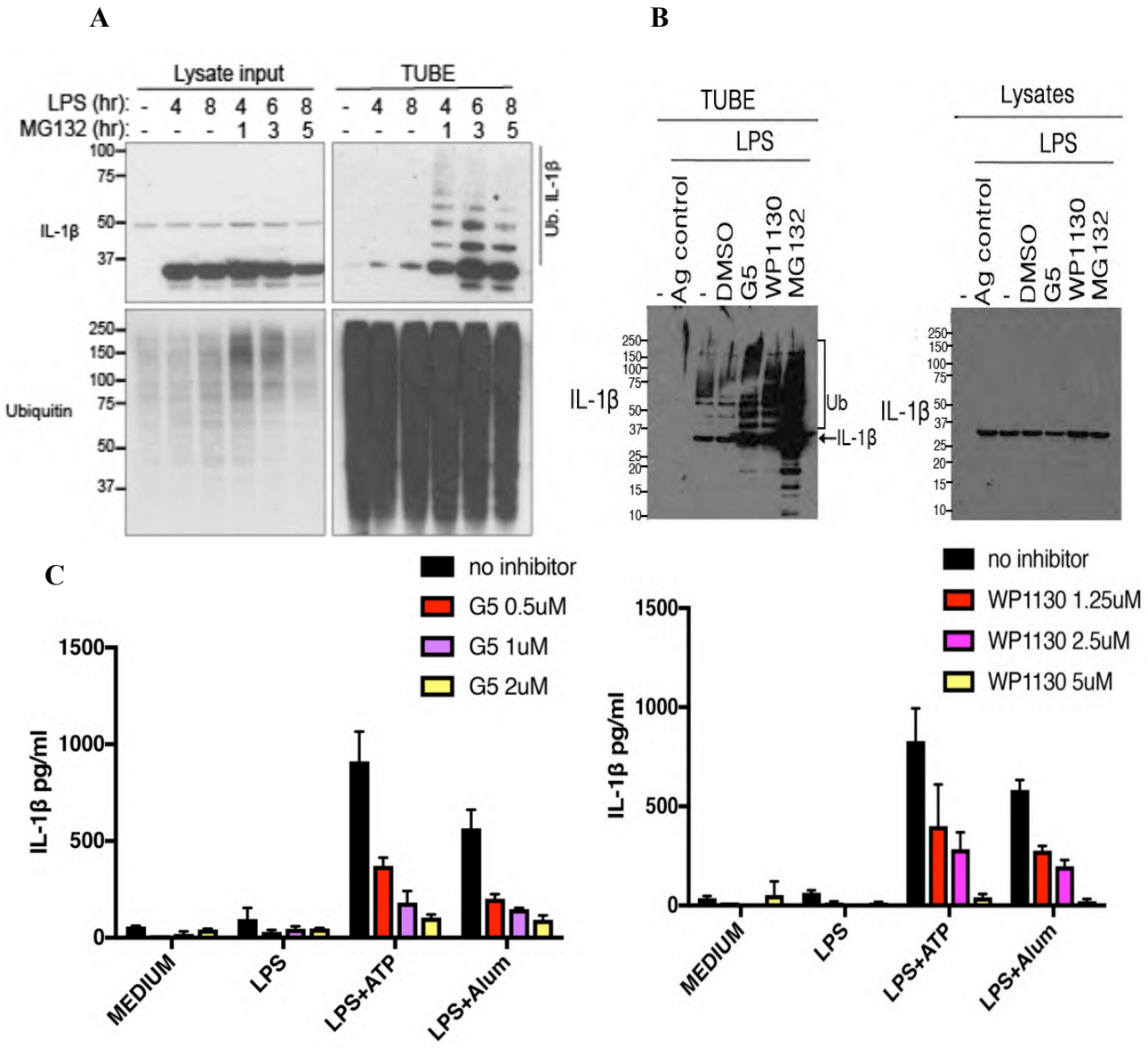


Figure 4.2.2 Precursor IL-1β ubiquitination is increased following the inhibition of proteasomal degradation or deubiquitinating enzyme activity.

A) Wildtype BMDMs were seeded in a 10 cm dish at a cell density of 10 x 10^6 cells and treated with LPS (50 ng/ml) for up to 8 hours as indicated and MG132 (20 μM) for the indicated time points, B) treated with LPS (50 ng/ml) for 4 hours followed with either G5 (2 μM) for 1 hour or WP1130 (2 μM) for 1 hour or MG132 (20 μM) for 2 hours. A) & B) ubiquitinated proteins were purified by TUBE and analysed by immunoblot. DMSO indicates vehicle control and Ag control indicates agarose beads control. Data representative of three independent experiments. C) Wildtype BMDMs were seeded in a 96-well plate at a cell density of 2 x 10^5 cells per well and treated with LPS (100 ng/ml for 3 hours) alone, or with LPS (100 ng/ml for 3 hours) and ATP (5 mM for 30 mins), or with LPS (100 ng/ml for 3 hours) and alum (320 μg/ml for 4 hours), in the presence or absence of DUB inhibitors G5 and WP1130 at the indicated concentrations for the last 30 mins of LPS priming. IL-1β levels were measured in cell supernatants by ELISA. n=3 independent experiments. Error bars represent the SD in all graphs.

4.2.3. Generation of IL-β Lysine 133 to Arginine (*IL-1β*$^{K133R/K133R}$) point mutant mice.

In the previous Chapter I demonstrated that IL-1β was ubiquitinated on lysine 133 (K133). In order to understand the functional consequence of IL-β ubiquitination, a point mutant mouse was generated (Melbourne Advanced Genome Editing Centre, WEHI; detailed in Chapter 2, section 2.8), in which IL-1β K133 was mutated to R133 (*IL-1β*$^{K133R/KI33R}$), which prevents ubiquitin being attached to this residue but allows retention of the positive charge and the salt bridge with D129. Founder mouse no.6 carried the desired IL-1β K133R point mutation without any off-target mutations (Figure 4.2.3A), therefore this mouse was selected and back crossed for two generations onto C57BL/6 wildtype mice. A snapshot of maintaining the *IL-1β*$^{K133R/KI33R}$ mouse colony is depicted in Figure 4.2.3B. Next generation sequencing (NGS) was performed on backcrossed mice (F1&F2 offspring; as detailed in Chapter 2, section 2.8.1) which confirmed that the IL-1β K133R mutation was transferred to the progeny. Further, routine genotyping analysis was carried out (as detailed in Chapter 2, section 2.8) to confirm *IL-1β*$^{K133R/KI33R}$ before they were utilized in experiments. Genotyping of progeny #90-#104 is shown in Figure 4.2.3C. The WT IL-1β allele PCR amplified a 254 bp DNA fragment from mice #91 and 93, whereas this DNA amplification was absent in the K133R KI allele PCR, and these animals were therefore confirmed as WT (Figure 4.2.3C). Conversely, PCR of DNA extracted from mice progeny #100, 102 and 104 resulted only in DNA amplification (of 254bp) of the K133R KI allele, but not the WT allele, and therefore were confirmed as *IL-1β*$^{K133R/KI33R}$ animals (Figure 4.2.3C). Mice #90, 92, 94, 95-99, 101 and 103 were confirmed as heterozygous as DNA amplification occurred in both WT and KI allele PCRs (Figure 4.2.3C). Thus, an *IL-1β*$^{K133R/KI33R}$ point mutant mouse line was successfully created and validated. Interestingly, *IL-1β*$^{K133R/KI33R}$ mice were phenotypically similar to wild type (WT) mice, and LPS treated BMDMs generated from *IL-1β*$^{K133R/KI33R}$ and WT resulted in equivalent expression of IL-1β and TNF mRNA (Figure 4.2.3D).

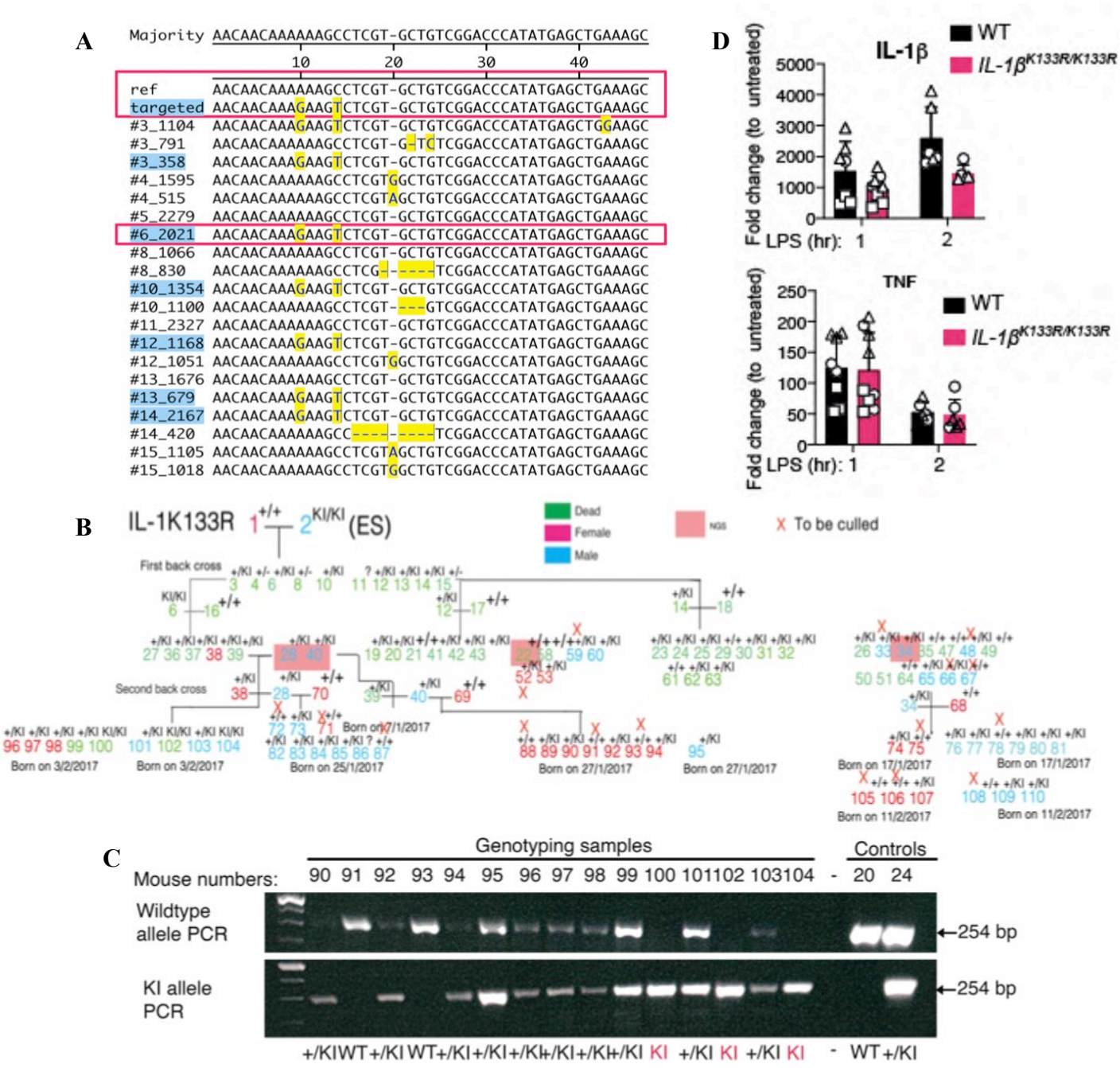


Figure 4.2.3 Generation of *IL-1β*$^{K133R/K133R}$ point mutant mice.

A) Mouse IL-1β gene sequence showing reference region targeted for the lysine to arginine mutation at lysine 133 residue (base pairs highlighted in yellow). Gene sequence of mouse #6 shows successful CRISPR-mediated mutation from lysine to arginine similar to the reference sequence with no other off-target mutations (boxed in red). B) A snapshot of *IL-β*$^{K133R/KI33R}$ mice line maintenance showing backcrossing with C57BL6 wildtype mice for two generations. C) Routine genotyping analysis showing wildtype (91 and 93), heterozygous (90,92,94,95,96,97,98,99,101and 103) and *IL-β*$^{K133R/KI33R}$ mice (100,102 and 104- highlighted in red) along with controls, as indicated. D) IL-1β & TNF mRNA levels from WT and *IL-1β*$^{K133R/KI33R}$ mice primed with LPS (50 ng/ml, 2 hours) and analysed by RT-qPCR. Data are the mean + SD. Individual mice/replicates are shown by symbols from 3 pooled independent experiments.

4.2.4. Ubiquitination of precursor IL-1β is similar in wildtype and *IL-1β*$^{K133R/KI33R}$ mice.

To examine if endogenous precursor IL-1β ubiquitination was altered in *IL-β*$^{K133R/KI33R}$ mice, BMDMs from wild type (WT) mice and *IL-1β*$^{K133R/KI33R}$ (K133R) mutant mice were treated with LPS (100 ng/ml for 5 hours) in the presence or absence of proteasomal degradation inhibitor MG132 (20 μM in the last 2 hours of LPS priming). TUBE purification and subsequent western blot analysis of IL-1β showed that *IL-1β*$^{K133R/KI33R}$ was ubiquitinated in the presence or absence of MG132 similar to WT IL-1β (Figure 4.2.4A). Both WT and *IL-1β*$^{K133R/KI33R}$ displayed increased ubiquitination upon proteasomal inhibition with MG132 treatment, suggesting that ubiquitination of IL-β is associated with proteasomal targeting (Figure 4.2.4A). Agarose control beads (Ag control) showed the specificity of TUBE approach. Ubiquitin blots demonstrated that the total levels of ubiquitin purified between the two genotypes were similar (Figure 4.2.4A). Therefore, IL-1β ubiquitination likely occurs on multiple residues, not just K133.

To examine the ubiquitin linkage types that decorate IL-1β BMDMs from WT, *IL-1β*$^{K133R/KI33R}$ and *IL-1β*$^{-/-}$ mice (negative control) were treated with LPS to induce the expression of precursor IL-1β and MG132 to enhance its ubiquitination. Ubiquitin specific linkage antibodies against K48- and K63-linked chains along with isotype controls IgG (from Genentech; (Newton, Matsumoto et al. 2008)) were used to pull down these ubiquitin linkage types under denaturing conditions. Western blot analysis showed that *IL-1β*$^{-/-}$ (KO) mice exhibited no IL-1β or IL-1β ubiquitin modification, as expected (Figure 4.2.4B). The bands corresponding to 25 kDa in the IL-1β knockout (KO) mice lanes represent antibody light (indicated with a * in Figure 4.2.4B). IL-1β ubiquitin modification containing both K48- and K63-linked chains were detected in WT and *IL-1β*$^{K133R/KI33R}$ mutant mice (Figure 4.2.4B). Ubiquitin blots showed similar total ubiquitin levels and purification in all genotypes and ponceau staining showed equal protein loading in the cell lysates (Figure 4.2.4B). Therefore, WT IL-1β and IL-1βK133R are decorated with K48- and K63-linked ubiquitin chains.

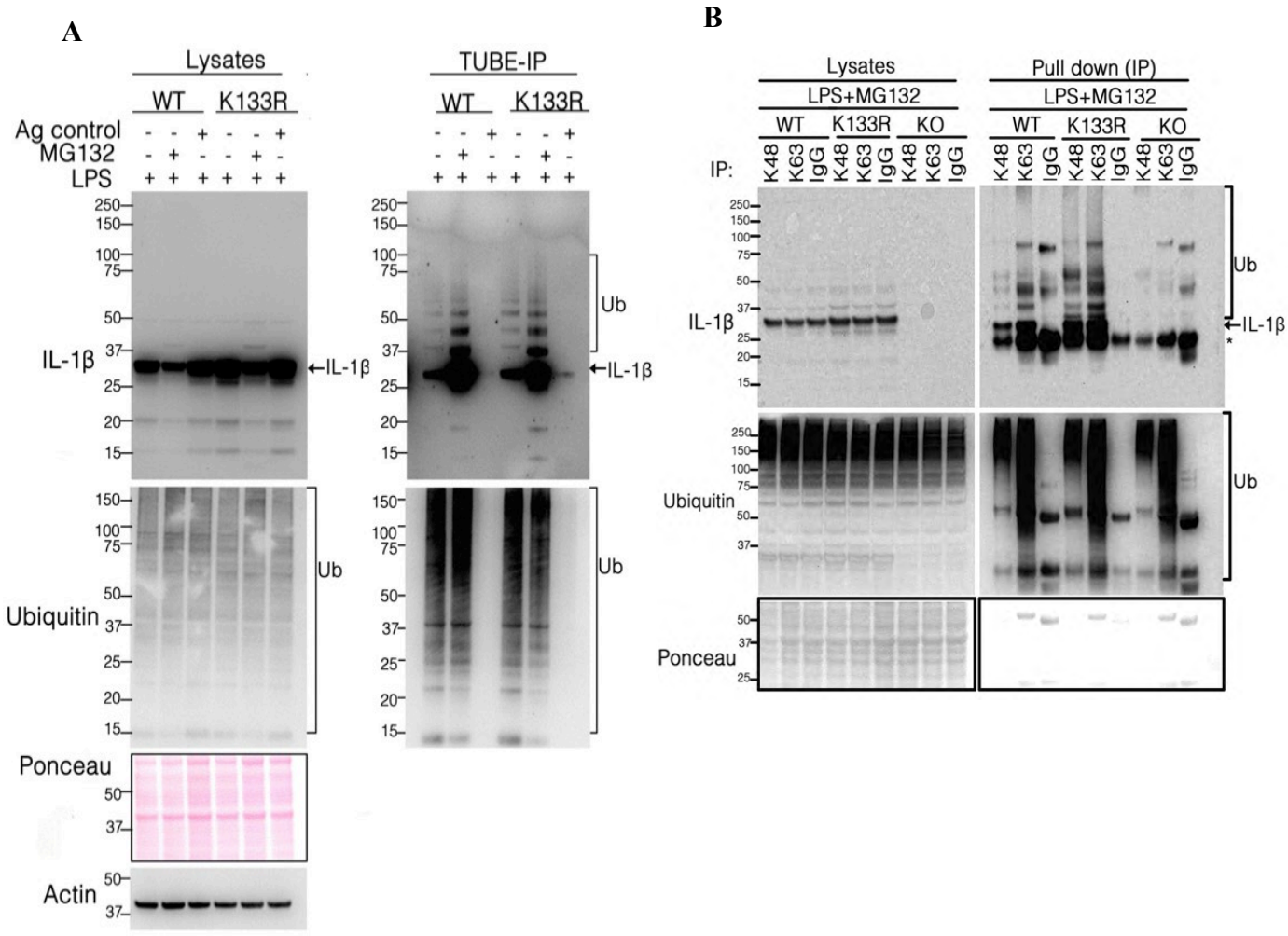


Figure 4.2.4 Ubiquitination of precursor IL-1β is similar in wildtype and *IL-1β*$^{K133R/KI33R}$ mice.

A) BMDMs from wildtype (WT) and *IL-1β*$^{K133R/KI33R}$ mutant mice (K133R) seeded in a 15 cm dish at a cell density of 2X10^7 were treated with 100 ng/ml LPS for 5 hours in the presence or absence of MG132 (20 μM in the last 2 hours of LPS priming). Ubiquitinated proteins were purified by TUBE and analysed by immunoblot. Ponceau staining and actin shows protein loading. Data is representative of three independent experiments. Ag control indicates agarose control. B) BMDMs from WT, *IL-1β*$^{K133R/KI33R}$ and *IL-1β*$^{-/-}$ mice were seeded in a 15 cm dish at a cell density of 2 X 10^7 were treated with 100 ng/ml LPS for 5 hours in the presence of MG132 (20 μM in the last 2 hours of LPS priming). Immunoprecipitations were carried out with ubiquitin specific linkage antibodies against K48- and K63-linked ubiquitin chains along with IgG control, followed by immunoblot analysis. * indicates light chain of the antibody that appears in all conditions. Ponceau staining shows protein input loading control. Data is representative of three independent experiments.

4.2.5. Endogenous IL-1β, but not other inflammasome components or associated cytokines, is a short-lived protein in macrophages

The above findings demonstrated that IL-1β expressed in 293T cells is rapidly turned over by proteasomal degradation. To examine the stability of endogenous IL-1β, WT BMDMs were treated with LPS (TLR4 ligand) followed with pan-caspase inhibitor, Q-VD-OPh added in the last 30 min to block the potential for LPS/CHX-induced caspase activation and cell death. Cells were then treated with the protein synthesis inhibitor CH for the indicated time points (in Figure 4.2.5A and B). Immunoblot analysis showed degradation of endogenous IL-1β between 2 and 4 hours post CHX treatment, with almost complete loss of IL-1β protein at 6 hours (Figure 4.2.5A). It is worth mentioning that although NLRP3 expression was also induced upon LPS treatment, NLRP3 degradation was not detected (Figure 4.2.5A). Similarly, caspase-1 and ASC were stable, long-lived, proteins relative to IL-1β (Figure 4.2.5A). Moreover, the other inflammasome-associated cytokines, IL-18 and IL-1α, were also significantly more stable when compared to IL-1β following CHX treatment (Figure 4.2.5B). Therefore, the rapid turnover of endogenous IL-1β stability appears unique among inflammasome related proteins, which may reflect its potent inflammatory potentially.

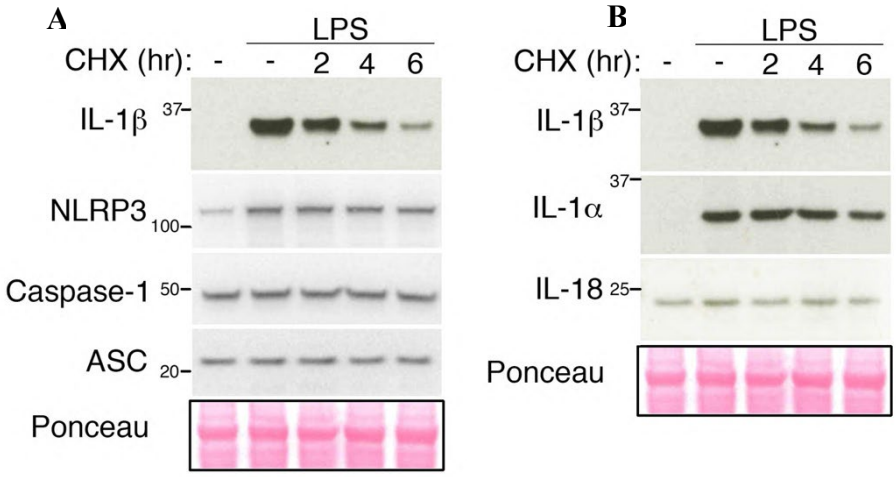


Figure 4.2.5 Endogenous IL-1β, but not other inflammasome components or associated cytokines, is a short-lived protein in macrophages.

Wildtype BMDMs were seeded in a 24-well plate at a cell density of $4x10^5$ cells per well and treated with LPS (100 ng/ml for 3 hours) followed with Q-VD-OPh (20 μM) added in the last 30 min. Cells were then treated with cycloheximide (CHX; 20 μg/ml) at indicated time points. Total cell lysates were analysed by immunoblot for A) NLRP3 inflammasome components and B) IL-1β related cytokines, as indicated. Ponceau shows protein loading. Data representative of two independent experiments.

4.2.6. Precursor IL-1β from *IL-1β*$^{K133R/KI33R}$ BMDMs is stabilised compared to WT IL-1β

Studies in 293T cells indicated that loss of IL-1β ubiquitination on K133 residue moderately stabilised precursor IL-1β, which is otherwise rapidly degraded in a proteasomal-dependent manner (Figure 4.2.1). Therefore, the stability of endogenous precursor IL-1β from *IL-1β*$^{K133R/KI33R}$ mice was examined. IL-1β expression was induced by LPS treatment in BMDMs from WT and *IL-1β*$^{K133R/KI33R}$ mice followed by CHX treatment for up to 8 hours (in Figure 4.2.6A). Notably, precursor IL-1β from *IL1-β*$^{K133R/KI33R}$ BMDMs showed increased stabilisation when compared to WT IL-1β (Figure 4.2.6A and C). Increased *IL-1β*$^{K133R/KI33R}$ stability was also observed in response to stimulation with the TLR1/2 ligand, Pam3Cys (Figure 4.2.6B and C). In contrast, caspase-1 and NLRP3 showed similar expression between WT and *IL-1β*$^{K133R/KI33R}$ BMDMs in all conditions (Figure 4.2.6A & B). These results demonstrate that the loss of ubiquitination on IL-1β K133 increases its stability, irrespective of the priming stimuli.

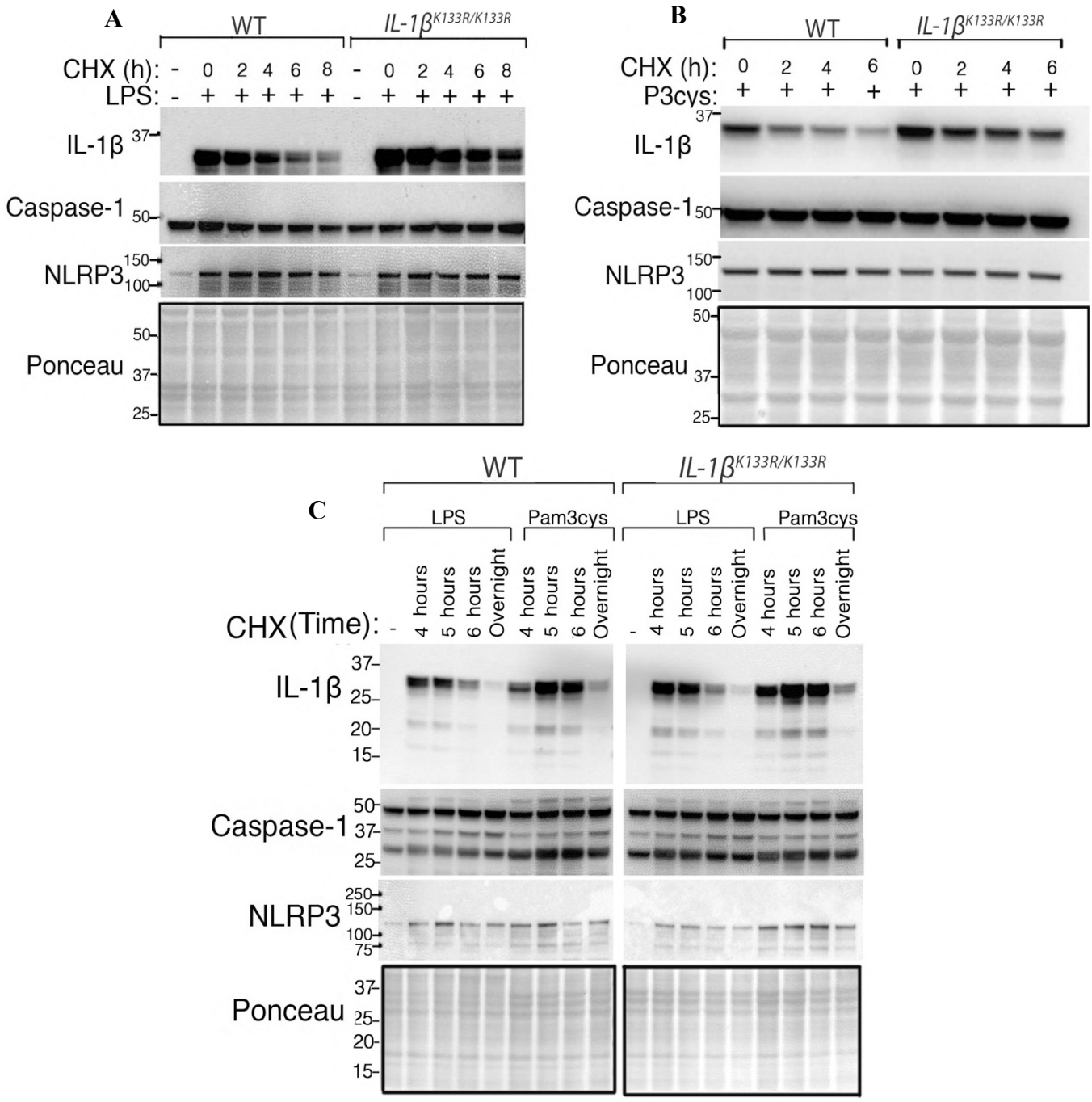


Figure 4.2.6 Precursor IL-1β from *IL-1β*$^{K133R/KI33R}$ BMDMs is stabilised compared to WT IL-1β

Wildtype and *IL-β*$^{K133R/KI33R}$ BMDMs were seeded in a 24-well plate at a cell density of 4x10^5 cells per well and treated with A) LPS (100 ng/ml for 3 hours), B) P3cys/Pam3cys (500 ng/ml for 3 hours) and C) LPS (100 ng/ml for 3 hours) & Pam3cys (500 ng/ml for 3 hours), as indicated, followed by cycloheximide; CHX (20 μg/ml) at indicated time points. Prior to priming stimuli, cells were pre-treated with QVD (20 μM for 15 mins). Cells were lysed and analysed on immunoblot for proteins as indicated. Ponceau shows protein loading. Data representative of three independent experiments.

4.2.7. Precursor IL-1β is degraded by the proteasome in WT and *IL-1β*$^{K133R/KI33R}$ BMDMs

To examine if the loss of endogenous wildtype IL-1β involved not just the proteasome but also the lysosome-autophagy pathway, BMDMs derived from WT and *IL-1β*$^{K133R/KI33R}$ mice were stimulated with either Pam3Cys or LPS to induce IL-1β expression and then treated in the presence of an inhibitor of lysosomal function, bafilomycin-A1 (BafA1), or MG132, during CHX chase assays. Interestingly, only MG132 treatment could rescue IL-1β degradation triggered by CHX treatment, while baliomycin-1A did not have any effect on IL-1β stability (Figure 4.2.7A and B). As shown before (Figure 4.2.6A, 4.2.6B), IL-1β K133R showed increased stability when compared to WT IL-1β (Fig. 4.2.7B), while caspase-1 and NLRP3 expression was similar between wildtype and *IL-1β*$^{K133R/KI33R}$ BMDMs (Figure 4.2.7B). This data demonstrates that IL-1β is regulated by ubiquitin-mediated proteasomal degradation, and that lysosomal targeting is not involved in precursor IL-1β turnover.

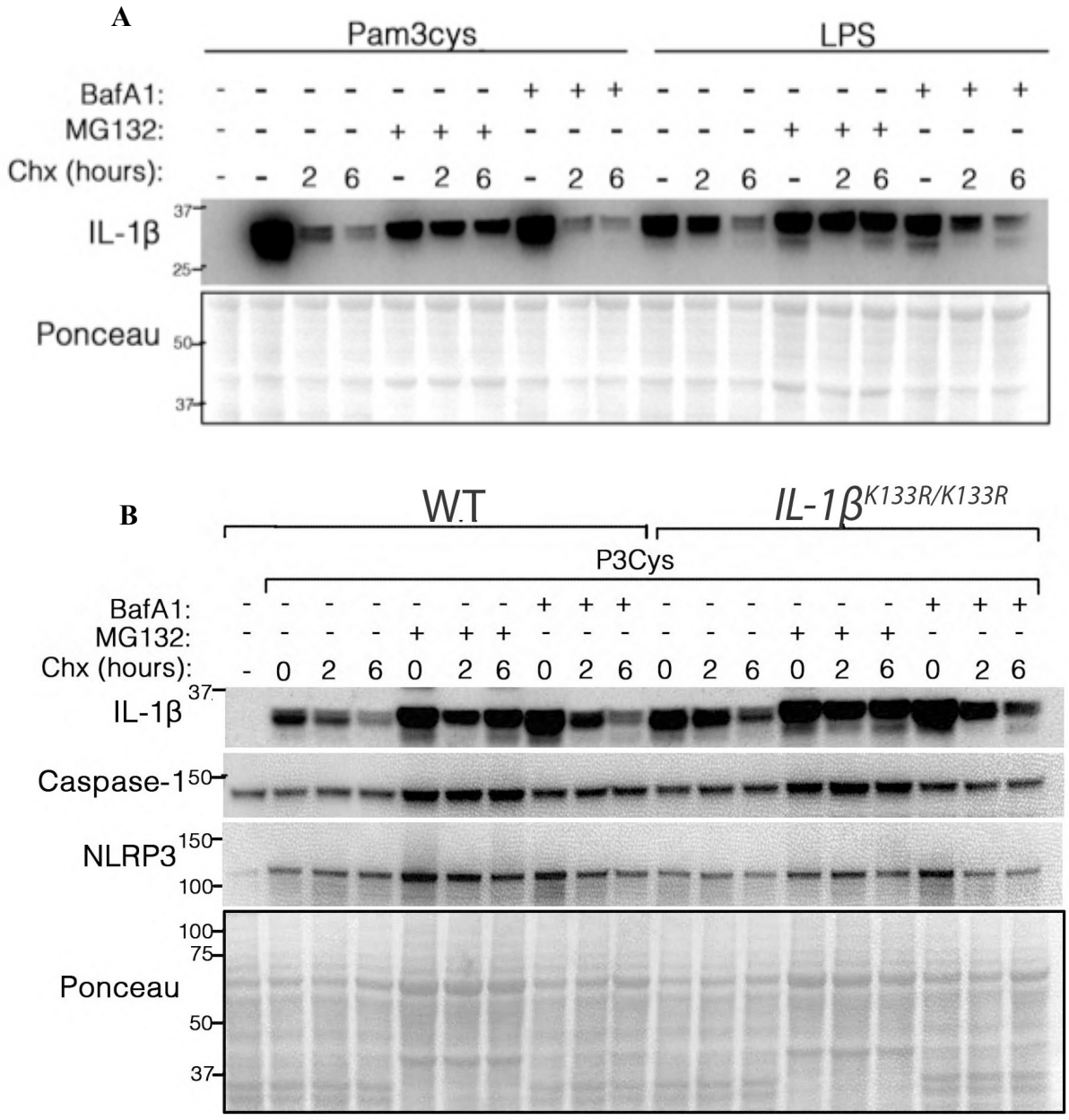


Figure 4.2.7 Precursor IL-1β is degraded by the proteasome in WT and *IL-1β*$^{K133R/KI33R}$ BMDMs.

A) Wildtype BMDMs were seeded in a 24-well plate at a cell density of 4 x 10^5 cells per well and treated with P3cys (0.5 μg/ml for 4 hours) or LPS (100 ng/ml for 4 hours) followed by CHX (20 μg/ml) at the indicated time points, in the presence or absence of MG132 (20 μM) or bafilomycin-1A, Baf1 (200 nM). B) Wildtype and *IL-1β*$^{K133R/KI33R}$ mutant BMDMs were seeded in a 24-well plate at a cell density of 4 x 10^5 cells per well and treated with P3cys (0.5μg/ml for 4 hours) followed by CHX (20 μg/ml) at the indicated time points in the presence or absence of MG132 (20 μM) or bafilomycin-1A, BafA1 (200 nM). Cells were lysed and analysed by immunoblot. Ponceau shows protein loading. Data representative of three independent experiments.

4.2.8 ATP and Nigericin treatment causes proteasome-mediated IL-1β degradation.

Canonical NLRP3 stimuli, ATP and nigericin, reduced IL-1β ubiquitination over time, which may result from pyroptotic, caspase-1-mediated, cell death (Figure 3.2.2). Therefore, to examine if NLRP3 activators might target IL-1β for proteasomal degradation to limit its inflammatory potential, BMDMs were derived from *Caspase-1*$^{-/-}$ mice, primed with LPS and treated with ATP or nigericin in the presence or absence of MG132 (Figure 4.2.8A). Interestingly, ATP or nigericin treatment reduced precursor IL-1β levels even in the absence of caspase-1 (i.e. pyroptosis), and MG132 treatment rescued this loss (Figure 4.2.8A). In contrast, IL-18 levels, a cytokine also activated by these NLRP3 stimuli, was similar across all conditions (Figure 4.2.8A).

Nlrp3$^{-/-}$ BMDMs were used to test if ATP induced loss of precursor IL-1β was dependent on NLRP3 signalling, but not caspase-1. Similar to caspase-1 loss, NLRP3 deficiency did not prevent ATP-mediated degradation of precursor IL-1β, which was rescued upon MG132 treatment (Figure 4.2.8B). Therefore, NLRP3 activators, such as ATP, can exacerbate precursor IL-1β ubiquitination which correlates with its degradation by the proteasome.

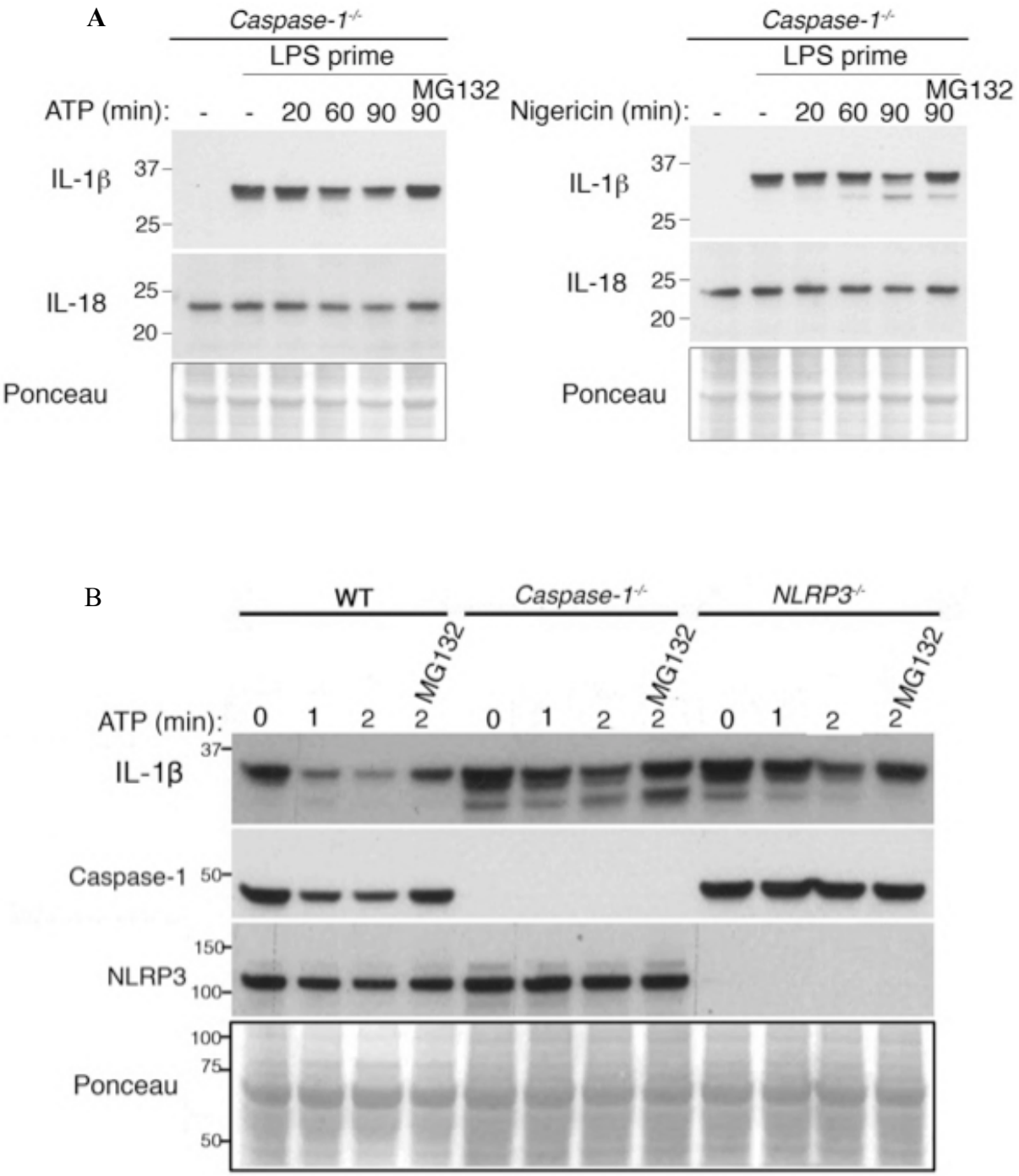


Figure 4.2.8 ATP and Nigericin treatment targets precursor IL-1β for degradation.

A) Caspase-$1^{-/-}$ BMDMs were seeded in a 24-well plate at a cell density of 4 x 10^5 cells per well and were LPS primed (100 ng/ml for 3 hours) followed by treatment with ATP (5 mM) -left panel - or nigericin (10 μM)-right panel - in the presence of MG132 (20 μM), as indicated. B) Wildtype (WT), *Caspase-1*$^{-/-}$ and *Nlrp3*$^{-/-}$ BMDMs were seeded in a 24-well plate at a cell density of 4 x 10^5 cells per well and were LPS primed (100 ng/ml for 3 hours) followed by treatment with ATP (5 mM) in the presence of MG132 (20 μM) as indicated. Cells were lysed and analysed by immunoblot for proteins, as indicated. Ponceau shows protein loading. Data representative of three independent experiments.

4.2.9 Enhanced stabilisation of precursor IL-1β in *IL-1β*$^{K133R/KI33R}$ macrophages increases IL-1β activation and release.

Previous experiments showed increased stability of precursor IL-1β from *IL-1β*$^{K133R/KI33R}$ BMDMs when compared to WT IL-1β (Figure 4.2.6A&B and 4.2.7B). Next, I wanted to examine if the increased stability of precursor IL-1β observed in *IL-1β*$^{K133R/KI33R}$ mouse macrophages impacted caspase-1-mediated IL-1β activation and release in response to NLRP3 inflammasome signalling. BMDMs derived from WT and *IL-1β*$^{K133R/KI33R}$ mice were subjected to ATP, alum, smac-mimetic (Cp. A), or nigericin-mediated NLRP3 activation and IL-1β secretion was analysed in the cell supernatants by ELISA. Remarkably, all these NLRP3 activators resulted in increased IL-1β secretion from *IL-1β*$^{K133R/KI33R}$ BMDMs compared to WT (Figure 4.2.9A). On the other hand, TNF-α secretion, as measured by ELISA in cell supernatants, was comparable between WT and *IL-1β*$^{K133R/KI33R}$ BMDMs (figure 4.2.9B). Thus, these data suggest that loss of IL-1β K133 ubiquitination and consequent IL-1β stabilization can result in moderately increased IL-1β secretion.

NLRP3 activation and IL-1β release is enhanced in serum free Opti-MEM media (Schneider, Thomas et al. 2013). CHX chase assays performed in Opti-MEM media documented that precursor IL-1β stability was enhanced in BMDMs from *IL-1β*$^{K133R/KI33R}$ mice when compared to WT cells (Figure 4.2.9D), similar to assays performed in serum containing media (Figure 4.2.9A). In contrast, MCL-1, another short-lived protein, showed similar expression and degradation between WT and *IL-1β*$^{K133R/KI33R}$ BMDMs (Figure 4.2.9C). Likewise, other inflammasome components, NLRP3, caspase-1 and ASC were comparably expressed between mutant and wildtype BMDMs (Figure 4.2.9C).

Next, a NLRP3 inflammasome activation assay was performed. BMDMs from WT and *IL-1β*$^{K133R/KI33R}$ mice were first cultured in complete DMEM (containing FBS) and primed with LPS or P3cys. Subsequently, cells were washed with Opti-MEM medium thrice to remove residual serum. All secondary NLRP3 activation signals; nigericin, alum and Cp.A were then provided in Opti-MEM (Figure 4.2.9D & E). Cell lysates and supernatants were collected and analysed by immunoblot. Cell lysates showed that upon LPS or P3cys priming alone, or in combination with NLRP3 inflammasome activation stimuli (Nigericin/ Alum/ Cp.A) precursor IL-1β showed increased stabilisation in *IL-1β*$^{K133R/KI33R}$ BMDMs compared to WT cells (Figure

4.29D). All the other inflammasome components, Caspase-1, ASC and NLRP3, showed similar expression between *IL-1β*$^{K133R/KI33R}$ mutant and WT cells (Figure 4.2.9D). Supernatants showed increased IL-1β secretion in *IL-1β*$^{K133R/KI33R}$ mutant BMDMs compared to wildtype (Figure 4.2.9E). However, caspase-1 cleavage in supernatants were similar between both genotypes (figure 4.2.9E). It is worth noting that while LPS and nigericin treatment appeared to result in marginally increased caspase-1 cleavage in *IL-1β*$^{K133R/KI33R}$ mutant, there was a more profound amount of IL-1β secreted from *IL-1β*$^{K133R/KI33R}$ mutant BMDMs compared to wildtype (Figure 4.2.9E). Thus, overall data suggests that enhanced stabilisation of pro IL-1β in *IL-1β*$^{K133R/KI33R}$ mutant macrophages results in increased IL-1β secretion.

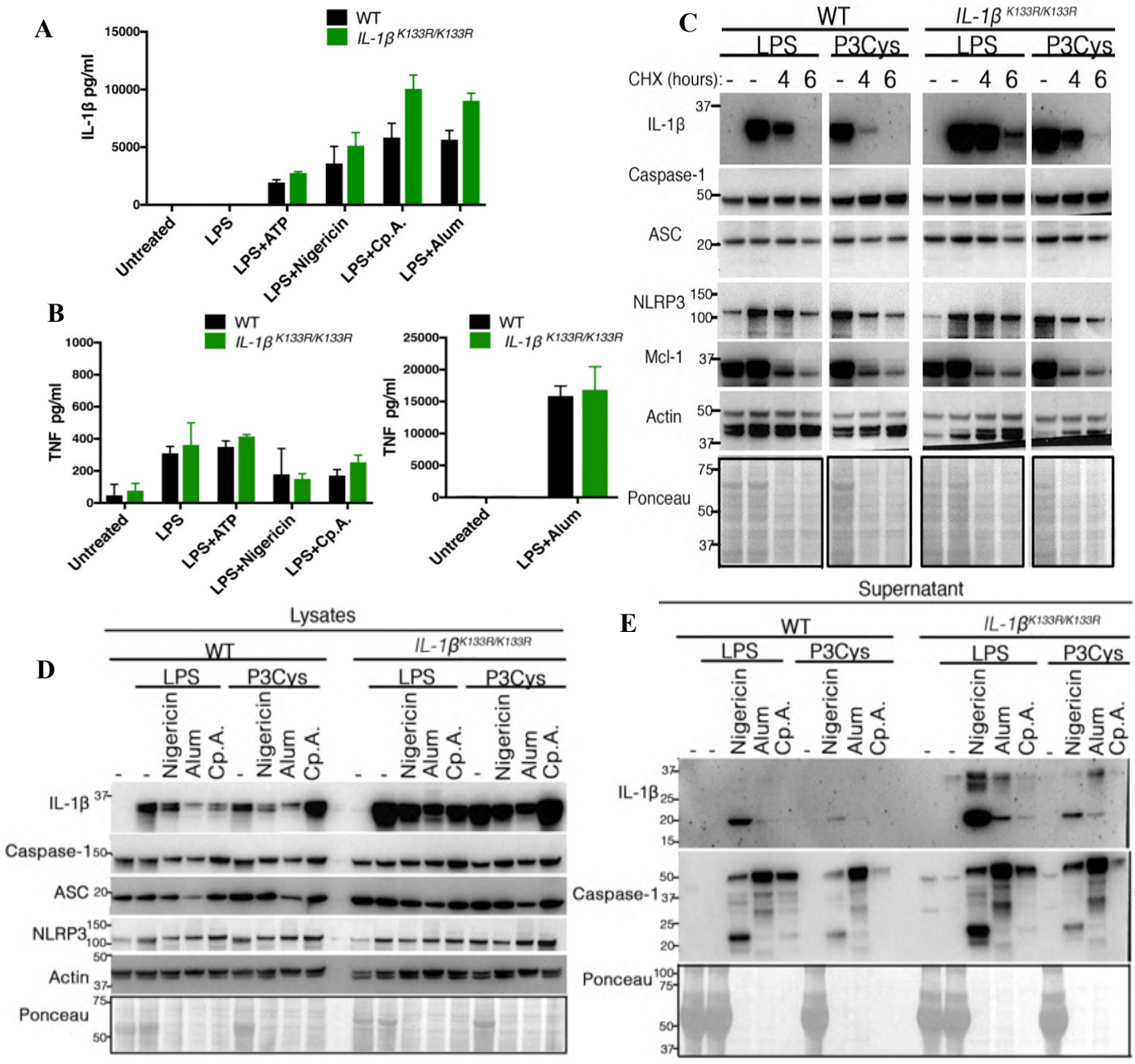


Figure 4.2.9 Enhanced stabilisation of precursor IL-1β in *IL-1β*$^{K133R/KI33R}$ mutant increases IL-1β secretion in BMDMs.

WT and *IL-1β*$^{K133R/KI33R}$ BMDMs were seeded in a 96-well plate at a cell density of 2 x 10^5 cells/well and treated with LPS alone (3 hours) and then with ATP, Nigericin, compound A (Cp.A) or Alum. A) IL-β & B) TNF secretion was measured in cell supernatants by ELISA. n=3 independent experiments. Error bars represent the SD in all graphs. C) WT and *IL-1β*$^{K133R/KI33R}$ BMDMs were seeded in a 24-well plate at a cell density of 4 x 10^5 cells/well cultured in complete DMEM and primed with either LPS or P3Cys and replaced with Opti-MEM media during cycloheximide (CHX) chases. Cells were lysed and analysed by immunoblot. WT and *IL-1β*$^{K133R/KI33R}$ BMDMs were cultured in complete DMEM and primed with either LPS or P3Cys. NLRP3 inflammasome activation stimuli treatments, as indicated, were added in Opti-MEM. D) Cell lysates and E) supernatants were analysed by immunoblot. C), D) & E) Ponceau and actin shows protein loading. Data representative of three independent experiments.

4.2.10. Enhanced stabilisation of precursor IL-1β in *IL-1β*$^{K133R/KI33R}$ mice increases IL-1β secretion in neutrophils.

As *IL-1β*$^{K133R/KI33}$ BMDMs showed increased IL-1β secretion in response to NLRP3 inflammasome activation (Figure 4.2.9D&E), I wanted to examine IL-1β secretion of *IL-1β*$^{K133R/KI33R}$ mice in another relevant cell type of innate immune system. Therefore, neutrophils ($CD11b^+$ $Ly6G^+$) sorted from bone marrow of WT and *IL-1β*$^{K133R/KI33R}$ mice, were subjected to inflammasome assays to examine IL-1β secretion. Pre-stimulation with IFNγ was performed prior to addition of an LPS priming signal to render neutrophils sensitive to inflammasome signalling (Chen, Lawlor et al. 2018), and cells were subsequently treated with nigericin (Figure 4.2.10A). Consistent with BMDMs, ELISA results showed increased IL-1β secretion in response to nigericin treatment in *IL-1β*$^{K133R/KI33R}$ neutrophils compared to WT cells, while TNF release was comparable between genotypes (Figure 4.2.10A and B). LPS treatment alone at 3 hours or 6 hours in the presence or absence of IFNγ, or untreated neutrophils, did not lead to any IL-1β secretion (Figure 4.2.10A). These results demonstrate that the increased IL-1β secretion in *IL-1β*$^{K133R/KI33R}$ neutrophils is specific to NLRP3 and IL-1β and does not result from altered TLR priming or NF-κB activation in *IL-1β*$^{K133R/KI33R}$ neutrophils.

Western blot analysis also showed that IL-1β expression induced by LPS was heightened in *IL-1β*$^{K133R/KI33R}$ neutrophils compared to WT cells in the presence of absence of IFNγ, while NLRP3, caspase-1 and ASC expression were similar in both genotypes across all treatments (Figure 4.2.10C). Interestingly, as in the case of macrophages, upon nigericin treatment increased IL-1β activation (cleavage to the bioactive p17 fragment) and secretion in supernatants of *IL-1β*$^{K133R/KI33R}$ neutrophils was enhanced compared to neutrophils isolated from WT mice (Figure 4.2.10D). Actin loading showed loss of actin with nigericin treatment indicative of cell death (Figure 4.2.10D). Thus, IL-1β K133 ubiquitination acts to decrease precursor IL-1β levels in both macrophages and neutrophils, and this limits available IL-1β pools for caspase-1 mediated cleavage upon NLRP3 inflammasome signalling.

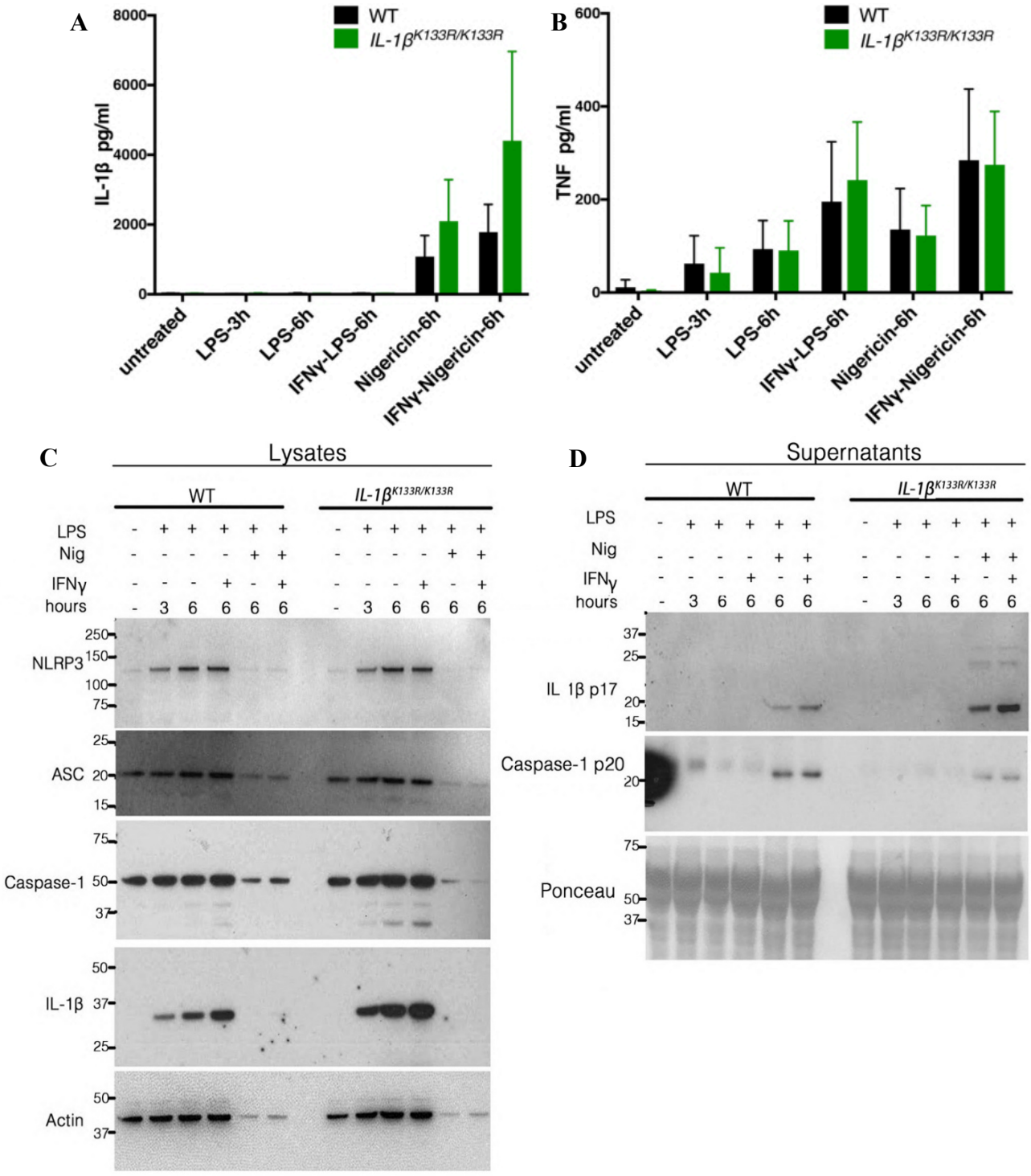


Figure 4.2.10 Enhanced stabilisation of precursor IL-1β in *IL-1β*$^{K133R/KI33R}$ neutrophils increases IL-1β levels and secretion.

WT and *IL-1β*$^{K133R/KI33R}$ CD11b^{+} Ly6G^{+} neutrophils sorted from bone marrow were pre-treated with IFNγ (50 ng/ml for 1 hour). Priming was provided with LPS (100 ng/ml for 3 hours) and NLRP3 inflammasome activation signal was provided with nigericin (2.5 μM for 6 hours). A) IL-β & B) TNF secretion was measured in cell supernatants by ELISA. n=3 independent experiments. Error bars represent the SD in all graphs. C) Cell lysates & D) supernatants were analysed by immunoblot. Ponceau and actin shows protein loading. Data representative of three independent experiments.

4.2.11. *IL-1β*$^{K133R/KI33R}$ mice exhibit increased IL-1β secretion in response to endotoxic shock *in vivo*

To examine if precursor IL-β stabilisation and increased IL-1β activation and secretion is phenocopied in *IL-1β*$^{K133R/KI33R}$ mice in an *in vivo* model of endotoxic shock, WT (C57BL/6) and *IL-1β*$^{K133R/KI33R}$ mice were injected with LPS (100 μg, 5mg/kg) intraperitoneally. IL-β, TNF-α and IL-6 secretion were measured by ELISA in the serum and peritoneal lavage fluid. Consistent with *in vitro* data, IL-1β secretion was increased in *IL-1β*$^{K133R/KI33R}$ mice compared to WT mice in both serum and peritoneal lavage fluid (Figure 4.2.11A). TNF-α and IL-6 secretion were similar in serum and peritoneal lavage fluids in both genotypes (Figure 4.2.11B&C). Further, analysis of peritoneal lavage fluids by immunoblot showed increased precursor IL-β stabilisation in *IL-1β*$^{K133R/KI33R}$ mice compared to WT animals (Figure 4.2.11D), while NLRP3 expression was similar across both genotypes (Figure 4.2.11D). Thus, enhanced precursor IL-1β stabilisation in *IL-1β*$^{K133R/KI33R}$ mice resulted in increased p17 IL-1β cleavage in *IL-1β*$^{K133R/KI33R}$ mice (Figure 4.2.11d). Overall, data suggests that ubiquitination of IL-1β on K133 inhibits LPS-induced inflammation *in vivo* by targeting precursor IL-1β for proteasomal degradation.

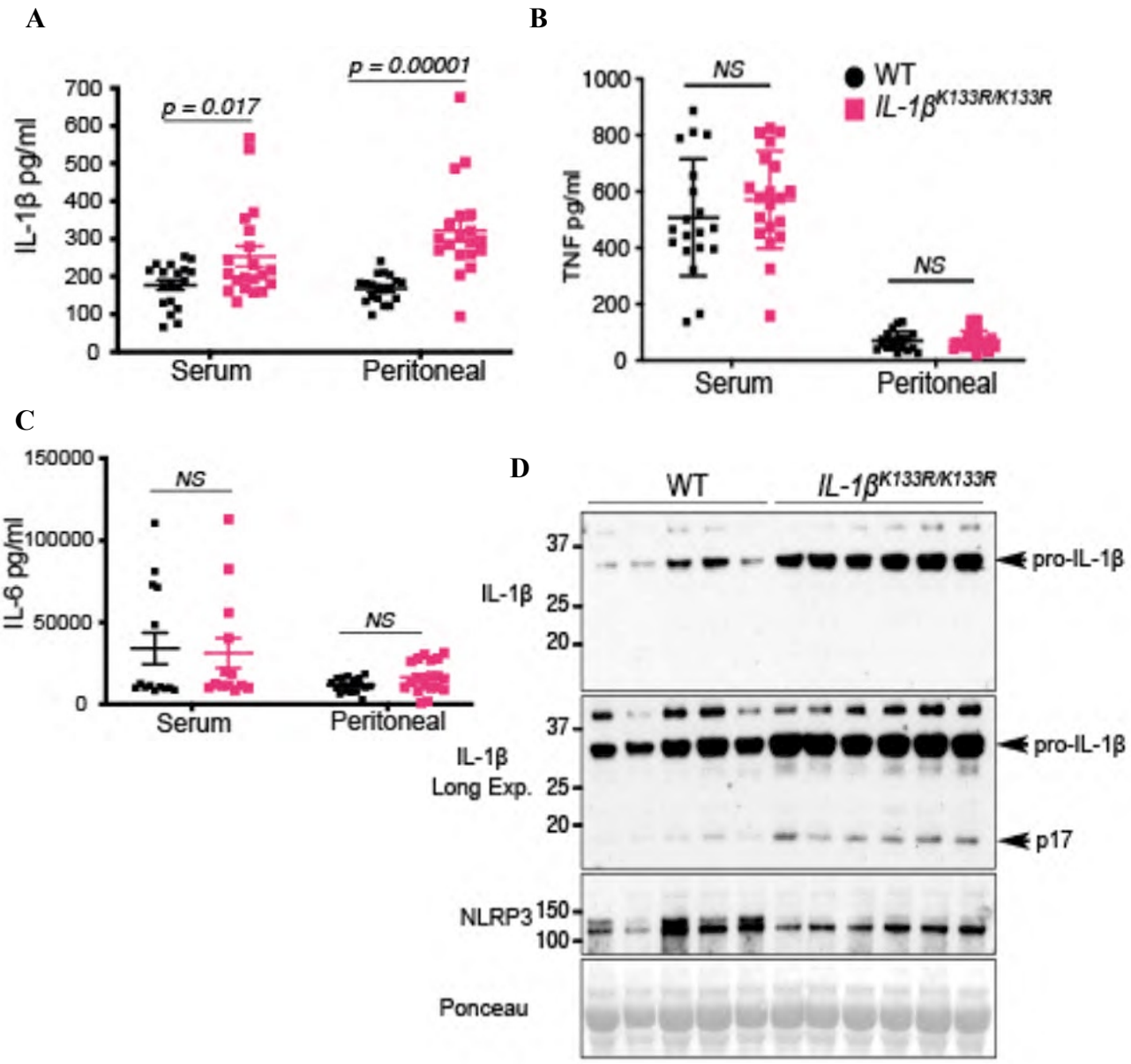


Figure 4.2.11 *IL-1β*$^{K133R/KI33R}$ mice exhibit increased IL-1β secretion in response to endotoxic shock in vivo.

Wildtype (C57BL/6) and *IL-1β*$^{K133R/KI33R}$ mice were injected with LPS (100 μg) intraperitoneally. After 2 hours serum and peritoneal lavage fluid was harvested and analysed for A) IL-1β, B) TNF-α and C) IL-6 levels by ELISA. Data are mean + SEM, symbols represent individual mice, n=19 mice per genotype from 3 pooled independent experiments. P-values calculated by an unpaired t-test. D) Lysates collected from peritoneal lavage fluids were analysed by immunoblot. Each lane represents a mouse. N=5-6 mice per genotype. Ponceau shows protein loading.

4.3 Discussion

There are no studies currently in the literature that describe the regulation of K133 IL-1β ubiquitination and its effects on IL-1β activation and secretion using primary cells, or in an *in vivo* animal model. This chapter aimed to study the role of K133 IL-1β ubiquitination and its physiological relevance via the generation of *IL-1β*$^{K133R/K133R}$ mice, using CRISPR/Cas9 targeted point mutation of K133 to arginine of precursor IL-1β.

Surprisingly, endogenous IL-1β was found to be a short-lived protein compared to other key inflammasome components and was rapidly turned over via ubiquitin mediated proteosomal degradation. The pivotal discovery in this chapter is that precursor IL-1β in *IL-1β*$^{K133R/K133R}$ mice displayed increased stability and consequently resulted in increased IL-1β secretion upon inflammasome activation in macrophages and neutrophils. Mirroring these findings, *IL-1β*$^{K133R/K133R}$ mice showed increased IL-1β secretion in response to LPS-induced inflammation *in vivo*.

Contrary to expectation, I found that the ubiquitination of precursor IL-1β was similar in wildtype (WT) and *IL-1β*$^{K133R/K133R}$ BMDMs in response to LPS priming alone or with proteasomal inhibitor (MG132) treatment. Therefore, K133 is not the only site targeted for IL-1β ubiquitination. Indeed, recently five lysine residues (including K133) were identified as ubiquitination sites of precursor murine IL-1β in 293T cells by Mass spectrometry analysis (Zhang, Liu et al. 2018). Overexpression of His tagged IL-1β with tagged versions of ASC, caspase-1 and K63-linked ubiquitin (specifically: Flag-ASC, V5-caspase-1 and HA-K63 Ubiquitin) constructs in 293T cells for 48 hours followed by immunoprecipitation with anti-His antibody for IL-1β isolation revealed K30, K133, K205, K209 and K247 ubiquitination sites by Liquid chromatography-electrospray tandem mass spectrometry analyses (LC-MS/MS analyses) (Zhang, Liu et al. 2018). Interestingly, in the same study, in a 293T overexpression system it was observed that IL-1β K133R ubiquitination was significantly reduced compared to WT (Zhang, Liu et al. 2018). This finding therefore contrasts my results showing that primary cells isolated from *IL-1β*$^{K133R/K133R}$ mice show no difference in IL-1β ubiquitination. Reasons for these discrepancies remain unclear. They could reflect 293T cells lacking the repertoire of ubiquitin E3 ligases present in innate immune cells that target IL-1 β for modification. Regardless, this emphasises the importance of validating key events in primary

cell and through *in vivo* studies, as overexpression of tagged versions of ubiquitin likely affect the physiology and bioactivity of ubiquitin leading to artefacts.
Further, the other ubiquitination sites (K30, K205 and K209) identified by Zhang et al were also mutated in the IL-1β K17A construct used in this study (Zhang, Liu et al. 2018). However, IL-1β K17A showed increased ubiquitination compared to WT IL-1β as presented in chapter 3, Figure 3.2.12. Interestingly, K247 that is a non-conserved lysine residue between human and mouse was not mutated in the IL-1β K17A construct and K247 was also identified as a ubiquitination site (Zhang, Liu et al. 2018). Therefore, it is possible that, in the absence of other key lysine residues, ubiquitination occurs on K247 and/or on the other available lysine residue, K224 of IL-1β K17A construct, as ubiquitination is previously reported to occur at any lysine residue in some proteins (Treier, Staszewski et al. 1994, King, Glotzer et al. 1996). In the future, it will be interesting to examine if the other IL-1β residues reported to be decorated by ubiquitin, K30, K205, K209 and K247, distinctively impact IL-1β signalling in relevant immune cell types.

In a separate study two KFERQ-like motifs (132-'QKSLV'-136 and 198-'QLESV'-202) of precursor IL-1β were identified to facilitate autophagy mediated IL-1β secretion under starvation conditions (Zhang, Kenny et al. 2015). KFERQ sequence motifs are recognised by cargos for lysosomal transport of proteins in chaperone-mediated autophagy (Dice, Chiang et al. 1986, Kaushik and Cuervo 2012) and one of the two identified IL-1β KFERQ-motifs, 132-'QKSLV'-136 is noteworthy as it contains the ubiquitination site K133. In this study, *IL-1β*$^{K133R/K133R}$ BMDMs show increased stability of precursor IL-1β and upon inflammasome activation subsequently secreted increased IL-1β in Opti-MEM medium (under starvation conditions) compared to WT. Future structural studies of IL-1β from *IL-1β*$^{K133R/K133R}$ mice may provide new insights into the mechanism of IL-1β secretion under starvation conditions.

This chapter also revealed that K48 and K63-linked ubiquitin chains decorate precursor IL-1β upon LPS priming (in the presence of proteasome inhibitor, MG132). Predominantly, K48-linked ubiquitin chains are associated with targeting proteins for proteosomal degradation contrary to K63-linked ubiquitin chains, which are mostly reported to regulate proteasome-independent processes, such as inflammatory signalling pathways, endocytosis and DNA repair (Komander and Rape 2012, Yau 2016). However, K63-linked ubiquitination has also emerged as targeting some proteins for proteasomal destruction (see manuscript for refs). Moreover, ubiquitin architecture is enormously complex, and in addition to homotypic chains,

heterotypic chains containing more than one linkage type have been recently reported. Heterotypic chains are classified in to i) mixed ubiquitin linkages, where each ubiquitin is modified by another ubiquitin molecule containing different linkages and ii) branched ubiquitin linkages, where a single ubiquitin molecule can be modified by two or more ubiquitin molecules, creating a branch (Swatek and Komander 2016). Recent technologies such as UbiChEM-MS (ubiquitin chain enrichment middle-down mass spectrometry) and Ubiquitin-clipping can expose heterotypic chains and provide quantitative analysis such as the ratio of branched to unbranched linkages (Wang, Wu et al. 2020).

Interestingly, using MS-based methods it was revealed that K63-linked polyubiquitin chains can recruit K48-linked chains for the subsequent assembly of K48/K63 branched ubiquitin chains targeting protein substrates to proteasome degradation (Ohtake, Tsuchiya et al. 2018). Strikingly, proteasomal inhibition with MG132 reportedly resulted in the enhancement of K48/K63 branched linkage and unbranched K48 linkage; while lysosomal inhibition with bafilomycin increased unbranched K63 linkage (Ohtake, Tsuchiya et al. 2018). In this chapter I demonstrated that precursor IL-1β from WT and *IL-1β*$^{K133R/KI33R}$ BMDMs were both targeted for proteosomal degradation and IL-1β turnover was not affected by bafilomycin treatment. Therefore, it is tempting to speculate that precursor IL-1β may contain branched K48/K63 branched linkage targeting it for proteosomal degradation. Future MS-based approaches, such as Ub-absolute quantification/parallel reaction monitoring (Ub-AQUA/PRM) and in vitro assays such as ubiquitin chain restriction assay (UbiCrest), may reveal and quantify the branched and/ unbranched ubiquitin linkage chains that regulate IL-1β. These studies will be important to address whether IL-1β ubiquitination may alter IL-1β activity other than targeting it for proteasomal degradation.

In accordance with previous studies, I showed that IL-1β secretion was blocked in a dose-dependent manner with inhibitors of deubiquitinases (DUBs) upon NLRP3 inflammasome activation (Juliana 2012, Lopez-Castejon, Luheshi et al. 2013, Py, Kim et al. 2013). DUB inhibitor treatments also increased precursor IL-1β ubiquitination upon LPS priming, similar to MG132 treatment, suggesting that in the presence of DUB inhibitors, enhanced IL-1β ubiquitination may lead to its degradation and therefore contribute to limiting IL-1β secretion upon NLRP3 inflammasome activation. Hence, this data expands on the known regulatory effect of ubiquitination on NLRP3 (Py, Kim et al. 2013, Ozen and Bilginer 2014). Strikingly,

my data also showed that canonical NLRP3 inflammasome activation stimuli, ATP and nigericin, can target IL-1β for proteasomal degradation independent of NLPR3 and caspase-1. However, we understand from previous studies caspase-8 can also cleave pro IL-1β and in the absence of caspase-1, caspase-8 mediated apoptosis is activated therefore the reduction of IL-1β expression at later time points could just reflect that (Vince, Wong et al. 2012, Antonopoulos 2015). Another hypothesis is that, DUB enzymes are required to deubiquitinate IL-1β upon ATP or nigericin treatment, similar to NLRP3 where deubiquitination is required for its activation (Juliana 2012, Py 2013). Therefore, further studies are required to make solid conclusions to whether ATP or nigericin treatment act independent of proteolysis regulation to ubiquitinate and target IL-1β for proteasomal degradation, operating as an additional protective mechanism to inhibit excessive inflammatory responses.

My findings indicate that IL-1β is a short-lived protein that undergoes rapid proteasomal degradation relative to other inflammasome components, and this rapid degradation mechanism occurs similarly in both 293T IL-1β overexpression systems and at the endogenous level in macrophages. Consistent with this, IL-1β K133 mutants that abrogate K133 modification with ubiquitin chains, stabilised precursor IL-1β in both 293T cells and in *IL-1β*$^{K133R/KI33R}$ BMDMs. Note, like IL-1β K133A mutant, IL-1β K133R mutant also stabilised precursor IL-1β in 293T cells (data not shown). In contrast, phospho-mutants of IL-1β, S134A and S134E, had no effect on IL-1β stability, suggesting that ubiquitination, but not phosphorylation, is primarily involved in regulating IL-1β stability. However, precursor IL-1β K133 mutant stability in 293T cells and in *IL-1β*$^{K133R/KI33R}$ BMDMs was not completely protected from degradation suggesting that there are other lysine residues that are likely targeted for modification consistent with ubiquitination of multi-lysine mutant (IL-1βK17A) in the previous chapter. Regardless, my findings are consistent with published literature indicating that the ubiquitin-proteasome system (UPS) is predominantly responsible for the degradation of polyubiquitinated, short-lived, proteins (Hershko 1983, Hershko 2005, Finley 2009).

As expected, the increased stabilisation of IL-1β derived from *IL-1β*$^{K133R/KI33R}$ BMDMs resulted in increased IL-1β, but not TNF, secretion in response to NLRP3 inflammasome activation induced by activation stimuli; ATP, nigericin, alum or Compound. A (Cp. A). Interestingly, our laboratory reported Cp.A., an Inhibitor of Apoptosis (IAP) antagonist, can induce both

caspase-8-mediated proteolysis of IL-1β directly and also caspase-8 induced NLRP3 inflammasome activation (Vince, Wong et al. 2012, Lawlor, Khan et al. 2015, Lawlor, Feltham et al. 2017), suggesting that increased IL-1β secretion is not limited to caspase-1 containing complexes.

Due to the distinctive differences in undergoing pyroptosis after canonical NLRP3 inflammasome activation between neutrophils and macrophages, stabilisation of precursor IL-1β and IL-1β secretion upon NLRP3 inflammasome activation were examined in *IL-1β*$^{K133R/KI33R}$ neutrophils. Nigericin, a pore-forming toxin, was chosen to activate the NLRP3 inflammasome in neutrophils, as previous studies indicated that crystal NLRP3 agonists are weak stimulus for NLRP3 activation in neutrophils (Karmakar 2015, Chen, Bezbradica et al. 2016). Despite the differences in neutrophil biology, precursor IL-1β in *IL-1β*$^{K133R/KI33R}$ neutrophils isolated from bone marrow cells was stabilized and resulted in enhanced IL-1β secretion upon NLRP3 inflammasome activation, similar to BMDMs. Thus, regulation of IL-1β ubiquitination in *IL-1β*$^{K133R/KI33R}$ BMDMs and neutrophils appear similar, and increased secretion of *IL-1β*$^{K133R/KI33R}$ from neutrophils is entirely dependent on increased stabilization of precursor IL-1β. Collectively, my data provides strong evidence that enhanced IL-1β secretion in *IL-1β*$^{K133R/KI33R}$ BMDMs can be solely attributed to the increased stabilisation of precursor IL-1β compared to WT BMDMs.

Both of the inflammasome and caspase-1 activated pro-inflammatory cytokines, IL-1β and IL-18, are implicated in the endotoxic shock model (Ohlsson, Bjork et al. 1990, Fischer, Marano et al. 1992, Netea, Fantuzzi et al. 2000). However, one of the hallmarks of endotoxic shock is neutrophilia, and since neutrophils are major contributors of IL-1β (Kovach and Standiford 2012), endotoxic (LPS) shock was selected as a suitable model to examine the stabilisation of IL-1β in *IL-1β*$^{K133R/KI33R}$ mice in the current study. Reflecting *in vitro* findings, both serum and peritoneal lavage fluid of *IL-1β*$^{K133R/KI33R}$ mice showed increased stabilisation of precursor IL-1β and enhanced IL-1β processing to its bioactive fragment when compared to WT animals. Future studies will examine differences in clinical responses such as injecting higher LPS doses and monitoring changes in temperature and organ pathology in this model.

Infection of *IL-1β*$^{K133R/KI33R}$ mice with *Salmonella Typhimurium* is another suitable and physiologically relevant model to examine the regulatory effects of IL-1β K133 ubiquitination

on IL-1β secretion, as at the early acute phase of the infection is dependent on IL-1β production contributed by inflammasome activation in neutrophils to control pathogens (Chen 2014). Therefore, future studies will be carried out in *Salmonella Typhimurium* infection or other physiologically relevant infection models dependent on IL-1β to examine IL-1β K133 ubiquitination regulation during pathogenic infection.

Overall, in this chapter I demonstrated that i) ubiquitination of endogenous precursor IL-1β upon TLR-mediated inflammasome priming is similar in wildtype and *IL-1β*$^{K133R/KI33R}$ mice, and endogenous IL-1β is decorated by K48 and K63-linked ubiquitin chains, ii) endogenous precursor IL-1β is a unique short-lived protein that is targeted for rapid proteasomal degradation compared to other NLRP3 inflammasome components, or related cytokines such as IL-18, iii) IL-1β K133 mutants (K133A in 293T cells and *IL-1β*$^{K133R/KI33R}$ BMDMs and neutrophils) displayed increased stability when compared to WT IL-1β, while IL-1β phosphorylation mutants, S134A, S134E IL-1β did not impact the stability of IL-1β, iv) NLRP3 agonists, ATP and nigericin, can alone target precursor IL-1β for proteasomal degradation independent of NLRP3 signalling, and v) *IL-1β*$^{K133R/KI33R}$ mice challenged with LPS injections intraperitoneally, showed increased stabilisation of precursor IL-1β, resulting in increased IL-1β pool for activation *in vivo*. Thus, from the results of this chapter it can be concluded that the K133 ubiquitination of IL-1β inhibits inflammation by enhancing IL-1β turn over by proteasomal degradation, and thereby limiting its activation by inflammasome complexes and other proteases. This level of complex regulation presumably exists to safe-guard against excessive, and potentially damaging, inflammatory responses.

Chapter 5: Overall Discussion

Innate immune cells, such as macrophages and neutrophils, are the first responders to pathogens or environmental threats and provide inflammatory responses to protect the host. Inflammasome signalling is an integral part of the innate immune system that contributes to the inflammatory response by promoting the activation and secretion of key proinflammatory cytokines, Interleukin-1β (IL-1β) and IL-18. Mechanisms that regulate the activity of IL-1β downstream of the NLRP3 inflammasome were the main focus of my thesis.

Tight control of innate immune responses is key for health, as excessive inflammation can lead to autoinflammatory diseases and inadequate responses can lead to immunosuppression. Post-translation modifications (PTMs), such as ubiquitination, are molecular switches that play an important role in regulating innate immune signalling to maintain innate immune equilibrium. Therefore, this thesis explored the interesting question of whether IL-1β is subject to PTMs and, if so, how these modifications impact IL-1β functionality within the scope of NLRP3 inflammasome signalling within the innate immune system.

In chapter 3, I examined the presence of PTMs on precursor IL-1β and determined that downstream of the caspase-1 cleavage site within the p17 subunit lysine133 (K133) is targeted for ubiquitination and serine134 (S134) is targeted for phosphorylation. While phosphorylation site IL-1β mutants (phospho-dead S134A and phospho-mimetic S134E) did not greatly impact IL-1β processing by caspase-1 or -8, the ubiquitination site IL-1β mutant (K133A) completely abrogated IL-1β activation and secretion. Structural modelling studies showed that K133 residue is capable of forming a salt bridge with aspartate 129 (D129), I generated the K133A and D129A mutants (consisting of lysine 133 residue mutated to arginine) and tested in caspase-1 and caspase-8 activation assays. Remarkably, both K133A IL-1β mutant and aspartate mutant D129A IL-1β mutant completely abrogated IL-1β secretion upon caspase-1 activation, while K133R IL-1β mutant did not perturb caspase-1 processing and IL-1β secretion. Thus, the ubiquitination of K133 may interfere with the electrostatic interaction between K133 and D129, inhibiting caspase-1 activation and IL-1β secretion. Future studies could define the structure of the IL-1 K133A and D129A mutants, including assessments for broad structural integrity (such as conducting circular dichroism on recombinant proteins) to help explain why this salt bridge is so important for caspase-1 processing.

Furthermore, TUBE experiments demonstrated that the mature p17 IL-1β fragment is unlikely to be ubiquitinated, and a previous study reported that phosphorylation events are also not detectable on mature IL-1β (Kobayashi, Appella et al. 1988). Therefore, it may be that caspase-1/8 mediated IL-1β secretion occurs only in the non-ubiquitinated/phosphorylated cellular pools of IL-1β. Obtaining pure fractions of ubiquitinated and non-ubiquitinated fractions of IL-1β can be problematic as ubiquitination has low-site specificity and ubiquitination can occur at nearby available lysine residue/s. Despite these issues, recent experiments using recombinant caspase-1 cleavage of cellular IL-1β, followed by TUBE purification of ubiquitylated IL-1β pools, suggest that ubiquitinated IL-1β is not able to be cleaved by caspase-1 (data not shown).

Very little information is present in the literature about phosphorylation of precursor IL-1β. An early study examining phosphorylation of human precursor IL-1β revealed that IL-1β was phosphorylated 10-fold less than related family member IL-1α, and most of the phosphorylation occurred at serine residues on IL-1β (Kobayashi, Appella et al. 1988). In line with this, and consistent with my findings via Mass Spectrometry, a search in the PhosphoSitePlus® database of reported phosphorylation sites of IL-1β revealed that the S134 site is phosphorylated. In addition, phosphorylation sites tyrosine141 (Y141), serine201 (S201) and serine269 (S269) of mouse precursor IL-1β are reported in the PhosphoSitePlus® database and all three residues are conserved between human and mouse species (www.phosphosite.org/uniprotAccAction?id=P10749). Therefore, future *in vitro* studies could be conducted to examine if the mutants of the putative phosphorylation sites, Y141, S201 and S269 of IL-1β, impact IL-1β processing and secretion during inflammasome activation and, if so, which kinases or phosphatases might be involved.

The reported S134 phosphorylation site of IL-1β was defined via a global phospho-proteomics analysis using Stable Isotope labelling by/with amino acids in cell culture (SILAC) approach in wildtype and Receptor interacting protein kinase-3 (RIPK3) knockout macrophages (Wu, Tian et al. 2012). In a further co-immunoprecipitation study, precursor IL-1β was also reported to be in a complex with RIPK3, RIPK1, capases-8 and caspase-1 (Duong, Onizawa et al. 2015). Further, in the absence of the DUB and ubiquitin E3 ligase A20, a regulator of ubiquitin dependent signalling and an NF-κB inhibitor, IL-1β ubiquitination was enhanced in a RIPK3-

dependent manner (Duong, Onizawa et al. 2015). Therefore, it will be interesting to examine if S134 phosphorylation of IL-1β is required for its complex interaction with RIPK3, RIPK1, caspase-8 and caspase-1, and if S134 phosphorylation, or any of the other identified sites, impacts IL-1β ubiquitination, either in WT or in A20 knockout cells. Moreover, despite my studies in 293T cells suggesting that IL-1β S134 phosphorylation does not impact caspase-1 recognition, IL-1β processing or precursor IL-1β stability, it will be important to validate these findings in innate immune cells.

One of the major findings of chapter 3, namely, that precursor IL-1β is ubiquitinated at K133 was also reported in the study examining how A20 restricts NLRP3 inflammasome activation (Duong, Onizawa et al. 2015). Strikingly, in this study it was reported via overexpression of an IL-1β K133R mutant in 293T cells that IL-1β secretion was reduced upon caspase-1 activation compared to the wildtype setting (Duong, Onizawa et al. 2015). However, in my thesis, overexpression of IL-1β K133R in 293T cells resulted in similar IL-1β secretion compared to wildtype. The only difference between my experiments and this study was that ASC was omitted in the overexpression experiments of my thesis. Therefore, it may be worth repeating the caspase-1 activation assay with IL-1β wildtype and K133R mutant constructs along with co-transfection of ASC to see if we can attain consistent results with the other group.

In chapter 4, the regulatory effects of IL-1β K133 ubiquitination on IL-1β stability and activation was examined further *in vitro* and *in vivo*. Consistent with previous studies, I showed that wildtype precursor IL-1β was ubiquitinated and underwent proteasomal degradation (Lopez-Castejon, Luheshi et al. 2013, Ainscough, Frank Gerberick et al. 2014). Further, I demonstrated that IL-1β has an extremely rapid turnover when compared to other inflammasome components. Notably, although IL-1β from *IL-1β*$^{K133R/KI33R}$ BMDMs was ubiquitinated upon priming and was decorated with both degradative K48- and activating K63-linked ubiquitin chains, it was more stable than wildtype precursor IL-1β. IL-1β from *IL-1β*$^{K133R/KI33R}$ neutrophils behaved in a similar manner, showing an increased stability and propensity for activation in response to canonical NLRP3 stimuli and cell death activators. Finally, upon endotoxin challenge, *IL-1β*$^{K133R/KI33R}$ mice exhibited increased precursor IL-1β stabilisation and subsequently, increased IL-1β secretion, reflecting my *in vitro* findings. Thus, by using a novel CRISPR/Cas9 generated knockin mouse model, I was able to perform, in a more physiological setting, an in-depth analysis of the impact of ubiquitination of K133, and

showed that ubiquitination of K133 in precursor IL-1β limits its bioavailability for activation and secretion by caspase-1 and caspase-8 by targeting precursor IL-1β for proteasomal degradation, providing valid evidence contrary to the previous study that reported K133 ubiquitination promotes caspase-1 processing and IL-1β secretion (Duong, Onizawa et al. 2015) (**Figure 5.1**).

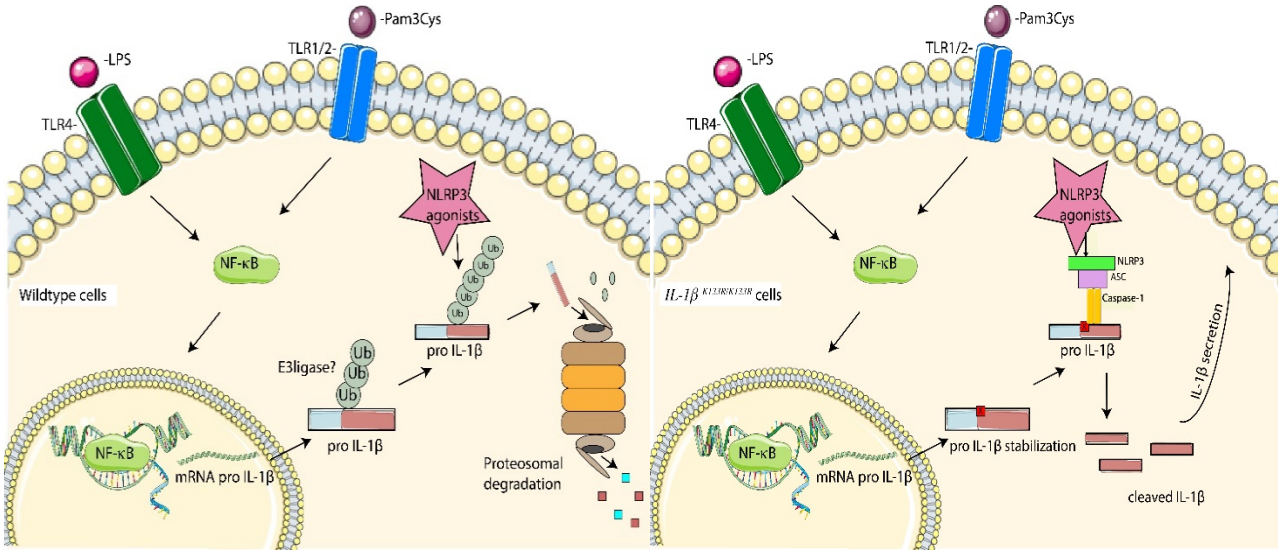


Figure 5.1: Schematic representation of ubiquitination mediated IL-1β activation in wildtype and *IL-1β*$^{K133R/K133R}$ cells. In WT cells on the left, upon TLR activation (TLR4 or TLR2), transcription and ubiquitination of precursor IL-1β on K133 occurs, targeting the protein for proteasomal degradation. NLRP3 agonists such as ATP can also independently cause IL-1β ubiquitination, possibly acting as a second layer of control to keep inflammation under check. On the other hand, in *IL-1β*$^{K133R/K133R}$ cells on the right, upon TLR activation (TLR4 or TLR2), transcription of precursor IL-1β occurs normally, however, K133 residue cannot be ubiquitinated and leads to increased IL-1β stabilisation, subsequently resulting in increased IL-1β secretion upon NLRP3 inflammasome activation, leading to inflammation. Thus, K133 ubiquitination of IL-1β limits the bioavailability of precursor IL-1β.

Recently, POH1/Rpn11, a K63-specific deubiquitinase (DUB) associated with the 19S regulatory particle lid of the proteasome was reported to deubiquitinate IL-1β by removing K63-linked ubiquitin chains and negatively regulate IL-1β processing (Zhang, Liu et al. 2018). Consistent with my results and others, IL-1β K133 was reported as the target ubiquitination

site. Additional IL-1β ubiquitination sites; K30, K205, K209 and K247 in precursor IL-1β were also reported. Mass spectrometry (MS) analysis in this study was performed using overexpression systems and co-transfections included caspase-1, ASC and K63-ubiquitin chains (Zhang, Liu et al. 2018), while the MS analysis in this thesis included only overexpression of IL-1β construct to reduce artefacts. Overexpression of K63-linked ubiquitin may significantly alter the physiology and bioactivity of ubiquitin, possibly leading to ubiquitination of sites that do not normally occur. Consistently, my data using the multi lysine mutant IL-1βK17A, where 17 lysines were mutated showed no defect in IL-1β ubiquitination. POH1-mediated deubiquitination was reported mainly at K133 and K247 residues, although the deubiquitination dominantly occurred at K133 residue (Zhang, Liu et al. 2018). Therefore, it will be important to examine IL-1β ubiquitination via MS-based approaches, such as absolute quantification (AQUA)/parallel reaction monitoring (PRM), that could provide a quantitative analysis of IL-1β ubiquitination, including the presence of other ubiquitin linkages (apart form K48, K63) and branched ubiquitin chains.

Similar to the study on A20-mediated IL-1β deubiquitination (Duong, Onizawa et al. 2015), POH1-mediated IL-1β deubiquitination reported that the K133R IL-1β mutant reduced IL-1β processing and in the presence of POH1, IL-1β processing was further inhibited (Zhang, Liu et al. 2018). These results are contrary to the data presented in this thesis. The mechanism that leads to the reduced IL-1β processing by deubiquitination of K63-linked ubiquitin chains of IL-1β mediated by POH1 are not clearly described. However it could be hypothesised that deleting the 19S proteasome subunit POH1, which removes ubiquitin to facilitate target protein entry into the 20S proteasome for degradation (Hao, Nanduri et al. 2013, Nanduri, Hao et al. 2015), could result in increased ubiquitination of IL-1β and subsequent perturbed processing. Moreover, K63-linked ubiquitination of IL-1β could be branched and contain K48-linked ubiquitin chains that target IL-1β for proteosomal degradation (as discussed in chapter 4.3). Thus, future studies are required to examine accurately the presence of various ubiquitin linkages on IL-1β and whether ubiquitination simply targets IL-1β for degradation, or whether it has additional functional roles.

Despite the discovery of putative DUBs that may remove ubiquitin from IL-1β, the native ubiquitin E3 ligase(s) that ubiquitinate IL-1β and promote its degradation remain ill-defined. In this thesis I performed MS analysis to identify novel ubiquitin E3 ligases that interact with

IL-1β. I have now identified several ubiquitin E3 ligases (Ari1, Dtx31, Rnf213 and Trim21) that potentially associate with IL-1β complexes. Future studies, including co-immunoprecipitation and knockout of the putative E3 ligases *in vitro* and *in vivo*, are required to validate the ubiquitin E3 ligase that interacts and promotes ubiquitination of IL-1β, and/or impacts inflammasome activation. Intriguingly, prior to my work, only one potential ubiquitin E3 ligase has been uncovered to date, the human papillomaviruses (HPV) associated, E6-oncoprotein associated protein (E6-AP), which is reported to induce IL-1β ubiquitination and proteasomal degradation in non-myeloid cells expressing the E6 oncoprotein, and thereby allow viral escape from innate immune surveillance (Niebler, Qian et al. 2013). In addition to this ligase, a ubiquitin E2 conjugating enzyme, UBE2L3, an indirect target of caspase-1, has also reported to enhance IL-1β ubiquitination and proteasomal degradation (Eldridge, Sanchez-Garrido et al. 2017). However, surprisingly in this study, LPS-induced IL-1β ubiquitination was not enhanced by proteasome inhibition. Furthermore, UBE2L3 was reported to only induce K48 ubiquitination, contrary to the results in this thesis and work by others (Duong, Onizawa et al. 2015, Zhang, Liu et al. 2018). Therefore, it will be interesting to investigate if any lysine residues in IL-1β are subject to K48 only-linked ubiquitination. Intriguingly, UBE2L3 itself appears to be regulated by canonical and noncanonical inflammasome activation, as capase-1 activity is reported to target UBE2L3 for proteasome degradation. Consequently, UBE2L3 depletion causes increased bioavailability of IL-1β for caspase-1 processing. Conversely, in the absence of caspase-1, UBE2L3 mediates IL-1β ubiquitination to promote its proteasomal degradation. Single nucleotide polymorphisms of UBE2L3 are associated with autoimmune inflammatory conditions including rheumatoid arthritis and Crohn's disease (Franke, McGovern et al. 2010, Orozco, Eyre et al. 2011). Therefore, investigating the E3 binding partners of UBE2L3 may provide some insights into the mechanism of IL-1β ubiquitination. One further study also suggested that an unknown inflammasome-independent host or pathogen ubiquitin E3 ligase triggered by Group A *Streptococcus* (GAS) pore-forming toxin streptolysin O (SLO) induces the ubiquitination and degradation of IL-1β to evade host defence (Hancz, Westerlund et al. 2019).

Overall, the results of this study and other emerging studies indicate that the ubiquitination of IL-1β plays a critical role in host protection and inflammation. Remarkably, no sequence variants of IL-1β have been reported to be associated with autoinflammatory disease according to the Infevers database (Touitou, Lesage et al. 2004), an open source collection of all sequence

variants associated with autoinflammatory diseases. This make sense as sequence variants of IL-1β could lead to deleterious effects hence the control of IL-1β is orchestrated at multiple levels (transcriptional and post-translational) by the host. Alternatively, as suggested by my data and recent studies, IL-1β is likely targeted for ubiquitination at multiple sites, meaning that a mutation in any single site may be insufficient for a strong phenotype. However, if a dominant ubiquitin E3 ligases which targets IL-1β is identified, it will be fascinating to examine if variants of this might be associated with autoinflammatory conditions, as my studies predict that their loss of function would increase the cellular pools of precursor IL-1β available for activation and initiating deleterious inflammatory responses. In conclusion, this thesis shows that K133 ubiquitination of IL-1β targets precursor IL-1β for proteosomal degradation, upon priming and inflammasome activation, keeping inflammation under check to maintain the health of the host.

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