Understanding the role of zinc in IL-1β production

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Contents

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Tables and Figures

Abstract

Zinc is an essential biological trace element required for proper immune functioning. Zinc deficient individuals have been reported to suffer compromised immune responses and increased levels of inflammatory cytokines. Inflammation is integral to the pathology of many disease states, ranging from pathogen dependent infectious disease to non-infectious disease such as cancer, heart disease, diabetes and stroke. One of the main mediators of inflammation is the pro-inflammatory cytokine interleukin-1β (IL-1β). Production of IL-1β occurs via a two step process; firstly the transcription of an inactive pro-form is initiated, followed by protease activation leading to the cleavage of IL-1β to a mature form. Here it is shown that *in vitro* zinc depletion of macrophages, using the zinc chelators TPEN and DTPA, leads to pro-IL-1β cleavage and furthermore to increased release of active IL-1β. This would suggest that zinc depletion induces activation of proteases that cleave IL-1β. Caspase-1, ASC, PP2A, cathepsin B and cathepsin G are all shown to regulate zinc depletion-induced IL-1β release in macrophages. The cell death proteins XIAP and caspase-8 have also been identified to be regulated by zinc depletion in macrophages and there is literature to suggest that these proteins may contribute to IL-1β processing and release. By identifying a role for zinc depletion in IL-1β processing we move closer to identifying potential therapeutic targets for zinc deficiency induced inflammatory disease.

Zinc also has regulatory roles in the expression of IL-1β. Here a systems biology approach is utilised to create an explicit representation of the pathways involved in IL-1β expression. In many *in vivo* and *in vitro* models, transcription of pro-Interleukin-1β is induced by the gram negative cell wall component lipopolysaccharide (LPS). A systematically curated network map of IL-1 transcription has been created. The map encompasses interactions at the macrophage cell membrane, where LPS binds Toll-like receptor 4 (TLR4); the resulting cytoplasmic signalling cascades, including MAPK and NF-κB; and finally the specific transcription factor interactions in the nucleus. By creating this model we aim to enable the production of dynamic models of IL-1 transcription.

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Thesis Title: Understanding the role of zinc in IL-1β production

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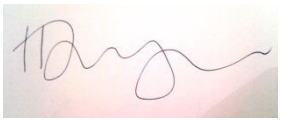
Signed: 

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Abbreviations

AcP	Accessory Protein
AIM2	Absent in melanoma 2
APS	Ammonium persulfate
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
BMDM	Bone marrow derived macrophage
CARD	Caspase recruitment *domains*
CD-14	Cluster of differentiation 14
cGMP	Cyclic guanosine monophosphate
Cu^{+}	Copper
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DTPA	Diethylenetriaminepentaacetic Acid
ECL	Enhanced chemi-luminescence
ER	Endoplasmic Reticulum
FCS	Foetal Calf Serum
FLIP	FLICE-like inhibitor protein
GLF	Z-Gly-Leu-Phe-chloromethyl ketone
H_2SO_4	Sulphuric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IAP	Inhibitor of apoptosis protein
ICE	Interleukin-1beta converting enzyme
IL-1	Interleukin-1
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-1Ra	Interleukin-1 Receptor antagonist
IL-18	Interleukin-18
IL-33	Interleukin-33
IL-36	Interleukin-36
IL-37	Interleukin-37
kD	kiloDalton
KO	Knock out
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
M-CSF	Macrophage colony-stimulating factor
MD-2	Lymphocyte antigen 96
MDP	Muramyl dipeptide
MIRIAM	Minimum Information Requested In the Annotation of biological Models
MT	Metallothionein
MyD88	Myeloid differentiation primary response gene (88)
NAIP5	NLR family, apoptosis inhibitory protein 5
NOD	Nucleotide Oligomerization Domain
NF-κB	Nuclear Factor-kappaB

NK cells	Natural killer cells
NLR	NOD-like receptor
NLRC4	NLR family, caspase recruitment domain (CARD) containing 4
NLRP1	NLR family, pyrin domain containing 1
NLRP3	NLR family, pyrin domain containing 3
p38	p38 MAP kinase
PAMP	Pathogen associated molecular pattern
PBS	Dulbecco's Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate
PP2A	Protein phosphatase 2
PRR	Pattern recognition receptor
RIPK	RIP kinase
ROS	Reactive oxygen species
RLR	RIG-I like receptor
SBGN	Systems Biology Graphical Notation
SBML	Systems Biology Markup Language
SDS	Sodium dodecyl sulfate
SIH	Salicylaldehyde isonicotinoyl hydrazone
TEMED	Tetramethylethylenediamine
TMB	3,3',5,5'-tetramethylbenzidine
TIR domain	Toll/IL-1R homology domain
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor alpha
TPEN	N,N,N',N'-Tetrakis(2pyridylmethyl)ethylenediamine
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF-related adapter molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
TTM	Ammonium tetrathiomolybdate
XIAP	X-linked inhibitor of apoptosis protein
YVAD	Ac-YVAD-CHO
Zn^{2+}	Zinc

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Chapter 1: Introduction

1.1 Inflammation

Inflammation is a host defence process that is initiated in response to infection or injury. This process co-ordinates the body's defence systems at the point of insult, assembling multiple immune cell types that function to clear pathogens or damage. There are two clear phases of inflammation: upregulation, associated with the key physiological signs of inflammation- pain, heat, redness and swelling [Nathan, 2002] and resolution [Serhan, 2009], associated with the downregulation of the inflammatory state.

Pathogen-induced inflammation has been well characterised [Takeuchi & Akira, 2010], [Lamkanfi & Dixit, 2011] and it involves an inflammatory process that leads to the clearance of pathogens, preventing spread and bringing about a return to the homeostatic state [Medzhitov, 2008]. Damage-induced or sterile inflammation, as it is more commonly known, occurs in response to signals produced by damaged or dying cells [Rock *et al.*, 2010]. The sterile inflammatory response functions to clear damaged tissue, but in doing so can also propagate tissue damage [Chen & Nuñez, 2010]. It appears this inflammatory response is a key contributor to the pathology of many non-infectious disease states such as atherosclerosis [Galkina & Ley, 2009], stroke [Lucas *et al.*, 2006], diabetes [Larsen *et al.*, 2007] and autoinflammatory diseases such as rheumatoid arthritis [Gabay *et al.*, 2010]. In many instances the downregulation of sterile inflammatory pathways may facilitate a more rapid return to the healthy homeostatic state. The nature of inflammatory pathways in disease will be discussed more thoroughly later.

An important mediator of the inflammatory response is the pro-inflammatory cytokine interleukin-1 (IL-1). Originally described as a fever-inducing pyrogen [Dinarello & Bernheim,

1981], IL-1 is released by cells of the monocytic lineage in response to inflammatory stimuli (pathogenic and sterile) and upregulates inflammation [Gabay *et al.*, 2010]. IL-1 exerts its effects upon multiple cell types. Endothelial cells are induced to express adhesion molecules which facilitate immune cell recruitment to the site of injury. IL-1 action then induces immune cells to express further cytokines and enzymes, which function both at the site of insult and systemically [Gabay *et al.*, 2010]. This central role in the inflammatory response therefore makes IL-1 an excellent therapeutic target for diseases associated with aberrant inflammation.

In keeping with the increasing interest in sterile stimuli of inflammation and the central role of IL-1 in the upregulation of this process, there is great interest in the role of sterile stimuli in IL-1 secretion. There are several well established sterile stimuli including uric acid crystals, cholesterol crystals, amyloid β, ATP and iron oxide [Rock *et al.*, 2010]; however there are many potentially undiscovered mechanisms for sterile induction of inflammation. Data in this thesis suggests zinc deficiency as a potential sterile stimulus of inflammation and IL-1 upregulation.

1.2 Interleukin-1 family cytokines

1.2.1 General

The IL-1 family is essential in the regulation of inflammatory processes. There are 11 members of the IL-1 family, which includes both pro- and anti-inflammatory cytokines [Dinarello, 2009]. Members include the well characterised IL-1α (IL-F1) [March *et al.*, 1985], IL-1β (IL-F2) [March *et al.*, 1985] and IL-1Ra (IL-F3) cytokines and the less well characterised IL-18 (IL-F4) [Udagawa *et al.*, 1997], IL-33 (IL-F11) [Dinarello, 2005], IL-36 (IL-F5,6,8,9)

[Mulero *et al.*, 1999] [Smith *et al.*, 2000] [Kumar *et al.*, 2000], IL-37 (IL-F7) [Smith *et al.*, 2000] and IL-F10 cytokines [Lin *et al.*, 2001] [Sims & Smith, 2010].

IL-18 and IL-33 are pro-inflammatory cytokines. IL-18 is cleaved to its mature form by the protease caspase-1 [Gu *et al.*, 1997] but maintained in an inactive complex with an IL-18 binding protein (IL-18BP) [Arend *et al.*, 2008]. The activity of IL-18 depends upon the balance between active cleaved IL-18 and abundance of its regulatory partner. In contrast to IL-18, full length IL-33 is active and this activity is increased by cleavage with the neutrophil serine proteases cathepsin G and elastase [Lefrançais *et al.*, 2012]. Unlike other IL-1 family members, IL-33 is inactivated by caspase cleavage [Cayrol & Girard, 2009][Lüthi *et al.*, 2009].

IL-36α (IL-1F6), IL-36β (IL-1F8) and IL-36γ (IL-1F9) are pro-inflammatory and bind the IL-1RL2 receptor. IL-136Ra (IL-1F5) functions as an antagonist to this pathway [Towne *et al.*, 2011]. The functions of the five isoforms of IL-37 have yet to be established. It has been suggested that these cytokines are anti-inflammatory and potentially exert their effects by regulating the pro-inflammatory actions of IL-18 [Boraschi *et al.*, 2011]. Of all the IL-1 family members least is known of IL-1F10, which due to structural similarities to IL-1Ra, a catalytically inactive competitor of IL-1α and IL-1β, has been suggested to have anti-inflammatory activity [Sims & Smith, 2010].

The two best characterised pro-inflammatory cytokines within the IL-1 family are IL-1α (IL-1F1) and IL-1β (IL-1F2). These cytokines both interact with the type I IL-1 receptor (IL-1RI) [Sims *et al.*, 1988]. In order to initiate signals at the receptor the cytokines also need to

interact with IL-1 receptor accessory protein (AcP) [Greenfeder *et al.*, 1995] so that a complex of IL-1, IL-1RI and AcP is formed at the membrane. The presence of all three proteins, IL-1, IL-1RI and ACP, is necessary for initiation of IL-1 dependent signalling.

IL-1α and IL-1β are distinct molecules produced from separate genes [March *et al.*, 1985]. The amino acid sequence homology between the two proteins is low (27%), but their tertiary structure is very similar which allows for the shared affinity to IL-1RI [Gabay *et al.*, 2010]. Both cytokines are produced by cells of monocytic lineage; monocytes, macrophages and dendritic cells, [Dinarello, 2010], although IL-1α may be also produced by epithelial cells and keratinocytes [Dinarello, 2009].

The main regulator of IL-1α and IL-1β is the IL-1 receptor antagonist (IL-1RA). IL-1RA binds the IL-RI receptor without binding AcP [Greenfeder *et al.*, 1995] consequently downstream pathways cannot be activated and the receptor is left unavailable for binding of IL-1α or IL-1β. Therefore IL-1Ra acts as a competitor to the classical IL-1 cytokines (IL-1α and IL-1β) and reduces their inflammatory effect [Greenfeder *et al.*, 1995]. Like IL-1α and IL-1β, IL-1Ra is also expressed in response to cell death [Palmer *et al.*, 2007] and inflammatory stimuli [Arend *et al.*, 1998]. This highlights the roles of the IL-1 family in both the upregulation and resolution of inflammation.

Both IL-1α and IL-1β are produced *via* a two-step process thus increasing the level of control of the initiation of the inflammatory response. They are not constitutively expressed. Firstly these cytokines are produced as a precursor proteins or pro-forms that can be subsequently cleaved to produce smaller proteins [Gabay *et al.*, 2010]. Activation of

NF-κB and p38 MAPK pathways lead to the transcription and translation of the 31 kD pro-forms of IL-1α and IL-1β [O'Neill, 2008] [Gabay *et al.*, 2010]. A second activation step is required to initiate the proteolytic cleavage of to the 17kD mature forms.

Both forms of IL-1α are biologically active [Dinarello, 2009]. The protease most associated with IL-1α cleavage is the calcium activated cysteine protease, calpain [Carruth *et al.*, 1991]. Typically IL-1α is associated with the cytoplasm [Kurt-Jones, 1985]. In microglia it has also been shown to be trafficked to the nucleus [Luheshi *et al.*, 2009b], leading to decreased IL-1α release following necrotic cell death [Luheshi *et al.*, 2009a]. IL-1α is found at low levels in the blood [Dinarello, 2009].

Pro-IL-1β is inactive and cell associated. The best characterised protease responsible for this is caspase-1 [Thornberry *et al.*, 1992]. After proteolytic cleavage mature IL-1β is immediately secreted. However, as yet a single mechanism for this secretion has not been identified [Lopez-Castejon & Brough, 2011]. The main focus of this thesis is the cytokine IL-1β. IL-1β does not contain a signal peptide [Auron *et al.*, 1984] and is not secreted by the conventional secretory pathway [Rubartelli *et al.*, 1990]. Also, in LPS activated monocytes IL-1β was determined to be absent from both the endoplasmic reticulum (ER) or Golgi apparatus, both of which are integral to the conventional secretory pathway [Singer *et al.*, 1988]. Further evidence for non-conventional secretion is the translation of IL-1β from free ribosomes as opposed to membrane bound ribosomes [Stevenson *et al.*, 1992]. This thesis will focus upon how zinc levels affect the production of this cytokine, IL-1β.

1.2.2 IL-1β expression

The expression of the pro-form of IL-1β occurs downstream of pattern recognition receptors (PRRs). PRRs recognise conserved patterns in microbial molecules otherwise known as pathogen associated molecular patterns (PAMPs) [Medzhitov & Janeway, 1997]. Identified classes of PRR include the Toll-like receptors (TLRs), the RIG-I like receptors (RLRs), NOD-like receptors (NLRs) and cytosolic DNA receptors [Kumar *et al.*, 2011].

The expression of IL-1β requires TLR activation. TLRs are the most well studied of the PRR families and were first identified in Drosophila [Lemaitre *et al.*, 1996]. In humans there are ten known TLRs and in mice there are twelve [Kawai & Akira, 2010]. The key features of these PRRs are an N-terminal leucine rich repeat (LRR) and a c-terminal cytoplasmic Toll/IL-1R homology (TIR) domain [Pålsson-McDermott & O'Neill, 2007]. This cytoplasmic domain is essential for interactions with downstream effectors.

Activation of both the TLR2 and TLR4 toll-like receptors have been reported to induce IL-1β expression [Akira, 2003][Ozören *et al.*, 2006][Segovia *et al.*, 2012]. The best characterised TLR4 ligand is lipopolysaccharide (LPS) [Miller *et al.*, 2005]. LPS binds the extracellular domain of TLR4, alongside MD-2 and CD-14 [Park *et al.*, 2009][Kim *et al.*, 2005]. This induces conformational changes that lead to TLR4 dimerisation [Lee *et al.*, 2004], which then cause intracellular changes leading to the recruitment of intracellular adaptor proteins. This occurs *via* interaction of the TIR domains of the TLRs with the TIR domains of cytoplasmic adaptor proteins [Kenny & O'Neill, 2008]. These adaptors are Myeloid differentiation primary response gene (88) (MyD88), toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP), TIR-domain-containing adapter-inducing

interferon-β (TRIF), and TRIF-related adapter molecule (TRAM) [Pålsson-McDermott & O'Neill, 2007]. TRAM and TRIF binding activates interferon signalling. Meanwhile, binding of MyD88 and TIRAP, initiates IL-1β expression *via* MAPK and NF-κB dependent transcription [Horng *et al.*, 2002] [Medzhitov *et al.*, 1998]. Activation of both MAPK pathways and the NF-κB pathway in IL-1β expression is dependent upon the signalling complex TRAF6 [Wang *et al.*, 2001]. Signal transduction in these pathways occurs *via* multiple phosphorylation and ubiquitination signalling events [Deng *et al.*, 2000] [Fukushima *et al.*, 2007] [Lamothe *et al.*, 2007]. A more in depth analysis of TLR4 induced IL-1β expression is presented in chapter 5 in the form of a systematically curated network map.

In addition to TLR stimulation, activation of intracellular NOD receptors also upregulates IL-1β expression. NOD1 and NOD2 are found in the cytoplasm. They possess a LRR domain at the c-terminus and one or two CARD domains respectively at the N-terminus [Kumar *et al.*, 2011]. Activation of NODs occurs *via* the binding of bacterial cell wall components, in particular muramyl dipeptide (MDP) [Brown & McIntyre, 2011]. Stimulation of NOD receptors with MDP upregulates IL-1β expression *via* NF-κB [Ferrero-Miliani *et al.*, 2007]. Agonists of TLR2, TLR4, NOD1 and NOD2 have been shown to function *via* TRAF6 to upregulate IL-1β transcription [Tang *et al.*, 2011]. In this paper it is shown that stimulation of the two separate PRRs has a synergistic effect upon IL-1β expression. This is representative of the *in vivo* situation where a pathogen could stimulate multiple inflammatory pathways.

After the induction of expression of the pro-form of IL-1β, proteolytic cleavage is required in order to produce the mature active form of the cytokine. This also occurs following stimulation of a PRR. The NOD-like receptors (NLRs) are essential in this process, which is described in more detail in the following section.

1.2.3 IL-1β processing

Proteolytic cleavage of inactive pro-IL-1β to mature IL-1β requires a second stimulus, separate from the activation of TLRs. This second stimulus activates another type of PRRs: the Nod-like receptors (NLRs). NLRs are an integral part of the multimeric activation platform, the inflammasome [Martinon *et al.*, 2002]. The inflammasome comprises Nod-like receptors (NLRs), the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), and caspase-1. Inflammasome assembly activates caspase-1 and then caspase-1 cleaves pro- IL-1β.

Caspase-1, previously known as Interleukin-1beta converting enzyme (ICE), was the first caspase to be identified [Black *et al.*, 1989][Kostura *et al.*, 1989]. It is produced as an inactive precursor or zymogen [Nadiri *et al.*, 2006]. Traditionally the caspases are associated with signalling cascades that lead to apoptosis, but caspase-1 is associated with inflammation [Riedl & Scott, 2009]. The main substrates of caspase-1 include pro-IL-1β, pro-IL-18, and potentially IL-33 [Schmitz *et al.*, 2005]. In the case of IL-33, cleavage leads to inactivation [Cayrol & Girard, 2009].

1.2.3.1 Inflammasomes

There are multiple NLRs which in turn may form multiple inflammasomes [Martinon *et al.*, 2009] [Pedra *et al.*, 2009]. These include NLR family, pyrin domain containing 1 (known as NLRP1 or NALP1), NLR family, pyrin domain containing 3 (NLRP3, NALP3 or cryopyrin), and NLR family, caspase recruitment domain (CARD) containing 4 (NLRC4 or IPAF). Additionally, non-NLR inflammasomes have also been identified; the PRRs absent in melanoma 2 (AIM2) and retinoic acid inducible gene-I (RIG-I)-like receptor (RLR) have also been shown to form inflammasomes [Poeck *et al.*, 2010] [Guarda & So, 2010] [Chen & Nuñez, 2010]. The precise stoichiometries of the inflammasomes are as yet unknown. NLRs, ASC and caspase-1 interact *via* CARD and PYRIN domains. Caspase-1 contains a CARD domain at its N-terminus [Martinon & Tschopp, 2004], ASC has a CARD and a PYRIN domain, and the NLRs all contain an N terminal CARD or PYRIN domain, depending upon the NLR [Schroder & Tschopp, 2010]. AIM2 contains a PYRIN domain (Fig 1.1).

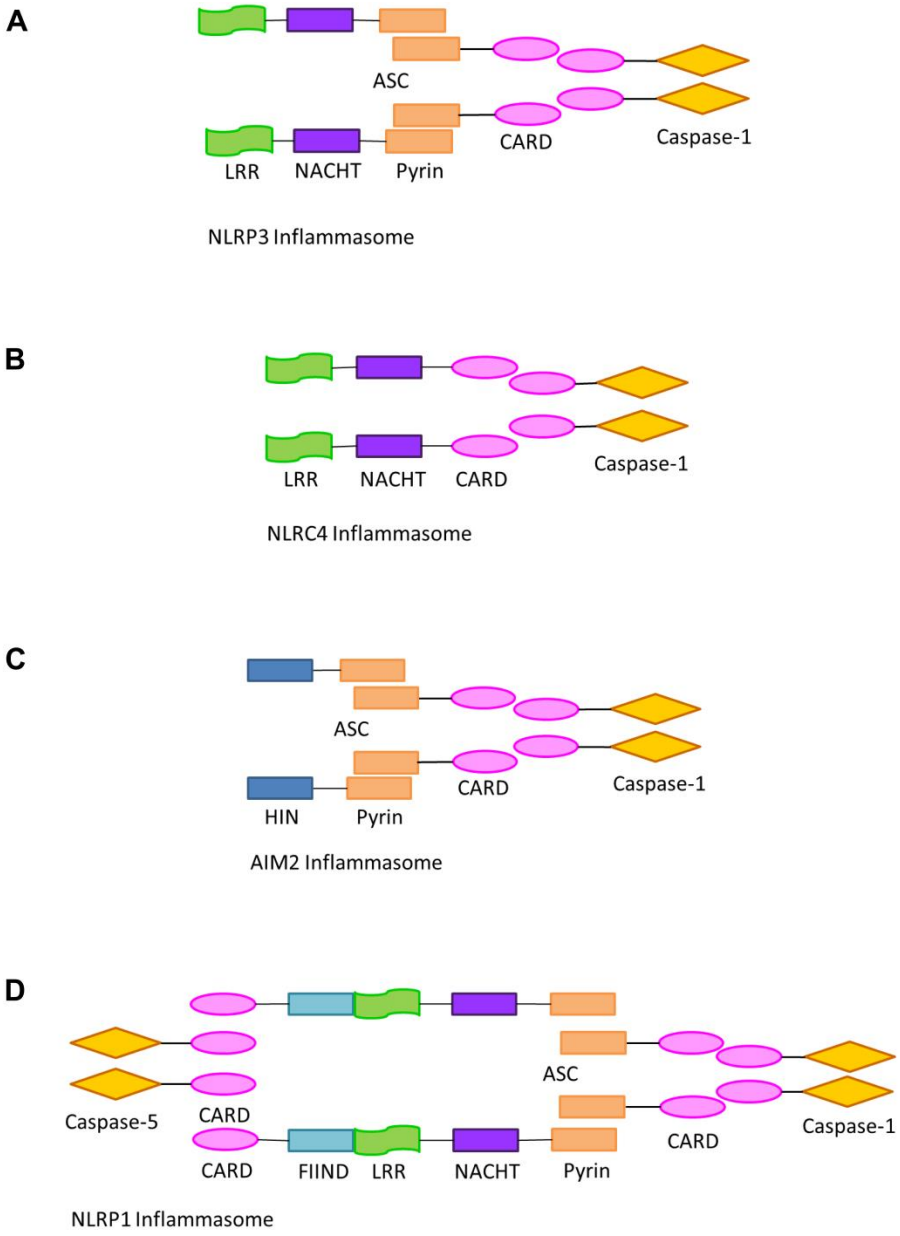

Figure 1.1: Domain structure of the best characterised inflammasomes

The structure of the NLRP3, NLRC4, AIM2 and NLRP1 inflammasomes are illustrated. **(A)** NLRP3 comprises a LRR, NACHT and PYRIN domain, which interacts with ASC (PYRIN and CARD domains) and pro-caspase-1 (CARD and caspase-1). **(B)** NLRC4 comprises a LRR, NACHT and CARD domain, which interacts pro-caspase-1 (CARD and caspase-1). **(C)** AIM2 comprises a HIN and a PYRIN domain, which interacts with ASC (PYRIN and CARD domains) and pro-caspase-1 (CARD and caspase-1). **(D)** NLRP1 comprises a CARD, FIIND, LRR, NACHT and PYRIN domain, which interacts with pro-caspase-5 (CARD and caspase-5), ASC (PYRIN and CARD domains) and pro-caspase-1 (CARD and caspase-1). Stoichiometries are not known for the inflammasomes. Figure adapted from [van de Veerdonk *et al.*, 2011].

Activation of inflammasomes occurs in response to specific PAMP and DAMP signals. The exact mechanisms of inflammasome activation however, remain unclear and appear to be complex. The NLRP3 inflammasome is the best characterised and is activated by a diverse range of PAMP and DAMP stimuli. NLRP3 activating PAMPs include MDP (bacterial muramyl dipeptide) [Martinon *et al.*, 2004], peptidoglycans, bacterial and viral RNA [Kanneganti *et al.*, 2006] and microbial toxins [Mariathasan *et al.*, 2006] such as the pore forming toxin streptolysin O [Harder *et al.*, 2009]. Examples of NLRP3 stimuli that could be classified as DAMPs include ATP [Mariathasan *et al.*, 2006], monosodium urate crystals and calcium pyrophosphate dehydrate crystals, which cause gout and pseudogout respectively [Martinon *et al.*, 2006].

These activators of the NLRP3 inflammasome are thought to initiate one of several overarching processes that in turn act upon the inflammasome. Lowered potassium (K^+) has been identified directly as an activator of the NLRP3 inflammasome in monocytes and macrophages in human and mouse respectively [Perregaux & Gabel, 1994] [Pétrilli *et al.*, 2007]. High levels of potassium and treatment of LPS stimulated monocytes with potassium channel blockers have been shown to inhibit processing of the pro-form of IL-1β to its mature form [Walev *et al.*, 1995]. K^+ efflux is essential for caspase-1 activation following stimulation with the pore forming toxin nigericin, ATP [Perregaux & Gabel, 1994] and MSU [Pétrilli *et al.*, 2007]. Furthermore *in vitro* NALP inflammasome assembly was inhibited in a high K^+ environment [Pétrilli *et al.*, 2007].

ATP stimulation of the P2X7 receptor has been identified to induce K^+ efflux [Perregaux & Gabel, 1994][Kahlenberg & Dubyak, 2004] [Pelegrin & Surprenant, 2006]. In addition to K^+

efflux ATP stimulation also leads to pore formation [Rassendren *et al.*, 1997], a process thought to be closely linked with IL-1β release. Pannexin-1 has been identified as the membrane protein that functions as a non-selective pore in response to P2X7 receptor activation [Pelegrin & Surprenant, 2006]. Blockade of pannexin-1 prevents IL-1β release but not K^+ efflux following stimulation of the P2X7 receptor [Pelegrin & Surprenant, 2007]. Furthermore the K^+ ionophore nigericin, which does not signal via P2X7, also requires a fully functioning Pannexin-1 channel to facilitate IL-1β release [Pelegrin & Surprenant, 2007]. This indicates that that both pannexin-1 and P2X7 are differentially required for inflammasome activation.

A further common mechanism for the activation of the NLRP3 inflammasome is the production of mitochondrial reactive oxygen species (ROS) [Tschopp, 2011]. It has been suggested that the ligand for the NLRP3 inflammasome is found within the mitochondria and the other NLRP3 activators induce its release from the mitochondria in addition to ROS [Leemans *et al.*, 2011]. NLRP3 activation is also induced by upstream activators via the induction of lysosomal destabilisation and subsequent release of cathepsins [Hornung *et al.*, 2008][Bauernfeind *et al.*, 2011]. Cathepsin B is a lysosomal protease that is commonly released in response to NLRP3 activators and inhibition of this protease can abrogate IL-1β release [Hentze *et al.*, 2003].

Release of Ca^{2+} from intracellular stores also been identified to contribute to IL-1β release [Brough *et al.*, 2003] and recently a central role for calcium (Ca^{2+}) signalling has been highlighted in NLRP3 activation. In cell free lysates Ca^{2+} induces NLRP3-ASC complex formation but not direct activation of caspase-1 [Lee *et al.*, 2012] and addition of Ca^{2+}

activates the NLRP3 inflammasome independently of the P2X7 receptor [Lee *et al.*, 2012]. This effect appears to be NLRP3 specific as extracellular Ca^{2+} does not activate the AIM2 or NLRC4 inflammasomes [Lee *et al.*, 2012].

The three main mechanisms identified to be responsible for NLRP3 activation, K^+ efflux, lysosomal destabilisation and mitochondrial ROS generation, can all be linked to Ca^{2+} dynamics. Initiators of the NLRP3 inflammasome that act via K^+ efflux, including ATP, MSU and nigericin, promote Ca^{2+} influx [Murakami *et al.*, 2012]. Following ATP stimulation Ca^{2+} is mobilised from both intracellular and extracellular pools [Stober *et al.*, 2001], both of which are required for ATP stimulated IL-1β release[Murakami *et al.*, 2012]. Ca^{2+} influx induced by ATP was reduced in the presence of elevated K^+ levels [Murakami *et al.*, 2012]. Lysosomes are known stores of intracellular Ca^{2+}[Haller *et al.*, 1996]. Induction of lysosomal rupture with Leu-Leu-OMe induces NLRP3 activation which can be blocked by Ca^{2+} signalling inhibitors [Murakami *et al.*, 2012]. Mitochondria are also important for the regulation of Ca^{2+} dynamics. Mitochondria release stored Ca^{2+} following IP_3 stimulation [Gilabert *et al.*, 2001]. Ca^{2+} signalling inhibitors have been shown to reduce the mitochondrial damage that occurs following ATP treatment. [Murakami *et al.*, 2012] observed reduced production of mROS, reduced loss of membrane potential and reduce release of mtDNA into cytoplasm.

The calcium sensing receptor (CASR) has been reported to be required for activation of the NLRP3 inflammasome via elevated levels of Ca^{2+}. Knock down of this receptor lead to reduced IL-1β release in response to Ca^{2+} and NLRP3 activators [Lee *et al.*, 2012]. CASR is a receptor for Ca^{2+} and functions upstream of phospholipase C (PLC) and adenylate cyclase (ADCY) [Hofer & Brown, 2003]. Phospholipase C cleaves phosphatidylinositol 4,5

bisphosphate (PIP_2)into diacylglycerol (DAG) and 1,4,5-inositol triphosphate (IP_3) [Clapham, 2007]. IP_3 signalling through IP_3 receptors (IP_3R) on the endoplasmic reticulum leads to Ca2+ efflux into the cytoplasm [deSouza *et al.*, 2007]. IL-1β secretion in response to ATP or elevated Ca^{2+} requires phospholipase C (PLC) activity [Lee *et al.*, 2012]. Inhibitors of IP_3 reduced the levels of IL-1β release in response to ATP or Ca^{2+}[Lee *et al.*, 2012]. PLC and IP_3R inhibitors have also been used to reduce Ca^{2+} flux. These inhibitors blocked IL-1β processing in response to ATP, nigericin, MSU and alum[Murakami *et al.*, 2012]. The inhibitors did not to affect NLRP3 or pro-IL-1β expression [Murakami *et al.*, 2012].

CASR negatively regulates adenylate cyclase (ADCY) function [Hofer & Brown, 2003]. Using the ADCY inhibitor, KH7, to mimic CASR function induced IL-1β release in WT macrophages but not in NLRP3-/, ASC-/- or caspase-1 -/- cells [Lee *et al.*, 2012]. In addition, concomitant knock down of the adenylate cyclases ADCY3, ADCY6, ADCY7 and ADCY9 also induces IL-1β release [Lee *et al.*, 2012]. ADCYs synthesise the second messenger cyclic AMP (cAMP) from ATP [Sunahara *et al.*, 1996]. Inhibiting or knocking down ADCY would result reduced levels of cAMP and it can be hypothesised that cAMP retains NLRP3 in an inactive conformation. Indeed cAMP has been shown to interact directly with the nucleotide binding domain of NLRP3 [Lee *et al.*, 2012]. This theory has also been tested in the context of human disease. Patients with Cryopyrin-Associated Periodic Syndromes (CAPS) often possess mutations in the NACHT (nucleotide binding domain) of NLRP3 [Masters *et al.*, 2009]. ADCY inhibitors blocked LPS induced IL-1β secretion in the cells of CAPS patients with NACHT mutations [Lee *et al.*, 2012]. Overall it appears that increases in intracellular Ca^{2+} may, via CASR, PLC, IP_3 ADCY and cAMP, function as a point of convergence of multiple molecules in the activation of the NLRP3 inflammasome.

The other inflammasomes are less promiscuous than the NLRP3 inflammasome. The NLRC4 (or IPAF) inflammasome responds to pathogens. In the gut NLRC4 is important in maintenance of homeostasis as it distinguishes between pathogenic and commensal bacteria, only producing IL-1β in response to pathogenic bacteria [Franchi *et al.*, 2012]. The best known activator of NLRC4 is bacterial flagellin [Miao *et al.*, 2006]. Flagellin initiates an interaction of the NLR protein NAIP5 with NLRC4 [Zhao *et al.*, 2011]. Some bacteria activate the NLRC4 inflammasome independent of flagellin expression [Sutterwala *et al.*, 2007]. *Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Shigella flexneri* are all gram negative bacteria which have been reported to activate NLRC4 through recognition of their Type III secretion system (T3SS)[Miao *et al.*, 2010], [Abdelaziz *et al.*, 2010]. A role for K^+ efflux, a known activator of the NLRP3 inflammasome, has been proposed in this T3SS-dependent NLRC4 activation [Arlehamn *et al.*, 2010]. Regardless of the initial trigger, an essential step in NLRC4 activation is phosphorylation at Ser533. Without this phosphorylation NLRC4 is inactive [Qu *et al.*, 2012].

In comparison to NLRP3 and NLRC4, relatively little is known about the NLRP1 inflammasome. Polymorphisms in the NLRP1 gene are associated with a wide range of disease states including the pigmentation disorder vitiligo [Jin *et al.*, 2010b], the fibrotic connective tissue disorder systemic sclerosis [Dieudé *et al.*, 2011], Kawasaki disease [Onoyama *et al.*, 2012] and Alzheimer's disease [Pontillo *et al.*, 2012]. To date the only identified activator of NLRP1 is the anthrax lethal toxin. Activation occurs by cleavage of NLRP1 by the anthrax lethal toxin which then induces IL-1β processing and the caspase-1 dependent cell death, pyroptosis [Levinsohn *et al.*, 2012]. This activation of the NLRP1 inflammasome is dependent upon its interaction with ASC [Finger *et al.*, 2012]. Another

event that is essential for NLRP1 activation is the autolytic cleavage of the protein at Ser1213 [Finger *et al.*, 2012] within the FIIND domain [D'Osualdo *et al.*, 2011].

It is interesting to note that the composition of this inflammasome varies across species. In mice there are three paralogs of NLRP1 and in rats there are two. Further to this human NLRP1 differs from the rodent, as the human protein possessing an N-terminal PYRIN domain [Moayeri *et al.*, 2012]. At present the endogenous activator of NLRP1 is still to be discovered and the relevance of the differences between rodent and human NLRP1 still to be elucidated.

AIM2 is a non-NLR inflammasome that responds to cytoplasmic DNA [Bürckstümmer *et al.*, 2009] [Rathinam *et al.*, 2010]. The AIM2 protein belongs to haemopoeitic interferon inducible nuclear (HIN) protein family [Ludlow *et al.*, 2005] [Guarda & So, 2010]. The crystal structure of the HIN domain shows DNA binding occurs via electrostatic interactions [Jin *et al.*, 2012]. In this way, AIM2 recognises both viral and bacterial infections, including *Listeria monocytogenes* [Sauer *et al.*, 2010], *Mycobacterium tuberculosis* [Saiga *et al.*, 2012], *Francisella tularensis* [Belhocine & Monack, 2012] and mouse cytomegalovirus [Rathinam *et al.*, 2010]. In addition to its role in activating caspase-1, leading to IL-1β processing and release, an AIM2/ASC complex has been shown to activate caspase-8 and caspase-9 leading to apoptotic cell death [Pierini *et al.*, 2012].

A common pathway has recently been identified for the activation of AIM2, NLRP3 and NLRP3. Inhibiting the PP1/PP2A signal reduces IL-1β release in mouse macrophages in response to DNA and *Salmonella thyphimurium,* which activate the AIM2 and NLRC4

inflammasomes respectively [Luheshi *et al.*, 2012]. IL-1β release was also reduced by inhibiting the PP1/PP2A signal following the activation of the NLRP3 inflammasome with ATP, uric acid crystals and sphingosine [Luheshi *et al.*, 2012]. The PP1/PP2A proteins are serine/threonine phosphatases. There are approximately 30 serine/threonine phosphatases in comparison to approximately 428 serine/threonine kinases, which suggests the serine/threonine phosphatases have many substrates [Shi, 2009]. PP1/PP2A can be further subcategorised as phosphoprotein phosphatases (PPP) [Cohen, 1997]. PPP rely upon the interaction of their catalytic subunit with multiple regulatory subunits to confer specificity [Shi, 2009]. The general inhibition of inflammasome activation would imply that a late stage dephosphorylation event occurs. Common substrates present at this time include pro-IL-1β, ASC and caspase-1β. Additionally some viral proteins target the PP1/PP2A proteins in order to promote survival [Guergnon *et al.*, 2011], it would be interesting to see if the inhibition of inflammasome activation contributes to this survival.

In addition to caspase-1 there are also other proteases that process IL-1β, although the literature on these proteases is less extensive. Proteases that have been linked with IL-1β processing include proteinase-3, cathepsin G and elastase [Netea *et al.*, 2010]. These are all components of leukocyte granules [Guma *et al.*, 2009]. Pro-IL-1β has also been reported to be cleaved by Granzyme A and chymase [Irmler *et al.*, 1995], [Lieberman, 2010] [Joosten *et al.*, 2009]. The pathways that induce IL-1β processing and the inhibitors that can block these pathways are summarised in Fig 1.2.

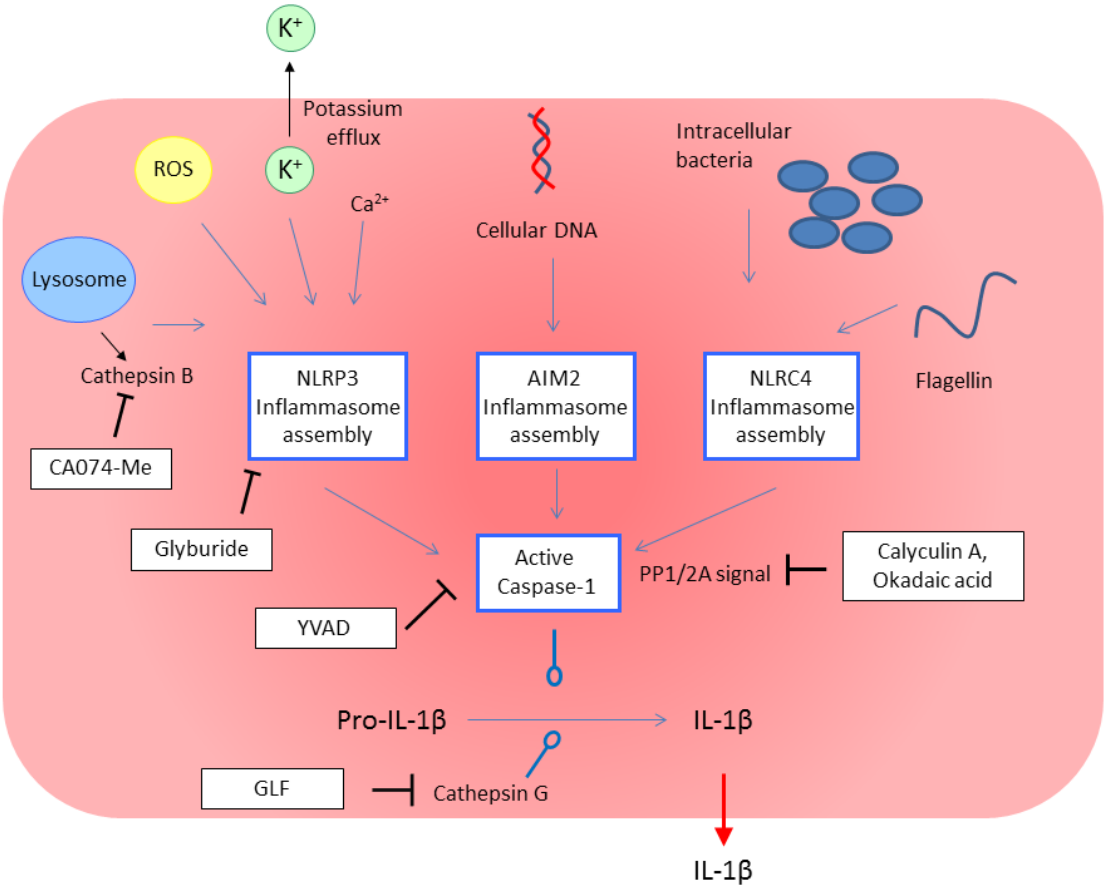

Figure 1.2: Mechanisms of IL-1β processing

A schematic diagram illustrating the processing of IL-1β as a consequence of NLRP3, AIM2 and NLRC4 inflammasome activation and the actions of cathepsin G. Inhibition of key pathway components are also illustrated, indicating the pathways targeted by intervention with YVAD, glyburide, calyculin A, okadaic acid, CA074-Me and GLF.

1.2.4 IL-1β release

Following cleavage mature IL-1β is rapidly secreted from the cell, although this does not occur via conventional pathways. Several mechanisms of IL-1β secretion have been proposed. In their recent review [Lopez-Castejon & Brough, 2011] categorised these secretory pathways into three, context-dependent, categories. These are: rescue and redirect, protected release and terminal release. This structure will be used to discuss the various mechanisms put forth for IL-1β release.

Rescue and redirect describes an IL-1β release where the cytokine is packaged in intracellular vesicles targeted for degradation. Under the influence of inflammatory stimuli, these vesicles are rescued from degradation and redirected for release into the extracellular space. This theory is supported by studies which identified storage of a small fraction of IL-1β in endolysosomal vesicles [Matsushima *et al.*, 1986], [Andrei *et al.*, 1999]. Furthermore, [Andrei *et al.*, 1999] observed elevated levels of secreted IL-1β following inhibition of proteases involved in degradation within these vesicles. When [Rubartelli *et al.*, 1990] first established the unconventional secretion of IL-1β, it was suggested that IL-1β may be packaged into vesicles via autophagy, a process that involves packaging of organelles into vesicles that are targeted for lysosomal degradation [Kroemer *et al.*, 2010]. More recently [Harris *et al.*, 2011] have shown IL-1β sequestration in autophagic vesicles. Enhanced activation of autophagy with rapamycin increased pro-IL-1β degradation and reduced IL-1β secretion. Conversely when autophagy was inhibited IL-1β secretion was upregulated [Harris *et al.*, 2011]. Taken together the evidence suggests that IL-1β is packaged in autophagic vesicles but released as active IL-1β following inflammatory stimuli.

The proposed mechanism of protected release suggests that IL-1β and the components necessary to induce its processing are protected within microvesicles which are then secreted to function at sites distant from the initial inflammatory insult. The short half-life of IL-1β within plasma [Kudo *et al.*, 1990], suggests that there must be mechanisms that facilitate IL-1β actions at a distance. In addition there is also a wealth of evidence that show microvesicles and exosomes - small vesicles produced from multi-vesicular bodies [Qu *et al.*, 2009] - can contain IL-1β and the components required for IL-1β processing. Microvesicles shed from the plasma membrane have been shown to contain bioactive IL-1β [MacKenzie *et al.*, 2001], and microvesicles from dendritic cells have been shown to contain

IL-1, caspase-1 and to express P2X7 receptors. ATP stimulation of these microvesicles also induces IL-1β release [Pizzirani *et al.*, 2007]. Exosomes containing IL-1, caspase-1 and other inflammasome components have also been reported [Qu *et al.*, 2007]. Interestingly the release of IL-1β from exosomes has been shown to be dependent on both ASC and NLRP3, but independent of caspase-1 [Qu *et al.*, 2009]. This suggests that inflammasomes may play roles in IL-1β release that do not involve activation of caspase-1. The final piece of evidence to support the protected release theory is the observation that microvesicles and exosomes are active at locations far from the point of initial inflammatory insult [Théry *et al.*, 2009].

Terminal release, IL-1β release following cell death, is the third and final proposed theory for IL-1β secretion. The regulation of IL-1β release and cell death are closely related. A caspase-1 dependent cell death, known as pyroptosis, occurs alongside IL-1β release, although lysis of the plasma membrane alone will not induce the release of mature IL-1β [Hogquist *et al.*, 1991]. Consequently it can be assumed that inflammatory stimuli initiate both cell death processes and activation of the proteases required for IL-1β processing. There is evidence to show that IL-1β release precedes this cell death [Brough & Rothwell, 2007]. It is entirely possible that the same network of regulatory mechanisms that control IL-1β processing also control inflammatory cell death and the existence of numerous instances of redundancy between the two processes would not be unexpected. When analysing cells that have been induced to release IL-1β it is important to consider the routes of IL-1β release. IL-1β released via terminal release or rescue and redirect mechanisms would release IL-1β directly into the supernatant and thus measured directly. IL-1β released via the protected release pathways may not be readily available and samples

may have to be freeze thawed in order to be measured. The different routes of IL-1β release are illustrated in Fig 1.3.

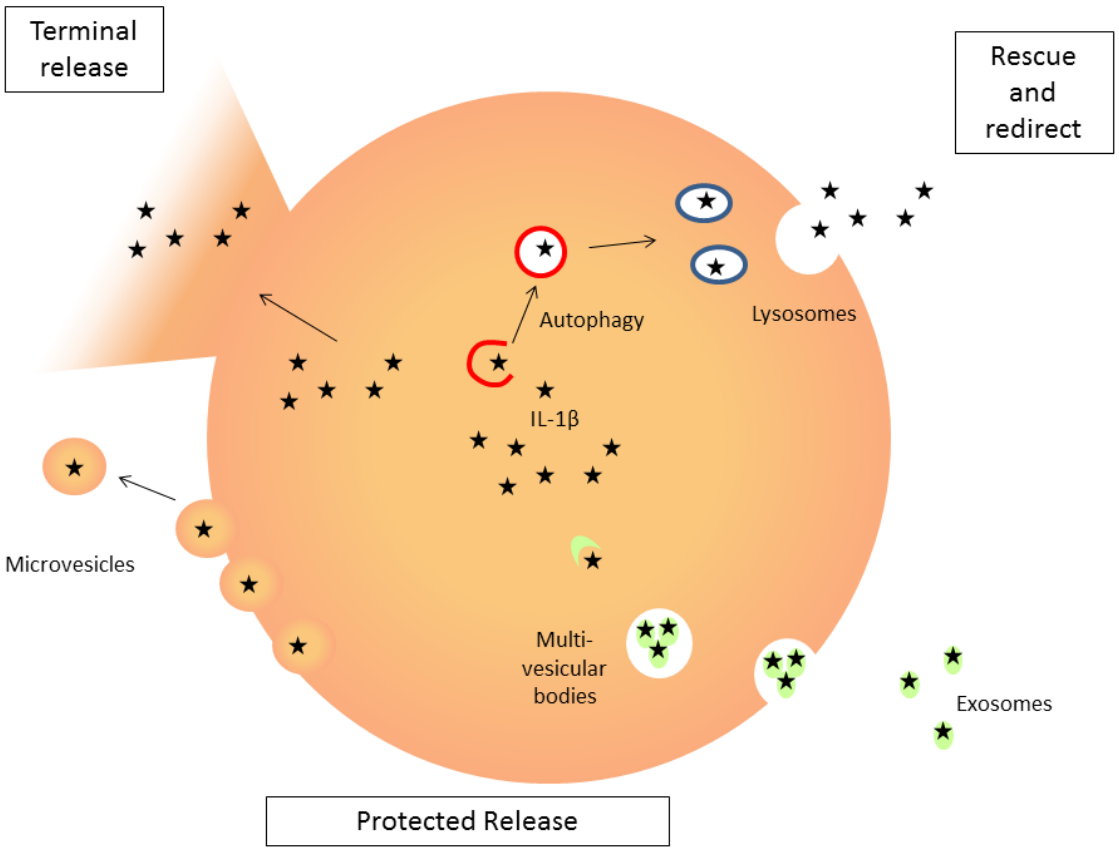

Figure 1.3: IL-1β release

A schematic diagram illustrating the different mechanisms of release of IL-1β; rescue and redirect, protected release and terminal release. Figure adapted from [Lopez-Castejon & Brough, 2011].

1.2.5 IL-1 and disease

As a key mediator of inflammation, IL-1 has become a target for the treatment of diseases with an inflammatory component. In particular there are a group genetic IL-1β activation disorders that display aberrant IL-1β expression in the absence of activation of high-titer autoantibodies or antigen-specific T cells [Masters *et al.*, 2009]. These disorders occur due

to mutations in components and regulators of the inflammasome. Mutations of NLRP3 are the most common and are referred to as Cryopyrin-associated periodic syndromes (CAPS) or cryopyrinopathies [Hoffman *et al.*, 2001a]. The main examples include familial cold autoinflammatory syndrome (FCAS) [Hoffman *et al.*, 2001b], Muckle-Wells syndrome (MWS) [Muckle, 1979], and neonatal-onset multisystem inflammatory disease (NOMID) otherwise known as Chronic infantile neurological cutaneous and articular (CINCA) syndrome [Torbiak *et al.*, 1989]. FCAS, MWS and NOMID mutations map to *CIAS1* [Hoffman *et al.*, 2001a] [Feldmann *et al.*, 2002] the gene for NLRP3 [Ting *et al.*, 2008]. The common symptom of these diseases include fevers and hives or rashes [Masters *et al.*, 2009]. FCAS patients are particularly sensitive to cold temperatures [Hoffman *et al.*, 2001a]. Additionally cells from CAPS patients respond to LPS alone without the requirement for secondary stimulation with ATP or other activators [Gattorno *et al.*, 2007]. The main locus for CAPS mutations is within the nucleotide binding domain or NACHT domain [Masters *et al.*, 2009] which highlights the importance of this domain in the control of IL-1β release.

A further inheritable inflammatory disease is Familial Mediterranean fever (FMF) [French FMF Consortium., 1997]. This condition occurs due to mutations in the MEFV gene which codes for the pyrin protein [Deng *et al.*, 1997] and is most frequently found in Mediterranean and middle eastern populations [Sohar *et al.*, 1967]. The protein Pyrin possesses the Pyrin domain with which it shares a name, B-box, bZIP basic, and coiled-coil domains [Nisole *et al.*, 2005]. Pyrin is known to interact with tubulin, colocalising to microtubules [Mansfield, 2001], and ASC [Richards *et al.*, 2001], [Yu *et al.*, 2006], [Chae *et al.*, 2003]. Symptoms include fever, pleural inflammation, rashes, arthritis and occasionally systemic amyloidosis [Masters *et al.*, 2009], and elevated IL-1β has been proposed to contribute to the pathology of this disease [Chae *et al.*, 2006].

Elevated IL-1 has been identified as a key contributor to many other disease states. These include stroke, cancer, type 2 diabetes and rheumatoid arthritis [Denes *et al.*, 2011][Dinarello, 2011][Soria *et al.*, 2011]. In humans polymorphisms in IL-1α [Um *et al.*, 2003], IL-1β [Dziedzic *et al.*, 2005][Kim *et al.*, 2009] and the anti-inflammatory IL-1Ra [Worrall *et al.*, 2003] genes have been correlated with elevated stroke risk. In rodents IL-1 has been reported to be upregulated in the brain following brain injury [Giulian & Lachman, 1985] [Woodroofe *et al.*, 1991] and inhibition of IL-1 with recombinant IL-1Ra reduces damage in focal cerebral ischemia [Relton & Rothwell, 1992]. Furthermore brain injury was reduced in IL-1α/β deficient mice in cerebral ischemia following middle cerebral artery occlusion [Boutin *et al.*, 2001].

IL-1β also promotes progression in tumours. IL-1 is often present in the tumour microenvironment as a consequence of expression by cancer cells [Portier *et al.*, 1993]. IL-1 has been reported to be expressed in colorectal adenocarcinoma, and melanoma tumour samples [Elaraj *et al.*, 2006], head and neck squamous cell carcinoma (HNSCC) [Chen *et al.*, 1999] and non- small-cell lung carcinoma [Gemma *et al.*, 2001], [Elaraj *et al.*, 2006]. IL-1β, IL-1α and IL-1Ra are all expressed by breast cancer cells [Miller *et al.*, 2000]. Furthermore mutations in IL-1 and related genes affect cancer risk and progression. A single nucleotide polymorphism (SNP) in an IL-1β promoter region, leads to reduced IL-β expression in lung tissues and a reduced risk of lung cancer [Landvik *et al.*, 2012]. The actions of IL-1β have been shown to promote tumour invasiveness which can be inhibited by the IL-1Ra treatment in fibrosarcoma cell lines [Apte *et al.*, 2006]. One mechanism by which IL-1β promotes tumour progression is the production of IL-1β following the stimulation of the growth factor receptor, mouse mammary tumour virus (MMTV)-driven inducible fibroblast

growth factor receptor 1 (iFGFR1), this in turn led to the increase of cyclooxygenase-2 which drove proliferation [Reed *et al.*, 2009].

IL-1β has been shown to contribute to underlying chronic inflammation associated with obesity [Speaker & Fleshner, 2012]. The saturated fatty acid palmitate activates the NLRP3 inflammasome leading to increased IL-1β secretion [Wen *et al.*, 2011]. In turn obesity-driven inflammation promotes insulin resistance eventually leading to the development of diabetes [Tack *et al.*, 2012]. Caspase-1 and inflammasome activation have also been shown to promote adipocyte differentiation to a more insulin resistant phenotype [Stienstra *et al.*, 2010]. Preadipocytes isolated from capase-1 -/- and NLRP3 -/- mice were determined to be more metabolically active [Stienstra *et al.*, 2010]. Finally obese patients with type 2 diabetes following a calorie restricted diet and exercise regime not only lost weight but had lowered expression of NLRP3 in their adipose tissue [Vandanmagsar *et al.*, 2011]. Elevated levels of IL-1 have also been associated with the synovial fluid of rheumatoid arthritis patients [Fontana *et al.*, 1982]. Degradation of collagen is also promoted by IL-1 presence by promoting the protein synthesis of collagenase enzymes [McCroskery *et al.*, 1985].

As a consequence of the integral part that IL-1 mediated inflammation plays in many disease states, several drugs targeting IL-1 have been developed. Due to its natural anti-inflammatory role IL-1Ra has been utilised in the treatment of autoinflammatory disease. A smaller, unglycosylated, version of the protein called anakinra is currently used as a treatment option for rheumatoid arthritis [Moltó & Olivé, 2010] [Fleischmann *et al.*, 2004] and there have been studies showing efficacy in other autoinflammatory diseases including

systemic-onset juvenile idiopathic arthritis (SOJIA) [Lequerré *et al.*, 2007]. Anakinra has also been used in clinical trials for the treatment of stroke [Emsley *et al.*, 2005].

Rilonacept and canakinumab are other drugs used to treat excessive IL-1β release. These drugs target IL-1β directly as opposed to competing for the interleukin-1 receptor [Goldbach-Mansky *et al.*, 2008] [Hoffman *et al.*, 2008] [Alten *et al.*, 2008]. Rilonacept is a dimeric fusion glycoprotein containing IgG and IL-1RI with IL-1R accessory protein domains [Economides *et al.*, 2003]. It targets both IL-1α and IL-1β [Stahl *et al.*, 2009]. Canakinumab is a human IgG monoclonal Ab directed to IL-1β [Church & McDermott, 2009a].

Anti-IL-1β therapy has also been shown to be an effective treatment for most CAPS [Koné-Paut & Piram, 2012]. Symptoms of MWD can be treated with anakinra [Hawkins *et al.*, 2003] [Hawkins *et al.*, 2004], rilonacept [McDermott, 2009]and canakinumab[Mueller *et al.*, 2011]. FCAS [Ross *et al.*, 2008] and NOMID [Goldbach-Mansky *et al.*, 2006] [Lovell *et al.*, 2005][Hedrich *et al.*, 2008]can also be effectively treated by blockade of IL-1β by anakinra. FMF, a hereditary disease that is not induced by NLRP3 mutation, has also been successfully treated by anakinra [Soriano *et al.*, 2013] [Roldan *et al.*, 2008][Calligaris *et al.*, 2008], canakinumab [Soriano *et al.*, 2013] and rilonacept [Hashkes *et al.*, 2012]. In each of the cases symptoms were resolved quickly. More general CAPS studies showed both rilonacept [Hoffman *et al.*, 2008][Hoffman *et al.*, 2012][Kapur & Bonk, 2009] and canakinumab [Lachmann *et al.*, 2009][Kuemmerle-Deschner *et al.*, 2011][Koné-Paut *et al.*, 2011]to be effective treatments.

The main side effects of anti-IL-1β therapy were local pain and inflammation at injection site and infections [Galeotti *et al.*, 2012][Hoffman *et al.*, 2008] [Zeft *et al.*, 2009] [Hedrich *et al.*, 2012] [Hoffman *et al.*, 2012]. In Anakinra treated patients the impact of injection site side effects is exacerbated as it has a short half-life therefore repeated injections are required [Kaiser *et al.*, 2012]. Canakinumab has a longer half-life than anakinra which may negate the impact of the injection site side effects [Abbate *et al.*, 2012]. Rilonacept is administered weekly, unlike Anakinra which is administered daily [Church & McDermott, 2009b]. Consequently the impact of injection site side effects is also reduced with rilonacept treatment when compared to anakinra.

1.3 Zinc and inflammation

Inflammation is involved in the pathology of many disease states. Zinc deficiency has been associated with increases in markers for inflammation [Haase *et al.*, 2006] [Prasad *et al.*, 2007] and zinc has been reported to reduce the incidence of inflammation when given as a supplement [Prasad *et al.*, 2004]. Here, the links between zinc deficiency and the inflammatory disease state will be explored.

1.3.1 Zinc

1.3.1.1 Cellular Zinc

Zinc is essential for cellular function. It is predicted to bind 10% of mammalian proteins [Andreini *et al.*, 2006]. Under physiological conditions zinc is found in the body in its cationic form (Zn^{2+}) [Cummings & Kovacic, 2009]. It is a ubiquitous trace element and can function as a second messenger [Yamasaki *et al.*, 2007] and as a structural or catalytic component of many cellular proteins. Zinc is associated with many classes of protein, from

enzymes to transcription factors. Furthermore, free zinc functions as an antioxidant reducing levels of free radicals [Prasad, 2008].

Within the cell levels of free intracellular zinc are low [Vallee & Falchuk, 1993], as most cellular zinc is bound to the sulphur and nitrogen atoms of cysteine and histidine residues of proteins. Ligand exchange of zinc from these residues occurs over a short period of time. This mechanism facilitates zinc depletion and acquisition allowing zinc to function as a regulatory element [Cummings & Kovacic, 2009].

1.3.1.2 Zinc and Nutrition

A 2005 study [Wuehler *et al.*, 2005] estimated that 20% of the world's population is zinc deficient. This figure was determined using a calculation that takes into account national food balance data, the available zinc within these foods, a model of zinc absorption and United Nations demographic data [Wuehler *et al.*, 2005]. This method is therefore not a direct measure of zinc deficiency but a prediction based upon the factors that contribute to zinc deficiency. This estimate is heavily reliant on the quality of the national food balance data and United Nations demographic data, the estimate of available zinc and the accuracy of the zinc absorption model.

Nutritionally the main dietary sources of zinc include meat, seafood, legumes and cereals [Cummings & Kovacic, 2009]. This is complicated by the fact that many plant-based sources of zinc contain a molecule called phytate which impairs the uptake of dietary zinc from these sources [O'Dell & Savage, 1960]. Consequently groups at risk of zinc deficiency include those in the developing world whose nutritional intake is mainly cereal based, the

elderly, as uptake of zinc decreases with age [Haase & Rink, 2009], and vegetarians as meats are one of the main dietary sources of zinc [Ibs & Rink, 2003].

Another form of zinc deficiency occurs as a result of insufficient zinc absorpbtion from the intestine. The inheritable condition acrodermatitis enteropathica is an example of this type of zinc deficiency [Maverakis *et al.*, 2007], which is caused by mutations in the ZIP14 zinc transporter [Wang *et al.*, 2002][Küry *et al.*, 2002]. The expression of ZIP14 is regulated in response to zinc levels; elevated in low zinc conditions and downregulated in high zinc [Kim *et al.*, 2004]. Point mutations lead to retention of ZIP4 in endoplasmic reticulum and prevent transport to the membrane [Dufner-Beattie *et al.*, 2003][Wang *et al.*, 2004]. Although acrodermatitis enteropathica can be defined as an inflammatory skin condition [Brocard & Dréno, 2011] to date there have been no reports of associated elevated IL-1. However ZIP14 expression can be induced by LPS and IL-1β stimulation [Lichten *et al.*, 2009]. It is unclear how this lack of ZIP14 expression in response to LPS and IL-1β would affect acrodermatitis enteropathica patients.

Plasma zinc levels are often used to detect zinc deficiencies [Kocyigit *et al.*, 2002]. However as zinc levels in blood plasma are generally buffered by cellular zinc levels it is often only possible to conclusively detect severe zinc deficiencies. Indeed when comparing cellular levels of zinc to plasma levels in individuals fed a low zinc diet, blood cell zinc levels dropped more rapidly than zinc within the plasma [Prasad, 1998]. Therefore mild zinc deficiency, which can have an effect on inflammatory status, is often difficult to detect [Prasad, 2008].

1.3.2 Zinc and disease

Zinc deficiency is a widespread global issue affecting people in both the developing and developed world. The 2002 World Health Report highlighted zinc deficiency as a major risk factor for disease and estimated that a third of the world's population suffered from mild to moderate zinc deficiency. Zinc supplementation has also been shown to be an effective treatment for many diseases. The incidence of diarrhoea [Sazawal *et al.*, 1995], lower respiratory tract infections [Sazawal *et al.*, 1998] and the duration of the common cold [Macknin *et al.*, 1998] have all been shown to be reduced via zinc supplementation. Additionally supplementation with zinc has been shown to promote survival and reduce bacterial load in a mouse model of sepsis [Nowak *et al.*, 2012].

Age-related immunosenescence is commonly associated with zinc deficiency [Fairweather-Tait *et al.*, 2008] and supplementing zinc in the elderly is an effective way of combating this zinc-deficiency induced reduced immune function [Haase & Rink, 2009]. Zinc supplementation may combat disease states by compensating for an existing zinc deficiency, which would contribute to the disease state by deregulation of the immune system.

1.3.3 Zinc and the immune system

The link between zinc deficiency and disease can at least be partly explained by the role zinc plays in regulating the actions of immune cells. As 10% of mammalian proteins bind zinc it is not surprising that zinc deficiency affects many regulatory systems within immune cells.

Zinc deficiency leads to lower levels of mature B-cells and T-cells [Prasad, 1998]. In T cells this is due to both impaired T cell development as a consequence of increased apoptosis [King *et al.*, 2002] and reduced T cell maturation due to a lack of thymulin [Prasad *et al.*, 1988]. The balance between the TH1 and TH2 response of T Helper cells is also altered in response to zinc deficiency, as the TH1 mediated responses are impaired [Hönscheid *et al.*, 2009]. Furthermore, dietary zinc deficiency has been shown to lead to reduced cytotoxic T killer activity in mice [Fernandes *et al.*, 1979].

Neutrophils, natural killer (NK) cells and macrophages are all affected by zinc deficiency [Prasad, 2008]. NK cell activity has been reported to decrease in zinc deficient individuals [Prasad, 1998]. Epithelial cell membrane damage and altered tight junctions in response to zinc deficiency have been shown to increase neutrophil infiltration [Finamore *et al.*, 2008]. In addition, zinc supplementation in mice treated with a common allergen reduced airway neutrophil infiltration [Morgan *et al.*, 2011].

Monocytes are precursor cells for macrophages and dendritic cells. In a screen by [Cousins *et al.*, 2003] 5% of genes in the monocytic cell line THP-1 were deemed to be zinc sensitive (responded either to zinc supplementation or zinc depletion). Of these genes, 104 displayed increased expression with increased cellular zinc and 86 displayed decreased expression with cellular zinc. 9% of the genes that responded to decreased zinc were associated with immune function compared to 6% of genes which upregulated cell death, a major response to zinc depletion [Cousins *et al.*, 2003]. This highlights the importance of zinc in the functioning of monocytic cells. Zinc deficiency has been linked with elevated

levels of pro-inflammatory cytokines [Bao *et al.*, 2003] and an important role of cells of the monocytic lineage is cytokine secretion.

1.3.4 Zinc and inflammation

Multiple studies in animals and humans have shown links between zinc and inflammation. There are many known mechanisms for the induction and propagation of inflammation; however, there are also many other mechanisms that are as yet poorly understood. The essential nutrient zinc has been reported to reduce the incidence of inflammation when given as a supplement [Prasad *et al.*, 2004], and zinc deficiency is associated with increases in markers for inflammation [Prasad *et al.*, 2007]. Specifically, zinc deficiency affects the expression of genes that control proinflammatory cytokines [Haase *et al.*, 2007] and cytokine secretion [Prasad, 2008].

IL-1β and TNF-α are key pro-inflammatory cytokines. Under conditions of zinc deficiency the secretion of these cytokines are elevated [Prasad *et al.*, 2002]. This has been described in elderly individuals, where subsequent zinc supplementation reduces inflammation [Prasad *et al.*, 2007]. Rheumatoid arthritis patients have also been shown to have significantly lower serum zinc levels than those with osteoarthritis and normal controls. In these patients, zinc levels were shown to correlate negatively with IL-1β and TNF-α levels [Zoli *et al.*, 1998a]. Zinc supplementation in healthy volunteers also reduces TNF-α and IL-1β [Prasad *et al.*, 2004]

In addition to promotion of inflammation by zinc deficiency, zinc levels may be further reduced by inflammatory processes. During inflammation the liver expresses increased

levels of ZIP14, a transporter that imports zinc into the liver, and metallothionein (MT), a zinc storage protein. This allows the liver to sequester zinc, effectively reducing the zinc levels within the plasma [Overbeck *et al.*, 2008]. This sequestration therefore initiates a positive feedback promoting the inflammatory state. It is clear that the balance of zinc within the body is a key regulator of inflammatory processes and it is possible that nutritional zinc deficiency induces inflammation by mimicking this natural regulation.

1.3.4.1 Zinc and inflammatory mechanisms

At a cellular level, there are several potential mechanisms that could lead to zinc deficiency-induced increases in pro-inflammatory cytokine expression and secretion. Zinc inhibits IL-1β and TNF-α transcription. Furthermore, [von Bülow *et al.*, 2005] have shown in LPS-stimulated monocytes that zinc treatment inhibits phosphodiesterases, leading to increased cellular cGMP, which in turn leads to the inhibition of TNF-α and IL-1β transcription. IL-1β and TNF-α are transcribed downstream of NF-κB. A20 (zinc finger transactivating factor) is a zinc dependent NF-κB inhibitor [Krikos *et al.*, 1992] [Opipari *et al.*, 1990], which has been reported to inhibit IL-1 production [Heyninck & Beyaert, 1999], [Jäättelä *et al.*, 1996] [Song *et al.*, 1996]. When these systems were zinc depleted the transcription of TNF-α and IL-1β would no longer be inhibited by cellular cGMP and A20. Interestingly, when dendritic cells are stimulated with the TLR4 ligand LPS, they express increased numbers of zinc transporters and consequently intracellular zinc decreases [Kitamura *et al.*, 2006]. This zinc depletion may function to activate pathways essential for IL-1β expression.

Cellular zinc depletion may also contribute to the processing of IL-1β from its pro-form to its mature form. Zinc depletion in mice using the zinc chelator N,N,N'N'-tetrakis(-)[2-pyridylmethyl]ethylenediamine (TPEN) *in vivo* and *in vitro* in peritoneal macrophages increases levels of IL-1β processing and release [Brough personal communication]. This would indicate that zinc depletion not only stimulates pro-IL-1β expression but also processing and subsequent secretion of the mature active form. Superficially these results appear to contrast with previous work published by the Brough group which show that zinc depletion can interfere with processes that induce IL-1β secretion, inhibiting release via these pathways [Brough *et al.*, 2009]. In this paper pre-treatment of peritoneal macrophages with TPEN leads to inhibition of pannexin-1, a hemi-channel that is required for ATP or nigericin-induced IL-1β secretion. It is therefore likely that the zinc depletion-dependent IL-1β processing observed in [Brough personal communication] does not occur via pannexin-1 and may activate different downstream pathways to ATP and nigericin. It should also be noted that the zinc depletion-induced IL-1β processing observed in [Brough personal communication] occurs after 4h, whereas [Brough *et al.*, 2009] deplete cells of zinc for 15min pre-treatment followed by 1h co-treatment with nigericin. Any effects observed at the 4h time point with TPEN alone may be observed with TPEN and ATP/ nigericin together if the timeframe were extended.

It has been discussed here, how zinc depletion could promote inflammatory disease. Another well characterised aspect of cellular zinc depletion is that it induces apoptotic cell death [Duffy *et al.*, 2001][Kolenko *et al.*, 2001][Guo *et al.*, 2012][Pang *et al.*, 2012][King *et al.*, 2002]. Inflammatory processes and cell death processes are also closely interlinked. This final section will explore the interplay between zinc, inflammation and cell death.

1.4 Cell death

A common underlying theme that permeates both the production of mature IL-1β and cellular responses to zinc depletion is the process of cell death. There are multiple cell death programmes that respond to different stimuli and produce different final cell death phenotypes.

1.4.1 Apoptosis

Apoptosis is a highly regulated, caspase dependent, cell death pathway. Morphologically apoptosis can be described as cell fragmentation into membrane bound vesicles which in turn are degraded by neighbouring phagocytic cells [Kerr *et al.*, 1972]. Induction of apoptosis occurs via the intrinsic, mitochondrial dependent, pathway or the extrinsic, death receptor dependent, pathway [Lavrik, 2010].

1.4.1.1 Extrinsic apoptosis

There are several key ligands involved in extrinsic pathway of apoptosis, these include FASL/CD95L [Suda *et al.*, 1993], TNF-α [Laster *et al.*, 1988] and the TNF-α ligand superfamily member 10 (TRAIL) [Wiley *et al.*, 1995]. These ligands interact with the receptors FAS/ CD95 [Itoh *et al.*, 1991], TNF-α receptor 1 (TNFR1)[Armitage, 1994] and TRAIL receptors (TRAILR) 1 and 2 [Pan *et al.*, 1997], respectively. In the native state FAS receptors are assembled as trimers via their pre-ligand assembly domain [Boldin *et al.*, 1995] [Siegel, 2000]. Receptor oligomerisation also occurs with TNFR1 and TRAILR [Schulze-Osthoff *et al.*, 1998]. Upon stimulation with the ligand, the trimers are stabilised and a dynamic multiprotein complex is formed at the cytoplasmic end of the receptor via the death domains (DD) [Kischkel *et al.*, 1995]. The death domains are conserved 80 residue domains

found in many of the proteins involved in apoptotic signalling pathways [Feinstein *et al.*, 1995]. The multiprotein complex, known as the Death Inducing Signalling Complex (DISC) [Kischkel *et al.*, 1995], is comprised of receptor-interacting protein kinase 1 (RIPK1)[Grimm *et al.*, 1996]; FAS associated proteins with a death domain (FADD)[Muzio *et al.*, 1996][Boldin *et al.*, 1996][Srinivasula *et al.*, 1996]; and pro-caspase-8 [Muzio *et al.*, 1998]; in addition to the apoptotic regulators c-FLIP [Thome *et al.*, 1997](a homologue of caspase-8 that lacks catalytic activity [Budd *et al.*, 2006]; and cellular inhibitors of apoptosis (cIAPs), E3 ubiquitin ligases that inhibit apoptosis by direct caspase inhibition [Deveraux *et al.*, 1998]and inhibition of RIPK1 via ubiquitination [Bertrand *et al.*, 2008].

Formation of this complex results in the activation of caspase-8 [Muzio *et al.*, 1998]. The subsequent activation steps are then dependent upon cell type. There are two types of cells, I and II, that induce mitochondrial independent and dependent cell death respectively [Scaffidi *et al.*, 1998]. In type I cells active capsase-8 is able to catalyse activation of the effector caspase, caspase-3 leading directly to apoptosis [Scaffidi *et al.*, 1998][Barnhart *et al.*, 2003]. In type II cells activation of caspase-8 is followed by cleavage of the BH3 interacting death domain agonist (BID) to produce truncated BID (tBID) [Scaffidi *et al.*, 1998][Barnhart *et al.*, 2003]. This in turn activates mitochondrial outer membrane permeabilisation (MOMP) [Crompton, 2000] which is an integral part of the apoptotic pathway.

Extrinsic apoptotic cell death can also be initiated by dependence receptors, which function initiate apoptosis when unbound. In the absence of their ligands the dependence receptors patched [Thibert *et al.*, 2003] and DCC (deleted in colorectal cancer) [Mehlen *et al.*, 1998]

interact with the cytoplasmic receptor DRAL which in turn activates caspase-9 [Mille *et al.*, 2009]. In the absence of netrin-1 the receptor UNC5B interacts with PP2A and death associated protein kinase 1 (DAPK1) which activates apoptotic pathways [Guenebeaud *et al.*, 2010].

1.4.1.2 Intrinsic apoptosis

Intrinsic apoptosis is induced by diverse cellular stress pathways, including oxidative stress [Madesh & Hajnóczky, 2001], excessive Ca^{2+}[Gincel *et al.*, 2001] and DNA damage [Lakin & Jackson, 1999]. These stress signals activate both pro and anti-apoptotic pathways [Veech *et al.*, 2000]. When the pro-apoptotic pathways overcome the anti-apoptotic pathways the outer membranes of the mitochondria are permeablised, in a process abbreviated to MOMP.

MOMP is a process common to both the extrinsic and intrinsic cell death pathways and functions as a point of no return in apoptotic cell death by initiating a set of terminal cell death pathways. Following MOMP the mitochondrial membrane potential is dissipated, preventing further mitochondrial function [Zamzami *et al.*, 1996]. Toxic proteins are also released from the permeablised mitochondria. These proteins include cytochrome c [Goldstein *et al.*, 2000], apoptosis inducing factor (AIF) [Susin *et al.*, 1999], endonuclease G [Li *et al.*, 2001], direct IAP binding proteins such as SMAC/DIABLO [Du *et al.*, 2000][Verhagen *et al.*, 2000] and high temperature requirement protein A2 (HTRA2)[Hegde *et al.*, 2002].

AIF and ENDOG enter the nucleus where they catalyse caspase independent DNA fragmentation [Susin *et al.*, 1999] [Li *et al.*, 2001][Joza *et al.*, 2001]. SMAC/ DIABLO and HTRA2 inhibit the antiapoptotic cIAP proteins, which are found bound to caspases, therefore promoting apoptosis [Chai *et al.*, 2000]. Released cytochrome c associates with APAF1 and dATP to form a macromolecular complex that associates with and activates caspase-9. This complex is known as the apoptosome. Following activation caspase-9 is released from the apoptosome and is available to activate the effector caspases [Zou *et al.*, 1999]. Caspase-3 [Fernandes-Alnemri *et al.*, 1994] and caspase-7 [Juan *et al.*, 1997] are the effector caspases of apoptosis. Caspase-3 is localised to the cytoplasm [Chandler *et al.*, 1998] and caspase-7 is targeted to the endoplasmic reticulum cytoplasm [Chandler *et al.*, 1998]. Substrates of these caspases include the DNA-repairing enzyme poly(ADP)ribose polymerase (PARP), U1-robonucleoprotein (U1-70 kD) and DNA dependent protein kinase (DNA-PK) [Casciola-Rosen *et al.*, 1996]. The processes activated by these cleavages constitute apoptotic cell death. These intrinsic and extrinsic pathways of apoptosis initiation are illustrated in Fig 1.4.

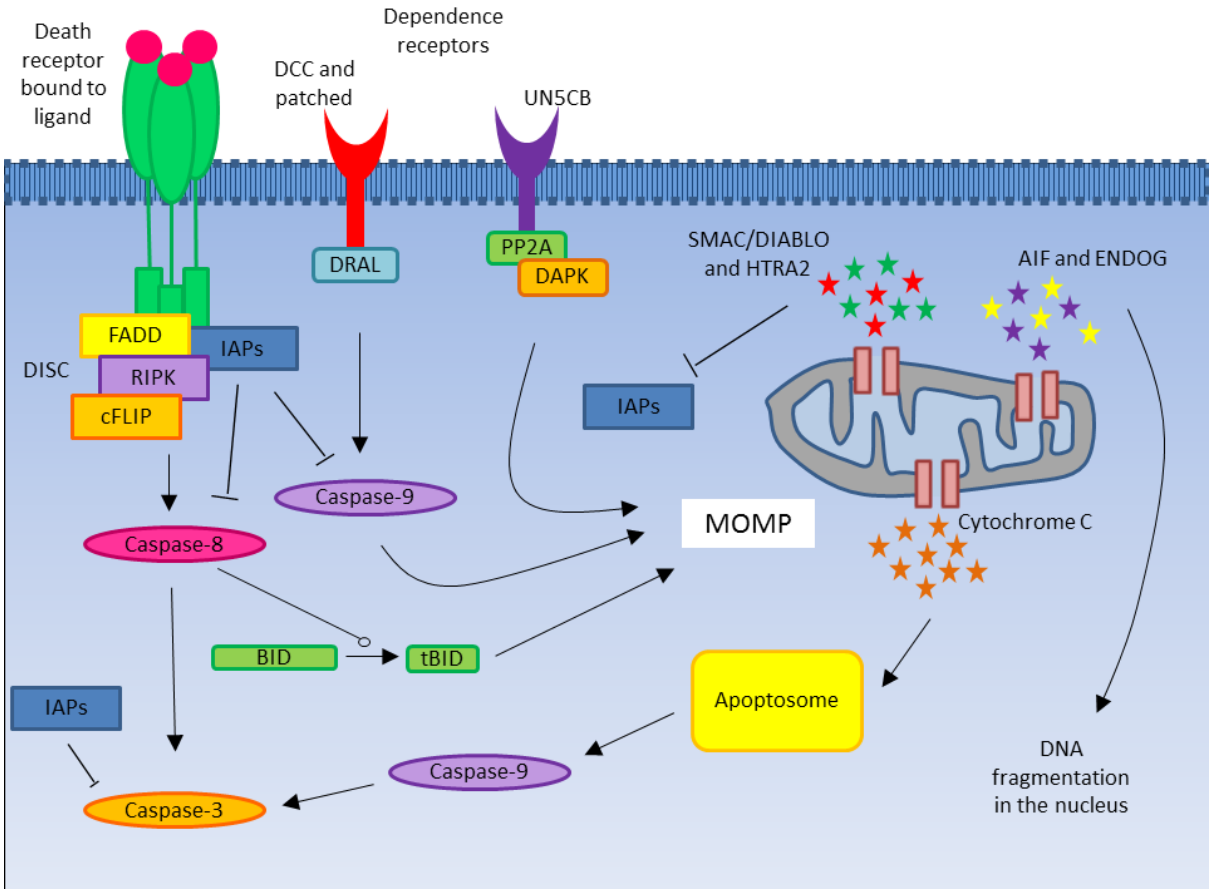

Figure 1.4: Apoptotic cell death

A schematic diagram illustrating the extrinsic and intrinsic pathways that initiate activation of effector caspases in apoptosis.

Zinc depletion has also been shown to initiate apoptosis. Inducing zinc deficiency in the HT-29 colorectal cell line with TPEN induces apoptotic cell death and an increase in transcription of caspase-3 [Gurusamy *et al.*, 2011]. Furthermore, in T lymphocytes TPEN treatment induced caspase-3, -8 and-9 expression [Kolenko *et al.*, 2001]. The inhibitor of apoptosis molecule, XIAP, potentially regulates apoptosis following zinc depletion as it is dependent upon zinc for its function and in the absence of zinc this inhibitor of apoptosis is depleted [Zuo *et al.*, 2012].

In addition to the role apoptosis plays in zinc deficiency, a condition commonly associated with an inflammatory phenotype, inducers of apoptosis have also been reported to upregulate IL-1β processing and release [Shimada *et al.*, 2012]. Mutations in the NLRP3 inflammasome have also been associated with delayed apoptosis [Blomgran *et al.*, 2012].

1.4.2 Necrosis and Necroptosis

Necrosis is an inflammatory cell death that can be characterised by cytoplasmic swelling, irreversible plasma membrane damage and breakdown of key organelles [Grooten *et al.*, 1993]. Following cytoplasmic and plasma membrane swelling the unpackaged contents of the cell are passively released into the extracellular space. These cell contents are inflammatory in nature and function as danger signals, activating key inflammatory processes [Raffray & Cohen, 1997]. Necrotic cell death with concomitant inflammation within a tissue leads to raised levels of pro-inflammatory cytokines [Cocco & Ucker, 2001]. A further characteristic of necrosis is random degradation of DNA as opposed to the ordered degradation observed in apoptosis [Shi *et al.*, 1990]. DNA is degraded by DNAse I [Napirei *et al.*, 2004] or lysosomal DNAse II [Tsukada *et al.*, 2001].

More recently regulatory processes have been identified in the initiation of necrosis. This regulated necrosis is referred to as necroptosis. Often initiated by the activators of apoptosis but redirected to an end process that is characterised by cell lysis and initiation of inflammatory processes. Specifically, necroptosis is defined as cell death dependent upon receptor- interacting protein kinase 1 (RIPK1) and RIPK3 [Cho *et al.*, 2009]. These kinases form part of a macromolecular complex known as the ripoptosome [Tenev *et al.*, 2011]. This complex is comprised of RIPK1, Fas-associated via death domain (FADD),

caspase-8 and RIPK3 [Tenev *et al.*, 2011]. Ripoptosome function is based upon the RIPK activities and it has been suggested that the switch to necroptotic cell death is determined by levels of RIPK [Cho *et al.*, 2009].

The ripoptosome is regulated by Inhibitor of apoptosis proteins (IAPs) and FLICE-like inhibitor proteins (FLIPs) which is similar to caspase-8 but does not possess catalytic activity [Wilson *et al.*, 2009]. IAPs inactivate the ripoptosome by binding and ubiquitinating and inactivating the RIP1 component [Tenev *et al.*, 2011][McComb *et al.*, 2012]. Ripoptosome-induced necroptosis can also be inhibited by the small molecule necrostatin. Necrostatin functions as an allosteric inhibitor of RIPK1 [Degterev *et al.*, 2008], which in turn prevents ripoptosome-induced activation of necroptotic cell death.

Ripoptosome formation and necroptosis have also been associated with IL-1β processing and release. [Vince *et al.*, 2012] showed by depleting IAPs (and consequently removing negative regulation of the ripoptosome) IL-1β processing and release was initiated. This IL-1β processing was determined to be caspase-8 dependent. This highlights the role that cell death mechanisms play in IL-1β release. As zinc depletion is commonly associated with increased cell death these mechanisms may potentially contribute to zinc-induced IL-1β production.

1.4.3 Pyroptosis

Pyroptosis is an inflammatory cell death, characterised by the activation of caspase-1. This activation can occur via any of the inflammasomes discussed previously or by a supramolecular assembly of ASC molecules known as the pyroptosome [Yeretssian *et al.*,

2008]. Described by [Chen *et al.*, 1996] and further characterised by [Hilbi *et al.*, 1998], pyroptosis is initiated by inflammatory stimuli, followed by an increase in osmotic pressure and membrane lysis [Miao *et al.*, 2011]. An evolutionary justification for pyroptosis is that the process may prevent the propagation of intracellular pathogens by removing their intracellular niche [Yeretssian *et al.*, 2008].

1.4.4 Autophagy

Autophagy is a cellular stress response that leads to the cannibalism of cellular components, in response to insufficient nutrients [Levine, 2005]. Autophagic vesicles are double membrane bound components [Baba *et al.*, 1995] that are delivered to lysosomes for degradation [Deter *et al.*, 1967]. This cell stress response pathway is not dependent upon the *de novo* synthesis of enzymes as inhibition of this process does not inhibit autophagy [Shelburne *et al.*, 1973]. It should also be noted that autophagy is not itself a cell death pathway, but as a cellular stress response it often leads to a cell death characterised by cytoplasmic vacuolisation [Galluzzi *et al.*, 2007]. Indeed, inhibition of autophagic processes can actually promote cell death [Boya *et al.*, 2005].

Autophagy can also be activated by PAMPs and TLR agonists [Xu *et al.*, 2007][Shi & Kehrl, 2008] and inhibition of autophagy has been shown to induce NLRP3 activation leading to the secretion of active IL-1β [Harris *et al.*, 2011]. Additionally autophagy is also a process that is dependent upon zinc. Zinc has been shown to be integral to the actions of the autophagy inducing drugs Tamoxifen and Clioquinol. Tamoxifen requires zinc to induce autophagy, and increased zinc was shown to be associated with acidic autophagic vacuoles

[Jin *et al.*, 2010a]. Furthermore cellular zinc increases are induced by the actions of the antibiotic Clioquinol which functions as a zinc ionophore [Park *et al.*, 2011].

1.4.6 Cell death summary

Having described the multiple forms of cell death, it is clear that each has differing implications for inflammation and involvement of zinc. The two original classifications of cell death, necrosis and apoptosis, traditionally fall into the roles of inflammatory and non-inflammatory cell death respectively. Pyroptosis is a more recently identified type of inflammatory cell death. This cell death is dependent upon caspase-1 and occurs alongside IL-1β processing and release. Autophagy, whilst not traditionally considered an inflammatory form of cell death, is also proposed to function as a mechanism of IL-1β release. Additionally, it is becoming clear that the multiple pathways of cell death have a considerable amount of cross over and regulatory mechanisms exist to transfer a cell from one route to another. The main example of this is the regulated cell death necroptosis. Apoptotic signals initiate this cell death but the final phenotype is an inflammatory necrotic phenotype.

Zinc is known to be required for autophagy and therefore would be necessary for, autophagy dependent IL-1β release. Conversely zinc depletion is known to induce apoptosis. If adhering to the traditional definitions of cell death it would be expected that zinc depletion would not induce an inflammatory response via cell death. However it is known that zinc deficiency is an inflammatory condition, associated with elevated levels of pro-inflammatory cytokines. It is possible therefore that zinc depletion may induce necroptosis via initiation of apoptotic pathways.

1.5 Summary and objectives

IL-1β production is an inflammatory process that influences many disease states and interacts with cell death processes. In turn zinc deficiency is also associated with an inflammatory phenotype and is known to induce cell death. Brough [personal communication] have shown IL-1β release to be induced by treating peritoneal macrophages with the zinc chelator TPEN. This data shows both an increase in overall levels of IL-1β and the initiation of a processing event which allows IL-1β to be released as a mature form of the cytokine. In this thesis I will investigate the interactions between zinc depletion and IL-1β production.

The link between zinc deficiency and inflammation has been well reported. Establishing mechanisms of the role of zinc in inflammation however is complicated by the fact that zinc is essential for many cellular processes and 10% of mammalian proteins bind zinc. Consequently the regulation of IL-1β production by zinc is an ideal problem to explore with integrative systems techniques, which take into account the actions of the network in addition to the actions of the pathway. This is known as emergence; the phenomena by which a network of interactions produces a property that could not have been predicted by studying all of the interactions individually [Pessa, 2006]. A systems biology approach benefits from the integration of information from individual interactions to give a more holistic picture.

In order to assess the contribution of zinc it would be beneficial to create a network of IL-1β production, processing and release. From here the proteins involved in the network could be assessed for their dependence upon zinc for functionality. It would then be

possible to assess the contribution of zinc to the pathway and investigate the varying roles of these pathway components. In order to complete such a project the networks that are to be studied need to be well defined. The networks for IL-1β transcription are well studied and are suitable for this kind of analysis, however the field of IL-1β processing and is release is somewhat younger. This field is developing and changing rapidly. Consequently, due to the volume of new literature being published on this subject, undergoing the time-consuming process of completing a network map would eventually produce an outdated piece of work. Therefore at present the characterisation of zinc depletion in IL-1β processing and release is best addressed via cellular biology techniques.

The main objectives of this project are as follows

1. Establish the mechanism by which zinc depletion induces IL-1β production and processing in mouse macrophages.
2. Compare the mechanisms of zinc depletion-induced IL-1β production and processing in human macrophage-like cells and mouse macrophages.
3. Assess the role cell death plays in the link between zinc depletion and inflammatory mechanisms.
4. Create a network model of IL-1β production and investigate the role of zinc binding proteins within this network.

Chapter 2: General Methods

2.1 Reagents

2.1.1 Chemicals and Reagents

All treatments and media used in this thesis are detailed in the table below (Table 2.1). All other chemicals and reagents are detailed within the text.

Table 2.1: Chemicals and reagents used with supplier details.

Chemical/ Reagent	Supplier
Treatment	
N,N,N',N'-Tetrakis(2 pyridylmethyl)ethylenediamine (TPEN)	**Sigma Aldrich (UK)**
ammonium tetrathiomolybdate (TTM),	**Sigma Aldrich (UK)**
Nigericin sodium salt	**Sigma Aldrich (UK)**
Salicylaldehyde isonicotinoyl hydrazone (SIH)	**ChemBridge (USA)**
Diethylenetriaminepentaacetic Acid (DTPA)	**Sigma Aldrich (UK)**
2-Mercaptopyridine N-oxide (Pyrithione)	**Sigma Aldrich (UK)**
phorbol 12-myristate 13-acetate (PMA)	**Sigma Aldrich (UK)**
Bacterial LPS (*Escherichia coli* 026:B6)	**Sigma Aldrich (UK)**
Media and Components	
Dulbecco's Modified Eagle's Medium (DMEM 4.5g/L glucose, 110mg Sodium Pyruvate and L-glutamine)	**Sigma Aldrich (UK)**
RPMI-1640 Medium (RPMI L-glutamine and $NaHCO_3$	**Sigma Aldrich (UK)**
Pen/Strep	
Foetal Calf Serum (FCS)	
Inhibitors	
Glyburide	**Sigma Aldrich (UK)**
Calyculin A	**Calbiochem (USA)**
Z-Gly-Leu-Phe-chloromethyl ketone (GLF)	**Sigma Aldrich (UK)**
Caspase-1 Inhibitor I (Ac-YVAD-CHO)	**Calbiochem (USA)**
CA-074 Me	**Calbiochem (USA)**

2.1.2 Antibodies

All antibodies used in this thesis are detailed in Table 2.2.

Table 2.2: Antibodies used in western blots.

Antibody	Supplier	Dilution	Vehicle
Anti-mouse IL-1β antibody, S329 (Sheep)	Gift from the National Institute of Biological Standards and Controls (UK)	1:1000	PBS, 0.1% Tween® 20 and 5% (w/v) dried milk
Anti-human IL-1β antibody, AF-201-NA (Goat)	R & D Systems Europe Ltd	1:1000	PBS, 0.1% Tween® 20 and 0.1% (w/v) BSA
Anti-XIAP antibody, 2042S (Rabbit)	Cell Signaling (USA)	1:100	PBS, 0.1% Tween® 20 and 0.1% (w/v) BSA
Anti-Cleaved Caspase-8 (Asp387) Antibody, 9496S (Rabbit)	Cell Signaling (USA)	1:100	PBS, 0.1% Tween® 20 and 0.1% (w/v) BSA
Rabbit anti-sheep HRP conjugated P0163	Dako (UK)	1:2000	PBS, 0.1% Tween® 20 and 5% (w/v) dried milk
Rabbit anti-goat HRP conjugated P0449	Dako (UK)	1:1000	PBS, 0.1% Tween® 20 and 5% (w/v) dried milk
Goat anti-rabbit HRP conjugated P0448	Dako (UK)	1:1000	PBS, 0.1% Tween® 20 and 5% (w/v) dried milk
Anti- β-actin. HRP conjugated.	Sigma Aldrich (UK)	1:20000	PBS, 0.1% Tween® 20 and 5% (w/v) dried milk

2.2 Cell Cultures

Cells were cultured in a humidified incubator at 37°C with 5 % CO_2. Cell lines and primary cells were maintained in either Dulbecco's Modified Eagle's medium (DMEM 4.5 g/L glucose, 110 mg/L Sodium Pyruvate and L-glutamine) or RPMI-1640 full (RPMI, L-glutamine and $NaHCO_3$) supplemented with penicillin/ streptomycin antibiotic solution (P/S, 100

µg/mL streptomycin and 100 IU penicillin, Sigma Aldrich, UK), and with varying concentrations of foetal calf serum (FCS, PAA Laboratories, UK).

2.2.1 Cell counting

Cells were counted using an Improved Neubaur counter (Hawksley, UK). 10 µl of cell suspension was added to the slide and a cover slip carefully placed over the cells, ensuring no air bubbles were formed over the grid. The number of cells was counted in each grid (4 by 4 squares) and the mean calculated. This number was then multiplied by 10 000 to give the number of cells in 1 ml of media. The total number of cells was established by multiplying this number by the volume of cells present. Where the cell number was too high to count, the cells were diluted 10 fold in media with this dilution taken in to account in the subsequent calculations. When counting bone marrow derived macrophages (BMDMs) the 10 fold dilution contained 1 part Trypan Blue Solution (0.4 %, Sigma Aldrich, UK). Trypan blue is a dye that stains only dead cells and therefore, when counting, any cells that have taken up the dye can be discounted.

2.2.2 Primary cell culture

C57BL/6J (WT) mice were supplied by Harlan, UK. ASC-/- C57BL/6N and NLRPP3 -/- C57BL/6N mice were generously provided by Dr Vishva Dixit, Genentech. All mice were maintained at the University of Manchester, BSF. Animals were kept in ventilated cages at 21°C ± 1°C, 55 % ± 10 % humidity and maintained in a 12 hour light-dark cycle with free access to food and water. Adult males were used at an approximate body weight of 25-30g. All animals used in this study were sacrificed according to the Animals (Scientific Procedures) Act, 1986, UK, by exposure to rising CO_2 concentrations. This is a schedule one method.

2.2.2.1 Peritoneal Macrophages

Mouse peritoneal macrophages were prepared as described previously [Perregaux *et al.*, 1992]. Adult, male C57BL/6 mice were sacrificed by exposure to rising CO_2 in accordance with schedule one as defined above. The abdomen of the mouse was then cleaned using 70% Industrial Methylated Spirit (IMS, Fisher Scientific, UK). Scissors and forceps were used to separate the skin covering the abdomen from the peritoneal cavity and a collection pocket formed at one side. 8 ml of RPMI 1640 medium (supplemented with 5 % FCS 1 % P/S) was then injected using a 1 0ml sterile syringe (Becton Dickenson, UK) with a sterile Microlance 3 needle (0.5 x 16 mm) (Becton Dickenson, UK) into the peritoneal cavity and the mouse abdomen gently massaged to maximise cell yield. The peritoneal cavity was then gently opened and the medium carefully collected. Cells were counted (see 2.2.1) and where required, the media recovered from multiple mice was pooled in order to achieve a required cell number. Cells were collected by centrifugation (80 g, 5 min, RT) and the pellet resuspended in RPMI 1640 medium (supplemented with 5 % FCS 1 % P/S). The macrophages were plated at a density of $1x10^6$ cells/ml in 48 or 24 well cell culture plates (Corning Incorporated, USA) and incubated overnight at 37°C, 5 % CO_2 for use on the following day.

2.2.2.2 Bone Marrow Derived Macrophages

Bone marrow derived macrophages (BMDMs) were obtained from adult, male C57BL/6J mice (Harlan, UK). The femur and tibia were removed and the flesh pulled away using forceps. The bones were then transferred to Dulbecco's Phosphate buffered saline (1X PBS, Sigma Aldrich, UK). Any bones that had been broken (exposing the bone marrow) were discarded as exposure would increase the risk of infection.

Differentiation of bone marrow cells to bone marrow derived macrophages requires the cytokine macrophage colony-stimulating factor (M-CSF). Here the L929 mouse fibroblast cell line is used to produce M-CSF. L929 cells were cultured in DMEM (10 % FCS, 1 % P/S). This media was removed and sterile filtered (Corning Incorporated, USA) and added to fresh DMEM (10 % FCS, 1 %P/S) at a ratio of 3:7 (L929 media: Fresh DMEM (10 % FCS, 1 % P/S)). This is referred to as differentiation media.

The bones were cut at both ends and the bone marrow washed out of the bone using a 10 ml sterile syringe (Becton Dickenson, UK) with a sterile Microlance 3 needle (0.5 x 16 mm) (Becton Dickenson, UK) of differentiation media. 10 ml of media was used for every 4 bones (1 mouse). The bone marrow was then resuspended using a 5 ml stripette (Corning Incorporated, USA). A further 30 ml differentiation media was added and the cells distributed between a T75 cell culture flask (Corning Incorporated, USA) and a T225 cell culture flask (Corning Incorporated, USA), 10 ml and 30 ml respectively.

Cells were incubated at 37°C, 5 % CO_2 for 3 days. On day three the cells were fed with differentiation media; 5 ml per T75 flask and 15 ml per T225 flask. Cells were suitable for use on day 6-9. Cells were dissociated by scraping with a cell scraper (Sarstedt, USA) into warm DMEM 10 % FCS, 1 % P/S. Cells were counted (described in 2.2.1) and seeded at 1x 10^6 cells/ml in 48 or 24 well cell culture plates (Corning Incorporated, USA). Cells were incubated overnight at 37°C at 5% CO_2 prior to treatment on the following day.

2.2.3 THP-1 Cell line

Cells analogous to human macrophages were produced by differentiating cells of the human monocytic cell line THP-1 with phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich, UK). THP-1 cells (Tsuchiya et al., 1980) were passaged and maintained in DMEM with P/S and 10 % volume/volume (v/v) FCS. When passaging, a 5 ml stripette (Corning Incorporated, USA) was used to break apart the cell aggregates by repeated aspiration and release.

One day before treatment the cells were passaged, counted (as described in 2.2.1) and seeded at 1x 10^6 cells/ml in 48 or 24 well cell culture plates (Corning Incorporated, USA). To stimulate the differentiation of the THP-1 cells to macrophage-like cells they were treated with 0.5 µM PMA (Sigma Aldrich, UK) for 3 h. After this time the media was removed and replaced with fresh DMEM (10 % FCS, 1 % P/S). The cells were incubated overnight at 37°C at 5 % CO_2 prior to treatment on the following day.

2.3 Cell culture treatment and inhibitor studies

2.3.1 Pro-IL-1β Expression

Pro-IL-1β expression was stimulated with bacterial lipopolysaccharide (LPS) (*Escherichia coli* 026:B6, 1 µg/ml, Sigma Aldrich) in peritoneal macrophages (2h), THP-1 (4h) and BMDM (4h).

2.3.2 Treatment

Cells were zinc depleted with 10µM N,N,N',N'-Tetrakis(2pyridylmethyl)ethylenediamine (TPEN, Sigma Aldrich, UK), 1 mM Diethylenetriaminepentaacetic Acid (DTPA, Sigma Aldrich,

UK) and/or 50 μM 2-Mercaptopyridine N-oxide (pyrithione, Sigma Aldrich, UK) following LPS treatment. Peritoneal macrophages were zinc depleted for 4h. THP-1 and BMDMs were zinc depleted for 24h. In a separate well, nigericin (Sigma Aldrich, UK) was added as a positive control for IL-1β processing and release at a final concentration of 20 μM. Cells were treated with nigericin for the duration of the zinc depletion experiments (4h in peritoneal macrophages, 24h in THP-1 cells and BMDMs). Vehicle controls were also used for each of the treatments. The vehicles for the treatments were as follows: Dimethyl sulfoxide (DMSO Sigma Aldrich, UK) for TPEN, 10 mM HEPES (Fisher Scientific, UK) buffered media (pH 7- 7.5) for DTPA, dH_2O for pyrithione and 100 % ethanol (Fisher Scientific, UK) for Nigericin. Excepting the 10mM HEPES buffered media (pH 7- 7.5) all vehicle final concentrations were 0.5 %.

2.3.3 Inhibitor Studies

Where inhibitors were used they were added to cells after LPS treatment and 15 min prior to zinc depletion. The inhibitors used in the experiments described in this thesis (for details and suppliers see table 2.1) were all diluted in DMSO. Appropriate DMSO controls were used in each experiment and are detailed in figure legends in chapters 3-6.

The caspase-1 inhibitor Ac-YVAD-CHO (YVAD) was added to a final concentration of 100 μM. Glyburide, a sulfonylurea drug which functions by inhibition of ATP-sensitive K^+ channels preventing the activation of the NLRP3 inflammasome [Lamkanfi *et al.*, 2009], was added to a final concentration of 100 μM. Ca074-Me, an inhibitor of cathepsin B, was added to a final concentration of 80 μM. Calyculin A, a Serine/Threonine Phosphatase Inhibitor that inhibits PP2A, was used at concentrations of 10 nM or 50 nM. Z-Gly-Leu-Phe-

chloromethyl ketone (GLF), a cathepsin G inhibitor, was added to a final concentration of 10 µM.

2.3.4 Sample Collection

Supernatants and lysates were collected at the end of the experiment. Plates were centrifuged at 400 g, 4°C for 5 min (Eppendorf centrifuge 5804R). The supernatant was then collected in eppendorf tubes and stored at -20°C. Cells were lysed in ice cold cell lysis buffer (1X PBS (Sigma Aldrich), 0.1 % Triton® X-100 (Sigma Aldrich, UK) and 1 % protease inhibitor cocktail (AEBSF hydrochloride, Aprotinin (bovine lung, crystalline), E-64 protease inhibitor, EDTA (disodium) and leupeptin hemisulphate) (Calbiochem, USA). Cell lysates (50 µl) were taken on ice by scraping with a sterile 200 µl pipette tip with a folded end, the well washed in the lysis buffer and the lysates taken in eppendorf tubes to be stored at -20°C.

2.4 Sample analysis

2.4.1 LDH assay

Lactate dehydrogenase (LDH) is a cytosolic protein that is only released after membrane rupture. LDH activity can be assayed rapidly and at low cost. Consequently measuring LDH activity is a good indication of cell death. This was measured in the cell culture medium using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, UK) according to the manufacturer's instructions. The assay is based upon LDH conversion of a tetrazolium salt into a red formazan product, where the amount of LDH is equivalent to the amount of red formazan product. An untreated sample was lysed using the lysis buffer provided to give a maximal LDH activity reading, which was considered to be indicative of 100 % cell death. A

baseline reading was taken using fresh media only. Absorbance readings were measured using the BioTek Synergy HT plate reader at 490 nm.

2.4.2 Bicinchonic acid (BCA) assay

Total protein content was measured in cell lysates using the BCA protein assay (Thermo Scientific, USA) according to the manufacturer's instructions. BCA is a copper chelator that chelates monovalent copper ion (Cu^{+}). The assay contains Cu^{2+} which is reduced to Cu^{+} by proteins present in the sample and the BCA chelates this Cu^{+} producing a purple product. Higher levels of protein lead to higher levels of Cu^{+} and consequently a larger amount of purple product. Standards and samples were diluted in PBS and measured in triplicate. 10 µl of standard or sample was incubated with 200 µl of assay buffer, at 60°C for 30 min. The final absorbance readings were measured using the BioTek Synergy HT plate reader at 570 nm.

2.4.3 Enzyme-linked immunosorbent assay (ELISA)

IL-1β levels were quantified in the lysates and supernatants using human and mouse IL-1β/IL-1F2 Duoset ELISA kits from R&D systems, according to the manufacturer's instructions. 96 well nunc-immuno plates (Nunc, Denmark) were coated with 50 µl/well of capture antibody (provided in kit) diluted in 1X PBS and incubated overnight (4°C). Parafilm (Pechiney Plastic Packaging Company, USA) was used to cover the plates at this stage and at each subsequent incubation step. On the following day plates were washed 4X with ELISA wash buffer (1x PBS (Sigma Aldrich), 0.05 % v/v Tween® 20 (Sigma Aldrich)) and dried thoroughly by banging the plate against a piece of polystyrene covered in blue roll. After each incubation step the plate was washed and dried in this way. The plates were then

blocked in 200 µl/well RD buffer (1X PBS (Sigma Aldrich), and 1 % weight/volume (w/v) Bovine serum albumin (BSA, Sigma Aldrich, UK) for 1h to prevent non-specific binding.

Standards (provided in kit) were serially diluted in RD buffer from 4000 pg/mL to 3.9 pg/mL. Standards (duplicate) and samples were loaded (50 µl/well) on the plate and incubated (either 2h RT or overnight at 4°C) on an orbital shaker. After this the plate was washed (x4) and dried before 50 µl/well detection antibody (provided in kit and diluted in RD buffer) was added to each well and incubated (RT 1h). After washing and drying, the next incubation was 50 µl/well Streptavidin-HRP (provided in kit) 30 min RT. HRP is an abbreviation for horseradish peroxidase. In the presence of hydrogen peroxide HRP will form oxygen radicals which oxidise substrates. In this instance the final incubation is with the 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Thermo Scientific, USA) (20 min, RT, protected from light). This oxidation creates a blue colour the intensity of which is dependent upon the amount of HRP present. The amount of blue product formed is proportional to the amount of the test protein. After 20 min the reaction was stopped with 1M sulphuric acid (H_2SO_4, Fisher Scientific, UK), this changes the colour from blue to yellow.

Absorbance readings were measured using the BioTek Synergy HT plate reader at 450 nm and 570 nm (to correct for volume). The readings at 570 nm were then subtracted from the 450 nm readings to give the final absorbance values. The absorbance values were analysed using the GraphPad Prism software. The assay values of the standards (y values) were tabulated against their known concentrations (x values), and the sample assay values (y values) entered into the same graph leaving the x values blank. The data was then logged, non-sigmoidal linear regression performed, and the unknown values interpolated from the curve.

2.4.4. Western blot analysis of IL-1β release

Levels of pro- and mature IL-1β cannot be distinguished by ELISA as the epitopes of the ELISA capture and detection antibodies are present on both the pro- and mature protein. In order to identify processing of IL-1β, samples therefore need to be separated via electrophoresis and visualised using Western blotting techniques.

The gel plates were cleansed thoroughly and placed in gel clamps (BioRad, UK). A 12 % SDS-polyacrylamide gel was prepared (Table 2.3) and set between the glass plates under water-saturated butan-1-ol (Fisher Scientific, UK). This prevents oxygen interfering with polymerisation process. After the gel was set the butan-1-ol was removed by washing with dH_2O (x5). A 5 % stacking gel (Table 2.3) was poured on top of the resolving gel and a plastic comb inserted to create either 10 or 15 lanes. The set gel was transferred to an electrophoresis chamber (Mini PROTEAN® Tetra Cell, BioRad, UK) and covered with running buffer (Table 2.3).

Protein denaturation with SDS abolishes secondary structure and imparts uniform charge; this facilitates separation of proteins on the basis of their electrophoretic mobility (a function of size). To denature the proteins 40 µl of sample was added to 10 µl of loading buffer (10 % w/v SDS, 50 % w/v glycerol, 400 mM Tris-HCl pH 6.8, 0.025 % w/v bromophenol blue, 5 % w/v β-mercaptoethanol) to a final volume of 50 µl, heated to 95°C for 5 min and vortexed to mix. 10 µl of molecular weight marker (Precision Plus Protein™ All Blue Standards, BioRad, UK) was loaded in the far left lane of each gel, followed by 40 µl of sample in each subsequent lane. All empty lanes were loaded with an equivalent volume of loading buffer.

Protein samples were resolved by electrophoresis at 70 V for 40 min (to allow the samples to travel through the stacking gel) and then 150 V for the remaining 1h until the solvent front had migrated to the bottom of the resolving gel. At this point it is important to ensure that the solvent front does not run off the end of the resolving gel as the sample could then be lost in the running buffer. Proteins were then transferred to a nitrocelluose membrane (Whatman, Germany) at 15 V for 45 min using a Trans-blot SD semi-dry transfer cell (BioRad, UK). The transfer stack comprised three filter papers and a nitrocellulose membrane soaked in transfer buffer (Table 2.3). Two pieces of filter paper were placed together, one on top of the other, and then rolled over gently with a stripette to remove any air bubbles. After carefully removing the stacking gel, the resolving gel was placed on the filter papers with the ladder on the right hand side. The nitrocellulose membrane was placed on top of this and the stack was again gently rolled with a stripette. A further filter paper was added and air bubbles rolled out of the stack for the final time. The stack was then inverted and placed on the bottom plate of the Trans-blot SD semi-dry transfer cell (BioRad, UK).

After the transfer step the membranes were blocked with PBS (and 0.1 % Tween® 20 with 5 % dried milk) for 1h to prevent any non-specific binding of the antibodies. Membranes were then incubated with primary antibodies (see table 2.2) overnight (4°C) on an orbital shaker. Membranes were washed extensively (30x, 30 min) with PBS (and 0.1 % Tween®20) before and after 1h incubation with Horse radish peroxidase (HRP)-conjugated secondary antibodies (see table 2.2). Finally membranes were incubated for 1 min with Enhanced chemi-luminescence (ECL) reagent (Amersham, UK) and light emission was measured using photosensitive film (KODAK BIOMAX MR-1,8 X 10 IN, Sigma Aldrich, UK). All Western blot images in this thesis are representative of three separate experiments.

Table 2.3: Recipes for gels and buffers required for western blotting

12% Running Gel **: Total volume 10mL**		
Component	**Volume (ml)**	**Supplier**
dH_2O	3.3	NA
30% acrylamide	4	National diagnostics, UK
1.5M Tris pH 8.8	2.5	Sigma Aldrich, UK
10% SDS	0.1	Fisher Scientific, UK
10% Ammonium persulfate	0.1	Sigma Aldrich, UK
TEMED	0.004	BioRad, UK
5% Stacking Gel**: Total volume 10mL**		
Component	**Volume (ml)**	**Supplier**
dH_2O	6.8	NA
30% acrylamide	1.7	National diagnostics, UK
1M Tris pH 6.8	1.25	Sigma Aldrich, UK
10% Sodium dodecyl sulphate	0.1	Fisher Scientific, UK
10% Ammonium persulfate	0.1	Sigma Aldrich, UK
TEMED	0.01	BioRad, UK
Running buffer (10X)**: Total volume 2L** To make 1X running buffer dilute 1 in 10 in dH_2O.		
Component	**Mass (g)**	**Supplier**
Trizma base	60.8	Sigma Aldrich, UK
Glycine	288	Fisher Scientific, UK
Sodium dodecyl sulfate	20	Fisher Scientific, UK
Transfer buffer (10X)**: Total volume 2L** To make 1X transfer buffer dilute 1:2:7 (10 X transfer buffer: Methanol (MeOH, Fisher Scientific): dH_2O).		
Component	**Mass (g)**	**Supplier**
Trizma base	58	Sigma Aldrich, UK
Glycine	290	Fisher Scientific, UK

2.4.5 β- Actin staining of western blots

In order to control for loading errors, gels can be blotted for β-Actin, which is considered to be constitutively expressed in all cells and should consequently be present at essentially constant levels in all cell lysate samples. β-Actin blots were completed after the initial Western blots for the primary protein of interest.

Following ECL treatment membranes were washed with PBS (and 0.1 % Tween®20) a minimum of five times and the blocked (1h) with PBS (and 0.1 % Tween® 20 with 5 % dried milk). Membranes were then incubated with β-Actin antibody (1h, (PBS and 0.1 % Tween® 20 with 5 % dried milk)), washed with PBS (and 0.1 % Tween®20) and incubated for 1 min with Enhanced chemi-luminescence (ECL) reagent (Amersham, UK). Light emission was measured using photosensitive film (KODAK BIOMAX MR-1,8 X 10 IN, Sigma Aldrich, UK). β-Actin was visible as a band of approximately 42 kD.

2.4.6 Densitometry

To assess the distribution of the 17 kD mature form of IL-1β in response to zinc depletion, the intensity of these bands were measured using the image analysis program Image J [Schneider *et al.*, 2012]. Using the gel analysis function each of the 17 kD bands was selected and the density plotted. The width of the peak represented the thickness of the band and the height of the peak the intensity. Each peak was enclosed, which normalised for background intensity, and then the area under the peaks calculated. For each blot the total of the areas under the peaks were considered to be 100% and a percentage of the total was calculated for each peak. Each value is an assessment of relative distribution of the 17 kD in a blot and cannot be considered in the absence of the other bands.

2.5 Network Map

In the fifth chapter I will describe how a network map of processes leading to IL-1β expression was constructed using a systematic procedure. This is a brief summary of the procedure which is described in more detail in chapter five. A literature search was carried out to return a set of articles satisfying an appropriately designed search string. The returned references were then assessed against certain criteria and any that did not fit were excluded. Following this the quality and suitability of the data for building a network map were assessed based on a further four criteria; reaction type, technique, replicates and presence or absence of a statistical test. Using these data a network map was created in CellDesigner4.2 [Funahashi *et al.*, 2003], exported in Systems Biology Markup Language (SBML) format [Hucka *et al.*, 2003], annotated according to Minimum Information Requested In the Annotation of biological Models (MIRIAM) [Le Novère *et al.*, 2005] standard and a Systems Biology Graphical Notation (SBGN) [Le Novère *et al.*, 2009] process diagram was created.

2.6 Statistical Analysis

Except where stated otherwise, all data presented are from a minimum of three separate experiments (n=3). Groups were analysed by one-way ANOVA and subsequent Bonferroni multiple comparison test or unpaired t-test. Data are the mean +/- standard error mean (SEM). ***P<0.001, **P<0.01,*P<0.05.

Chapter 3: Zinc depletion and IL-1β in mouse macrophages

3.1 Introduction

Correlations between elevated levels of pro-inflammatory cytokines and low zinc status are well established [Prasad, 2008][Prasad, 2009]. Zinc depletion is biologically relevant in inflammation in both the context of systemic zinc deficiency and localised zinc depletion induced by intracellular pathogens [Corbett *et al.*, 2012]. Additionally, a wide body of evidence suggests that IL-1β levels are associated with low zinc levels in disease [Overbeck *et al.*, 2008].

Our lab has shown previously that an acute zinc depletion in macrophages inhibits the activity of pannexin-1, which is necessary for ATP- and nigericin-induced IL-1β processing and release through the NLRP3 inflammasome [Brough *et al.*, 2009]. Conversely, further work from our lab indicated that sustained zinc depletion may induce IL-1β processing both *in vitro* and *in vivo.* Injection of TPEN into the peritoneal cavity of C57BL/6 mice induced elevated levels of processed IL-1β in the plasma [Brough personal communication]. 4h treatment of peritoneal macrophages with the TPEN zinc chelator induced IL-1β processing *in vitro*, where copper and iron chelation did not [Brough personal communication]. This would suggest that zinc depletion is an activator of the inflammasome and initiates IL-1β processing and release.

The objective of this study was to understand the mechanisms by which zinc depletion induced IL-1β processing and release. Here it was shown that zinc depletion activates multiple mechanisms associated with IL-1β processing.

3.2 Methods

Details of all methods used in this study are described in Chapter 2. In brief, C57BL/6 (WT) adult males were used at an approximate body weight of 25-30g, to generate peritoneal macrophages. Endogenous IL-1β was induced by 2h LPS treatment. Zinc-depletion-dependent IL-1β processing was induced by 4h treatment with TPEN, DTPA and a DTPA pyrithione combination. Nigericin was used as a positive control for IL-1β processing (4h). This K^+/H^+ ionophore induces potassium efflux activating the NLRP3 inflammasome [Perregaux *et al.*, 1992] [Mariathasan *et al.*, 2006]. In some experiments inhibitors of cellular proteases were used. These inhibitors were added following LPS treatment and 15 min prior to zinc depletion or nigericin treatment. IL-1β release was measured in cell culture supernatants by ELISA. IL-1β processing was measured in cell culture supernatants by western blot and the proportion of the 17 kD form assessed by densitometry. Levels of intracellular proteins were measured by western blot of the cell lysate.

3.3 Results

3.3.1 Zinc depletion induced IL-1β release and cell death in the absence of LPS

To assess whether zinc depletion alone, in the absence of LPS, could induce IL-1β release, mouse peritoneal macrophages were treated for 4h with varying concentrations of the membrane permeable zinc chelator TPEN (2.5 μM, 5 μM, 10 μM) and equivalent DMSO vehicle controls (0.125 %, 0.25 %, 0.5 %). Macrophages treated with 10 μM TPEN had elevated IL-1β release when compared with the DMSO vehicle control. Significant IL-1β release does not occur following treatment with 2.5 μM and 5 μM TPEN for 4h. This data shows that LPS priming may not be required to induce low levels of IL-1β release. LDH levels, which are measured as an indicator of cell death, are also increased following 10 μM TPEN treatment (Fig 3.1C). As IL-1β release levels were low it was not possible to assess IL-1β processing by western blot.

It is also possible that IL-1β expression is induced by zinc depletion. Cell lysates were measured for IL-1β using ELISA and then normalised for protein content (assessed with a BCA assay). No significant increase in IL-1β expression was observed following zinc depletion, however there is a trend of increasing IL-1β in cell lysates followed by a drop in IL-1β present in cell lysates at 10 μM TPEN (Fig 3.1 B).

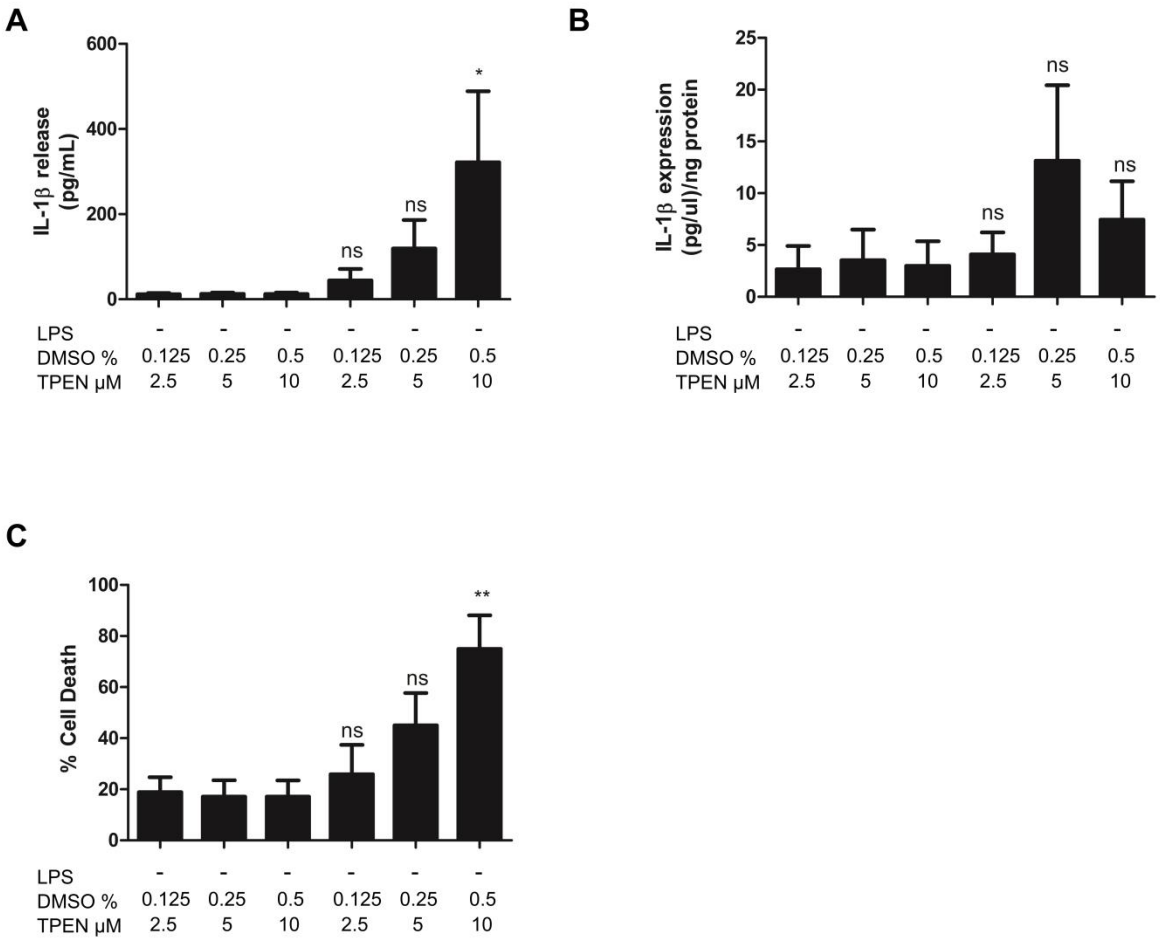

Figure 3.1: IL-1β release and expression following 4h zinc depletion of peritoneal macrophages.

Mouse peritoneal macrophages were treated with 0.125%, 0.25% 0.5% DMSO vehicle, TPEN (2.5µM, 0.125% DMSO; 5µM, 0.25% DMSO; 10µM, 0.5% DMSO) and IL-1β release was measured by ELISA in the supernatant **(A)** and cell lysate **(B)** (normalised with total protein value, measured by BCA). % total LDH was measured as an indicator of cell death **(C).** Error bars ±SEM of 5 **(A),** 4 **(B)** and 5 **(C)** independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; DMSO (0.125%) with TPEN (2.5 µM), DMSO (0.25%) TPEN (with 5 µM), DMSO (0.5%) with TPEN (10 µM) (*). **p<0.01, *p<0.05.

3.3.2 Zinc depletion induces IL-1β release and processing

The intracellular zinc pools of LPS-primed (1 μg/ml, 2h) mouse peritoneal macrophages were depleted using zinc chelators for 24h and 4h. The membrane permeable zinc chelator TPEN (10 μM) induced cell death alongside processing and release of IL-1β at both 24h and 4h (Fig 3.2A, Fig 3.2B, Fig 3.2D, Fig 3.3A, Fig 3.3B, Fig 3.3D). When compared with DMSO vehicle treated cells the IL-1β release following TPEN treatment was significantly increased by 3.36 fold after 4h and by 10.74 fold after 24h. Processing from the 31 kD pro form to the 17 kD mature form can be observed in cells treated with TPEN (10 μM) but not in cells treated with DMSO vehicle alone (Fig 3.2B, Fig 3.3B).

To ensure that TPEN-induced processing and release of IL-1β was specific to zinc depletion and didn't occur due to an 'off target' artefact, a further zinc depletion method was utilised. Intracellular zinc depletion was modelled with a combination of the extracellular zinc chelator DTPA (1 mM) and the zinc ionophore pyrithione (50 μM). Treatment of peritoneal macrophages using this second method for both 24h and 4h also induced cell death alongside IL-1β processing and release (Fig 3.2A, Fig 3.2B, Fig 3.2D, Fig 3.3A, Fig 3.3B, Fig 3.3D). At 4h DTPA (1 mM) treated cells released 14.77 fold more IL-1β into the supernatant than cells treated with HEPES vehicle. Cells treated with DTPA (1 mM) and pyrithione (50 μM) at 4h and 24h showed no significant difference in IL-1β release, although fold changes of 26.73 and 16.3 were observed respectively, when compared to vehicle. Similarly there was no significant difference in IL-1β release between DTPA and HEPES vehicle treated cells at 24h. Processing to mature 17 kD IL-1β can be seen following DTPA treatment alone and in conjunction with pyrithione at both 4h (Fig 3.2B) and 24h (Fig 3.3B).

IL-1β release is assessed by measuring total IL-1β in the supernatant via IL-1β ELISA. This measures both the pro-form and the mature form of the cytokine. It is not possible to use this ELISA to assess the proportion of pro and mature IL-1β present within the sample; however elevated levels of released IL-1β appear to correlate with IL-1β processing events when compared with western blot data. Densitometry can be used to assess the intensity of bands of one size on a western blot. In this way the changes in the 17 kD form of IL-1β following zinc depletion can be assessed. This is not a direct measurement of quantity but a measure of proportion. Band intensity was measured and then assessed as percentage of all 17 kD bands on a particular blot. In this way the values presented represent the distribution 17 kD forms but are only relevant when presented with all lanes present and only compiled with data from blots with the same lanes.

Following assessment of 17 kD band distribution, higher percentages of the 17 kD form were seen following zinc depletion than vehicle treatment. There was a significantly higher proportion of 17 kD form in TPEN treated lanes than DMSO treated lanes at both 4h and 24h (Fig 3.2C, Fig 3.3C). As with the IL-1β release data, there was no significant difference in the proportion of 17 kD IL-1β in the DTPA and pyrithione treated lanes compared with treatment with pyrithione alone at 4h or 24h (Fig 3.2C, Fig 3.3C). At 4h there was a significantly higher proportion of 17 kD IL-1β following DTPA treatment when compared HEPES, although this was not seen at 24h (Fig 3.2C, Fig 3.3C). Following zinc depletion the pattern of 17 kD band distribution is similar to the pattern of IL-1β release suggesting that patterns seen in IL-1β release are indicative of processing activity.

At 4h cell death is significantly higher in DTPA treated cells when compared with HEPES vehicle treated cells (Fig 3.2D). Cell death is significantly higher in DTPA and pyrithione treated cells when compared with pyrithione treated cells at this time point (Fig 3.2D). At 24h cell death is significantly higher in TPEN treated cells when compared with DMSO vehicle treated cells and in DTPA treated cells when compared with HEPES vehicle treated cells (Fig 3.3D). As there is a high level of cell death in cells treated with pyrithione alone at 24h (Fig 3.3D) there is no significant difference with DTPA and pyrithione treated cells. This elevated cell death was not associated with increased levels of IL-1β release or any observable IL-1β processing (Fig 3.3A, Fig 3.3B). This supports the hypothesis that zinc depletion initiates an IL-1β processing event that is associated with IL-1β release, and whilst cell death may occur in the absence of processing this does not increase IL-1β release. After assessing all of the data described above and noting that a response was observed at 4h, all subsequent experiments were treated for 4h with zinc chelators.

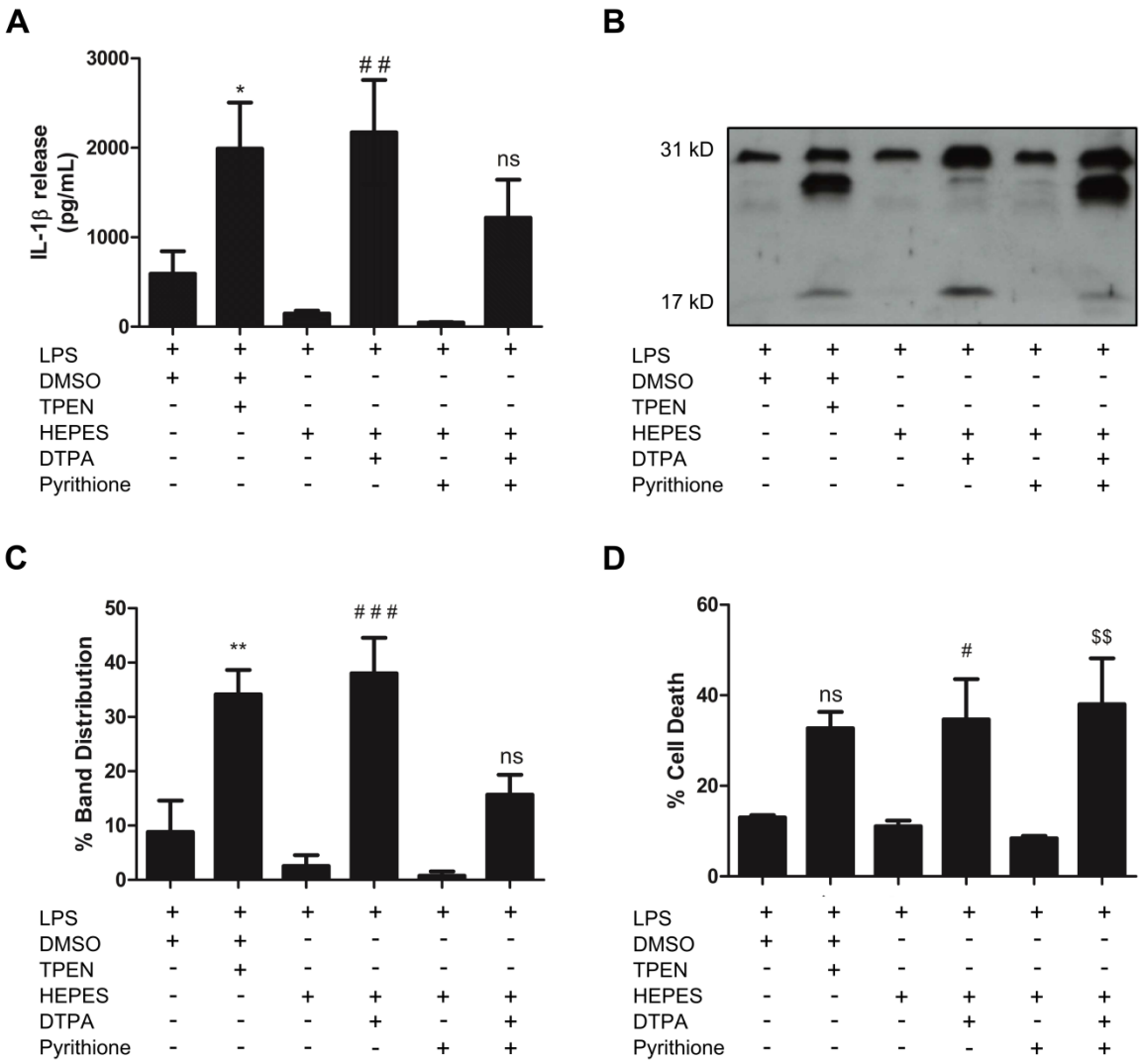

Figure 3.2: IL-1β processing and release following 4h zinc depletion of LPS primed peritoneal macrophages.

LPS-primed (1 µg/ml, 2h) mouse peritoneal macrophages were treated with 0.5 % DMSO vehicle, TPEN (10 µM, 0.5 % DMSO), HEPES (10 mM, pH 7-7.5), DTPA (1 mM, 10 mM HEPES media) or Pyrithione (50 µM, 10 mM HEPES media) and IL-1β release was measured in the supernatant was by ELISA **(A)** IL-1β processing from pro to mature (31 to 17 kD) was measured by western blot **(B),** % distribution of the 17 kD IL-1β western blot band was analysed as an indicator of IL-1β processing **(C)** and % total LDH was measured as an indicator of cell death **(D).** Error bars ±SEM of 5 **(A),** 3 **(C)** and 4 **(D)** independent experiments. Western blot is representative of 3 independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; DMSO with TPEN (*), HEPES with DTPA (#) and pyrithione with DTPA pyrithione ($). *** p<0.001, **p<0.01, *p<0.05.

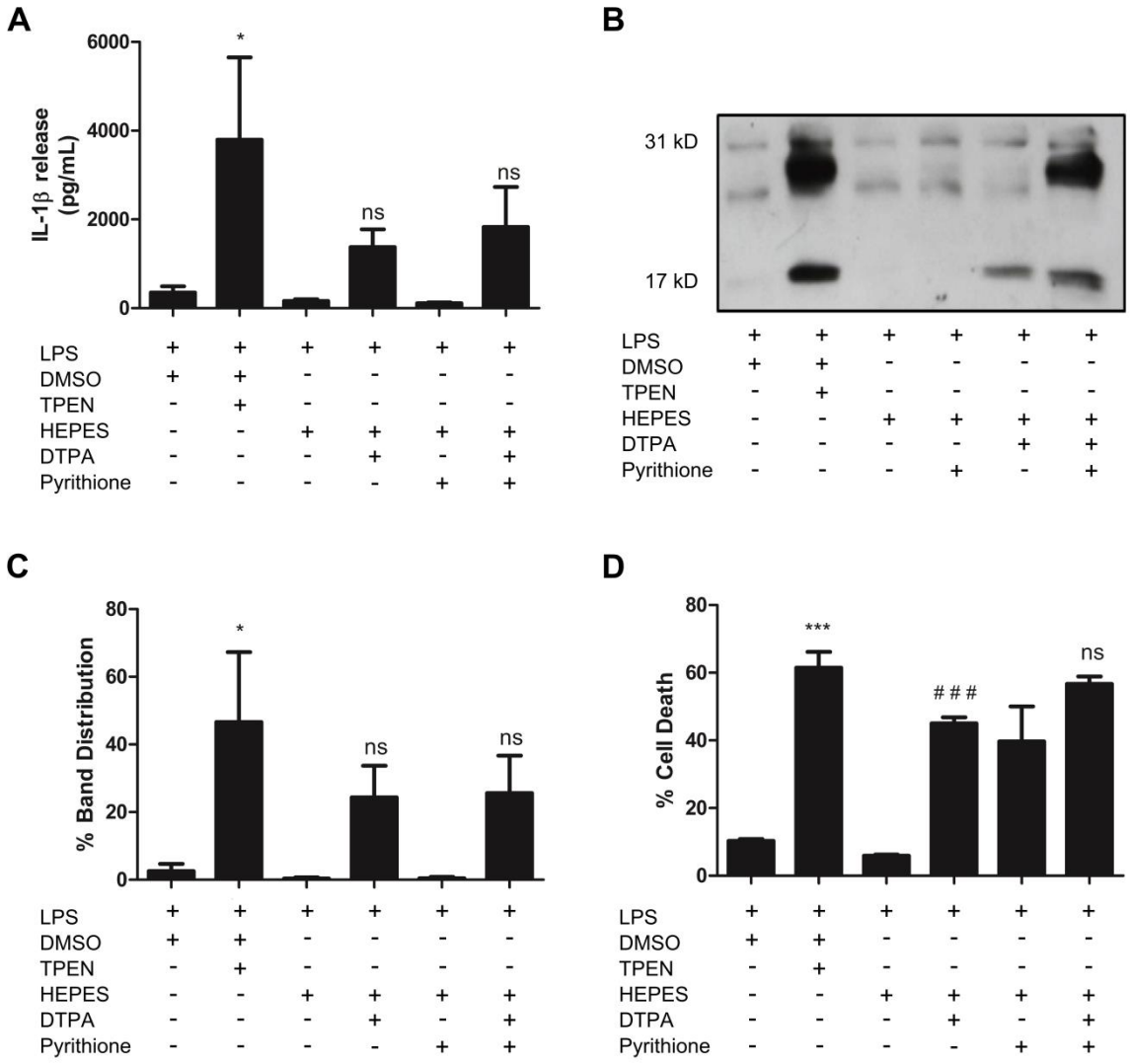

Figure 3.3: IL-1β processing and release following 24h zinc depletion of LPS primed peritoneal macrophages.

LPS-primed (1µg/ml, 2h) mouse peritoneal macrophages were treated with 0.5% DMSO vehicle, TPEN (10µM, 0.5% DMSO), HEPES (10mM, pH7-7.5), DTPA (1mM, 10mM HEPES media) or Pyrithione (50µM, 10mM HEPES media) and IL-1β release was measured in the supernatant was by ELISA **(A)** IL-1β processing from pro to mature (31 to 17kD) was measured by western blot **(B),** % distribution of the 17 kD IL-1β western blot band was analysed as an indicator of IL-1β processing **(C)** and % total LDH was measured as an indicator of cell death **(D).** Error bars ±SEM of 3 **(A),** 3 **(C)** and 3 **(D)** independent experiments. Western blot is representative of 3 independent experiments. Please note that the order of this western blot differs from the graphs, with the pyrithione lane positioned before the DTPA lane. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; DMSO with TPEN (*), HEPES with DTPA (#) and pyrithione with DTPA pyrithione. *** $p<0.001$, * $p<0.05$.

3.3.3 Zinc depletion induced IL-1β processing and release is caspase-1 dependent.

Caspase-1 is the best characterised protease recognised to cleave IL-1β from its pro-to its mature form [Gross *et al.*, 2011], although additional proteases have been identified [Netea *et al.*, 2010]. Peritoneal macrophages that were to be zinc depleted with TPEN were pre-treated with an inhibitor for caspase-1 (YVAD-cho) in order to establish whether zinc depletion induced caspase-1-dependent IL-1β release. Inhibition of caspase-1 reduced IL-1β release (Fig 3.4A) by 70%. This suggests that zinc depletion by TPEN induced caspase-1 activation, which in turn cleaved pro-IL-1β. Although it should be noted that YVAD inhibition did not reduce IL-1β release to vehicle levels, therefore other mechanisms may contribute to IL-1β release induced by zinc depletion.

The hypothesis of zinc depletion induced caspase-1 dependent processing and release is further supported by western blot data. Fig 3.4 B shows decreased IL-1β processing, which is also shown by the assessment of % distribution of the 17 kD IL-1β band (Fig 3.4 C). Although this data cannot be considered to be conclusive as it is only representative of a single repeat. Inhibiting caspase-1 with YVAD did not reduce the levels of cell death (Fig 3.4D). It can therefore be surmised that TPEN induced cell death is not caspase-1 dependent.

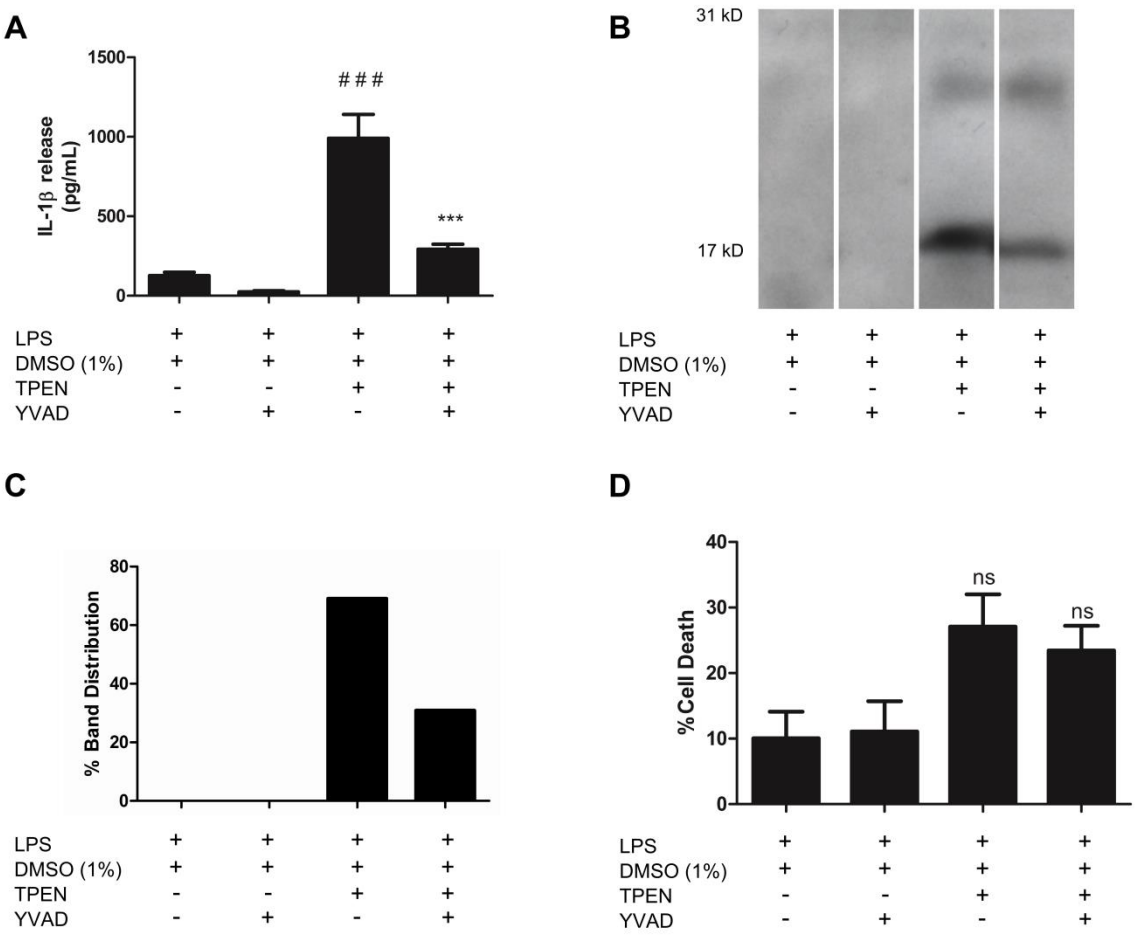

Figure 3.4: YVAD inhibition reduces IL-1β release in zinc depleted peritoneal macrophages.

LPS-primed (1µg/ml, 2h) mouse peritoneal macrophages were treated with YVAD (100 µM, 0.5 % DMSO), 0.5 % DMSO vehicle and TPEN (10 µM, 0.5 % DMSO). IL-1β release was measured in the supernatant was by ELISA **(A)** IL-1β processing from pro to mature (31 to 17kD) was measured by western blot **(B),** % distribution of the 17 kD IL-1β western blot band was analysed as an indicator of IL-1β processing **(C)** and % total LDH was measured as an indicator of cell death **(D).** Error bars ±SEM of 3 **(A)** and 3 **(D)** independent experiments. Western blot **(B)** shows 4 separate lanes from one repeat. This n of 1 was assessed for % distribution of the 17 kD IL-1β band as an indicator of IL-1β processing **(C).** Comparison pairs were as follows; DMSO with TPEN (#), and TPEN with TPEN and YVAD (*). *** $p<0.001$

3.3.4 Inhibition of cathepsin B reduces zinc depletion induced IL-1β processing and release.

Cathepsin B is a lysosomal protease that is associated with NLRP3 inflammasome activation following its release from destabilised lysosomes [Hornung & Latz, 2010]. To investigate whether zinc depletion activates IL-1β processing via this process, cathepsin B was inhibited with CA074-Me in combination with TPEN induced zinc depletion (4h). Inhibition of cathepsin B reduced IL-1β release by 67 % in TPEN treated cells (Fig 3.5A) and IL-1β processing induced by TPEN was also reduced to vehicle levels (Fig 3.5B). This was supported by analysis of the distribution of the 17 kD band, which showed a decreased proportion of processed IL-1β following cathepsin B inhibition (Fig 3.5C). This would suggest that the activity of cathepsin B is necessary for zinc depletion induced IL-1β processing and release. Cell death levels in macrophages treated with both the cathepsin B inhibitor and TPEN were not significantly lower than the levels of cell death in macrophages treated with TPEN alone (Fig 3.5D), therefore zinc depletion induced cell death is not dependent upon cathepsin B.

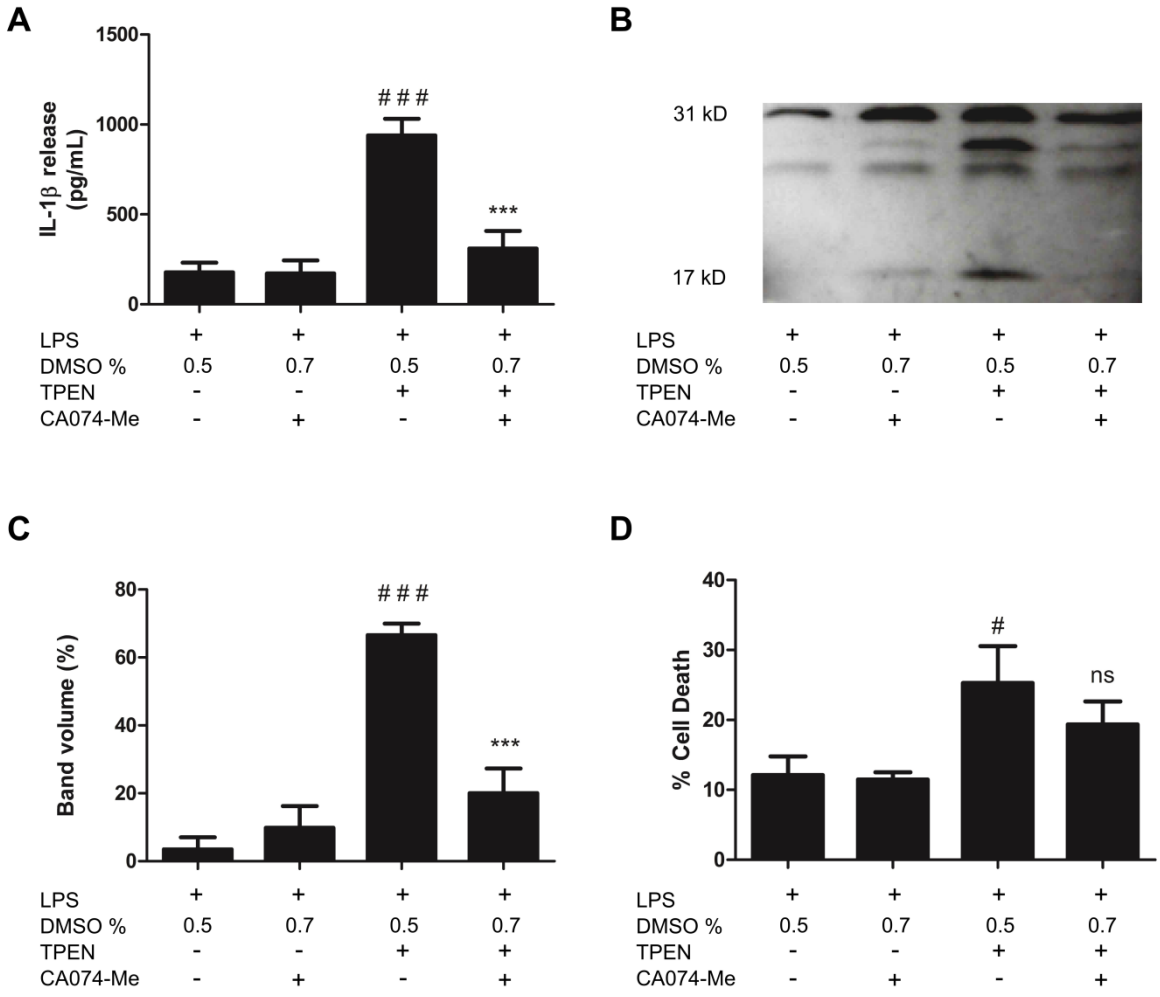

Figure 3.5: Cathepsin B inhibition reduces IL-1β processing and release in zinc depleted peritoneal macrophages.

LPS-primed (1µg/ml, 2h) mouse peritoneal macrophages were treated with CA074-Me (80µM, 0.2% DMSO), 0.5% DMSO vehicle, TPEN (10µM, 0.5% DMSO), and IL-1β release was measured in the supernatant was by ELISA **(A)** IL-1β processing from pro to mature (31 to 17kD) was measured by western blot **(B),** % distribution of the 17 kD IL-1β western blot band was analysed as an indicator of IL-1β processing **(C)** and % total LDH was measured as an indicator of cell death **(D).** Error bars ±SEM of 4 **(A),** 3 **(C)** and 4 **(D)** independent experiments. Western blot **(B)** is representative of 3 independent experiments. Comparison pairs were as follows; DMSO with TPEN (#), and TPEN with TPEN and CA074-Me (*). *** $p<0.001$ *$p<0.05$.

3.3.5 Inhibition of PP1/PP2A reduces zinc depletion induced IL-1β processing and release.

It has been shown that a PP1/PP2A signal is necessary for the activation of the NLRP3, NLRC4 and AIM2 inflammasomes [Luheshi *et al.*, 2012]. TPEN (10 μM) treated peritoneal macrophages were treated with the PP1/PP2A inhibitors calyculin A and okadaic acid to assess whether zinc-depletion induced IL-1β processing and release was dependent upon a PP1/PP2A signal. At calyculin A concentrations of 10 nM and 50 nM TPEN-induced IL-1β release was reduced by 83% and 77% respectively to levels of release that were equivalent to vehicle treated cells (Fig 3.6A). TPEN-induced IL-1β processing was also inhibited by calyculin A (Fig 3.6B). The proportion of 17 kD IL-1β was also lower than vehicle controls for macrophages treated with calyculin A (Fig 3.6C). IL-1β release was also reduced by 45% in macrophages treated with TPEN (10 μM) and okadaic acid (2 μM) (Fig 3.6A). Okadaic acid with 0.5 % DMSO vehicle control induces some IL-1β processing (Fig 3.6B), although to a lesser extent than TPEN treatment. When macrophages are treated with both TPEN and okadaic acid IL-1β processing is reduced to vehicle control levels (Fig 3.6B). This would suggest that a PP1/PP2A signal is essential for zinc depletion induced IL-1β processing and release, and is consistent with the involvement of an inflammasome.

PP1/PP2A inhibition does not inhibit release of mature IL-1β by promoting the survival of the macrophages. Treatment of peritoneal macrophages with the PP1/PP2A inhibitors calyculin A and okadaic acid induced cell death levels similar to those observed with TPEN treatment (Fig 3.6D). Consequently there is no significant reduction in cell death in cells treated with TPEN and PP1/PP2A inhibitors (Fig 3.6D). As both the PP1/2A inhibitors and the TPEN treatment induce cell death it is not possible to determine the cause of cell death following a combination of both treatments.

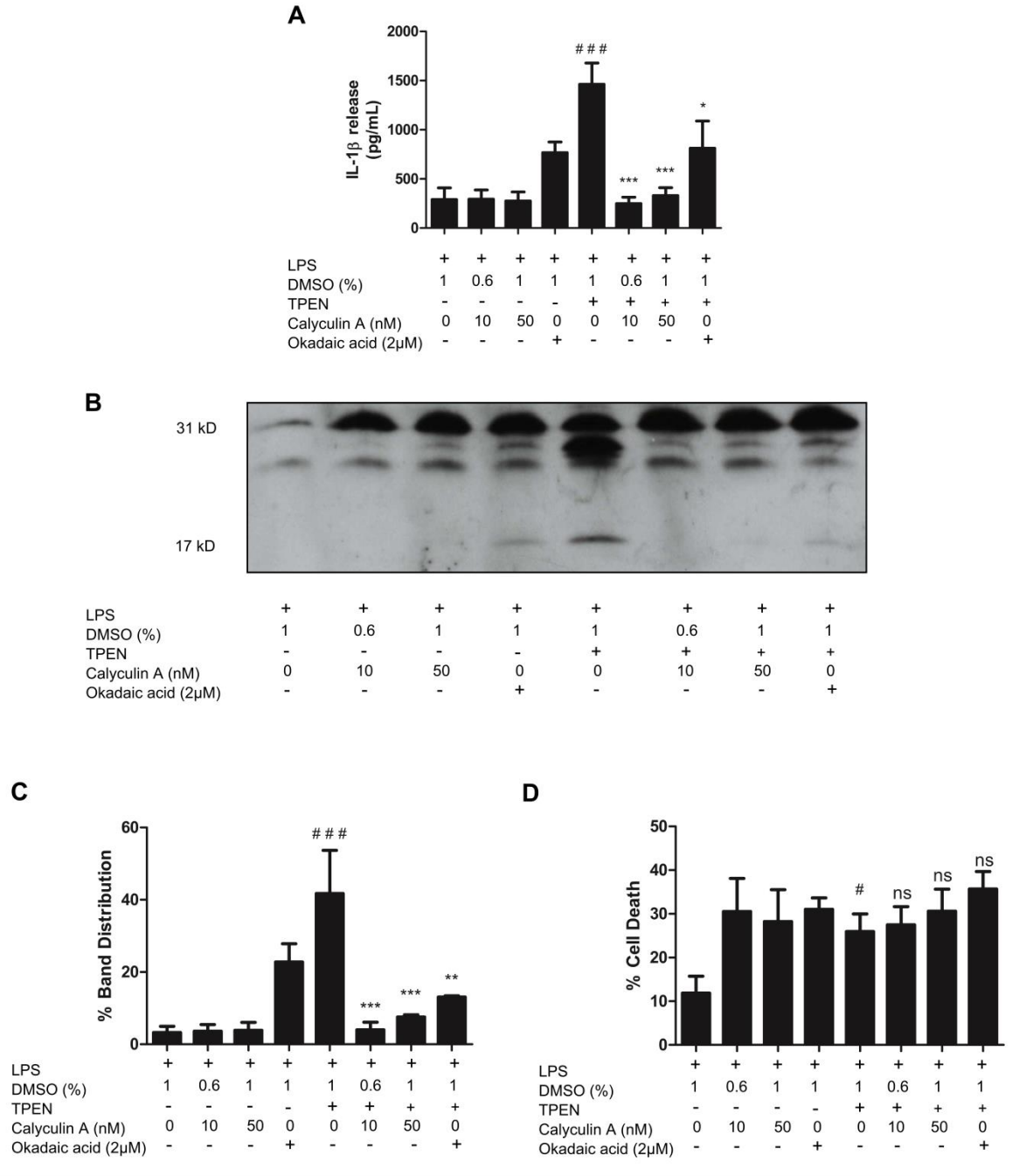

Figure 3.6: PP1/PP2A inhibition reduces IL-1β processing and release in zinc depleted peritoneal macrophages.

LPS-primed (1 μg/ml, 2h) mouse peritoneal macrophages were treated with Calyculin A (DMSO vehicle), Okadaic acid (2 μM, 0.5 % DMSO vehicle), TPEN (10 μM, 0.5 % DMSO vehicle), and IL-1β release was measured in the supernatant was by ELISA **(A)** IL-1β processing from pro to mature (31 to 17 kD) was measured by western blot **(B)** % distribution of the 17 kD IL-1β western blot band was analysed as an indicator of IL-1β processing **(C)** and % total LDH was measured as an indicator of cell death **(D).** Error bars ±SEM of 3 **(A),** 3 **(C)** and 3 **(D)** independent experiments. Western blot **(B)** is representative of 3 independent experiments. Comparison pairs were as follows; DMSO with TPEN (#), TPEN with TPEN and Calyculin A (10 nM), Calyculin A (50 nM), and Okadaic acid respectively (*). *** p<0.001 **p<0.01 *p<0.05.

3.3.6 ASC is required for zinc depletion induced IL-1β processing and release

ASC is central to caspase-1 activation as a central component of many of the inflammasomes and the pyroptosome. To establish whether ASC was involved in zinc depletion-induced IL-1β processing and release, zinc depletion experiments were undertaken in ASC knockout (-/-) mice. TPEN, DTPA alone and DTPA and pyrithione in combination induced IL-1β release in WT mice by 9.99, 5.34 and 8.56 fold respectively (Fig 3.7). TPEN-induced release was reduced by 63% in ASC -/- (Fig 3.7A). The IL-1β release was reduced by 89% in DTPA treated ASC-/- macrophages and by 75% in the ASC -/- treated with a combination of DTPA and pyrithione (Fig3.7B). These data suggest that zinc-depletion-induced IL-1β release occurs via an ASC dependent inflammasome. As a positive control for ASC-/- behaviour WT and ASC-/- macrophages were treated with nigericin, which is known to be dependent upon ASC. Nigericin induced IL-1β release was reduced by 99% in ASC-/- macrophages (Fig 3.7C). As abolition of IL-1β release in ASC-/- macrophages following zinc depletion is not complete it is possible that ASC dependent processes may not be entirely responsible for this effect.

The IL-1β processing that occurs in response to zinc depletion in WT macrophages is down regulated in ASC -/- cells. This is seen in response to TPEN, DTPA and DTPA in combination with pyrithione (Fig 3.8A, Fig 3.8B). Treatment of ASC KO macrophages with nigericin (a control for ASC dependent IL-1β processing) fails to induce IL-1β processing (Fig 3.8 C) which confirms the ASC -/- cells are behaving as expected. Assessment of distribution of the 17 kD band in western blots shows decreased proportions of the 17 kD form in response to zinc depletion in ASC-/- macrophages when compared to WT macrophages. The pattern of distribution of the 17 kD form of IL-1β is very similar to the pattern of IL-1β release, highlighting the strong link between processing and release. In addition to the 17

kD form, a larger band of approximately 28 kD is often observed in conjunction with the 17 kD form. In the zinc depleted ASC -/- macrophages the 17 kD form is not observed but the 28 kD form remains (Fig 3.8A, Fig 3.8B), although this 28 kD form is not present in nigericin treated ASC-/- macrophages. This would imply that in the absence of ASC zinc depletion is still inducing some inflammasome independent IL-1β processing events.

Cell death levels in both the WT and ASC -/- cells were similar, with no significant reductions in cell death in the zinc depleted ASC-/- macrophages. This would indicate that the cell death induced by zinc depletion is not dependent upon ASC (Fig 3.9A, Fig 3.9B) or caspase-1 (Fig 3.4D). In the nigericin treated cells the level of cell death in the ASC KO was also similar to cell death levels in WT cells (Fig 3.9C).

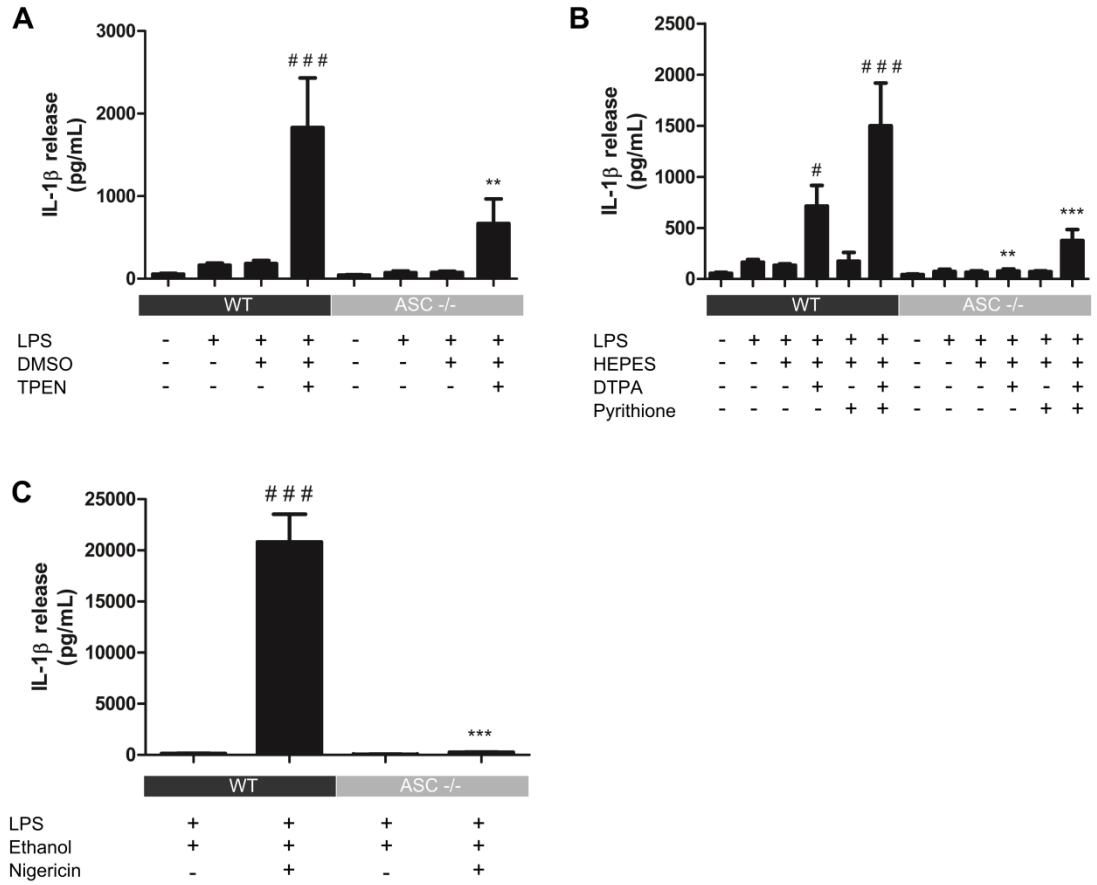

Figure 3.7: IL-1β release in zinc depleted WT and ASC -/- peritoneal macrophages

LPS-primed (1µg/ml, 2h) mouse peritoneal macrophages (WT and ASC -/-) were treated with 0.5% DMSO vehicle, TPEN (10µM, 0.5% DMSO) **(A),** HEPES (10mM, pH7-7.5), DTPA (1mM, 10mM HEPES media) or Pyrithione (50µM, 10mM HEPES media) **(B)** and 0.5% Ethanol vehicle, Nigericin (20µM, 0.5% Ethanol vehicle) **(C).** IL-1β release was measured in the supernatant was by ELISA. Error bars ±SEM of 6 **(A)** 6 **(B)** and 6 **(C)** independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; **(A)** WT DMSO with WT TPEN (#) and WT TPEN with ASC-/- TPEN (*); **(B)** WT HEPES with WT DTPA (#), pyrithione with WT DTPA pyrithione (#), WT DTPA with ASC-/- DTPA (*),WT DTPA pyrithione with ASC-/- DTPA pyrithione (*);**(C)** WT ethanol with WT nigericin (#) and WT nigericin with ASC-/- nigericin (*); *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

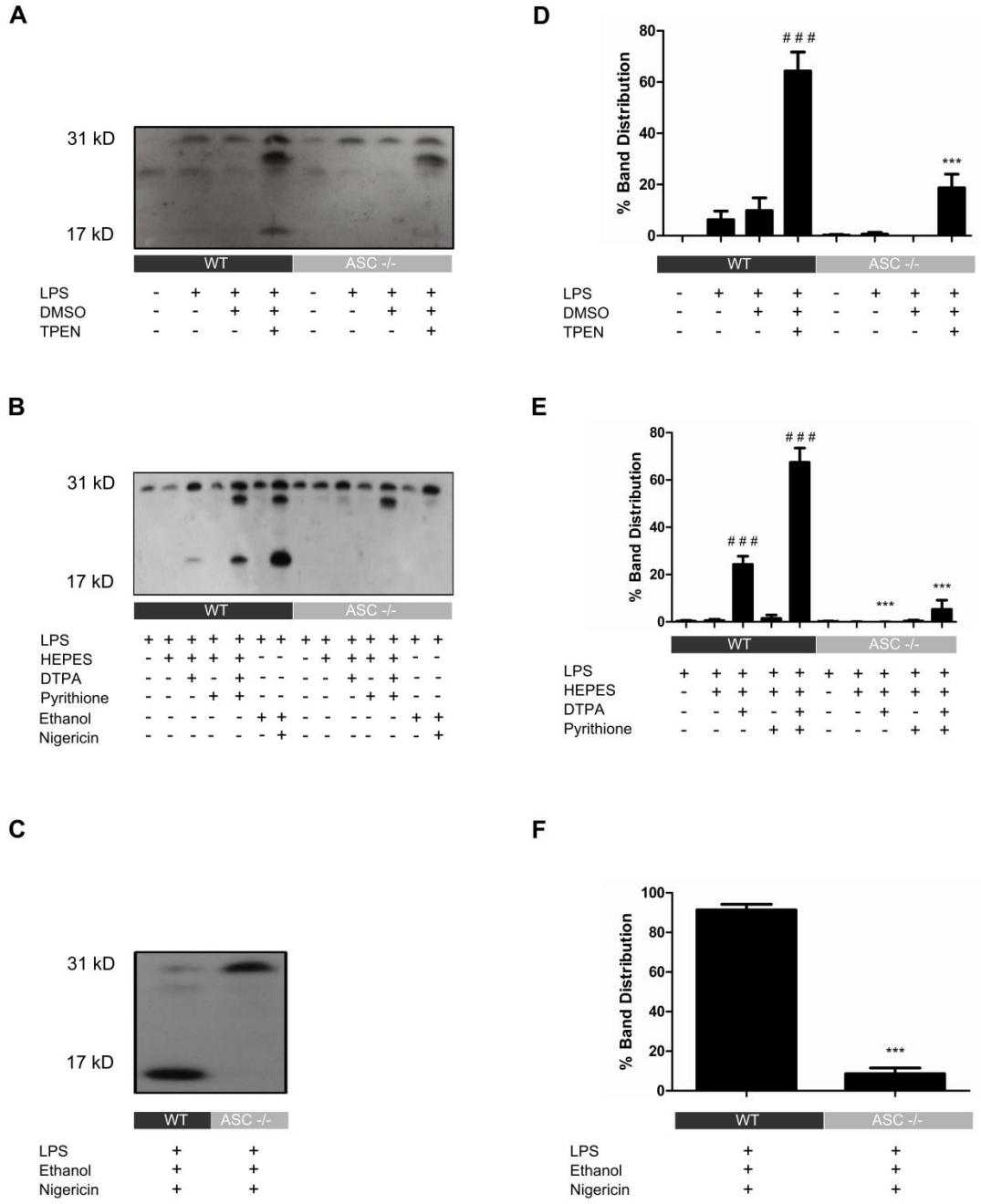

Figure 3.8: IL-1β processing in zinc depleted WT and ASC -/- peritoneal macrophages

LPS-primed (1µg/ml, 2h) mouse peritoneal macrophages (WT and ASC -/-) were treated with 0.5% DMSO vehicle, TPEN (10µM, 0.5% DMSO)**,** HEPES (10mM, pH7-7.5), DTPA (1mM, 10mM HEPES media) or Pyrithione (50µM, 10mM HEPES media) and Nigericin (20µM, 0.5% Ethanol vehicle)**.** IL-1β processing from pro to mature (31 to 17kD) was measured by western blot (**A), (B)** and **(C).** Western blots are representative of 3 independent experiments. % distribution of the 17 kD IL-1β western blot band was analysed as an indicator of IL-1β processing **(D), (E)** and **(F)**. Error bars ±SEM of 3 **(D),** 3 **(E)** and 3 **(F)** independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; **(D)** WT DMSO with WT TPEN (#) and WT TPEN with ASC-/- TPEN (*); **(E)** WT HEPES with WT DTPA (#), pyrithione with WT DTPA pyrithione (#), WT DTPA with ASC-/- DTPA (*),WT DTPA pyrithione with ASC-/- DTPA pyrithione (*). An unpaired t test was undertaken to identify significant differences between WT nigericin and ASC-/- nigericin **(F)** (*); *** p<0.001.

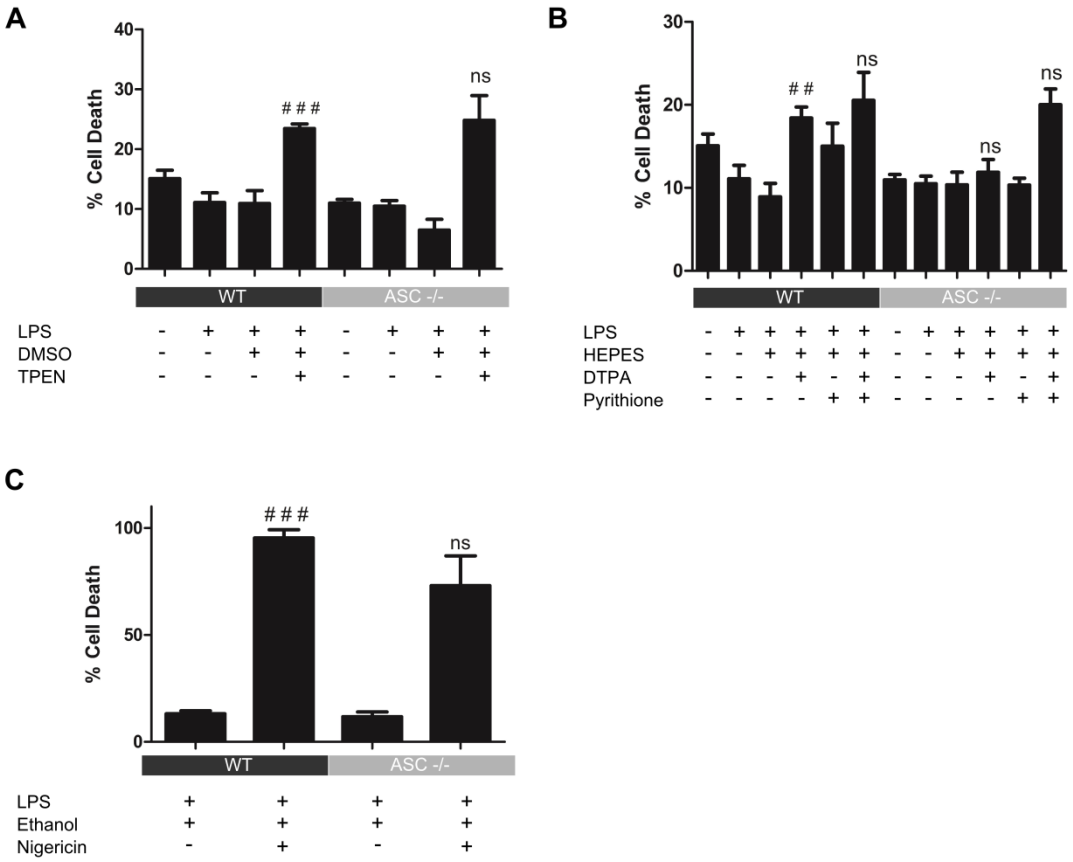

Figure 3.9: Cell death in zinc depleted WT and ASC -/- peritoneal macrophages

LPS-primed (1µg/ml, 2h) mouse peritoneal macrophages (WT and ASC -/-) were treated with 0.5% DMSO vehicle, TPEN (10µM, 0.5% DMSO) **(A),** HEPES (10mM, pH7-7.5), DTPA (1mM, 10mM HEPES media) or Pyrithione (50µM, 10mM HEPES media) **(B)** and 0.5% Ethanol vehicle, Nigericin (20µM, 0.5% Ethanol vehicle) **(C).** % total LDH was measured as an indicator of cell death. Error bars ±SEM of 4 **(A)** 4 **(B)** and 4 **(C)** independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; **(A)** WT DMSO with WT TPEN (#) and WT TPEN with ASC-/- TPEN (*); **(B)** WT HEPES with WT DTPA (#), pyrithione with WT DTPA pyrithione (#), WT DTPA with ASC-/- DTPA (*),WT DTPA pyrithione with ASC-/- DTPA pyrithione (*);**(C)** WT ethanol with WT nigericin (#) and WT nigericin with ASC-/- nigericin (*); *** $p<0.001$, ** $p<0.01$.

3.3.7 NLRP3 is not essential for zinc depletion induced IL-1β processing and release

The best characterised inflammasome is the NLRP3 inflammasome [Leemans *et al.*, 2011] [De Nardo & Latz, 2011]. To test whether the NLRP3 inflammasome was also essential for the zinc-depletion-induced IL-1β release, peritoneal macrophages from NLRP3 -/- mice were zinc depleted in an experiment analogous to the ones described above.

Depleting zinc in NLRP3 -/- cells using TPEN, DTPA or DTPA in combination with pyrithione induced IL-1β release equivalent to the release from WT cells (Fig 3.10A, Fig 3.10B). However the standard error mean of each of the data sets is large and may be masking other effects. Similar patterns were observed in relation to IL-1β processing. In TPEN and DTPA-pyrithione treated cells IL-1β processing was observed to occur in WT and NLRP3 -/- macrophages (Fig 3.11A, Fig 3.11B). Distribution of the 17 kD band in western blots followed a similar pattern to IL-1β release (Fig 3.11D, Fig 3.11E). This data showed a partial reduction in IL-1β processing in TPEN stimulated NLRP3 cells when compared with TPEN simulated WT cells (Fig 3.11D). A significant reduction was also observed following DTPA stimulation of NLRP3 -/- cells when compared to WT (Fig 3.11E). Although not conclusive this data would support a role for NLRP3 in zinc depletion induced IL-1β processing. Cell death levels were similar in both NLRP3 -/- macrophages and WT macrophages following zinc depletion (Fig 3.12A, Fig 3.12B).

IL-1β processing and release was reduced in NLRP3 -/- macrophages in comparison with WT macrophages in response to nigericin treatment (Fig 3.11C, Fig 3.11F, 3.10C). Nigericin-induced IL-1β release is known to depend upon the NLRP3 inflammasome [Mariathasan *et al.*, 2006]. Therefore the loss of IL-1β processing and release in the NLRP3 -/- cells in

response to this stimulus indicates that the NLRP3 -/- cells were behaving normally. Overall, the evidence presented on zinc depletion induced IL-1β processing and release in NLRP3 -/- macrophages is not conclusive and cannot prove or disprove involvement of the NLRP3 inflammasome.

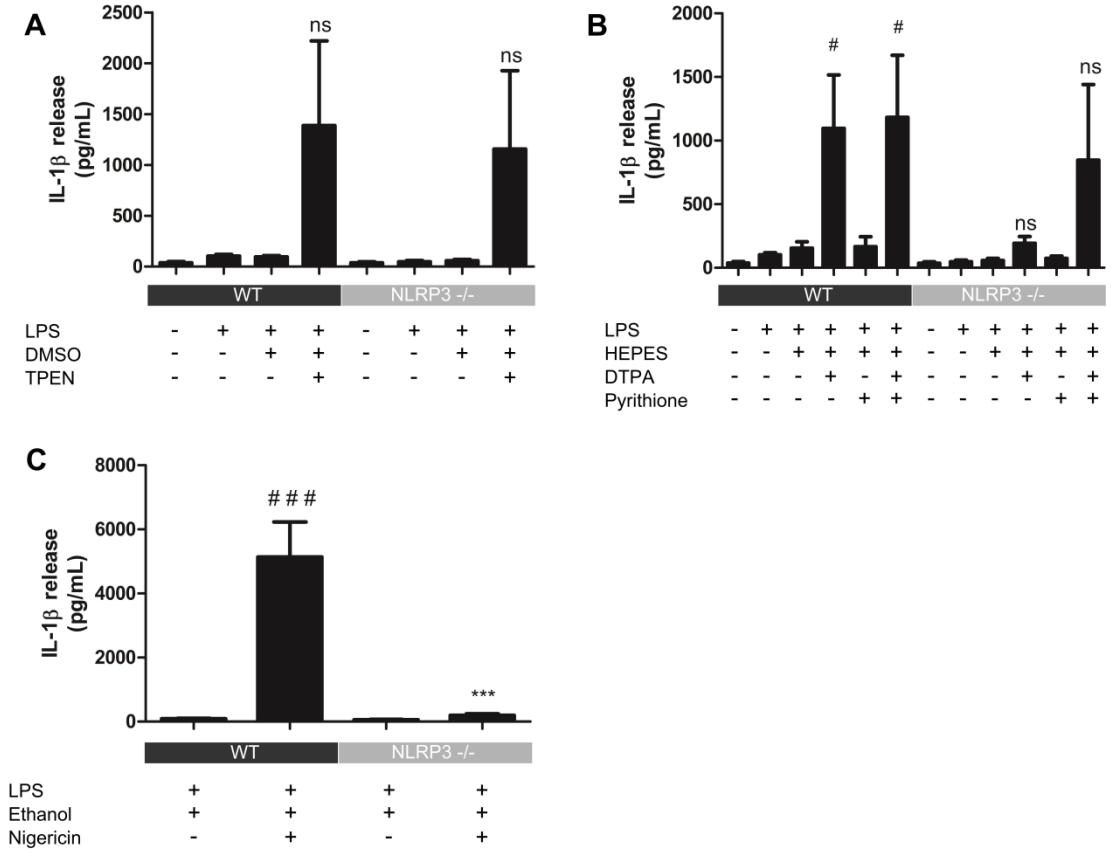

Figure 3.10: IL-1β release in zinc depleted WT and NLRP3 -/- peritoneal macrophages

LPS-primed (1µg/ml, 2h) mouse peritoneal macrophages (WT and NLRP3 -/-) were treated with 0.5% DMSO vehicle, TPEN (10µM, 0.5% DMSO) **(A),** HEPES (10mM, pH7-7.5), DTPA (1mM, 10mM HEPES media) or Pyrithione (50µM, 10mM HEPES media) **(B)** and 0.5% Ethanol vehicle, Nigericin (20µM, 0.5% Ethanol vehicle) **(C).** IL-1β release was measured in the supernatant was by ELISA. Error bars ±SEM of 6 **(A)** 6 **(B)** and 6 **(C)** independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; **(A)** WT DMSO with WT TPEN (#) and WT TPEN with NLRP3 -/- TPEN (*); **(B)** WT HEPES with WT DTPA (#), pyrithione with WT DTPA pyrithione (#), WT DTPA with NLRP3 -/- DTPA (*),WT DTPA pyrithione with NLRP3 -/- DTPA pyrithione (*);**(C)** WT ethanol with WT nigericin (#) and WT nigericin with NLRP3 -/- nigericin (*); *** $p<0.001$, * $p<0.05$.

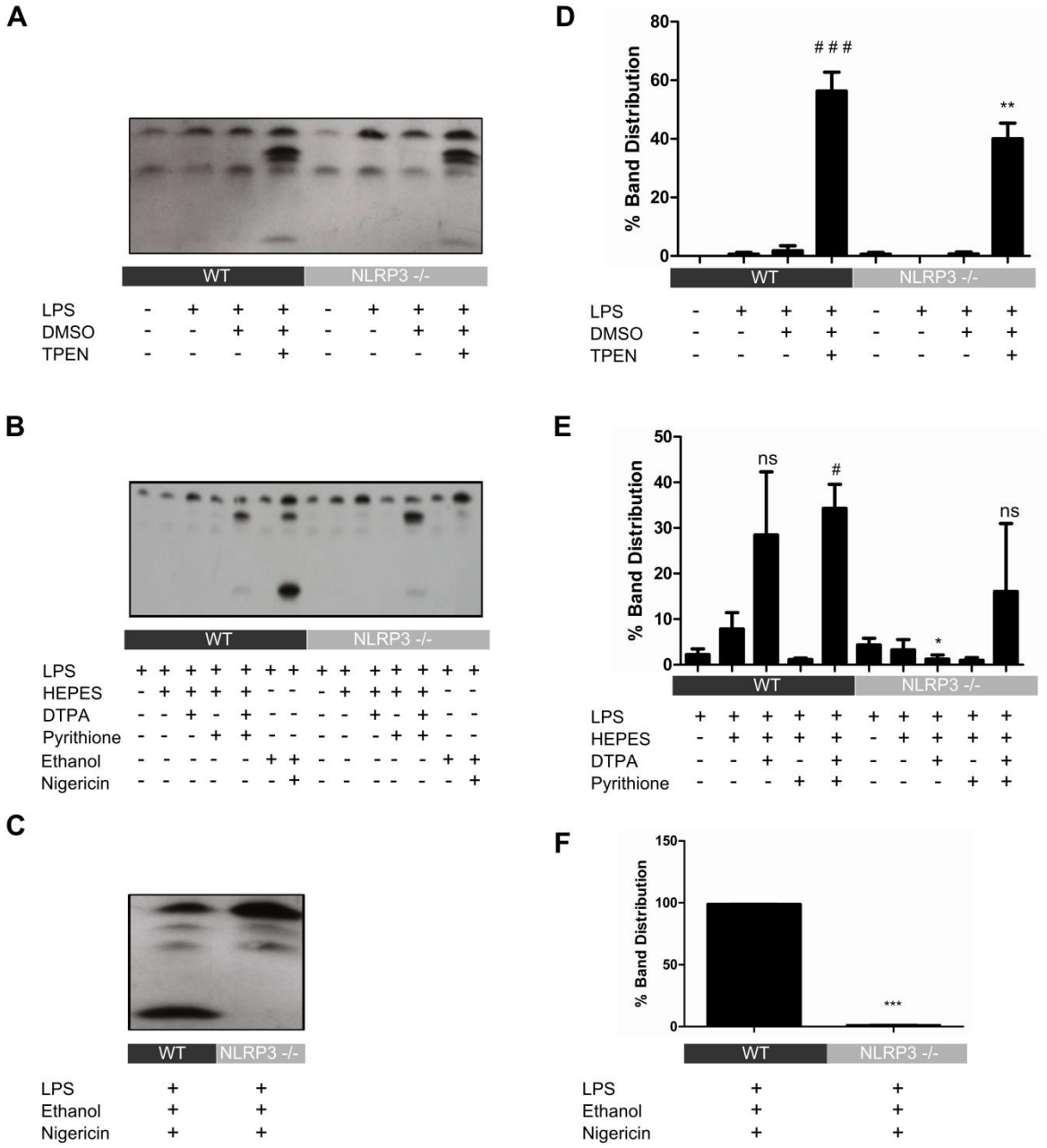

Figure 3.11: IL-1β processing in zinc depleted WT and NLRP3 -/- peritoneal macrophages

LPS-primed (1µg/ml, 2h) mouse peritoneal macrophages (WT and NLRP3 -/-) were treated with 0.5% DMSO vehicle, TPEN (10µM, 0.5% DMSO)**,** HEPES (10mM, pH7-7.5), DTPA (1mM, 10mM HEPES media) or Pyrithione (50µM, 10mM HEPES media) and Nigericin (20µM, 0.5% Ethanol vehicle)**.** IL-1β processing from pro to mature (31 to 17kD) was measured by western blot (**A), (B)** and **(C).** Western blots are representative of 3 independent experiments. % distribution of the 17 kD IL-1β western blot band was analysed as an indicator of IL-1β processing **(D), (E)** and **(F)**. Error bars ±SEM of 3 **(D),** 3 **(E)** and 3 **(F)** independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; **(D)** WT DMSO with WT TPEN (#) and WT TPEN with NLRP3-/- TPEN (*); **(E)** WT HEPES with WT DTPA (#), pyrithione with WT DTPA pyrithione (#), WT DTPA with NLRP3-/- DTPA (*),WT DTPA pyrithione with ASC-/- DTPA pyrithione (*). An unpaired t test was undertaken to identify significant differences between WT nigericin and ASC-/- nigericin **(F)** (*); *** p<0.001.

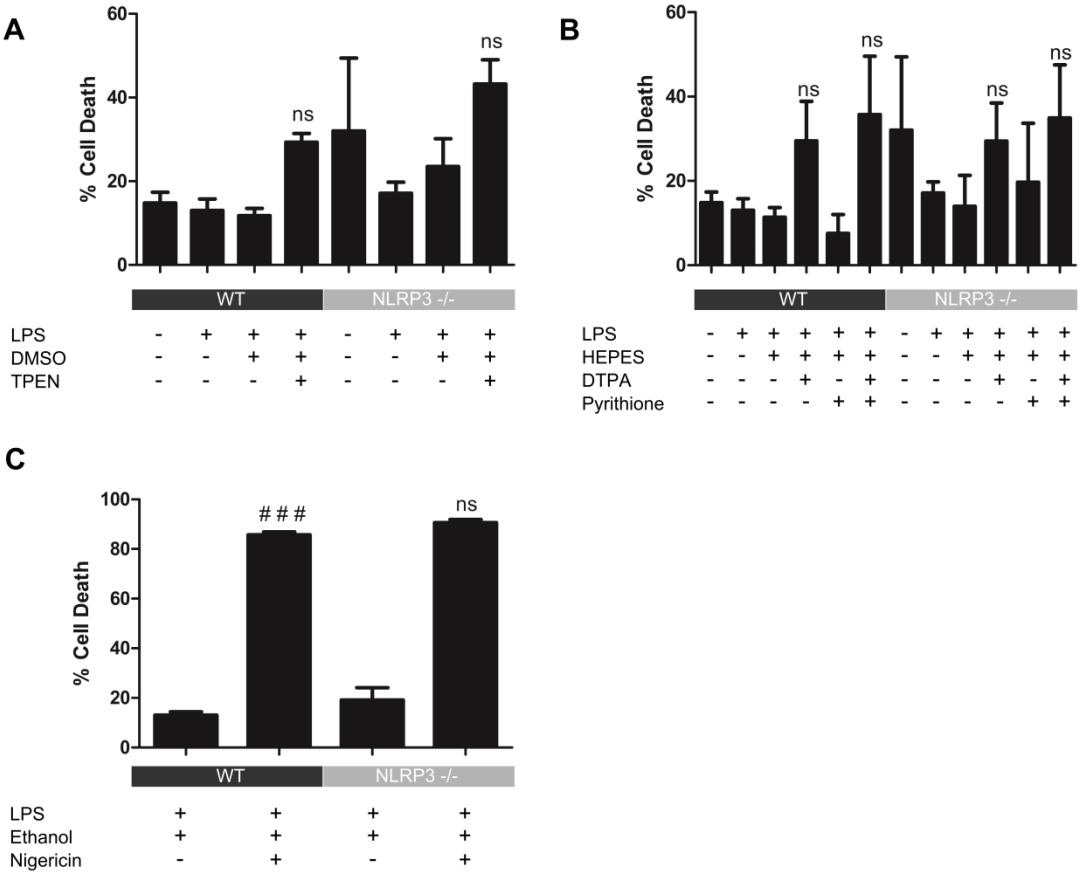

Figure 3.12: Cell death in zinc depleted WT and NLRP3 -/- peritoneal macrophages

LPS-primed (1µg/ml, 2h) mouse peritoneal macrophages (WT and NLRP3 -/-) were treated with 0.5% DMSO vehicle, TPEN (10µM, 0.5% DMSO) **(A),** HEPES (10mM, pH7-7.5), DTPA (1mM, 10mM HEPES media) or Pyrithione (50µM, 10mM HEPES media) **(B)** and 0.5% Ethanol vehicle, Nigericin (20µM, 0.5% Ethanol vehicle) **(C).** % total LDH was measured as an indicator of cell death. Error bars ±SEM of 3 **(A)** 3 **(B)** and 3 **(C)** independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; **(A)** WT DMSO with WT TPEN (#) and WT TPEN with NLRP3 -/- TPEN (*); **(B)** WT HEPES with WT DTPA (#), pyrithione with WT DTPA pyrithione (#), WT DTPA with NLRP3 -/- DTPA (*),WT DTPA pyrithione with NLRP3 -/- DTPA pyrithione (*);**(C)** WT ethanol with WT nigericin (#) and WT nigericin with NLRP3 -/- nigericin (*); *** p<0.001, **P<0.01, *P<0.05

3.3.8 Zinc depletion induces XIAP depletion and caspase-8 cleavage.

Whilst zinc depletion-induced IL-1β processing and release was partially ASC dependent, the ASC KO did not completely ablate IL-1β processing and release (Fig 3.7, 3.8). There is literature to suggest that depletion of inhibitor of apoptosis proteins (IAPs) and subsequent activation of caspase 8 results in caspase-8 dependent IL-1β processing and release [Vince *et al.*, 2012]. Cellular levels of X-linked inhibitor of apoptosis (XIAP) were measured in parallel with cellular levels of caspase-8. XIAP is a protein known to be degraded in low zinc conditions [Makhov *et al.*, 2008]. In peritoneal macrophages treated with TPEN and a combination of DTPA and pyrithione there was evidence for XIAP degradation and caspase-8 upregulation and cleavage (Fig 3.13).

As XIAP was decreased and caspase-8 activated in my zinc depleted macrophages, it was possible that the inflammasome independent IL-1β processing observed following TPEN treatment may be induced by ripoptosome formation. It would be interesting to investigate further using inhibitors of the ripoptosome. Overall these data suggest that zinc depletion activates inflammasome-dependent caspase-1 activation and depletion of XIAP to activate caspase-8 activity.

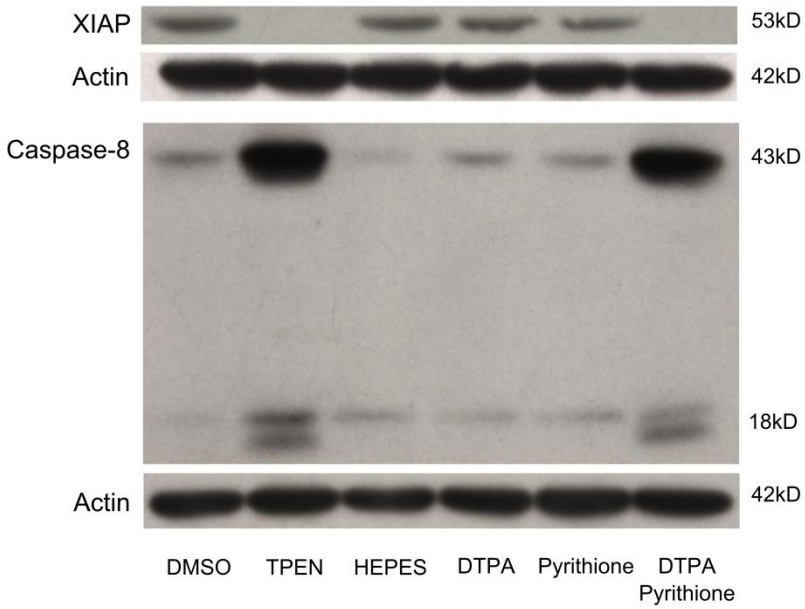

Figure 3.13: Cell death in zinc depleted WT and NLRP3 KO peritoneal macrophages

LPS-primed (1µg/ml, 2h) mouse peritoneal macrophages were treated with 0.5% DMSO vehicle, TPEN (10µM, 0.5% DMSO), HEPES (10mM, pH7-7.5), DTPA (1mM, 10mM HEPES media) or pyrithione (50µM, 10mM HEPES media). Intracellular XIAP and caspase-8 measured by western blot. Western blots are representative of 3 independent experiments.

Table 3.1: Summary table of IL-1β and cell death response to zinc depletion

Figure	LPS	Zinc depletion	Inhibitor or knockout cell	Time of treatment (h)	Processing	Release	Cell Death
3.1	-	TPEN	NA	4	NA	Yes	Yes
3.2	+	TPEN	NA	4	Yes	Yes	Not significant
3.2	+	DTPA	NA	4	Yes	Yes	Yes
3.2	+	DTPA and pyrithione	NA	4	Yes	Not significant	Yes
3.3	+	TPEN	NA	24	Yes	Yes	Yes
3.3	+	DTPA	NA	24	Yes	Not significant	Yes
3.3	+	DTPA and pyrithione	NA	24	Yes	Not significant	Not significant
3.4	+	TPEN	YVAD	4	NA (note n=1)	Inhibited	No significant reduction
3.5	+	TPEN	CA074-Me	4	Inhibited	Inhibited	No significant reduction
3.6	+	TPEN	PP1/PP2A inhibitors calyculin A and okadaic acid.	4	Inhibited	Inhibited	No significant reduction
3.7, 3.8, 3.9	+	TPEN	ASC -/-	4	Inhibited	Inhibited	No significant reduction
3.7, 3.8, 3.9	+	DTPA	ASC -/-	4	Inhibited	Inhibited	No significant reduction
3.7, 3.8, 3.9	+	DTPA and pyrithione	ASC -/-	4	Inhibited	Inhibited	No significant reduction
3.10, 3.11, 3.12	+	TPEN	NLRP3 -/-	4	Partial inhibition	Not significant	No significant reduction
3.10, 3.11, 3.12	+	DTPA	NLRP3 -/-	4	Partial inhibition	Not significant	No significant reduction
3.10, 3.11, 3.12	+	DTPA and pyrithione	NLRP3 -/-	4	Yes	Not significant	No significant reduction

TPEN, DTPA and DTPA with pyrithione induce cell death, IL-1β processing and release. Inhibiting caspase-1 (YVAD), cathepsin B (CA074-Me) and PP1/2A (calyculin A and okadaic acid) inhibits IL-1β processing and release but does not reduce cell death. TPEN, DTPA and DTPA with pyrithione induced IL-1β processing and release is reduced in ASC -/- cells but cell death is not affected. Data for zinc depletion of NLRP3 -/- macrophages is variable and consequently no conclusions can be made from this data.

3.4 Discussion

In this study the role of zinc depletion as a novel activator of IL-1β processing and release has been confirmed. Furthermore the mechanism by which this happens has also been identified. Treatment of mouse peritoneal macrophages with TPEN (10 μM) in the absence of LPS induced low levels of IL-1β release (Fig 3.1). In the presence of LPS several methods of zinc depletion were shown to activate IL-1β processing and release at early (4h Fig 3.2) and late (24h Fig 3.3) time points. This observation is supported by existing research that suggests that inflammatory phenotypes are common in zinc deficient individuals [Zoli *et al.*, 1998b] [Wong & Ho, 2012] particularly in response to infection [Prasad *et al.*, 2007]. Using multiple forms of zinc depletion confirmed the role of zinc depletion in TPEN-induced zinc depletion. This data was further supported by an unpublished experiment carried out previously in this lab in which zinc chloride was added to the treatment media to counteract the zinc depletion properties of TPEN, and IL-1β release was reduced to vehicle levels [Brough, personal communication].

3.4.1 Zinc depletion, Inflammasomes and caspase-1-dependent processing

The canonical pathway for IL-1β processing is the inflammasome-dependent activation of IL-1β. Consequently it was not unexpected that zinc depletion induced IL-1β processing and release was, at least in part, dependent on caspase-1 as shown by inhibition of YVAD (Fig 3.4). To further confirm activation of caspase-1, an activity assay or caspase-1 western blot would be useful, although caspase-1 westerns are known to have variable success. Sterile insults have generally been associated with upregulation of IL-1β via the NLRP3 inflammasome [Leemans *et al.*, 2011]. The effects of zinc deficiency and consequent low intracellular zinc represent a sterile inflammatory insult and therefore activation of the NLRP3 inflammasome would not be unexpected.

3.4.1.1 Cathepsin B

Lysosomal destabilisation and the enzymes released as a result of this process have been recognised to be involved in the activation of the NLRP3 inflammasome [Hornung & Latz, 2010]. Cathepsin B is integral to this activation. Inhibition of cathepsin B with the inhibitor CA074-Me has been shown to reduce NLRP3 activation in response to multiple inflammatory insults including *Listeria monocytogenes* infection [Meixenberger *et al.*, 2010], serum amyloid A [Niemi *et al.*, 2011], silica particles [Morishige *et al.*, 2010], *Neisseria gonorrhoeae* infection [Duncan *et al.*, 2009], islet amyloid polypeptide [Masters *et al.*, 2010] and polyene macrolide antifungal drugs [Darisipudi *et al.*, 2011]. When cathepsin B was inhibited in zinc depleted cells, a reduction in IL-1β processing and release was observed (Fig 3.5) indicating that like many NLRP3 activating inflammatory insults, the effects of zinc depletion on IL-1β levels were cathepsin B dependent. It should be noted however that CA074-Me treatment also inhibits the activation of the NLRP1b inflammasome in response to anthrax lethal toxin [Newman *et al.*, 2009], suggesting that this could be a pathway involved in the activation of multiple inflammasomes.

3.4.1.2 PP2A

In comparison with cathepsin B inhibition, the role of PP2A inhibition in IL-1β processing and release is not well characterised. PP2A inhibition has been identified as a method of preventing the activation of multiple inflammasomes [Luheshi *et al.*, 2012], although the mechanism by which this occurs has not been defined. Inhibition of PP2A has previously been shown to increase JNK activity and consequent c-jun phosphorylation, AP-1 activity and IL-1β expression [Shanley *et al.*, 2001]. Therefore in this study, reduction of IL-1β levels in response to PP2A inhibition is likely to occur as a result of modulating pathways specific to IL-1β processing as opposed to IL-1β expression. In agreement with [Luheshi *et al.*,

2012], it was observed that inhibition of PP2A with calyculin A , reduced levels of IL-1β processing and release in zinc depleted macrophages (Fig 3.6).

3.4.1.3 Inflammasome components

The evidence discussed thus far supported the hypothesis that an inflammasome dependent pathway initiated the processing and release of IL-1β in response to zinc depletion. Using mice deficient in the integral inflammasome component ASC confirmed that the above hypothesis was at least partially true (Figs 3.7, 3.7). The evidence from macrophages lacking NLRP3 was less conclusive however. It appears that there is little reduction in IL-1β processing and release following zinc depletion in NLRP3 deficient macrophages (Figs 3.10, 3.11). However, the error bars in the IL-1β release experiment (Fig 3.10) are particularly large so it is not possible to conclude definitively that there is no reduction in IL-1β release. Although it is unlikely that NLRP3 is responsible for the majority of IL-1β processing and release, if at all. It should also be noted that the genetic backgrounds of the knockout mice and the WT mice, whilst both being C57BL/6 are N and J respectively. Consequently there may be intrinsic genetic differences that lead to a differential zinc depletion response; however there is no evidence to suggest this is the case (Fig 3.7, Fig 3.8, Fig 3.9, Fig 3.10, Fig 3.11, Fig 3.12).

3.4.2 Cell death, Caspase-8 and alternative processing of IL-1β

It is apparent that zinc depletion of mouse peritoneal macrophages induces cell death (Fig 3.1, Fig 3.2, Fig 3.3). This cell death is not dependent upon caspase-1, cathepsin B or the ASC component of the inflammasome (Fig 3.4, Fig 3.5, Fig 3.9), therefore cell death is occurring independently of inflammasome dependent IL-1β processing and release. As the

cell death was independent of caspase-1 it could not be classified as pyroptosis [Labbé & Saleh, 2008][Kepp *et al.*, 2010], however the concomitant release of IL-1β indicates that an inflammatory cell death is being initiated. Zinc depletion is known to induce apoptosis in cells [Kolenko *et al.*, 2001], however traditionally apoptosis has been considered to be non-inflammatory form of cell death [Franc *et al.*, 1999]. TPEN has been shown to reduce XIAP levels in prostate and breast cancer cells [Zuo *et al.*, 2012]. In this paper it is proposed that this occurs due to XIAP destabilisation due to lack of zinc binding at the BIR domains of the IAP proteins.

Whilst inflammasome components contribute to IL-1β processing and release in response to zinc depletion, it is clear that some IL-1β processing and release occurs in their absence. A mechanism for IL-1β processing with a partial NLRP3 dependence has been proposed recently [Vince *et al.*, 2012]. Depleting levels of IAPs may activate both NLRP3/caspase-1 and caspase-8, and these proteases together induce IL-1β processing and release. In light of these two publications levels of XIAP and caspase-8 were measured in the lysates of zinc depleted macrophages (Fig 3.13). As in breast and prostate cancer cells, XIAP was depleted in cells treated with the intracellular zinc chelators TPEN and DTPA in combination with pyrithione. There was also a concomitant increase in caspase-8 activation. Interestingly this was not observed in cells treated with DTPA alone, although this treatment had induced IL-1β processing (Fig3.9). This may suggest that the caspase-8 dependent pathway is not induced in response to this extracellular zinc depletion.

Whilst the cell death initiated by zinc depletion probably occurs in a XIAP dependent manner, the role of caspase-8 contribution will require more careful study. The relationship

of caspase-8 with the inflammasome is complicated. A recent publication has shown that caspase-8 deficiency activates the NLRP3 inflammasome [Kang *et al.*, 2013], and in contrast [Vince *et al.*, 2012] state that depletion of IAPs induce both caspase-8 and NLRP3 function. Caspase-8 is known to be activated by a complex known as the ripoptosome [Oberst & Green, 2011], IAPs have also been reported to modulate the ripoptosome and the integral ripoptosome component RIPk1 [Darding & Meier, 2012][Tenev *et al.*, 2011]. To investigate the contribution of this pathway to zinc-depletion-induced IL-1β processing and release, RIPK1 could be inhibited. Necrostatin is known to be an inhibitor of RIP1 kinase (RIP1K) and the consequent necroptotic cell death induced by this kinase [Degterev *et al.*, 2008].

Cell death data presented alongside IL-1β processing and release data, suggest that cell death processes are closely linked to mechanisms of IL-1β release. In all of the experiments presented above cell death in zinc depleted cells is generally constant regardless of pathway manipulation by inhibitors. This would indicate that the cell death processes are separate to the pathways of previously identified modulators of IL-1β processing (PP2A, cathepsin B, inflammasomes and caspases). This is in contrast to the caspase-1 induced pyroptotic cell death [Miao *et al.*, 2011].

3.4.3 Conclusions

Zinc depletion has been identified as a novel activator of IL-1β processing and release. This process occurs via pathways that involve PP1/2A signals, cathepsin B, caspase-1 and ASC. It is not known if NLRP3 contributes to this IL-1β processing and release as the data was inconclusive. Two caspases that cleave IL-1β, caspase-1 and caspase-8, are activated by zinc depletion. XIAP an inhibitor of apoptosis is depleted in response to zinc depletion in

macrophages and is probably responsible for the initiation of cell death observed throughout this investigation. The depletion of XIAP and activation of caspase-8 suggest that necroptotic processes may be important in zinc deficiency associated inflammation.

Chapter 4: Zinc depletion and IL-1β in human and mouse cells.

4.1 Introduction

In humans zinc status is closely interlinked with the inflammatory state [Bleackley & Macgillivray, 2011]. Consequently it is important to identify the role zinc depletion plays in the activation and release of the inflammatory mediator IL-1β. In the previous chapter the role of zinc depletion in IL-1β processing and release was investigated in mouse peritoneal macrophages. In this chapter the role of zinc depletion in human macrophage-like cells was investigated. Establishing the mechanism of zinc-depletion-induced IL-1β processing and release in human cells is an important step towards producing data with relevance to human health and disease.

In this investigation the human monocytic cell line THP-1 was used. THP-1 cells were originally derived from an acute monocytic leukaemia [Tsuchiya *et al.*, 1980]. These cells can be differentiated to macrophage-like cells with phorbol 12-myristate 13-acetate (PMA) [Daigneault *et al.*, 2010]. This induces the cells to become adherent like macrophages. The level of IL-1β release in response to zinc depletion at 4 hours was low in these cells, although not below detection limits, (Summersgill, unpublished); therefore in this investigation the cells will be depleted of zinc for 24h. To compare the zinc-depletion-induced effects on IL-1β release between species at this time point mouse bone marrow derived macrophages (BMDMs) were also used. These mouse cells are more similar to PMA differentiated THP-1s than mouse peritoneal macrophages are as both BMDMs and THP-1s are differentiated to macrophage like cells in *vitro.* BMDMs also do not release IL-1β in response to zinc depletion as rapidly as peritoneal macrophages. Comparing the two cell types over the 24h period will allow comparisons of zinc depletion induction of IL-1β release between mouse and human cells. In addition, as these cells are less responsive than peritoneal macrophages, a longer LPS treatment of 4h was used to induce IL-1β expression.

4.2 Methods

The methods used in this study are summarised below. For more detail please refer to Chapter 2. The cells used in these experiments were maintained in a humidified incubator at 37°C, 5%CO_2. THP-1 cells were maintained and passaged in DMEM 10%FCS 1%Penicillin/streptomycin (V/V). The THP-1 cells were seeded in cell culture plates and differentiated with PMA for 3h the day before they were used. Bone marrow for BMDM culture was obtained from C57BL/6 (WT) adult males of an approximate body weight of 25-30g. BMDMs were generated by differentiating the bone marrow cells with M-CSF in DMEM 10%FCS 1%Penicillin/streptomycin media for approximately 1 week prior to use. Differentiated BMDMs were then removed by cell scraping and seeded into cell culture plates. Endogenous IL-1β was induced by 4h LPS treatment. Zinc-depletion-dependent IL-1β processing was induced by 24h treatment with TPEN, DTPA or a DTPA Pyrithione combination. Nigericin was used as a positive control for IL-1β processing and release (24h). Inhibitors of caspase-1, cathepsin B, and cathepsin G were used in conjunction with these zinc chelators. These inhibitors were added following LPS treatment and 15 min prior to zinc depletion or nigericin treatment. IL-1β release was measured in cell culture supernatants by ELISA. IL-1β processing was measured in cell culture supernatants by western blot.

4.3 Results

4.3.1 Zinc depletion and inhibition of nigericin induced IL-1β processing and release

A previous Brough lab publication [Brough *et al.*, 2009] had identified a role for zinc in the regulation of the pannexin-1 hemichannel and its subsequent induction of caspase-1 activation. In this study nigericin induced pannexin-1 activity was abolished by pre-treatment with the zinc chelator TPEN. To investigate whether human cells responded to zinc depletion in the same way PMA differentiated THP-1 cells were zinc depleted for 15 min following LPS induction of IL-1β expression (4h) and prior to nigericin treatment (1h). Whilst not significant, a trend of lowered levels of IL-1β following treatment with the intracellular zinc chelator TPEN was observed in comparison to those pre-treated with 0.5 % DMSO vehicle only (Fig 4.1A). In contrast cells zinc depleted with a combination of the extracellular zinc chelator DTPA and the zinc ionophore pyrithione did not show reduced levels of IL-1β release in comparison to those pre-treated with vehicle (HEPES buffered media) (Fig 4.1B).

Similar effects were also observed in relation to IL-1β processing. Western blot data showed that cells pre-treated with TPEN prior to nigericin treatment, released less of the mature 17 kD IL-1β than cells treated with DMSO vehicle (Fig 4.1C). Cells pre-treated with DTPA and pyrithione in combination, showed similar levels of nigericin-induced IL-1β processing to the non-zinc depleted cells (Fig4.1D). The inability of the extracellular zinc chelator DTPA to inhibit nigericin induced IL-1β processing and release is interesting. The data implies that intracellular zinc depletion is necessary for inhibition of pannexin-1 activity. The reduction of nigericin induced IL-1β processing and release in TPEN pre-treated THP-1 cells, suggests that the mechanisms observed in peritoneal macrophages in [Brough *et al.*, 2009] also occur in THP-1 cells. Therefore this example of zinc regulation of IL-1β processing and release is consistent in both human and mouse.

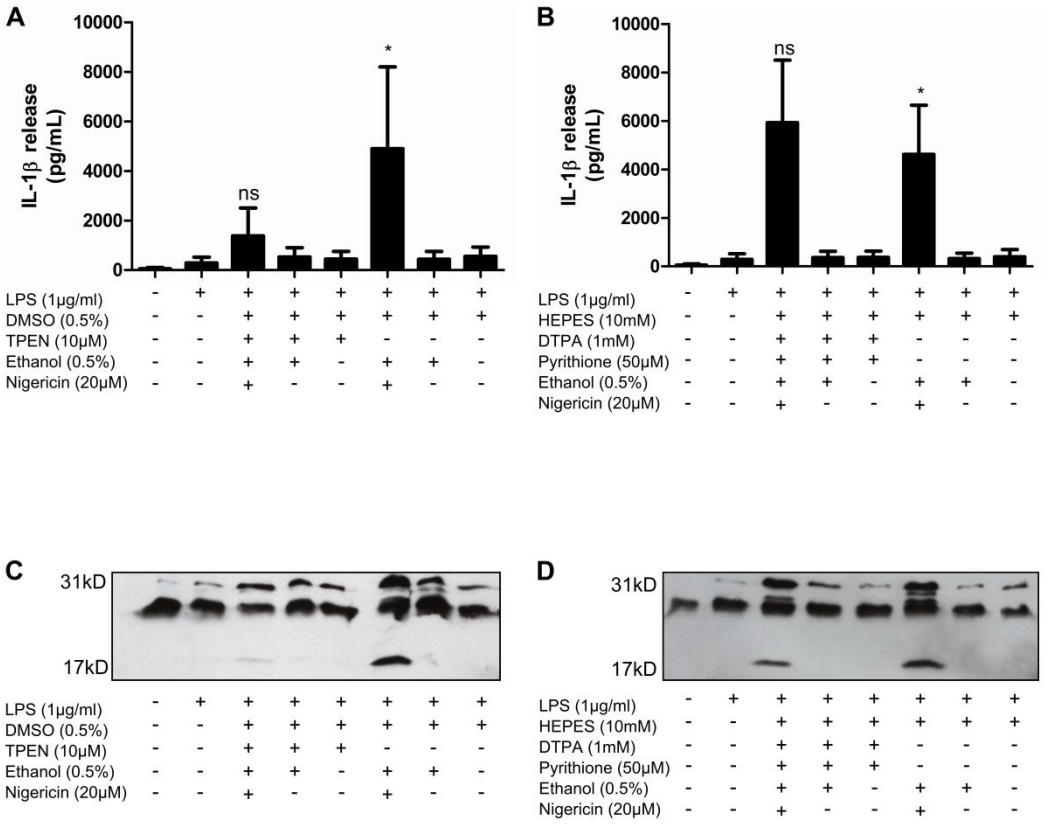

Figure 4.1: IL-1β processing and release induced by the potassium ionophore Nigericin is inhibited by 15min pre-treatment with TPEN but not with DTPA and Pyrithione.

LPS-primed (1 μg/ml, 4h) PMA differentiated THP-1 cells were pre-treated (15 min) with TPEN (10 μM), 0.5 % DMSO vehicle **(A, C)**; or DTPA (1 mM), and Pyrithione (50 μM), HEPES media vehicle (10 mM, pH 7-7.5) **(B, D)**; prior to treatment (1h) with Nigericin (20 μM), 0.5 % Ethanol vehicle. Supernatant samples were assayed for IL-1β release by ELISA **(A, B)** and IL-1β processing from pro to mature (31 to 17 kD) by western blot **(C, D)**. Error bars ±SEM of 3 **(A),** and 3 **(B)** independent experiments. Western blots **(C)** and **(D)** are representative of 3 independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; **(A)** DMSO and ethanol vehicle controls with nigericin and DMSO vehicle control (*), nigericin and DMSO vehicle control with TPEN and nigericin (#), and **(B)** HEPES and ethanol vehicle controls with nigericin and HEPES vehicle control (*), nigericin and HEPES vehicle control with DTPA, pyrithione and nigericin (#).*$p<0.05$.

4.3.2 Zinc depletion induces IL-1β processing and release in both human macrophage-like cells and mouse macrophages

As macrophage-like, PMA differentiated THP-1 cells behaved in the same way as peritoneal macrophages in response to a combination of TPEN and nigericin, it was necessary to investigate whether zinc depletion induced IL-1β processing and release in these cells. LPS primed (4h) PMA differentiated THP-1 cells were zinc depleted for 24h. After 24h THP-1 cells that were zinc depleted with 100 μM TPEN showed an increase of 25.33 fold in IL-1β release and cells treated with 10 μM TPEN showed a 31.59 fold increase when compared with DMSO vehicle controls (Fig 4.2A). Zinc depletion of THP-1 cells with a combination of DTPA and pyrithione also induced 41.28 fold increased IL-1β release when compared to HEPES vehicle treated cells (Fig 4.2B). Nigericin served as a positive control for IL-1β processing and release in this experiment (Fig 4.2.C, Fig 4.2D, Fig 4.2E). IL-1β release from the nigericin positive control was 22.17 fold higher than release from the THP-1 cells treated with ethanol vehicle control.

IL-1β processing was induced in both TPEN treated cells and those treated with a combination of DTPA and pyrithione (Fig 4.2D, Fig 4.2E). This processing however was unlike the processing induced by nigericin which produced a single band 17 kD. The zinc depleted cells produced three forms of processed IL-1β at approximately 17 kD. This could be an indication that zinc depletion may activate different pathways to those induced by nigericin in the processing and release of IL-1β.

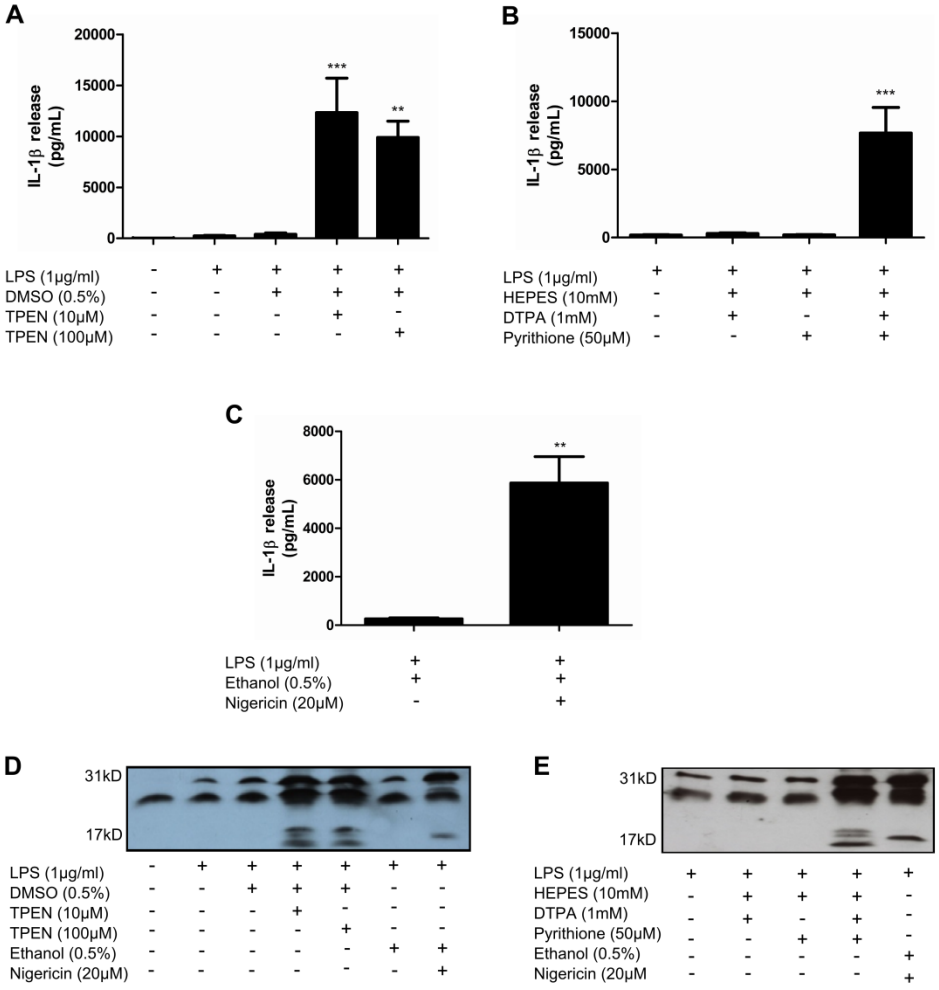

Figure 4.2: IL-1β processing and release following 24h zinc depletion of LPS primed PMA differentiated THP-1 cells

LPS-primed (1 µg/ml, 4h) PMA differentiated THP-1 cells were treated (24h) with 0.5 % DMSO vehicle ,TPEN (10 µM, 0.5 % DMSO)**(A,D)**; HEPES (10 mM, pH7-7.5), DTPA (1 mM, 10 mM HEPES media) or Pyrithione (50 µM, 10 mM HEPES media) **(B,E)**; 0.5 % Ethanol vehicle or Nigericin (20 µM, 0.5 % Ethanol) **(C,D,E)**. Supernatant samples were assayed for IL-1β release by ELISA **(A, B, C)** and IL-1β processing from pro to mature (31 to 17 kD) by western blot **(D, E)**. Error bars ±SEM of 4 **(A),** 5 **(B)** and 4 **(C)** independent experiments. Western blots **(D)** and **(E)** are representative of 3 independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; **(A)** DMSO with TPEN (10 µM) (*), DMSO with TPEN (100 µM) (*), **(B)** HEPES with DTPA and pyrithione (*), **(C)** nigericin with ethanol (*). *** p<0.001, **p<0.01.

This induction of IL-1β processing and release in zinc depleted macrophage-like THP-1 cells is consistent with the findings presented in chapter 3 on zinc depletion in mouse peritoneal macrophages. To ensure that any differences highlighted between species in this chapter were not artefacts of the longer incubation time of the cells with the zinc chelators, experiments were also completed in mouse bone marrow derived macrophages (BMDMs). Zinc depletion with both TPEN (10 μM) and DTPA (1 mM) in combination with pyrithione (50 μM) induced elevated levels of IL-1β release in BMDMs (Fig 4.3A, Fig 4.3B). IL-1β release in TPEN treated macrophages was 8.17 fold higher than in macrophages treated with DMSO vehicle control. Following treatment with DTPA in combination with pyrithione BMDMs released 24.66 times more IL-1β than cells treated with HEPES vehicle control alone. IL-1β release was also induced following treatment with the nigericin positive control (Fig 4.3C). Nigericin treated macrophages released 112.5 times more IL-1β than the ethanol vehicle control, which in turn is five times higher than the increase following this treatment in THP-1 cells (Fig 4.2C). This would indicate that whilst nigericin is a good positive control for both cell types, it is most effective in mouse cells.

Following 24h zinc depletion of BMDMs, with TPEN and DTPA in combination with pyrithione, IL-1β was processed from the pro-form to the mature single 17 kD form only (Fig 4.3D, Fig 4.3E). In this way the BMDM cells behave more similarly to peritoneal macrophages than THP-1 cells, and the production of the multiple mature bands following zinc depletion in THP-1 cells cannot be attributed to the long incubation time.

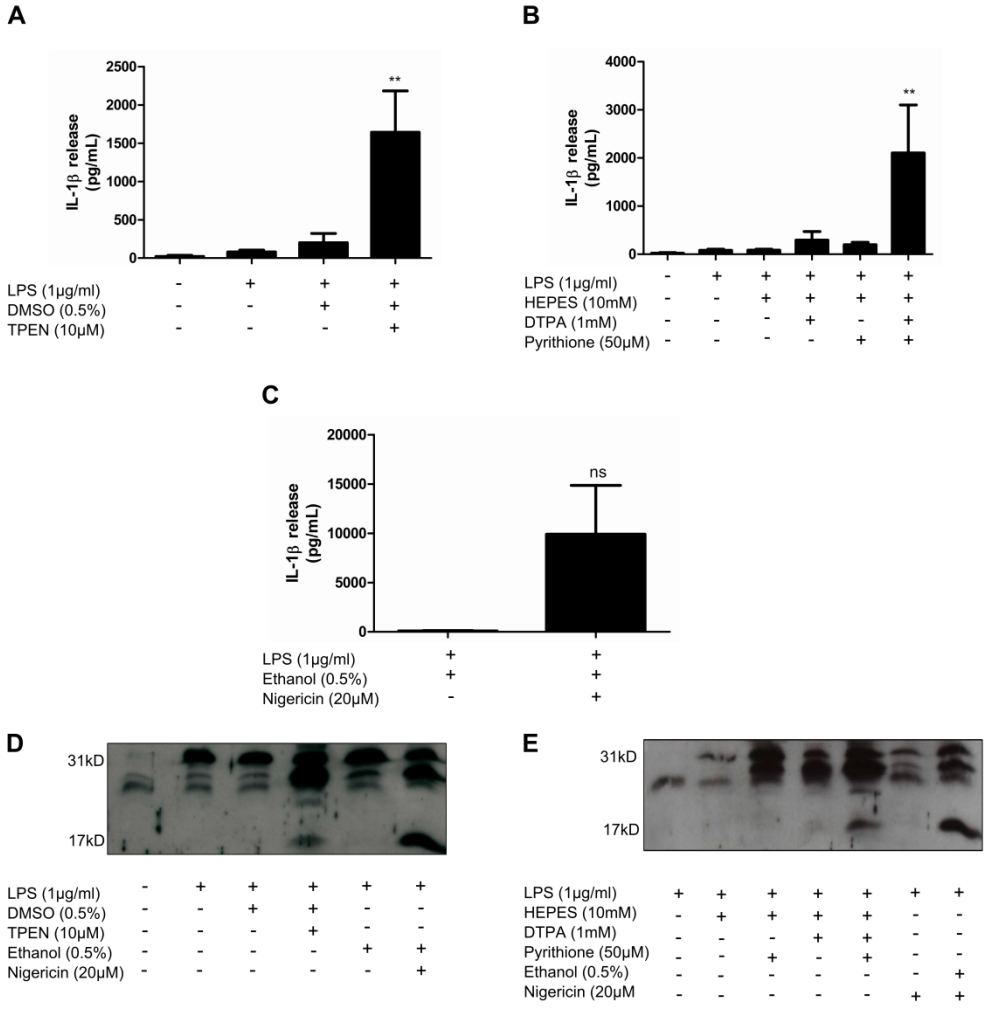

Figure 4.3: IL-1β processing and release following 24h zinc depletion of LPS primed BMDMs

LPS-primed (1 μg/ml, 4h) BMDMs were treated (24h) with 0.5 % DMSO vehicle ,TPEN (10 μM, 0.5 % DMSO)**(A,D)**; HEPES (10 mM, pH7-7.5), DTPA (1 mM, 10 mM HEPES media) or Pyrithione (50 μM, 10 mM HEPES media) **(B,E)**; 0.5 % Ethanol vehicle or Nigericin (20 μM, 0.5 % Ethanol) **(C,D,E)**. Supernatant samples were assayed for IL-1β release by ELISA **(A,B,C)** and IL-1β processing from pro to mature (31 to 17kD) by western blot **(D,E).** Error bars ±SEM of 4 **(A),** 4 **(B)** and 4 **(C)** independent experiments. Western blots **(D)** and **(E)** are representative of 3 independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; **(A)** DMSO with TPEN (*), **(B)** HEPES with DTPA and pyrithione (*), **(C)** nigericin with ethanol (*). **p<0.01.

4.3.3 Addition of zinc sulphate inhibits IL-1β release from zinc depleted THP-1 cells and BMDMs

To confirm that the IL-1β release induced in both macrophage-like THP-1 cells and BMDMs occurred as a consequence of the zinc chelation, the cells were treated with a combination of zinc sulphate and zinc chelator. Cells treated with 10 µM TPEN were supplemented with 50 µM $ZnSO_4$ and cells treated with 1mM DTPA with 50 µM pyrithione were supplemented with 1mM $ZnSO_4$.

Supplementation of zinc sulphate to the media of the zinc depleted cells reduced the levels of IL-1β release to vehicle levels. The reduction in IL-1β release in TPEN treated THP-1 cells was 97% (Fig 4.4A). IL-1β release in DTPA and pyrithione treated THP-1 cells was reduced by 86% (Fig 4.4B). In zinc supplemented BMDMs IL-1β release was reduced 91% in TPEN treated cells and 68% in DTPA and pyrithione treated cells. This confirmed that the increased IL-1β release is occurring as a direct result of zinc depletion.

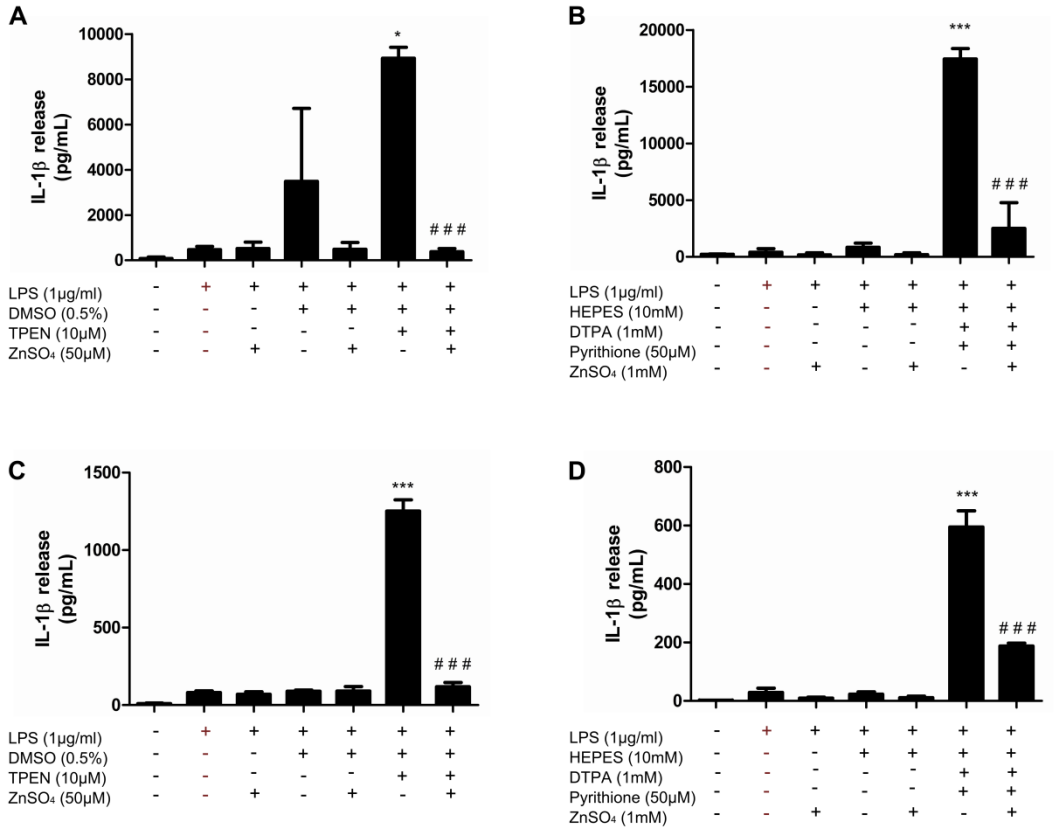

Figure 4.4: IL-1β release following 24h zinc depletion and zinc treatment of LPS primed BMDMs and PMA differentiated THP-1s

LPS-primed (1 µg/ml, 4h) PMA differentiated THP-1 cells were treated (24h) with 0.5 % DMSO vehicle, TPEN (10 µM, 0.5 % DMSO)in the presence or absence of $ZnSO_4$ (50 µM) **(A)**, or with HEPES (10 mM, pH 7-7.5), DTPA (1 mM, 10 mM HEPES media) or Pyrithione (50 µM, 10 mM HEPES media) in the presence or absence of $ZnSO_4$ (1 mM) **(B)**. LPS-primed (1 µg/ml, 4h) BMDMs were treated (24h) with 0.5 % DMSO vehicle, TPEN (10 µM, 0.5 % DMSO) in the presence or absence of $ZnSO_4$ (50 µM) **(C)** or with HEPES (10 mM, pH 7-7.5), DTPA (1 mM, 10 mM HEPES media) or Pyrithione (50 µM, 10 mM HEPES media) in the presence or absence of $ZnSO_4$ (1 mM) **(D)**. Supernatant samples were assayed for IL-1β release by ELISA. Error bars ±SEM of 3 **(A),** 3 **(B)**, 3 **(C)** and 3 **(D)** independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; **(A)** DMSO with TPEN (*), TPEN with TPEN and $ZnSO_4$ (#), **(B)** HEPES with DTPA and pyrithione (*), DTPA and pyrithione with DTPA, pyrithione and $ZnSO_4$ (#), **(C)** DMSO with TPEN (*), TPEN with TPEN and $ZnSO_4$ (#), **(D)** HEPES with DTPA and pyrithione (*), DTPA and pyrithione with DTPA, pyrithione and $ZnSO_4$ (#). *** $p<0.001$, *$p<0.05$.

4.3.4 Zinc depletion induced IL-1β release is partially inflammasome dependent in mouse macrophages and inflammasome independent in human macrophage-like cells

In mouse peritoneal macrophages zinc depletion induced IL-1β release that was inhibited by YVAD, an inhibitor of caspase-1. Caspase-1 is the best characterised protease known to activate IL-1β by proteolytic cleavage. THP-1 cells were pre-treated with YVAD (15 min) prior to zinc depletion (24h). YVAD did not reduce IL-1β release in response to TPEN (Fig 4.5A) or DTPA and pyrithione treatment (Fig 4.5B). Although significantly reduced IL-1β release was not observed following treatment with the nigericin positive control and YVAD (Fig 4.5C). However YVAD inhibition of nigericin treated cells did reduce IL-1β processing (Fig 4.5 D, Fig 4.5E). Zinc depletion induced IL-1β processing in THP-1 cells was not inhibited by YVAD (Fig 4.5 D, Fig 4.5E).

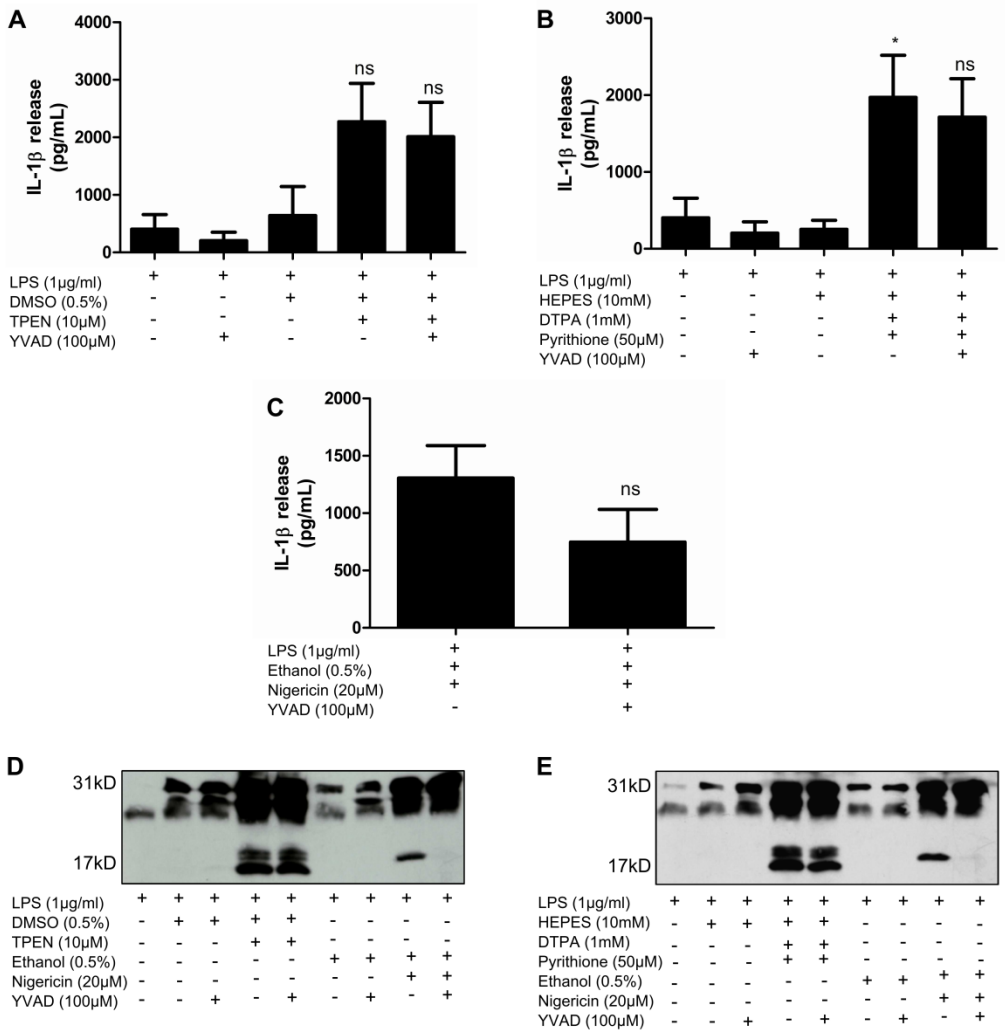

Figure 4.5: IL-1β processing and release partially inhibited by YVAD in zinc depleted PMA differentiated THP-1s.

LPS-primed (1 μg/ml, 4h) PMA differentiated THP-1s were pre-treated (15 min) with YVAD (100 μM, 0.5 % DMSO), 0.5 % DMSO vehicle prior to treatment (24h) with TPEN (10 μM, 0.5 % DMSO), 0.5 % DMSO vehicle **(A,D)**; HEPES (10 mM, pH 7-7.5), DTPA (1 mM, 10 mM HEPES media), Pyrithione (50 μM, 10 mM HEPES media) **(B,E)**; or 0.5 % Ethanol vehicle or Nigericin (20 μM, 0.5 % Ethanol) **(C,D,E)**. Supernatant samples were assayed for IL-1β release by ELISA. Error bars ±SEM of 3 **(A),** 3 **(B)** and 3 **(C)** independent experiments. Western blots **(D)** and **(E)** are representative of 3 independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; **(A)** DMSO with TPEN (*), and TPEN with TPEN and YVAD (#), **(B)** HEPES with DTPA and pyrithione (*), and DTPA and pyrithione with DTPA, pyrithione and YVAD (#). An unpaired t test was undertaken to identify significant differences between nigericin with nigericin and YVAD (#) **(C)**. * $p<0.05$.

IL-1β release in zinc-depleted THP-1s is not reduced by YVAD; however IL-1β release following zinc depletion is reduced by YVAD in peritoneal macrophages. To assess whether this apparent difference in caspase-1 dependence occurs due to a species difference, BMDMs were pre-treated with YVAD prior to zinc depletion.

YVAD treatment did not reduce IL-1β processing and release in TPEN treated BMDMs (Fig 4.6 A, Fig 4.6D) or DTPA and pyrithione treated BMDMs (Fig 4.6B, Fig 4.6E). Although IL-1β processing and release induced by the positive control nigericin, was not significantly reduced by YVAD either (Fig 4.6C, Fig 4.6D, Fig 4.6E). This would suggest that these treatments induce caspase-1 independent processing and release of IL-1β in this cell type.

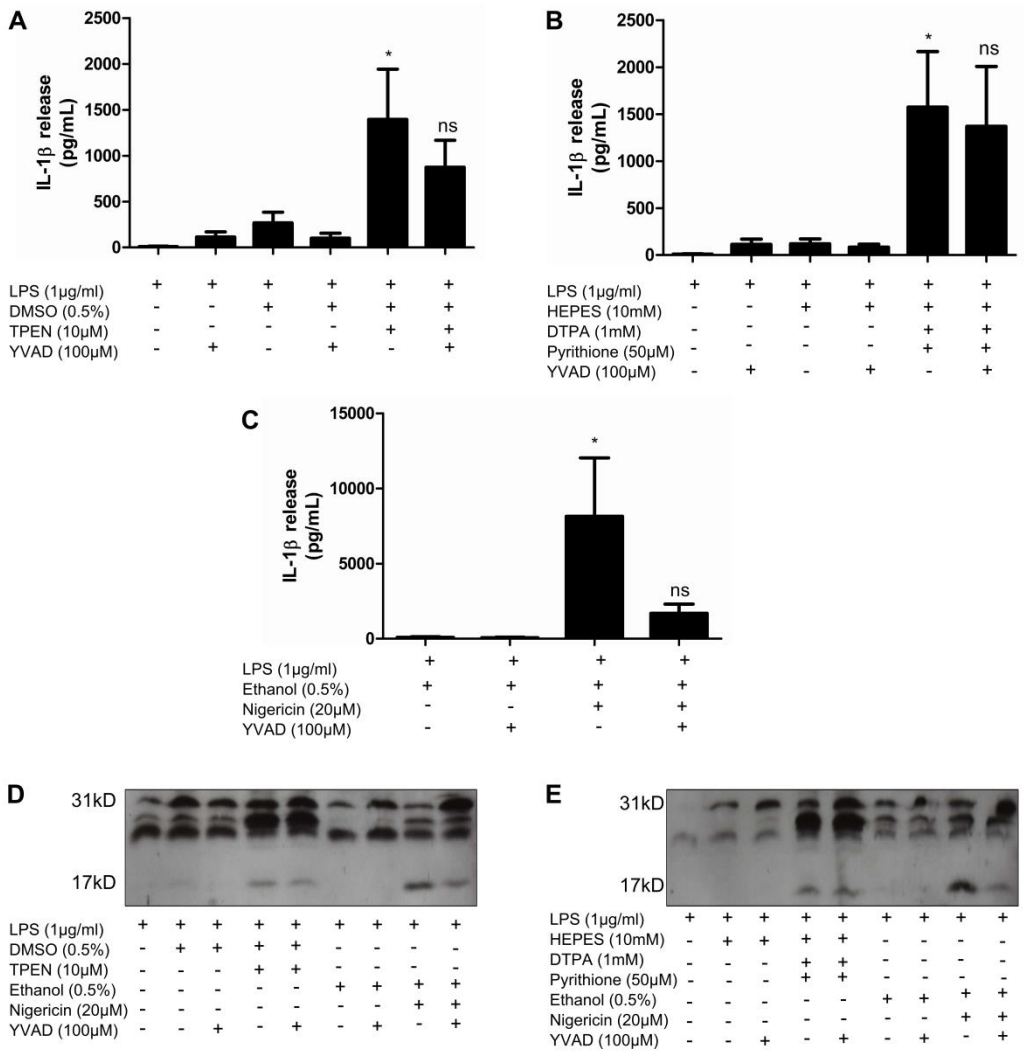

Figure 4.6: IL-1β processing and release partially inhibited by YVAD in zinc depleted BMDMs.

LPS-primed (1 μg/ml, 4h) BMDMs were pre-treated (15 min) with YVAD (100 μM, 0.5 % DMSO), 0.5 % DMSO vehicle prior to treatment (24h) with TPEN (10 μM, 0.5 % DMSO), 0.5 % DMSO vehicle **(A,D)**; HEPES (10 mM, pH 7-7.5), DTPA (1 mM, 10 mM HEPES media), Pyrithione (50 μM, 10 mM HEPES media) **(B,E)**; or 0.5 % Ethanol vehicle or Nigericin (20 μM, 0.5 % Ethanol) **(C,D,E)**. Supernatant samples were assayed for IL-1β release by ELISA. Error bars ±SEM of 3 **(A),** 3 **(B)** and 3 **(C)** independent experiments. Western blots **(D, E)** are representative of 3 independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; **(A)** DMSO with TPEN (*), and TPEN with TPEN and YVAD (#), **(B)** HEPES with DTPA and pyrithione (*), and DTPA and pyrithione with DTPA, pyrithione and YVAD (#), **(C)** ethanol with nigericin (*), and nigericin with nigericin and YVAD (#) * $p<0.05$.

Glyburide is known to inhibit the NLRP3 inflammasome [Lamkanfi *et al.*, 2009]. In PMA differentiated THP-1 cells glyburide pre-treatment did not reduce IL-1β release in cells treated with TPEN and DTPA in combination with pyrithione (Fig 4.7A, Fig 4.7B). It should be noted however that glyburide did not completely inhibit nigericin induced IL-1β release either (Fig 4.7C) and nigericin induced IL-1β release is known to be dependent upon NLRP3. In BMDMs TPEN induced IL-1β release was reduced by 27% after pre-treatment with glyburide (Fig 4.8A). Glyburide also reduced IL-1β release in cells treated with DTPA and pyrithione by 46% (Fig 4.8B) and in nigericin treated cells by 67% (Fig 4.8C). This suggests a potential difference in NLRP3 activation in these two cell types, although this cannot be stated outright as the positive control did not show reduced IL-1β release. It should be noted that glyburide treatment did not reduce IL-1β release to the levels induced by vehicle treatments alone in any of the experiments, indicating that a proportion of IL-1β release in BMDMs is independent of NLRP3.

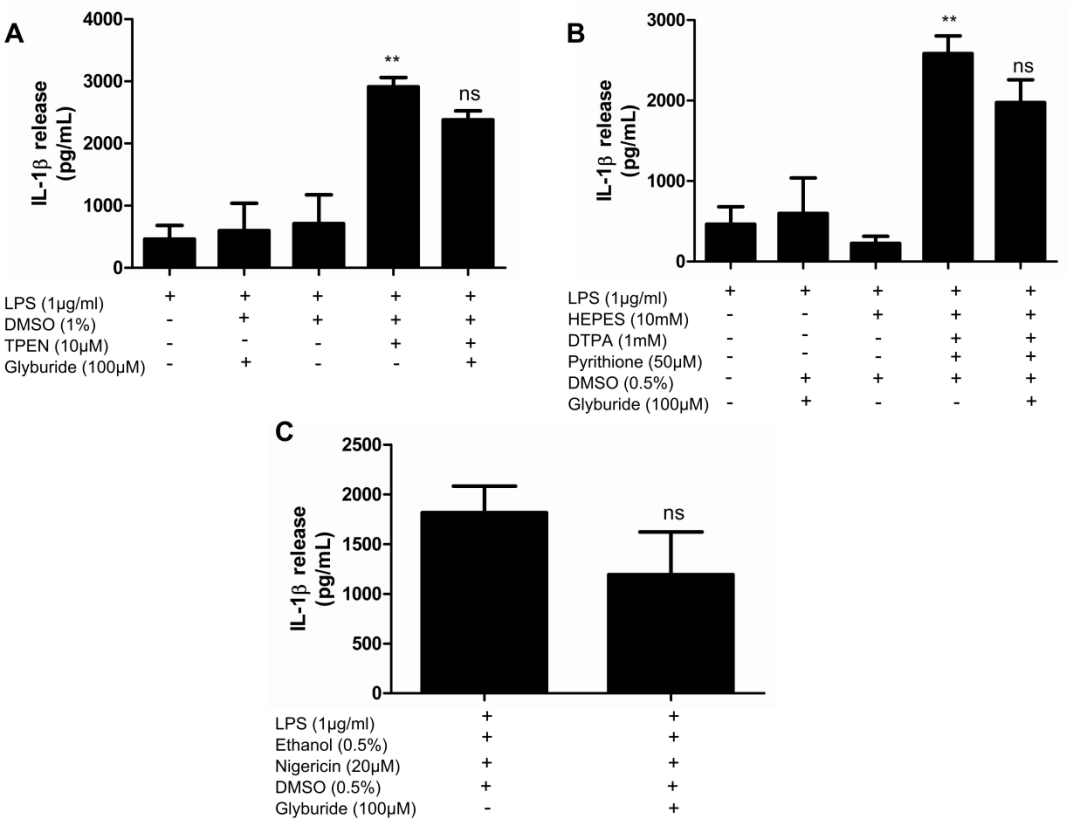

Figure 4.7: IL-1β release partially inhibited by glyburide in zinc depleted PMA differentiated THP-1s.

LPS-primed (1 µg/ml, 4h) PMA differentiated THP-1s were pre-treated (15 min) with Glyburide (100 µM, 0.5 % DMSO), 0.5 % DMSO vehicle prior to treatment (24h) with TPEN (10 µM, 0.5 % DMSO), 0.5 % DMSO vehicle **(A)**; HEPES (10 mM, pH 7-7.5), DTPA (1 mM, 10 mM HEPES media), Pyrithione (50 µM, 10 mM HEPES media) **(B)**; or 0.5 % Ethanol vehicle or Nigericin (20 µM, 0.5 % Ethanol) **(C)**. Supernatant samples were assayed for IL-1β release by ELISA. Error bars ±SEM of 3 **(A),** 3 **(B)** and 3 **(C)** independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; (A) DMSO with TPEN (*), and TPEN with TPEN and glyburide (#), (B) HEPES with DTPA and pyrithione (*), and DTPA and pyrithione with DTPA, pyrithione and glyburide (#), (C) ethanol with nigericin (*), and nigericin with nigericin and glyburide (#). **p<0.01.

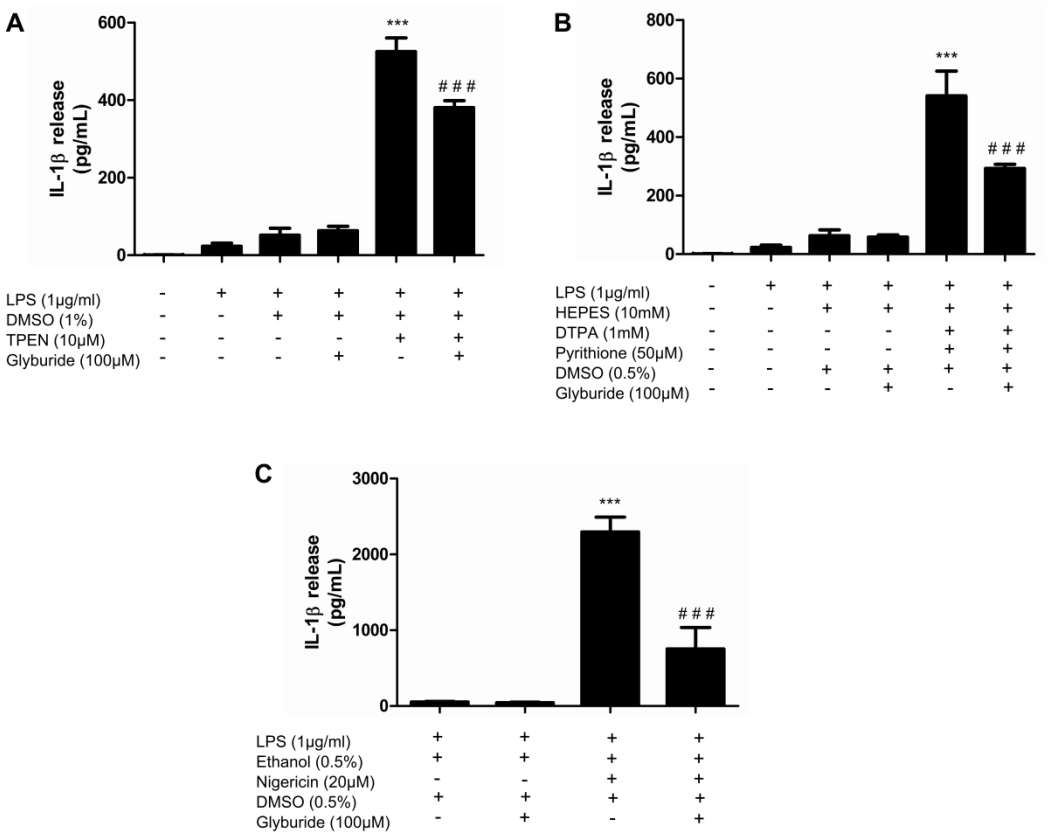

Figure 4.8: IL-1β release inhibited by glyburide in zinc depleted BMDMs.

LPS-primed (1 μg/ml, 4h) BMDMs were pre-treated (15 min) with glyburide (100 μM, 0.5 % DMSO), 0.5 % DMSO vehicle prior to treatment (24h) with TPEN (10 μM, 0.5 % DMSO), 0.5 % DMSO vehicle **(A)**; HEPES (10 mM, pH 7-7.5), DTPA (1 mM, 10 mM HEPES media), Pyrithione (50 μM, 10 mM HEPES media) **(B)**; or 0.5 % Ethanol vehicle or Nigericin (20 μM, 0.5 % Ethanol) **(C)**. Supernatant samples were assayed for IL-1β release by ELISA. Error bars ±SEM of 3 (A), 3 (B) and 3 (C) independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; (A) DMSO with TPEN (*), and TPEN with TPEN and glyburide (#), (B) HEPES with DTPA and pyrithione (*), and DTPA and pyrithione with DTPA, pyrithione and glyburide (#), (C) ethanol with nigericin (*), and nigericin with nigericin and glyburide (#). ***p<0.001.

4.3.5 Cathepsin B is not involved in zinc depletion induced IL-1β release in human macrophage-like cells

In peritoneal macrophages zinc depletion induced IL-1β release was significantly reduced after treatment with the cathepsin B inhibitor CA074-Me (Fig 3.5). THP-1 cells that were pre-treated with CA074-Me prior to zinc depletion, however did not show reduced levels of IL-1β release. This effect was observed in both TPEN treated THP-1 cells (Fig 4.9A) and DTPA and pyrithione treated THP-1 cells (Fig 4.9B). Nigericin induced IL-1β release was reduced 83% by CA074-Me treatment (Fig 4.9C). In contrast, zinc depletion induced release in BMDMs was reduced by inhibition of cathepsin B (Fig 4.10A). TPEN induced IL-1β release was reduced by 75% and IL-1β release induced by the nigericin positive control was reduced by 92% (Fig 4.10C). IL-1β release induced by DTPA in combination with pyrithione was not significantly reduced by cathepsin B inhibition (Fig 4.10B). The difference in the response to cathepsin B inhibition highlights a key difference in the mechanisms of zinc-depletion-induced IL-1β release in human and mouse cells. Differing involvement of cathepsin B may also explain the difference in response to glyburide as cathepsin B release is known to activate the NLRP3 inflammasome [Hentze *et al.*, 2003][Hornung *et al.*, 2008].

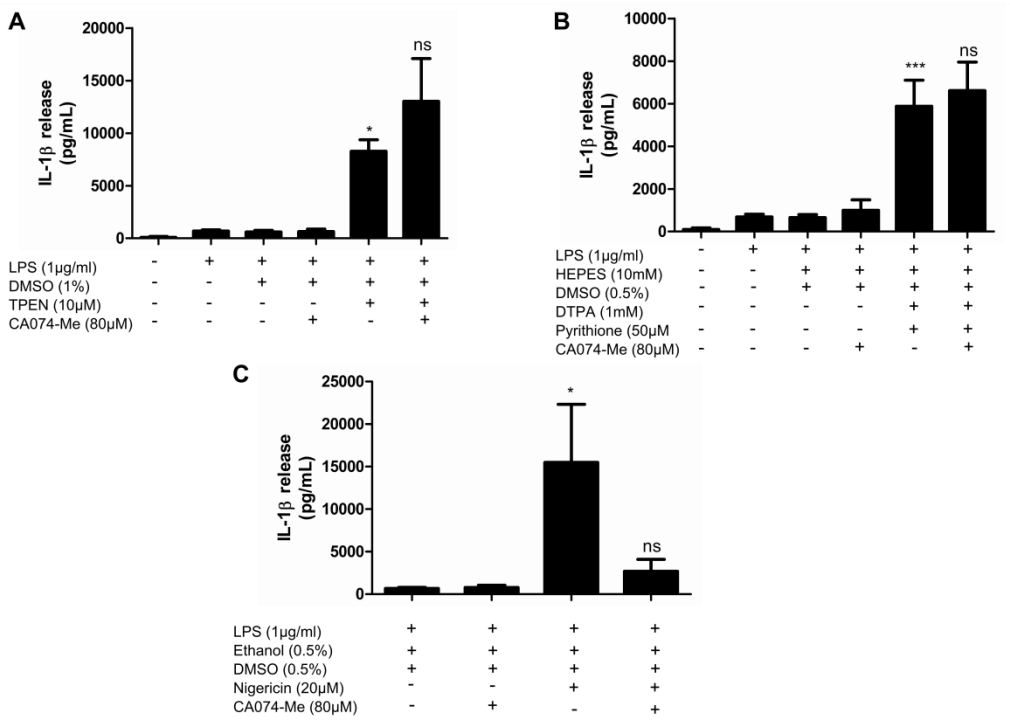

Figure 4.9: IL-1β release potentiated by Cathepsin B inhibition in zinc depleted PMA differentiated THP-1s.

LPS-primed (1 µg/ml, 4h) PMA differentiated THP-1s were pre-treated (15 min) with CA074-Me (80 µM, 0.5 % DMSO), 0.5 % DMSO vehicle prior to treatment (24h) with TPEN (10 µM, 0.5 % DMSO), 0.5 % DMSO vehicle **(A)**; HEPES (10 mM, pH 7-7.5), DTPA (1 mM, 10 mM HEPES media), Pyrithione (50 µM, 10 mM HEPES media) **(B)**; or 0.5 % Ethanol vehicle or Nigericin (20 µM, 0.5 % Ethanol) **(C)**. Supernatant samples were assayed for IL-1β release by ELISA. Error bars ±SEM of 3 (A), 3 (B) and 3 (C) independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; (A) DMSO with TPEN (*), and TPEN with TPEN and CA074-Me (#), (B) HEPES with DTPA and pyrithione (*), and DTPA and pyrithione with DTPA, pyrithione and CA074-Me (#), (C) ethanol with nigericin (*), and nigericin with nigericin and CA074-Me (#). *** $p<0.001$, * $p<0.05$**.**

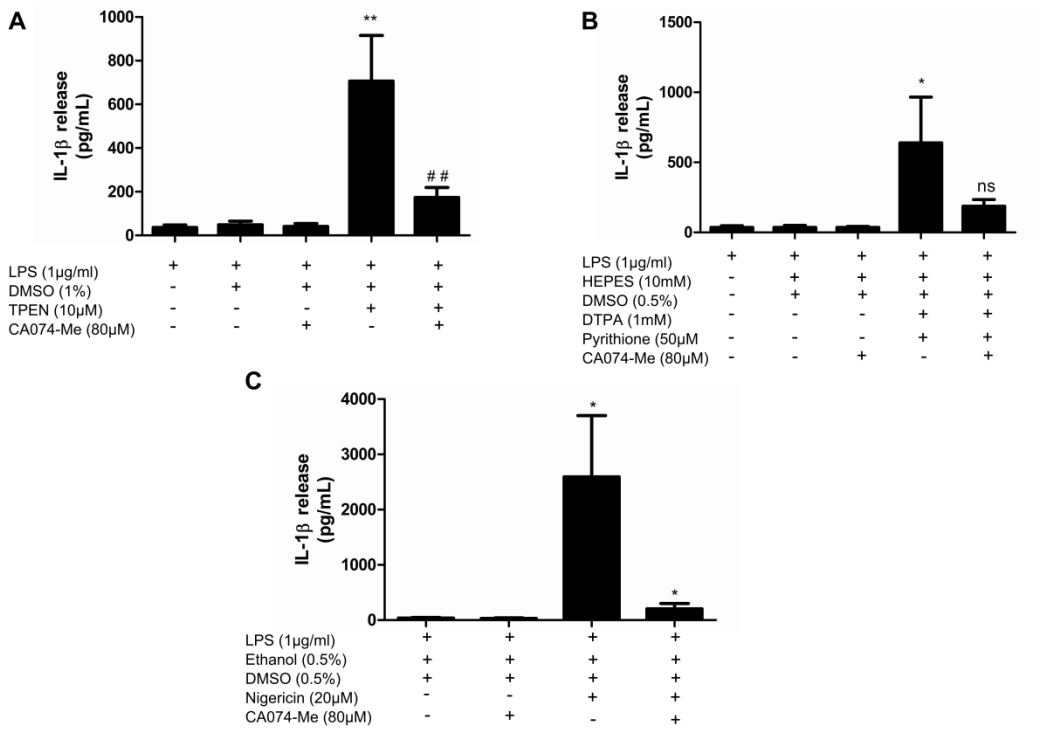

Figure 4.10: IL-1β release reduced by Cathepsin B inhibition in zinc depleted BMDMs.

LPS-primed (1 μg/ml, 4h) BMDMs were pre-treated (15 min) with CA074-Me (80 μM, 0.5 % DMSO), 0.5 % DMSO vehicle prior to treatment (24h) with TPEN (10 μM, 0.5 % DMSO), 0.5 % DMSO vehicle **(A)**; HEPES (10 mM, pH 7-7.5), DTPA (1 mM, 10 mM HEPES media), Pyrithione (50 μM, 10 mM HEPES media) **(B)**; or 0.5 % Ethanol vehicle or Nigericin (20 μM, 0.5 % Ethanol) **(C)**. Supernatant samples were assayed for IL-1β release by ELISA. Error bars ±SEM of 3 (A), 3 (B) and 3 (C) independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; (A) DMSO with TPEN (*), and TPEN with TPEN and CA074-Me (#), (B) HEPES with DTPA and pyrithione (*), and DTPA and pyrithione with DTPA, pyrithione and CA074-Me (#), (C) ethanol with nigericin (*), and nigericin with nigericin and CA074-Me (#). **p<0.01, *p<0.05**.**

4.3.6 Cathepsin G is involved in zinc-depletion-induced IL-1β release in both human macrophage-like cells and mouse macrophages

The data suggests that IL-1β release in THP-1 cells occurs independently of caspase-1. Other proteases that have been proposed to cleave IL-1β include the neutrophil granule serine proteases [Netea *et al.*, 2010]. One of those proteases is cathepsin G, which in monocytes is associated with the plasma membrane [Avril *et al.*, 1995] and is also expressed in monocytic azurophilic granules [Scott *et al.*, 1999]

Cathepsin G inhibition prior to zinc depletion reduced IL-1β release in TPEN treated THP-1 cells by 73% (Fig 4.11 A), and IL-1β release in DTPA and pyrithione treated THP-1 cells by 74% (Fig 4.11B). Inhibition of cathepsin G also reduced nigericin-induced IL-1β release by 85% (Fig 4.11C). In BMDMs the effects of cathepsin G inhibition were even more pronounced. Inhibition of cathepsin G prior to treatment reduced IL-1β release in TPEN by 80% (Fig 4.12A), in DTPA and pyrithione by 90% (Fig 4.12 B), and in nigericin treated cells by 99% (Fig 4.12C). In BMDMs cathepsin G inhibition was also observed to change the morphology of the cell. Following GLF treatment cells appeared more rounded and lost the characteristic elongated phenotype (Fig 4.13). In cells treated with TPEN, DTPA in combination with pyrithione and nigericin membrane disruption was observed. This membrane disruption appeared to be inhibited in cells treated with the cathepsin G inhibitor GLF. Taken together this would suggest that cathepsin G controls an integral IL-1β processing or release pathway, possibly via regulation of the plasma membrane.

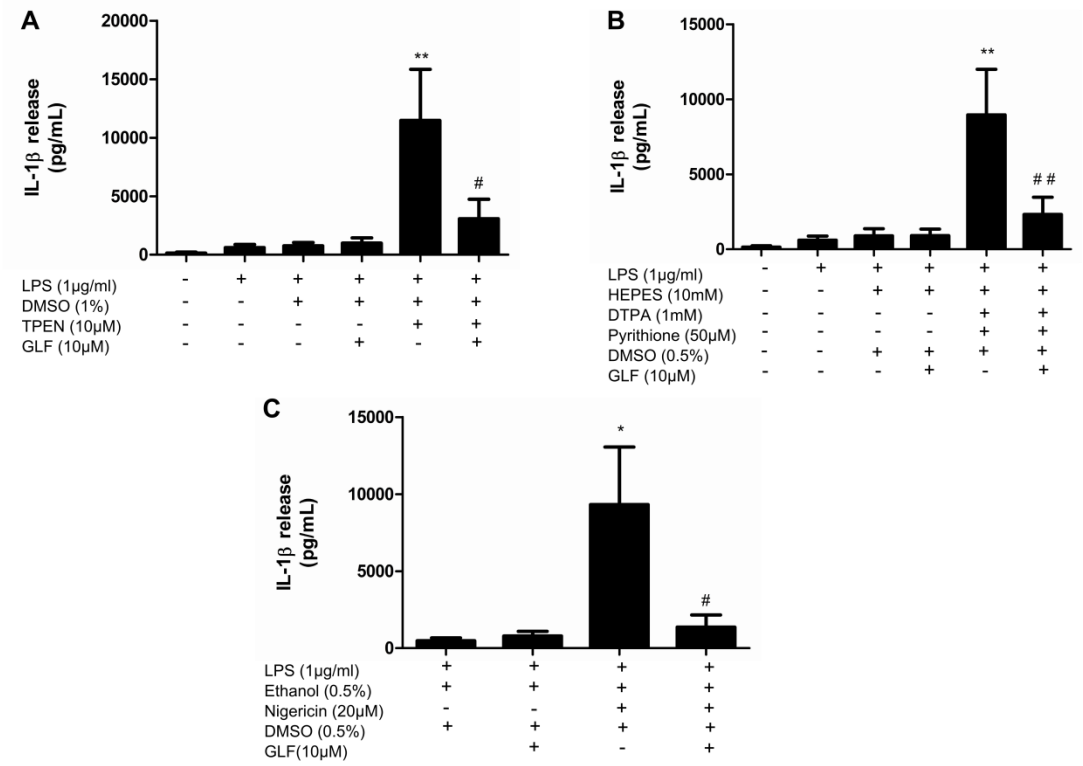

Figure 4.11: IL-1β release inhibited by GLF in zinc depleted PMA differentiated THP-1s.

LPS-primed (1 µg/ml, 4h) PMA differentiated THP-1s were pre-treated (15 min) with GLF (10 µM, 0.5 % DMSO), 0.5 % DMSO vehicle prior to treatment (24h) with TPEN (10 µM, 0.5 % DMSO), 0.5 % DMSO vehicle **(A)**; HEPES (10 mM, pH 7-7.5), DTPA (1 mM, 10 mM HEPES media), Pyrithione (50 µM, 10 mM HEPES media) **(B)**; or 0.5 % Ethanol vehicle or Nigericin (20 µM, 0.5 % Ethanol) **(C)**. Supernatant samples were assayed for IL-1β release by ELISA. Error bars ±SEM of 3 (A), 3 (B) and 3 (C) independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; (A) DMSO with TPEN (*), and TPEN with TPEN and GLF (#), (B) HEPES with DTPA and pyrithione (*), and DTPA and pyrithione with DTPA, pyrithione and GLF (#), (C) ethanol with nigericin (*), and nigericin with nigericin and GLF (#). **p<0.01, *p<0.05**.**

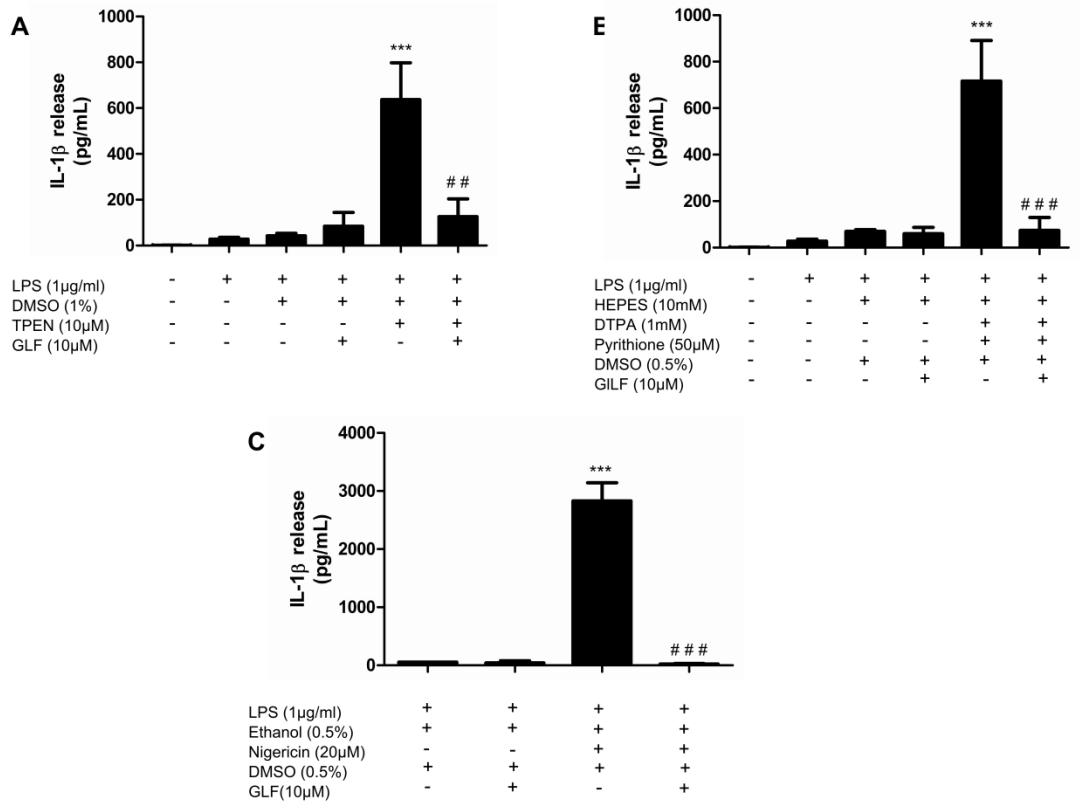

Figure 4.12: IL-1β release inhibited by GLF in zinc depleted BMDMs.

LPS-primed (1 µg/ml, 4h) BMDMs were pre-treated (15 min) with GLF (10 µM, 0.5 % DMSO), 0.5 % DMSO vehicle prior to treatment (24h) with TPEN (10 µM, 0.5 % DMSO), 0.5 % DMSO vehicle **(A)**; HEPES (10 mM, pH 7-7.5), DTPA (1 mM, 10 mM HEPES media), Pyrithione (50 µM, 10 mM HEPES media) **(B)**; or 0.5 % Ethanol vehicle or Nigericin (20 µM, 0.5 % Ethanol) **(C)**. Supernatant samples were assayed for IL-1β release by ELISA. Error bars ±SEM of 3 (A), 3 (B) and 3 (C) independent experiments. A one-way ANOVA with a post-hoc Bonferroni multiple comparison test was used to identify significant differences between groups. Comparison pairs were as follows; (A) DMSO with TPEN (*), and TPEN with TPEN and GLF (#), (B) HEPES with DTPA and pyrithione (*), and DTPA and pyrithione with DTPA, pyrithione and GLF (#), (C) ethanol with nigericin (*), and nigericin with nigericin and GLF (#). ***p<0.001, **p<0.01.

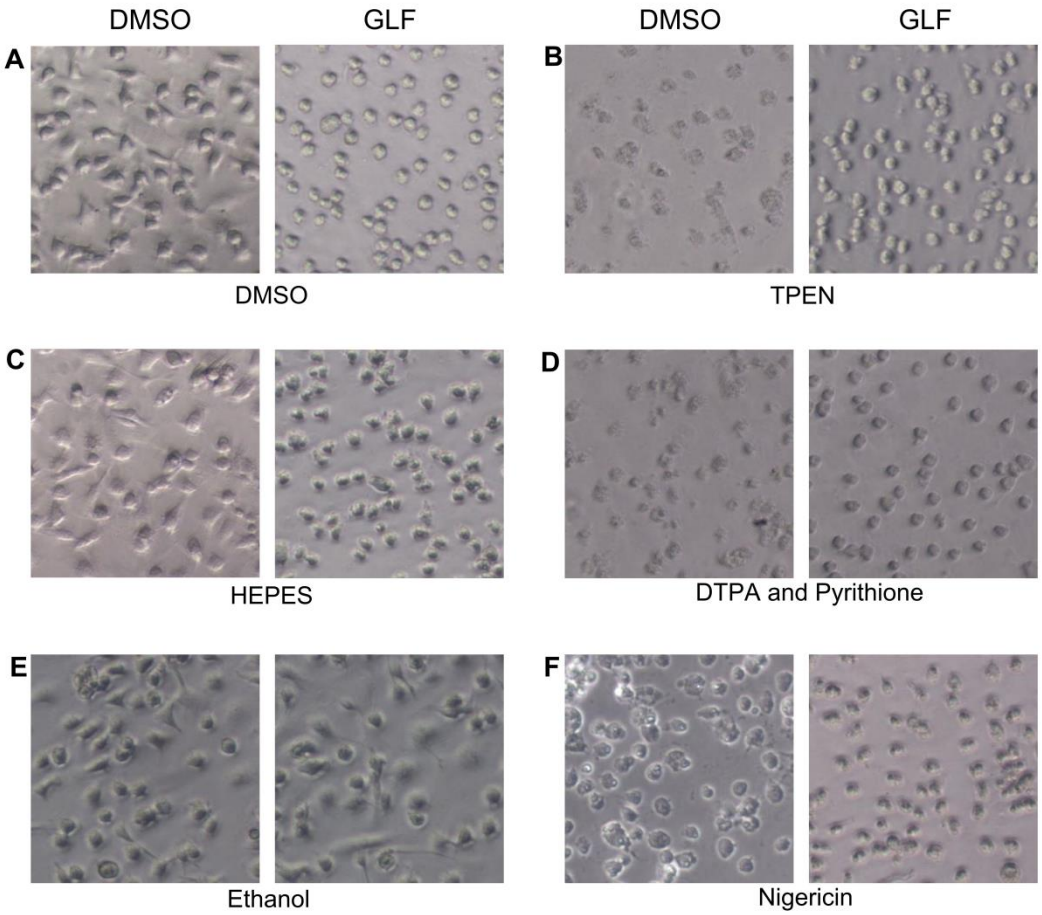

Figure 4.13: GLF treatment of zinc depleted and nigericin treated BMDMs.

LPS-primed (1 µg/ml, 4h) BMDMs were pre-treated (15 min) with GLF (10 µM, 0.5 % DMSO), 0.5 % DMSO vehicle prior to treatment (24h) with TPEN (10 µM, 0.5 % DMSO), 0.5 % DMSO vehicle **(A, B)**; HEPES (10 mM, pH 7-7.5), DTPA (1 mM, 10 mM HEPES media), Pyrithione (50 µM, 10 mM HEPES media) **(C,D)**; or 0.5 % Ethanol vehicle or Nigericin (20 µM, 0.5 % Ethanol) **(E,F)**. Images are representative of 3 independent experiments.

Table 4.1: Summary table of IL-1β response to zinc depletion

Figure	Cell Type	Zinc depletion	Inhibitor	Time of treatment (h)	Processing	Release
4.1	THP-1	TPEN with Nigericin	NA	1	Inhibited by TPEN	Not significantly reduced
4.1	THP-1	DTPA_Py with Nigericin	NA	1	Not inhibited by DTPA Py	Not reduced
4.2	THP-1	TPEN	NA	24	Induced. 3 bands at approximately 17 kD.	Induced
4.2	THP-1	DTPA_Py	NA	24	Induced. 3 bands at approximately 17 kD.	Induced
4.2	THP-1	Nigericin	NA	24	Induced	Induced
4.3	BMDM	TPEN	NA	24	Induced	Induced
4.3	BMDM	DTPA_Py	NA	24	Induced	Induced
4.3	BMDM	Nigericin	NA	24	Induced	Induced
4.4	THP-1	TPEN	$ZnSO_4$	24	NA	Inhibited by $ZnSO_4$
4.4	BMDM	DTPA_Py	$ZnSO_4$	24	NA	Inhibited by $ZnSO_4$
4.4	BMDM	TPEN	$ZnSO_4$	24	NA	Inhibited by $ZnSO_4$
4.4	BMDM	DTPA_Py	$ZnSO_4$	24	NA	Inhibited by $ZnSO_4$
4.5	THP-1	TPEN	YVAD	24	Not reduced by YVAD.	Not significantly reduced.
4.5	THP-1	DTPA_Py	YVAD	24	Not reduced by YVAD.	Not significantly reduced.
4.5	THP-1	Nigericin	YVAD	24	Reduced by YVAD	Not significantly reduced.
4.6	BMDM	TPEN	YVAD	24	Not reduced by YVAD.	Not significantly reduced.
4.6	BMDM	DTPA_Py	YVAD	24	Not reduced by YVAD.	Not significantly reduced.
4.6	BMDM	Nigericin	YVAD	24	Not completely reduced by YVAD.	Not significantly reduced.
4.7	THP-1	TPEN	Glyburide	24	NA	Not significantly reduced.
4.7	THP-1	DTPA_Py	Glyburide	24	NA	Not significantly reduced.
4.7	THP-1	Nigericin	Glyburide	24	NA	Not significantly reduced.
4.8	BMDM	TPEN	Glyburide	24	NA	Reduced
4.8	BMDM	DTPA_Py	Glyburide	24	NA	Reduced
4.8	BMDM	Nigericin	Glyburide	24	NA	Reduced
4.9	THP-1	TPEN	CA074-Me	24	NA	Not significantly reduced.
4.9	THP-1	DTPA_Py	CA074-Me	24	NA	Not significantly reduced.
4.9	THP-1	Nigericin	CA074-Me	24	NA	Not significantly reduced.
4.10	BMDM	TPEN	CA074-Me	24	NA	Reduced
4.10	BMDM	DTPA_Py	CA074-Me	24	NA	Not significantly reduced.

4.10	BMDM	Nigericin	CA074-Me	24	NA	Reduced
4.11	THP-1	TPEN	GLF	24	NA	Reduced
4.11	THP-1	DTPA_Py	GLF	24	NA	Reduced
4.11	THP-1	Nigericin	GLF	24	NA	Reduced
4.12	BMDM	TPEN	GLF	24	NA	Reduced
4.12	BMDM	DTPA_Py	GLF	24	NA	Reduced
4.12	BMDM	Nigericin	GLF	24	NA	Reduced

TPEN and DTPA with pyrithione IL-1β processing and release in THP-1 and BMDMs. Zinc depletion induces IL-1β release in both THP-1 cells and BMDMs and IL-1β processing to 3 mature forms of approximately 17 kD in THP-1 cells and a single 17 kD form in BMDMs. In THP-1 cells this IL-1β release is only reduced by inhibition of cathepsin G. In BMDMs this release is reduced by inhibition of NLRP3, cathepsin B and cathepsin G.

4.4 Discussion

The aim of this study was to identify the differences and similarities between zinc-depletion-induced IL-1β processing and release in human and mouse macrophage-like cells. Initially the response to zinc depletion in THP-1 cells and BMDMs was similar, both released IL-1β. However a difference in IL-1β processing in response to zinc depletion was observed in human THP-1 cell and mouse BMDMs. Whilst zinc depletion induced IL-1β processing to a single mature 17 kD form, three bands of approximately 17kD size were observed following zinc depletion of THP-1 cells. The significance of this is unknown. There is a potential that the multiple processed forms have differing activities, however determining this is difficult. Identifying the bands would require extensive separation and characterisation and would be costly. Additionally the biological relevance of these results is also in some doubt. A zinc depletion experiment was undertaken in immortalised BMDMs for 4h (following 4h LPS priming) and the supernatant condensed. IL-1β western blots of these concentrated samples of zinc depleted BMDM cell line macrophages also showed multiple bands of approximately 17kD in size (Summersgill, unpublished). This suggests that the multiple bands may be some artefact of prolonged cell culture.

Caspase-1 appears not to play a role in zinc-depletion-induced IL-1β release in either THP-1 cells or in BMDMs, as YVAD failed to inhibit processing or release. Although, it should be noted that YVAD did not significantly inhibit IL-1β release following treatment with nigericin in either cell type. As nigericin is known to activate IL-1β release via caspase-1 [Perregaux *et al.*, 1992][Kahlenberg & Dubyak, 2004] YVAD inhibition should have reduced the release, therefore as the positive control failed caspase-1 dependency cannot be excluded in zinc-depletion-induced IL-1β release. To determine whether the caspase-1 is activated in THP-1s and BMDMs following zinc depletion it would be useful to complete a caspase-1 activity assay.

A difference was observed in response to treatment with glyburide, a drug known to inhibit NLRP3 [Lamkanfi *et al.*, 2009]. In BMDMs glyburide reduced IL-1β release following both nigericin treatment and zinc depletion, indicating that these processes are, at least partially dependent upon glyburide. This reduction in IL-1β release following treatment with glyburide was not observed in THP-1 cells. Both zinc depleted cells and nigericin treated cells were both unaffected by glyburide. Glyburide should have inhibited the nigericin induced IL-1β release as nigericin induced IL-1β processing is NLRP3 dependent [Mariathasan *et al.*, 2006]. Due to failures of positive controls it cannot be stated that THP-1 induced IL-1β release is inflammasome dependent or independent. Further work would be required to determine this. Due to the reduction of zinc-depletion-induced IL-1β release in response to glyburide it is probable that in BMDMs zinc depletion initiates inflammasome dependent IL-1β release.

Cathepsin B release from lysosomes is suggested to be one of the major pathways of NLRP3-inflammasome activation [Hornung & Latz, 2010]. Inhibiting cathepsin B has been shown to inhibit IL-1β release in response to activation of the NLRP3 inflammasome [Niemi *et al.*, 2011][Duncan *et al.*, 2009]. Cathepsin B and ASC have also been shown to be involved in a pro-inflammatory necrotic cell death [Willingham *et al.*, 2007]. In BMDMs, like peritoneal macrophages, the inhibition of cathepsin B reduced levels of IL-1β released. In contrast, in THP-1 cells inhibition of cathepsin B did not affect IL-1β release. This would strongly suggest that some of the mechanisms of zinc-depletion-induced IL-1β processing and release are different in humans and mice. This difference would be an important area to study further when investigating the role zinc depletion plays in upregulating human inflammatory disease.

An interesting observation was the role of cathepsin G in the release of IL-1β. Cathepsin G is a serine protease, commonly associated with neutrophil granules [Korkmaz *et al.*, 2010] but also expressed in azurophilic granules of monocytes [Scott *et al.*, 1999]. Cathepsin G has also been reported to process IL-1β to its mature form [Netea *et al.*, 2010]. Inhibition of this protease dramatically reduces IL-1β release induced by nigericin or zinc chelator treated THP-1s and BMDMs. The extreme reduction in IL-1β release would suggest that essential pathways in either processing or release are blocked.

In monocytes cathepsin G is also associated with the plasma membrane and is the mechanism that permits the entry of HIV into cells [Avril *et al.*, 1995]. The role of cathepsin G at the plasma membrane of monocytes has not been well defined, with most of the research in this area focussed upon the actions of cathepsin G in neutrophil granules [Korkmaz *et al.*, 2010]. In this study cathepsin G inhibition changed the morphology of BMDMs from an elongated and spread morphology to a more spherical morphology (Fig 4.13). In cells treated with TPEN, DTPA in combination with pyrithione or nigericin, the disruption to membrane integrity induced by these treatments was abolished by cathepsin G inhibition (Fig 4.13). This would imply that cathepsin G is responsible for IL-1β release processes. Although, cathepsin G has been identified to play a role in caspase-1 activation and apoptosis [Danelishvili *et al.*, 2011], which would the hypothesis of direct inhibition of processing. It would be interesting to analyse cathepsin G inhibited lysates by IL-1β western blot to identify whether processing had occurred. If processing had occurred this would further support the hypothesis that inhibition of cathepsin G prevents IL-1β release. In addition caspase-1 activity assays of GLF treated cells would identify whether caspase-1 activity is affected by cathepsin G inhibition. Cathepsin G may prove to be an important therapeutic target.

The processing and release of IL-1β are controlled at many points. These sites of regulation differ depending upon the manner in which processing and release of IL-1β are induced and the cell type in which they are induced. It appears there may be differences between human and mouse responses to zinc depletion in terms of both processing and mechanisms of action. The differences in regulation of IL-1β release by cathepsin B and the production of multiple mature forms of IL-1β in THP-1 cells would need to be investigated further in other human monocytic cells to confirm that apparent differences occur due to species differences. Most striking is the inhibition of IL-1β release in cells treated with a cathepsin G inhibitor. It would be interesting to see the effects of this inhibitor on IL-1β processing and identify the mechanisms of action as cathepsin G could be an important therapeutic target for inflammatory disease.

Chapter 5: A network map of IL-1β expression

This work was carried
out in collaboration with Dr. Ben Small.

Ben and I contributed equally to the following tasks;

- *Determination of inclusion and exclusion criteria.*
- *Creation and optimisation of the search string.*
- *Assessment of papers returned from the literature search against the inclusion and exclusion criteria.*
- *Determination of data assessment criteria.*
- *Map assembly.*
- *Annotation of the map.*

I completed all other work within this chapter, including interpretation and analysis of the map, independently.

5.1 Introduction

5.1.1 Zinc and IL-1β expression

Having established a clear role for zinc deficiency in IL-1β processing, it would be interesting to investigate the role of zinc and zinc deficiency in IL-1β expression. In Chapter 3 TPEN treatment in the absence of LPS was shown to induce some IL-1β release and potentially upregulate IL-1β expression. Zinc is predicted to bind 10% of mammalian proteins [Andreini *et al.*, 2006] functions as a regulatory cofactor in many instances [Cummings & Kovacic, 2009]. In addition, elevated IL-1β expression has also been reported in regions of the intestine in zinc deficient rats [Vignolini *et al.*, 1998], and in pro-myeloid cells [Wessels *et al.*, 2013]. This highlights the potential role of zinc regulation in the LPS induced pathways that control IL-1β expression.

5.1.2 IL-1β expression

IL-1β transcription is an essential step in the inflammatory process. Stimulation of Toll-like receptor 4 (TLR4) initiates this transcription via a complex signalling system with multiple layers of control. There are many regulatory factors within this system, reflecting the need for tight control of the initiation of inflammatory processes. Assessing the contribution of any one regulatory mechanism to this process is made difficult by the complexity of the system.

The main ligand for TLR4 is LPS [Kawai & Akira, 2010]. Lipopolysaccharide (LPS) is an integral component of the Gram-negative bacterial cell wall and is an essential factor in mediating mammalian responses to endotoxin [Lozano-Torres *et al.*, 2012]. LPS recognition by TLR4 was first identified by [Poltorak *et al.*, 1998]. This study utilised an LPS

unresponsive strain of mouse called C3H/HeJ which contains naturally occurring mutations in a locus necessary for LPS sensitivity. This LPS sensitive allele maps to the *Tlr4* locus, suggesting that TLR4 is the LPS receptor [Poltorak *et al.*, 1998]. Since then the binding of LPS to TLR4 at the membrane has been well established [Park *et al.*, 2009], [da Silva Correia & Ulevitch, 2002][Kobayashi *et al.*, 2006][Saitoh *et al.*, 2004].

Pathways downstream of TLR4 binding have also been studied [Fitzgerald *et al.*, 2004][Banerjee & Gerondakis, 2007], however as yet these pathways have not been integrated into a single network that encompasses the LPS signalling at the membrane to IL-1β transcription. The paper that comes closest to achieving this aim, [Oda & Kitano, 2006], is a comprehensive map of all TLR signalling. In this paper a bow tie structure is highlighted showing the signals to converge upon the same few MAPK and NF-κB molecules that control the transcription of a vast number of downstream signalling molecules. This impressive piece of work is useful for understanding the similarities in the multiple TLR pathways; however from this map it is very difficult to isolate the LPS-induced signals and the signals that lead to the transcription of IL-1β. Here we have produced a map that achieves this by focussing on LPS specific induction of IL-1β transcription. Using the map it will be possible to assess the contributions of regulatory mechanisms to IL-1β transcription and produce dynamic models of IL-1β expression that can be used to further explore the mechanisms behind this process.

5.1.3 Standards

The map adheres to the current standards observed within the systems biology community. Data standards give details of standard practices that have been agreed upon

by a particular community [Brazma *et al.*, 2006]. Use of standards prevents data from being published in different formats which would impede data sharing and reduce the usefulness of a particular endeavour. In biology this is a relatively new idea in comparison to industry where the use of standards is common practice. In systems biology there are three major standards, the systems biology markup language (SBML)[Hucka *et al.*, 2003], systems biology graphical notation (SBGN) [Le Novère *et al.*, 2009] and the minimum information required in the annotation of models (MIRIAM) [Le Novère *et al.*, 2005].

SBML is a XML based format used for storage and communication of computational biological models. It allows for the particular features unique to biological models to be explicitly described and shared. Further to this, most programs developed for the creation and analysis of biological models support SBML, thus creating a network map in SBML increases the potential for development of the model using a wide range of tools.

SBGN is the standard for the way in which models are displayed [Le Novère *et al.*, 2009]. Before the introduction of this standard the nodes and edges of a network were drawn in many different ways. Symbols that represented genes in one diagram may have represented proteins in another. This complicated understanding and comparisons of models. By adhering to a standard the map becomes more easily readable for the wider systems biology community, and accessing model data becomes easier for those unfamiliar with this area.

MIRIAM describes the minimum information required for inclusion within the model and what should be reported when mentioning models in the literature. The aim of this set of

standards is to ensure models have sufficient information to be reused and developed by others [Le Novère *et al.*, 2005]. An example of where these standards are particularly useful is defining the molecules within a model. Many of molecules have been named in multiple ways and different molecules occasionally share the same names. This can be problematic when building models that need to be understood and reused by others. The situation can be further complicated when different modellers use different abbreviations for the same molecules. Thus annotation of models using databases such as UniProt [The Uniprot Consortium, 2012] and ChEBI [de Matos *et al.*, 2010], is particularly useful in ensuring the identity of the molecules within the model is properly defined. By adhering to current community standards in annotation (MIRIAM), visualisation (SBGN) and format (SBML) it is hoped a base will be provided which will facilitate further exploration of this network using a systems approach.

5.1.4 Using the map to analyse zinc regulation

There are many potential applications for a network map detailing the induction of IL-1β transcription downstream of LPS. One example is assessing the role of zinc in this system. Zinc is known to bind to many proteins within the cell and zinc has also been shown to play a regulatory role in IL-1β expression. Following LPS stimulation of dendritic cells the expression of zinc transporters is upregulated, leading to an overall reduction in intracellular free zinc [Kitamura *et al.*, 2006]. LPS stimulation activates dendritic cells and this was shown to be dependent upon the consequent zinc depletion, as activation could be induced by treatment with the zinc chelator TPEN [Kitamura *et al.*, 2006]. There is also a potential role for A20 , a zinc binding protein [Verstrepen *et al.*, 2010] this is expressed in response to pro-inflammatory stimuli and functions as a negative regulator of NF-κB. Elevated levels of zinc have been shown to increase A20, and consequently reduce NF-kB

activity [Bao *et al.*, 2010a] [Prasad *et al.*, 2011]. Removal of this negative feedback mechanism by zinc deficiency may lead to higher levels of IL-1β. In contrast, [Haase *et al.*, 2008] describe an intracellular rise in Zn^{2+} following stimulation of monocytes with *E.coli* LPS. In this paper chelation of zinc with TPEN blocks LPS-induced activation of p38 MAPK, ERK1/2, and NF- κB and subsequent transcription of TNF-α. Whilst none of the papers specifically refer to the role of zinc in LPS induced IL-1β transcription, the evidence given highlights potential opposing zinc regulatory mechanisms within this network. The network map could function as a starting point from which zinc binding proteins involved in the regulation of IL-1β expression may be identified. Further work could then be completed to assess the interplay between these mechanisms.

5.2 Materials and Methods

5.2.1 Building a network map

A network map for IL-1β expression was created using a combination of literature search, data assessment from the returned literature, and assembly of the information into a series of interactions using the CellDesigner4.2 software [Funahashi *et al.*, 2003]. One of the main objectives of this project was to build the map to a set of predefined criteria in order to prevent inclusions of bias and inconsistencies. The ideal information selected to create the network map is detailed in the criteria below.

1) Provides details of direct interactions
2) Reactions occur as a result of LPS stimulation of TLR4
3) Reactions lead to the expression of IL-1β
4) Data was obtained from cells of the monocytic lineage.
5) Data was obtained from human or mouse background.

Using these criteria a search string (Appendix 1) was created and used to search the NCBI PubMed database. This search, completed on 28-3-2012, returned 799 references. The abstracts of all the papers returned from this search were exported from the database and assessed for relevance to the original set of criteria. Papers that were deemed to be inappropriate were then excluded. It was essential that the papers detailed a LPS induced effect or an effect that described the production of IL-1β, although not all papers detailed both. All the papers retained detailed reactions in either mouse or human cells. In some instances papers in which experiments were conducted in non-monocytic cells, were

retained as these reactions had not been investigated in monocytic lines. At this stage 529 papers were excluded and 270 papers were included.

Assessing the literature using the information in the abstracts relied heavily upon the description of the data by the authors. Data presented in the figures of each of the remaining 270 papers were therefore assessed to ensure an evidence based network map was produced, avoiding inclusion of author bias (at times authors assume things that are not supported by the data). We assessed the evidence in each paper for relevance to the map and quality of data in this context. The data assessment criteria comprised scores for the level of interaction between entities, the relevance of technique, the number of replicates and the use of statistics (Table 5.1).

Levels of interaction ranged from direct binding leading to a direct measured reaction to descriptions of an entity with no details of direct binding given. One example of direct binding leading to a direct measured reaction is the binding of a protein that in turn leads to a direct fluorescence output [Thompson *et al.*, 2003]. Most of the data analysed fell into the second category of direct binding leading to an indirect measured reaction. In this case direct binding maybe characterised by techniques such as co-immunoprecipitation and X-ray crystallography and then combined with reporter assays or immunoaffinity measurements. In the absence of evidence for direct binding reporter assays or immunoaffinity measurements were classified as being involved in the reaction. The final category describes the characterisation of a protein in LPS treated cells but in the absence of evidence for binding to another protein or involvement in specific reaction.

These scores were then summed to provide a basis for assessing weak to strong evidence for relevant interactions. Assessing the data in this way allowed for construction of the map based upon the weight of evidence for a particular interaction. The interactions were then assembled together in a network map using the CellDesigner4.2 software. The map was annotated according to MIRIAM standards and exported to SBML and SBGN formats. This workflow is summarised in Figure 5.1.

Table 5.1: Data assessment scoring criteria

Reaction	**Score**
Direct binding leading to a direct measured reaction	5
Direct binding leading to an indirect measured reaction	4
Direct Binding	3
Involved in reaction	2
Description of entity / No binding	1
Technique Relevance	**Score**
High	3
Average	2
Low	1
Replicates	**Score**
N=3+	3
N=2+	2
N=1+	1
Statistics	**Score**
Stats used	1
Stats not used	0

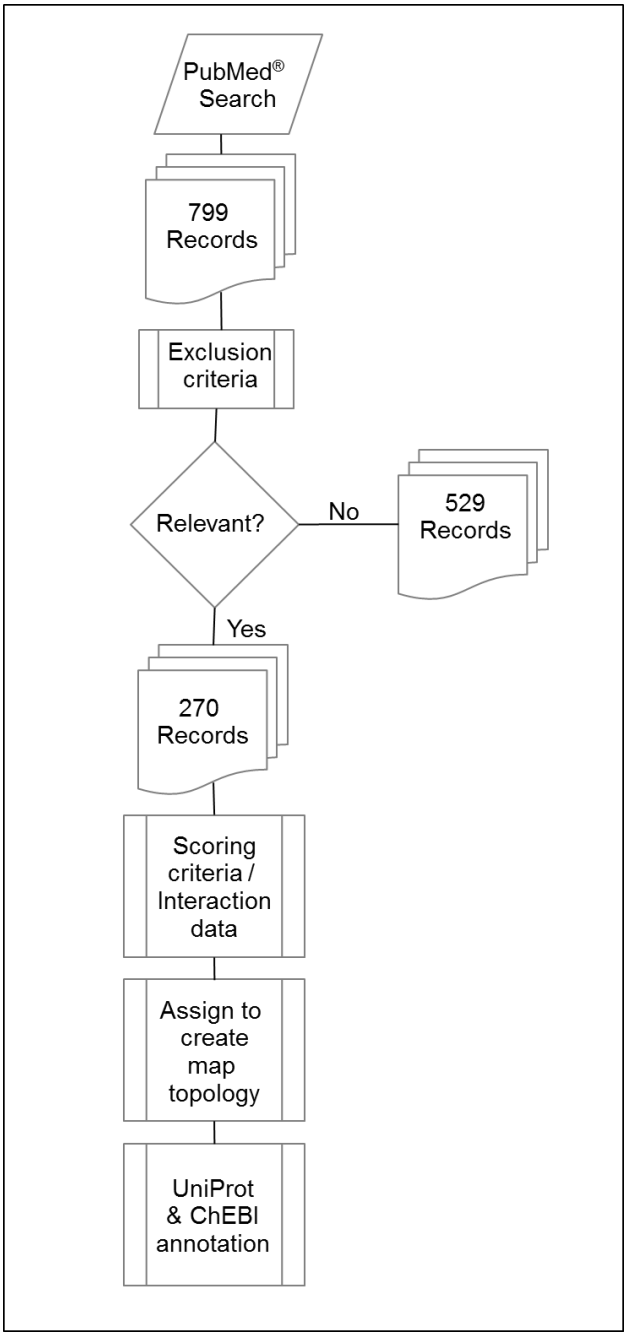

Figure 5.1: Workflow providing an overview of the methods, criteria, decisions, data and annotation used for the systematic curation of the network map.

799 references were returned from a defined search of the NCBI PubMed database. Of these 799 references 270 were retained and assessed for data quality. The network map was created using this data and then the species were annotated using the UniProt and ChEBI databases.

5.3 Results

The completed network map is shown in SBGN format in Figure 5.2. An outline of the interactions encompassed by the map is given below, although it should be noted that this is not intended to be an in depth description. To do so would detract from the visual simplicity that is one of the major aims of this project. The literature cited in this section will comprise only those papers returned from the stringent literature search detailed in section 5.2.

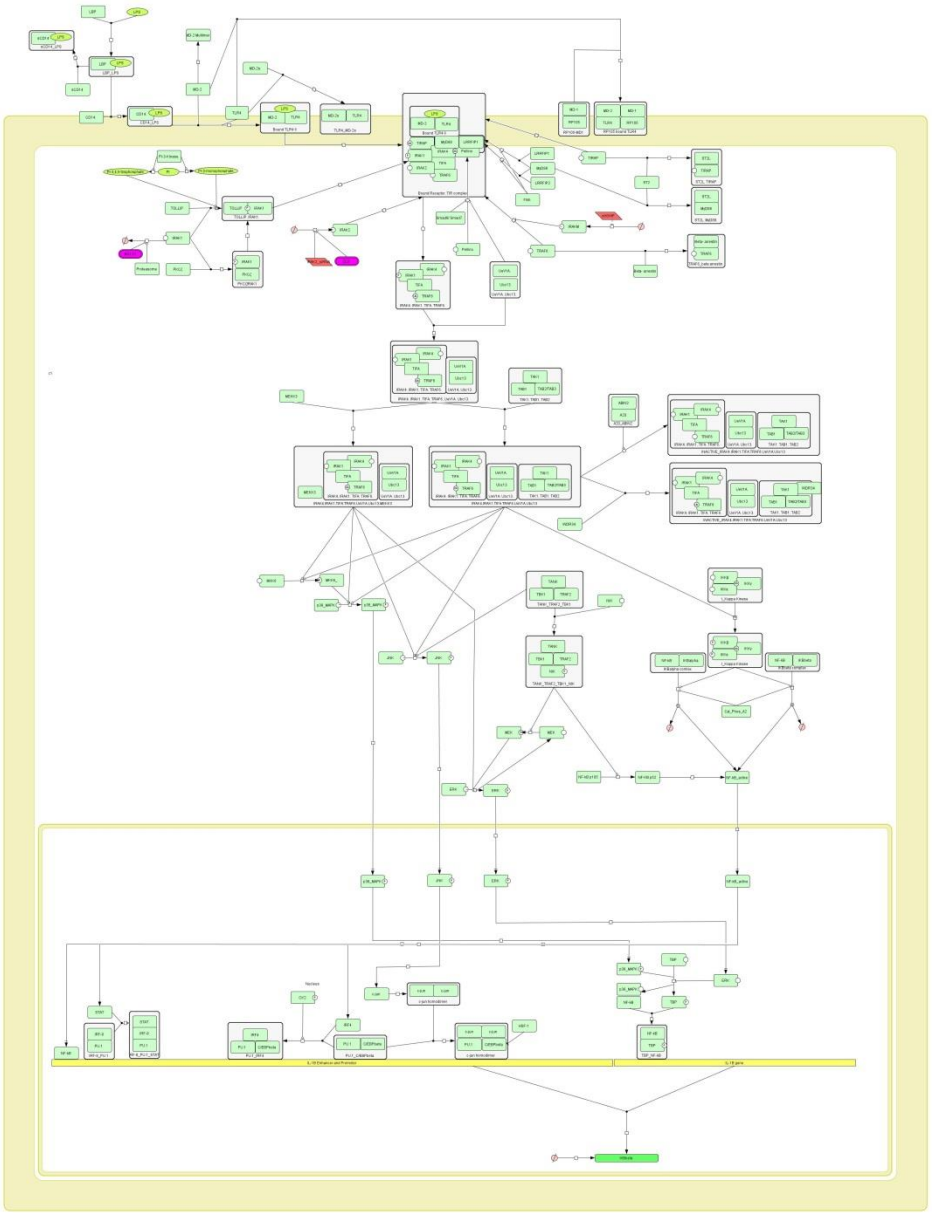

Figure 5.2: A systematically curated network map of LPS stimulated IL-1β transcription.

Map created in CellDesigner4.2. Visualised in SBGN compliant format.

5.3.1. Actions of LPS at the membrane

LPS is presented to plasma membrane bound proteins by the lipopolysaccharide binding protein (LBP)[Kohara *et al.*, 2006][Li *et al.*, 2007][Thompson *et al.*, 2003][Shawkat *et al.*, 2008]. In turn LPS is transferred from the LBP to CD14. Membrane bound CD-14 is a glycosylphosphatidylinositol (GPI)-linked glycoprotein [Kitchens *et al.*, 1998]. CD14 interacts with MD-2 and via this interaction passes on the bound LPS to MD-2 [da Silva Correia & Ulevitch, 2002][Gioannini *et al.*, 2004][Resman *et al.*, 2009].

MD-2 is essential for TLR4-dependent signalling, since without MD-2 TLR4 cannot become active and consequently cannot respond to LPS [Fujimoto *et al.*, 2004]. MD-2 and TLR4 bind with LPS at the membrane to form the active LPS bound TLR4 complex [Visintin *et al.*, 2006][Meng *et al.*, 2010]. MD-2 binds TLR4 through the formation of disulphide bonds [Mullen *et al.*, 2003]. The crystal structure of TLR4:MD-2:LPS [Park *et al.*, 2009], highlights that MD-2 binding of TLR4 creates a cavity to which LPS binds.

LPS induced signalling is negatively regulated by multimerisation of MD-2 [Teghanemt *et al.*, 2008], reducing the availability of active MD-2. MD-2s, a splice variant of MD-2 sequesters TLR4 in an inactive complex [Gray *et al.*, 2010]. A further regulatory step is shown in this map. Active TLR4-MD-2 complex is bound by a RP105-MD-1 complex rendering it inactive [Divanovic *et al.*, 2005]. All of these regulatory mechanisms highlight the importance for control at the initiation of this inflammatory signalling pathway.

Although it is apparent that some internalisation of LPS and the receptor complex occurs [Kitchens *et al.*, 1998] [Latz *et al.*, 2002], there is insufficient evidence to distinguish

between the pathways downstream of the internalised and non-internalised receptors in this network map. Consequently LPS binding of TLR4 is depicted in the map as occurring at the plasma membrane[Yanagimoto *et al.*, 2009][Panter & Jerala, 2011]. This is clearly an area where more research is needed to clarify the mechanistic details.

5.3.2 Intracellular TLR4: TIR domains and signalling complexes

The Toll-like/IL-1 receptor (TIR) domains are the main functional components of intracellular TLR4. These domains interact with the equivalent TIR domains on intracellular adaptor proteins. These TIR:TIR interactions are necessary for propagation of the LPS induced TLR4 signal [Ohnishi *et al.*, 2009] [Bovijn *et al.*, 2012]. The TIR domains of signalling mediators have been shown to confer TLR selectivity on the adaptor proteins [Jiang *et al.*, 2006]. The mediators, or adaptors, possessing TIR domains shown in this map are Myeloid differentiation primary response gene (88) (MyD88) [Avbelj *et al.*, 2011] [Nishiya *et al.*, 2007] and toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP)[Yamamoto *et al.*, 2002a][Horng *et al.*, 2002][Horng *et al.*, 2002]. TIR-domain-containing adapter-inducing interferon-β (TRIF), and TRIF-related adapter molecule (TRAM) binding activates interferon signalling and therefore these adaptors are not detailed in the map [Kagan *et al.*, 2008][Yamamoto *et al.*, 2002b][Kawai *et al.*, 2001].

Many proteins bind to the intracellular component of TLR4, interacting with the signalling adaptors, and leading to phosphorylation and ubiquitination dependent signalling events. MyD88 and TIRAP binding results in the recruitment of interleukin receptor-associated kinase 1(IRAK1), IRAK4 and tumor necrosis factor receptor-associated factor 6 (TRAF6) to the receptor complex [Neumann *et al.*, 2007].

5.3.2.1 IRAK

Activation of the IRAK proteins occurs via phosphorylation of key serine, proline and threonine residues. IRAK4 is upstream of IRAK1 and is activated by autophosphorylation [Cheng *et al.*, 2007]. Three phosphorylation sites present in the activation loop of this protein have been identified as being involved in IRAK4 activation. These are T^{342}, T^{345}, and S^{346} [Cheng *et al.*, 2007]. The kinase activity of IRAK4 is required for TLR induced NF-κB activation [Fraczek *et al.*, 2008][Koziczak-Holbro *et al.*, 2007].

Upon binding the receptor complex IRAK1 is phosphorylated at T^{209} and T^{387} following which autophosphorylation is initiated in the proline-, serine-, and threonine-rich ProST region of the protein [Kollewe *et al.*, 2004]. Hyperphosphorylation of this region then induces dissociation from both MyD88 and the negative regulatory protein, Tollip [Kollewe *et al.*, 2004]. Interaction of IRAK-1 with Myd88 and Tollip is also dependent upon the N terminal death domain (DD) [Neumann *et al.*, 2007]. The C terminal domain of IRAK-1 is required for interaction with the downstream signalling protein, TRAF6, although this domain has also been identified as being responsible for maintaining the protein in an inactive conformation [Nguyen *et al.*, 2009].

Ubiquitination of IRAK1 is also an essential process in LPS induced signaling. IRAK-1 is polyubiquitinated via K^{63} lined chains [Windheim *et al.*, 2008]. K^{63} linked poly-ubiquitin chains are not associated with degradation of the protein by the proteasome such as occurs with K^{48} linked polyubiquitin chains, suggesting therefore that this ubiquitination of IRAK-1 occurs as part of a signaling mechanism. The sites of ubiquitination on the IRAK protein itself have been identified as K^{134} and K^{180} [Conze *et al.*, 2008].

Prior to LPS stimulation, IRAK1 is held in its inactive state by the inhibitory protein Tollip [Neumann *et al.*, 2007]. Tollip remains bound until IRAK1 autophosphorylates, at which point Tollip rapidly dissociates from the kinase. Tollip expression is induced by LPS and functions as a negative regulator of LPS induced NF-κB signalling [Li *et al.*, 2004].

It is clear that the control the IRAK proteins exert at the receptor complex involves a complicated interplay between the processes of phosphorylation and ubiquitination. In addition to this IRAK4 and IRAK1 not only activate the receptor by phosphorylation, but also in target the adaptor protein TIRAP for degradation. This process leads to the inactivation of the receptor complex and thus the IRAK proteins also function as endogenous negative regulators of IL-1β transcription [Dunne *et al.*, 2010]. In this way IRAK proteins can be viewed to modulate their own activity.

A third IRAK protein, whose interactions at the TLR4 receptor complex are less well understood, is IRAK-2. In human cells IRAK-2 is required for NF-κB activation downstream of TLR4 and has been proposed to be essential for TRAF6 ubiquitination [Keating *et al.*, 2007][Flannery *et al.*, 2011]. In contrast the mouse homolog of IRAK-2 does not induce NF-κB activity [Rosati & Martin, 2002a] although interactions with TRAF6 are still observed [Wan *et al.*, 2009]. In the map the IRAK-2 interactions displayed are representative of human IRAK-2 as this was deemed most beneficial in supporting the future application of investigating human health and disease.

An inhibitory member of the IRAK family is IRAK-M [Rosati & Martin, 2002b][Wesche *et al.*, 1999]. Inhibition by IRAK-M is independent of IRAK-1 and has been shown to lower

activation levels of p38, but not JNK or ERK [Su *et al.*, 2007]. IRAK-M is ubiquitously expressed but localises to the cytoplasm upon endotoxin stimulation [Su *et al.*, 2007].

5.3.2.2 Pellino

Another family of proteins that interact with the IRAKs at the TLR4 receptor complex are the Pellino proteins. Pellinos are E3 ubiquitin ligases that catalyse the Lys63 polyubiquitination of IRAK1 [Butler *et al.*, 2005]. Pellino proteins possess two functionally relevant structural motifs- a C-terminal RING domain that confers the E3 ubiquitin ligase activity [Schauvliege *et al.*, 2006] and a Forkhead-associated (FHA) domain with a phospho-threonine binding module, which facilitates IRAK1 interaction [Lin *et al.*, 2008a]. Pellino-1 has been reported to be activated by IKK-related kinases in response to TLR activation [Goh *et al.*, 2012]. Members of the Pellino family activate both the NF-κB and MAPK pathways [Butler *et al.*, 2005][Jensen & Whitehead, 2003]. Pellino activity is inhibited by binding of the Smad6 and Smad7 proteins [Choi *et al.*, 2006][Lee *et al.*, 2010]. There are three known Pellino proteins; Pellino-1, Pellino-2 and Pellino-3. In this map they are all represented as Pellino.

5.3.3 TRAF6

TRAF6 is an integral component of the TLR4 receptor network, essential for activation of JNK, p38 and NF-κB [Gohda *et al.*, 2004]. *Via* interactions with IRAK at the receptor complex [Neumann *et al.*, 2007] TRAF6 integrates the signal received at TLR4 with downstream activators of NF-κB and MAPKs such as Transforming growth factor-β activated kinase-1(TAK1). Structurally, TRAF6 possess a C-terminal tumour necrosis factor receptor (TNFR) associated factor (TRAF) domain, zinc finger domains and a RING domain that confers E3

ubiquitin ligase activity [Yang *et al.*, 2004][Muroi & Tanamoto, 2008]. This RING domain is essential for TRAF6 interaction with the E2 enzyme Ubc13/Uev1A, which in turn is necessary for the K^{63} ubiquitination of TRAF6 [Yang *et al.*, 2004]. Interaction between TRAF6 and Ubc13/Uev1A is also required for the downstream activation of IKK [Yang *et al.*, 2004]. It has been shown that TRAF6 activation promotes the production of unanchored Lys63 polyubiquitin chains, which can activate TAK1 [Lamothe *et al.*, 2007] [Xia *et al.*, 2009].

TAK1 activation is also associated with the TAK1 binding proteins TAB1 and TAB2/3. The TAB proteins function as ubiquitin receptors, which when activated and bound to TAK1, induce TAK1 autophosphorylation [Xia *et al.*, 2009]. TAK1 then activates IκB kinase (IKK) leading to downstream NF-κB activity [Xia *et al.*, 2009]. Of the components TAK1:TAB1:TAB2/3 complex TAK1 appears to be essential for NF-kB and MAPK induced signalling [Shim *et al.*, 2005][Takaesu *et al.*, 2003]. Control mechanisms that are active in this area of the network include the TAK1 binding protein WDR34, which was identified in a yeast-2-hybrid screen. The siRNA knockdown of this protein increased NF-κB activity [Gao *et al.*, 2009]. TAB1 also possesses a negative regulatory function in addition to its role in activating TAK1. Once activated p38 induces phosphorylation of TAB1 at the S^{423}, T^{431} and S^{438} sites. Once this phosphorylation has occurred TAB1 inhibits TAK1 instead of activating it [Cheung *et al.*, 2003].

The complex containing TRAF6 and the IRAK proteins can also bind and be activated by the serine/threonine MAPK kinase kinase, MEKK3 [Huang *et al.*, 2004]. TRAF6 binding of MEKK3 also induces NF-κB activity [Nakamura *et al.*, 2010].

5.3.4 Transcriptional Activation

LPS induces p38, JNK and ERK phosphorylation [Marantos *et al.*, 2008], and increases NF-κB activity [Yang *et al.*, 2000]. The various NF-κB molecules, referred to collectively as NF-κB, are activated *via* the IκB kinases (IKKs). IKK activity is involved in the upregulation of IL-1β expression. IKKα/IKKβ catalyse the dissociation of the regulatory IκB subunits (both α and β) from NF-κB molecules, activating the molecules and facilitating transport to the nucleus [Clark *et al.*, 2011]. Increased stability of the IκBα subunit has been reported to suppress IL-1β transcription [Xia *et al.*, 1999]. NF-κB activation may also involve another complex comprising the TRAF binding protein TANK, TANK Binding protein (TBK1) and TRAF2 [Pomerantz & Baltimore, 1999].

There is also evidence for interplay between p38 MAPK and NF-κB inducing IL-1β expression. The p38 inhibitor SB203580 decreases binding of the TATA-binding protein (TBP) to the TATA box region in the IL-1β gene. TBP is shown to bind NF-kB at the IL-1β gene and is required for NF-κB activation of IL-1βexpression. This paper shows that p38 phosphorylates and activates TBP, and is thus a necessary step in NF-κB induced IL-1β expression [Carter *et al.*, 1999]. Prior to LPS stimulation, constitutively active ERK functions as an inhibitor of TBP phosphorylation by inhibiting the kinase activity of p38 [Carter & Hunninghake, 2000].

In addition, within the enhancer and promoter regions of the IL-1β gene there are several mechanisms in place to regulate expression. The transcription factors C/EBPβ and PU.1 are bound in an inactive but primed structure. Following LPS stimulation CK-2 phosphorylates PU.1 and IRF-4 is recruited to the complex, initiating IL-1β expression [Liang *et al.*, 2006]. A

further poised complex, comprising PU.1, interferon-responsive factor 8 (IRF8) and STAT-1, has also been described. This complex interacts with a C/EBPβ transcription factor bound at an adjacent site upon LPS stimulation and thus activates IL-1β expression [Unlu *et al.*, 2007]. The similarities between these two mechanisms would indicate that there may be some redundancy between the two and they may represent a larger mechanism involving all of these factors. There is, however, insufficient evidence to join the two mechanisms within this map. Homodimers of c-jun have also been reported activate IL-1β transcription *via* interactions with the DNA bound C/EBPβ: PU.1 complex [Grondin *et al.*, 2007].

5.3.5 The role of zinc

Table 5.2: Zinc binding proteins in the IL-1β network map

Protein List	UniProt Human	UniProt Mouse
A20	O76080	O88878
TRAF6	**Q9Y4K3**	**P70196**
TAB2	Q9NYJ8	Q99K90
TAB3	Q8N5C8	Q571K4

Each protein present in the network map was annotated with UniProt ids. These UniProt entries were then used to assess the proteins present in the map for zinc binding capabilities. The proteins within the map that were identified in UniProt as having zinc binding capabilities are given in the table above.

The proteins presented in the table above possess zinc binding capabilities by means of a zinc finger binding domain. This information was obtained from the UniProt database [The Uniprot Consortium, 2012]. A20 is a regulatory protein that functions to inactivate the LPS initiated signal. It is a deubiquitinase that removes K^{63} linked ubiquitin chains from TRAF6 which is consequently inactivated [Lin *et al.*, 2008b]. This results in the inactivation of NF-

κB and the consequent downregulation of IL-1β transcription. A20 is itself regulated by binding to another protein ABIN-2, which binds at the C terminal zinc finger domain [Van Huffel *et al.*, 2001]. Overexpression of ABIN-2 inhibits ligand stimulated NF-κB activity [Van Huffel *et al.*, 2001].

TRAF6, TAB2 and TAB3 are essential for the propagation of the LPS induced signal for IL-1β expression. The zinc finger domains of TRAF6 are integral to its RING domain which is required for TRAF6 autoubiquitination [Lamothe *et al.*, 2007]. TRAF6 autoubiquitination is needed for both downstream NF-κB activation [Lamothe *et al.*, 2007] and inhibitory negative regulation of TRAF6 [Wang *et al.*, 2010]. Consequently it is not clear how zinc deficiency would affect TRAF6 functioning and thus downstream IL-1β expression. The zinc finger domains of TAB2 and TAB3 are required for binding to K^{63} linked polyubiquitin chains, which in turn is necessary for NF-κB activation *via* TAK1 and IKK [Kanayama *et al.*, 2004]. As mutations of this domain inhibit NF-κB activation via TAB2 and TAB3, it can also be assumed that zinc deficiency would have the same effect upon these molecules. It is not clear from the map whether there is an overall positive or negative influence of zinc in IL-1β expression.

5.4 Discussion

5.4.1 Control and regulation in the TLR4-IL-1β transcriptional network

Whilst activation of the TLR4 signalling pathway is an integral mechanism in the host response to infection, uncontrolled IL-1β production can contribute to chronic inflammatory disease. In the TLR4 network there are many instances of negative regulation, which function to dampen LPS dependent TLR4 signalling [Liew *et al.*, 2005].

The majority of the regulatory and feedback mechanisms reduce the expression of IL-1β induced by LPS. This highlights the potency of the cytokine. These inhibitory mechanisms are present throughout all levels of the signalling network, from inhibition of LPS binding at the plasma membrane due to MD-2 multimerisation [Teghanemt *et al.*, 2008] and RP105:MD-1 sequestration of the TLR4:MD-2 receptor complex [Divanovic *et al.*, 2005], to ERK regulated inhibition of the transcriptional machinery at the IL-1β gene [Carter & Hunninghake, 2000].

Further inhibitors control the assembly of the intracellular complex. These inhibitory mechanisms mainly function to reduce IRAK and TRAF6 activity. The E3 ubiquitin ligase Pellino is a binding partner of IRAK and is inhibited by differential Smad6 and Smad7 binding [Lee *et al.*, 2010]. An inhibitory member of the IRAK family, IRAK-M [Rosati & Martin, 2002b], is expressed in monocytic cells, preventing formation of an active TRAF6 complex.

In a dynamic reconstruction of the network model, event based modelling could be used to investigate the temporal element of this control, adding a further dimension to the recreation of the signalling network. By creating this in depth network map of TLR4 signalling we have produced a platform from which to mathematically model this essential pathway in the inflammatory response.

5.4.2 Signal transduction in the TLR4-IL-1β transcriptional network

There are several key features of this TLR4 network map. The roles of ubiquitination and phosphorylation are particularly apparent. These post-translational modifications are the primary methods of signal transduction, and appear to function in a complementary manner. For the most part the proteins that constitute the TLR4 signalling pathway possess either E2 ubiquitin-conjugating activity, E3 ubiquitin ligase activity or kinase activity. The ubiquitin chains in this system are Lys63 polyubiquitin chains which are known to activate kinases [Hunter, 2007]. Furthermore phosphorylation appears to activate the RING domains of E3 ligases. This interplay between the two modes of signal transduction and direct protein: protein interactions are the signalling mechanisms by which the inflammatory message induced by LPS stimulation is propagated.

5.4.3 The role of zinc in the TLR4-IL-1β transcriptional network

In studying this network it has become apparent that zinc binding plays a central role to the functioning of many of its components. The zinc binding motifs present within the network modulate ubiquitin signalling, either positively or negatively. A20, TRAF6 and TAB2/3 each possess different zinc binding motifs. The TRAF6 zinc binding motif possesses a RING domain, which binds zinc in order to function [Lamothe *et al.*, 2008]. In this domain zinc

functions as a structural component [Deshaies & Joazeiro, 2009]. The zing finger domain of the A20 protein is a distinct domain first identified in this protein [Opipari *et al.*, 1990]. This zinc finger domain interacts with ubiquitin at D^{58} which differs from other zinc finger interactions with ubiquitin including the RanBP zinc fingers, found within TAB2 and TAB3 [Gamsjaeger *et al.*, 2007]. The presence of so many different forms of zinc finger motif in the binding of ubiquitin highlights a conserved relationship between zinc status and ubiquitin signalling.

In a dynamic model using data detailing the zinc affinities of these proteins it would be possible to investigate which signalling pathways would predominate under different zinc conditions. It would be interesting to see whether the integral signalling pathway components such as TRAF6 bound zinc more tightly than the regulatory proteins such as A20. In this case it would be predicted that zinc deficiency would have a lesser effect upon the functioning of the signalling proteins compared to the regulatory proteins, which in turn would result in the zinc status of the regulatory proteins having more control over the network.

5.4.4 Zinc, NF-κB and other cytokines

As zinc deficiency has been shown to increase NF-κB activity at a tissue level [Bao *et al.*, 2010b] it would be interesting to investigate the effect that zinc depletion has on the expression of other pro-inflammatory cytokines. NF-κB activity is linked to both IL-1β expression [Cogswell *et al.*, 1994] and expression of other key proinflammatory cytokines such as IL-6 and TNF-α [Zhang *et al.*, 1995][Edelman *et al.*, 2007]. Zinc deficiency has been shown to increase both the expression of IL-1β and TNF-α in pro-myeloid cells [Wessels *et*

al., 2013]. Zinc supplementation reduced levels of IL-1β and TNF-α [Bao *et al.*, 2008] [Prasad *et al.*, 2004]. In contrast, IL-6 levels measured in LPS stimulated zinc deficient infant blood samples were lower than in zinc sufficient samples [Wieringa *et al.*, 2004]. The effects of zinc supplementation on IL-6 levels are inconsistent. Supplementation with 45mg zinc/ day has been shown to decrease IL-6 [Bao *et al.*, 2010a], whilst supplementation with 10mg/ day zinc increases IL-6 levels [Mocchegiani *et al.*, 2008]. Whilst it appears that the effects of zinc deficiency on TNF-α are similar to the effects upon IL-1β, the role of zinc regulation of IL-6 appears more complicated. Elucidating the complex interplay between zinc regulation and these cytokines would be greatly enhanced by the use of network maps and dynamic models.

5.4.5 Methodology

The development of method for selection and integration of literature into a network map was one of the major undertakings of this work. Creating a literature search that included as much of the relevant data as possible whilst excluding irrelevant data to minimise the number of reference returned was difficult. Hierarchical arrangements of “AND” and “OR” terms were used to select papers that referred to LPS binding and IL-1β transcription in monocytic cells. These search terms were returning several thousand papers. In order to reduce this number to something more manageable we relied heavily on the use of “NOT” terms to exclude irrelevant papers. In this way we also selected against review articles, ensuring everything included in the final set of literature was a primary source of data.

The refinement methods described in 5.2 to produce a final set of data were undertaken. These data refinement methods were entirely manual and did not involve the use of text

mining software. Furthermore the assessment of data was undertaken by two separate individuals. This manual approach has a distinct advantage in that humans can understand the nuances of context and can interpret data in figures in addition to data given in the text of a publication. The disadvantage of this approach is that it is labour intensive and consequently not suitable for assessing data from a rapidly changing field or a field with a larger body of data.

5.4.6 Future work

In addition to analysing the effect of zinc on IL-1β transcription there are many other potential applications of this network. Through analysis of the IL-1β transcription network other regulatory mechanisms can also be explored. In the future, building a dynamic model from this network map will enable us to identify any emergent properties in IL-1β transcription that would not have been obvious from studying individual interactions. Here modelling would be used to analyse the non-linear properties of the network, thus reducing the constraints of linear thought processes.

It would also be possible to investigate the roles of regulatory mechanisms and individual proteins in endotoxin tolerance. The integration of signalling, expression and feedback in this phenomenon makes it prime candidate for a systematic investigation into its causes.

5.5 Conclusions

The network map has highlighted many important themes related to LPS induced IL-1β expression, namely the role of phosphorylation and ubiquitination. By further investigation

a role for zinc status in regulating the ubiquitin signal responsible for delivering the signal for IL-1β transcription was identified. This opens up exiting potential areas of investigation using either cell culture and “wet lab” techniques, or by developing the network map into a dynamic model and modelling the effects of zinc status. In this way this project can be perceived to epitomise an investigation that fully utilises the modelling cycle, where information gained from investigative lab work is inputted into a dry computational project and the information gained from this can feed further “wet lab” investigation.

Chapter 6: General Discussion

6.1 Introduction

IL-1β has been shown to be an important modulator of inflammatory phenotypes and has proved to be an important therapeutic target in diseases with a large inflammatory component such as stroke and rheumatoid arthritis [Emsley *et al.*, 2005][Mertens & Singh, 2009]. Zinc deficiency has been shown to be associated with an increased inflammatory phenotype. In rheumatoid arthritis a negative correlation between zinc status and IL-1β has already been identified [Zoli *et al.*, 1998b]. In this thesis it is shown that cellular zinc deficiency can directly influence the production of IL-1β, thus providing a mechanism to explain the long recognised link between low zinc status and inflammation. This is the first time that zinc depletion has been identified as a sterile initiator of IL-1β processing and release.

6.2 Key themes

Zinc regulation of inflammation occurs at the cellular level. In both human and mouse cells, depleting cellular zinc leads to an upregulation of the release of mature IL-1β. Zinc supplementation has also been shown to reduce IL-1β expression *via* the NF-κB inhibitor A20 [Morgan *et al.*, 2011][Prasad *et al.*, 2011]. Investigation into the mechanisms that produce these effects has further developed understanding of the links between inflammation and zinc deficiency.

6.2.1 IL-1β processing: inflammasome and non-inflammasome

The most well characterised mechanisms for IL-1β processing and release are those modulated by macromolecular complexes known as inflammasomes. This work shows that zinc depletion induction of IL-1β release is partially dependent upon inflammasomes. Other

modulators of IL-1β processing such as XIAP, RIPK, caspase-8 and cathepsin G have been highlighted to contribute to zinc depletion-induced IL-1β processing.

ASC is an adaptor molecule that is required for the assembly of some inflammasomes. Inflammasomes that are known to have some ASC dependence are NLRP3, NLRC4, NLRP1 and AIM2, although NLRP1 and NLRC4 can also bind directly to procaspase-1 [Case, 2011]. Zinc depletion-induced IL-1β processing and release was significantly reduced in ASC KO peritoneal macrophages. This highlighted an essential role for ASC in the zinc depletion-induced processing of IL-1β. Interestingly, levels of cell death were not reduced, indicating that the cell death induced by zinc depletion is not pyroptotic, as ASC is an essential mediator of this cell death process [Brodsky & Medzhitov, 2011].

The role of the NLRP3 inflammasome in zinc depletion- induced IL-1β processing and release is more uncertain. In mouse bone marrow derived macrophages (BMDMs) inhibition with glyburide shows reduced levels of IL-1β release. Glyburide has been shown to inhibit NLRP3 inflammasome activation [Lamkanfi *et al.*, 2009]. Additionally, in both mouse peritoneal macrophages and BMDMs, treatment with CA074-Me, (a cathepsin B inhibitor) reduced IL-1β release. Cathepsin B is known to be an activator of the NLRP3 inflammasome [Hornung & Latz, 2010]. This would indicate that NLRP3 plays a role in IL-1β release in response to zinc depletion; however zinc depletion experiments in NLRP3 KO peritoneal macrophages proved inconclusive. In human macrophage-like, PMA differentiated THP-1 cells the potential contribution of the NLRP3 inflammasome to zinc-depletion induced processing seems less likely. Neither inhibition with glyburide or CA074-Me in zinc-depleted THP-1 cells reduced IL-1β release. More investigation is required to

draw any clear conclusions from this data; however, additional experiments may show a difference in the zinc depletion-dependent regulation of IL-1β processing at the level of NLRP3.

An interesting observation in human and mouse BMDMs was the reduction in IL-1β release following the inhibition of the serine protease, cathepsin G. Cathepsin G inhibition reduced both nigericin-induced release and zinc depletion-induced release. Cathepsin G has also been reported to cleave and activate the interleukin-1 family member IL-33 [Lefrançais *et al.*, 2012]. *Mycobacterium tuberculosis* infection of THP-1 cells showed a downregulation of cathepsin G mRNA, which was suggested as a bacterial mechanism of evading the host immune response [Rivera-Marrero *et al.*, 2004]. More recently the *Mycobacterium tuberculosis* protein Rv3364c has been shown to bind cathepsin G, subsequently inhibiting caspase-1 activity and pyroptosis [Danelishvili *et al.*, 2011]. This highlights a potential role for cathepsin G in upregulating IL-1β in response to inflammatory stimuli. Reduction in zinc depletion-induced IL-1β release was far greater following cathepsin G inhibition than caspase-1 inhibition. This would suggest that following zinc depletion, cathepsin G activates other mechanisms in addition to activation of caspase-1 activity.

6.2.2 Roles of zinc in cell death

Cell death is central to zinc depletion-dependent IL-1β processing and release. Zinc depletion is a well-known activator of apoptosis [Chimienti *et al.*, 2001] and classical processing of IL-1β *via* inflammasome assembly is associated with an upregulation in the inflammatory cell death pyroptosis [Bergsbaken *et al.*, 2009]. Increasingly cell death mechanisms are being identified as contributing to inflammatory pathways. One pertinent

example of the occurrence of crossover of cell death and inflammation is illustrated by the caspase family. The caspase family has been historically divided into two groups- the inflammatory caspases and the apoptotic caspases. However, it is becoming more apparent that this delineation between the two groups is not as clear cut as it first appeared, with both groups seeming to possess the ability to induce both cell death and inflammatory processes to varying extents.

As described previously, Caspase-1 is characterised as an inflammatory caspase [Riedl & Scott, 2009]. In humans the main inflammatory caspases are caspase-1, caspase-4 and caspase-5, whereas in mice caspase-1, caspase-11 and caspase-12 are the main inflammatory caspases [Martinon & Tschopp, 2007]. Human caspases-4 and 5 [Lin *et al.*, 2000] in are thought to be orthologues of the mouse caspase-11. Caspase-12 is mostly non-functional in humans but is retains its function in mice [Saleh *et al.*, 2004].

The role of caspase-1 to IL-1β processing has been well characterised, and this protease has been identified as contributing in zinc depletion-dependent processing. However it is clear that following zinc depletion there is also a significant contribution from other proteases to the processing and release of IL-1β.

In this thesis an inflammatory role is proposed for caspase-8, which is traditionally viewed as an apoptotic caspase. The primary pro-apoptotic role of caspase-8 is the cleavage of the main effector caspase, caspase-3 [Brenner & Mak, 2009]. Caspase-8 has also been reported to cleave the pro-inflammatory cytokine interleukin-1β (IL-1β) at the same site as caspase-1, activating the cytokine to a mature secreted form [Maelfait *et al.*, 2008]. This cleavage

was reported after prolonged stimulation with LPS or poly[I:C], leading to activation of interferon-β pathways and subsequent induction caspase-8 [Maelfait *et al.*, 2008]. The assembly of an ASC-containing caspase-8 scaffold, stimulated by fungi, has also been shown to lead to cleavage of pro-IL-1β to its mature form [Gringhuis *et al.*, 2012]. Here it is shown that zinc depletion leads to activation and cleavage of caspase-8, potentially *via* a mechanism involving ripoptosome assembly. Given the evidence of caspase-8 catalysis of IL-1β cleavage at the same site as caspase-1 [Maelfait *et al.*, 2008], it is likely that this activation of caspase-8 induces IL-1β cleavage following zinc depletion.

Caspase-8 and caspase-10 cleavage have also been reported to be involved in antiviral activation of NF-κB dependent signalling [Takahashi *et al.*, 2006] and TLR4 activation of NF-κB dependent signalling. Following TLR4 stimulation, caspase-8 is recruited to IKKαβ, facilitating NF-κB transcriptional activity [Lemmers *et al.*, 2007]. Here we show that zinc depletion of LPS-primed macrophages upregulates caspase-8 activity. This mechanism was not identified in the network map, although the actions of caspase-8 did not fall within the scope of the search term. Once a dynamic model of the network map has been created, however, reactions that have been identified as modulating the TLR4 stimulated pathway can be added and the effects of these additions assessed for the contribution to the final IL-1β expression. In this way the effect of zinc upon IL-1β expression can be further explored.

Here the activation of caspase-8 as a consequence of zinc depletion is hypothesised to occur *via* the inactivation of the x-linked inhibitor of apoptosis protein (XIAP). Inhibitors of apoptosis are constitutively expressed regulators of cell death. They function to prevent cell death *via* their interactions with caspases. In humans there are three IAPs, CIAP1 and

CIAP2, which possess CARD domains and XIAP [Yang & Li, 2000]. The IAPs have three BIR domains at their N terminus and possess RING finger domains that have E3 ubiquitin ligase activity [Gyrd-Hansen & Meier, 2010].

cIAPs have also been reported to have a role in promoting inflammation as well as cell death, as they have been shown to be required for inflammasome activation and consequently caspase-1 activation. This process involves the Lys63 ubiquitination of caspase-1 without degradation [Labbé *et al.*, 2011], although this is contradictory to the findings of [Huang *et al.*, 2000] who did not see evidence of cIAP-induced caspase-1 ubiquitination.

A further link between IAPs and inflammation is the requirement for IAP function in NF-κB activation and subsequent transcription of pro-inflammatory cytokines [Gyrd-Hansen *et al.*, 2008] [Gyrd-Hansen & Meier, 2010].This is illustrated by the requirement of XIAP for the innate immune response to Listeria infection *via* the NF-κB and JNK signalling pathway [Bauler *et al.*, 2008]. Whether this function of XIAP occurs *via* caspase-8 remains to be seen.

6.2.3 Zinc in inflammatory disease.

The relevance of this work is highlighted by the increasing interplay of zinc deficiency with inflammatory disease. One example is the effect that zinc has upon metabolic disease, which is itself a major risk factor for developing other diseases such as diabetes, heart disease and stroke [Chakraborty *et al.*, 2010]. Zinc supplementation has shown to be an effective treatment for reducing markers of oxidative stress and inflammation in metabolic

syndrome in children [Kelishadi *et al.*, 2010]. In addition, low zinc status has also been observed to exacerbate inflammation in obese adults, contributing to the progression of the obese state [Costarelli *et al.*, 2010]. This interplay between zinc and inflammation in disease is complicated. By identifying one of the root causes of increased inflammation observed with decreased zinc status, the data presented in this thesis has contributed to unravelling the mechanisms behind the phenotypes of this disease state.

6.3 Systematic approaches

Two different approaches to conducting scientific investigation are represented in this thesis. The first approach begins with an observation which is then explored via further investigation of mechanisms that have been previously recognised to take part in similar reactions. The focus of this approach is upon the observation. Using inhibitors the contributions of targeted proteins to the overall effect are assessed. Whilst this approach relies upon the presence of a network of interactions this network is not made explicit. Instead the focus of this approach remains with the actions of the individual proteins.

The focus of the second approach is the network of interactions that produce a particular observation. By assembling the network of interactions that contribute to a particular observation, emergent behaviours and regulatory features of networks can be identified. In assembling a network and making reactions and interactions explicit, areas of networks that require further study can also be identified. Importantly, it is clear that this second approach cannot be undertaken without first characterising the biological field with the first approach.

A key tool of systems biology is quantitative mathematical modelling. In this way quantitative experimental data can be used to model a particular biological system. Modelling can be used for many applications, but in systems biology there are three main categories of model: predictive, exploratory and models created for the engineering of biological systems (synthetic biology). Models also function as an effective tool for communicating and analysing biological knowledge.

The network map of IL-1β emphasised the high level of regulation controlling the expression of IL-1β. A considerable portion of this regulation was in the form of negative feedback. It was also interesting to identify a role for zinc in this network that upon building the map was not immediately obvious, but upon further probing a role for zinc regulation of ubiquitination was highlighted. In this way the building the network map is just the beginning to using a systematic approach to explore IL-1β expression. By further analysing the properties of the components of the map I believe that other themes would emerge.

6.4 Future directions

The work described in this thesis could be developed further in several ways. The roles of the ripoptosome and caspase-8 function in zinc depletion-induced IL-1β could be further elucidated. Moreover, differences between the human and mouse response could be additionally characterised. Firstly, to confirm the differences observed are species dependent, the zinc depletion experiments with inhibitors of caspase-1 and cathepsin B should be repeated in another human macrophage cell line. If this is the case then mechanisms of NLRP3 activation in human and mouse zinc depletion-induced IL-1β processing could be explored. Another interesting avenue of investigation would be to

study the effects of cathepsin G in IL-1β release. As both nigericin and zinc depletion-induced IL-1β release were affected it would be interesting to see if IL-1β release induced by other stimuli, including cytoplasmic DNA, *Salmonella* infection and *Listeria monocytogenes* infection could be reduced by cathepsin G inhibition. As the network map was built as a tool to investigate IL-1β expression there is still much that can be done with the map. The most natural progression from building the network map would be to produce a dynamic model which could be used in conjunction with experimental data to investigate IL-1β production.

6.5 Summary

Zinc and zinc depletion have been shown to have a profound effect upon the production of IL-1β. Numerous cellular proteins function to induce and regulate the expression and release of this cytokine. In this thesis caspase-1, ASC, XIAP, caspase-8, cathepsin B and cathepsin G have all been shown to be essential to the regulation of zinc depletion-induced IL-1β release. Increasing awareness of the contribution of inflammation to disease states and the global prevalence of zinc deficiency make this research extremely relevant to human health and disease. Zinc supplementation in combination with anti-IL-1β drugs may in the future prove to be an effective treatment for inflammatory disease.

Appendix 1

Network Map Search String

(((Toll-Like Receptor 4 OR Lipopolysaccharides) AND (TLR4-MD2 protein complex OR Lymphocyte Antigen 96 OR Antigens, CD14 OR Lipopolysaccharide-binding protein))
OR
((Toll-Like Receptor OR Lipopolysaccharides OR TLR/IL-1R or Interleukin-1) AND (Myeloid Differentiation Factor 88 OR Interleukin-1 Receptor-Associated Kinase OR Tollip OR Pellino OR "TNF Receptor-Associated Factor 6"[Mesh] OR "Tbk1 protein, mouse" [Supplementary Concept] OR "TBK1 protein, human" [Supplementary Concept] OR Casein Kinase II OR p38 Mitogen-Activated Protein Kinases))
OR
((Interleukin-1 OR Interleukin 1 precursor) AND (Gene Expression AND (Transcription, Genetic OR Cloning, Molecular* OR Base Sequence))))

AND

((macrophages OR monocytes OR dendritic cells or Cell line or fibroblasts) OR (Toll like receptor AND (Protein binding or phosphorylation or signal transduction)))

AND

(human OR mouse OR Escherichia coli)

AND English [Language]

NOT (equine OR bovine OR sheep OR swine OR rats OR guinea pigs or hamster OR rabbits OR mollusc* OR zebrafish OR baboon or avian OR worm OR Arabidopsis OR viral OR fungi OR gram-positive bacteria OR plant OR Oncorhynchus mykiss or plasmodium)

NOT (clinical OR patient OR Children OR disease or sepsis OR Diabetes Mellitus OR cardiovascular diseases or cardiac OR arthritis OR depression OR anxiety OR ageing OR ovulation OR cigarette OR obesity OR melanoma OR asthma OR encephalitis OR paraplegia or implant or HIV or diet)

NOT (stem cells OR endothelial cells OR muscle cells OR granulocytes OR mast cells OR B cells OR T cells OR keratinocytes OR chondrocytes OR hepatocytes OR splenocytes OR osteoblast or odontoblast OR luteal cells OR keratinocyt* OR adipocyt* OR astrocyt* OR satellite cells OR foam cells or Caco-2 or PC12 or HL-60)

NOT (prostate OR liver OR nervous system OR synapse OR lung OR skeletal muscle OR ocular OR colorectal OR whole blood OR intestinal OR glomerular OR cervical OR vascular OR renal OR cardiovascular* OR pulmonary OR neurolo* OR axon OR hippocampus OR ganglion OR neurons OR synovial OR "P bodies" OR Tight Junctions OR corneal OR ovarian OR periodont* OR endometri* OR Fibrosarcoma OR Muscle Fibers)

NOT (inflammasomes OR caspase OR haemoglobin OR serum OR P2X7 OR IL-4 OR glucose OR nitric oxide OR adhesion molecule OR "ADAM proteins" OR Nrf2 OR lipid rafts OR laminin OR Glycosphingolipids OR CAP18 OR Adenosine A3 receptor OR elastase OR

calcium OR chaperone OR RhoA OR Taxol OR Immunoglobulin Fab Fragments OR Connexin 43 OR Anthocyanins OR Chemokine CCL2 OR Fut2 OR Prostaglandin OR "14-3-3" OR Collagenases OR Particulate Matter OR IL32 protein, human [Supplementary Concept] OR Receptors, Scavenger OR Receptors, Aryl Hydrocarbon OR Cathepsins OR Ceruloplasmin OR Serum Amyloid A Protein OR Aphidicolin OR glucocorticoid OR breast milk or histamine or prolactin or collagen or retinoic acid OR hormone)

NOT (chemotaxis OR synthetic OR hypoxia OR Gene Expression Regulation, Plant[Mesh] or evolution or stress or homeostasis or oncogenic)

NOT (review [publication type] OR Retracted Publication [Publication Type])

Searched on 28-3-12.

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