# Interleukin-1 signalling in disease

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Michelle E. Edye

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# Abbreviations

| | |
|---|---|
| ABC | ATP-binding cassette |
| AED | Anti-epileptic drug |
| AIM2 | Absent in melanoma 2 |
| AMPA | 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid |
| ANOVA | Analysis of variance |
| ASC | Apoptosis-associated speck-like protein containing a CARD |
| ASIC | Acid-sensing ion channel |
| ATP | Adenosine triphosphate |
| BBB | Blood brain barrier |
| BSA | Bovine serum albumin |
| CAPS | Cryopyrin associated periodic syndromes |
| CARD | Caspase recruitment domain |
| CPPD | Calcium pyrophosphate dihydrate crystals |
| CLR | C-type lectin receptors |
| CNS | Central nervous system |
| COP | CARD only protein |
| COX-2 | Cyclooxygenase-2 |
| CPPD | Calcium pyrophosphate dihidrate |
| CREB | cAMP response element-binding protein |
| CSF | Cerebrospinal fluid |
| CXCL1 | Chemokine (CXC subtype) ligand 1 |
| DAMP | Damage-associated molecular pattern |
| DMEM | Dulbecco's modified Eagle's medium |
| ECL | Enhanced chemiluminescence |
| EEG | Electroencephalogram |

| | |
|---|---|
| ELISA | Enzyme-linked immunosorbance assay |
| FBS | Foetal bovine serum |
| FS | Febrile seizure |
| GABA | Gamma-aminobutyric acid |
| h | Hour |
| HBSS | HEPES-buffered salt solution |
| i.c.v. | Intracerebroventricular |
| i.n. | Intranasal |
| i.p. | Intraperitoneal |
| i.v. | Intravenous |
| IFN | Interferon |
| IκB | Inhibitor of NF-κB |
| IKK | IκB kinase |
| IL-1 | Interleukin-1 |
| IL-6 | Interleukin-6 |
| IL-1Ra | Interleukin-1 receptor antagonist |
| IL-1RAcP | Interleukin-1 receptor I accessory protein |
| IL-1RI | Interleukin-1 receptor I |
| IL-1RII | Interleukin receptor II |
| IL-1α | Interleukin-1 alpha |
| IL-1β | Interleukin-1 beta |
| iNOS | Inducible nitric oxide |
| IRAK | IL-1R-associated serine/threonine kinase |
| KA | Kainic acid |
| KO | Knock-out |
| L | Litre |
| LDH | Lactate dehydrogenase |

| | |
|---|---|
| LPS | Lipopolysaccharide |
| LRR | Leucine-rich repeat |
| LTP | Long-term potentiation |
| MAPK | Mitogen-activated protein kinase |
| MCT4 | Monocarboxylate transporter 4 |
| Min | Minute |
| miR | Micro RNA |
| MMP | Matrix metalloproteinase |
| MSU | Monosodium urate |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MyD88 | Myeloid differentiation primary response protein |
| NACHT | Nucleotide-binding and oligomerisation domain |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NDS | Normal donkey serum |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NLR | NOD-like receptor |
| NLRC4 | NOD-like receptor family, CARD domain-containing |
| NLRP | NOD-like receptor family, pyrin domain-containing |
| NMDA | N-methyl-D-aspartic acid |
| NO | Nitric oxide |
| NOD | Nucleotide-binding oligomerisation domain |
| NR2B | NMDA receptor 2B subunit |
| ns | Not significant |
| nSMase | Neutral sphingomyelinase |
| PAMP | Pathogen-associated molecular pattern |
| PB | Phosphate buffer |
| PBMC | Peripheral blood mononuclear cells |

| | |
|---|---|
| PBS | Phosphate buffered saline |
| PD | Primary diluent |
| PepA | Pepstatin A |
| PI3K | Phosphoinositide 3-kinase |
| PKA | Protein kinase A |
| PMA | Phorbol 12-myristate 13-acetate |
| POLY-IC | Polyinosinic:polycytidylic acid |
| POP | PYRIN-domain only protein |
| PRR | Pattern recognition receptor |
| PYD | Pyrin domain |
| PYN | Pyrin |
| RA | Rheumatoid arthritis |
| RD | Reagent diluent |
| RIG | Retinoic acid-inducible gene |
| RLR | RIG-like receptor |
| ROS | Reactive oxygen species |
| RT | Room temperature |
| s.c. | Sub-cutaneous |
| SE | Status epilepticus |
| SEAP | Secreted embryonic alkaline phosphatase |
| sIL-1 | Soluble IL-1 |
| SOCS | Suppressor of cytokine signalling |
| TLE | Temporal lobe epilepsy |
| TLR | Toll-like receptor |
| TNF | Tumour necrosis factor |
| TRAF | TNF receptor-associated factor |
| UT | Untreated |

| | |
|---|---|
| Veh | Vehicle |
| WHO | World health organisation |
| WT | Wild type |
| YVAD | Ac-YVAD-CHO |
| YVAD | Ac-YVAD-cmk |

# Abstract

## Interleukin-1 signalling in disease

Michelle E. Edye
Doctor of Philosophy
The University of Manchester
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The pro-inflammatory cytokine interleukin 1 (IL-1) is involved in numerous physiological and pathological processes. It contributes to thermoregulation, sleep, feeding behaviour and notably to the exacerbation of non-communicable disorders such as cancer, heart disease, stroke and epilepsy, which are the greatest cause of mortality worldwide. Given this important role, IL-1 is tightly regulated, with regulation mechanisms present at the level of its synthesis, activation and receptor engagement. However, when studying IL-1 *in vitro*, little notice is taken of the disease microenvironment in which it acts. Acidosis is a hallmark of disease, often due to poor perfusion resulting in a shift to anaerobic respiration, a build-up of lactic acid and poor clearance of $CO_2$. Additionally, highly active infiltrating immune cells favour anaerobic respiration and can contribute to this local acidosis.

This thesis utilised primary cell cultures, cell lines and reporter cells to explore the mechanisms of IL-1 signalling under disease-relevant acidic conditions. Subsequently, a murine seizure model was developed to further explore IL-1 signalling in disease conditions *in vivo*.

This work demonstrated that acidic pH itself did not induce IL-1β release, however, it did promote release of minimally active 20 kDa IL-1β in response to damage associated molecular patterns (DAMPs) such as ATP, monosodium urate crystals or calcium pyrophosphate dihydrate crystals. The cleavage of pro-IL-1β into 20 kDa IL-1β was mediated by cathepsin D and was also induced on addition of lactic acid to the culture media. This 20 kDa IL-1β was not further cleaved to the active mature 17 kDa IL-1β thus its production limits the spread of inflammation. The intranasal administration of kainic acid induces seizures in C57Bl/6J mice, however, IL-1β was not observed acutely in this model thus the presence of 20 kDa IL-1β *in vivo* was not confirmed.

In recent years, the contribution of IL-1 to disease has become well established. However, despite successes in the development of novel therapeutics targeted at blocking IL-1 activity, such as anakinra, canakinumab or rilonacept to treat cryopyrin associated periodic syndromes, a number of studies have demonstrated poor efficacy and only minor improvements in patients when targeting IL-1. Thus further knowledge of the mechanisms of IL-1 signalling in disease is required to understand this system and develop improved novel therapeutics.

# Declaration

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Michelle E. Edye 25th September 2015

# Copyright statement

## Alternative format submission

This thesis is being presented in the alternative format to allow for incorporation of published papers. The format consists of a general introduction, three results chapters presented in manuscript form but reformatted to form a cohesive body of work, a general discussion, references and appendices containing supplementary methods and data. This format has been approved by the Faculty of Life Sciences for submission (Presentation of Theses Policy, Chapter 7, June 2014).

## Publications

**Edye ME**, Brough D, Allan SM. Acid-dependent interleukin-1 (IL-1) cleavage limits available pro-IL-1β for caspase-1 cleavage. J Biol Chem. 2015 Aug 31. pii: jbc.M115.667162. [Epub ahead of print]

**Edye, ME**., Walker LE., Sills GJ., Allan SM., Brough D. Epilepsy and the inflammasome: Targeting inflammation as a novel therapeutic strategy for seizure disorders. Inflammasome, 2014Jun; 1(1):36-43. doi:10.2478/infl-2014-0004

England H, Summersgill HR, **Edye ME**, Rothwell NJ, Brough D. Release of interleukin-1α or interleukin-1β depends on mechanism of cell death. J Biol Chem. 2014 Jun 6;289(23):15942-50. doi: 10.1074/jbc.M114.557561.

**Edye ME**, Lopez-Castejon G, Allan SM, Brough D. Acidosis drives damage-associated molecular pattern (DAMP)-induced interleukin-1 secretion via a caspase-1-independent pathway. J Biol Chem. 2013 Oct 18;288(42):30485-94. doi: 10.1074/jbc.M113.478941.

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I'm sure everyone in the Brain Inflammation Group past and present has helped me in some way or other over the years, whether providing reagents, advice, or light entertainment when times were tough. Thanks also to the many many cakes of Friday lab meetings/birthdays/general Mondays… which I have enjoyed probably too much! Special mentions, however, must go to Emily Robinson for her patience, particularly during her final year, to teach me recovery surgery; Cat Savage for teaching me cell culture; and the rest of the Brough lab for general advice on how to do science! Kat Smedley and Graham Coutts were invaluable in answering all lab-based and ordering questions and additionally Graham for teaching me immunos. Regardless of how many stats lectures I have been to, I am very grateful for the arrival of Jack Rivers-Auty in our lab with his endless knowledge and understanding of the right way to do stats. I'd also like to acknowledge all the BSF staff who helped to look after my animals and especially Brian Landamore for always having the right tool and know-how to find or fix anything!

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# Chapter 1

# Introduction

# 1. Introduction

## 1.1. Inflammation & disease

Inflammation has been known about for thousands of years, with the classical description of rubor, tumor, calor, and dolor coined in the first century AD by Celsus. Since then we have learned much about the components and mechanisms involved in inflammation. The traditional role of inflammation is as a rapid response of the innate immune system to target invading pathogens for destruction and to initiate the repair process, however, more recently, the role of inflammation when there is no pathogen, 'sterile inflammation', or where inflammation persists, (chronic inflammation,) have been described (Chen and Nunez, 2010). Non-communicable diseases (NCD) are chronic disorders such as cancer, cardiovascular disease, respiratory disorders and diabetes that are not caused by an infectious agent. Inflammation contributes to most non-communicable diseases and together they are the greatest cause of mortality worldwide (Daar et al., 2007).

## 1.2. Mechanisms of inflammation

The traditional theory of the immune response was based on the recognition of self vs non-self, where anything recognised as 'self' by the immune system is ignored but 'non-self' is attacked. However, this doesn't explain findings such as the presence of antibodies against DNA, or the lack of response to silicone, thus in 1994, Matzinger proposed the 'Danger hypothesis' (Matzinger, 1994). This suggested that it was a danger signal that initiated an immune response and this could be from something foreign, or an endogenous signal exposed or released during cellular damage. This could occur either be an active signal of cellular stress, or exposure of an internal molecule during cell death that under healthy conditions is not found outside of the cell (Matzinger, 1994). The process of inflammation therefore starts with the detection of something dangerous, either an exogenous pathogen-associated molecular pattern (PAMP) or endogenous damage-associated molecular pattern (DAMP) by pattern recognition receptors (PRRs). These can be toll-like receptors (TLRs), nucleotide oligomerisation domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs) or retinoic acid-inducible gene (RIG)-1-like receptors (RLRs) typically expressed on tissue resident macrophages. Activation of PRRs results in the release of cytokines, chemokines and adhesion molecules to recruit further inflammatory cells to resolve the threat (Chen and Nunez, 2010). Neutrophils are rapidly recruited to the site of injury followed by a later arrival of monocytes which can differentiate into macrophages

(Kataoka et al., 2014). One of the key pro-inflammatory cytokines that drives inflammation is interleukin-1 (IL-1).

## 1.3. IL-1

The IL-1 family comprises of 11 proteins, both pro-inflammatory and anti-inflammatory. The pro-inflammatory cytokines IL-1α and IL-1β represent the most widely studied. IL-1β was initially termed 'endogenous pyrogen' due to its ability to induce fever and is now known to be involved in a number of physiological and pathological processes. In addition to inducing fever via upregulating cyclooxygenase-2 (COX-2 (Horai et al., 1998), IL-1β is involved in long term potentiation (LTP), sleep regulation, regulation of insulin levels and feeding behaviour, bone formation and resorption, and proliferation of $CD4^+$ Th2 cells (Lichtman et al., 1988; Dinarello, 1996; Matsuki et al., 2003; Lee et al., 2010; Shimizu et al., 2015). Additionally, evidence showing elevated levels of IL-1β in disease tissue and exacerbation of animal models with IL-1β treatment, highlights its widespread involvement in sterile inflammation in a host of disorders, such as arthrosclerosis, diabetes, Alzheimer's disease, Parkinson's disease, psychiatric disorders, stroke and epilepsy (Zhao and Schwartz, 1998; Allan et al., 2005).

Despite only having 26 % (human) or 30 % (murine) homology in the amino acid sequence, IL-1α and IL-1β both act at the same IL-1 receptor (IL-1R1). There is some redundancy in their actions suggested by the lack of effect of individual IL-1α or β KO mice but protection in double KOs in a murine model of stroke (Boutin et al., 2001), but they do also have distinct roles. For example, in an acute model of DSS-induced colitis, IL-1α KO mice are more resistant to colitis, whereas IL-1β KO mice experience severe colitis without resolution. Here IL-1α was proposed to mediate early inflammation, whereas IL-1β was involved in repair (Bersudsky et al., 2014). IL-1α contains a highly conserved nuclear localisation sequence so can be sequestered in the nucleus to prevent its release during necrosis (Luheshi et al., 2009a) or can regulate transcription within the cell where it is produced (Werman et al., 2004). IL-1α can be membrane bound or if released on cell death, act on neighbouring cells as a DAMP to trigger inflammation and further promote the expression of IL-1 thereby amplifying the inflammatory response (Horai et al., 1998). Unlike IL-1β, IL-1α is constitutively expressed in epithelial cells, endothelial cells and keratinocytes and so can be rapidly released during necrosis and induce an early pro-inflammatory response (Rider et al., 2011). At 24 h post subcutaneous injection of supernatants from hypoxia-induced necrotic keratinocytes, IL-1α initiates the recruitment

of neutrophils, whereas at 5 days, IL-1β was more important for the recruitment of mature macrophages (Rider et al., 2011).

### 1.3.1. IL-1 expression

In the periphery, IL-1α and β are synthesised predominantly by macrophages and monocytes. In the brain, microglia (and to a lesser extent astrocytes, neurons and endothelial cells (Pinteaux et al., 2009)) produce IL-1α and IL-1β to exert central inflammatory actions (Allan et al., 2005; Luheshi et al., 2011). Endogenous expression of IL-1β is low but activation by bacterial or viral components or endogenous pro-inflammatory molecules induces the expression of 31 kDa pro-IL-1β (Allan et al., 2005). Pro-IL-1β is not biologically active and requires cleavage, for example via caspase-1, into a 17 kDa mature IL-1β to activate IL-1R1 (Dinarello, 1996). IL-1β is released in its mature form through as yet unconfirmed secretory pathway(s) (Lopez-Castejon and Brough, 2011). Pro-IL-1α is cleaved by calpain into a 17 kDa mature form (Carruth et al., 1991). Although both pro- and mature IL-1α are biologically active, pro-IL-1α has a lower affinity for IL-1R1 and at physiological levels is less active at this receptor than mature IL-1α (Zheng et al., 2013).

### 1.3.2. IL-1 release

IL-1β lacks a signal sequence thus does not follow the conventional secretion route through the endoplasmic reticulum and Golgi (Rubartelli et al., 1990). Instead, a number of secretion mechanisms have been proposed which are suggested to form a continuum depending on the type and severity of the stimulus (Figure 1.1) (Lopez-Castejon and Brough, 2011). IL-1β is synthesised on free polyribosomes and is predominantly located within the cytosol, although some is sequestered in vesicles (Lopez-Castejon and Brough, 2011). Pro-IL-1β co-localises with markers of lysosomes and late endosomes and exocytosis of these endolysosomal vesicles is one proposed mechanism of IL-1β release (Andrei et al., 1999). Similarly, IL-1β is co-localised with autophagosomal markers and starvation-induced autophagy increases IL-1β secretion from murine bone marrow-derived macrophages in response to a variety of NLRP3 (nuclear oligomerisation domain (NOD)-like receptor (NLR) pyrin domain-containing protein 3) activating stimuli (Dupont et al., 2011). Conversely, Harris and colleagues demonstrate increased IL-1β secretion if autophagy is inhibited (Harris et al. 2011). These differences were suggested to be due to different mechanisms of basal vs induced autophagy (Dupont et al., 2011).

Release of IL-1β within microvesicles or exosomes are an alternative mechanism of release that would protect IL-1β from rapid degradation in the plasma and allow IL-1β to exert its affects at a site distal to the initiating insult (Lopez-Castejon and Brough, 2011). The release of microvesicles is rapid, with IL-1β-containing microvesicles detected in the supernatant within 2 minutes following BzATP treatment of primed THP1 cells (MacKenzie et al., 2001). These microvesicles contain bioactive IL-1β and are able to stimulate IL-1R1 on other cells and induce downstream signalling (MacKenzie et al., 2001). $Ca^{2+}$ is required for exocytosis of lysosomes or microvesicles but not for IL-1β release. Components of plasma membrane (MHC-II) are released concomitantly with IL-1β, thus Qu and colleagues propose a prominent route of IL-1β secretion from ATP treated murine macrophages is the release of IL-1β in exosomes following fusion of multivesicular bodies with the plasma membrane (Qu et al., 2007). However, they, and subsequent studies have been unable to directly show the presence of IL-1β in exosomes (Monteleone et al., 2015).

A final mechanism of IL-1β release is directly through the plasma membrane either passively following cell lysis or actively via transporters. Activation of caspase-1 induces pyroptosis, a form of cell death that induces pores in the membrane which may provide a direct route for IL-1β out of the cell (Lopez-Castejon and Brough, 2011). Pro-IL-1β release is inhibited in the presence of glycine which prevents cytolysis, however, the release of mature IL-1β is not affected (Verhoef et al., 2004). Thus pyroptosis is likely to be a method for the passive release of pro-IL-1β but not involved in mature IL-1β secretion. Inhibition of ATP-binding cassette (ABC) transporters prevents IL-1β release and has therefore been suggested to mediate the transport of IL-1β across the plasma membrane. However, there is no direct evidence for this and they may instead promote IL-1β release via modulating $Cl^-$ concentration or IL-1β uptake into lysosomes for its release via lysosomal exocytosis (Andrei et al., 1999; Eder, 2009).

The mechanisms of IL-1β release need not be mutually exclusive and may depend on the stimulus, its intensity and the cell type. There remains conflicting literature on the existence and importance of each route thus further investigation is still required to determine what dictates the mechanism of release (Monteleone et al., 2015). IL-1α also does not contain a signal sequence so does not undergo conventional secretion. Unlike IL-1β, IL-1α is not found in vesicles (Rubartelli et al., 1990) and its release is likely passive during cytolytic cell death (Monteleone et al., 2015). This would fit with its role as a danger signal following cell death and an early mediator of inflammation (Luheshi et al., 2011).

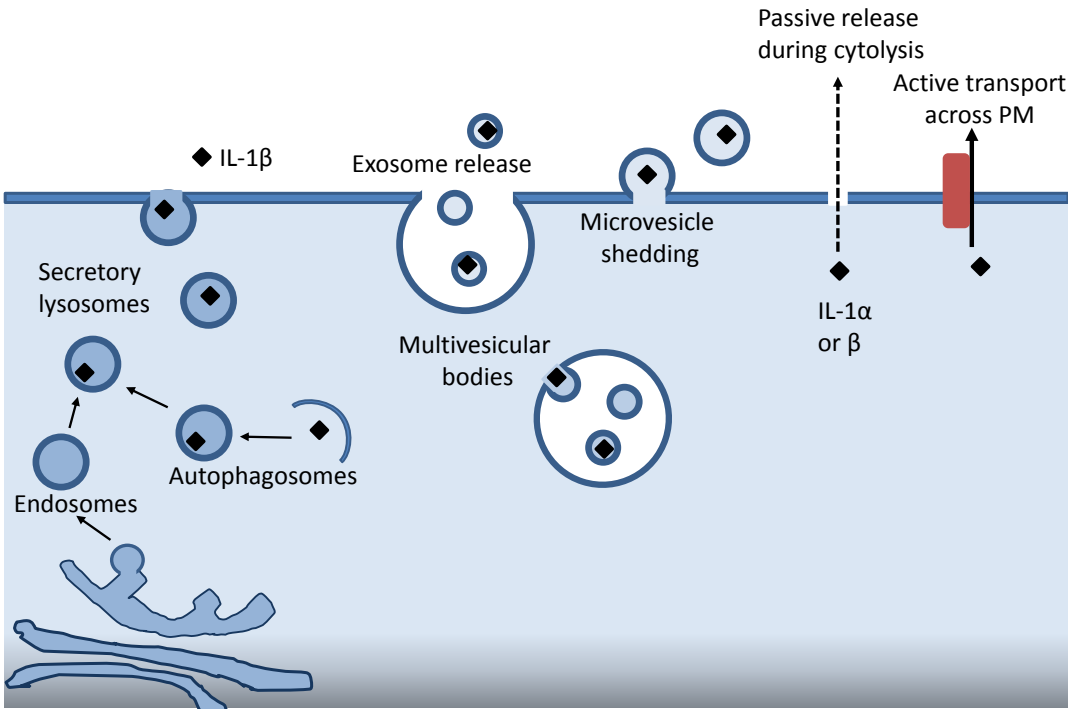


*Figure 1.1 Proposed mechanisms of IL-1 release.* Adapted from (Lopez-Castejon and Brough, 2011).

### 1.3.3. IL-1 signalling

IL-1α and IL-1β are agonists at IL-1R1 and IL-1 receptor antagonist (IL-1Ra) is an endogenous antagonist of this receptor. A decoy receptor, IL-1R2, which binds IL-1α and β but does not induce intracellular signalling has also been described. Activation of IL-1R1 by IL-1α or IL-1β recruits the accessory protein IL-1RAcP (Greenfeder et al., 1995). The formation of an IL-1R1/IL-1RAcP heterodimer results in the recruitment of myeloid differentiation primary response protein (MyD88) and IL-1R-associated serine/threonine kinase (IRAK) 4 which activates IRAK1 inducing its autophosphorylation. Dissociation from the receptor complex and association with tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF6) follows and subsequent poly-ubiquitination and recruitment of additional proteins including TAK1 results in activation of inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (IκB) kinase (IKK) 2 which phosphorylates IκB causing it to release its inhibition on NF-κB (Wesche et al., 1997; Wang et al., 2001; O'Neill, 2008). This allows the translocation of NF-κB to the nucleus where it can initiate transcription of a number of pro-inflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteinases (MMPs), COX-2, inducible nitric oxide (iNOS) and even expression of further IL-1 to amplify the inflammatory response (Figure 1.2, left panel)(Dinarello, 1996; Horai et al., 1998; Gosselin and Rivest, 2007).

### 1.3.4. Alternative IL-1β signalling

As described above, IL-1β induces NF-κB mediated transcription of pro-inflammatory signalling. However, despite expressing IL-1 receptors, hippocampal neurons do not activate NF-κB on stimulation with IL-1β as they lack the adapter protein TRAF6 (Srinivasan et al., 2004). Instead they can activate the transcription factor cAMP response element-binding protein (CREB) via activation of p38 mitogen-activated protein kinase (MAPK) (Srinivasan et al., 2004). As CREB transcribes a number of genes involved in LTP, this pathway been proposed to mediate the effects of IL-1β on LTP (Srinivasan et al., 2004). A similar activation of p38 MAPK by IL-1β has been observed in cortical neurons where it induces tau phosphorylation and reduces synaptophysin which could contribute to the IL-1β-mediated exacerbation of Alzheimer's disease (Li et al., 2003).

Low concentrations of IL-1β have been shown to exert rapid downstream signalling independent of transcription. IL-1β induces Src phosphorylation resulting in the phosphorylation of the N-methyl-D-aspartic acid (NMDA) receptor 2B (NR2B) subunit and enhanced $Ca^{2+}$ conductance (Viviani et al., 2003). An alternative central nervous system-(CNS-) specific accessory protein, IL-1RAcPb, has been described in neurons in areas across the brain and spinal cord (Smith et al., 2009). This brain-specific accessory protein is thought to co-localise with a truncated version of the IL-1R1 termed IL-1R3 (not to be confused with some papers that refer to IL-1RAcP as IL-1R3), also preferentially expressed in the brain (Qian et al., 2012). IL-1RAcPb, rather than IL-1RAcP, is required for phosphorylation of Src and NR2B and potentiation of NMDA-induced $Ca^{2+}$ currents (Huang et al., 2011). Thus the rapid IL-1β signalling described by Viviani and colleagues is likely mediated by the neuronal specific IL-1R3 and IL-1RAcPb, however, as IL-1RAcPb can complex with IL-1R1 or IL-1R3, this is unconfirmed. Additionally, Src phosphorylation has been suggested to be mediated via MyD88, neutral sphingomyelinase (nSMase) and ceramide (Davis et al., 2006b), whereas it was shown elsewhere that IL-1RAcPb is unable to recruit MyD88 (Smith et al., 2009). Thus the contribution of IL-1 receptor components and accessory proteins in rapid signalling requires further study. Recent literature has also shown that IL-1β can modulate gamma-aminobutyric acid $(GABA)_A$ signalling, reducing GABA currents up to 30 % in tissue from patients with temporal lobe epilepsy (TLE) (Roseti et al., 2015). Together this enhanced excitatory signalling and reduced inhibitory signalling

could lead to increased excitability and has been suggested to drive the IL-1β-mediated exacerbation of seizures (Balosso et al., 2008).

In addition to Src phosphorylation, IL-1β is also able to induce phosphorylation of Akt via activation of phosphoinositide 3-kinase (PI3K) which can directly interact with IL-1R1 (Davis et al., 2006a). Phosphorylation of Akt causes phosphorylation of $GABA_A$Rs resulting in an increased insertion into the plasma membrane and subsequent increase in $GABA_A$ chloride currents (Serantes et al., 2006). This is different to what is observed in tissue from people with TLE described above highlighting changes in response to IL-1β in disease. IL-1β-mediated activation of Akt also reduces $K^+$ and $Na^+$ currents and this protects against retinal ganglion cell death following optic nerve transection (Diem et al., 2003). The effects of IL-1β on channel conductance were independent of IL-1R1 and this again could suggest a role for an alternative receptor such as IL-1R3. Although despite activation of IL-1R3/IL-1RAcPb resulting in the phosphorylation of Akt, it was shown to increase an outward voltage-gated $K^+$ current rather than decrease outward $K^+$ as Diem and colleagues show (Qian et al., 2012). Some of these discrepancies may be due to differences in the experimental conditions as the effect of IL-1β is cell type, time and dose dependent (Pinteaux et al., 2009). This is exemplified by a brief incubation with IL-1β reducing $Na^+$ currents in trigeminal nociceptive neurons but addition for 24 h resulting in increased $Na^+$ currents (Liu et al., 2006).

The discovery of alternative promoter sites on the IL-1R gene and the expression of CNS-specific IL-1RAcP has furthered our understanding of IL-1 signalling (Qian et al., 2012) in immune cells and non-immune cells such as neurons. However, as IL-1R3 has only recently been described, it is unknown whether some previous IL-1β activities attributed to IL-1R1 were actually dependent on IL-1R3. Now we are aware of IL-1R3, and know it lacks the IL-1Ra binding site and is retained in IL-1R1 KO mice, these differences can be explored (Qian et al., 2012). The contribution of each receptor to IL-1 signalling can now be put together to form a comprehensive picture of the time course of IL-1β signalling across all cell types at the concentrations observed in health and disease.

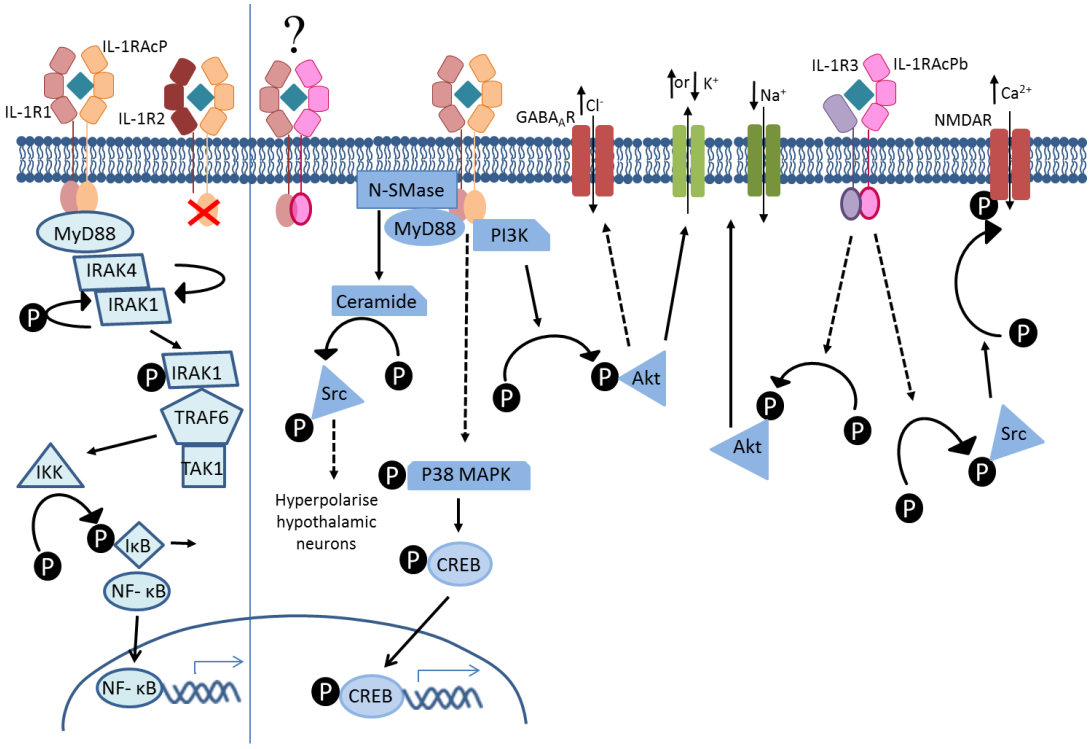


*Figure 1.2. Proposed IL-1β signalling.* The left panel describes classical IL-1β-mediated activation of IL-1R1 and NF-κB-mediated transcription. The right panel brings together literature on IL-1β-induced CREB-mediated transcription and IL-1β-induced rapid signalling alongside the contribution of neuronal IL-1R3 and IL-1RAcPb.

## 1.4. IL-1β activation

As described above, IL-1β is synthesised as a 31 kDa pro form which requires cleavage into its 17 kDa mature form for activation. This cleavage is typically carried out by the serine protease caspase-1, which itself is activated following recruitment to a large macromolecular inflammasome (Schroder and Tschopp, 2010). IL-1α release is also proposed to be part dependent on the inflammasome. This has been suggested as IL-1R2 can bind and sequester IL-1α but IL-1R2 is cleaved by caspase-1, therefore inflammasome activation would result in the release IL-1α from IL-1R2 (Zheng et al., 2013). Alternatively, inflammasome activation results in cytolysis and subsequent passive release of IL-1α (Monteleone et al., 2015).

Inflammasomes contain PRRs that can respond to a variety of PAMPs or DAMPs. Different PRRs form different inflammasomes which are activated by diverse stimuli (see Table 1.1). Most inflammasomes are activated by a specific PAMP or DAMP, i.e. double stranded DNA for AIM2 (absent in melanoma 2), but NLRP3 can be activated by a diverse array of PAMPs (such as *listeria monocytogenes* or nigericin,) and DAMPs (such as ATP or monosodium urate (MSU) crystals,) thus this inflammasome has been widely studied (Schroder and Tschopp, 2010). The activation of the NLRP3 inflammasome by DAMPs under sterile

conditions makes it a likely player in IL-1β-dependent exacerbation of NCDs. Indeed, NLRP3 KOs are protected in a number of animal models of neurodegenerative disease including Alzheimer's disease and multiple sclerosis (Gustin et al., 2015) and gain of function mutations in NLRP3 produce the autoinflammatory disorders cryopyrin associated periodic syndromes (CAPS; (Yu and Leslie, 2011)). Conversely, NLRP3 is redundant in a model of stroke where NLRC4 (NOD-like receptor family, CARD domain containing protein 4) and AIM2 were the more important inflammasomes (Denes et al., 2015). Thus NLRP3 is likely to be involved in NCDs but there may be further involvement of other inflammasomes that have not yet been described.

| PRR | Alias | Activator | Ref |
|---|---|---|---|
| NLRP1 | NALP1(b), CARD7, CLR17.1, DEFCAP, VAMAS1 | Muramyl dipeptide, *Bacillus anthracis* lethal toxin | (Boyden and Dietrich, 2006; Faustin et al., 2007) |
| NLRP2 | NALP2 | ATP | (Minkiewicz et al., 2013) |
| NLRP3 | NALP3, cryopyrin, CLR1.1, PYPAF1 | ATP, crystals (MSU, CPPD, cholesterol, silica), alum, Aβ, nigericin, *Listeria monocytogenes, Candida albicans* | (Mariathasan et al., 2006; Martinon et al., 2006; Gross et al., 2009; Schroder and Tschopp, 2010) |
| NLRP6 | NALP6 | ? | |
| NLRP7 | NALP7 | Bacterial acylated lipopeptides | (Khare et al., 2012) |
| NLRP12 | NALP12, RNO, PYPAF7, Monarch-1 | ? | |
| NLRC4 | IPAF, CARD12, CLR2.1 | Bacteria/flagellin e.g. *Salmonella typhimurium, Pseudomonas aeruginosa, Listeria monocytogenes* | (Mariathasan et al., 2004; Franchi et al., 2007; Warren et al., 2008) |
| AIM2 | PYHIN4 | Double stranded DNA, *Listeria monocytogenes* | (Roberts et al., 2009; Kim et al., 2010) |

*Table 1.1 Inflammasomes and their activators. PRR, pattern recognition receptor; NLR, NOD-like receptor; NLRP, NLR family, pyrin domain-containing; NLRC, NLR CARD domain-containing; AIM, absent in melanoma; CPPD, calcium pyrophosphate dihydrate; monosodium urate, MSU; Aϐ, amyloid beta;*

The NLR containing PRRs are comprised of a nucleotide-binding and oligomerisation domain (NACHT), a leucine rich repeat (LRR) and a caspase activation and recruitment domain (CARD) or pyrin domain (PYD), whereas the AIM2 inflammasome is composed of a PYD and HIN (Schroder and Tschopp, 2010). In the resting state, the LRR domain of NLRP3 is suggested to interact with the NACHT domain preventing the formation of a protein complex. On sensing a DAMP or PAMP, NLRP3 oligomerises and recruits the adapter protein ASC (apoptosis-associated speck-like protein containing a CARD) through pyrin-pyrin interactions. ASC then polymerises forming short filaments. The CARD domain of ASC binds the CARD domain of pro-caspase-1 which polymerises forming its own filaments stretching out from the central ASC filament. The pro-caspase-1 molecules are close enough together to induce auto cleavage and the formation of active caspase-1. Caspase-1 can then cleave pro-IL-1β into 17 kDa mature IL-1β (or pro-IL-18 into IL-18) (Schroder and Tschopp, 2010; Lu et al., 2014).

The NLRP3 inflammasome is typically expressed in myeloid cells following stimulation. In the brain, the NLRP3 inflammasome is primarily expressed in microglial cells with conflicting literature on its expression in astrocytes and neurons (Walsh et al., 2014; Gustin et al., 2015; Johann et al., 2015). The presence of active extracellular inflammasome components and caspase-1 have also been described so although pro-IL-1β released from cells is not active, it can undergo extracellular cleavage by active caspase-1 (Baroja-Mazo et al., 2014). Extracellular inflammasome components can be taken up into other cells thereby spreading inflammation (Baroja-Mazo et al., 2014). Other IL-1β-activating inflammasomes such as NLRP1, NLRP2, NLRP6, NLRP12, NLRC4 and AIM2 are expressed in myeloid cells or in differing amounts in microglia, astrocytes and neurons in control conditions or following stimulation (Gustin et al., 2015).

### 1.4.1. Priming

Although the most widely studied inflammasome, the mechanisms involved in the activation of NLRP3 remain unclear. Endogenous levels of NLRP3 are not sufficient to induce significant IL-1β activation thus the cell needs to initially undergo priming to induce the expression of NLRP3 inflammasome components and pro-IL-1β. This priming step can occur via PAMPs or DAMPs acting on TLRs, IL-1R1, TNF receptor, interferon (IFN) receptors or complement receptors typically resulting in activation of NF-κB-mediated transcription. To induce expression of inflammasome components and pro-IL-1β *in vitro*, LPS, a component of gram negative bacteria cell wall, is typically used to prime cells, however less

is known about what primes cells *in vivo* under sterile conditions. S100B, the acute phase protein serum amyloid A, complement component C5a, extracellular matrix components biglycan and hyaluronic acid, oxidised low density lipoprotein, TNFα and IL-1 itself have all been shown to induce expression of pro-IL-1β and NLRP3 to prime cells (Horai et al., 1998; Allan et al., 2005; Liu et al., 2005; Iyer et al., 2009; Tiwari et al., 2014; Cumpelik et al., 2015).

In addition to inducing the transcription of pro-IL-1β and NLRP3, LPS can induce rapid non-transcriptional priming of NLRP3 via deubiquitination of NLRP3. This results in the activation of caspase-1 which could then cleave and activate the endogenously expressed pro-IL-18 or induce other IL-1β-independent effects (Juliana et al., 2012). However, transcriptional priming to induce expression of pro-IL-1β is required before NLRP3- and caspase-1-mediated activation of IL-1β can occur.

### 1.4.2. NLRP3 inflammasome activation

Once all the NLRP3 inflammasome components are sufficiently expressed, formation of the NLRP3 inflammasome can be activated by a multitude of structurally diverse ligands, both exogenous and also endogenous (Table 1.1). It is thought that rather than directly act on NLRP3, these ligands converge on an endogenous signalling pathway. The main factors proposed in this pathway include mitochondrial stress (release of reactive oxygen species (ROS), mitochondrial DNA or expression of cardiolipin on the mitochondrial membrane), translocation of NLRP3 to the mitochondria, lysosomal disruption and release of cathepsin B, or $K^+$ efflux (Schroder and Tschopp, 2010; Muñoz-Planillo et al., 2013; Guo et al., 2015).

A number of NLRP3 activators including ATP, MSU, nigericin, asbestos and alum induce the production of ROS. Initial evidence suggested ROS produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases was key but this has been recently contradicted using human peripheral blood mononuclear cells (PBMCs) with mutated NADPH oxidase activity (van Bruggen et al., 2010). Instead, the source of these ROS is likely mitochondrial. Indeed, inhibiting complex I or III of the respiratory chain induces mitochondrial ROS and the production of mature IL-1β in a NLRP3-dependent manner (Zhou et al., 2011). Stimulation of NLRP3 causes it to translocate to endoplasmic reticulum/mitochondrial structures and translocation to the mitochondria has been suggested itself as necessary for optimal NLRP3 activation (Subramanian et al., 2013). This would bring it in contact with the mitochondrial ROS which are short-lived and do not migrate far from their source (Zhou et

al., 2011) providing further support for this method of activation. However, Iyer and colleagues have shown that direct interaction with the mitochondrial phospholipid cardiolipin could activate the NLRP3 inflammasome in a $K^+$ efflux-dependent but ROS-independent manner (Iyer et al., 2013). Additionally, treatment of LPS-primed cells with ATP or nigericin induces the release of oxidised mitochondrial DNA into the cytoplasm which can then directly interact with NLRP3 and activate it. Highlighting the interlinking nature of the NLRP3 activating pathways, LPS plus ATP results in increased mitochondrial ROS and this is able to oxidise mitochondrial DNA and the oxidised mitochondrial DNA-induced IL-1β release is dependent on $K^+$ (Shimada et al., 2012). This suggests that activation of NLRP3 via disruption of mitochondria may converge on $K^+$ efflux.

Lysosomal destabilisation and cathepsin B release into the cytosol have been suggested as pre-requisites for NLRP3 activation in response to a number of stimuli. Inhibition of cathepsin B results in an impaired NLRP3 response to silica crystals, MSU, alum or nigericin (Hentze et al., 2003; Hornung et al., 2008). Additionally, artificial disruption of lysosomes stimulates NLRP3 activity, which is not observed in cells isolated from NLRP3 KO mice (Hornung et al., 2008). However, inhibition of cathepsin B does not impair ATP-induced IL-1β release (Hornung et al., 2008) and IL-1β release on stimulation with $Al(OH)_3$, $SiO_2$, CPPD (calcium pyrophosphate dihydrate) or the lysosomal damaging dipeptide LL-OMe is not impaired in cathepsin B KO mice, suggesting lysosomal damage is not a conserved mechanism of action for all NLRP3 activators and is dispensable (Muñoz-Planillo et al., 2013). Interestingly, lysosomal damage induced by $Al(OH)_3$, $SiO_2$, CPPD or LL-OMe caused a reduction in intracellular $K^+$ prior to IL-1β release thus as with mitochondrial disruption described above, lysosomal damage-mediated activation of NLRP3 may also converge on $K^+$ efflux (Muñoz-Planillo et al., 2013).

As suggested above, movement of ions appears key to inflammasome regulation (Muñoz-Planillo et al., 2013). Increased extracellular concentrations of $K^+$, $Ca^{2+}$ and $Mg^{2+}$ inhibit LPS plus ATP-induced activation of NLRP3 (Lee et al., 2012) and $Ca^{2+}$ released from intracellular stores is required for LPS plus ATP- or nigericin-induced IL-1β release, but is not sufficient to induce IL-1β release alone (Brough et al., 2003). ATP, the prototypical DAMP, induces $K^+$ efflux and inflammasome activation through activation of P2X7 receptors and opening of the pannexin-1 hemichannel (Kanneganti et al., 2007), although there is conflicting data on the requirement of pannexin-1 on inflammasome activation. Opening of pannexin-1 allows the release of ATP during apoptosis but NLRP3, NLRC4 and AIM2 inflammasome activation

can occur independently of pannexin-1 (Qu et al., 2011). LPS and ATP-induced mature IL-1β release is prevented with inhibition of pannexin-1, despite this still resulting in low intracellular $K^+$ (Pelegrin and Surprenant, 2006). However, the majority of literature suggests $K^+$ efflux is required for NLRP3 activation and is prevented with high extracellular $K^+$. Low intracellular $K^+$ is sufficient to induce the formation of ASC specks (Fernandes-Alnemri et al., 2007) and activation of caspase-1 (Pétrilli et al., 2007) and similarly, Muñoz-Planillo and colleagues demonstrate that reducing extracellular $K^+$ is sufficient to reduce intracellular $K^+$, activate caspase-1 and induce IL-1β release (Muñoz-Planillo et al., 2013). They go on to show that unlike ROS, cell swelling, lysosomal damage, mitochondrial damage, pore formation, extracellular calcium and membrane depolarisation, a reduction in intracellular $K^+$ prior to inflammasome activation was conserved across all NLRP3-activating DAMPs studied, again supporting its role as the common trigger for NLRP3 activation (Muñoz-Planillo et al., 2013).

It has been difficult to tease apart the contribution of each cellular event to the activation of NLRP3 as lysosomal permeabilisation, mitochondrial damage, ROS and elevated intracellular $Ca^{2+}$ are all interlinked. ROS induces lysosomal damage, lysosomal damage induces ROS, lysosomal enzymes induce mitochondrial ROS generation and oxidants increase intracellular $Ca^{2+}$ which increases lysosomal permeability (Guicciardi et al., 2004). However, the current consensus seems to be heading towards $K^+$ efflux as a point of convergence on the NLRP3 activation pathway, although how $K^+$ efflux activates NLRP3 remains to be solved (Muñoz-Planillo et al., 2013).

As activation of the inflammasome seems inextricably linked to IL-1β release, it is hard to separate processes involved in NLRP3 activation and IL-1β release. Recent literature suggests that cathepsin B is involved in the starvation-induced autophagy-mediated IL-1β secretion. Thus the proposed involvement of cathepsin B in IL-1β activation could actually be due to its role in IL-1β release (Dupont et al., 2011). Additionally, IL-1α release can be independent or dependent on inflammasome activation. ATP, nigericin and *C. albicans* induce inflammasome-dependent IL-1α release (although independent of caspase-1 protease activity), whereas particulates or crystals could induce IL-1α release independently of the inflammasomes (Gross et al., 2012). One explanation for this was suggested to be that particulates and crystals induce cell death (necrosis) independently of NLRP3, whereas the other factors require NLRP3 activation to induce cell death (pyroptosis)

leading to the passive release of IL-1α. This further corroborates the proposal that IL-1α release is passive following cell death (Monteleone et al., 2015).

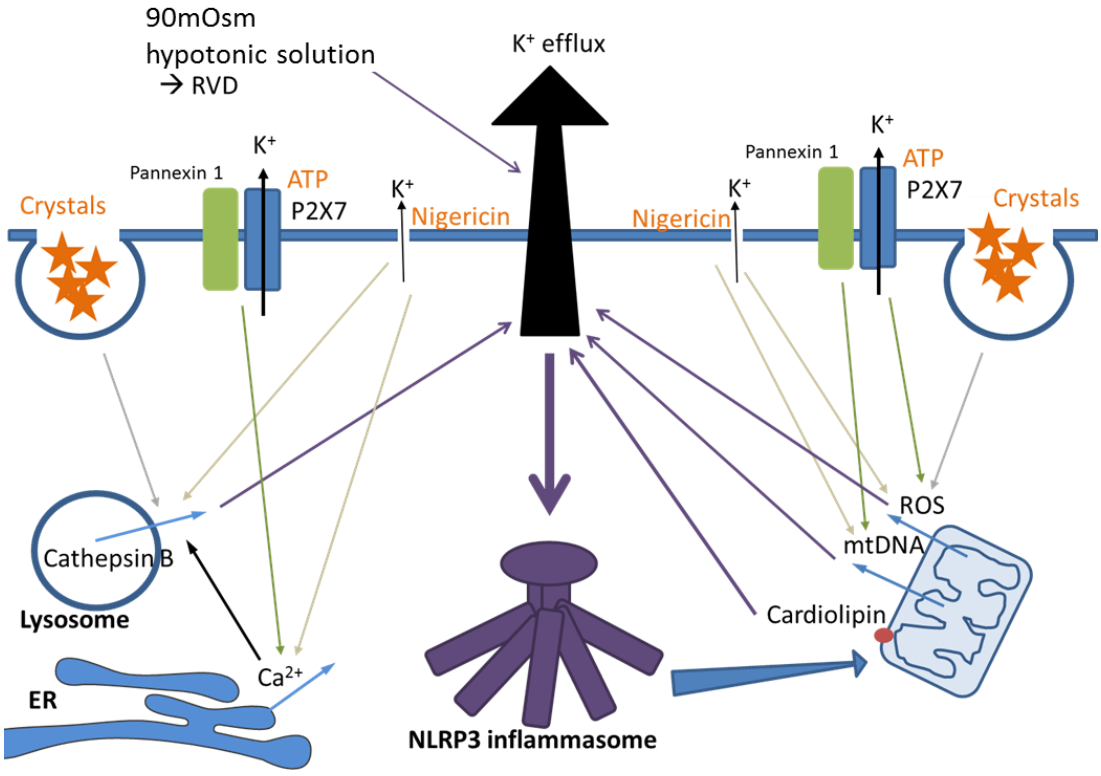


*Figure 1.3 NLRP3 inflammasome activation.* PAMPs and DAMPs induce cellular changes that are suggested to converge on $K^+$ efflux resulting in the formation and activation of the NLRP3 inflammasome.

## 1.4.3. Non-canonical inflammasomes

Non-canonical inflammasomes, which contain components in addition to the typical PRR, adaptor protein and pro-caspase-1, have recently been described. In 1998, Wang and colleagues first demonstrated the importance of caspase-11 in IL-1β release and septic shock and showed that caspase-11 could directly interact with caspase-1 (Wang et al., 1998). Interest in this area was recently re-ignited when it was found that caspase-11 is mutated in strain 129 mice and so the caspase-1 KO mice produced from this background were actually caspase-1/caspase-11 double KOs (Kayagaki et al., 2011). Caspase-11 is required for NLRP3/ASC/caspase-1-mediated activation of IL-1β on stimulation by cholera toxin B, live *E. coli*, *C. rodentium* and *V. cholerae* in LPS-primed murine bone marrow derived macrophages. Caspase-11 co-precipitates with caspase-1 suggesting it forms part of a non-cannonical inflammasome with NLRP3, ASC and caspase-1. Caspase-11 is not required for the NLRP3-mediated IL-1β release on stimulation with ATP, MSU, CPPD, nigericin, *C. difficile* toxin B, adenylcyclase toxin or listeriolysin O toxin suggesting separate

roles for the canonical caspase-1 inflammasome and non-canonical caspase-1/caspase-11 inflammasome (Kayagaki et al., 2011). Similarly, caspase-11 was not required for ATP- or *C. difficile* toxin B-mediated cell death but was required for cell death following infection with the gram negative bacteria. Interestingly, ASC was not required for cell death induced by any non-canonical stimuli suggesting caspase-11 induces cell death independently of the inflammasome. *In vivo*, caspase-11 not caspase-1 was required for resistance to mortality from LPS-induced sepsis and this is likely mediated via its induction of cell death, independent of IL-1β production (Kayagaki et al., 2011). LPS, when transfected into cells, was sufficient alone to activate the non-canonical inflammasome, independent of TLR activation (Kayagaki et al., 2013) and was later shown to directly bind caspase-11 (Shi et al., 2014).

Caspase-4 and 5 are human orthologs to murine caspase-11. As with caspase-11, caspase-4 and caspase-5 can both directly bind LPS and LPS binding induces caspase oligomerisation and activation (Shi et al., 2014), however Casson and colleagues show that intracellular LPS induces processing of pro-caspase-4 but not pro-caspase-5, suggesting that caspase-4 may be the main mediator of human non-canonical inflammasomes (Casson et al., 2015). Similar to caspase-11, caspase-4 is required for intracellular LPS-induced cell death independently of NLRP3, ASC and caspase-1, but intracellular LPS-induced IL-1β release is dependent on NLRP3 and caspase-4 in human monocytes and macrophages (Casson et al., 2015; Schmid-Burgk et al., 2015). As with canonical IL-1β release, non-canonical IL-1β release requires $K^+$ efflux (Schmid-Burgk et al., 2015). However, in human macrophages, unlike caspase-11, caspase-4 is not required for caspase-1-mediated activation of IL-1β in response to gram negative bacteria (Casson et al., 2015).

Caspase-8 is also involved in mechanisms of cell death and has separately been shown to cleave pro-IL-1β directly, independently of caspase-1 (Maelfait et al., 2008). A caspase-8 inflammasome has been described in dendritic cells where activation of Dectin 1 by various fungal pathogens or mycobacteria can induce the formation of a CARD9, Bcl-10, MALT1, ASC and caspase-8 complex that results in the release of mature IL-1β (Gringhuis et al., 2012). Additionally, a RIP1-FADD-caspase-8 complex has been described in response to Fas activation, resulting in release of mature IL-1β independent of caspase-1 (Bossaller et al., 2012), and there is contradicting literature on the involvement of RIP3 in caspase-8-mediated IL-1β release (Latz et al., 2013). Much of the mechanisms involved in IL-1β activation, particularly with respect to non-canonical inflammasomes, are yet to be fully

elucidated thus further investigation is required to understand exactly where functional inflammasomes are expressed and all the components that make them up.

## 1.5. IL-1 regulation

IL-1β is a highly potent cytokine able to induce a maximal response with less than 5 % of its receptors on a cell occupied (Arend et al., 1990). Activation of IL-1R1 results in expression of many pro-inflammatory factors and amplification of pro-inflammatory signalling, thus the production of this cytokine, its activation and receptor engagement needs to be tightly regulated (Figure 1.4 and Figure 1.5). There also needs to exist a balance to turn off the innate immune system once the adaptive immune system is activated in order to limit damage to healthy tissue (Mishra et al., 2013).

### 1.5.1. Regulation of IL-1 signalling

The endogenous IL-1R antagonist, IL-1Ra, binds IL-1R1 with high affinity but does not recruit IL-1RAcP and activate downstream signalling (Greenfeder et al., 1995). Due to the high potency of IL-1 (as mentioned above), IL-1Ra is required in a great excess, up to 100 fold higher than IL-1 to reduce 50 % activity (Arend et al., 1990). In the brain, however, this regulation is less tightly controlled as the increase in IL-1Ra is delayed compared to IL-1α or IL-1β, and it is not produced in excess (Vezzani et al., 2002). Splice variants of IL-1Ra include intracellular forms which have been described in keratinocytes and other epithelial cells which may be released on cell death or act to oppose any intracellular effects of IL-1α (Arend et al., 1998).

Soluble IL-1RAcP (sIL-1RAcP) is produced via alternative splicing. It can bind IL-1R1 but not induce further NF-κB-mediated signalling. Additionally, sIL-1RAcP can bind IL-1R2, increasing its affinity for IL-1 (Smith et al., 2003). Soluble IL-1R1 can be formed by cleavage of the extracellular domain of IL-1R1 where again it could bind IL-1β but not induce downstream intracellular signalling (Gabay et al., 2010).

IL-1R2 is similar in structure to IL-1R1 except it has a truncated intracellular signalling domain which prevents it from initiating any downstream signalling following IL-1 binding and so is considered a decoy receptor (McMahan et al., 1991; Colotta et al., 1993). It is present on macrophages, monocytes, neutrophils and B cells at rest and the soluble form (sIL-1R2) is present at high levels in plasma (Peters et al., 2013). IL-1R2 is also present on microglia and activated regulatory T cells (Pinteaux et al., 2002; Mercer et al., 2010). IL-1R2

binds IL-1β with greatest affinity, then IL-1α and IL-1Ra to a similar extent. IL-1Ra rapidly dissociates from IL-1R2 leaving it free to bind IL-1α or β. IL-1β dissociates much more slowly from IL-1R2 almost forming an irreversible bond (Arend et al., 1994). These factors contribute to the success of IL-1R2 binding and blocking IL-1α or β activity rather than binding IL-1Ra. Additionally, IL-1R2 can bind IL-1RAcP preventing it from forming functional receptors with IL-1R1 (Smith et al., 2003). Unlike IL-1R1, sIL-1R2 is also able to bind pro-IL-1β thereby preventing any extracellular cleavage to mature IL-1β (Symons et al., 1995). Intracellular IL-1R2 has also been shown to bind pro-IL-1α, thereby preventing its cleavage and activation (Zheng et al., 2013). The importance of regulation by IL-1R2 has been confirmed in disease models as IL-1R2 KO mice show increased arthritis severity (Shimizu et al., 2015) and mice over expressing IL-1R2 in keratinocytes are protected in acute and chronic models of skin inflammation (Rauschmayr et al., 1997).

The orphan receptor TIR8 (SIGIRR) acts as a negative regulator of the IL-1 and TLR systems. It interacts with IL-1R1 and downstream signalling components such as MyD88, TRAF6 and IRAK causing a reduction in IL-1-induced NF-κB signalling (Wald et al., 2003). It also disrupts the interaction between IL-1R1 and IL-1RAcP (Qin et al., 2005). As IL-1 treatment can induce the interaction between TIR8/SIGIRR and IL-1R1 components, this can act as a negative feedback mechanism for IL-1 to limit its own inflammatory effects (Qin et al., 2005).

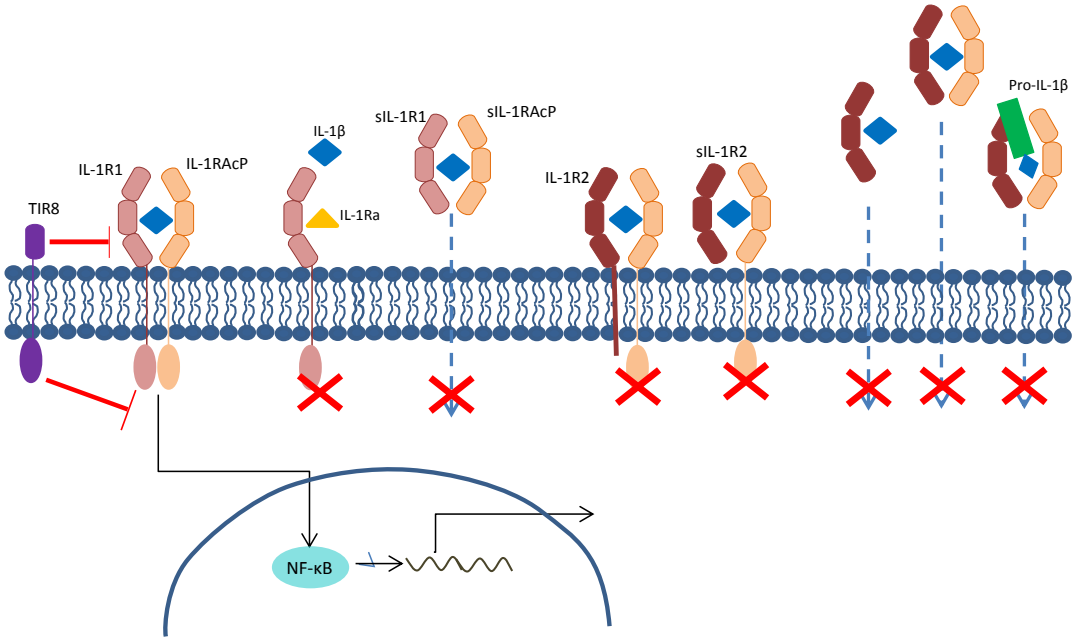


*Figure 1.4. Regulation of interleukin 1 signalling.* IL-1 can be regulated at the level of receptor activation. Multiple combinations of receptor complexes can form and bind IL-1 but only activation of IL-1R1 and IL-1RAcP results in NF-κB-mediated downstream signalling.

## 1.5.2. Regulation of IL-1 activation

IL-1 production is also regulated at the level of its activation by caspase-1 and the inflammasomes. A number of regulators can directly act on the inflammasome, either to inhibit the expression of NLRP3 components (priming) or the formation of the inflammasome complex and its activation (Latz et al., 2013).

At the level of priming, microvesicles released from neutrophils inhibit the expression of NLRP3 and pro-IL-1β. These ectosomes express phosphatidylserine on their surface which can engage the receptor tyrosine kinase MerTK, activating SOCS (suppressor of cytokine signalling) 3 and inhibiting expression of pro-IL-1β and NLRP3 (Cumpelik et al., 2015). Addition of exosomes after priming also reduces MSU-induced mature IL-1β release (Cumpelik et al., 2015).

Micro RNAs (miRs) are endogenous non-coding short strands of RNA that can bind to the 3' untranslated region of mRNA, decreasing mRNA levels. miR-223 is expressed in myeloid cells where increased expression of miR-223 inversely correlates with NLRP3 expression (Bauernfeind et al., 2012). Overexpression of miR-223 leads to a reduction in NLRP3 and a subsequent reduction in IL-1β release on stimulation with ATP or nigericin. Accordingly, blocking miR-223 has the opposite effect. As miR-223 is differentially expressed in different cell types, this can set the threshold for NLRP3 activation in different cells (Bauernfeind et al., 2012). Several miRs have also been described to negatively regulate TLR signalling components which could further inhibit priming (O'Neill et al., 2011).

Indirect inhibition of the NLRP3 inflammasome is possible by blocking one of the NLRP3 activation pathways described earlier. For example, Youm and colleagues show that the ketone body β-hydroxybutarate inhibits NLRP3 inflammasome by inhibiting $K^+$ efflux thereby preventing ASC oligomerisation and formation of the NLRP3 inflammasome (Youm et al., 2015).

Direct inhibition of the formation of the inflammasome complex can also occur. This can occur at the level of pro-caspase-1 by proteins only expressing CARD domains (CARD only proteins; COPs) such as CARD16 and CARD18. These bind the CARD domain of caspase-1 preventing it's interaction with ASC (Latz et al., 2013). Similar PYRIN-domain only proteins such as POP1 and POP2 exist and these prevent the interaction between ASC and NLRP3 (Latz et al., 2013). Additionally, splice variants of ASC that have different effects on inflammasome formation and activation have been described. ASCb lacks the linker domain between PYD and CARD and forms less active NLRP3 inflammasomes than ASC. ASCc just

contains the CARD domain so binds caspase-1 but not NLRP3 thereby inhibiting NLRP3 activity by acting as a COP (Bryan et al., 2010).

Additionally, activation of IL-1R1 or TLRs can induce the expression of iNOS which increases nitric oxide (NO). NO thio-nitrosylates NLRP3 preventing its oligomerisation and therefore activation of caspase-1 (Mishra et al., 2013). The fact that IL-1β can activate this pathway suggests another mechanism of negative feedback by IL-1. Alternatively, the production of IL-1 can be regulated via interferon-mediated production of iNOS. IFNβ and IFNγ are released from T cells or NK cells following activation of the adaptive immune system so this pathway could protect healthy tissue from further non-specific damage by shutting down the innate immune response once the adaptive immune system is activated.

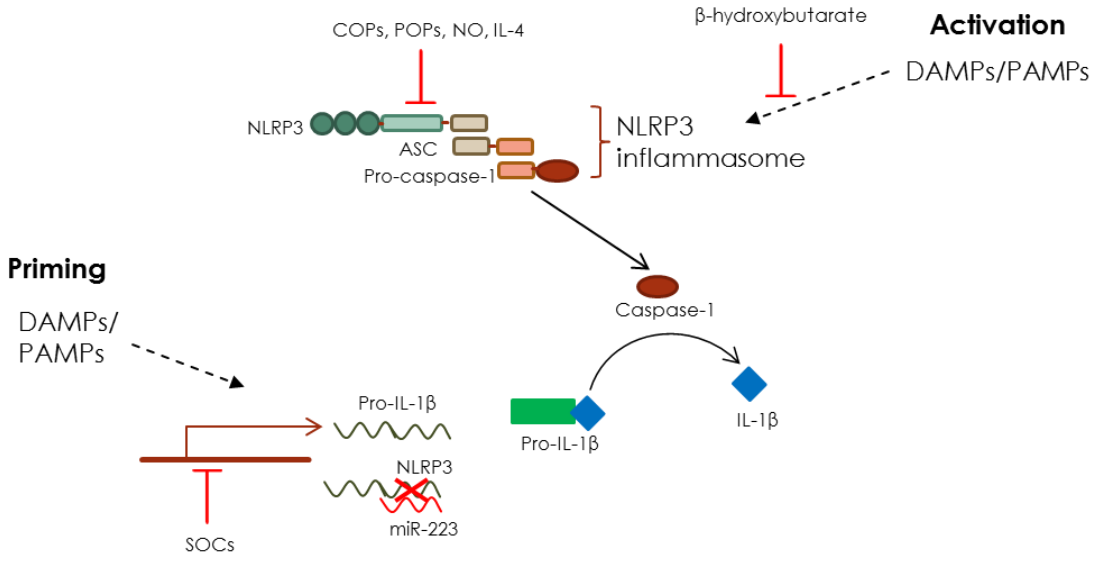


*Figure 1.5. Regulation of inflammasome activation.* Production of mature IL-1β can be regulated at the level of expression of pro-IL-1β and NLRP3, and activation of NLRP3 inflammasome formation.

Recent literature suggests crosstalk between the adaptive and innate immune systems. Direct contact of macrophages or dendritic cells by activated $CD4^+$ T cells specifically inhibits NLRP1- or NLRP3- mediated caspase-1 activation and release of IL-1β, without affecting other inflammatory mediators. The authors speculate that this could help with the transfer from an initial non-specific inflammatory response to a more directed one (Guarda et al., 2009). IL-4, a Th2 anti-inflammatory cytokine reduces transcription of pro-inflammatory genes but also has a recently described transcription-independent role. IL-4 disrupts NLRP3 oligomerisation, ASC recruitment and ASC polymerisation. It may do this through disruption of microtubule polymerisation and subsequent inhibition of NLRP3

relocation to mitochondria. Although whether inhibition of inflammasome formation or relocation comes first is unclear (Hwang et al., 2015). Interestingly, a novel role for NLRP3 in regulating transcription of IL-4 independently of inflammasome formation and IL-1β activation has been suggested. Bruchard and colleagues show that NLRP3, present in the nucleus of Th2 cells, can bind DNA alongside the transcription factor IRF4 to induce IL-4 expression and Th2 differentiation (Bruchard et al., 2015). Taken together, this would suggest that NLRP3 can induce a negative feedback pathway where it increases IL-4 expression which in turn reduces NLRP3 formation. This would be another way that the innate immune system could be turned off once the adaptive immune system is activated.

### 1.5.3. Caspase-1-independent IL-1β

Pro-IL-1β contains cleavage sites for multiple proteases in addition to caspase-1 (Figure 1.6). Thus a number of proteases are able to regulate IL-1β activation by cleaving pro-IL-1β into mature proteins. As IL-1β activity decreases the further from the caspase-1 cleavage site and the larger the IL-1β fragment produced (Black et al., 1988; Hazuda et al., 1991), it would therefore be intriguing to postulate whether any of the larger IL-1β proteins could in fact be partial agonists or antagonists at IL-1R1. Indeed, a recent paper by Davaro and colleagues shows that statin-induced 28 kDa IL-1β inhibits mature 17 kDa IL-1β signalling (Davaro et al., 2014).

Caspase-1-dependent cleavage of IL-1β was the focus of much early research into the role of IL-1β in disease. However, it was repeatedly found that knocking-out caspase-1 was not protective in disease models (such as arthritis, peritonitis, lung inflammation, osteomyelitis and epidermolysis bullosa acquisita,) despite protective effects of blocking IL-1β (Guma et al., 2009; Joosten et al., 2009; Provoost et al., 2011; Lukens et al., 2014; Sadeghi et al., 2015). This suggested that other pro-IL-1β cleavage proteases may be relevant in disease. Neutrophils contain multiple proteases and are rapidly recruited to sites of inflammation, particularly in diseases such as arthritis, gout and pulmonary inflammatory disorders (Afonina et al., 2015). Joosten et al. show inhibition of neutrophil protease proteinase 3, alongside genetic ablation of caspase-1 is required for protection in acute arthritis, as caspase-1 KOs alone were not protected (Joosten et al., 2009). At day 28 in a model of chronic arthritis, caspase-1 KO mice do display protection against joint inflammation, cartilage damage and bone erosion likely reflecting the more prevalent role of

macrophages over neutrophils at this late stage in disease (Joosten et al., 2009). Similarly, mast cells contribute to inflammation, and inhibition of mast cell chymase in caspase-1 KO mice is also protective in arthritis (Guma et al., 2009). Inhibition of elastase and chymase are also protective in models of gout and peritonitis, however these proteases also display redundant roles as inhibition of multiple proteases is required for effective protection (Guma et al., 2009). This suggests that targeting one protease alone may not been a viable therapeutic option, rather combined treatments may be required. Additionally, these proteases have multiple targets therefore using combined low dose inhibitors would also have the benefit of utilising lower doses of each inhibitor thereby potentially not completely inhibiting other beneficial effects of the proteases (Afonina et al., 2015).

In addition to neutrophil and mast cell proteases, the ubiquitous lysosomal protease cathepsin D can also cleave pro-IL-1β, although this results in a 20 kDa fragment rather than mature 17 kDa IL-1β (Takenouchi et al., 2011). 20 kDa IL-1β has also been observed in response to hypotonic solution (Perregaux et al., 1992), cleavage by HIV protease (Hazuda et al., 1991) or in mouse submandibular gland following i.p. LPS (Yao et al., 2005). However, the relevance of this cathepsin D-mediated IL-1β in health or disease is not yet known.

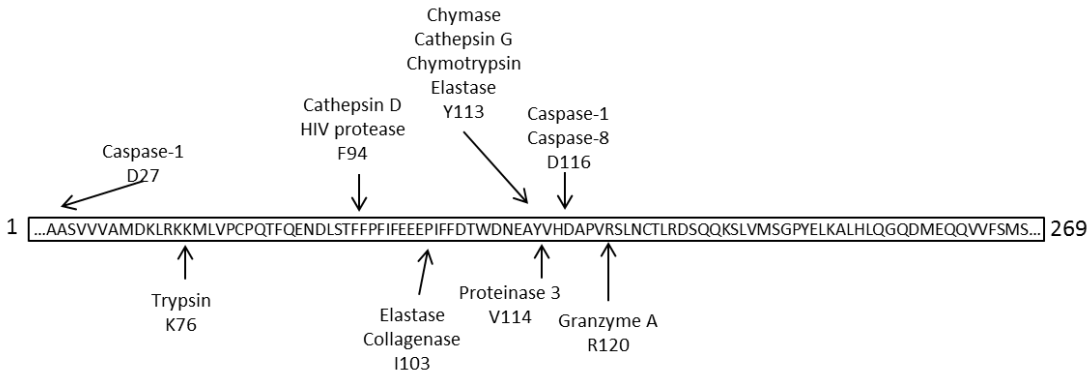


*Figure 1.6. Pro-IL-1β cleavage sites.* Proposed pro-IL-1β cleavage enzymes and their sites of cleavage. Adapted from (Dinarello, 2011b).

Despite caspase-1 inhibitors demonstrating effective reduction in mature IL-1β *in vitro*, the studies described above emphasise the complexity of the intact animal and perhaps suggest caspase-1 is less important in IL-1β activation than may have originally been thought (Netea et al., 2014). This highlights the importance of considering the complete disease microenvironment when performing *in vitro* studies.

## 1.6. Disease microenvironment

Cells are surrounded by, and interact with, extracellular matrix, extracellular fluid, neighbouring cells and a vast array of soluble factors or cell-associated molecules. Insult or injury can induce changes in the local microenvironment including changes to cell morphology, cell-cell interactions and changes to the composition of the extracellular matrix and extracellular fluid. For example structural changes in the extracellular matrix, changes to the vasculature, infiltrating immune cells, infiltrating pathogens, cytokines, chemokines, DAMPs and alteration in surface receptors can all be observed (Knezevic et al., 2001; Sands and Mooney, 2007). This disease microenvironment is often associated with a reduction in pH. The inflammation that occurs as a response to injury results in infiltration of active immune cells which can result in a reduction in pH via increased glycolysis and lactate production through TLR-mediated upregulation of genes involved in glycolysis and downregulation of those involved in the Krebs cycle (Menkin and Warner, 1937). If a disruption to blood flow is observed, for example during stroke or myocardial infarction, oxygen supply to the tissue cannot meet demand and $CO_2$ is not effectively removed. Poor oxygen delivery causes a shift to anaerobic respiration and lactic acid is produced (Orlowski et al., 2011) resulting in a drop in pH as low as pH 6.2 (Nemoto and Frinak, 1981). During a seizure, neurons display increased firing and this can also result in anaerobic respiration and a fall in pH (Wang and Sonnenschein, 1955; Duffy et al., 1975; Somjen, 1984). Similarly, tumours, characterised by their proliferative nature, are also associated with anaerobic respiration and acidic pH, although this can occur even in the presence of sufficient oxygen (Warburg effect; (Vander Heiden et al., 2009). Interestingly, increased glycolytic activity and subsequent drop in pH has been shown to correlate with disease severity in patients with rheumatoid arthritis (RA) (Fujii et al., 2015). Mechanistically, this reduction in pH is driven by up-regulation of monocarboxylate transporter 4 (MCT4) on synovial fibroblasts resulting in increased transport of lactate from the fibroblasts into the synovial fluid as a way to control the increase in lactate production due to the increased activity as these cells proliferate (Fujii et al., 2015). Low pH is also associated with asthma, sepsis, diabetes, meningitis, COPD, gouty arthritis and Alzheimer's disease (Hunt et al., 2000; Wang and Xu, 2011).

### 1.6.1. Inflammation under acidic pH

As described above, the inflammatory microenvironment is often associated with a drop in pH, leading researchers to investigate if this has an effect on inflammatory signalling.

Indeed an acidic pH has been shown to affect multiple cells of the innate and adaptive immune system. At pH 6.5-7.0, activation of neutrophils was observed as demonstrated by a transient increase in intracellular calcium, a shape change and enhanced surface expression of CD18 (involved in neutrophil adhesion). Activation of neutrophils in response to traditional agonists was also enhanced at acidic pH, and this was observed alongside a reduction in apoptosis and increase in cytotoxicity (Trevani et al., 1999). Acidic pH also induces maturation of dendritic cells and enhances their activation of T cells (Martinez et al., 2007). Whereas acidic pH (pH 6.4 and 6.7) was associated with a reduction in activity of natural killer cells (Loeffler et al., 1991). Meanwhile eosinophils show opposing effects to acidic pH. pH 6.2 has been shown to increase necrosis (Hunt et al., 2000) whereas pH 6.0-7.0 has been shown elsewhere to increase viability of eosinophils in a GPR65-, cAMP-dependent manner (Kottyan et al., 2009).

### 1.6.2. IL-1 and low pH

Unsurprisingly, as cytokines and specifically pro-inflammatory IL-1β are involved in inflammatory signalling by a number of the above described cell types, the role of acidic pH in the expression and release of cytokines has been explored. In monocytes, but not macrophages, a reduction in pH to 6.5 increases expression of pro-IL-1β and the release of mature caspase-1-dependent IL-1β, with no change to IL-6 or TNF-α release or cell viability. The reduction in extracellular pH was associated with a reduction in intracellular pH, and reducing intracellular pH with pharmacological inhibition of the sodium-hydrogen exchanger, mimicked the extracellular pH 6.5-mediated release of IL-1β. This suggests that the low pH-induced IL-1β release is mediated, at least in part, via an intracellular decrease in pH causing an increase in pro-IL-1β expression (Jancic et al., 2012).

On stimulation with LPS, monocytes induce IL-1β release without the need for a second stimulus. This is due to the ubiquitous expression of caspase-1, thought to be induced by the continuous release of ATP (Netea et al., 2009). Thus in monocytes, low pH-induced expression of pro-IL-1β would result in release of mature IL-1β due to the presence of active caspase-1, however, macrophages, which do not constitutively express active caspase-1, would not, thereby explaining cell-specific differences observed by Jancic and colleagues (Jancic et al., 2012). In addition to increasing expression, caspase-1- and NLRP3-dependent IL-1β release is also induced at acidic pH. Again this was mimicked by blocking $H^+$ export from the cell, highlighting the role for intracellular acidic pH (Rajamäki et al., 2013). Rajamäki and colleagues did not observe any change in IL-1β expression with low

pH, although this was in cells already primed thus additional expression may have been hard to determine. They did however, observe an increase in NLRP3 expression with low pH thus pro-IL-1β expression may have been increased in a similar manner (Rajamäki et al., 2013). Conversely, in microglia, acidic pH (pH 7.0-6.4) does not induce expression of pro-IL-1β but does reduce LPS-mediated IL-1β expression of pro-IL-1β and IL-1β release, which at pH 6.8-7.0 was mediated via inhibition of LPS-induced phosphorylation of ERK/JNK through the proton-sensitive receptor TDAG8, cAMP and protein kinase A (PKA). At pH 6.4 reduction in IL-1β was independent of this pathway (Jin et al., 2014). The effect of low pH on the release of mature IL-1β from LPS-primed microglia following NLRP3 activation was not investigated in this study. Takenouchi and colleagues similarly showed that a reduction in pH reduced the release of pro-IL-1β from LPS-primed mouse microglial MG6 cell line. They further showed that the response of microglia to traditional DAMPs, such as ATP, under acidic conditions is altered compared to neutral pH. At acidic pH, a reduction in release of caspase-1 is observed concomitantly with the release of a caspase-1-independent, but cathepsin D-dependent 20 kDa form of IL-1β in addition to mature IL-1β (Takenouchi et al., 2011). These reports suggest a cell type- and primed state-dependent effect of pH on IL-1β expression and release.

Besides a direct drop in pH of the cell culture media, addition of lactate, or a reduction of oxygen to induce hypoxia (both associated with a fall in pH) have also been shown to potentiate IL-1 release (Ghezzi et al., 1991; Andersson et al., 2005). Although similar to the results described above, a reduction in pH alone did not increase IL-1 release from microglia (Andersson et al., 2005). Hypoxia potentiated LPS-induced IL-1 (α and β) and TNFα release but did not significantly increase IL-1β expression (Ghezzi et al., 1991).

Although microglia have been referred to as the ‘macrophages of the brain’, there are differences between macrophages and microglia which may reflect differences observed in IL-1 signalling in response to acidic pH (Greter et al., 2015). Little is known about the effect of low pH on IL-1α expression and release and further research is required to fully understand the effect of hypoxia and low pH on the expression of IL-1β and the form of IL-1β released; whether this is cell type specific or dependent on the type of stimulus and whether the low pH is present during priming or release. Some of the results observed may have been due to species differences in IL-1 signalling pathways. IL-1 expression is regulated by NF-κB-mediated transcription. NF-κB is typically composed of p50 and p65 subunits which induce the expression of multiple inflammatory genes. The p50 subunit

does not have a transactivation domain thus needs to be paired with a subunit that does to induce transcription. A p50 homodimer does exist which therefore acts as a transcriptional repressor. In mouse but not human macrophage cells, a reduction in pH causes increased NF-κB binding to DNA in response to TNF which was more pronounced for the p50 homodimer. This was associated with a reduction in LPS-mediated TNF release at acidic pH in mouse macrophages but not human. Overexpression of the p50 subunit in human macrophages resulted in similar a reduction in LPS-mediated increase in TNF (Gerry and Leake, 2014).

Whether the acidic pH observed in so many diseases is protective or detrimental likely depends on a number of factors. There is considerable literature on the low pH observed in seizure exerting a protective role by terminating the seizure (Ziemann et al., 2008) and lactate has also shown to be protective in organ damage in murine models of hepatitis or pancreatitis (Hoque et al., 2014).

## 1.7. Epilepsy

IL-1 is involved in the exacerbation of multiple disorders including stroke, chronic heart disease, diabetes, Alzheimer's disease and epilepsy (Allan et al., 2005). Epilepsy is a highly prevalent brain disorder characterised by recurrent spontaneous seizures that affects over 50 million people worldwide. The term epilepsy covers a broad spectrum of diseases with varying severity, duration and frequency of attacks (World Health Organisation (WHO), 2009). Over 40 types of epilepsy have been described depending on whether they involve generalised or partial seizures, have a known cause or not, and on their location of origin. Epilepsy can develop following brain trauma or infection but the majority of seizures have no known cause and are termed idiopathic (Roger et al., 1989). Due to the idiopathic nature of epilepsy and the lack of understanding of the mechanisms underlying the development of epilepsy, antiepileptic drugs (AEDs) remain ineffective treatments for nearly a third of people with epilepsy. Seizures are driven by hyperexcitability and/or synchronicity of neurons and so AEDs typically target this imbalance in excitation and inhibition to redress the balance and reduce the chance of a seizure (Sills, 2011). This however does not target the underlying changes that may have led to the development of epilepsy. Further understanding of pathways involved in the development and exacerbation of epilepsy have uncovered a key role for inflammation.

### 1.7.1. Inflammation in epilepsy

Interest in the role of inflammation in epilepsy stemmed from a study by McQuarrie et al., where desoxycorticosterone acetate, a steroid hormone with mineralocorticoid activity, prevented seizures in a patient with intractable epilepsy (Mcquarrie et al., 1942). More recently, pro-inflammatory molecules such as IL-1 and inflammasomes have been implicated in the exacerbation of seizure and epilepsy (reviewed in (Edye et al., 2014).

### 1.7.2. Interleukin-1 in epilepsy

IL-1β and IL-1R1 are upregulated in experimental models of seizure and epilepsy (Eriksson et al., 1999; Vezzani et al., 1999; Ravizza and Vezzani, 2006; Dube et al., 2010). Similar increases in IL-1 or inflammasome expression post seizure have also been observed clinically in human brains from patients with TLE (Ravizza et al., 2008a) and other seizure disorders such as Rasmussen's encephalitis and malformations of cortical development (Ravizza et al., 2006a; Ramaswamy et al., 2013). Additionally, elevated IL-1 has been observed in the cerebrospinal fluid of children with febrile seizures (Haspolat et al., 2002).

In rodents, addition of exogenous IL-1β exacerbates febrile seizures, kainic acid-, bicuculline- or electrically-induced seizures which can be prevented with pre-treatment of IL-1Ra (Vezzani et al., 1999, 2000, 2002; Heida and Pittman, 2005). Additionally, seizure onset is delayed in mice lacking IL-1R1 (Vezzani et al., 2000). Taken together, these studies provide compelling evidence for a role for IL-1β in the exacerbation of epilepsy. Therefore, targeted treatments against these molecules are being developed, with the caspase-1 inhibitor VX-765 in trials for treatment resistant partial epilepsy (ClinicalTrials.gov, 2010). However less is known about molecules upstream of caspase-1 in the exacerbation of seizures and development of epilepsy and whether specific inflammasome components are involved.

### 1.7.3. Mechanisms of IL-1β-mediated exacerbation of seizure

The actions of IL-1β on seizure are thought to be driven in part by activating the fast signalling pathways that increase neuronal excitability, described earlier (Balosso et al., 2008). Additionally, IL-1β has been shown directly or indirectly to reduce glutamate uptake, increase translocation of AMPA receptors into neuronal membranes or increase the internalisation of GABA receptors (Ye and Sontheimer, 1996; Bezzi et al., 2001; Stellwagen et al., 2005). Additionally, IL-1β can cause disruption to the blood-brain barrier resulting in albumin infiltration which decreases expression of Kir4.1 and the astrocytic glutamate

transporters SLC1A2 and SLC1A3 thereby further increasing extracellular $K^+$ and glutamate and therefore excitability (David et al., 2009). Activation of IL-1R1 can also result in more long-term changes to transcription which may alter neurogenesis, cell death and subsequent synaptic reorganisation inducing an epileptogenic brain (Maroso et al., 2011).

IL-1β is often detected in areas of neuronal death following seizures and has been suggested to contribute to the cell death. The activation of IL-1β through the formation of the inflammasome induces caspase-1-mediated pyroptosis (Bergsbaken et al., 2009) and IL-1β potentiates NMDA-mediated cell death (Viviani et al., 2003). Cell death occurs in a number of animal models of epilepsy and can mimic the hippocampal sclerosis observed in TLE. Cell death has been shown to peak at 24h and 4 days following kainic acid induced seizures (Eriksson et al., 1999). This suggests a prolonged effect that may contribute to the long term changes in neuronal circuits in epilepsy. Ben-Ari and Dudek summarised the potential effect of cell death on epileptogenesis suggesting that inhibitory cell loss and the formation of new excitatory circuits with abnormal excitatory synapses may contribute to hyperexcitability and susceptibility to spontaneous seizures (Ben-Ari and Dudek, 2010).

## 1.8. Experimental seizures & epilepsy

Of the many types of epilepsy described to date, TLE is the most common form and accounts for 30-50% of all epilepsies. It is frequently associated with hippocampal sclerosis and is one of the most treatment resistant forms of epilepsy (Ben-Ari, 1985). Thus it is a relevant form of epilepsy to try to model to help further understand the mechanisms involved in treatment resistant epilepsies and uncover new targets for development of novel treatments. TLE is often found in patients with a history of prolonged febrile seizures (Mathern et al., 1995). Complex febrile seizures have been associated with an upregulation of IL-1 and there is evidence to suggest that complex febrile seizures increase the likelihood of developing TLE later in life (Annegers et al., 1987; Mathern et al., 1995; Dube et al., 2006) thus some experimental models mimic infection and fever early in life to study subsequent seizure development. However, this requires long studies from birth to adulthood so in addition to these febrile models, genetic mutations in mice that reduce seizure threshold or induce spontaneous seizures are commonly used (Grone and Baraban, 2015). Alternatively, chemically- or electrically-induced seizure models can be a simple way to induce recurrent spontaneous seizures that can mimic treatment resistant TLE with hippocampal sclerosis (Nissinen et al., 2000; Lévesque and Avoli, 2013). Recording differences in spontaneous seizures, however, can be expensive and time consuming so

initial studies may focus on changes in acute seizure parameters. Alternatively, kindling, following repetitive chemical or electrical stimulation over a short time period, can be a way to show changes in seizure threshold and the development of generalised seizures although its relationship to human epilepsy is unclear (White, 2002).

With the development of more sophisticated hardware, electrical activity in the brain during seizure events can be recorded in addition to observing behavioural seizures. This is more sensitive than behaviour to detect small changes in electrical activity (Bergstrom et al., 2013) thus milder seizure models can be used and systems now exist that allow remote recording from freely moving animals in their home cage thereby reducing stress for the experimental animal.

Despite differences in brain architecture and complexity across species, mathematical models suggest that the fundamental mechanisms underlying the generation and termination of seizures are conserved from flies and fish to mice and man (Jirsa et al., 2014).

## 1.9. Targeting IL-1 – success and concerns

Compounds aimed at the IL-1 system are successfully used for the treatment of a number of inflammatory disorders and there are a great many more undergoing trials. Recombinant IL-1Ra, anakinra, is licenced for use in RA and CAPS and has shown positive effects in clinical trials for many more conditions such as gout, stroke, diabetes, Behçet's disease and heart failure (Dinarello et al., 2012). The soluble IL-1R1/AcP complex rilonacept is also approved for use in some CAPS and again further clinical trials have been completed for disorders such as gout, arthrosclerosis and chronic kidney disease (Dinarello et al., 2012). The humanised monoclonal antibody against IL-1β, canakinumab, which would spare the early IL-1α-induced sterile inflammation, is approved for CAPS and systemic juvenile idiopathic arthritis. It is also in clinical trials for multiple indications including diabetes, gout, RA, osteoarthritis and chronic obstructive pulmonary disorder (Dinarello et al., 2012). No IL-1α specific antibodies have been approved but trials are underway in cancer and type 2 diabetes (Dinarello et al., 2012). In clinical trials, the long acting IL-1R1 blocking antibody AMG108 significantly but only moderately improves RA symptoms (Cardiel et al., 2010) and does not offer significant improvement in osteoarthritis measurements (Cohen et al., 2011) suggesting targeting IL-1 alone may not always provide sufficient therapeutic benefit in these disorders.

Targeting further up the IL-1-activating pathway with P2X7R antagonists or inhibiting caspase-1 or NLRP3 has also been investigated. P2X7R antagonists AZD9056 and CE 224, 535 have had variable success and some reports of side effects in clinical trials for RA, osteoarthritis, Crohn's disease and COPD with significant improvements observed for patients with RA and Crohn's disease taking AZD9056 (Arulkumaran et al., 2011). Caspase-1 has also been investigated therapeutically with the caspase-1 inhibitor VX-765 in phase II trials for treatment resistant partial epilepsy, however, the latest trial has been terminated early (ClinicalTrials.gov, 2010). Targeting NLRP3 may have additional benefits over IL-1 as in addition to IL-1β, it is also involved in IL-1α activation and that of other pro-inflammatory cytokines such as IL-18 and HMGB1, as well as preventing other IL-1-independent but caspase-1-dependent effects (Edye et al., 2014). However, although IL-18 has been implicated in auto-immune and inflammatory diseases (Sedimbi et al., 2013), IL-18 is ubiquitously expressed and has a role in homeostasis (Netea et al., 2006) and treatment with IL-18 has shown positive outcomes for cancer treatment, thus targeting NLRP3 would also disrupt these positive effects (Ozaki et al., 2015).

Inflammation does have a beneficial role in fighting infection; removing debris, pathogens and initiating the repair process. Thus as with any anti-cytokine treatment, patients on anti-IL-1 therapy are likely to be more susceptible to infection; however opportunistic infections are rarely seen with anakinra (Fleischmann et al., 2003). This may be explained by a recent paper demonstrating the brain-specific, but periphery-independent, requirement for IL-1 to induce an innate immune response following LPS treatment (Giles et al., 2015). A balance is required between reducing the harmful persistent inflammation but not destroying the beneficial effects and IL-1β has in fact been investigated as a treatment in its own right and has shown success in improving bone marrow function and inducing tumour regression. However, the side effects were numerous preventing its successful use as a therapeutic (Ozaki et al., 2015).

Our knowledge of IL-1 has come a long way since 1944 when Menkin described pyrexin as the chemical basis of fever isolated from inflammatory exudates (Menkin, 1944). We now extend the IL-1 family to include 11 members, both pro- and anti-inflammatory, we are aware of its activation through the formation of inflammasomes (canonical and non-canonical) and via caspase-1-independent mechanisms (Latz et al., 2013). Furthermore, we appreciate its role in the exacerbation of all NCDs and have developed and licensed IL-1-based therapies (Dinarello et al., 2012). However, there are still gaps in our understanding

of IL-1 activation, regulation and its role in disease and this is emphasised by the fact that very few successful IL-1-targeted treatments have been approved for use in any of the many diseases IL-1 has been implicated in.

## 1.10. Research Aims

The general aim of this thesis is to investigate IL-1 signalling under disease-relevant conditions, specifically acidic pH. This will be addressed by three separate papers and supplementary work contained in an appendix.

**Paper 1: Acidosis drives DAMP-induced interleukin-1 secretion via a caspase-1-independent pathway**

This paper aims to address how IL-1 signals in conditions experienced in disease at sites of inflammation, specifically low pH. It investigates IL-1 release from murine glial cells and human macrophages in response to different stimuli at different pH and additionally describes the ability of lactic acid to induce IL-1 release.

**Paper 2: Acid-dependent interleukin-1 (IL-1) cleavage limits available pro-IL-1β for caspase-1 cleavage**

This paper builds on paper 1 and explores the activity of IL-1 under acidic conditions and the interactions of acid-dependent 20 kDa IL-1β with other members of the IL-1 signalling system (i.e. mature IL-1α and β, IL-1R1, IL-1R2 and caspase-1).

**Paper 3: Interleukin-1β is not required for acute seizures in mice following intranasal kainic acid administration**

As caspase-1-dependent and independent IL-1 has been shown to be relevant in multiple disorders, the final paper explores the role of IL-1 in an *in vivo* disease model, investigating the requirement for inflammasomes and IL-1β in acute seizures.

### 1.10.1. Contribution of Authors

Below are the details of each paper including the contribution of each author to the work presented.

**Chapter 2: Acidosis drives DAMP-induced interleukin-1 secretion via a caspase-1-independent pathway**

Journal: Journal of Biological Chemistry

Status: Accepted

Authors: Michelle E. Edye, Gloria Lopez-Castejon, Stuart M. Allan, David Brough

The experiments were devised in collaboration with David Brough and Stuart Allan. I performed all experiments and subsequent analysis in glial cells and then collaborated with Gloria Lopez-Castejon for her to repeat the experiments in THP1 cells. I wrote the paper with David Brough with critical review by Stuart Allan and Gloria Lopez-Castejon. I combined the comments and modified the manuscript for re-submission following reviewers' comments.

**Chapter 3: Acid-dependent interleukin-1 (IL-1) cleavage limits available pro-IL-1β for caspase-1 cleavage**

Journal: Journal of Biological Chemistry

Status: Accepted

Authors: Michelle E. Edye, David Brough, Stuart M. Allan

I devised the experiments, analysed the data and wrote the first draft of the manuscript with advice and guidance from David Brough and Stuart Allan. I responded to the reviewers' comments and modified the manuscript for re-submission.

**Chapter 4: Interleukin-1β is not required for acute seizures in mice following intranasal kainic acid administration**

Authors: Michelle E. Edye, David Brough, Stuart M. Allan

The experiments were devised in collaboration with Stuart Allan and David Brough. I performed all the experiments and analysis and wrote the first draft of the manuscript

which was critically reviewed by Stuart Allan. Automated analysis programs were written by Dean Plumbley.

# Chapter 2

## Acidosis drives DAMP-induced interleukin-1 secretion via a caspase-1-independent pathway

Michelle E. Edye, Gloria Lopez-Castejon, Stuart M. Allan, David Brough

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**Acidosis drives DAMP-induced interleukin-1 secretion via a caspase-1-independent pathway**

Michelle E. Edye, Gloria Lopez-Castejon, Stuart M. Allan, David Brough

*Faculty of Life Sciences, University of Manchester, AV Hill Building, Oxford Road, Manchester, M13 9PT, U.K.*

*Running title: *Acidosis regulated IL-1 release*

To whom correspondence should be addressed: Stuart Allan, Faculty of Life Sciences, University of Manchester, AV Hill Building, Oxford Road, Manchester, M13 9PT, U.K. Tel: +44 (0)161 275 5255. Fax: +44 (0)161 275 5948. Email: stuart.allan@manchester.ac.uk

**Key words:** Acidosis, interleukin-1, inflammation, inflammasome

**Capsule**

**Background:** Interleukin-1β is a 'master' pro-inflammatory cytokine central to host responses to injury and infection. Danger molecules (DAMPs) activate IL-1β via activation of a protease called caspase-1.

**Results:** Acidosis promotes alternative DAMP-induced processing of IL-1β independent of caspase-1.

**Conclusion:** Acidosis is a regulator of inflammatory pathways.

**Significance:** Multiple pathways may contribute to the activation of IL-1 during disease.

# 2. Acidosis drives DAMP-induced interleukin-1 secretion via a caspase-1-independent pathway

## 2.1. ABSTRACT

The pro-inflammatory cytokine interleukin-1β (IL-1β) is a key mediator of inflammatory responses that contribute to and exacerbate brain injury. IL-1β is synthesised by microglia in the brain as an inactive precursor (pro-IL-1β). Cleavage of pro-IL-1β to a mature form is stimulated by damage associated molecular patterns (DAMPs). These DAMPs are sensed by a pattern recognition receptor called NLRP3, which forms an inflammasome resulting in the activation of caspase-1 and cleavage of pro-IL-1β. To-date, regulation of the inflammasome in culture has been studied under normal culture conditions and it is not known how DAMPs signal under disease relevant conditions such as acidosis. Given the presence of acidosis in pathological states, our objective was to test the hypothesis that acidic conditions modify DAMP-induced IL-1β release from cultured primary mouse glial cells. When LPS-primed glial cells were stimulated with DAMPs under acidic conditions (pH 6.2) the predominant IL-1β form secreted was 20 kDa rather than the 17 kDa caspase-1-dependent species. Lactic acidosis, induced by the addition of 25 mM lactic acid, also induced the release of 20 kDa IL-1β. This 20 kDa product was produced independently of NLRP3 and caspase-1 but was inhibited by the cathepsin D inhibitor pepstatin A. These data suggest that under disease relevant acidosis, DAMPs and lactic acid induce the secretion of IL-1β independently of the inflammasome. Therapeutic strategies directed to the inhibition of IL-1β processing should therefore consider alternative processing of IL-1β in addition to caspase-1-dependent processing.

## 2.2. INTRODUCTION

Non-communicable diseases (cardiovascular disease, diabetes, stroke, cancer) kill more people than all other causes combined and are thus recognised as a global healthcare priority (Reardon, 2011; Angell et al., 2012). Inflammation has been strongly implicated in non-communicable diseases (Rock et al., 2010), and in this context (the absence of infection) is considered "sterile" and represents a major drug target (Dinarello, 2010). IL-1β is a primary mediator of sterile inflammatory responses and, given its established contribution to disease (Dinarello, 2011a), understanding the mechanisms of its processing and release could lead to the identification of new therapeutic targets. IL-1β is an established contributor to acute brain injury, such as caused by stroke (Allan et al., 2005).

In the brain, IL-1β is produced primarily by microglia in response to an inflammatory stimulus as an inactive precursor - pro-IL-1β. Pro-IL-1β requires proteolytic cleavage to an active, mature form that signals at the type I IL-1 receptor (IL-1RI) on responsive cells (Luheshi et al., 2009b). Activation of IL-1β is regulated by inflammasomes which are multi-molecular complexes defined by the presence of a cytosolic pattern recognition receptor (PRR). Of particular relevance to sterile inflammation is the PRR NLRP3 (NLR family, pyrin domain-containing 3) (Strowig et al., 2012). After activation, NLRP3 interacts with the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) and pro-caspase-1 to form an inflammasome resulting in caspase-1 activation and its processing of pro-IL-1β to a mature active form (Schroder and Tschopp, 2010). NLRP3 senses molecules released from dead and dying cells, so called damage associated molecular patterns (DAMPs) (Chen and Nunez, 2010). Treatment of activated macrophages with necrotic cells also drives NLRP3-inflammasome-dependent IL-1β secretion (Iyer et al., 2009). How diverse DAMPs activate NLRP3 is not known, but several host signals are thought to integrate these stimuli (Schroder and Tschopp, 2010). To-date, studies investigating regulation of the inflammasome *in vitro* have been conducted under physiological or 'normal culture' conditions. However, during disease there are profound changes in the Intercellular milieu (such as a drop in pH), and how DAMPs signal under these particular conditions is not known.

Lactic acidosis commonly occurs during disease conditions, and results from an increase in anaerobic glycolysis due to tissue hypoxia. In the brain, following a stroke, parenchymal pH can drop as low as 6.2 (Nemoto and Frinak, 1981). It was reported recently that P2X7-receptor-

dependent release of IL-1β from cultured microglia is affected by acidosis (Takenouchi et al., 2011). Stimulation of microglia with the P2X7 receptor agonist ATP at pH 6.2 results in the secretion of a 20 kDa species of IL-1β as opposed to the more conventionally reported 17 kDa form. The 20 kDa cleavage product did not require caspase-1 but rather depended upon the protease cathepsin D (Takenouchi et al., 2011). Several other non-caspase proteases also cleave pro-IL-1β (Fantuzzi et al., 1997; Stehlik, 2009), and proteinase 3 has been implicated in acute arthritis (Joosten et al., 2009). Acidosis was also recently described as a danger signal that could activate the NLRP3 inflammasome (Rajamäki et al., 2013). Thus during disease, pro-IL-1β could be cleaved by a variety of proteases that may act to shape the inflammatory response.

Here we sought to determine if acidic conditions modified DAMP-induced IL-1β release from cultured primary glial cells. We report that acidosis induced a shift from the 17 to the 20 kDa IL-1β product in response to DAMP stimulation and that this was independent of the NLRP3 inflammasome. We also report that addition of lactic acid directly to the culture induced the secretion of 20 kDa IL-1β raising the possibility that lactic acidosis could drive IL-1-dependent inflammatory responses during disease.

## 2.3. EXPERIMENTAL PROCEDURES

### 2.3.1. Materials.

RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), penicillin streptomycin solution, LPS (*Escherichia coli* O26:B6), ATP, L-lactic acid, cathepsin D inhibitor pepstatin A and cathepsin B inhibitor CA-074-Me were all purchased from Sigma. Caspase-1 inhibitor Ac-YVAD-CHO (YVAD) and calpain inhibitor III were purchased from Calbiochem. Monosodium urate (MSU) and calcium pyrophosphate (CPPD) were purchased from InvivoGen. Foetal bovine serum (FBS) was purchased from PAA Laboratories. HEPES-buffered salt solution comprised 145 mM NaCl, 2.5 mM KCl, 1mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose and 0.01 % BSA as described previously (Takenouchi et al., 2011). The pH of the HEPES-buffered salt solution was adjusted using 1M NaOH. Antibodies against human and mouse IL-1β and mouse IL-1α were purchased from R&D systems. Secondary antibody HRP conjugates were from DAKO.

### 2.3.2. Cell culture.

Initial studies used the human monocytic cell line THP-1, which provides a well characterised secretion model of IL-1β. THP-1 cells were cultured in RPMI 1640 media supplemented with 10 % FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were plated in 24-well plates at a density of $5x10^5$ cells/well and treated with phorbol 12-myristate 13-acetate (PMA, 0.5 μM). After 3 h, the medium was removed, fresh media was added, and cells were incubated overnight (37 °C, 5 % $CO_2$). Cells were then LPS-primed (1 μg/ml, 4 h) to induce pro-IL-1β and NLRP3 expression. The culture medium was then replaced with HEPES-buffered salt solution for subsequent treatments.

DAMP-dependent inflammatory responses in cultures of mixed glia reflect inflammatory responses produced by an ischemic brain (Savage et al., 2012), so we considered these cultures to represent an appropriate system for examining the effects of pH on DAMP-induced inflammation in the brain. Primary mixed glia were cultured from 1-4 day old mice (C57BL/6J or NLRP3 KO) as described previously (Savage et al., 2012). Briefly, meninges were removed and cells were dissociated by trituration. Cells were seeded at 1 brain/60 $cm^2$ and maintained in DMEM growth medium supplemented with 10 % FBS, 100 units/ml penicillin, and 100 μg/ml

streptomycin. Cells were incubated at 37 $^{o}$C and 5 % $CO_2$ until confluent (~14 DIV). Cultures were then incubated with LPS (1 μg/ml, 18-24 h). The growth medium was then replaced with serum free media or HEPES-buffered salt solution prior to treatment. All procedures were performed in accordance with the Animal (Scientific Procedures) Act 1986.

### 2.3.3. Treatments.

Prior to treatment with DAMPs (ATP, MSU, CPPD), the culture medium was replaced with HEPES-buffered salt solution that had its pH adjusted to 7.4 or 6.2. Prior to lactic acid treatment, the culture media was replaced with serum free media. Primed human THP-1 or mouse mixed glial cultures were treated with ATP (5.5 mM, 1 h), MSU (250 μg/ml, 1 h), CPPD (250 μg/ml, 1 h), or lactic acid (25 mM, 8 h). In subsequent studies, cells were pre-treated with caspase-1 (Ac-YVAD-CHO, 100 μM), cathepsin B (CA-074-Me; 50 μM) cathepsin D (pepstatin A, 50 μM) inhibitors or their respective vehicles (1 % DMSO or 1 % MeOH) for 15 min prior to DAMP/lactic acid treatment. Supernatants were collected and stored at -20 $^{o}$C until required.

### 2.3.4. Western blots.

Supernatants were collected and, if required, concentrated using 10 kDa cutoff filters (Millipore). Samples of this supernatant were resolved on 12 % polyacrylamide gels for detection of IL-1β or IL-1α. Proteins were transferred to a nitrocellulose membrane and specific proteins were detected by western blot with anti-hIL-1β, anti-mIL-1β or anti-mIL-1α followed by a secondary HRP-conjugated antibody and subsequently detected using enhanced chemiluminescence reagents (ECL, Amersham Biosciences).

### 2.3.5. ELISA.

Supernatants were analysed for IL-1β and IL-1α using specific ELISA kits for human or mouse IL-1 from R&D Systems according to the manufacturer's instructions. The IL-1β ELISA is designed against the mature 17 kDa form of IL-1β and has been suggested to have increased affinity for 17 kDa IL-1β over pro-IL-1β. This may result in an underestimate of pro-IL-1β concentration (Herzyk et al., 1992).

### 2.3.6. Cell death assay.

Lactate dehydrogenase (LDH) assay was used as an indicator of cell death. LDH release from cells was detected using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to manufacturer's instructions.

### 2.3.7. Statistics.

Statistical analysis was performed using GraphPad Prism v5. For comparisons between 2 groups, Students' t-test was used. For comparisons involving 3 or more experimental groups, a one-way ANOVA with Bonferroni multiple comparisons post hoc test was used. Data are expressed as mean ± SEM for the number of repeats indicated in the figure legends. $P \leq 0.05$ was considered significant.

## 2.4. RESULTS

### 2.4.1. DAMPs induced alternative IL-1β processing at acidic pH in THP-1 cells.

In LPS-primed THP-1 cells incubated at pH 6.2, mature IL-1β release was not observed in the absence of DAMP, in contrast to a previous report (Rajamäki et al., 2013). However, at pH 6.2, both 20 and 17 kDa forms were present after treatment with the NLRP3-inflammasome activating DAMPs CPPD or MSU (Figure 2.1A). At pH 7.4, only the 17 kDa form was present in the culture supernatants after DAMP treatment (Figure 2.1A). In these THP-1 cells CPPD proved to be a powerful inducer of IL-1β release with levels readily detected by ELISA (Figure 2.1B). The lack of reduction in IL-1β release shown by the ELISA at pH 7.4 compared to pH 6.2 may be due to its enhanced affinity for 17 kDa IL-1β over larger forms of IL-1β (Herzyk et al., 1992). There was no difference in DAMP-induced toxicity between cells maintained at pH 7.4 and 6.2 (Figure 2.1C) suggesting the effect cannot be explained by toxicity of the pH change. Thus these data indicate that at acidic pH (pH 6.2) DAMPs induced the release of 20 kDa IL-1β from THP-1 cells.

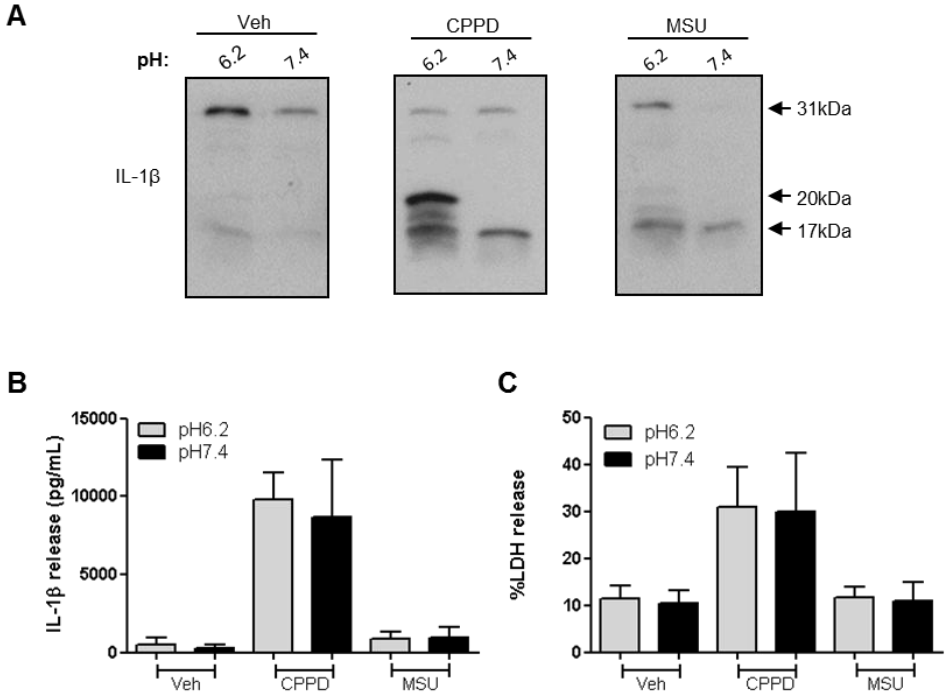


*Figure 2.1. Effect of extracellular pH on IL-1β processing in THP-1 cells*

LPS-primed THP-1 cells were treated with vehicle (Veh), CPPD (250 µg/ml) or MSU (250 µg/ml) for 1 h at pH 6.2 or 7.4. Processing of IL-1β was observed by western blot of the supernatants (A) with quantification of levels released by ELISA (B). The effect of pH and DAMPs on cell death was measured by the release of LDH (C). ELISA and LDH data are the mean ± SEM of three separate experiments. Western blots are representative of 3 separate experiments.

YVAD has previously been shown to robustly inhibit caspase-1 activity in THP-1 cells (Lopez-Castejon et al., 2013). We showed here that neither the caspase-1 inhibitor YVAD (Figure 2.2A), or the cathepsin B inhibitor Ca074-Me (Figure 2.2B) blocked the production of 20 kDa IL-1β from LPS-primed THP-1 cells induced by CPPD at pH 6.2, thereby suggesting alternative processing of IL-1β independent of caspase-1 in THP-1 cells at acidic pH. Consistent with the previously reported effect of acidic conditions on P2X7-receptor-dependent IL-1β release (Takenouchi et al., 2011), the cathepsin D inhibitor pepstatin A inhibited CPPD-induced release of 20 kDa IL-1β from LPS-primed THP-1 cells, whilst the release of 17 kDa IL-1β was unaffected (Figure 2.2C). Overall, these data suggest that in THP-1 cells at pH 6.2, CPPD induces the release of a cathepsin D-, and not caspase-1-dependent IL-1β that is 20 kDa in size.

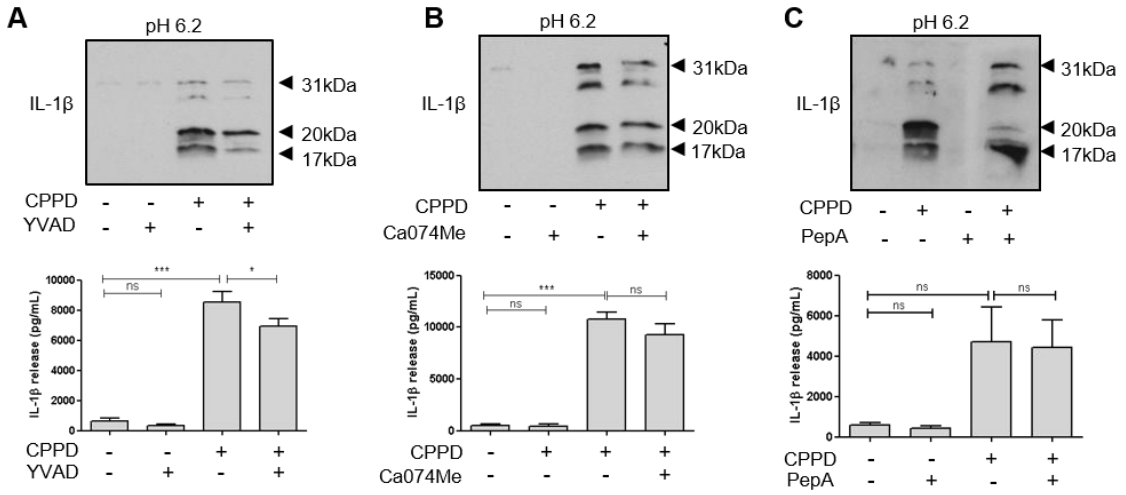


*Figure 2.2. DAMP-induced release of 20 kDa IL-1β is caspase-1 independent.*
LPS-primed THP-1 cells were incubated at pH 6.2, treated with the caspase-1 inhibitor Ac-YVAD-CHO (YVAD; 100 µM; A), the cathepsin B inhibitor Ca074Me (50 µM; B), or the cathepsin D inhibitor pepstatin A (PepA; 50 µM; C) for 15 mins prior to CPPD treatment (250 µg/ml, 1 h). Processing of IL-1β was observed by western blot of the supernatants (blots) with quantification of levels released by ELISA (graphs). ELISA data are the mean ± SEM of three separate experiments. Western blots are representative of 3 separate experiments. Statistical analysis was performed using a one-way ANOVA with Bonferroni's post hoc. ns = not significant, *P<0.05, ***P<0.001.

### 2.4.2. DAMPs induced alternative IL-1β processing at acidic pH in mixed glial cultures.

We next determined whether the alternative caspase-1-independent processing of IL-1β in response to DAMPs at acidic pH occurred in primary cultures of glia. Incubation of LPS primed mouse mixed glia at pH 7.4 with the NLRP3-inflammasome activating DAMPs ATP, MSU or CPPD induced the secretion of 17 kDa IL-1β as expected (Figure 2.3A). However, when the cultures were incubated under acidic conditions at pH 6.2, these DAMPs also induced the secretion of 20 kDa IL-1β (Figure 2.3B), consistent with a previous report on the effects of ATP (Takenouchi et al., 2011) and our data presented above in the THP-1 cells (Figure 2.1). There were no obvious differences in DAMP-induced toxicity between the experiments at different pH (Figure 2.3C) suggesting the effect cannot simply be explained by toxicity of the pH change.

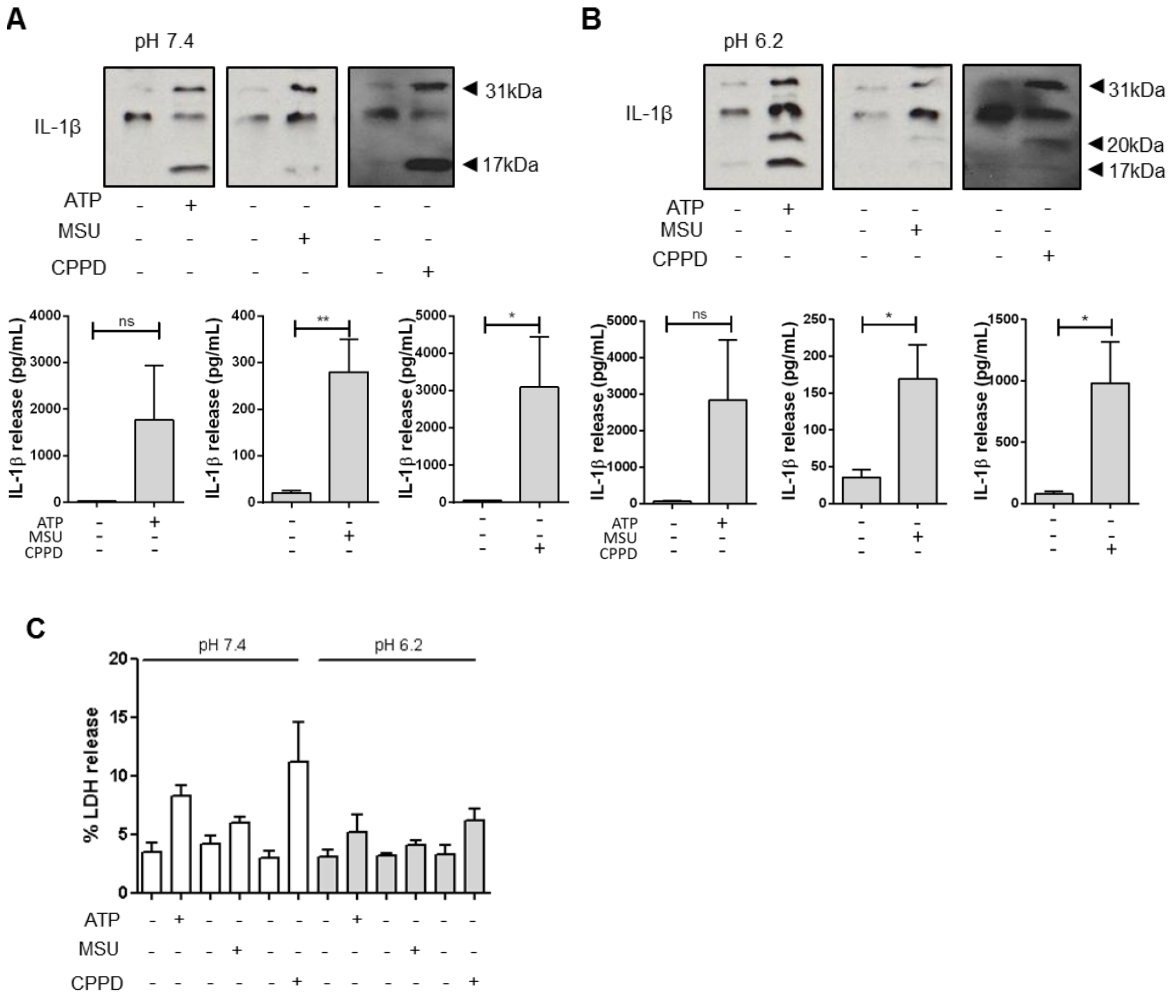


*Figure 2.3. Effect of extracellular pH on IL-1β processing in primary mixed glia.*

LPS-primed mixed glia were treated for 1 h with ATP (5.5 mM), MSU (250 µg/ml) or CPPD (250 µg/ml) at pH 7.4 (A) or 6.2 (B). Processing of IL-1β was observed by western blot of the supernatants (blots) with quantification of levels released by ELISA (graphs). The effect of pH and DAMPs on cell death was measured by the release of LDH (C). ELISA and LDH data are the mean ± SEM of between 5 and 6 separate experiments. Western blots are representative of 3 separate experiments. Statistical analysis was performed using a Student's t-test. ns = not significant, *P<0.05, **P<0.01.

To investigate if the 20 kDa IL-1β released from mixed glia was processed in the same way as that from THP-1 cells, we pre-treated cells with the caspase-1 inhibitor YVAD. CPPD treatment of LPS-primed mixed glia at normal pH (7.4) resulted in the release of mature 17 kDa IL-1β that was completely inhibited with YVAD pre-treatment (Figure 2.4A). At pH 6.2, YVAD still inhibited the appearance of 17 kDa IL-1β in response to CPPD, but had no effect on 20 kDa IL-1β (Figure 2.4B). Conversely, pepstatin A had no effect on CPPD-induced IL-1β (17 kDa) secretion at pH 7.4 (Figure 2.4C), but at pH 6.2, pepstatin A inhibited the release of 20 kDa IL-1β (Figure 2.4D). This confirmed that production of 20 kDa IL-1β was independent of caspase-1 and suggested that it required cathepsin D.

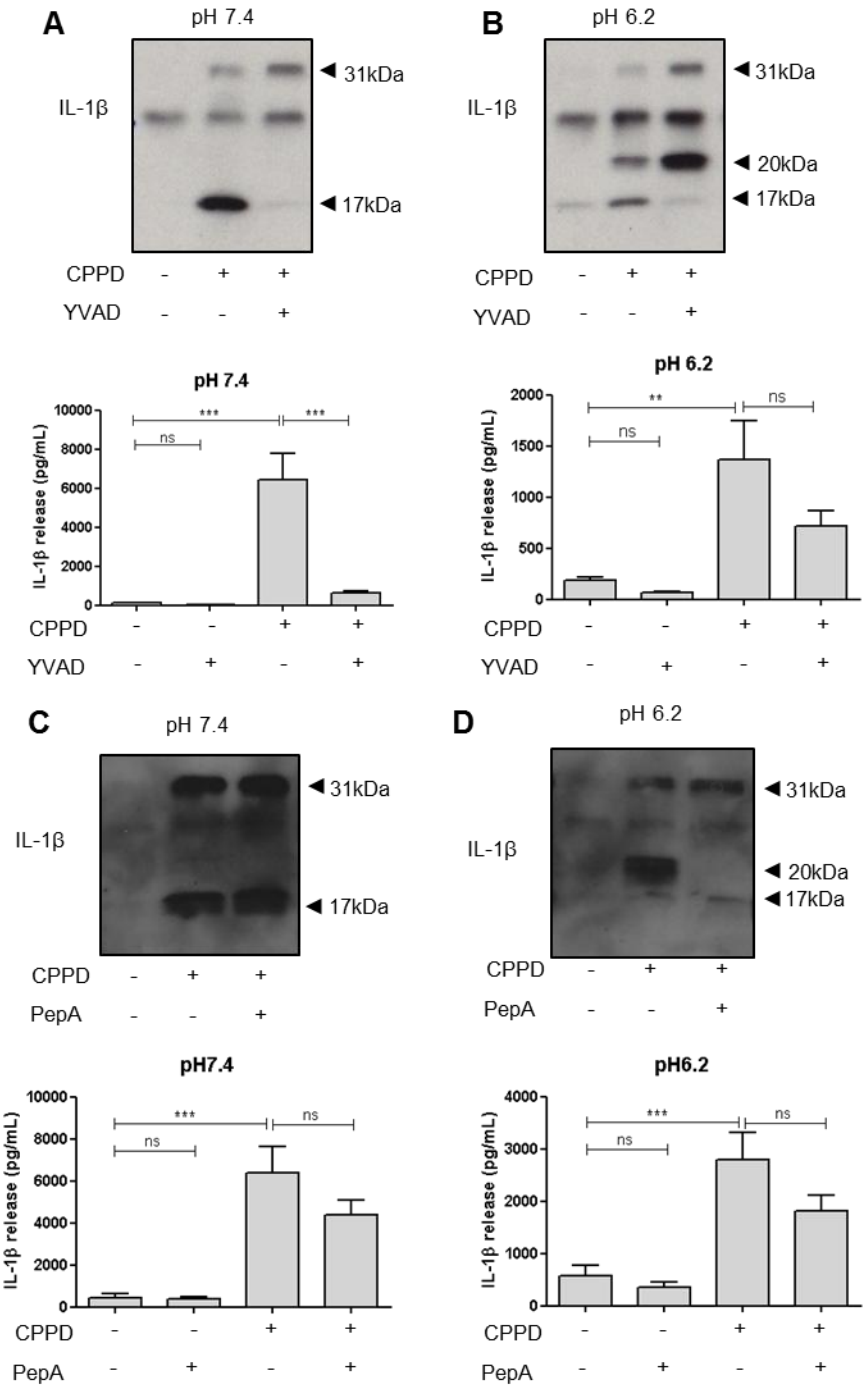


*Figure 2.4. DAMP-induced release of 20 kDa IL-1β is caspase-1 independent in primary mixed glia.*
LPS-primed mixed glia were pre-treated with caspase-1 inhibitor Ac-YVAD-CHO (YVAD; 100 μM) or cathepsin D inhibitor pepstatin A (PepA; 50 μM) for 15 mins prior to CPPD treatment (250 μg/ml, 1 h) at pH 7.4 (A, C) or pH 6.2 (B, D). Processing of IL-1β was observed by western blot of the supernatants (blots) with quantification of levels released by ELISA (graphs). ELISA data are the mean ± SEM of between 5 and 6 separate experiments. Western blots are representative of 3 separate experiments. Statistical analysis was performed using a one-way ANOVA with Bonferroni's post hoc. ns = not significant, **P<0.01, ***P<0.001.

To confirm that the effects of the NLRP3 activating DAMP CPPD at acidic pH were independent of the NLRP3 inflammasome and caspase-1, we compared the effects of CPPD on IL-1β release in cultures of mixed glia isolated from WT and NLRP3 KO mice. At pH 6.2, CPPD induced the secretion of 20 kDa IL-1β from both WT and NLRP3 KO mixed glia (Figure 2.5), confirming that NLRP3-inflammasome activating DAMPs induced the secretion of IL-1β from LPS-primed cells independently of the NLRP3 inflammasome under acidic conditions.

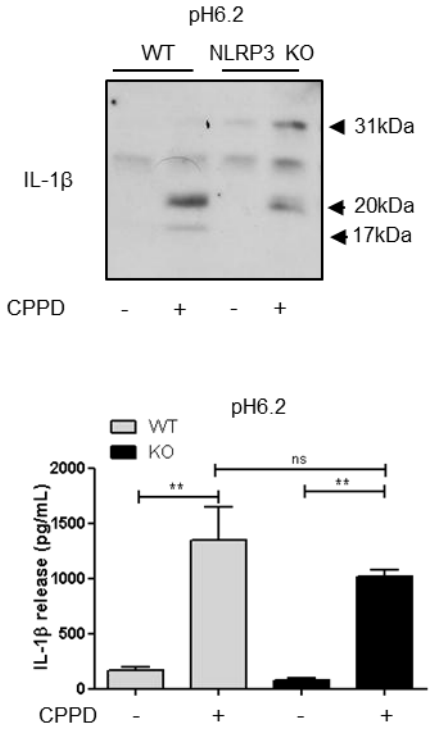


*Figure 2.5. CPPD-induced 20 kDa IL-1β is NLRP3 independent in primary mixed glia.*
Mixed glial cultures prepared from WT and NLRP3 KO mice were treated with CPPD (250 μg/ml, 1 h) at pH 6.2. Processing of IL-1β was observed by western blot of the supernatants (blots) with quantification of levels released by ELISA (graphs). ELISA data are the mean ± SEM of 3 separate experiments. Western blots are representative of 3 separate experiments. Statistical analysis was performed using a one-way ANOVA with Bonferroni's post hoc. ns = not significant, **P<0.01.

## 2.4.3. Lactic acidosis induced IL-1β release from mixed glial cultures.

During cerebral ischemia there is a marked elevation in lactic acid which contributes to the drop in tissue pH (Nemoto and Frinak, 1981; Sun et al., 2011). Thus we hypothesised that lactic acid could stimulate the release of IL-1β in an ischemic brain. Treatment of LPS-primed mixed glia with lactic acid induced the release of 20 kDa IL-1β and a minor amount of 17 kDa IL-1β (Figure 2.6A). This effect required LPS priming since lactic acid failed to induce expression or release of IL-1β in its absence (Figure 2.6B, C). Again there was no obvious change in toxicity with lactic acid treatment (Figure 2.6D) suggesting that the IL-1β release observed with lactic acid cannot simply be explained by toxicity.

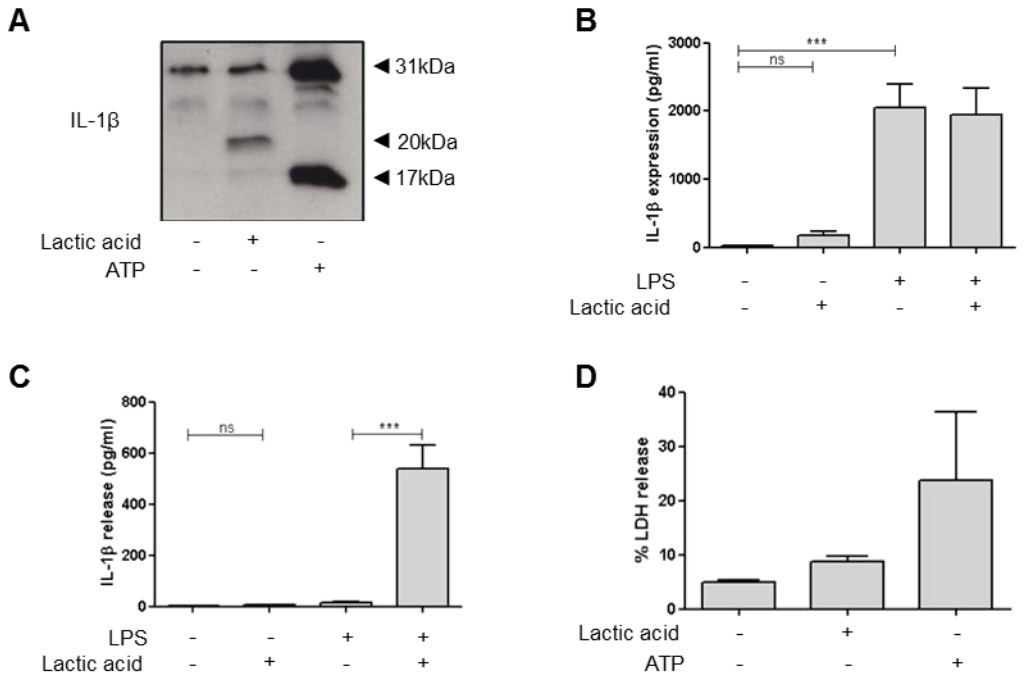


*Figure 2.6. Lactic acid induces the release of 20 kDa IL-1β from primary mixed glia.*

LPS-primed mixed glia were treated with lactic acid (25 mM; 8 h) or ATP (5.5 mM; 1 h). Processing of IL-1β was observed by western blot of the supernatants (A) with quantification of levels of IL-1β in cell lysates (B), and released into the supernatants (C) measured by ELISA. The effect of lactic acid and ATP on cell death was measured by the release of LDH (D). ELISA data are the mean ± SEM of 6 separate experiments. Western blots are representative of 3 separate experiments. LDH data are the mean ± SEM of 3 separate experiments. Statistical analysis was performed using a one-way ANOVA with Bonferroni's post hoc. ***P<0.001, ns=not significant.

Lactic acid-induced release of 17 kDa IL-1β was inhibited by YVAD, whilst release of 20 kDa IL-1β was unaffected (Figure 2.7A). Lactic acid also induced the release of 20 kDa IL-1β from NLRP3 KO mixed glia, confirming the NLRP3-inflammasome and caspase-1 independent release (Figure 2.7B). Consistent with data presented above, lactic acid-induced 20 kDa IL-1β was inhibited by the cathepsin D inhibitor pepstatin A (Figure 2.7C).

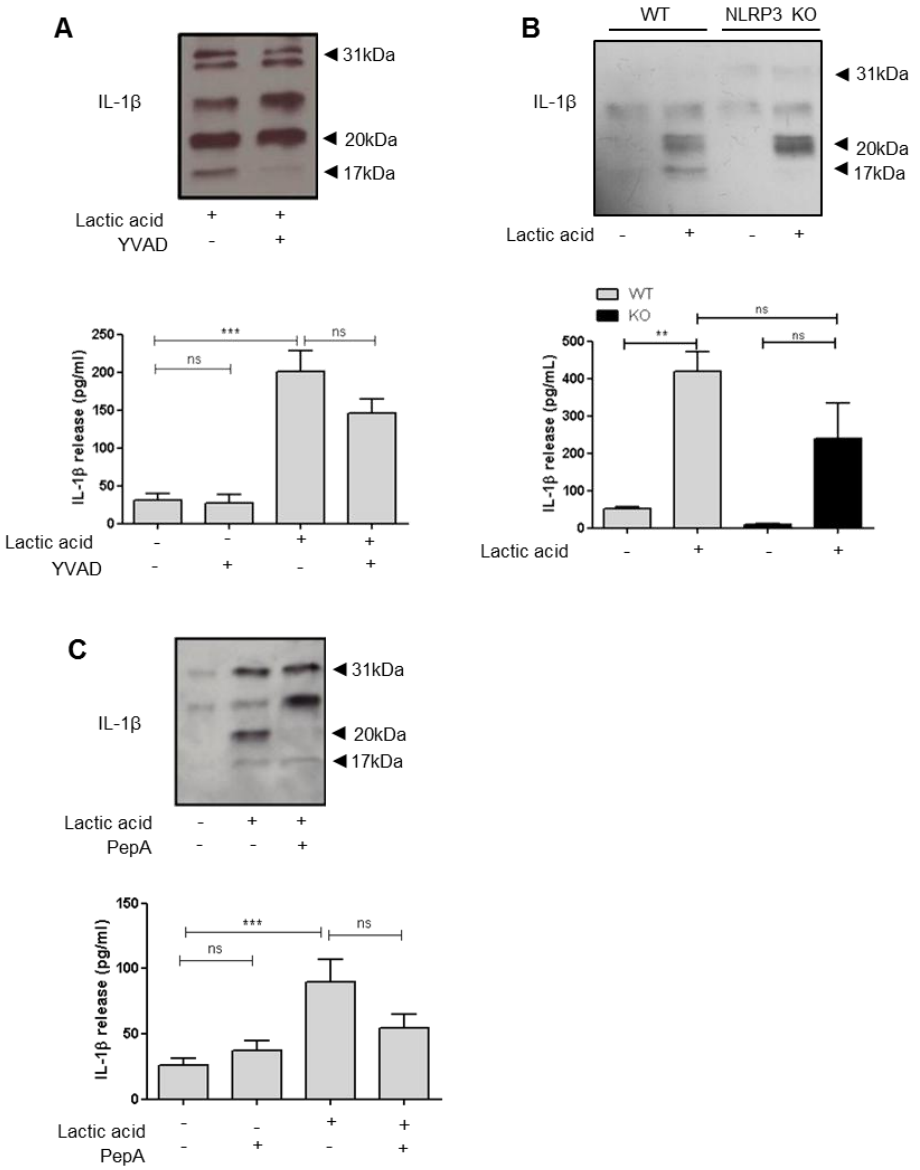


*Figure 2.7. Lactic acid-induced release of 20 kDa IL-1β occurs independently of caspase-1 in primary mixed glia.* LPS-primed mixed glia were pre-treated with 100 µM Ac-YVAD-CHO (YVAD; A) or 50 µM pepstatin A (PepA; C) for 15 mins prior to 25 mM lactic acid treatment (8 h). Mixed glia cultures prepared from WT and NLRP3 KO mice were treated with 25mM lactic acid (8 h; B). Processing of IL-1β was observed by western blot of the supernatants (blots) with quantification of levels released by ELISA (graphs). ELISA data are the mean ± SEM of between 3 and 6 separate experiments. Western blots are representative of 3 separate experiments. Statistical analysis was performed using a one-way ANOVA with Bonferroni's post hoc. ns = not significant, **P<0.01, ***P<0.001.

### 2.4.4. Lactic acid induced IL-1α processing and release from mixed glial cultures.

The effects of lactic acid on the IL-1 system were not limited to IL-1β since treatment of LPS-primed mixed glia with lactic acid also induced the release of processed IL-1α (Figure 2.8A), which we have reported previously to be a key early mediator of inflammation following acute brain injury (Luheshi et al., 2011; Greenhalgh et al., 2012). Lactic-acid-induced IL-1α release was also independent of the NLRP3 inflammasome (Figure 2.8B), but required the calcium dependent protease calpain since calpain inhibitor III inhibited lactic acid-induced IL-1α processing and release (Figure 2.8C). Thus in line with the NLRP3-independent actions of lactic acid on IL-1β release described above, these data suggest that lactic acid also induced the release of NLRP3-independent, but calpain-dependent, IL-1α release from glia.

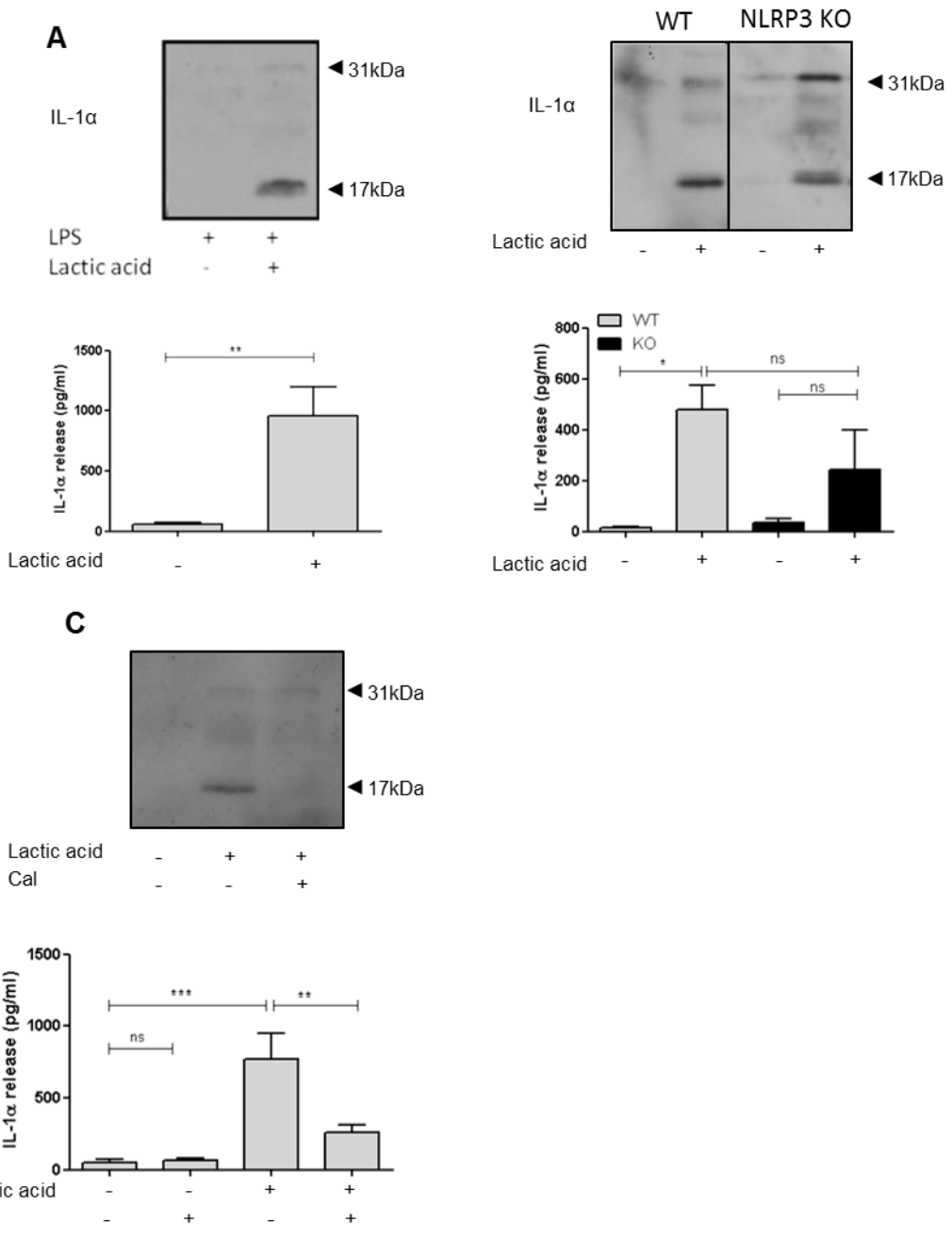


*Figure 2.8. Lactic acid induced release of IL-1α from primary mixed glia.*

LPS-primed WT mixed glia were treated with 25 mM lactic acid for 8 h and IL-1α processing and release was measured (A). LPS-primed mixed glia from WT and NLRP3 KO mice were treated with lactic acid (25 mM; 8 h; B). LPS-primed WT mixed glia were pre-treated with calpain inhibitor III (Cal; 50 μM) for 15 mins prior to lactic acid treatment (25 mM, 8 h; C). Processing of IL-1α was observed by western blot of the supernatants (blots) with quantification of levels released by ELISA (graphs). ELISA data are the mean ± SEM of between 3-6 separate experiments. Western blots are representative of 3 separate experiments. Statistical analysis was performed using Student's t-test (A), or one-way ANOVA with Bonferroni's post hoc test (B, C). ns = not significant, *P<0.05, **P<0.01, ***P<0.001.

## 2.5. DISCUSSION

Most studies on IL-1β release in *in vitro* systems are conducted under physiological conditions and in peripheral immune cells such as macrophages. The objective of this study was to investigate IL-1β secretion under disease relevant conditions, with particular reference to ischemic stroke, for which lactic acidosis is relevant (Sun et al., 2011). Under acidic conditions, 'classical' NLRP3-inflammasome activating DAMPs such as ATP (Mariathasan et al., 2006) or MSU and CPPD crystals (Martinon et al., 2006) induced the release of a processed IL-1β that was completely independent of the NLRP3 inflammasome and caspase-1, and this occurred in both peripheral macrophage-like cells and also glial cells from the central nervous system. Although we, and others, have reported that MSU and CPPD stimulate IL-1-independent inflammatory responses (Guerne et al., 1989; Bouchard et al., 2002; Savage et al., 2012), when used to stimulate IL-1 production they are associated with the NLRP3 inflammasome and caspase-1-dependent processing (Martinon et al., 2006). We also showed that lactic acid induced the release of processed IL-1β that was again independent of the NLRP3 inflammasome and caspase-1. Caspase-1-independent processing of IL-1β has been reported previously and may impact certain diseases even more significantly than caspase-1 processed IL-1β (Fantuzzi et al., 1997; Joosten et al., 2009; Stehlik, 2009).

In our experiments, priming to induce the synthesis of pro-IL-1β was required prior to lactic acid- (or DAMP) induced IL-1β release. The TLR4 agonist LPS is a tool for priming cells in culture but little is known about priming *in vivo* in an injured brain. Brain injury often results in disruption of the blood-brain barrier (Allan et al., 2005) which would allow circulating peripheral molecules to enter the brain and act on glia. The acute phase protein serum amyloid A is elevated in plasma during inflammation and has been shown to prime glial cells to release IL-1 following subsequent DAMP stimulation *in vitro* in a similar way to LPS (Savage et al., 2012).

The contribution of IL-1  to ischemic brain injury is well established (Allan et al., 2005) with the utility of the IL-1 receptor antagonist (IL-1Ra) as a therapeutic under clinical investigation (Emsley et al., 2005). Brain acidosis after ischemia contributes to neuronal injury, and the damaging effects are mediated, at least in part, by acid-sensing ion channels (ASICs) (Xiong et

al., 2004; Gao et al., 2005). Our data provide the rationale that acidosis and neuronal injury could also be linked *via* the IL-1 system. Levels of lactic acid increase in an ischemic brain due to a lack of oxygen and the upregulation of anaerobic glycolysis. Stimulation of LPS-primed cultures of glial cells with lactic acid, or with NLRP3-inflammasome activating DAMPs under acidic conditions induced cathepsin D-dependent, release of 20 kDa IL-1β, consistent with a previous report showing ATP-induced release of 20 kDa IL-1β from microglia under acidic conditions (Takenouchi et al., 2011). This cathepsin-D cleavage of IL-1β adds to a growing number of proteases now known to cleave pro-IL-1β. These proteases cleave IL-1β at different sites to produce different size fragments corresponding to differing activities (Hazuda et al., 1991). The putative cathepsin D-dependent product at 20 kDa is suggested to be approximately 5 fold less active at the type I IL-1 receptor (IL-1RI) than the caspase-1 generated 17 kDa product (Hazuda et al., 1991). Thus, if generated *in vivo* it could act as an agonist, or maybe a partial agonist of IL-1RI, and contribute to inflammatory responses during ischemia; therefore further experiments to understand the exact nature of its role *in vivo* are required to help direct any future targeting of this pathway. Acidosis was also recently described as an inducer of the NLRP3 inflammasome (Rajamäki et al., 2013). Whilst we did observe 17 kDa IL-1β following lactic acid treatment (e.g. Figure 2.7A), it was the minor species compared to the 20 kDa form. Together these data strongly suggest that acidosis is an important disease relevant regulator of IL-1β processing pathways.

Until recently, the precursor of IL-1α, pro-IL-1α, was thought to be as biologically active as the mature 17 kDa form at IL-1RI. However, recent research has shown that processed IL-1α is significantly more biologically active than the pro-form (Afonina et al., 2011; Zheng et al., 2013), which highlights IL-1α processing as a biologically important mechanism. As well as its effects on IL-1β, lactic acid also induced the release of processed IL-1α, which was 17 kDa, and was inhibited by a calpain inhibitor, consistent with the involvement of a calpain protease in its processing (Luheshi et al., 2009b). DAMPs are known to induce the secretion of processed IL-1α (Gross et al., 2012) and DAMP-induced processing and secretion of IL-1α can be dependent, partially dependent, or independent of the NLRP3 inflammasome and caspase-1, depending upon the DAMP (Gross et al., 2012). Our data suggest that lactic acid-induced IL-1α secretion occurs independently of the inflammasome. Further to the effects of IL-1β in acute brain injury, IL-1α is also known to play an important role in brain injury (Boutin et al., 2001; Luheshi et al., 2011; Greenhalgh et al., 2012). IL-1α is upregulated before IL-1β and as early as 4 h post

ischemic brain injury in areas of subsequent neuronal loss, suggesting it may be the key driver of the damaging early inflammatory response (Luheshi et al., 2011). Thus the lactic acid-induced IL-1α release we have described may contribute to damage post ischemic injury.

In summary, these data provide new insights into the mechanisms of IL-1 production during disease relevant conditions. We show that under acidic conditions both pro-inflammatory forms of the IL-1 family (IL-1β and IL-1α) are regulated independently of the NLRP3 inflammasome. Thus consideration of this observation should be made when investigating inhibitors of the NLRP3 inflammasome to target IL-1 in disease, particularly where changes in the local environment may dictate the mechanism of IL-1β processing.

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# Chapter 3

## Acid-Dependent Interleukin-1 (IL-1) Cleavage Limits Available Pro-IL-1β for Caspase-1 Cleavage

Michelle E. Edye, David Brough[1], Stuart M. Allan

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Acid-Dependent Interleukin-1 (IL-1) Cleavage Limits Available Pro-IL-1β for Caspase-1 Cleavage*

**Michelle E. Edye, David Brough[1], Stuart M. Allan**

From the Faculty of Life Sciences, University of Manchester, Manchester, M13 9PT, UK

*Running title: *Regulation of IL-1 signaling*

[1] To whom correspondence should be addressed**:** David Brough, Faculty of Life Sciences, University of Manchester, AV Hill Building, Oxford Road, Manchester, M13 9PT, UK
Tel: 44-161-275-5039; Fax: 44-161-275-5948; Email: david.brough@manchester.ac.uk

**Keywords:** Interleukin-1 (IL-1); acidosis; inflammation; caspase-1; innate immunity; cathepsin D; IL-1R2.

**Capsule**

**Background:** Interleukin-1β (IL-1β) is an inflammatory cytokine that is activated by cleavage of an inactive precursor. Multiple proteases can cleave IL-1β but their contribution under disease conditions is unknown.

**Results:** Cathepsin D cleaved IL-1β to a less active form than caspase-1.

**Conclusion:** During disease relevant acidic conditions, cathepsin D cleavage of IL-1β reduces IL-1β activity.

**Significance:** Cathepsin D cleaved IL-1β may limit IL-1-dependent inflammation.

# 3. Acid-Dependent Interleukin-1 (IL-1) Cleavage Limits Available Pro-IL-1β for Caspase-1 Cleavage

## 3.1. ABSTRACT

Non-communicable diseases such as cardiovascular disease (stroke and heart attack), cancer, chronic respiratory disease and diabetes are a leading cause of death and disability worldwide and are worsened by inflammation. Interleukin-1 (IL-1) is a driver of inflammation and implicated in many non-communicable diseases. Acidosis is also a key feature of the inflammatory microenvironment therefore it is vital to explore IL-1 signaling under acidic conditions. A HEK-IL-1 reporter assay and brain endothelial cell line were used to explore activity of mature IL-1α and IL-1β at pH 7.4 and pH 6.2, an acidic pH that can be reached under inflammatory or ischemic conditions, alongside cathepsin-D-cleaved 20 kDa IL-1β produced under acidic conditions. We report that mature IL-1 signaling at IL-1R1 is maintained at pH 6.2 but the activity of the decoy receptor, IL-1R2, is reduced. Additionally, cathepsin-D-cleaved 20 kDa IL-1β was minimally active at IL-1R1 and was not further cleaved to highly active 17 kDa IL-1β. Therefore formation of the 20kDa form of IL-1β may prevent the generation of mature bioactive IL-1β and thus may limit inflammation.

## 3.2. INTRODUCTION

Inflammation worsens multiple non-communicable diseases such as stroke, heart attack, diabetes and cancer (Coussens and Werb, 2002; Allan et al., 2005; Rock et al., 2010), which are the greatest cause of mortality worldwide and a key priority for ongoing research (Angell et al., 2012). Interleukin-1 (IL-1) α and β are pro-inflammatory cytokines and key drivers of the inflammation observed in non-communicable diseases. Much of the work on the mechanisms of IL-1 production and signaling has been performed on cells cultured under standard culture conditions, not relevant to disease.

Disease is associated with a change in the local environment. Following a disruption in blood flow for example during stroke, or in the metabolically active environment of a proliferating tumor or epileptic brain undergoing a seizure, a drop in pH is observed (Wang and Sonnenschein, 1955; Nemoto and Frinak, 1981; Webb et al., 2011). Inflammation is also associated with a decrease in pH. As inflammation progresses, there is an increase in infiltrating immune cells, an increase in glycolysis and subsequent elevation in lactic acid and drop in pH (Menkin and Warner, 1937; Rajamäki et al., 2013). Whether this acidosis affects the inflammatory signaling observed in disease is unknown.

IL-1α and β are potent inducers of inflammation, thus their activation and release are tightly regulated. Both IL-1α and β are synthesized as pro forms and although initial studies suggested pro-IL-1α possessed activity at its receptor (IL-1 receptor type 1; IL-1R1), it is now known that both pro-IL-1α and pro-IL-1β require a second activating step to induce their cleavage in order to exert maximal activity at IL-1R1 (Zheng et al., 2013). Pro-IL-1α is cleaved via calcium-dependent cysteine proteases of the calpain family to produce 17 kDa IL-1α (Zheng et al., 2013). Cleavage of pro-IL-1β is typically performed by the cysteine protease caspase-1 to produce 17 kDa mature IL-1β. Caspase-1 itself requires activation via association with a protein complex called an inflammasome before it can cleave pro-IL-1β (Schroder and Tschopp, 2010).

In addition to regulation of IL-1α and β at the level of processing, a decoy receptor (IL-1R type II; IL-1R2), endogenous receptor antagonist (IL-1Ra) and intracellular negative regulators (TIR8/SIGIRR or micro RNAs) exist to control IL-1 activity (Garlanda et al., 2013). Mutations in these regulators could result in excessive IL-1 signaling, contributing to the development or exacerbation of inflammatory diseases. Indeed, a gain of function mutation in the NLRP3 inflammasome results in the autoinflammatory diseases cryopyrin associated periodic syndromes (CAPS) (Dinarello, 2011b), and the protective role of IL-1R2 has been shown in a

mouse collagen-induced arthritis model where IL-1R2 knockout (KO) mice have increased arthritis severity (Shimizu et al., 2015).

Due to the established role of IL-1 in disease, it has been targeted therapeutically (Dinarello et al., 2012). However, targeting IL-1β activation with caspase-1 inhibitors has not always been effective in preventing IL-1β signaling, and recent evidence has emerged for a role of caspase-1-independent IL-1β processing in disease (Guma et al., 2009; Joosten et al., 2009). While much work has been done on caspase-1-dependent IL-1β processing, research into mechanisms of caspase-1-independent IL-1β processing is less studied. Under acidic conditions, caspase-1-independent 20 kDa IL-1β is produced (Edye et al., 2013). The 20 kDa form of IL-1β has been suggested to be 5-fold less active than mature 17 kDa IL-1β (Hazuda et al., 1991). However, the activity of 20 kDa IL-1β has not been investigated under the acidic conditions in which it is produced. Additionally, the activity of mature IL-1 has not been studied under acidic conditions, nor have the interactions between these forms of IL-1. HEK-Blue IL-1β reporter cells and a brain endothelial cell line were used to explore the role of 20 kDa IL-1β in IL-1 signaling pathways. The cells were used to determine the activity of 20 kDa IL-1β and investigate its signaling alongside mature 17 kDa IL-1 α and β at pH 6.2. Our data suggest that 20 kDa IL-1β is a negative regulator of IL-1β that limits the amount of pro-IL-1β available for caspase-1 cleavage.

## 3.3. EXPERIMENTAL PROCEDURES

### 3.3.1. IL-1 activity assay.

HEK-Blue IL-1β cells (InvivoGen) were used to determine IL-1β activity. Cells were cultured in selective media [DMEM (Sigma) 10 % FBS (Life Technologies), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma), 100 µg/ml Normocin (InvivoGen), 100 µg/ml Zeocin (InvivoGen), 200 µg/ml HygroGold (InvivoGen)] to express IL-1R1. Cells were seeded at 3.3 x $10^5$ cells per ml in a 96-well plate (~50,000 cells/well). Treatments were made up in HEPES-buffered salt solution (HBSS; 145 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM HEPES, 10mM glucose, 0.01 % BSA as described previously,) (Edye et al., 2013) buffered to the indicated pH with 1 M NaOH and incubated with HEK-IL-1β reporter cells overnight. In these cells, activation of IL-1R1 results in NF-kB-driven expression of secreted embryonic alkaline phosphatase (SEAP), which causes a color change when incubated with QUANTI-Blue. Absorbance was measured at 630 nm on a Synergy HT plate reader (BioTek).

Little reporter activity was observed with cells incubated with IL-1 at pH 6.2 overnight (data not shown) but in line with a transient reduction in pH to model acute acidosis during inflammation, cells were treated at pH 6.2 for up to 60 min before the media was changed to DMEM 100 U/ml penicillin 100 µg/ml streptomycin overnight. Control cells treated at pH 7.4 alongside pH 6.2-treated cells also underwent a media change to DMEM 100 U/ml penicillin 100 µg/ml streptomycin overnight. When a media change was required, cells were seeded at least 4 h prior to use. IL-1R2 and IL-1RAcP were purchased from R&D Systems, IL-1Ra (Kineret) was obtained from the hospital pharmacy and phosphate-buffered saline (PBS) was purchased from Sigma.

### 3.3.2. bEnd.5 cells.

Brain endothelial cell line bEnd.5 (from Health Protection Agency Culture Collections, UK) was cultured in DMEM (Sigma) supplemented with 10 % FBS (Life Technologies), and 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma). Cells were seeded at 2 x $10^5$ cells per ml in a 96-well plate overnight. Treatments were made up in reducing media (100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1mM EDTA, 10 % glycerol, 1% CHAPS) at pH 6.2. Pro-IL-1β (100 ng/ml; Sino Biological Inc.) was incubated with 100 U/ml caspase-1 (Calbiochem) and/or 1 U/ml cathepsin D

(Calbiochem) for 8 h then incubated with cells for 60 min before being replaced with DMEM 100 U/ml penicillin 100 µg/ml streptomycin for a further 23 h. Supernatants were collected on ice and stored at -20$^{o}$C until required.

### 3.3.3. Cathepsin D cleavage assay.

Human pro-IL-1β (100 ng/ml; Sino Biological Inc.) was incubated with human cathepsin D (1 U/ml; Calbiochem) in HBSS at the indicated pH for 60 min unless stated otherwise.

### 3.3.4. Caspase-1 cleavage assay.

To ensure all pro-IL-1β had been cleaved to the 20 kDa from prior to the addition of caspase-1, pro-IL-1β was incubated with cathepsin D for 8 h. Caspase-1 (100 U/ml; catalogue number 218783 Calbiochem) was incubated overnight with 20 kDa IL-1β, pro-IL-1β or vehicle in reducing media (see above) buffered to indicated pH with 1 M NaOH.

### 3.3.5. Western Blot.

Samples were denatured at 95$^{o}$C for 5 min prior to separation on a 12 % acrylamide gel and transfer to a nitrocellulose membrane on a Trans-Blot SD Semi-Dry Transfer Cell (BioRad). Membranes were blocked with 5 % milk before incubation with goat anti-human IL-1β primary antibody (0.1 µg/ml; R&D Systems; AF-201) overnight at 4$^{o}$C. Membranes were washed, incubated with HRP-conjugated rabbit anti-goat secondary antibody (1/1000; Dako; P0449) then enhanced chemiluminescence (Amersham). Film was processed on a JP-33 automatic film processor.

### 3.3.6. ELISA.

Supernatants were analyzed for IL-6 and CXCL1 using specific ELISA kits (R&D Systems).

### 3.3.7. Statistical Analysis.

Statistical analysis was performed using GraphPad Prism v6. For comparisons between multiple groups, a one-way analysis of variance (ANOVA) with a Bonferroni multiple comparison post-hoc test was used. When unequal variance was observed (SEM proportional to the mean value), data was log transformed prior to statistical analysis. For comparisons between time

course data, a two-way ANOVA with Bonferroni multiple comparison post-hoc test was used. For competition experiments, data was expressed as relative activity/release compared to IL-1-induced activity/release which was normalized to 100 %. As this removes the variance in the control group, for normalized results, a one-sample t-test against a hypothetical value (100 %) with a Bonferroni multiple comparison post-hoc test was used. Data are expressed as mean +/- S.E.M. and $p \leq 0.05$ was considered significant.

## 3.4. RESULTS

### 3.4.1. Acidic pH does not affect the activity of IL-1 at IL-1R1.

Under inflammatory or ischemic conditions, local pH can drop as low as pH 6.2 (Nemoto and Frinak, 1981). A HEK-Blue IL-1β reporter assay that measures NF-κB mediated SEAP release after IL-1R1 activation by IL-1β was used to measure the activity of IL-1 at neutral and acidic pH. As expected, at pH 7.4, activity increased with increasing IL-1β concentrations (Figure 3.1A; squares). Activity also increased with increasing IL-1α concentrations at pH 7.4 (Figure 3.1A; triangles) suggesting that this assay can additionally be used to measure IL-1α activity. When cells were incubated with 10 ng/ml IL-1 at pH 6.2 for times up to 60 min, to model acute acidosis, IL-1β- and IL-1α-induced activity was not significantly altered when measured 16-18 h later compared to pH 7.4 (Figure 3.1B). To confirm that the IL-1 activity recorded was due to actions of IL-1 during the initial 60 min incubation at pH 6.2 or 7.4, and not the activity of residual IL-1 remaining during the subsequent overnight incubation, cells were treated with IL-1 for 60 min as in Figure 3.1B, but replaced with media containing IL-1Ra for the subsequent overnight incubation to block any further activation of IL-1R1. Cells treated in this way continued to produce IL-1-induced SEAP activity suggesting that a 60 min incubation is sufficient to induce activation of IL-1R1 and subsequent downstream activation of NF-κB signaling. Treatment with IL-1Ra for the duration of the initial IL-1 treatment and subsequent overnight incubation prevented any IL-1-induced activity, confirming that the dose used was sufficient to block IL-1 activity at IL-1R1 in this model (Figure 3.1C). These data confirm that IL-1 signaling was not affected by acidic pH.

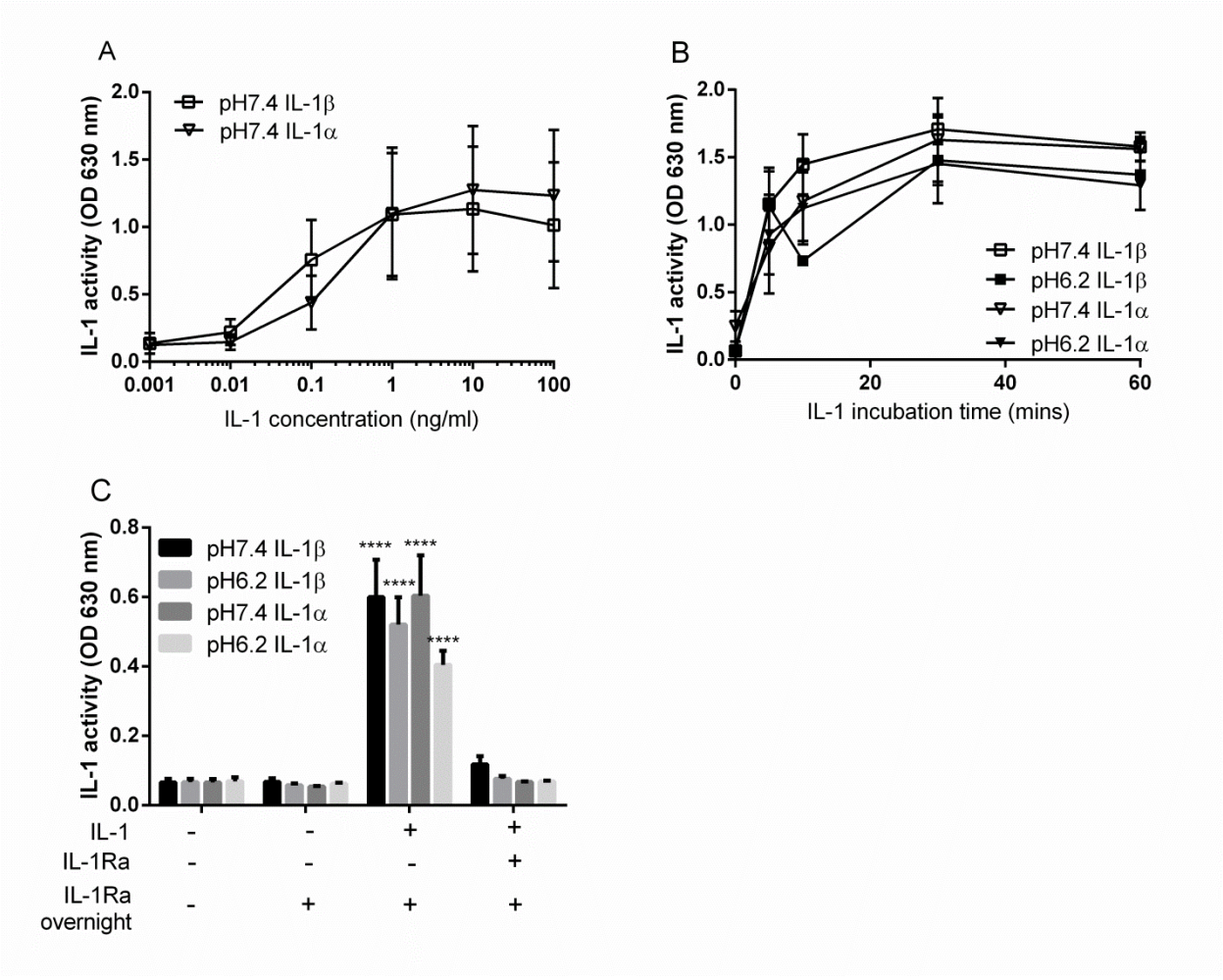


*Figure 3.1. 17 kDa IL-1α and IL-1β activity at IL-1R1 at pH 7.4 and pH 6.2.*

A HEK-IL-1 reporter assay (InvivoGen) was used to determine the activity of IL-1, expressed as optical density at 630 nm. Serial dilutions of IL-1α and β at pH 7.4 were incubated with HEK-IL-1 cells overnight before IL-1 activity was recorded at 630 nm (**A**). 10 ng/ml IL-1α or β was incubated with HEK-IL-1 cells for 0-60 min at pH 7.4 or 6.2 prior to a further incubation with DMEM 100 U/ml penicillin 100 µg/ml streptomycin overnight before IL-1 activity was recorded at 630 nm (**B**). 1 µg/ml IL-1Ra (or 0.1 % BSA in PBS vehicle) was incubated with the cells overnight following the initial 60 min incubation with 10 ng/ml IL-1 as in B. IL-1Ra was also included for the entire duration of the experiment as a positive control (**C**). Data are expressed as mean + SEM, n=3-4. Statistical analysis for (**A**) and (**B**) did not find any significance between groups using a two-way ANOVA with Bonferroni's post hoc test. Statistical analysis for **(C**) was performed with a one-way ANOVA followed by Bonferroni's post hoc test following a log transformation to adjust for unequal variance. No significant difference was detected in IL-1 activity between pH 7.4 and pH 6.2 with 60 min IL-1α or β treatment plus IL-1Ra overnight. No significant difference was observed between IL-1 plus IL-1Ra for the duration of the experiment and the control (IL-1Ra overnight). **** $p < 0.0001$ compared to control (IL-1Ra overnight).

### 3.4.2. Cathepsin D cleaves pro-IL-1β to a 20 kDa IL-1β species.

We have previously reported that at pH 6.2, caspase-1-independent 20 kDa IL-1β is released from mixed glia and human macrophages (Edye et al., 2013). Here we confirmed that recombinant cathepsin D was able to cleave pro-IL-1β into a 20 kDa form under acidic conditions (Figure 3.2A) and that this displayed activity at IL-1R1 (Figure 3.2B, C) but this was reduced compared to mature IL-1β (Figure 3.2D). This is in line with previous literature that shows HIV protease-cleaved 20 kDa IL-1β is approximately 5-fold less active than mature IL-1β at neutral pH (Hazuda et al., 1991).

To confirm that any activity shown with the 20 kDa IL-1β was acting through IL-1R1, we incubated HEK-IL-1 reporter cells with the 20 kDa IL-1β produced from 60 min incubation of pro-IL-1β and cathepsin D +/- IL-1Ra (Figure 3.2C). 20 kDa IL-1β activity was completely abolished on co-incubation with IL-1Ra, confirming that 20 kDa IL-1β was acting at IL-1R1.

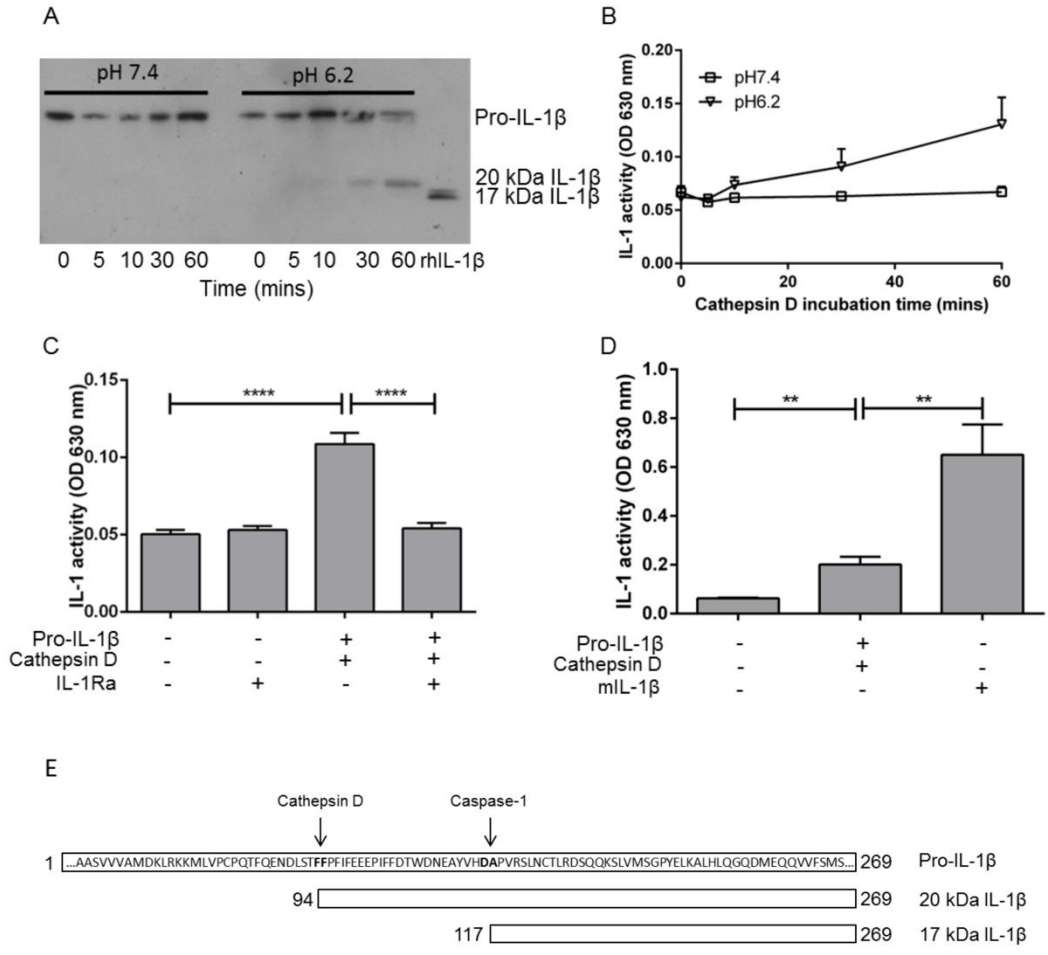


*Figure 3.2. Production and activity of cathepsin D-cleaved 20 kDa IL-1β.*

Recombinant pro-IL-1β (100 ng/ml) was incubated with 1 U/ml cathepsin D for 0-60 min at pH 7.4 or 6.2. Samples were processed by western blot analysis alongside recombinant human 17 kDa IL-1β (rhIL-1β) in the final lane (**A**). These same samples were also incubated with HEK-IL-1 reporter cells (**B**). For **B**, samples from 0-60 min pro-IL-1β plus cathepsin D incubation were added to HEK-IL-1 cells for 60 min before being replaced with fresh media overnight. Squares represent samples of pro-IL-1β incubated with cathepsin D for 0-60 min at pH 7.4; triangles, pH 6.2. For **C**, samples from 60 min pro-IL-1β plus cathepsin D incubation were added to HEK-IL-1 cells +/- 1 μg/ml IL-1Ra for 60 min before being replaced with fresh media overnight. In **D**, samples from 60 min pro-IL-1β plus cathepsin D incubation were added to HEK-IL-1 cells and compared alongside mature 17 kDa IL-1β for 60 min before being replaced with fresh media overnight. **E**, schematic of pro-IL-1β and putative cathepsin D(Takenouchi et al., 2011) and caspase-1 cleavage sites adapted from Hazuda et al (Hazuda et al., 1991). Western blot is representative of n=3, graphical data expressed as mean + SEM, n=3-4. Statistical analysis was performed with a one-way ANOVA with Bonferroni's multiple comparison test. Significance was determined as $p \leq 0.05$. $^{**}p < 0.01$, $^{****}p < 0.0001$.

### 3.4.3. 20 kDa IL-1β does not affect mature IL-1α or IL-1β signaling at IL-1R1.

To understand the relevance of 20 kDa IL-1β in IL-1 signaling under disease relevant acidic conditions, we investigated whether 20 kDa IL-1β would affect active 17 kDa IL-1 signaling at pH 6.2. 20 kDa IL-1β (generated by the incubation of 100 ng/ml pro-IL-1β and 1 U/ml cathepsin D at pH 6.2) was added to HEK-IL-1 cells with 10 ng/ml mature 17kDa IL-1β (Figure 3.3A, C) or IL-1α (Figure 3.3B) at pH 6.2 for 60 min before being replaced with serum free media overnight. The activity induced by 17 kDa IL-1 alone was normalized to 100 % and the other treatments were expressed relative to this. Under these conditions 20 kDa IL-1β did not alter the activity of 17 kDa IL-1β or IL-1α at IL-1R1. To more closely mimic a potential inflammatory scenario, cathepsin D and caspase-1 were incubated together with pro-IL-1β at pH 6.2. This resulted in complete processing of pro-IL-1β producing predominantly 20 kDa IL-1β with a small amount of 17 kDa IL-1β (lane 2, Figure 3.3D). When this was added to HEK-IL-1 cells, there was still no significant change in activity from caspase-1 cleaved 17 kDa alone (Figure 3.3E), again suggesting that the 20 kDa form is not able to influence the 17kDa species at IL-1R1. The 20 kDa form may however act to remove available pro-IL-1β for further caspase-1-cleavage (see below).

To investigate 20 kDa IL-1β signaling in brain endothelial cells, which are known to respond to IL-1β and contribute to the inflammatory response in central nervous system disorders (Denes et al., 2011), samples from a 8 h incubation of pro-IL-1β with cathepsin D and/or caspase-1 were added to the mouse brain endothelial cell line, bEnd.5, for 60 min before being replaced with serum free media for a further 23 h. Incubation of pro-IL-1β with caspase-1 and cathepsin D predominantly resulted in the production of 20 kDa IL-1β with some 17 kDa IL-1β (Figure 3H). IL-1-mediated activity (as measured by IL-6 and CXCL1 release) with the caspase-1- plus cathepsin D-cleaved 17 kDa and 20 kDa IL-1β was not significantly different from caspase-1-cleaved 17 kDa IL-1β alone (Figure 3.3F, G), again suggesting that 17 kDa IL-1β signaling at IL-1R1 is not directly influenced by the 20 kDa form.

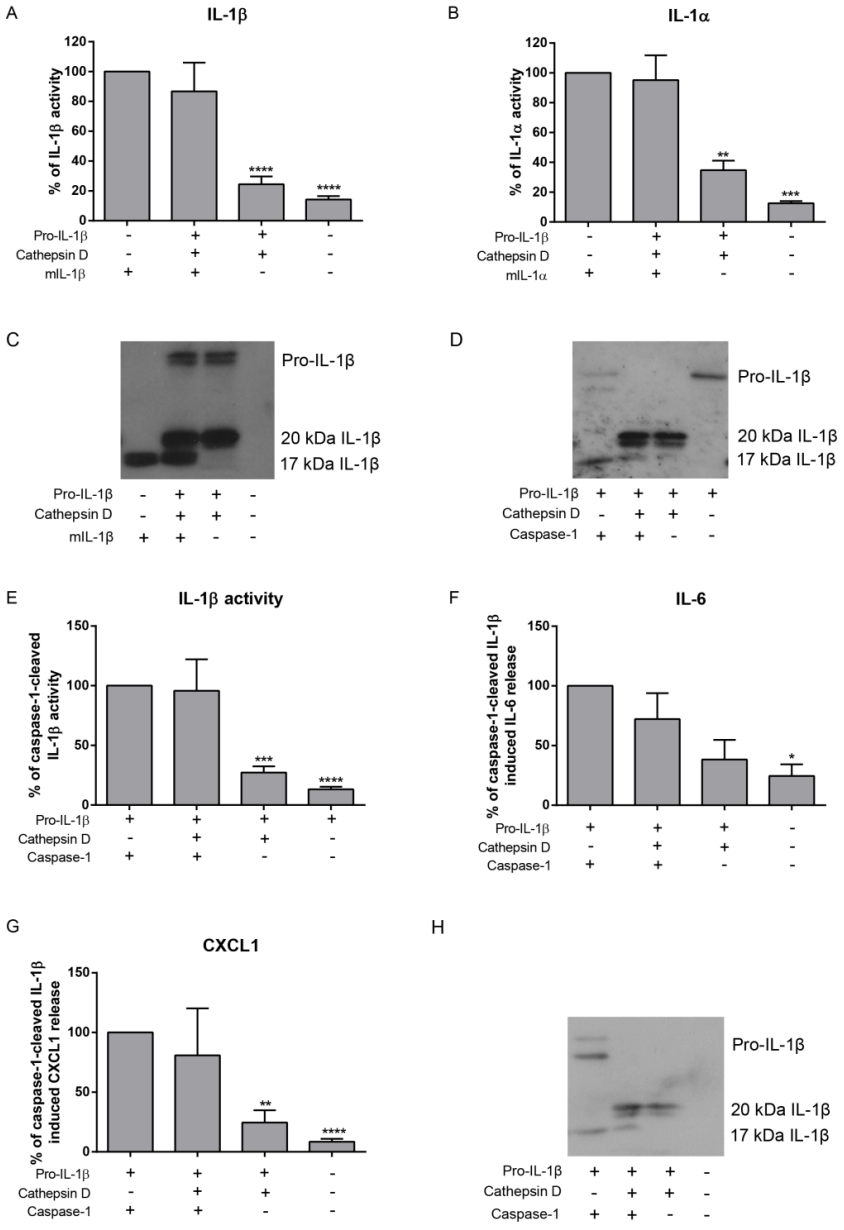


*Figure 3.3. IL-1 activity in the presence of 20 kDa IL-1β.*

100 ng/ml pro-IL-1β was incubated with 1 U/ml cathepsin D for 60 min to produce 20 kDa IL-1β before this was added to HEK-IL-1 cells with 10 ng/ml recombinant mature IL-1β (mIL-1β; **A**) or IL-1α (mIL-1α; **B**) for 60 min. This was then replaced with fresh media overnight. Pro-IL-1β cleavage following 60 min incubation with 1 U/ml cathepsin D was confirmed by western blot alongside 10 ng/ml recombinant mature IL-1β (**C**). 100 ng/ml pro IL-1β was incubated with caspase-1 (100 U/ml), cathepsin D (1 U/ml) or both overnight at pH 6.2 and cleavage was confirmed by western blot (**D**). The resulting samples were added to HEK-IL-1 cells for 60 min before being replaced with fresh media overnight (**E**). Samples from an 8 hour incubation of 100 ng/ml pro-IL-1β with 100 U/ml caspase-1 and/or 1 U/ml cathepsin D were added to brain endothelial cells (bEnd.5) for 60 min before being replaced with fresh media overnight. Levels of IL-6 (**F**) and CXCL1 (**G**) released were quantified by ELISA. Pro-IL-1β cleavage during the 8 h incubation with caspase-1 and cathepsin D was confirmed by western blot (**H**). Western blots are representative of n=3. Data are presented relative to activity induced by mature 17 kDa IL-1 which was normalized to 100%. Data are the mean + SEM n=3-5. Statistical analysis was performed with a one-sample t-test against a hypothetical value (100 %) with a Bonferroni multiple comparison post-hoc test. $p \leq 0.05$ was considered significant. * $p< 0.05$, ** $p< 0.01$,*** $p< 0.001$**** $p< 0.0001$.

### 3.4.4. IL-1R2 decoy activity is reduced at pH 6.2.

The decoy receptor IL-1R2 binds IL-1 with different affinities, binding IL-1β with high affinity, but requiring the presence of IL-1R accessory protein (IL-1RAcP) for efficient IL-1α binding (Smith et al., 2003; Garlanda et al., 2013). Whether IL-1R2 signaling is altered under disease-relevant acidic conditions is unknown, as is the ability of this receptor to bind 20 kDa IL-1β. Using the HEK-IL-1 reporter assay, IL-1β activity was reduced with soluble IL-1R2 at pH 7.4 (Figure 3.4A) but not pH 6.2 (Figure 3.4C). Under these conditions IL-1R2 did not affect IL-1α signaling alone or in combination with IL-1RAcP at pH 7.4 or pH 6.2 (Figure 3.4B, D). At pH 6.2, 20 kDa IL-1β signaling was reduced with IL-1R2 and IL-1RAcP combined (Figure 3.4E). These data suggest that IL-1R2 may only have a minor role in the regulation of mature IL-1 under acidic conditions.

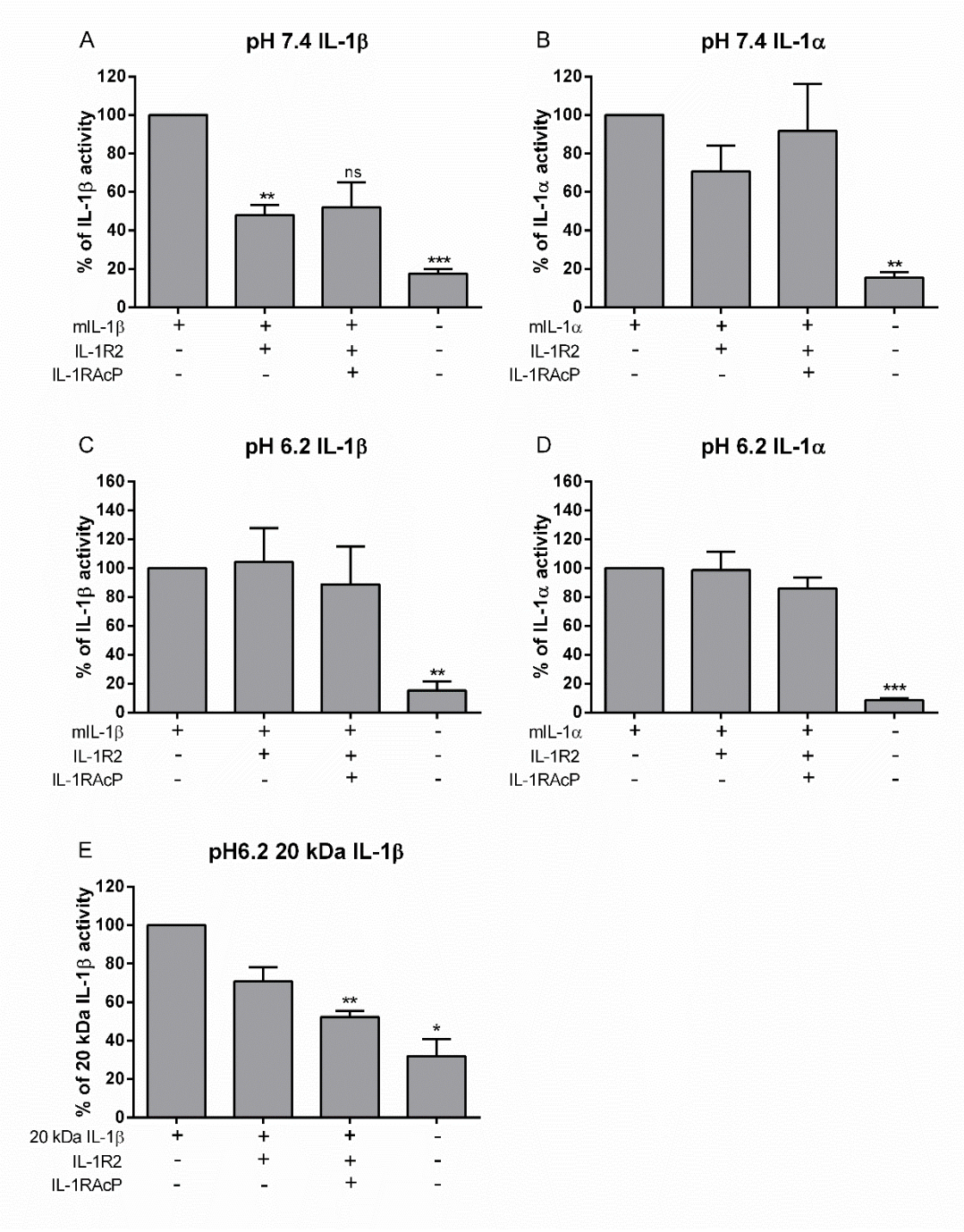


*Figure 3.4. Effect of IL-1R2 on IL-1 activity at pH 7.4 and pH 6.2.*

10 ng/ml 17 kDa mature IL-1β (mIL-1β; **A**, **C**), 10 ng/ml 17 kDa mature IL-1α (mIL-1α; **B, D**) or the product of a 60 min incubation of 100 ng/ml pro-IL-1β with 1 U/ml cathepsin D to produce 20 kDa IL-1β (20 kDa IL-1β; **E**) was added to HEK-IL-1 cells at the indicated pH with 2.5 µg/ml IL-1R2, 2 µg/ml IL-1RAcP or vehicle (PBS for IL-1, IL-1RAcP, cathepsin D or 0.1 % BSA in PBS for IL-1R2) for 60 min before being replaced with fresh media overnight. Data are presented relative to activity induced by IL-1 alone (17 kDa IL-1β **A, C**; 17 kDa IL-1α **B, D**, 20 kDa IL-1β, **E**) which was normalized to 100 % + SEM, n=3-6. Statistical analysis was performed with a one-sample t-test against a hypothetical value (100 %) with Bonferroni's multiple comparison test. Significance was determined as $p \leq 0.05$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

## 3.4.5. 20 kDa IL-1β is not further cleaved by caspase-1.

In order to assess if 20 kDa IL-1β is a temporary holding state before further cleavage by caspase-1, 20 kDa IL-1β was incubated with recombinant caspase-1 at pH 6.2 and 7.4. Caspase-1 was able to cleave pro-IL-1β into 17 kDa mature IL-1β at pH 7.4 and to a lesser extent at pH 6.2 but it did not cleave 20 kDa IL-1β (produced from complete cleavage of pro-IL-1β following 8 h incubation with 1 U/ml cathepsin D) into 17 kDa IL-1β at either pH (Figure 3.5). These data suggest that the formation of 20 kDa IL-1β may act to limit IL-1β signaling by reducing the pro-IL-1β pool available for caspase-1 cleavage.

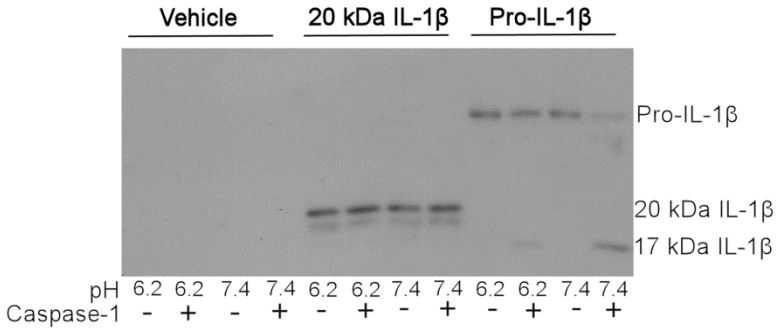


*Figure 3.5. Western blot analysis of caspase-1 cleavage.*

Vehicle (PBS), 20 kDa IL-1β (produced from 8 h incubation of 100 ng/ml pro-IL-1β with 1 U/ml cathepsin D in reducing media at pH 6.2) or 100 ng/ml pro-IL-1β were incubated with 100 U/ml caspase-1 or vehicle (PBS) overnight in reducing media buffered to indicated pH with 1 M NaOH. Samples were collected on ice and analyzed by western blot. Image is representative of n=3.

## 3.5. DISCUSSION

Acidosis is a key feature of disease and yet few researchers take this into account when designing *in vitro* studies. Our findings show that although mature 17 kDa IL-1α and β signaling at IL-1R1 is not altered at low pH or in the presence of cathepsin D-cleaved 20 kDa IL-1β, production of 20 kDa IL-1β may act to limit IL-1β signaling by reducing the pool of pro-IL-1β available for caspase-1 cleavage thus preventing the appearance of further mature 17kDa IL-1β. We also show that IL-1R2 was able to reduce IL-1β signaling at IL-1R1 at pH 7.4 but not at pH 6.2, thus the production of 20 kDa IL-1β may partly compensate for this lack of IL-1R2-mediated control at sites of inflammation where there is reduced pH.

As 20 kDa IL-1β binds the same receptor as 17 kDa IL-1, but only acts as a partial agonist (Figure 2), we questioned whether 20 kDa IL-1β would compete with 17 kDa IL-1 and therefore reduce activity at IL-1R1. 20 kDa IL-1β did not have any significant effect on mature IL-1 signaling in our model, even when produced in excess compared to 17 kDa IL-1β. This may be due to the sensitivity of cells to respond to mature IL-1 and the excess of IL-1R1 receptors on their surface. It is due to this excess of receptors that IL-1Ra is produced at up to 1000-fold the amount of IL-1 in order to effectively inhibit IL-1 (Arend et al., 1998). The absolute amounts of 20 kDa IL-1β produced *in vivo* would need to be analyzed in order to determine if 20 kDa IL-1β could act as an antagonist like IL-1Ra, however, our data suggest its regulatory role is more likely to be in removing pro-IL-1β to prevent further cleavage by caspase-1.

There was a significant reduction in IL-1β activity with IL-1R2 at pH 7.4 but not at pH 6.2, thus our data suggests IL-1R2 is less effective at blocking IL-1 activity at low pH. A significant reduction in 20 kDa IL-1β activity was observed with IL-1R2 and IL-1RAcP at pH 6.2 and this may in part be due to the lower activity of 20 kDa IL-1β requiring less binding by IL-1R2 to induce an observable effect. However, once 20 kDa IL-1β has been formed, it has performed its regulatory role in preventing the formation of mature 17 kDa IL-1β. Thus binding IL-1R2/IL-1RAcP would further help reduce IL-1-mediated inflammation as it would block any minor signaling induced by 20 kDa IL-1β.

Activation of caspase-1 can induce pyroptosis resulting in loss of plasma membrane integrity and release of intracellular components. Nearly 50 % of pro-IL-1β produced in response to nigericin is released from bone marrow derived macrophages without any further cleavage (Baroja-Mazo et al., 2014). Although pro-IL-1β is not active, it is now known that inflammasome components and mature caspase-1 can be released from cells and cleave

extracellular pro-IL-1β into its 17 kDa active form (Baroja-Mazo et al., 2014). Unlike IL-1R1, IL-1R2 is able to bind pro-IL-1β thereby preventing any subsequent cleavage to mature IL-1β. If IL-1R2 is unable to do this at low pH, cathepsin D-dependent cleavage of pro-IL-1β to 20 kDa IL-1β could fulfil this IL-1-limiting role and prevent the spread of inflammatory signaling.

Cathepsin D is an aspartyl protease that resides in the lysosome and is maximally active at low pH. The cellular location of cathepsin D cleavage of pro-IL-β (i.e. within lysosomes or extracellularly following pro-IL-1β release) remains to be elucidated (Edye et al., 2013; Takenouchi et al., 2014).

In addition to caspase-1, multiple other proteases are now known to cleave pro-IL-1β (Netea et al., 2014). Proteinase 3, a neutrophil protease, cleaves pro-IL-1β near to the caspase-1 binding site to produce an active protein (Coeshott et al., 1999) and inhibiting this protease is protective in a mouse model of arthritis (Joosten et al., 2009). These alternatively cleaved IL-1β forms appear to be pro-inflammatory and therefore blocking their activity could prove beneficial in disease. Moving away from the caspase-1 cleavage side towards the N-terminus results in increasingly larger and less active forms of IL-1β (Hazuda et al., 1991) with Black et al., (Black et al., 1988) suggesting no further activity observed at greater than 18.4 kDa in size. Whether other larger alternatively cleaved forms of IL-1β are protected from further cleavage to active 17 kDa and could therefore represent an additional negative regulation of IL-1β signaling is yet to be elucidated.

Lactic acid is able to induce the release of 20 kDa IL-1β (Edye et al., 2013) and has also been suggested to exert anti-inflammatory effects through a reduction of LPS-induced NF-κB signaling (Kellum et al., 2004). Mature 17 kDa IL-1β signaling through IL-1R1 also activates NF-κB-induced expression of pro-inflammatory cytokines. Thus it would follow that a reduction in mature 17 kDa IL-1β, due to lactic acid-mediated production of 20 kDa IL-1β, would lead to a reduction in IL-1β signaling at IL-1R1 and therefore a subsequent reduction in IL-1-induced pro-inflammatory cytokines. Alternatively Rajamäki and colleagues (Rajamäki et al., 2013) show that acidosis can activate the inflammasome, inducing mature 17 kDa IL-1β release. These differences may be due to the type of acid used as this has previously been shown to determine whether a pro- or anti-inflammatory effect is observed, as lactic acid exerts different effects to hydrochloric acid (Kellum et al., 2004).

## 3.6. CONCLUSION

Our data suggest that the formation of 20 kDa IL-1β under acidic conditions provides negative regulation of IL-1 signaling. Cleavage of pro-IL-1β to 20 kDa IL-1β inhibits its further cleavage to the highly active 17 kDa IL-1β thereby limiting the inflammatory cascade and dampening pro-inflammatory signaling at sites of inflammation where a reduction in pH is observed. Additional mechanisms of regulation of this system are particularly important at low pH as the ability of IL-1R2 to inhibit IL-1 signaling is reduced. The *in vitro* system used here is a valuable tool for exploring signaling pathways, and the use of acidic pH to more closely mimic the disease microenvironment is an improvement on physiological culture conditions. However, it is not a perfect model of human disease thus further work *in vivo* and in disease tissue is required to fully understand the role of 20 kDa IL-1β.

**Acknowledgments**

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**Conflicts of interest**

The authors declare that they have no conflicts of interest with the contents of this article.

**Author contributions**

MEE, DB and SMA designed the study. MEE performed and analyzed the experiments and wrote the manuscript with critical review by DB and SMA. All authors reviewed the results and approved the final version of the manuscript.[1]

[1] Abbreviations: ANOVA, analysis of variance; IL-1, interleukin-1; IL-1R, interleukin-1 receptor; IL-1RAcP, interleukin-1 receptor accessory protein; NLRP3, NLR family, pyrin domain-containing protein 3; PBS, phosphate-buffered saline

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# Chapter 4

## Interleukin-1β is not required for acute seizures in mice following intranasal kainic acid administration

Michelle E. Edye, David Brough, Stuart M. Allan

# 4. Interleukin-1β is not required for acute seizures in mice following intranasal kainic acid administration

## 4.1. ABSTRACT

Epilepsy is a widely prevalent disorder but remains inadequately treated with nearly a third of patients resistant to current treatments. Inflammation has been recognised as an important contributor to seizures, with the pro-inflammatory cytokine interleukin-1 beta (IL-1β) exacerbating seizure activity in rodent models and increased expression of IL-1β observed in human seizure tissue. IL-1β is produced as an inactive precursor that requires cleavage for its activation; typically by caspase-1. Caspase-1 is itself activated through association with the NLRP3 inflammasome protein complex. Intranasal kainic acid in rodents induces seizures and hippocampal cell death reminiscent of temporal lobe epilepsy, one of the more treatment-resistant forms of epilepsy. We sought to determine if the NLRP3 inflammasome, caspase-1 or IL-1 was involved in the exacerbation of acute intranasal kainic acid-induced seizures. Following intranasal kainic acid (30 mg/kg) administration to C57Bl/6J mice, time to seizure onset, number of seizures and duration of seizure activity was recorded by visual inspection of the EEG, seizure severity was scored based on behaviour and automated seizure analysis of the EEG trace was performed. Seizure parameters were unchanged in NLRP3 knock-out and inflammasome adaptor protein ASC knock-out mice compared to wild-type mice. Similarly, caspase-1 inhibition did not reduce any seizure parameters studied and no increase in IL-1 expression was observed 3 h following intranasal kainic acid treatment. This suggests that the NLRP3 inflammasome, caspase-1 and IL-1 are not involved in the exacerbation of acute seizures following intranasal kainic acid treatment in this model, thus further work is required to determine the precise involvement of the IL-1β system in seizures and epilepsy.

## 4.2. INTRODUCTION

Epilepsy is one of the most common brain disorders worldwide but nearly a third of epilepsy patients still remain resistant to current treatments (World Health Organisation, 2009). Epilepsy is defined by the occurrence of at least two spontaneous seizures and these are driven by hyperexcitability and/or aberrant synchronicity of neuronal firing (Stafstrom and Carmant, 2015). Standard antiepileptic drugs (AEDs) typically target ion channels to address this imbalance in excitability but do not address the underlying mechanisms that cause epilepsy (Sills, 2011). Thus, there is a need for further research into the mechanisms involved in seizure generation and its development into epilepsy (epileptogenesis) to discover novel therapeutic targets that may provide relief for treatment resistant epilepsy.

Inflammation is known to exacerbate seizures and specifically the pro-inflammatory cytokine interleukin-1 beta (IL-1β) has been implicated in multiple neurological disorders including Alzheimer's disease, Parkinson's disease, stroke, and traumatic brain injury, in addition to epilepsy (Allan et al., 2005). Elevated expression of IL-1β and its receptor IL-1R1 in the brain have been shown in multiple seizure models (Eriksson et al., 1999; Vezzani et al., 1999; Ravizza and Vezzani, 2006; Dube et al., 2010). Similar increases in IL-1 expression post seizure have been observed in human samples from patients with temporal lobe epilepsy (TLE)(Ravizza et al., 2008a) and elevated IL-1 has also been observed in the cerebrospinal fluid of children with febrile seizures (Haspolat et al., 2002). In rodents, addition of exogenous IL-1β increases kainic acid-induced seizure activity which is prevented by pre-treatment with the endogenous IL-1R1 antagonist, IL-1Ra (Vezzani et al., 1999). Seizure behaviour is also increased in IL-1β-treated animals following bicuculline-induced seizures (Vezzani et al., 2000) or febrile seizures (Heida and Pittman, 2005). Together these provide strong evidence for IL-1β involvement in seizures.

Production and release of active IL-1β is a two-step process. An initial stimulus is required to induce the expression of the inactive pro-IL-1β, followed by a second stimulus to induce cleavage into the active form. This cleavage is typically performed by the cysteine protease caspase-1, following its own activation via association with an inflammasome complex (Schroder and Tschopp, 2010). A number of inflammasomes have been characterised and these differ in their pattern recognition receptor and binding partners. The NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome is comprised of the NLRP3 pattern recognition receptor, the adapter protein apoptosis-associated, speck-like protein containing a CARD (ASC) and pro-caspase-1. NLRP3 is able to respond to a variety of pathogen associated molecular pathogens (PAMPS) and endogenous damage associated

molecular patterns (DAMPS) (Schroder and Tschopp, 2010). This ability to respond to DAMPs under sterile conditions makes it a likely target for IL-1β production in seizure.

Due to the compelling data on the role of IL-1β in the exacerbation of seizures, this pathway has been explored as a therapeutic target for the treatment of seizures. Intra-cerebral infusion of IL-1Ra delays seizure onset and reduces seizure behaviour following bicuculline treatment (Vezzani et al., 2000) and intravenous IL-1Ra delays onset of status epilepticus following pilocarpine treatment (Marchi et al., 2009). This pathway has also been targeted at the level of IL-1β activation where pre-treatment with caspase-1 inhibitors delays seizure onset and decreases seizure number and time in seizure activity following kainic acid treatment (Ravizza et al., 2006b). The role of NLRP3 in the activation of danger signals such as HMGB1 that may also exacerbate seizure, in addition to IL-1β, makes it a promising therapeutic target (Edye et al., 2014). Indeed, recent literature demonstrates a reduction in spontaneous recurrent seizures post status epilepticus (SE) in mice with NLRP3 knocked-down (Meng et al., 2014). These data suggest that targeting the IL-1 pathway may produce effective anticonvulsant treatment. Accordingly, the caspase-1 inhibitor VX-765 has reached phase IIb trials for treatment resistant partial epilepsy (ClinicalTrials.gov, 2010).

TLE, where the seizure focus is in the temporal lobe, is often clinically associated with hippocampal sclerosis and forms one of the more treatment resistant forms of epilepsy (Ben-Ari, 1985). Modelling this clinically relevant disease to help further elucidate the role of IL-1β signalling in seizures will provide information about potential novel targets for treatment resistant epilepsy.

Kainic acid is a well characterised chemical convulsant and is used to model TLE (Lévesque and Avoli, 2013). However, mouse strains respond differently to kainic acid. C57Bl/6J mice, which form the basis for many knock-out (KO) mice strains, are more resistant to peripheral treatment with kainic acid and do not display the characteristic cell death associated with clinical TLE when dosed in this way (Schauwecker and Steward, 1997). To overcome this, kainic acid has previously been injected directly into the hippocampus. However, intrahippocampal kainic acid is still required at high doses to induce neuronal cell loss in C57Bl/6J mice that is comparable to other mouse strains (Schauwecker, 2002), thus this route is predominantly used when a C57Bl/6J background is not required or when studying seizure onset and duration regardless of cell death (Ravizza et al., 2006b). Intrahippocampal injections also require implantation of a dosing cannula during surgery which can become blocked, thereby preventing the use of that animal. Hippocampal

damage, similar to that observed in patients with TLE, is however, observed when kainic acid is administered via the intranasal (i.n.) route (Chen et al., 2002). Dosing i.n. is a simple, minimally invasive dosing route which also prevents the variability and low bioavailability observed with systemic dosing due to first pass metabolism and the need to cross the blood-brain barrier (Pardeshi and Belgamwar, 2013). Additionally, dosing via the i.n. route has repeatedly shown enhanced delivery to the brain compared to other routes (intravenous, i.v.; intraperitoneal, i.p.; or sub-cutaneous, s.c.) (Lochhead and Thorne, 2012). Here we used i.n. kainic acid to induce seizures in wild-type (WT) C57Bl/6J mice and mice with NLRP3 inflammasome components knocked-out (NLRP3 or ASC), in addition to pre-treatment with ac-YVAD-cmk, a caspase-1 inhibitor, to investigate the role of IL-1, caspase-1 and the NLRP3 inflammasome in this model of acute seizure.

## 4.3. EXPERIMENTAL PROCEDURES

### 4.3.1. Experimental Animals

Male C57Bl/6J mice were purchased from Charles River. NLRP3 and ASC KO mice generated on a C57Bl/6J background were kindly provided by Dr Vishva Dixit (Genentech, CA) and bred in house. Animals were housed on a 12 hour light/dark cycle under temperature, humidity and light-controlled conditions with free access to food and water. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and approved by the local Animal Welfare and Ethical Review Board. Wild-type and KO mice were age-matched and KO status has been confirmed previously (Summersgill et al., 2014). Animals from each strain (WT, NLRP3 KO, ASC KO) or treatment group (Vehicle (PBS), YVAD or GYKI) were randomly assigned an ID with strains or treatments blocked for surgery day. An individual animal was counted as an experimental unit and sample size was selected using power calculations based on previous data from our group showing the effect of caspase-1 deficiency on kainic acid-induced seizures (Ravizza et al., 2006b). Type 1 error was set at 0.05 and power at 0.8. The mean standard deviation of WT and KO animals from this study was 983 s for seizure onset, 118 s for time in seizure activity and 2.6 for number of seizures, and the expected difference in means between the two groups was set at 1000 s, 110 s and 2.5 respectively. This produced sample sizes of 8, 10 and 9 for seizure onset, time in seizure and number of seizures respectively.

### 4.3.2. Surgical implantation EEG transmitter and i.c.v. cannula

Surgery was performed using stereotaxic guidance on mice over 20 g. Anaesthesia was induced with 4 L/min isoflurane 0.2 L/min O2, 0.4 L/min $NO_2$ and maintained at approximately 2.5 L/min isoflurane 0.2 L/min O2, 0.4 L/min $NO_2$ during surgery depending on the response of the animal. Reflexes and breathing were monitored to ensure surgical anaesthesia was maintained during the procedure. Body temperature was maintained with a heated blanket and Buster sterile op cover. Buprenorphine (0.1 mg/kg s.c.) was administered at the start of surgery and the dose was repeated 4-6 h later. During recovery from surgery, animals were monitored on a heated blanket then in a heated cabinet set to 30 $^{o}$C until they were deemed fit to return to normal housing. Animals were singly housed post-surgery to minimise suture or electrode removal and allowed to recover for at least 3 days before seizure induction. Animals were monitored and body weight was recorded daily.

ETA-F20 small animal transmitters (Data Sciences International, St Paul, MN) were implanted in a sub-cutaneous pocket on the flank. Electrodes were tunnelled subcutaneously to an incision over the skull where the reference electrode was glued to the nasal sinus and the EEG recording electrode was implanted into the dorsal hippocampus (-1.9 mm posterior, -1.5 mm lateral to Bregma and 1.5 mm below the dura). For stability, a 1.4 mm screw (Precision Technology Supplies, UK) was attached via a shallow hole on the skull and the whole area secured with dental cement. When required, an i.c.v. cannula (23 gauge; 1 mm from surface of skull) was implanted at -0.4 mm, +1.0 mm from Bregma.

### 4.3.3. Seizure induction

Animals were housed in their home cage over a receiver pad. Seizure induction was performed between 8.30 and 11 am with kainic acid administration staggered to allow time for terminal sample collection. At least 30 min baseline EEG and video were recorded prior to seizure induction, and up to 3 h EEG and video post seizure induction. Kainic acid (30 mg/kg; Tocris, UK) dissolved in PBS (10 mg/ml for repeated dosing or 15 mg/ml for subsequent studies; pH 7.2) was administered i.n. using a P200 Gilson pipette under brief anaesthesia (isoflurane) to induce seizures in all animals. Doses lower than this do not consistently induce seizures (correspondence with Xiangyu Zheng) (Chen et al., 2002). PBS was administered i.n. under brief isoflurane anaesthesia as a control. If severe behavioural seizures were repeated or prolonged (greater than 10 per h), animals were culled.

Pre-treatment with the caspase-1 antagonist Ac-YVAD-cmk (Sigma, UK; 200 ng in 1 µl; i.c.v.) as has been shown to be protective in a mouse model of intracerebral haemorrhage (Wu et al., 2010), its vehicle (1 µl; 0.2 % DMSO in PBS; i.c.v.), or the AMPA/kainate antagonist GYKI 52466 (Sigma, UK; 30 mg/kg, 6 mg/ml $H_2O$; i.p. shown to be protective in multiple seizure models (Yamaguchi et al., 1993)) occurred 15 min prior to seizure induction. For i.c.v. injection, animals were lightly anaesthetised with isoflurane, the injection cannula inserted into the guide cannula to extend 2.5 mm into the brain and the animals returned to their home cage above the receiver pad. 1 µl was administered over 30 s and the cannula left in place for a further 30 s post dose to prevent reflux.

### 4.3.4. Sample collection

At the end of the study, mice were culled via a schedule 1 method or terminally anaesthetised with isoflurane and intracardially perfused with 0.9 % NaCl with 0.1 % diethyl

pyrocarbonate. Half brains were immerse-fixed in PFA overnight followed by 30 % sucrose then rapidly frozen in isopentane at -30 to -40 $^{o}$C. All brains were stored at -80 $^{o}$C until use.

## 4.3.5. Immunohistochemistry

Paraformaldehyde-fixed half brains were sliced at 30 µm on a sledge microtome (8000 Microtome, Bright, UK) and collected sequentially in 12. One set was used per animal per staining protocol. Sections were blocked with 10 % normal donkey serum (NDS; Sigma, UK) before overnight incubation with primary antibodies (rabbit anti-IL-1β (1/100 R&D Systems, UK), rabbit anti-IL-1α (1/100 R&D Systems, UK), goat anti-Iba1 (1/500 Wako, VA) and mouse anti-GFAP (1/1000 Millipore, UK). Slices were then PBS washed before being incubated in fluorophore-conjugated secondary antibodies (Alexa Fluor 488 and 594 (1/500), Invitrogen, UK). Images were acquired using a 20x/0.80 Plan Apo objective using the 3D Histech Pannoramic 250 Flash II slide scanner and specificity of the antibodies was demonstrated previously (Girard et al., 2012; Denes et al., 2015).

## 4.3.6. EEG analysis

EEG data were collected using DSI A.R.T. Acquisition 4.0 at a sample rate of 1000 Hz.

### By eye

EEG traces were analysed by eye using DSI A.R.T. Analysis 4.0 by an experimenter blind to strain or treatment. Baseline and 60 min post kainic acid EEG traces were viewed and seizures were determined as discrete events with a profile distinct from baseline recording i.e. increased amplitude and/or frequency and a duration greater than 5 s (e.g. Bergstrom 2013). Seizure onset, number and total duration were recorded. When seizure onset was greater than 60 min from kainic acid treatment, a value of 60 was awarded.

### Automated

Automated seizure analysis was adapted from White et al (White et al., 2006). 10 or 15 min baseline EEG data and 60 min post kainic acid EEG data was exported to a text file and analysed with EEG analyser v1.0 written in house.

*Coastline method*

Coastline analysis compares the actual length of the EEG trace as if it were stretched out into a flat line. As seizures are represented by increased amplitude and frequency, it would therefore follow that the EEG would be longer for those experiencing most seizures. The

sum of the absolute value between consecutive points was calculated per 3 s / 3000 point window as follows where x represents the EEG voltage and ABS, the absolute value. These were then summed across the recording period (baseline or 60 min post kainic acid). To remove inter-mouse variability in seizure parameters and any artefacts from noisy baselines, values calculated from 60 min post-seizure were normalised to the mouse's own baseline (10-15 min pre-treatment recording).

$$CoastLine = \sum_{i=1}^{3000} ABS(x_i - x_{i-1})$$

*Autocorrelation – sum difference*

Again, using the fact that seizures are characterised by repeated spikes, or increased amplitude and frequency, autocorrelation can be used to detect seizures. The maximum to minimum vector of a 125 ms (125 point) window was calculated and compared to that of the next two windows. The area shared between all three windows was calculated and summed to produce the sum of the difference as follows:

$$Sum\ Difference = \sum_{i=1}^{125} (HVi - LVi)$$

HVi is the smallest maximum point of the 3 windows and LVi the largest minimum point of the 3 (i.e. HVi = min [max Si, max(max (Si+1), max (Si+2))], LVi = max [min Si, min(min(Si+1), min(Si+2))]).

The sum difference for each segment was summed over the duration of the recording period to give a total value. Sum difference post kainic acid was again normalised to the animal's pre-treatment baseline.

## 4.3.7. Behavioural analysis

Maximum seizure severity reached during the recording period was determined following a modified Racine's scale; Stage 1: Mouth and facial movements; Stage 2: Head nodding; Stage 3: Forelimb clonus; Stage 4: Rearing; Stage 5: Rearing and falling; and Stage 6: Repeated stage 5 seizures and early termination (Racine, 1972).

### 4.3.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism v6. For normalized results, a one-sample t-test against a hypothetical value (0 for seizure severity, number and duration, 60 for maximum seizure onset, or 100 % for automated analysis change from baseline). For comparisons between multiple groups, a one-way analysis of variance (ANOVA) with a Bonferroni multiple comparison post-hoc test was used. Data are expressed as mean +/- S.E.M or median +/- interquartile range (for severity scores) and $p \leq 0.05$ was considered significant.

## 4.4. RESULTS

### 4.4.1. Intranasal kainic acid induces seizures in C57Bl/6J mice

With literature suggesting a reduced response to kainic acid by C57Bl/6J mice, a pilot study was performed to confirm that i.n. dosing of kainic acid was able to induce seizures in C57Bl/6J mice. All animals dosed with kainic acid displayed behavioural and/or EEG seizures whereas those dosed with PBS displayed normal behaviour and no seizures (Figure 4.1). When kainic acid administration was divided over 2 doses, mean +/- SEM seizure onset time (from final dose) was 7.10 mins +/- 6.05, duration of seizure activity 2.53 mins +/- 0.37, number of seizures 6.67 +/- 0.88 and median seizure severity (classified using a modified Racine's scale) was 3. When the kainic acid was administered in one dose, seizure onset time was 13.58 mins +/- 0.63, duration of seizure activity 3.86 mins +/- 1.40, number of seizures 9.33 +/- 1.86 and median seizure severity was 4. The one mouse that didn't reach stage 1 seizures (mouth or facial movements) was immobile post kainic acid treatment which some experimenters describe as stage 1 seizure behaviour (Schauwecker and Steward, 1997). Due to improvements in formulation and the difficulty in determining accurate seizure onset when a repeated dose was used, single dose kainic acid was used for future experiments.

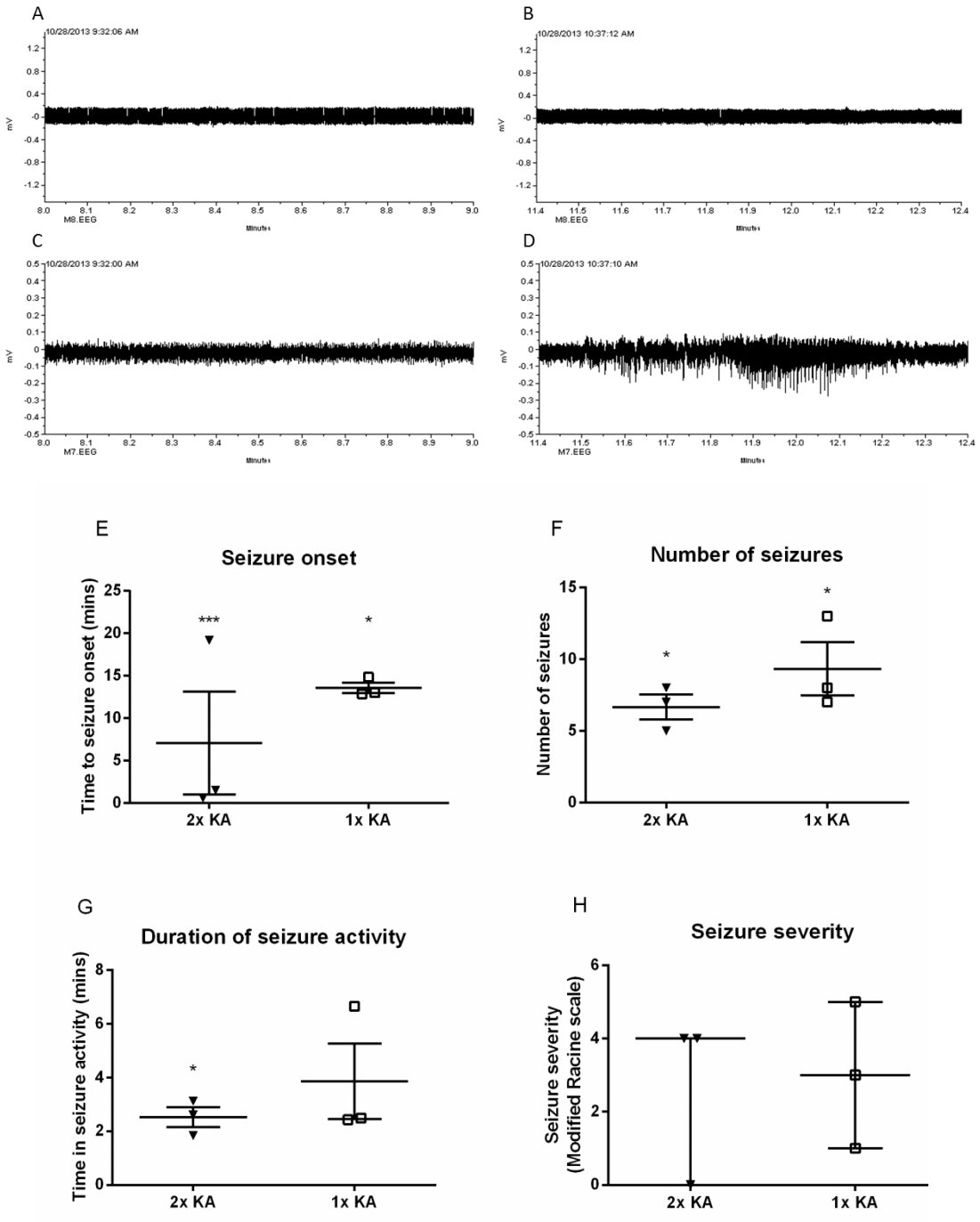


*Figure 4.1. Intranasal kainic acid induces seizures.* Following 30 min baseline EEG recording, animals were dosed with PBS or 30 mg/kg i.n. kainic acid either as one dose (1x KA) or split over two doses 15 min apart (2x KA). EEG was recorded for a further 60-180 min post final dose. Example EEG traces from baseline (A, C) or post dose (B, D) are shown for PBS-treated (A, B) and kainic acid-treated (C, D) mice. Time to seizure onset (from the end of dosing; E) number of seizures (F) and duration of seizure activity (G) over 60 min post kainic acid was recorded. Maximum severity seizure stage reached over the recording period was also analysed (H; classified using modified Racine's scale; Stage 1: Mouth and facial movements; Stage 2: Head nodding; Stage 3: Forelimb clonus; Stage 4: Rearing; Stage 5: Rearing and falling and Stage 6: Repeated stage 5 seizures and early termination or death). N= 3, data displayed as individual points with mean +/- SEM, except H which is median +/- interquartile range. Statistical analysis was performed with a one-sample t-test of kainic acid treatment against a hypothetical value (60.0 for E, 0.0 for F, G and H). $p \leq 0.05$ was deemed significant, * $p < 0.05$, *** $p < 0.001$.

Detecting seizures by eye is subjective and although bias was reduced with blinding, alternative analysis methods may be preferable. Automated analysis programs were developed to provide an objective measure of EEG seizure behaviour. Coastline provides a measure of total length of the EEG trace, a larger value indicative of enhanced activity, i.e. seizure-like behaviour. Autocorrelation measure 'sum difference' reflects increased high frequency, high amplitude spikes (i.e. seizure-like behaviour) with a large value (White et al., 2006). Automated analysis performed on the EEG traces from Figure 4.1 produced larger values for coastline and sum difference with kainic acid treatment compared to baseline (Figure 4.2), thereby confirming the seizure activity observed with analysis by eye. This suggests that i.n. kainic acid is a suitable model for investigating acute seizures and that automated analysis can aid in objective detection of EEG seizure-like activity.

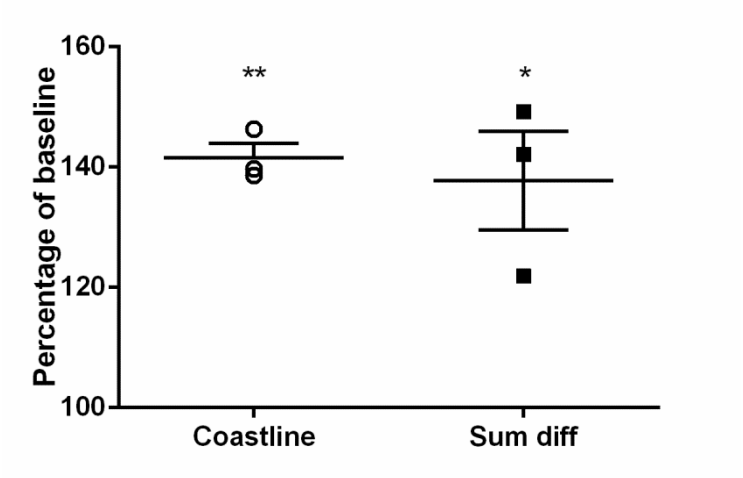


*Figure 4.2. Automated seizure analysis following i.n. kainic acid.* Automated coastline analysis (open circles) or sum difference (black squares) was performed on the EEG data from Figure 4.1 following a single dose of kainic acid. Data was normalised to baseline (100 %) and a one-sample t-test against a hypothetical value (100 %) was used for statistical analysis. Lines represent mean +/- S.E.M., n=3, $p \leq 0.05$ was considered significant. * $p < 0.05$; ** $p < 0.01$.

### 4.4.2. NLRP3 and ASC KO mice are not protected from intranasal kainic acid-induced seizures

The pro-convulsant roles of caspase-1 and IL-1β in seizure have been well described. Following work from Annamaria Vezzani's group and ours demonstrating that caspase-1 KOs are resistant to seizure (Ravizza et al., 2006b), the i.n. kainic acid model was used to investigate if NLRP3 or ASC KOs also conferred seizure resistance. EEG activity was recorded following i.n. kainic acid and no significant differences in seizure parameters were observed in NLRP3 or ASC KO mice compared to WT mice either using analysis by eye (Figure 4.3A-D) or automated analysis (Figure 4.3E, F).

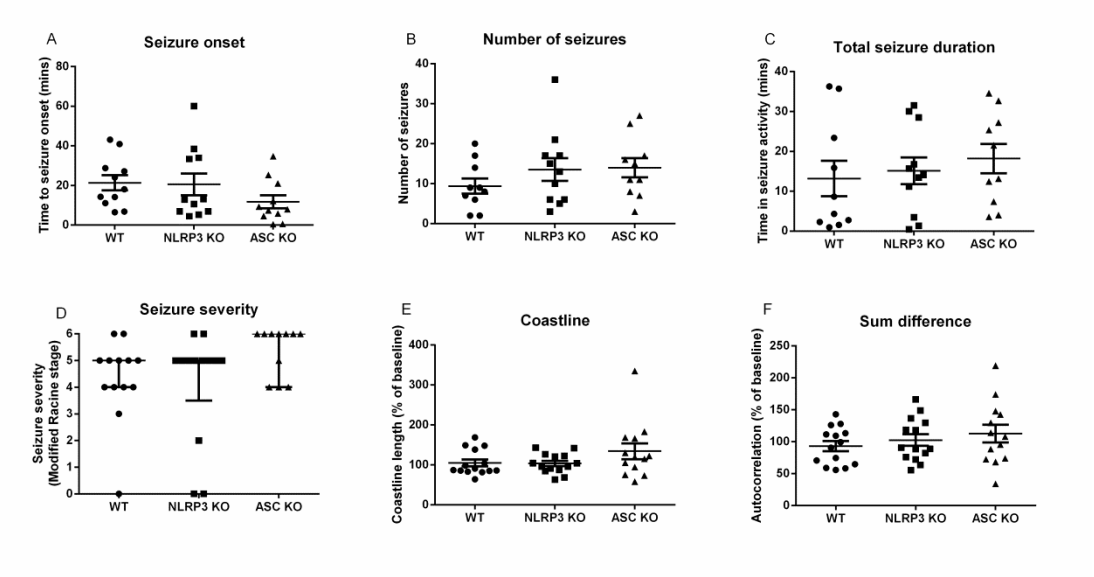


*Figure 4.3. Inflammasome KO does not protect against intranasal kainic acid-induced seizures.* Following a 30 min baseline period, WT, NLRP3 KO or ASC KO mice were dosed with 30 mg/kg i.n. kainic acid. EEG was recorded and time to seizure onset (A), number of seizures (B) and seizure duration (C) over 60 min post dose was calculated. Automated analysis (coastline, E and sum difference, F) was also performed over the same period. Maximum seizure severity reached over the recording period (modified Racine scale, see Methods) was also recorded (D). N=10-14, data is displayed as individual points with mean +/- SEM, except D which is median +/- interquartile range. Statistical analysis was performed using a one-way ANOVA to compare seizure activity between strains. No significant differences were observed between strains ($p > 0.05$).

### 4.4.3. Caspase-1 inhibition does not protect against intranasal kainic acid-induced seizures

Caspase-1 inhibitors have previously been shown to reduce seizures following intrahippocampal administration of kainic acid. As inflammasome KOs were not protected in this acute i.n. kainic acid seizure model, we next investigated if the caspase-1 inhibitor Ac-YVAD-cmk (YVAD) showed anti-convulsant effects in this model. C57Bl/6J mice were pre-treated with YVAD 15 min prior to i.n. kainic acid. Pre-treatment with YVAD did not alter time to seizure onset, number of seizures, duration of seizure activity or seizure severity compared to vehicle pre-treatment (Figure 4.4A-D). Again, automated seizure analysis was performed and similarly showed no significant difference in any seizure parameter tested between those pre-treated with drug or vehicle (Figure 4.4E, F). GYKI 52466, a kainate/AMPA receptor antagonist also did not affect seizure parameters (Figure 4.4).

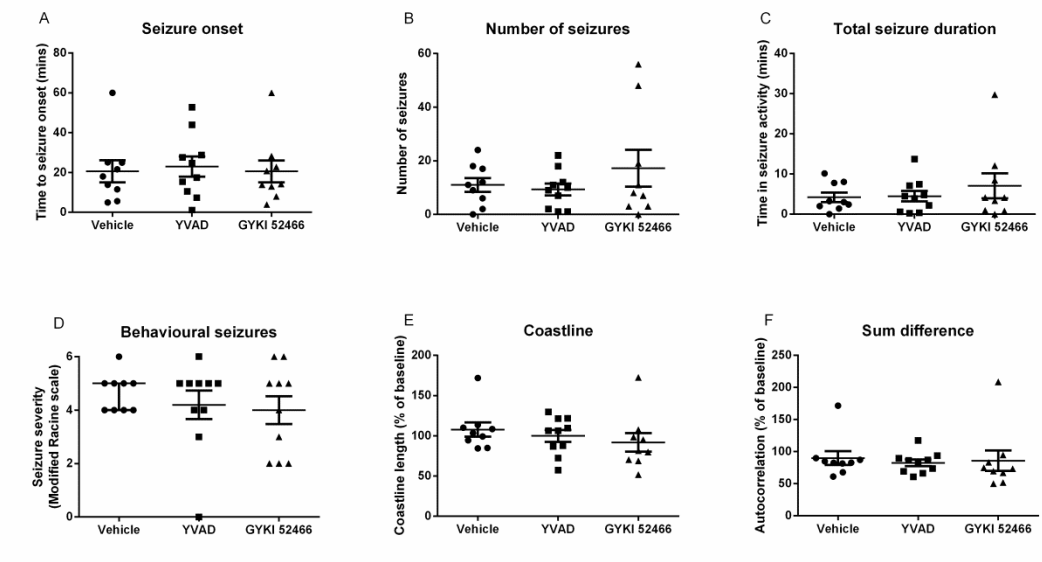


*Figure 4.4. Caspase-1 inhibition does not protect against intranasal kainic acid-induced seizures.* Following a 30 min baseline period, WT mice were dosed with the caspase-1 inhibitor Ac-YVAD-CMK (200 ng in 1 µl, i.c.v; Sigma) or the AMPA/kainate receptor antagonist GYKI 52466 (30 mg/kg i.p.; Sigma) 15 mins prior to 30 mg/kg i.n. kainic acid. EEG was recorded and time to seizure onset (A), number of seizures (B) and seizure duration (C) over 60 min post dose was calculated. Automated analysis (coastline, E and sum difference, F) was also performed over the same period. Maximum seizure severity reached over the total recording period (modified Racine scale, see Methods) was also recorded (D). N=9-10, data is displayed as individual points with mean +/- SEM, except D which is median +/- interquartile range. Statistical analysis was performed using a one-way ANOVA to compare seizure activity between treatments. No significant differences were observed between strains ($p > 0.05$).

### 4.4.4. Acute intranasal kainic acid does not induce hippocampal expression of IL-1

To further explore if IL-1 was involved in this model, brains were collected at the end of the study (1-3 h post seizure) and expression of IL-1β in the hippocampus and its co-localisation with microglia (Iba1) or astrocytes (GFAP) was investigated using immunohistochemistry. Little IL-1β expression was observed in the hippocampus and no co-localisation of IL-1β with Iba-1 was observed at this time point (Figure 4.5A). Co-localisation of IL-1β with GFAP was only observed in the hippocampus surrounding areas of damage, likely caused by implantation of the electrode (Figure 4.5B). This suggests that IL-1β expression may increase in astrocytes following brain injury rather than acutely post i.n. kainic acid-induced seizures.

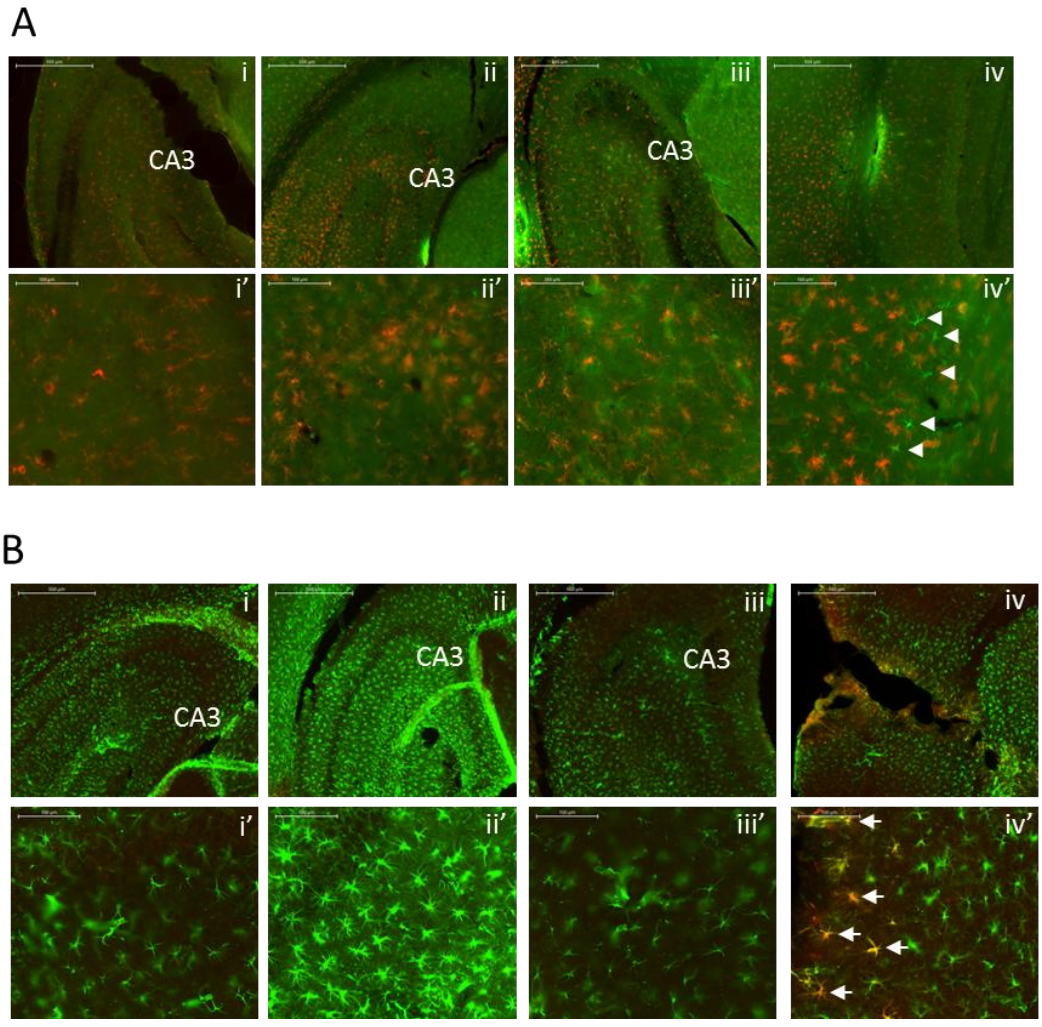


*Figure 4.5. Immunofluorescene micrographs of IL-1β in the hippocampus following i.n. kainic acid-induced acute seizures.* Representative double immunofluorescence micrographs of IL-1β and Iba1 (A) or GFAP (B). IL-1β (green in A, red in B) was not observed in the hippocampus of mice up to 3 h post 30 mg/kg i.n. kainic acid in any treatment group (15 min pre-treatment with vehicle (i), YVAD (ii), or GYKI (iii)). IL-1β staining was observed around electrode track but did not show co-localisation with Iba1 positive (red; microglial) cells (Aiv). Arrowheads mark IL-1β staining. IL-1β (red) was co-localised with GFAP-positive cells (astrocytes; green) show co-localisation along area of damage (yellow; arrows Biv). Scale bar i-iv = 500 µm, i'-iv' = 100 µm.

IL-1α has been suggested to mediate early inflammatory events so IL-1α expression was also investigated post seizure. IL-1α staining was not observed up to 3 h post seizure (Figure 4.6) suggesting that IL-1 may not be involved in acute i.n. kainic acid-induced seizures.

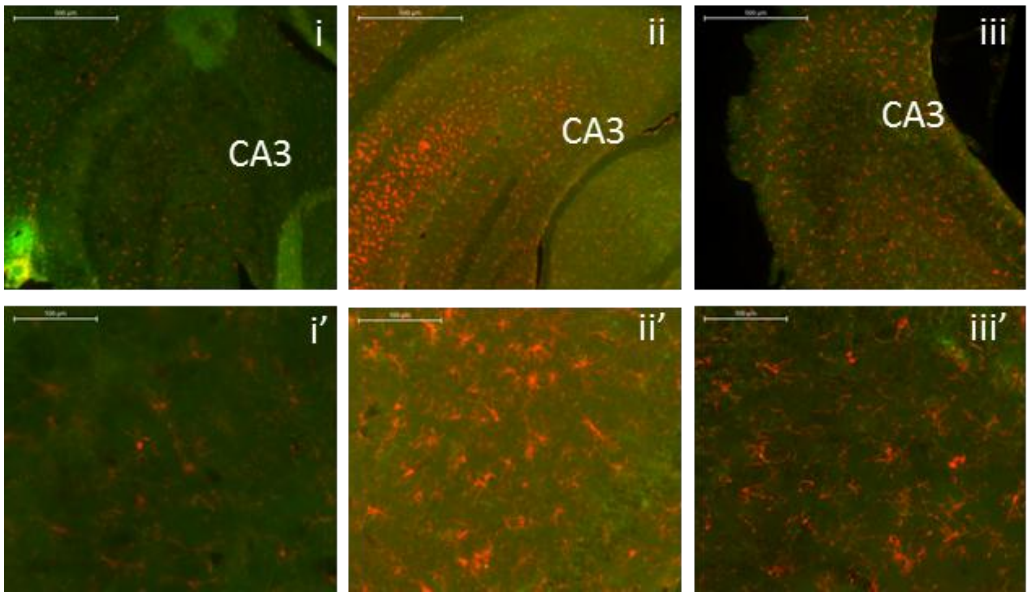


*Figure 4.6. Immunofluorescene micrographs of IL-1α in the hippocampus following i.n. kainic acid-induced acute seizures.* Representative double immunofluorescence micrographs show Iba1-positive microglia-like cells (red; A) but no IL-1α (green) in the hippocampus of mice up to 3 h post 30 mg/kg i.n. kainic acid for any treatment group (i, vehicle; ii, YVAD, iii, GYKI). Scale bar = 500 µm i-iii, 100 µm i'-iii'.

## 4.5. DISCUSSION

The pro-convulsant role of IL-1β has been well described and has instigated clinical trials with the caspase-1 inhibitor VX-765 for treatment resistant epilepsy (ClinicalTrials.gov, 2010). However, little is known about the contribution to seizures of proteins involved in IL-1β activation upstream of caspase-1, such as NLRP3- or ASC-containing inflammasomes.

No difference was observed in the response of NLRP3 KO or ASC KO mice compared to WT mice for any seizure parameter assessed following i.n. kainic acid. The NLRP3 inflammasome is activated by endogenous danger signals and has been implicated in the exacerbation of a number of diseases involving sterile inflammation, making it a likely candidate for caspase-1- and IL-1β-mediated exacerbation of seizures; although other inflammasomes, such as NLRC4 and AIM2, have also recently been implicated in the sterile inflammatory response (Denes et al., 2015). As there were no differences observed in any parameter tested with ASC KO mice or caspase-1 inhibition in this model, it is unlikely that these (NLRP3, NLRC4, AIM2) or any other any inflammasomes are involved in the acute response to i.n. kainic acid-induced seizures in this model.

Neither YVAD, nor GYKI 52466 were protective in this model despite both having shown anticonvulsant properties previously (Yamaguchi et al., 1993; Ravizza et al., 2006b). This may have been due to the specific experimental model used here - the route of administration, the severity, the variability observed or the dose of putative anticonvulsant used.

A reduction in seizure number, time in seizure activity and delayed seizure onset have previously been observed with i.c.v. or i.p. injection of caspase-1 inhibitor (pralnacasan or VX-765 respectively) following intrahippocampal kainic acid (Ravizza et al., 2006b). With the focal injection of kainic acid, a low dose that results in EEG seizures without severe behavioural phenotype can be used. Many animals in our studies experienced severe generalised seizures associated with rearing and falling behaviours thus this may have been too strong an effect to observe any protection with NLRP3 or ASC KOs, or with the inhibitor doses used. Indeed 50 mg/kg GYKI 52466 is required to halt seizures during status epilepticus (Fritsch et al., 2010), despite lower doses proving effective in a number of other models (Yamaguchi et al., 1993). Additionally, repeated doses of the caspase-1 inhibitors were used in the above study by Ravizza et al., to get maximal anticonvulsant effect (Ravizza et al., 2006b) and repeated high doses of VX-765 were also required to prevent

kindling following electrical stimulation (Ravizza et al., 2008b). Thus despite the caspase-1 inhibitor used in our study previously showing reduced IL-1β and neuroprotective effects when dosed once at 200 ng/mouse (Wu et al., 2010), higher or repeated doses may have been required to prevent seizures in this i.n. kainic acid seizure model.

Due to repeatedly blocked hippocampal cannulas and lack of robust seizures when kainic acid was dosed via the intrahippocampal route (data not shown), we explored the use of i.n. kainic acid to induce seizures as this has been suggested as a model of TLE (Chen et al., 2002). I.n. dosing is favoured for a number of drug treatments due to its rapid delivery and non-invasive administration (Pardeshi and Belgamwar, 2013) and has shown positive outcomes against a number of disorders both pre-clinically and clinically (Lochhead and Thorne, 2012). However, the route by which i.n. kainic acid enters the brain and induces seizures is still not fully understood. It must first cross the epithelial barrier via the transcellular or paracellular route or directly into olfactory sensory neurons via the intracellular route. From here it may enter blood vessels, lymphatic vessels, the cerebrospinal fluid or olfactory bulb or continue to diffuse further into the brain (Lochhead and Thorne, 2012). Kainic acid may activate receptors in the olfactory bulb that project to areas of the temporal lobe i.e. amygdala and hippocampus, thereby inducing further excitation there. Neuronal damage has been observed in the olfactory bulb following i.n. kainic acid suggesting at least part of the effects of kainic acid may be mediated in this way (Chen et al., 2002).

The amount of kainic acid that travels via each route and the speed at which it reaches the brain may depend on the administration and the individual animal thus this may explain some of the variability in the results. The specific site of administration affects compound uptake as this is not uniform across the nasal mucosa (Garcia et al., 2009). Inter-animal variation may also be produced due to different age, sex, body size, genetic background, activity level or health as these can alter parameters such as nasal anatomy, blood flow in the nasal mucosa and ventilation, which would all affect i.n. uptake (Garcia et al., 2009). However, as age-matched, inbred male mice were used, these differences should be minimal.

The i.n. seizure model has previously been used successfully to detect changes in seizure parameters in KO mice, where TNF receptor 1 KOs show significantly increased clinical score following i.n. kainic acid (Lu et al., 2008). However, data here was presented in a categorical manner, showing the percentage of animals that passed a determined

threshold, thus the variability in their data cannot be observed and the only significant change reported was an increase in clinical score.

Increased IL-1β expression has been observed immunohistologically in rats 3 h post intrahippocampal kainic acid treatment (Vezzani et al., 1999). However, we did not detect elevated IL-1 in our mice up to 3 h post i.n. kainic acid (except for around sites of damage). This may have been due to a species difference, particularly as C57Bl/6J mice have been shown to be more resistant to the effects of kainic acid (Schauwecker and Steward, 1997). However, elevated IL-1β has been observed in C57Bl/6J mice as early as 1 h following status epilepticus (SE; (Engel et al., 2012) thus our lack of IL-1β immunostaining may have alternatively been due to the indirect route of administration of the kainic acid to the brain. IL-1β staining was observed in the dorsal hippocampus of some animals surrounding areas of local damage caused by implantation of the recording electrode. In these areas, IL-1β was not co-localised with Iba1 but some co-localisation was observed with GFAP-positive cells (astrocytes). IL-1β expression has previously been observed co-localised with astrocytes, but not microglia, from 36 h post pilocarpine-induced status epilepticus or in both cell types at 18 h post SE (Ravizza et al., 2008a); which is consistent with the IL-1β expression observed here being present prior to seizure induction.

As it was only the acute response to kainic acid measured here, we do not know if NLRP3- or ASC-containing inflammasomes are involved in the long term development of spontaneous seizures and epilepsy. Recent work from Meng et al., found elevated NLRP3 expression at 12 h post SE (Meng et al., 2014) providing evidence that the NLRP3 inflammasome may be involved in the production of IL-1β also observed at this stage post seizure. They also demonstrate that knocking down NLRP3 with siRNA is protective against developing spontaneous seizures following electrically-induced status epilepticus (Meng et al., 2014). This suggests a role for NLRP3 in epileptogenesis, highlighting the need for further research into this pathway to determine the exact role and time frame that NLRP3 and IL-1β play in the development of seizures and epilepsy.

Behavioural seizures have been classified into stages based on severity (Racine, 1972), however, these do not reflect mild changes to brain activity. EEG can therefore provide a more sensitive measure of seizure activity and additionally detect spikes and interictal activity that may not translate to behaviour (Bergstrom et al., 2013). Despite seizures having been studied for centuries, there is still no definitive consensus on what classifies EEG activity as seizure activity compared to EEG spikes or noise; rather seizure detection is usually performed by visual inspection of the EEG trace by an expert (Bergstrom et al.,

2013). Analysing EEG traces by eye can be time consuming, particularly for the detection of spontaneous seizures for which weeks' worth of data may have been recorded. Thus people have been developing automated analysis programs that are higher throughput and less subjective than analysis by eye. The autocorrelation and coastline analysis used in our study was consistent with that obtained by eye in that no significant differences were observed between strains or treatment groups. However, as no changes in seizure parameters were observed, we were unable to test the sensitivity of these methods to small changes in EEG activity. Additionally, apart from in the pilot study, coastline and sum difference were approximately 100 % of baseline values suggesting no seizure activity over baseline following kainic acid treatment. As seizures were observed by eye in the EEG trace and behaviourally, this would suggest that these analysis methods are not sensitive enough to detect seizures in this way. One reason may have been that the baseline EEG was reduced following dosing thereby masking any seizure-induced increases from pre-treatment baseline (Fritschy, 2004). If seizures had subsided prior to the end of the recording period, this may have been a preferable period for recording baseline EEG.

Seizures are associated with an increase in amplitude and frequency of the EEG trace. However, for rodents implanted with EEG electrodes, artefacts may be produced from physiological behaviour such as eating or grooming which may also display as a period of increased frequency and amplitude (Wykes et al., 2012). This highlights the complex nature of seizure detection from EEG traces and thus automated analysis may require more sensitive detection methods than the crude methods used in this study. Machine learning is being used to train software to detect seizures as distinct episodes from noise or spiking behaviour and this is a promising way forward, although this is typically limited to detecting the same type of seizure as the data set it was trained on thereby limiting its use across general seizure paradigms (Fergus et al., 2015).

The pro-convulsant role of IL-1β has been well described and has instigated clinical trials in treatment resistant epilepsy with a caspase-1 inhibitor (ClinicalTrials.gov, 2010). However, the Phase IIb trial was terminated early and pre-clinical treatment of mice with caspase-1 inhibitors or in combination with IL-1Ra was not successful in reducing seizures when given after seizure induction (Noe et al., 2013). Although reducing IL-1β with a P2X7R antagonist alongside lorazepam treatment is protective when given 60 min post kainic acid (Engel et al., 2012). This highlights the complexity of the IL-1β involvement in the exacerbation of seizures and the need for further research to understand the precise mechanisms involved, the time course of action and the specific pathway components involved.

## Acknowledgements

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# Chapter 5

# Discussion

# 5. Discussion

IL-1 is a potent inflammatory cytokine implicated in multiple diseases including cancer, arthritis, stroke, and epilepsy (Allan et al., 2005; Dinarello et al., 2012). Due to its potential for wide-reaching damaging effects, IL-1β is highly regulated through its expression, activation and target receptors (Garlanda et al., 2013). Together the work presented in this thesis adds to our knowledge on IL-1 signalling and its regulation in disease conditions (Figure 5.1). A drop in pH is a hallmark of disease, and inflammation underlies most diseases, thus it is important to study inflammation under disease conditions such as acidosis. Here it is shown that pH affects IL-1β processing. A reduction in pH caused the cathepsin D-dependent cleavage of pro-IL-1β into 20 kDa IL-1β on stimulation with NLRP3-activating DAMPs. Additionally this work has highlighted a role for lactic acid in the production of 20 kDa IL-1β. 20 kDa IL-1β is only minimally active at IL-1R1 and is not further cleaved to mature 17 kDa IL-1β by caspase-1, thus may act to limit mature IL-1β production and limit inflammation. This is particularly relevant as the decoy activity of IL-1R2 is reduced at low pH. IL-1β expression was not seen in the acute seizure model used here, in contrast to previously published work, so the presence of 20 kDa IL-1β *in vivo* is therefore still to be confirmed and further research is required to elucidate the exact role of 20 kDa IL-1β in health and disease.

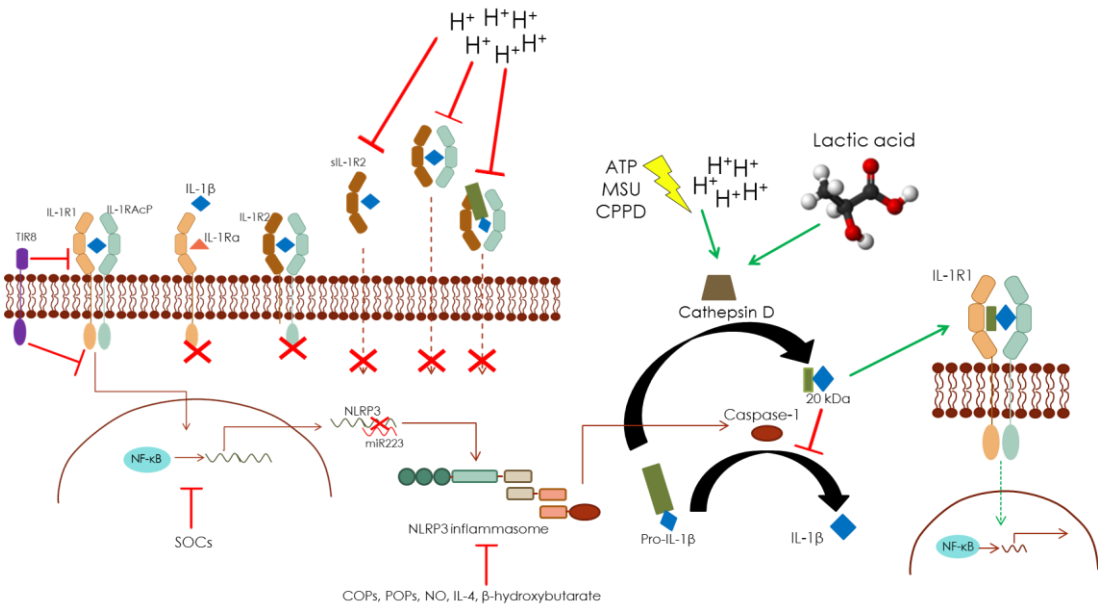


*Figure 5.1. Thesis contribution to mechanisms of IL-1β regulation*

ATP, MSU or CPPD under acidic conditions favours the production and release of 20 kDa IL-1β (1). Lactic acid induces the release of 20 kDa IL-1β and 17 kDa IL-1α (2). 20 kDa IL-1β induces minimal NF-κB-mediated expression via IL-1R1 (3). Low pH reduces the ability of sIL-1R2 to inhibit IL-1β activity (4).

## 5.1. How is acid-dependent IL-1 released?

The mechanisms by which acidic pH induced the release of 20 kDa IL-1β on stimulation with DAMPs are unknown. The key questions with regards to this are how does pro-IL-1β come into contact with cathepsin D and how is the IL-1β released (either as pro-IL-1β for extracellular cleavage or as 20 kDa IL-1β following cathepsin D-mediated cleavage). Cathepsin D is present in lysosomes whereas pro-IL-1β is typically expressed in the cytosol, thus whether pro-IL-1β is taken up into lysosomes, cathepsin D is released into the cytosol or if both are released from the cell for extracellular cleavage is unknown. The classical role of cathepsin D is in general protein breakdown and turnover (Benes et al., 2008), however, additional roles in activating cleavage of proteins such as prolactin (Lkhider et al., 2004), parathyroid hormone (Diment et al., 1989) or angiostatin (Morikawa et al., 2000) have been reported. The location of these cathespsin D-mediated cleavages is protein-dependent with released cathepsin D cleaving prolactin and angiostatin, whereas parathyroid hormone is cleaved within endosomes (Diment et al., 1989; Morikawa et al., 2000; Lkhider et al., 2004). There is conflicting literature on whether IL-1β could be present within lysosomes and therefore available for cleavage by cathepsin D therein. IL-1β is observed alongside cathepsin D in endolysosomes of dendritic cells prior to exocytosis (Gardella, 2001). However, co-localization of IL-1β with cathepsin D was not observed in macrophages (Brough and Rothwell, 2007). Andrei and colleagues detected pro-IL-1β co-localised with cathepsin D in a subset of endolysosomes within human monocytes and suggest pro-IL-1β is taken up into endolysosomes via an ABC1 transporter-mediated mechanism (Andrei et al., 1999) (Figure 5.2). In line with this theory, ABC1 proteins (ABCA5) are expressed on endolysosomes (Kubo et al., 2005) and pro-IL-1β release is inhibited with the ABC1 inhibitor glybenclamide (Lottaz et al., 2001). This inhibition of pro-IL-1β release is independent of a more recent function ascribed to glybenclamide as an inhibitor of NLRP3 (Lamkanfi et al., 2009) as it inhibited the release of pro-IL-1β independently of caspase-1 activation (Lottaz et al., 2001). Interestingly, cathepsin D has been suggested to mediate the degradation of IL-1β in the kidney and here IL-1β accumulated in lysosomes (Kudo et al., 1990). However, this resulted in the degradation of IL-1β to inactive fragments of less than 3.5 kDa, as measured using gel filtration chromatography (Kudo et al., 1999). Thus there is some evidence for pro-IL-1β coming into contact with cathepsin D in lysosomes.

Following expression, pro-IL-1β mainly resides in the cytosol. If no secondary signal is encountered, this pro-IL-1β is polyubiquinated and degraded by the proteasome (Ainscough et al., 2014). For cathepsin D-mediated cleavage of pro-IL-1β in the cytosol, cathepsin D should be released from lysosomes into an area sufficiently acidic for it to maintain its cleavage abilities. Phagocytosis of crystals such as MSU and silica induce lysosomal destabilisation and leakage of lysosomal contents into the cytosol (Hornung et al., 2008). Additionally, ROS can induce lysosomal membrane destabilisation (Appelqvist et al., 2013) and so DAMPs that induce ROS may also be able to induce the release of cathepsin D into the cytosol. ATP activation of P2X7R reduces intracellular pH (Henriksen and Novak, 2003) and during ischaemia, intracellular pH can drop to pH 6.2 (Combs et al., 1990). Chapter 3 demonstrated that cathepsin D can cleave pro-IL-1β at pH 6.2. Thus DAMPs may be able to induce the release of cathepsin D into the cytosol where acidic conditions allow it to cleave pro-IL-1β (Figure 5.2).

Release of cathepsin D and pro-IL-1β into the extracellular fluid would be required for extracellular cleavage of pro-IL-1β into 20 kDa IL-1β. Pro-IL-1β may be released on cell death (Verhoef et al., 2004) and indeed a recent study by Cullen and colleagues suggests that all NLRP3-activators tested (nigericin, ouabain, valinomycin, streptolysin O, ATP and LLOMe) induce necrosis and release of IL-1β (Cullen et al., 2015) and Baroja-Mazo and colleagues show release of pro-IL-1β and inflammasome components on stimulation with LPS and DAMPs (Baroja-Mazo et al., 2014). Thus extracellular pro-IL-1β would be available for cleavage into 20 kDa IL-1β if cathepsin D was also released.

Cathepsin D release from lysosomes is induced with elevated $Ca^{2+}$ (Rodriguez et al., 1997). Acid-sensing ion channels (ASICs) are expressed on many cell types including astrocytes (Huang et al., 2010), microglia (Yu et al., 2015), and macrophages (Kong et al., 2013), and activation of ASIC1 by acidic pH results in influx of $Ca^{2+}$. Thus there is evidence to support pro-IL-1β cleavage by cathepsin D in lysosomes, the cytosol and extra-cellularly, but which is the predominant location of cathepsin D-mediated cleavage (if there is one,) is unknown. As pro-IL-1β can be cleaved intracellularly or extracellularly by caspase-1 (Baroja-Mazo et al., 2014), wherever the cathepsin D cleavage of pro-IL-1β occurs would limit IL-1β signalling.

The second question proposed was, ‘how is acid-dependent IL-1 released?’ As described above, it is unknown whether pro-IL-1β is released and then cleaved to 20 kDa IL-1β, or if it is cleaved intracellularly and then released. There are a number of mechanisms proposed

for release of 17 kDa IL-1β (see Chapter 1, Figure 1.1) and so whether 20 kDa IL-1β is released by any of these mechanisms, or a separate one entirely, is yet to be elucidated. Removal of extracellular $Ca^{2+}$ inhibits the release of 20 kDa IL-1β (Takenouchi et al., 2011) and inhibition of $Ca^{2+}$ release from intracellular stores also prevented 20 kDa IL-1β release (Appendix 2,Figure 7.3), thus it is likely at least part of the production or release of 20 kDa IL-1β is dependent on elevated cytosolic $Ca^{2+}$. However, Chapter 2 demonstrated that a reduction in extracellular pH alone was not sufficient to induce 20 kDa IL-1β release and furthermore, Takenouchi and colleagues show that $Ca^{2+}$ influx with reduced extracellular pH is also not sufficient to induce 20 kDa IL-1β release (Takenouchi et al., 2011). Therefore, as 20 kDa IL-1β release was independent of NLRP3 and caspase-1, it is likely that ATP, MSU and CPPD are required to contribute to the release of 20 kDa IL-1β at low pH but in a manner distinct from their activation of NLRP3. MSU, CPPD and ATP can all induce increases in intracellular $Ca^{2+}$ (Naccache et al., 1991; Pelegrin and Surprenant, 2009), and ATP, MSU and CPPD have been shown to induce the release of lysosomal enzymes including cathepsin D (Takenouchi et al., 2009; Savage et al., 2012). Similarly, lactic acid can also enhance ASIC activity (Immke and McCleskey, 2001) thereby increasing intracellular $Ca^{2+}$, which again could lead to exocytosis of cathepsin D or 20 kDa IL-1β (Figure 5.2).

In the studies described in Chapter 2, the pH of the extracellular fluid was artificially reduced. In disease, poor oxygen perfusion and/or increased activity induce a shift to anaerobic respiration. This results in increased lactic acid and therefore $H^+$, but reduced ATP in the cell. If there is insufficient ATP to fuel transporters such as the plasma membrane $Na^+/K^+$ pump, a build-up of intracellular $Na^+$ may induce the reversal of the $Na^+/Ca^{2+}$ exchanger thereby increasing $Ca^{2+}$ influx (Avkiran and Marber, 2002). Similarly, if there is a lack of ATP to fuel the lysosomal $H^+$ pump/ATPase, then the lysosomes will not be able to maintain their acidic pH and increased lysosomal pH is also known to induce lysosome exocytosis (Mindell, 2012). Thus the acidosis experienced in disease is likely to increase intracellular $Ca^{2+}$ and induce lysosome exocytosis in a similar manner to that induced by an artificial reduction in extracellular pH. The release of 17 kDa IL-1β from monocytes and macrophages at low pH is dependent on a reduction of intracellular pH (Jancic et al., 2012; Rajamäki et al., 2013). However, in our system, the intracellular pH was not recorded thus it is unknown if 20 kDa IL-1β release is also reliant on a reduction in intracellular pH.

The experiments described in Chapter 2 used LPS to prime cells to express pro-IL-1β. Non-communicable diseases such as chronic heart disease, stroke and epilepsy are suggested to be sterile and able to occur without a pathogenic insult. Therefore, the inflammation experienced in these situations would be unlikely to be driven by LPS. However, whether any tissue is truly sterile has recently been called into question. The presence of bacteria has been described in blood and elevated not only in those with infectious diseases, but also in people who develop non-communicable diseases (Potgieter et al., 2015). Regardless of whether LPS is present in non-communicable diseases, endogenous molecules have also been shown to induce pro-IL-1β expression. MSU and CPPD can increase IL-1β expression (Roberge et al., 1991) and lactate decreases IκB, increases NF-κB transcriptional activity and potentiates LPS-induced IL-1β expression in macrophages (Samuvel et al., 2009), although this was not observed in microglia (see Chapter 2, Figure 2.6B). Whether ATP-induced activation of P2X7R could induce expression of pro-IL-1β is unconfirmed, although under hypoxic conditions, activation of P2X7R induced translocation of NF-κB into the nucleus where it could induce transcription (Tafani et al., 2011). However, DAMPs have been shown to induce cell death independently of inflammasome activation (Cullen et al., 2015), so whether there is time to induce pro-IL-1β expression prior to cell death should be considered. The literature on the effect of low pH on the expression of pro-IL-1β is not conclusive. Acidic pH increases pro-IL-1β expression in monocytes (Jancic et al., 2012) but inhibits LPS-induced pro-IL-1β expression in microglia (Jin et al., 2014). In the work presented in this thesis, a reduction in pH during LPS-induced priming was not investigated thus we cannot comment on the effect of low pH on pro-IL-1β expression without priming. However, no reduction of LPS-induced IL-1β within cell lysates was observed following any treatment at pH 6.2 compared to pH 7.4; although as this was measured by ELISA, pro-IL-1β cannot be distinguished from mature IL-1β (Appendix 2, Figure 7.4).

Taken together, the data presented in this thesis in combination with published literature suggests that a reduction in extracellular pH alongside ATP or crystal induced $Ca^{2+}$ influx, pro-IL-1β expression, exocytosis of lysosomes and release of lysosomal enzymes (such as cathepsin D) may be sufficient to induce 20 kDa IL-1β release. However, whether IL-1β is released in its pro- form for extracellular cleavage by cathepsin D or if the cleavage to 20 kDa IL-1β occurs prior to release is unknown and suggestions of how to determine this will be described later (Chapter 5.5).

Less literature is present on IL-1α signalling at low pH. Endothelial cells expressing the proton-sensitive receptor GPR4, increase IL-1α expression at pH 6.4 (Dong et al., 2013) and lactic acid was shown to induce the release of mature calpain-cleaved IL-1α (Chapter 2, Figure 2.8). Calpain is a $Ca^{2+}$ -activated protease and lactic acid-mediated increase in $Ca^{2+}$ has been described above, thus increased lactic acid during disease may induce release of mature IL-1α as observed in Chapter 2.

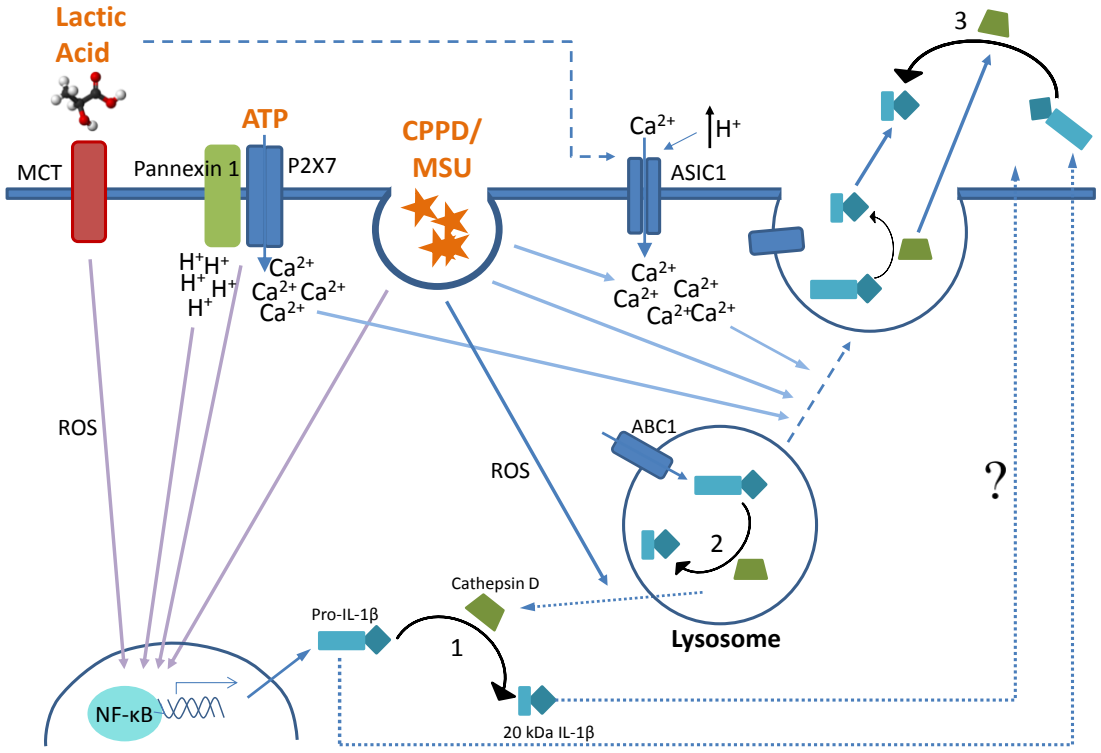


*Figure 5.2. Location of cathepsin D-cleavage.* 1. Cathepsin D can leak from lysosomes into the cytosol where it could cleave pro-IL-1β. 2. Pro-IL-1β could be taken up into lysosomes where it could be cleaved by cathepsin D. 3. Cathepsin D released during lysosomal exocytosis could cleave extracellular pro-IL-1β.

## 5.2. What is the true impact of acid-dependent IL-1 *in vivo*?

Chapter 3 demonstrates that 20 kDa IL-1β is only minimally active at IL-1R1 but does not out-compete 17 kDa IL-1β at this receptor, but how does this relate to the *in vivo* or disease situation? Mature 17 kDa IL-1β is a potent cytokine which is able to induce a maximal response with less than 5 % of its receptors on a cell occupied (Arend et al., 1990). Following kainic acid-induced seizures, hippocampal IL-1β levels increase 16-fold to approximately 24 pg/ml (Vezzani et al., 1999) and even in patients with rheumatoid arthritis (RA) or in rats post stroke (disorders where blocking IL-1β activity is protective), levels of IL-1β in the plasma do not exceed 250 pg/ml (Tolusso et al., 2006; Tu et al., 2010).

However, due to the sensitivity of the HEK assay, the level of IL-1 required was in the ng/ml range (Chapter 3, Figure 3.1A). With less 17 kDa IL-1β available to bind to IL-1R1 *in vivo*, 20 kDa may be able to out-compete 17 kDa IL-1β and therefore reduce IL-1R1-mediated signalling. However, as we do not yet know what levels of 20 kDa IL-1β are produced *in vivo* we cannot determine the relative effect of 17 KDa and 20 kDa IL-1β *in vivo*. Additionally, without *in vivo* data we cannot know for sure the impact of a small amount of 20 kDa-IL-1β activity at IL-1R1 – whether its impact is negligible or if that small amount of activation is sufficient to induce a physiologically relevant response. As 20 kDa IL-1β (formed from 100 ng/ml pro-IL-1β incubated with 1 U/ml cathepsin D,) did not induce a significant increase in release of IL-6 or CXCL1 over vehicle treated brain endothelial cells, one may consider it unlikely to significantly contribute to pro-inflammatory signalling *in vivo,* although this needs to be confirmed (Chapter 3, Figure 3.3). We have not explored the binding kinetics of 20 kDa IL-1β thus we do not know the affinity of 20 kDa IL-1β for IL-1R1 or IL-1R2. Greater affinity and/or slower release from IL-1R1 with low affinity and/or rapid release from IL-1R2, similar to IL-1Ra, would suggest a role for the 20 kDa form in the negative regulation of the IL-1 system.

The data presented in this thesis suggests that acidosis limits mature IL-1β signalling but promotes mature IL-1α. Chapter 4 shows that low pH reduces the decoy activity of IL-1R2. Acidosis may also reduce the intracellular binding of IL-1R2 to pro-IL-1α thereby providing more available pro-IL-1α for cleavage by calpain (Zheng et al., 2013). Could acute acidosis, as occurs in disease, therefore contribute to the early responsiveness of IL-1α compared to the typically delayed response of caspase-1 cleaved IL-1β (Luheshi et al., 2011; Rider et al., 2011)? Or is the response of IL-1α naturally dampened by its sequestration in the nucleus, whereas IL-1β requires cathepsin-mediated cleavage to limit its inflammatory response (Luheshi et al., 2009a)?

Alternatively, 20 kDa IL-1β may be a substrate for the neuronal IL-1R3 and induce alternative IL-1β signalling through this pathway (Huang et al., 2011). Lactic acid has been described, particularly in the brain, to have multiple signalling roles in addition to being a by-product of metabolism (Mosienko et al., 2015). It enhances $Ca^{2+}$ conductance via modulating ASICs (Immke and McCleskey, 2001) and NMDA receptors (Yang et al., 2014) and increases intracellular $Ca^{2+}$ causing depolarisation and release of noradrenaline via adenylate cyclase and protein kinase A (Tang et al., 2014). If the lactic acid-induced 20 kDa IL-1β was an agonist of IL-1R3, it could contribute to the potentiation of NMDA-mediated

$Ca^{2+}$ currents and increased intracellular $Ca^{2+}$ described above and contribute to enhanced neuronal excitability and exacerbation of seizures described by Balosso and colleagues (Balosso et al., 2008). Conversely, lactate has also been shown to activate GPR81 and reduce neuronal $Ca^{2+}$ spiking frequency (Bozzo et al., 2013), thus whether lactic acid contributes to protection against, or exacerbation of seizures and what contribution, if any, 20 kDa IL-1β would make in mediating the described effects of lactic acid is as yet unknown.

## 5.3. Acidosis and cleavage during inflammation

A number of papers have now described the effects of acidosis on IL-1β (Takenouchi et al., 2011; Jancic et al., 2012; Rajamäki et al., 2013; Jin et al., 2014) and a recent review highlights the combined effect of ATP and acidosis on inflammasome activation (Takenouchi et al., 2014), but as acidosis is a central phenomenon in disease, what is the effect of low pH on other cytokines, chemokines and receptors and does acidosis induce alternative cleavage elsewhere?

Chapter 3 shows that acidosis not only affects IL-1β production by NLRP3-activating DAMPs but also reduces the efficacy of sIL-1R2 to inhibit IL-1β activity and could therefore promote IL-1-mediated inflammation. Other receptors are also modulated by changes in pH. Acidic pH inhibits AMPA and NMDA receptors (Traynelis and Cull-Candy, 1990; Ihle and Patneau, 2000), whereas low pH is required for optimal binding of CpG-DNA to TLR9 (Rutz et al., 2004).

In line with lactic acid inducing the release of mature IL-1α, (Chapter 2, Figure 2.8) kidney cells exposed to pH 7.0 for 24 h also increase expression of a number of inflammatory proteins including IL-1α, matrix metalloprotease 9 and 13 and chemokine (C-C motif) ligand 2, 7 and 9 (Raj et al., 2013). The reduction in extracellular pH translated to a reduction in intracellular pH, although what mechanisms induced the increase in inflammatory gene expression under acidic conditions were not investigated further (Raj et al., 2013). IL-18 is upregulated in synovial tissue from RA patients and contributes to inflammation (Gracie et al., 1999). Arthritic joints are acidic and Rajamäki and colleagues show that low pH induces IL-18 release (Rajamäki et al., 2013). Although this was only measured by ELISA, thus whether alternative processing of IL-18 occurs during acidosis, similar to the cathepsin D-mediated processing of IL-1β, is unknown.

Similar to the conflicting literature available on the effect of low pH on IL-1, little consensus is present on the effect of acidosis on other inflammatory responses. This is exemplified by data describing TNF expression and secretion. Incubation of peritoneal macrophages with lactic acid increases TNF mRNA and secretion (Jensen et al., 1990), whereas Jancic and colleagues show no effect of pH on TNF secretion from monocytes (Jancic et al., 2012). Conversely, pH 6.5 and 5.5 significantly reduced release from macrophages at 18 h (Heming et al., 2001) and lactic acid or pH 6.6 media reduces LPS-induced TNF expression and secretion from human monocytes (Dietl et al., 2010) but pH 6.5 and 5.5 had no effect on LPS-mediated TNF expression (Heming et al., 2001). These experiments were performed in different cell types (monocytes vs macrophages) which are known to behave differently (i.e. constitutive caspase-1 activation in monocytes); used different methods to reduce the pH (lactic acid vs acidic media) and HCl and lactic acid have shown opposing effects on inflammation (Kellum et al., 2004); and were carried out under different conditions (i.e. with/without LPS pre-treatment). LPS induces activation of myeloid cells and a concurrent shift to glycolysis to meet the extra energy requirements this demands. Addition of extracellular lactic acid reduces glucose uptake and lactate release which would impair glycolysis (Dietl et al., 2010). Additionally, blocking glycolysis with 2-deoxyglucose also inhibits LPS-mediated TNF release from human monocytes. Thus lactic acid or a reduction in pH may affect LPS-mediated expression and release via disrupting glycolysis (Dietl et al., 2010), or via inhibition of ERK/JNK phosphorylation as was suggested for the effect of low pH reducing LPS-mediated expression of IL-1β (Jin et al., 2014), and this may be separate to a direct effect on expression without LPS pre-treatment. Thus the response of a cell to a reduction in pH may be determined by its activation state.

There is much literature, including the work described above, that demonstrates that acidosis affects cell behaviour and cytokine signalling. However, further investigation is required to tease out the effect of low pH compared to lactic acid or lactate at neutral pH. Dietl and colleagues show that either lactic acid or both reducing the pH and addition of sodium lactate (which did not reduce pH) was required to induce a significant reduction in LPS-mediated TNF release (Dietl et al., 2010), whereas Samuvel and colleagues show lactate (at neutral pH) potentiates LPS-induced expression of IL-1β, IL-6, IL-8, GM-CSF and CXCL10 via increasing expression of the MD-2 TLR4 accessory protein (Samuvel et al., 2009). Interestingly, lactic acid and sodium lactate are selectively transported by Slc16a1 on $CD8^+$ T cells or Slc5a12 on $CD4^+$ T cells respectively (Haas et al., 2015), thus the transport and signalling effect of lactate may depend on what form is present.

IL-1 family members in addition to IL-1β are able to undergo caspase-1-independent cleavage which can enhance or reduce their activity (Afonina et al., 2015). Reminiscent of the cathepsin D cleavage of IL-1β, IL-18 is cleaved by mast cell chymase to a form that displays approximately 20 % activity of caspase-1-cleaved IL-18 (Omoto et al., 2006). IL-33 exerts some activity in its pro-form but can also be cleaved by caspase 3 or 7 which reduce its activity (Lüthi et al., 2009), cathepsin G or elastase which enhance its activity (Lefrançais et al., 2012) or proteinase 3 which can either cleave it into bioactive or inactive forms depending on the duration of incubation (Bae et al., 2012). However the preference for different protease-mediated cleavages under acidic conditions has not been characterised.

The body of evidence on proteases controlling inflammatory activity is growing thus specific IL-1 cleavage proteases could provide therapeutic targets. Protection from inflammatory disorders by targeting caspase-1 has been demonstrated pre-clinically and this has led to clinical trials (Denes et al., 2012). More recently the role of caspase-1-independent cleavage of IL-1β has been implicated in disease (Guma et al., 2009) and further understanding of the contributions of different proteases in disease could help to develop selective inhibitors thereby acutely targeting a specific phase of inflammation (i.e. early neutrophil proteases versus later macrophage caspase-1). This may help to retain the initial protective inflammation whilst preventing chronic inflammation and long-term damage. Or in cancer, inhibit neutrophil proteases that activate IL-1 and induce angiogenesis and tumourigenesis but leave granzyme B-mediated cleavage of IL-1α and IL-18 that can stimulate cytotoxic cells (Afonina et al., 2015).

## 5.4. Experimental models of disease

The discrepancy between results from animal models and clinical studies has been a major downfall in the development of successful treatments, with approximately 89 % of all compounds entering clinical trials failing before reaching the patient (Kola and Landis, 2004). Although this is not exclusively due to the failings in animal models, continual improvements in pre-clinical models are being made to try and more closely mimic the human (disease) situation to improve translatability. The use of co-morbid animals such as aged, obese, infected or diseased animals should be used in pre-clinical studies to more closely represent the likely target population (van der Worp et al., 2010). This can further be extended to *in vitro* studies in which the disease microenvironment can be modelled. Cell culture models are becoming increasingly complex with co-culture systems and 3D cell culture complete with microfluidics now routinely used, although careful consideration is

required to ensure the relevance of these complex models to the *in vivo*/human situation (Fitzgerald et al., 2015). We show here that modelling the disease environment such as changes in pH can demonstrate alterations in signalling that may not have been considered otherwise.

The ability to generate complex models that may more closely mimic the *in vivo*/human situation is a great step forward, however early preclinical studies may still initially require an inexpensive, simple, high throughput model. This may be described as reductionist but can be necessary to determine fundamental interactions between specific proteins for example. Chapter 3 used a reporter cell line (HEK293) selectively expressing IL-1R1. With this we could determine the activity of different forms of IL-1 at IL-1R1 in combination with specific components of the IL-1 system. HEK cells are not representative of naturally expressing IL-1R1 cells but did show similar responses to brain endothelial cells that do endogenously express IL-1R1, thus can be used as a tool to model more physiological situations.

Animal models rarely directly represent human disease; instead they model different aspects of the disease as best as possible. Modelling epilepsy presents further complications as the underlying aetiology is still fairly elusive. Many models have been described, some better for exploring acute seizures, seizure threshold or epileptogenesis, but all come with pros and cons (Grone and Baraban, 2015). The model used in Chapter 4 looked at the acute response to an intranasal (i.n.) kainic acid challenge. Although no differences were observed with NLRP3 or ASC KO mice at the acute time point studied, these mice may possess an altered ability to develop subsequent spontaneous seizures and epilepsy and the role of 20 kDa IL-1β in this long-term effect could be explored.

Rodents are typically used for seizure models, however, C57Bl/6 mice which form the basis of many KO strains are more resistant to the standard chemiconvulsant kainic acid, particularly when administered peripherally (Schauwecker and Steward, 1997). I.n. kainic acid is a novel seizure model that has many similarities with human TLE (Chen et al., 2002). However, further work is required to fully elucidate the i.n. route to the brain and to reduce the variability observed in this model. Acute delivery of chemiconvulsant to the brain may be preferable over i.n. delivery as the exact dose reaching the area of interest is known and that dose can be tailored to produce minimal local effects thus adhering to the 3Rs of humane animal research by minimising any distress the animal may experience.

Alternatively one may consider first studying seizure activity in model organisms, such as *Drosophila melanogaster* and zebrafish. Zebrafish are vertebrates and share approximately 70 % protein coding genes with humans (Howe et al., 2013). These systems provide simple, high-throughput models that are relatively easy to manipulate genetically which has led to the identification of a number of genes involved in human epilepsies (Grone and Baraban, 2015).

## 5.5. Future directions

The key unanswered question coming out of this work is 'what is the relevance of 20 kDa IL-1 signalling *in vivo*?' In the short term, more physiological *in vitro* models that mimic the hypoxia and reduced glucose that induces lactic acid release and the drop in pH encountered in disease could be investigated. If oxygen glucose deprivation increased lactic acid and reduced pH and produced similar results to those obtained in this thesis by directly adding lactic acid or reducing pH, this would add confidence to the physiological relevance of 20 kDa IL-1β.

Although using NLRP3 KO mice did not cause any change in seizure parameters tested here, we cannot be convinced that this is due to a lack of need for NLRP3 over an effect of the model itself. As no seizure-induced IL-1β was observed, we could not detect 20 kDa IL-1β and cannot extrapolate the lack of effect in NLRP3 and ASC KOs as a role for caspase-1-independent IL-1β in seizure. As we did not observe any reduction in seizure parameters with any treatment or strain used, we cannot be confident of the sensitivity of this model to detect changes in seizure parameters. Thus the role of NLRP3 in epilepsy remains uncertain. The protection exerted by caspase-1 inhibitors or KOs against intrahippocampal kainic acid-induced seizures suggests that this pathway can be important in seizure (Ravizza et al., 2006b) and as NLRP3 is the key caspase-1-activating inflammasome implicated in sterile responses, it would follow that NLRP3 is important in the caspase-1-mediated exacerbation of seizures. Additionally with NLRP3 siRNA providing protection against SE, there is cause to suggest that this inflammasome is important in seizures (Meng et al., 2014). However, recent work showing that NLRC4 and AIM2, rather than NLRP3, are critical mediators of sterile inflammation in the exacerbation of stroke (Denes et al., 2015), and the presence of ATP-activated NLRP2 in astrocytes (Minkiewicz et al., 2013), highlights the potential for other inflammasomes to be involved in the caspase-1-mediated exacerbation of seizures under sterile conditions and should not be ignored. Although caspase-1-

dependent IL-1 appears to be important in seizure, no work has investigated the presence of caspase-1-independent IL-1 in such models thus this remains to be elucidated.

In addition to determining 20 kDa IL-1β expression *in vivo*, its role in disease requires investigation. Does the expression of 17 kDa vs 20 kDa IL-1β alter with disease and does this correlate with outcome? With the development of small molecule inhibitors of NLRP3 (Coll et al., 2015), the role of NLRP3-dependent and NLRP3-independent IL-1β in seizures can be explored in more detail. Additionally, as KO animals would not be required, experiments could be performed in rats, or other species of mice which are not resistant to kainic acid (Schauwecker and Steward, 1997). With this thesis demonstrating the generation of NLRP3-independent 20 kDa IL-1β under acidic conditions that would be present during seizure, and previous literature highlighting a role for caspase-1-independent IL-1β in disease, it is important to determine the specific contribution of NLRP3-dependent and independent 17 kDa and 20 kDa IL-1β to seizures and other inflammatory diseases. A profile of the contribution of NLRP3 and IL-1 in seizure induction, generalisation and subsequent development of epilepsy induced via a variety of convulsants would further our knowledge on when and how IL-1 is involved in epilepsy. Addition of NLRP3 inhibitors in combination with IL-1Ra (to inhibit all forms of IL-1 activity at IL-1R1,) or specific IL-1-blocking antibodies (targeted at IL-1α or IL-1β,) can be used to determine what form of IL-1 is involved and when.

IL-1β detection in the brain is difficult as levels are low, can be transient and the presence of sIL-1Rs would further reduce levels of free IL-1β (Arend et al., 1994). Thus novel techniques are being developed to increase the sensitivity of new technology to detect low abundant proteins such as IL-1β. Circulating IL-1β is typically below 1 pg/ml in healthy humans, whereas the sensitivity of standard ELISA kits and western blot is typically greater than this. An immunoassay system from Singulex is able to detect IL-1β as low as 0.03 pg/ml and IL-1α from 0.25 pg/ml (Singulex). However, it is unlikely that such technology could distinguish 20 kDa IL-1β from 17 kDa IL-1β. To get a more concentrated sample of IL-1β following seizures or other brain injury models, microdissection of the specific area of insult or suspected region of IL-1β upregulation could be performed which could then be homogenised and run on a western blot to detect if 20 kDa IL-1β is observed (Engel et al., 2012). Alternatively, if IL-1β is detected in sufficient quantity via immunohistochemistry, laser capture microdissection could be performed to specifically excise IL-1β-producing

cells to observe what form of IL-1β is present (Chen et al., 2008; Koob et al., 2012). Although this would require 20 kDa IL-1β to be present within the cell rather than released.

As the levels of IL-1β have been hard to detect in the brain, an acute peripheral model of inflammatory disease may be preferable to detect 20 kDa IL-1β. *In vitro* infection with *Pseudomonas aeruginosa* at pH 6.7 induces release of 20 kDa IL-1β from bone marrow derived dendritic cells. Additionally *in vivo* intraperitoneal injection of *Pseudomonas aeruginosa* in an acidic pH buffer also induces IL-1β release (Torres et al., 2014), although *in vivo* IL-1β release was only measured via ELISA thus whether 20 kDa IL-1β was released remains to be confirmed. Also, the contribution to disease of any 20 kDa IL-1β released *in vivo* was not explored as mice were culled 2 h after infection and no mention of sickness behaviour was described (Torres et al., 2014). This study could be repeated to include western blot analysis of the size of IL-1β released *in vivo* and also whether cathepsin D inhibition or overexpression alters IL-1 response or disease phenotype.

As 20 kDa IL-1β is less active than 17 kDa IL-1β at IL-1R1, if a protective role of 20 kDa IL-1β is determined *in vivo*, this may be exploited therapeutically. Targeting NLRP3 may provide a preferable target to IL-1 or caspase-1, particularly if it prevented the release of the inflammatory mature 17 kDa IL-1β but left the putative protective cathepsin D-cleaved 20 kDa IL-1β. Furthermore, specifically inhibiting NLRP3 would mean that the response of other inflammasomes would be spared thus the host could still respond to invading pathogens such as bacteria detected by NLRC4 (Mariathasan et al., 2004), or double stranded DNA by AIM2 (Roberts et al., 2009) without exacerbating the response to sterile insults and causing collateral damage.

If 20 kDa IL-1β was to be targeted therapeutically, further understanding of the mechanisms of its production and release should be investigated. The location of cathepsin D mediated cleavage of pro-IL-1β could be explored via a number of methods. Studies could include the use of digitonin to selectively lyse the plasma membrane to observe if IL-1β co-localises to vesicles when treated at low pH, and to determine what form of IL-1β is released from the cytosol (Brough and Rothwell, 2007). Cytolysis can be inhibited to prevent pro-IL-1β release to determine if this also alters 20 kDa IL-1β in the supernatant (Verhoef et al., 2004) or lysosome exocytosis can be inhibited or measured in KO mice with defective lysosome secretion and western blots performed to determine if 20 kDa IL-1β is present in cell lysates (Brough and Rothwell, 2007). Additionally, improvements in microscopy could utilise single cell imaging to observe the time course of 20 kDa IL-1β

production alongside cell death or the requirement for a concurrent reduction in intracellular pH can be determined via pH sensing dyes and inhibition of the sodium-hydrogen exchanger (Jancic et al., 2012). Additionally, whether 20 kDa IL-1β is an agonist for IL-1R3 can be investigated in cells from IL-1R1 KO mice, where the phosphorylation of Src, Akt or NR2B can be determined simply via western blot (Huang et al., 2011; Qian et al., 2012).

In summary, this thesis has contributed to our knowledge on the regulation of IL-1 under disease conditions. It has shown that an acidic environment, which is a hallmark of disease, contributes to alternative processing of IL-1β. Cathepsin D-mediated 20 kDa IL-1β produced under acidic conditions is less active than mature 17 kDa IL-1β at IL-1R1 and is not further cleaved to 17 kDa IL-1β thus may act to limit the spread of inflammation. Although we were unable to confirm the presence of 20 kDa IL-1β *in vivo*, the relative presence of 17 kDa and 20 kDa IL-1β may modulate IL-1β signalling in disease and therefore provide a novel therapeutic target.

# Chapter 6

# References

# 6. References


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# Chapter 7

# Appendix

# 7. Appendix

## 7.1. Appendix 1. Supplementary Methods

### 7.1.1. Cell culture

**Primary mixed glia**

Mixed glia were obtained from the brains of 1-4 day old mouse pups. Cells were used from wild type C57Bl/6J or NLRP3 KO mice from a C57Bl/6J background. Pups were decapitated and heads were collected in a petri dish on ice. Heads were secured to a wax-filled petri dish with four 25 gauge needles. An incision was cut along the midline through the skin and skull layer from the base of the skull to the frontal lobe, before using forceps to collect the brain tissue. The brain was then rolled on filter paper to remove the meninges before being collected in a falcon tube (separate tubes per 'n', one litter is one 'n') containing 5-10 ml warm media (37 $^{o}$C; Dulbecco's modified Eagle's medium (DMEM; Sigma, UK) with 10 % foetal bovine serum (FBS; Life Technologies, UK) and 100 units/ml penicillin, and 100 μg/ml streptomycin; Sigma, UK). Brains were then triturated with stripettes of decreasing size. Cell suspensions were centrifuged at 1000 rpm for 10 min before the media was aspirated and the pellet re-suspended in 10-12 ml per pup of the media described above. Cells were seeded at 500 μl per well of a 24 well plate and incubated at 37 $^{o}$C and 5 % $CO_2$ until a confluent layer of astrocytes was present (approximately 2 weeks). The growth medium was initially changed after 5 days followed by every 3-4 days. Growth media was replaced with serum free media (DMEM with 1 % penicillin/streptomycin) or HEPES-buffered salt solution (HBSS; 145 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM HEPES, 10 mM glucose and 0.01 % BSA. pH altered to 7.4 or 6.2 with 1 M NaOH) prior to treatment. All procedures were performed in accordance with the Animal (Scientific Procedures) Act 1986.

**THP1 cells**

THP1 cells are a human monocytic cell line that can be differentiated into macrophage-like cells with phorbol 12-myristate 13-acetate. Cells were maintained in RPMI 1640 (Sigma, UK) supplemented with 10 % FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were counted using a haemocytometer (improved double neubauer bright; SLS, UK), centrifuged at 2000 rpm for 5 min, seeded in 24-well plates at a density of $5x10^5$ cells/well and treated with phorbol 12-myristate 13-acetate (PMA, 0.5 μM). After 3 h, the medium was removed, fresh maintenance media was added, and cells were incubated overnight (37

°C, 5 % $CO_2$). Cells were primed with LPS (1 μg/ml, 4 h) to induce pro-IL-1β and NLRP3 expression. The culture medium was replaced with HEPES-buffered salt solution for subsequent treatments.

**bEnd.5 cells**

bEnd.5 cells (from Health Protection Agency Culture Collections, UK) are a mouse brain endothelial cell line that express the IL-1R1 and behave similarly to primary brain endothelial cells (Allen et al., 2012). Cells were maintained in DMEM (Sigma, UK) supplemented with 10 % FBS, 100 U/ml penicillin, 100 μg/ml streptomycin. To passage the cells, maintenance media was removed, cells were washed with phosphate-buffered saline (PBS; Sigma, UK) before being incubated with trypsin (Sigma, UK) until detached. Maintenance media was added to neutralise the trypsin and cells were centrifuged at 2000 rpm for 5 min. Cells were counted using a haemocytometer and seeded at 2 x $10^5$ cells per ml of maintenance media in a 96-well plate overnight.

**HEK-Blue IL-1β cells**

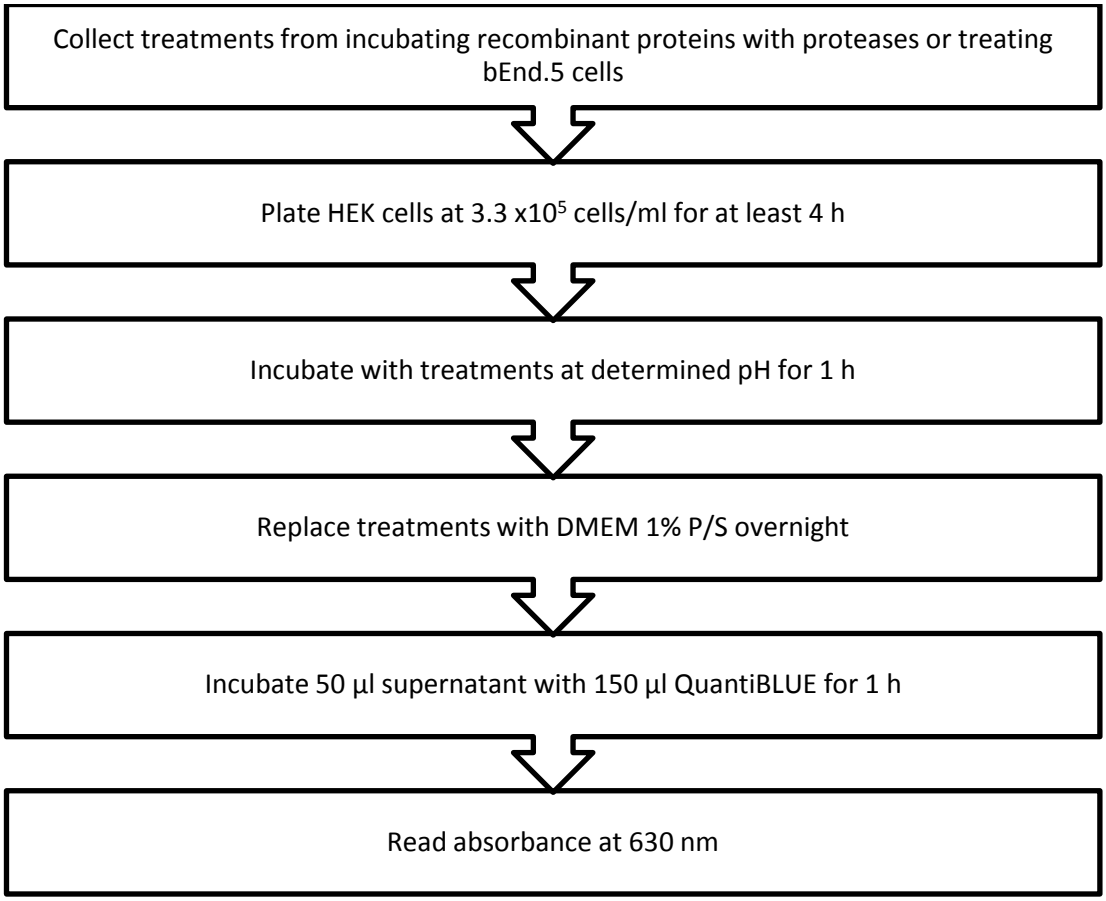
HEK-Blue IL-1β cells (InvivoGen, UK) were used to determine IL-1 activity. Cells were cultured in growth media (DMEM, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 mg/ml Normocin (InvivoGen, UK)) for at least two passages before being transferred to selective media (DMEM, 10 % FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml Normocin, 100 μg/ml Zeocin (InvivoGen, UK), 200 μg/ml HygroGold (InvivoGen, UK)) to express IL-1R1. To passage, media was removed and cells were washed with phosphate-buffered saline (PBS). A cell scraper was used to detach cells and cell number was counted using a haemocytometer. Cells were seeded at 3.3 x $10^5$ cells per ml selective media in a 96-well plate (~50,000 cells/well).

**Sample collection**

Following treatment, supernatants were collected on ice and stored at -20 $^oC$ until required. Lysates were collected in PBS with 0.1 % triton (Sigma, UK) and 1 % protease inhibitor cocktail (Set 1; Calbiochem, UK) and stored at -20 $^oC$ until required.

## 7.1.2. HEK-Blue IL-1β activity assay

IL-1 activity was determined using HEK-Blue IL-1β cells (InvivoGen, UK) as follows:




## 7.1.3. Enzyme-linked immunosorbance assay (ELISA)

IL-1α, IL-1β, IL-6 and CXCL1 levels were recorded in culture supernatants and/or cell lysates using enzyme-linked immunosorbance assay (ELISA) DuoSets (R&D Systems, UK). A 96 well immuno plate (Nunc, UK) was coated with the relevant capture antibody in PBS (50 µl/well) and placed on an orbital shaker at 4 $^{o}$C overnight. All subsequent incubations were performed at room temperature (RT) on an orbital shaker. Plates were washed 4 x with wash buffer (0.05 % tween in PBS) and blocked with 200 µl reagent diluent (RD; 1 % bovine serum albumin (BSA) in PBS) per well for at least 1 h. A 12 point standard curve was generated using 2-fold serial dilutions in RD and a high standard of 4000 pg/ml. Plates were washed 4 x and 50 µl/well samples were loaded either neat or diluted in RD to ensure they fell on the linear part of the standard curve, and incubated for 2 h. Plates were washed 4 x, incubated in 50 µl/well of the relevant detection antibody in RD for 1 h. Plates were again washed 4 x, incubated with 50 µl/well streptavidin-HRP in RD for 30 min, washed 4 x and incubated with 50 µl/well substrate solution (1:1 mixture of hydrogen peroxide and tetramethylbenzidine) protected from the light for 20 min. 1 M $H_2SO_4$ was added to each well to stop the reaction and absorbance was read at 450 nm (corrected at 570 nm; Synergy HT plate reader, BioTek, UK). Data were analysed using GraphPad Prism v5 or 6.

Optical density values of standards were log-transformed and fitted to a sigmoidal 4 parameter logistic standard curve. Sample values were also log-transformed and interpolated from the curve before being transformed to determine concentration values.

## 7.1.4. Western blot

**Solutions:**

**Sample buffer**

10 % w/v sodium dodecyl sulphate (Fisher Scientific, UK), 50 % v/v glycerol (Fisher Scientific, UK), 400 mM Tris-HCl (Fisher Scientific, UK) pH 6.8, 0.025 % w/v bromophenol blue (Sigma, UK), 5 % β mercaptoethanol (Sigma, UK) in $dH_2O$.

**10 x Running buffer**

30.8 g tris base (Fisher Scientific, UK), 144 g glycine (Fisher Scientific, UK), 20 g sodium dodecyl sulphate (Fisher Scientific, UK) in 1 L $dH_2O$.

**10 x Transfer Buffer**

29 g tris base (Fisher Scientific, UK), 145 g glycine (Fisher Scientific, UK ) 1 L $dH_2O$.

To make up 1 L Transfer buffer, add 200 ml methanol and 100 ml 10 x transfer buffer to 700 ml $dH_2O$.

**Sample concentration**

If required, supernatants were concentrated prior to being run on a western blot. Equal amounts of supernatant (up to 500 µl) were added to 10 k Amicon Ultra 0.5 ml centrifugal filters (Millipore, UK). These were centrifuged at 14, 000 x g for 30 min at 4 $^{o}$C. Tubes were inverted and centrifuged at 1000 x g for 2 mins to collect the concentrate.

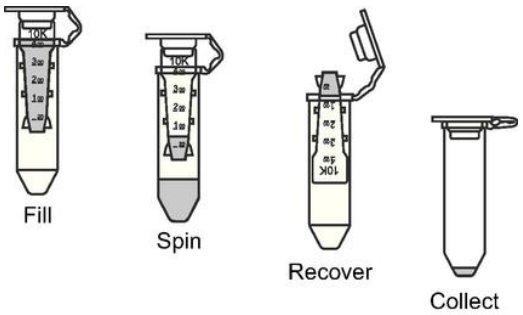



*Figure 7.1. Sample concentration protocol from Millipore*

## Protocol

5 % acrylamide stacking gels and 12 % acrylamide resolving gels were made with the components listed below (Table 7.1). Samples buffer was added 1/5 to each sample. Samples were denatured at 95 $^{o}$C for 5 min before equal volumes were loaded onto the gel alongside 10 µl Precision Plus Protein molecular weight ladder (BioRad, UK; 10-150 kDa). Samples were run until they reached the bottom of the resolving gel and then transferred onto a nitrocellulose membrane at 15 V for 45 mins (Trans-Blot SD Semi-Dry Transfer Cell, BioRad, UK). The membrane was then blocked in 5 % dried skimmed milk powder in PBS (Sigma, UK) 0.1 % tween (Sigma, UK) for 1 h at RT (all incubation steps are performed on a rotator or orbital shaker). Membranes were then washed with PBS 0.1 % tween before being transferred into primary antibody in PBS 0.1 % tween, 0.1 % BSA (Sigma, UK). Membranes were incubated in primary antibody (see Table 7.2) on an orbital shaker for 1 h at RT or overnight at 4 $^{o}$C before being washed as above and transferred into secondary antibody (see Table 7.3) for 1 h at RT. Membranes were washed a final time before being exposed to enhanced chemiluminescene (ECL, Amersham) for 1 min, placed in a cassette and taken to a dark room for exposure. Films were exposed on a JP-33 automatic film processor (JPI Healthcare solutions, NY) and images were captured using Northern eclipse software (Northern Eclipse, UK) or a CanoScan LiDE 700F scanner (Canon, UK).

| Component | 12 % Resolving Gel (5 ml) | 5 % Stacking Gel (1 ml) |
|---|---|---|
| $H_2O$ | 1.6 | 0.68 |
| 30 % Acrylamide mix | 2.0 | 0.17 |
| 1.5 M Tris pH8.8<br>1.5 M Tris pH6.8 | 1.3<br>- | -<br>0.13 |
| 10 % Sodium dodecyl sulphate | 0.05 | 0.01 |
| 10 % Ammonium persulphate | 0.05 | 0.01 |
| TEMED | 0.002 | 0.001 |

*Table 7.1. Components for making Western blot gels in Chapters 2 and 3*

| Primary Antibody | Concentration | Solution | Catalogue number |
|---|---|---|---|
| Goat anti-mouse IL- | 0.1 µg/ml | 0.1 % BSA in PBS 0.1 | R&D Systems; AF- |

| | | | |
|---|---|---|---|
| 1β | | % tween | 401-NA |
| Goat anti-mouse IL-1α | 0.1 µg/ml | 0.1 % BSA in PBS 0.1 % tween | R&D Systems; AF-400-NA |
| Goat anti-human IL-1β | 0.1 µg/ml | 0.1 % BSA in PBS 0.1 % tween | R&D Systems; AF-201-NA |

*Table 7.2. Details of Western blot primary antibodies in Chapters 2 and 3*

| Secondary Antibody | Concentration | Solution | Catalogue number |
|---|---|---|---|
| HRP-conjugated rabbit-anti goat | 1/1000 | 5 % Milk in PBS 0.1 % tween | Dako P0449 |

*Table 7.3. Details of Western blot secondary antibody in Chapters 2 and 3*

## 7.1.5. Lactate dehydrogenase (LDH) assay

Lactate dehydrogenase (LDH) assay (Promega, UK) was performed to determine cell viability. Living cells with intact membranes do not release LDH so levels of LDH in supernatants reflect release from dead cells. 25 µl lysis buffer was added to an untreated well 15 min prior to the end of the experiment to induce total cell lysis. 25-50 µl supernatant from this well was added to a 96 well plate in triplicate and this was used as a control for maximum cell death. 25-50 µl media alone was added in triplicate to control for absorbance of the media alone and 25-50 µl supernatant from each treated well was added to remaining wells to measure cell death. 25-50 µl substrate was added to each well and the plate covered and placed on an orbital shaker for 30 min. 25-50 µl Stop solution (1 M acetic acid) was added to each well before absorbance was read at 490 nm (Synergy HT plate reader, BioTek, UK). Percentage of LDH released compared to that following total lysis (after subtracting for media absorption) was plotted as a marker of cell death.

## 7.1.6. In vivo

**Power calculations**

Sample size was selected using power calculations based on previous data from our group showing the effect of a caspase-1 KO on kainic acid-induced seizures (Ravizza et al., 2006b).

Formula was as follows:

$$N = (E\alpha + E\beta)^2 \times [\, 2 \times SD^2 / (\mu B - \mu A)^2 \,]$$

Where 'N' is number of animals. α refers to type 1 error (set at 0.05), β is type 2 error (set at 0.2), SD is standard deviation, µB-µA is the expected difference in mean between the two groups. The mean standard deviation of WT and KO animals from this study was 983 s for seizure onset, 118 s for time in seizure activity and 2.6 for number of seizures, and the

expected difference in mean between the two groups were set at 1000 s, 110 s and 2.5 respectively. This produced sample sizes of 8, 10 and 9 for seizure onset, time in seizure and number of seizures respectively.

## 7.1.7. Stereotaxic surgery

Animals were prepared for surgery by shaving the head and flank under anaesthesia (2.5 L/min isoflurane 0.2 L/min O2, 0.4 L/min $NO_2$) before being transferred to the stereotaxic frame. Lubrithal eye gel was applied to each eye to prevent drying during surgery and 0.1 mg/kg buprenorphine was injected sub-cutaneously (s.c.; Vetergesic; Alstoe, UK) for analgesia. The surgical area on the head and flank was prepared with povodine-iodine. A midline incision was made across the skin to expose the skull surface and mark bregma. The skin above the flank was cut to insert borer through to the opening above the skull and transmitter electrodes (ETA-F20 small animal transmitter; Data Sciences International, St Paul, MN) were fed through. The reference electrode was glued between the eyes and a shallow burr hole drilled to attach a screw (Precision Technology Supplies, UK) for stability. Co-ordinates from bregma for intracerebroventricular cannula and hippocampal electrode were -0.4 mm, +1 mm, and -1.9 mm, -1.5 mm, (anterior/posterior, medial/lateral) respectively (Paxinos and Franklin, 2004). The recording electrode was lowered in place 1.5 mm below the dura and secured in place with the cannula (if required) with dental cement. The incision on the flank was extended and blunt dissection performed to create a s.c. pocket for the transmitter body. Incisions were closed with suture (6-0; Ethicon, UK), EMLA cream was spread over sutures for local anaesthesia and 0.5 ml-1 ml saline was injected s.c. to replace fluid lost during surgery.

## 7.1.8. Intra-cardial perfusion and brain collection

**Solutions**

**0.4 M Phosphate buffer (PB)**

Dissolve 57.2 g $Na_2HPO_4 * 2H_2O$ (Fisher Scientific, UK) and 11.035 g $NaH_2PO_4 * H_2O$ (Fisher Scientific, UK) in 1 L $dH_2O$.

**30 % Sucrose**

Dissolve 30 g sucrose (Fisher Scientific, UK) in 100 ml 0.4 M PB

**3.7-4.1 % Paraformaldehyde (PFA)**

Dilute 37-41 % PFA (Fisher Scientific, UK) 1/10 in 0.4 M PB

**Perfusion solution**

Dissolve 9 g NaCl (Fisher Scientific, UK) in 999 ml $dH_2O$, add 1 ml diethyl pyrocarbonate (Sigma, UK) and autoclave twice.

### Protocol

Mice were anaesthetised at 4 L/min isoflurane, 0.2 L/min $O_2$, 0.4 L/min $NO_2$ and maintained at approximately 3 L/min isoflurane 0.2 L/min O2, 0.4 L/min $NO_2$ during perfusion. Pedal reflexes were checked to confirm depth of anaesthesia. Skin over the chest and abdomen was removed, the diaphragm was cut away and the ribs were cut to expose the heart. A 21 gauge butterfly needle was inserted into the left ventricle and clamped and a small incision was made in the right atrium. 0.9 % NaCl 0.1 % diethyl pyrocarbonate (Sigma, UK) was perfused at a rate of 10 ml/min using a peristaltic pump (Harvard Apparatus, UK).

Brains were removed and cut in half along the midline with a scalpel blade. Half was collected in 3.7-4.1 % paraformaldehyde and stored at 4 $^{o}$C overnight. Brains were then transferred to 30 % sucrose for up to 2 days before being rapidly frozen in isopentane cooled to -30 to -40 $^{o}$C and stored at -80 $^{o}$C until required.

## 7.1.9. Immunofluorescence

### Solutions

**Cryoprotectant (1 L)**

Dissolve 6.6 g $Na_2HPO_4$ * $2H_2O$ (Fisher Scientific, UK) and 0.79 g $NaH_2PO_4$ * $H_2O$ (Fisher Scientific, UK) in 500 ml $dH_2O$. Then add 300 ml Ethylene glycol (Sigma, UK) and 200 ml Glycerol (Fisher Scientific, UK). Mix well.

**Primary diluent (PD; 100 ml)**

Heat 20 ml PBS (Sigma, UK) to 40 – 50 $^{o}$C and dissolve 300 µl triton in it. Top up with 80 ml cool PBS, 10 mg $NaN_3$ (Sigma, UK) and 1 g BSA (Sigma, UK)

**Block**

10 % Normal donkey serum (NDS; Sigma, UK) in PD

| Primary Antibody | Concentration | Solution | Catalogue number |
|---|---|---|---|
| Rabbit-anti Iba-1 | 1 µg/ml | 2 % NDS in PD | Wako; 019-19741 |
| Mouse-anti GFAP | 1/1000 | 2 % NDS in PD | Millipore; MAB360 |

| | | | |
|---|---|---|---|
| Goat anti-mouse IL-1β | 1 µg/ml | 2 % NDS in PD | R&D Systems; AF-401-NA |
| Goat anti-mouse IL-1α | 1 µg/ml | 2 % NDS in PD | R&D Systems; AF-400-NA |

*Table 7.4. Primary antibodies for immunofluorescence in Chapter 4*

| Secondary Antibody | Concentration | Solution | Catalogue number | Primary used against |
|---|---|---|---|---|
| Donkey-anti rabbit Alexa fluor 594 | 1/500 | 2 % NDS in PD | Life technologies, UK; A21207 | Rabbit-anti Iba-1 |
| Donkey-anti mouse Alexa fluor 488 | 1/500 | 2 % NDS in PD | Life technologies, UK; A21202 | Mouse-anti GFAP |
| Donkey-anti goat Alexa fluor 594 | 1/500 | 2 % NDS in PD | Life technologies, UK; A11058 | Goat anti-mouse IL-1β |
| Donkey-anti goat Alexa fluor 488 | 1/500 | 2 % NDS in PD | Life technologies, UK; A11055 | Goat anti-mouse IL-1β or IL-1α |

*Table 7.5. Secondary antibodies for immunofluorescence in Chapter 4*

### Brain sectioning

A sledge microtome (8000 Microtome, Bright, UK) and freezing unit (Bright solid state freezer, Bright, UK) were used for sectioning brains. Half brains were secured on the platform using distilled water and OCT embedding matrix (Thermo scientific, UK), frozen in place using crushed dry ice. The blade was fixed at 15$^{o}$ to cut 30 µm slices. Twelve sets of slices per brain were collected in cryoprotectant and stored at -20$^{o}$C until use.

### Protocol

Immunofluorescence was performed on free-floating sections prepared as described above. A set of brain slices from each animal was transferred to a separate well of a 24-well plate and washed 2 x in PBS for 10 min each on an orbital shaker. Non-specific binding was blocked with 450 µl 10 % normal donkey serum (see above) for 1 h at RT. All incubations were performed on an orbital shaker. Sections were incubated in 450 µl primary antibody (see Table 7.4) at 4 $^{o}$C overnight. Sections were washed 3 x with PBS and incubated with 450 µl fluorescently-conjugated secondary antibody (see Table 7.5) for 2 h protected from the light. Sections were washed 3 x in PBS and transferred to $dH_2O$, one set at a time, to be mounted onto glass slides (coated with 0.5 % gelatine (BDH Laboratory Supplies, UK) and 0.05 % chromium potassium sulphate (BDH Laboratory Supplies, UK). Slides were left to dry before being coverslipped using Prolong Gold Anti-fade Reagent with DAPI (Invitrogen, UK).

Images were viewed on an upright microscope (BX51, Olympus, UK) and acquired using a 20x/0.80 Plan Apo objective using the 3D Histech Pannoramic 250 Flash II slide scanner. Filter sets for DAPI, FITC and Texas Red were used to visualise fluorescence.

## 7.2. Appendix 2. Seizure model development

The following section contains data on model development supplementary to Chapter 4.

### 7.2.1. Introduction

Intrahippocampal injection of kainic acid has been shown to induce changes in EEG representative of seizure activity (Vezzani et al., 1999). Seizure activity is exacerbated with addition of exogenous IL-1β and reduced with IL-1Ra, caspase-1 inhibitors or in caspase-1 KO mice (Vezzani et al., 1999; Ravizza et al., 2006b). The aim of this work was to develop a reproducible model of acute seizures using intrahippocampal kainic acid for future work to explore the role of NLRP3 inflammasome components in seizure.

### 7.2.2. Methods

**Experimental Animals**

Male C57Bl/6J mice were purchased from Charles River, UK. Animals were housed on a 12 hour light/dark cycle under temperature, humidity and light-controlled conditions with free access to food and water. All procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and approved by the local Animal Welfare and Ethical Review Board. For studies taking longer than a day, animals from each treatment group (Vehicle (PBS) or KA) were randomly assigned an ID with treatments blocked for surgery day.

**Surgical implantation EEG transmitter**

Surgery was performed using stereotaxic guidance on mice over 20 g as in Chapter 4. Anaesthesia was induced with 4 L/min isoflurane 0.2 L/min O2, 0.4 L/min $NO_2$ and maintained at approximately 2.5 L/min isoflurane 0.2 L/min O2, 0.4 L/min $NO_2$ during surgery depending on the response of the animal. Reflexes and breathing were monitored to ensure surgical anaesthesia was maintained during the procedure. Body temperature was maintained with a heated blanket and Buster sterile op cover. Buprenorphine (0.1 mg/kg s.c.) was administered at the start of surgery and again 4-6 h later. During recovery from surgery, animals were monitored on a heated blanket then in a heated cabinet set to 30 $^{o}$C until the second dose of buprenorphine had been administered and they were deemed fit to return to normal housing (3-5 h post-surgery). Animals were singly housed post-surgery to minimise suture or electrode removal and allowed to recover for at least 5

days before seizure induction. Animals were monitored and body weight was recorded daily.

Hippocampal guide cannulas were made from a 23 gauge needle (Beckton Dickenson, UK) cut to desired length (6 mm). These were attached to the side of the electrode with superglue so they ended 1.5 mm before the end of the electrode. ETA-F20 small animal transmitters (Data Sciences International, St Paul, MN) were implanted in a subcutaneous pocket on the flank. Electrodes were tunnelled subcutaneously to an incision over the skull where the reference electrode was glued to the nasal sinus and the EEG recording electrode was implanted into the dorsal hippocampus (-1.9 mm posterior, -1.5 mm lateral to Bregma and 1.5 mm below the dura so the cannula rests on the dura). For stability, a 1.4 mm screw (Precision Technology Supplies, UK) was attached via a shallow hole on the skull and the whole area secured with dental cement.

**Seizure induction**

Animals were housed in their home cage over a receiver pad. Seizure induction was performed between 9 and 11.30 am with at least 30 min baseline EEG recorded prior to seizure induction and up to 3 h post seizure induction. To induce seizures, 14-30 ng kainic acid (Tocris, UK) dissolved in PBS (0.5 µl) was administered using an intrahippocampal dosing cannula (made from fine bore polythene tubing (Smiths Medical, UK), and 31 gauge wire (STN, UK)) under brief anaesthesia (isoflurane). 14 ng/mouse has previously been used to induce seizures in C57Bl/6 mice (Ravizza et al., 2006b). If severe behavioural seizures were repeated or prolonged (greater than 10 per h), animals were culled.

**Seizure analysis**

EEG data were collected using DSI A.R.T. Acquisition 4.0 at a sample rate of 1000 Hz. EEG traces were analysed by eye using DSI A.R.T. Analysis 4.0 by an experimenter blind to treatment. Baseline and post kainic acid EEG traces were viewed and seizures were determined as discrete events with a profile distinct from baseline recording i.e. increased amplitude and/or frequency and a duration greater than 5 s (e.g. Bergstrom 2013).

## 7.2.3. Results

Initial experiments with 14 ng kainic acid did not induce clear EEG seizures and hippocampal cannulas were repeatedly blocked (data not shown). To confirm that EEG seizure activity could be observed in this model, a pilot study was performed where 60 min after 14 ng intrahippocampal kainic acid, (when no further seizure activity was observed,) 30 ng intrahippocampal kainic acid was administered to induce behavioural seizures to

observe if these correlated with EEG seizure activity. In Mouse 1, no EEG or behavioural changes were observed following 14 ng kainic acid (Figure 7.2B). Nine mins post 30 ng kainic acid, a clear behavioural seizure was observed in Mouse 1 which correlated with EEG seizure activity (Figure 7.2C). Intermittent seizure activity followed. Mouse 2 displayed little seizure behaviour following 14 ng or 30 ng kainic acid i.e. immobile and staring and no clear EEG seizures were observed (data not shown).

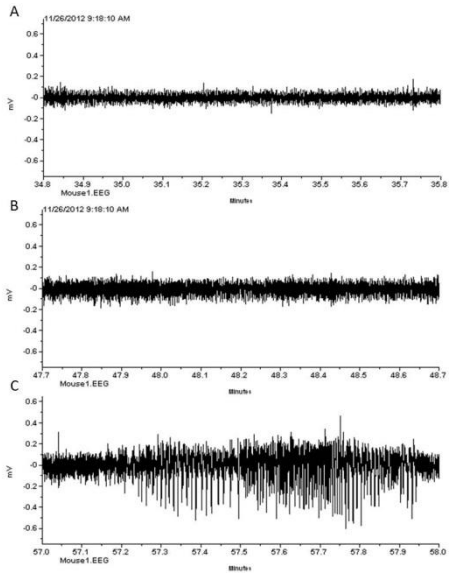



*Figure 7.2 Intrahippocampal kainic acid induces EEG seizure activity in C57Bl/6 mice.* Following at least 30 min baseline EEG recording, animals were dosed with 14 ng/0.5 µl intrahippocampal kainic acid. After 60 min, to correlate behavioural seizure activity with EEG seizure activity, 30 ng/0.5 µl intrahippocampal kainic acid was administered. EEG was recorded for a further 60 min. Example EEG traces from baseline (A), post 14 ng kainic acid (B) or post 30 ng kainic acid (C) are shown.

As EEG seizures were observed in the pilot study following intrahippocampal kainic acid and these correlated with behaviour, a dose response study was performed to establish a dose that could produce reliable EEG seizures without severe behavioural seizures.

PBS, 14 ng, 22 ng or 30 ng intrahippocampal kainic acid was administered and seizure activity recorded. To prevent cannulas getting blocked during and post-surgery (as had been observed initially with 14 ng kainic acid), at the end of surgery, wire was used to confirm that the cannulas were clear. Two animals were not included in the EEG analysis due to poor outcome post-surgery. Despite clearing the cannulas, six out of fourteen animals had cannulas at least partially blocked at the time of dosing. Of those dosed successfully, seizure activity was not consistently observed with kainic acid treatment at any dose (only 3/6 mice dosed with kainic acid), although animal numbers were low (Table 7.6).

| Animal ID | Dose (ng KA) | Cannula blocked? | Seizure behaviour/ EEG observed? |
|---|---|---|---|
| 1 | 14 | Yes | N/A |
| 2 | 0 | Yes | N/A |
| 3 | 22 | No | Yes |
| 4 | 30 | No | No |
| 5 | 14 | No | Yes |
| 6 | 0 | Yes | N/A |
| 7 | 22 | No | Yes |
| 8 | 30 | Yes | N/A |
| 9 | 14 | No | No |
| 11 | 0 | No | No |
| 12 | 30 | Yes | N/A |
| 13 | 14 | Yes | N/A |
| 15 | 0 | No | No |
| 16 | 22 | No | No |

*Table 7.6. Data from intrahippocampal kainic acid dose response. Intrahippocampal kainic acid (14-30 ng) or PBS was administered following at least 30 min baseline EEG recording. Seizure activity was determined for up to 3 h post dose. KA, kainic acid*

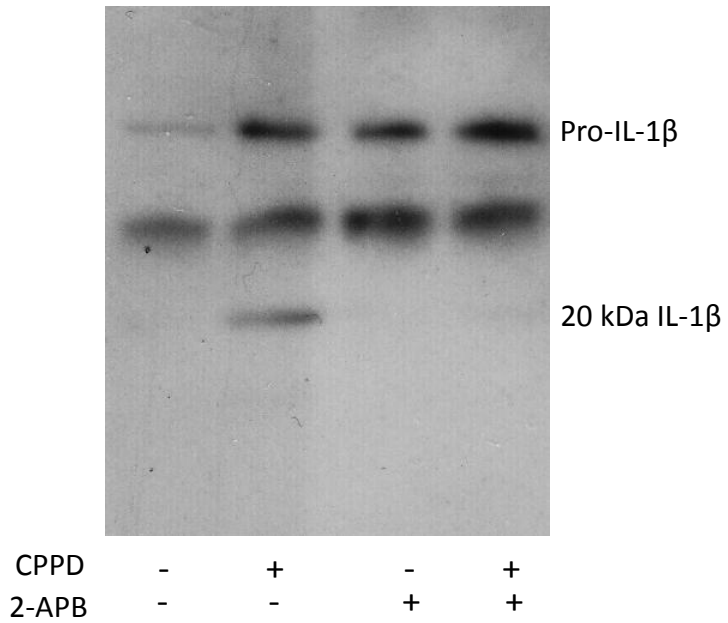
## 7.2.4. Discussion

Although used previously to induce seizures in C57Bl/6J mice (Ravizza et al., 2006b), 14-30 ng intrahippocampal kainic acid did not induce reproducible seizures here. This was in part due to technical challenges with intrahippocampal dosing. As large animal numbers would likely be required to induce reproducible seizures and to observe a treatment effect using this model, alternative dosing methods were investigated, as described in Chapter 4.

## 7.3. Appendix 3. Supplementary data

### 7.3.1. Effect of intracellular calcium on 20 kDa IL-1β

Inhibition of $Ca^{2+}$ release from intracellular stores (via inhibition of inositol triphosphate ($IP_3$) receptors with 2-APB) prevented CPPD-mediated release of 20 kDa IL-1β.



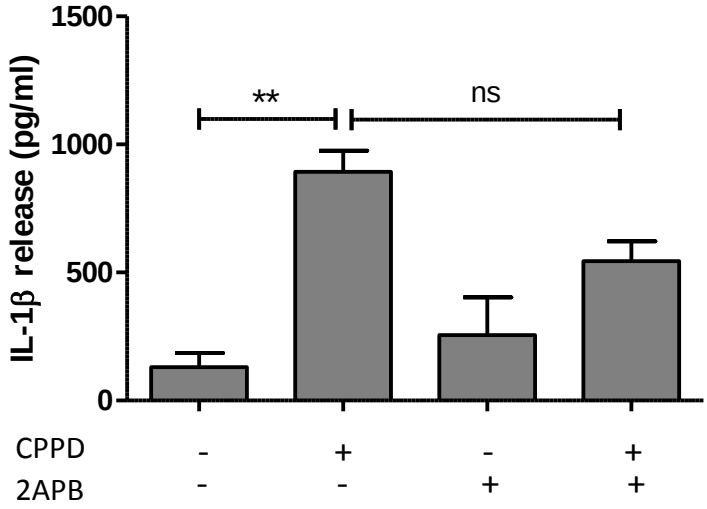




*Figure 7.3. Effect of intracellular calcium on 20 kDa IL-1β.* LPS-primed mixed glia were pre-treated with 2-aminoethoxydiphenyl borate (2-APB; 100 µM; R&D Systems, UK) or vehicle (0.5 % DMSO) for 15 min prior to 250 µg/ml calcium pyrophosphate dihydrate (CPPD; InvivoGen, UK) or PBS (Sigma, UK) in pH 6.2 HBSS (See Appendix 1 for details). Supernatants were run on a Western blot (A) or mouse IL-1β ELISA (R&D Systems; B). N=3. A is representative of 2 separate blots. For B, data is presented as mean + SEM and statistical analysis was performed using a one-way ANOVA with a Bonferroni's multiple comparison test *post hoc.* ns, not significant; ** $p \leq 0.01$.

## 7.3.2. IL-1β expression at low pH.

Treating cells at pH 6.2 for 1 h does not affect IL-1β expression in mixed glia.

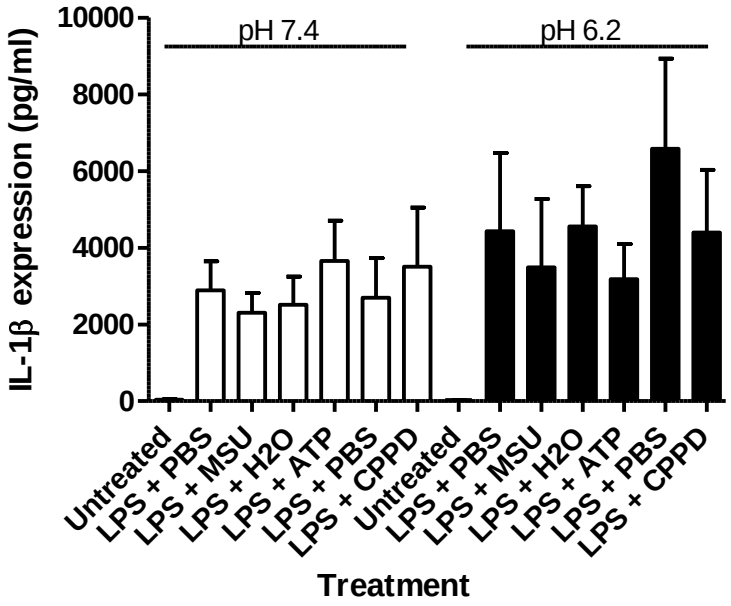



*Figure 7.4 IL-1β expression at low pH.* LPS primed mixed glia were treated for 1 h with 5.5 mM ATP (Sigma, UK), 250 μg/ml monosodium urate (MSU; InvivoGen, UK), 250 μg/ml calcium pyrophosphate dihydrate (CPPD; InvivoGen, UK) or vehicle in HBSS pH 7.4 or 6.2. Cell lysates were collected as described in Appendix 1 and run on an IL-1β ELISA (R&D Systems, UK). Data is plotted as mean + SEM, n=3-8.