Understanding the regulation of interleukin-1

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Biology, Medicine and Health

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Abbreviations

3xTgAD  Triple transgenic
AD      Alzheimer's disease
AIM2    Absent in melanoma 2
ALR     Absent in melanoma 2-like receptor
Alum    Aluminium hydroxide
ANOVA   Analysis of variance
AP-1    Activator protein 1
APC     Antigen-presenting cell
APP     Amyloid Precursor Protein
ASC     Apoptosis-associated speck-like protein containing a caspase activation
        and recruitment domain
ATP     Adenosine triphosphate
Aβ      Amyloid beta
BCA     Bicinchoninic acid
BHB     β-hydroxybuturate
BMC     Bone-marrow cell
BMDM    Bone marrow-derived macrophage
BSA     Bovine serum albumin
C57BL/6 C57 Black 6
cAMP    Cyclic adenosine monophosphate
CANTOS  Canakinumab Antiinflammatory Thrombosis Outcome Study
CAPS    Cryopyrin-Associated Autoinflammatory Syndrome
CARD    Caspase activation and recruitment domain
Casp1   Caspase-1
CASP8   Caspase-8
CC      Coiled coil
CD      Cluster of differentiation
cDNA    Complementary deoxyribonucleic acid
CFTR    Cystic fibrosis transmembrane conductance regulator
cGAS    Cyclic GMP-AMP Synthase
CLIC    Chloride intracellular channel
CLR     C-type lectin receptors
CNS     Central nervous system
COX     Cyclooxygenase
CPPD    Calcium pyrophosphate dihydrate
CRID    Cytokine release inhibitory drug
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRISPR</td>
<td>Clustered, regularly interspaced, short palindromic repeat</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>ddH2O</td>
<td>Double distilled H2O</td>
</tr>
<tr>
<td>DIDS</td>
<td>4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPBA</td>
<td>Diphenylborinic anhydride</td>
</tr>
<tr>
<td>DPH</td>
<td>Diphenhydramine</td>
</tr>
<tr>
<td>DPI</td>
<td>Diphenyleneiodonium</td>
</tr>
<tr>
<td>DPTTF</td>
<td>2,2-diphenyltetrahydrofuran</td>
</tr>
<tr>
<td>DSB</td>
<td>Double stranded break</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double stranded ribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPPS</td>
<td>4-(2-hydroxyethyl)-1-Piperazinepropanesulfonic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain protein</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FCAS</td>
<td>Familial Cold Autoinflammatory Syndrome</td>
</tr>
<tr>
<td>FIIND</td>
<td>Function to find domain</td>
</tr>
<tr>
<td>GSDMD</td>
<td>Gasdermin-D</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
</tr>
<tr>
<td>HAX1</td>
<td>HS-1-associated protein X-1</td>
</tr>
<tr>
<td>HDR</td>
<td>Homology directed repair</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-Piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia-inducible factor</td>
</tr>
<tr>
<td>HIN</td>
<td>Hematopoietic interferon-inducible nuclear antigens with a 200-amino-acid repeat</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High mobility group box 1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
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</tr>
<tr>
<td>iBMDM</td>
<td>Immortalised bone marrow-derived macrophage</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>ICE</td>
<td>Interleukin converting enzyme</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of nuclear factor-κB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin-1 receptor 1</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IL-1RACP</td>
<td>Interleukin-1 receptor accessory protein</td>
</tr>
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<td>IL-18</td>
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</tr>
<tr>
<td>IL-18BP</td>
<td>Interleukin-18 binding protein</td>
</tr>
<tr>
<td>IL-18Rα</td>
<td>Interleukin-18 receptor alpha</td>
</tr>
<tr>
<td>IL-18Rβ</td>
<td>Interleukin-18 receptor beta</td>
</tr>
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<td>IRAK</td>
<td>Interleukin-1 receptor-associated kinase</td>
</tr>
<tr>
<td>Kapβ</td>
<td>Karyopherin-β</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>lincRNA</td>
<td>Long intergenic noncoding ribonucleic acid</td>
</tr>
<tr>
<td>IncRNA</td>
<td>Long noncoding ribonucleic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich repeat</td>
</tr>
<tr>
<td>LRRC8A</td>
<td>Leucine rich repeat containing 8 A</td>
</tr>
<tr>
<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MD</td>
<td>Myeloid differentiation factor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLKL</td>
<td>Mixed lineage kinase domain like pseudokinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MNS</td>
<td>3,4-Methylenedioxy-β-nitrostyrene</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSU</td>
<td>Monosodium urate</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>MWS</td>
<td>Muckle-Wells Syndrome</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
</tr>
<tr>
<td>NACHT</td>
<td>Nucleotide-binding and oligomerisation</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAIP</td>
<td>Neuronal apoptosis inhibitory protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NAT</td>
<td>Natural antisense transcript</td>
</tr>
<tr>
<td>NBC</td>
<td>Novel boron compound</td>
</tr>
<tr>
<td>NDS</td>
<td>Normal donkey serum</td>
</tr>
<tr>
<td>NEK</td>
<td>Mammalian never in mitosis gene a-related kinase</td>
</tr>
<tr>
<td>NEMO</td>
<td>Nuclear factor-κB essential modulator</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NHEJ</td>
<td>Non-homologous end joining</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding oligomerization domain-like receptor</td>
</tr>
<tr>
<td>NLRC</td>
<td>Nucleotide-binding oligomerisation domain-like receptor, CARD-containing</td>
</tr>
<tr>
<td>NLRP</td>
<td>Nucleotide-binding oligomerisation domain-like receptor, pyrin-containing</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localisation sequence</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>NOMID</td>
<td>Neonatal-Onset Multisystem Inflammatory Disease</td>
</tr>
<tr>
<td>NOR</td>
<td>Novel object recognition</td>
</tr>
<tr>
<td>NPPB</td>
<td>5-Nitro-2-(3-phenylpropyl-amino) benzoic acid</td>
</tr>
<tr>
<td>NRTI</td>
<td>Nucleoside reverse transcriptase inhibiting drug</td>
</tr>
<tr>
<td>ns</td>
<td>Not significant</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OTE</td>
<td>Off-target effect</td>
</tr>
<tr>
<td>OxATP</td>
<td>Oxidised Adenosine Triphosphate</td>
</tr>
<tr>
<td>OxLDL</td>
<td>Oxidised low-density lipoprotein</td>
</tr>
<tr>
<td>OxPAPC</td>
<td>Oxidised 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>OxPL</td>
<td>Oxidised phospholipid</td>
</tr>
<tr>
<td>PAAND</td>
<td>Pyrin-Associated Autoinflammation with Neutrophilic Dermatosis</td>
</tr>
<tr>
<td>PAM</td>
<td>Protopspacer adjacent motif</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate-buffered saline with Tween</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PenStrep</td>
<td>Penicillin, streptomycin</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PHENY</td>
<td>Phenytoin</td>
</tr>
<tr>
<td>PITS</td>
<td>Pore-induced intracellular traps</td>
</tr>
<tr>
<td>PolydA:dT</td>
<td>Poly(deoxyadenylic-thymidylic)</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
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<td>-----------</td>
</tr>
<tr>
<td>PS1</td>
<td>Presenilin-1</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin domain</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised controlled trials</td>
</tr>
<tr>
<td>RD</td>
<td>Reaction diluent</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>RIPK</td>
<td>Receptor-interacting serine/threonine-protein kinase</td>
</tr>
<tr>
<td>RLR</td>
<td>Retinoic acid-inducible gene I-like receptor</td>
</tr>
<tr>
<td>ROCS</td>
<td>Rapid overlay of chemical structures</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RVD</td>
<td>Regulatory volume decrease</td>
</tr>
<tr>
<td>SALA</td>
<td>Selective Aβ42-lowering agent</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SpCas9</td>
<td><em>Streptococcus pyogenes</em> Cas9</td>
</tr>
<tr>
<td>STING</td>
<td>Stimulator of interferon genes</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type three secretion system</td>
</tr>
<tr>
<td>TAB</td>
<td>Transforming growth factor-β –activating kinase-binding protein</td>
</tr>
<tr>
<td>TAK</td>
<td>Transforming growth factor-β –activating kinase</td>
</tr>
<tr>
<td>TALEN</td>
<td>Transcription activator-like effector nuclease</td>
</tr>
<tr>
<td>TBI</td>
<td>Traumatic brain injury</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll-Interleukin receptor</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin-layer chromatography</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>Tumour necrosis factor receptor-associated factor</td>
</tr>
<tr>
<td>TRIF</td>
<td>Toll or interleukin-1 receptor domain-containing adapter inducing interferon-beta</td>
</tr>
<tr>
<td>TSPO</td>
<td>Translocator Protein-18 kDa</td>
</tr>
<tr>
<td>TXNIP</td>
<td>Thioredoxin-interacting protein</td>
</tr>
<tr>
<td>UT</td>
<td>Untreated</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Veh</td>
<td>Vehicle</td>
</tr>
<tr>
<td>VRAC</td>
<td>Volume-regulated anion channel</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>ZFN</td>
<td>Zinc finger nuclease</td>
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</tbody>
</table>
Abstract

Inflammation is a highly conserved process utilised by an organism to protect itself against damage or infection. However, inflammation can also be extremely damaging and is implicated in many diseases. Indeed, targeting inflammation can be of great therapeutic benefit. Inflammation is initiated and propagated by pro-inflammatory cytokines such as interleukin-1 (IL-1). The two major pro-inflammatory members of the IL-1 family are IL-1α and IL-1β, both of which signal through the IL-1 type 1 receptor following secretion via unconventional mechanisms. IL-1 signalling is stringently regulated. IL-1β, which is relatively well researched, is translated as an inactive pro form which must be cleaved by a multi protein complex called an inflammasome. The mechanisms governing the formation of inflammasomes and subsequent IL-1β processing are not completely understood. IL-1α is poorly researched in comparison to IL-1β and undergoes subcellular transport to the nucleus of the cell as a result of the presence of a nuclear localisation sequence (NLS); the biological significance of this is poorly understood.

In order to effectively target IL-1 signalling in disease it is crucial to understand how this signalling is regulated. The aim of this thesis was to improve the understanding of IL-1 regulation by focusing on formation of the inflammasome and importance of the IL-1α NLS.

I contributed to the discovery that the fenamate class of nonsteroidal anti-inflammatory drugs (NSAIDs) prevents formation of the NLRP3 inflammasome via reversible blockade of the VRAC Cl⁻ channel and may be effective therapeutics in Alzheimer’s disease (AD). Building on this discovery, I was also involved in the design and development of novel NLRP3 inhibiting compounds using the Ca²⁺ blocker 2APB as a starting point. After multiple rounds of structure-activity relationships and phenotypic screening NBC6 was developed, a molecule 100-fold more potent than 2APB and with no effect on Ca²⁺-signalling. Finally, CRISPR/Cas9 was used to neutralise the NLS on IL-1α which resulted in an observed loss of IL-1α transcription. Further analysis revealed that this procedure had inadvertently disrupted the secondary structure of the crucial regulatory long-noncoding RNA AS-IL1α, the most likely explanation for the loss of IL-1α expression.

Together, these data greatly improve our understanding of the cellular and molecular mechanisms underpinning the regulation of IL-1. Not only do these studies pave the way for novel therapeutics to treat inflammatory disease but they also open new avenues of research for the rapidly accelerating field of inflammation.
Declaration

Work included in Chapter 2 this thesis has previously been submitted as part of MSci and BSc dissertations to the University of Manchester by Shi Yu and Claire Latta respectively, both of whom worked under my supervision. Work included in Chapter 2 of this thesis has also previously been submitted to the University of Manchester as part of a BSc dissertation by Sophie Booth, an MSci dissertation by Victoria Fasolino and PhD theses by William Watremez and Joshua Jackson. Work included in Chapter 3 this thesis has previously been submitted as part of a PhD thesis to the University of Manchester by Alex Baldwin. I declare that no other portion of 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 institute of learning.

Michael JD Daniels

22nd March 2018
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Publications


Daniels MJD, Adamson AD, Humphreys N, Brough D. (2017). CRISPR/Cas9 mediated mutation of mouse IL-1α nuclear localisation sequence abolishes expression. Scientific reports. 7, 17077


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Chapter 1: Introduction

1.1 Inflammation

It is not a ground-breaking observation that injury, for example in the form of a sprain or burn, results in rapid and local swelling, redness, heat and pain. These signs, first documented two thousand years ago, make up Roman encyclopaedist Celcus’ four cardinals of inflammation and today remain relevant in recognition of the inflammatory response. Inflammation can therefore be defined as the response of an organism to damage or infection, a process which is highly conserved from mammalian, non-mammalian and even invertebrate clades. Despite the clear importance in defining the mechanisms underlying the inflammatory response, it was not until the 19th century that our understanding began to broaden. Augustus Waller (1846) and Julius Cohnheim (1867) isolated a crucial cellular mediator of the inflammatory response – the leukocyte; and characterised the swelling phenomenon described by Celcus – vasodilation (Medzhitov, 2010). Nobel laureate Élie Metchnikoff discovered the process of phagocytosis and suggested that inflammation could be a protective response (Tauber, 2003) whilst Paul Ehrlich, who shared the Nobel prize with Metchnikoff, developed key methods to visualise immune cells in the blood by microscopy (Drews, 2004). Finally, Robert Koch, a good friend of Ehrlich’s, built on work by Louis Pasteur in the 1850s to affirm the germ theory of disease (in contrast to the now obsolete miasma theory) and demonstrated how inflammation can be initiated by microbial agents (Blevins and Bronze, 2010). In this way a platform was built for researchers to expand upon. Following years of ground-breaking research and multiple Nobel prizes, we now have basic knowledge of the key events of the inflammatory response and begin to pick further into the crucial mechanisms underpinning the fundamental process of inflammation.

1.1.1 The inflammatory response

The acute inflammatory reaction to damage, injury or infection forms part of the innate immune system. The innate immune response is a rapid and phylogenetically-ancient system that begins with the detection of generic patterns found either on pathogenic organisms (pathogen-associated molecular patterns, PAMPs) or on agents jettisoned by our own cells under stress (damage-associated molecular patterns, DAMPs). Examples of PAMPs include lipopolysaccharide (LPS), a component of the outer cell membrane on Gram-negative bacteria whilst DAMPs can include DNA or extracellular ATP amongst many others. These motifs are detected by germline-encoded pattern recognition receptors
(PRRs) located on sentinel cells such as macrophages, dendritic cells or microglia, known as antigen-presenting cells (APCs), which patrol the tissues of the body (Janeway and Medzhitov, 2002).

There are currently five characterised families of PRRs. Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are membrane-bound receptors and are located either on the cell surface or in intracellular compartments. Nucleotide-binding domain, leucine-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs), RIG-I-like receptors (RLRs), and AIM2-like receptors (ALRs) are located in the cytoplasm, where they detect intracellular PAMPs and DAMPs (Brubaker et al., 2015). When triggered, PRRs initiate a downstream signalling cascade that results in expression and secretion of a host of secondary mediators such as cytokines and chemokines. These diverse molecules bind their cognate receptors to initiate a range of secondary effects.

Initially, vasodilating factors such as histamine and nitric oxide increase the diameter of nearby blood vessels. It is this effect that was observed by Celsus so long ago. Chemokines such as CXCL1 and CXCL2 initiate the recruitment and extravasation of circulating leukocytes, the majority of which are made up of neutrophils, from the dilated blood vessels. The infiltrating leukocytes, arriving within minutes, release degrading factors such as reactive oxygen species (ROS), matrix metalloproteinases (MMPs) and sodium hypochlorite designed to destroy harmful invading species. Monocyte cells are also recruited to the site and can differentiate into macrophages or dendritic cells capable not only of phagocytosing and destroying dangerous material but also presenting components of microbes on their own cell surface via major histocompatibility complexes (MHCs) (Chaplin, 2010).

Antigen presentation via MHCs is required for the recruitment of the highly-tuned adaptive immune system, a network of differing T and B-cell subsets found only in higher order organisms which offers a slower but far more specific complement to innate immune activation. The adaptive immune system, activated by the binding of T-cell receptors presented on naïve T-cells to MHC complexes previously mentioned, initiates specific destruction of the defined threat and resolution of the innate immune response. Crucially, the adaptive immune system also provides memory of the molecular identity of the foreign invader thus allowing for a swifter, specific reaction in subsequent infections (Chaplin, 2010). Memory can also be provided by the innate immune system. Trained immunity is a recently-described phenomenon by which innate immune cells can ‘remember’ previous insults and has become a major research field in itself (Netea et al., 2016). In this way, the
immune system is activated in response to injury or infection and restores homeostasis to
the host tissue.

1.1.2 Sterile inflammation

It is important to note that inflammation, although likely driven evolutionarily by the necessity
to fight microbial infection and disease, is not necessarily a response to a foreign agent. In
fact, the inflammatory response is also initiated in response to sterile stimuli such as
monosodium urate, cholesterol, sphingosine, amyloid-β (Aβ) and DNA (Rock et al., 2010).
By reacting to sterile signs of danger and damage the innate immune system is capable of
a rapid response to resolve injury or infection without the necessity to directly identify a
foreign agent. However, this ability comes at a price. Innate immune activation can lead to
extensive collateral tissue damage. In this way the innate immune system is indeed a
double-edged sword, offering crucial protection from immune invasion as the cost of risking
damage to our own healthy cells. Thus, the erroneous initiation of sterile inflammation can
be extremely dangerous and is a major causative factor behind non-communicable
diseases such as stroke, atherosclerosis, Alzheimer’s disease, schizophrenia and cancer
amongst many others (Rock et al., 2010). These non-communicable diseases make up the
majority of mortalities globally (Daar et al., 2007) and it is therefore of huge importance that
our understanding of the mechanisms behind sterile inflammation is improved.

There has been substantial focus on the mechanisms that instigate and propagate the
signals that result in sterile inflammation. These inflammatory mediators, known as
cytokines, form a highly regulated and crucial part of the innate immune response and are
major culprits in sterile inflammatory disease. This introduction will outline the current
knowledge of one of the major proinflammatory cytokine families, the interleukin-1 (IL-1)
family, with particular focus on the two major members IL-1α and IL-1β. This chapter will
cover the numerous ways in which these potent molecules are regulated in homeostasis
and how this regulation goes awry in disease.

1.2 Interleukin-1 (IL-1)

IL-1 is in many ways the master pro-inflammatory cytokine. Prior to the discovery of its
precise molecular identity and subsequent nomenclature, IL-1 was recognised for its ability
to induce fever (one of Celsus’ cardinals of inflammation) and was known, amongst
numerous other names, as ‘pyrogen’. From its initial discovery by Paul Beeson in 1948
(Beeson, 1948) to its eventual cloning 36 years later, ‘endogenous pyrogen’ was studied
by researchers in hugely varied fields for its seemingly crucial involvement in areas such as immunology, metabolism, cancer (Dinarello, 1994).

Since the successful DNA sequencing and cloning of both mouse (Lomedico et al., 1984) and human (Auron et al., 1984) IL-1, research has accelerated to continue to elucidate the role of this cytokine in almost all aspects of mammalian systems. Despite this, a vast amount of information still remains unknown.

There are 11 members of the IL-1 superfamily, classified as such largely by shared structural features (Rivers-Auty et al., 2018). Two of the best characterised members, IL-1α and IL-1β, occur side by side on chromosome 2 in humans and most likely arose as a result of a gene duplication event that took place approximately 200 million years ago. Additionally, IL-36α, IL36β, IL-36γ, IL-36Ra, IL-37, IL-38 and IL-1Ra also all reside in the same chromosome cluster. The remaining members of the IL-1 superfamily, IL-33 and IL-18, are found on chromosomes 11 and 9 respectively. The IL-1 superfamily bind and signal through 10 different receptors, however, both IL-1α and IL-1β signal through the major IL-1 receptor IL-1R1(Dinarello, 2018; Rivers-Auty et al., 2018).

IL-1R1 consists of three immunoglobulin (Ig) domains at the extracellular side and a TIR domain located intracellularly (Figure 1.1). Binding IL-1R1 however is not sufficient for transmission of a signal, the accessory protein (IL-1RAcP) must also be recruited to the complex. The TIR domain is the same as that found in TLRs as described above and testament to its importance is the proposal that this may be one of the most conserved sequences across the kingdoms of both flora and fauna (O’Neill, 2008). In addition to this binding complex, the IL-1 ligands can also bind the Type II IL-1 receptor (IL-1R2); however, in this case no signal is transmitted as IL-1R2 is a decoy receptor (which can be secreted) and may act as a ‘sink’ to control levels of active IL-1, particularly IL-1β (Colotta et al., 1993). There also exists a naturally-occurring inhibitor to the IL-1 signalling system. IL-1 receptor antagonist (IL-1Ra) binds to IL-1R1 but does not propagate a signal and thus acts to regulate IL-1 signalling (Figure 1.1) (Dinarello, 1996).

Upon IL-1 binding and formation of the IL-1R1/ IL-1RAcP complex (leading to dimerization of TIR domains), the accessory protein MyD88 is recruited (Medzhitov et al., 1998). MyD88 also contains a TIR domain however, in contrast to the Ig domains found in IL-1R1, the TIR is coupled to a death domain (DD) at the N terminus. The forming of the ligand-TLR complex is thought to bring the two TIR domains together to form a platform on which MyD88 can be recruited. Following binding of the adapter protein to the complex, the serine-threonine kinase IRAK4 is recruited which subsequently activates IRAK1 by stimulating autophosphorylation. The two IRAK complexes then dissociate from MyD88 and activate
tumour necrosis factor receptor (TNFR)-associated factor 6 (TRAF6) which, as an E3-Ubiquitin ligase, auto-ubiquitinates itself. This then recruits transforming growth factor β-activated protein kinase (TAK1) by promoting binding with TAK1-binding protein 2 (TAB2) and TAB3. The complex then binds to the inhibitor of NFκB (IκB) kinase (IKK) complex (which consists of IKKβ and NFκB essential modulator (NEMO)) and promotes K63 ubiquitination of the latter subunit. This allows the active IKKβ subunit to phosphorylate IκBα leading to K48 ubiquitination which targets the complex for proteasome degradation. With the nuclear localisation sequences on NFκB factors unmasked they are then able to translocate to the nucleus and begin gene transcription.
Upon ligand binding, an intracellular cascade occurs via multiple factors resulting in NFκB-dependent transcription of pro-inflammatory genes. LPS, lipopolysaccharide; TLR4, toll-like receptor 4; MD2, myeloid differentiation factor 2; LRR, leucine-rich repeat; TIR, toll/interleukin-1 receptor; CD14, cluster differentiation factor 14; IgG, immunoglobulin; IL-1, interleukin-1; IL-1R, interleukin-1 receptor; IL-1RacP, interleukin-1 receptor accessory protein; IRAK, interleukin-1 receptor-associated kinase; TRAF, tumour necrosis factor-receptor associated factor; pUb, poly-ubiquitinated; TAK1, transforming growth factor-β activated kinase 1; TAB, transforming growth factor-β activated kinase 1 binding proteins; IKK, inhibitor of NF-kB (IκB) kinase; NEMO, NF-kB essential modulator; IKKβ, inhibitor of nuclear factor kappa-B kinase subunit beta; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; NFκB, nuclear factor kappa-light-chain-enhancer of activated B-cells.

The downstream effects of IL-1 binding (and the effector genes following NFκB transcription) are extremely potent and include cytokines such as IL-6 (Libermann and Baltimore, 1990), cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) (Yang et al., 2010), metalloproteinase enzymes (Zwerina et al., 2007), chemokines such
as GRO-α (also known as CXCL1 or KC) and monocyte chemotactic protein 1 (MCP1, also known as CCL2) (Hall et al., 1989; Sica et al., 1990) and even IL-1 itself (Hiscott et al., 1993).

The overall result of these downstream signalling events must be tightly regulated. Dysregulation of IL-1 signalling, leading to chronic inflammation and irreparable damage, has been implicated in a plethora of sterile inflammatory diseases including stroke, atherosclerosis, arthritis, dermatitis, cancer, epilepsy and many more (Dinarello, 1996; Allan et al., 2005). It is for these reasons that IL-1 signalling is tightly regulated by a number of mechanisms. The nature of this regulation is of interest in both clinical and preclinical research as IL-1 may be targeted to treat non-communicable disease. In the following sections I will outline current knowledge of the diverse ways in which IL-1 signalling is regulated with a focus on IL-1α and IL-1β.

1.3 Regulation of IL-1 signalling

1.3.1 Expression

Despite residing in the same gene cluster and evolving likely as a result of duplication of an ancestor gene (Rivers-Auty et al., 2018), IL-1α and IL-1β differ in their patterns of expression and subcellular localisation.

In humans, gene atlas data suggest both IL-1α and IL-1β mRNA is expressed in immune cell compartments such as the bone marrow, spleen and lymph nodes. However, IL-1α is also highly expressed in barrier tissues such as the skin and the gastro-intestinal tract (Uhlén et al., 2015). This is also corroborated in mouse where IL-1β mRNA is more associated with immune compartments such as the bone marrow and thymus whilst IL-1α mRNA is expressed in higher levels in the stomach, tongue and skin (Petryszak et al., 2016). Interestingly, both IL-1α and IL-1β mRNA is highly expressed in reproductive tissue such as the testis, cervix and vagina, suggesting a possible role in reproduction.

The cellular expression patterns of IL-1α and β largely reflect the tissue-level patterns described above. IL-1β is rarely expressed basally and must be induced by a stimulus (discussed in detail below). When induced, IL-1β expression is limited largely to cells of myeloid lineage such as monocytes, macrophages and microglia. However, IL-1β expression has also been detected in neurones (Watt and Hobbs, 2000), oligodendrocytes (Moyon et al., 2015), astrocytes (Lieberman et al., 1989) and epithelial cells (Waterhouse and Stadnyk, 1999) amongst others. In contrast to the inducible expression of IL-1β, IL-1α
is reported to be constitutively expressed at low levels in barrier cell types such as fibroblasts (McCarthy et al., 2013), epithelial cells (Suwara et al., 2014), keratinocytes (Kong et al., 2006) and vascular smooth muscle cells (Zheng et al., 2013). However, like IL-1β, the bulk of the studies have been performed in myeloid cells and suggest that IL-1α expression is inducible and only observed following stimulation (Hawn et al., 2002; Fettelschoss et al., 2011; Luheši et al., 2011; Rider et al., 2012).

As previously mentioned, both IL-1α and IL-1β require an independent stimulus to induce expression in the majority of cases. In most experimental models, this stimulus comes in the form of LPS. LPS is a potent endotoxin which mediates its effects via the essential component Lipid A (Darveau, 1998). LPS binds to the prototypical PRR TLR4 and co-receptors CD14 and MD2 and signals via MyD88 resulting in NFκB-dependent gene transcription as with IL-1R1 signalling described previously (Figure 1.1). Although LPS is often used to induce IL-1 expression experimentally, a step known as ‘priming’, it may not be the trigger in sterile disease. In fact, there have been numerous reported priming stimuli independent of LPS. A large class of proposed sterile priming stimuli are the endogenous TLR ligands – a group of molecules that act as DAMPs to activate TLR4 and induce inflammatory cytokine expression (Yu et al., 2010). Notable examples of endogenous TLR4 ligands include fibronectin (Okamura et al., 2001), heparan sulphate (Johnson et al., 2002), hyaluronan (Termeer et al., 2002), HMGB1 (Andersson and Tracey, 2011), S100 proteins (Vogl et al., 2007), DNA (Leadbetter et al., 2002), RNA (Karikó et al., 2004), the oxidized phospholipids (OxPL) OxPAPC (Imai et al., 2008) and OxLDL (Stewart et al., 2010), monosodium urate crystals (MSU) (Liu-Bryan et al., 2005), and aggregated species of the amyloid-β peptide (Walter et al., 2007; Reed-Geaghan et al., 2009). It should be noted, however, that the existence of these endogenous TLR4 ligands is not without controversy. In fact, there is a large body of evidence that many of these proposed ligands activate TLR4 only through endotoxin contamination (Tsang and Gao, 2004; Erridge, 2010). It remains unclear to what extent endogenous molecules can activate TLR4 but it does appear that TLR4 is activated in sterile disease and can be activated by a wide variety of molecules. Via these diverse triggers, NFκB signalling commences and IL-1 transcription is initiated. IL-1 can also be transcribed independently of TLRs. Notable examples of these pathways include the complement system (Fu et al., 2016), receptor for advanced glycation end products (RAGE) (He et al., 2012), HIF-1α (Fang et al., 2009; Palsson-McDermott et al., 2015) and ROS (Bauernfeind et al., 2011). IL-1 transcription can also be induced by proinflammatory cytokines such as TNFα (Franchi et al., 2009) or IL-1 itself (Hiscott et al., 1993), leading to an autocrine positive feedback loop which amplifies inflammation.
In their inducible forms, the molecular mechanisms determining IL-1α and IL-1β transcription have significant overlap. Extensive research carried out in the 1990s and early 2000s demonstrated the involvement of NFκB, PU.1, C/EBPβ and AP-1 for IL-1β transcription (Hiscott et al., 1993; Shirakawa et al., 1993; Tsukada et al., 1994; Kominato et al., 1995; Roman et al., 2000). More recently, a crucial role for HIF-1α has also been identified (Tannahill et al., 2013). Research into the factors controlling IL-1α expression is far less extensive. However, studies indicate that, as with IL-1β, AP-1 and NFκB are required for inducible IL-1α expression (Alheim et al., 1996; Mori and Prager, 1996) in addition to HIF-1α in some cells (Rider et al., 2012). The constitutive expression of IL-1α (see above) is believed to be regulated by the transcription factor Sp1 which binds between −52 and −45 bp at the 5’ end of the IL1A gene (McDowell et al., 2005; Enya et al., 2008). Recently, an additional mechanism regulating IL-1α expression has been discovered in the form of a long noncoding (Inc)RNA located on the anti-sense strand of the gene (Chan et al., 2015). This IncRNA - itself induced by immune stimulus - has been shown to be crucial in promoting transcription and expression of IL-1α in murine macrophages. Recently, it has also been shown that the AS-IL1α IncRNA can also be regulated at the level of secondary structure (Daniels et al., 2017) (see chapter Chapter 4). In this study, we demonstrated that CRISPR/Cas9-mediated mutation of the nuclear localisation sequence (NLS) (see section 1.3.2) on the Il1a gene led to an unexpected loss of transcription. Further investigation showed that the mutations made in the gene may have led to a change in the secondary structure of AS-IL1α and preventing IL-1α transcription. Overall, these studies demonstrate multiple layers of regulation by which IL-1 expression is controlled.

Following the described forms of IL-1 transcription both proteins are translated into 31 kDa forms consisting of a conserved signature β-trefoil fold comprising 12 anti-parallel β-strands arranged in a three-fold symmetric pattern whereupon they are distributed within the cell.

1.3.2 Subcellular localisation

Once translated, IL-1α and IL-1β differ in their patterns of subcellular distribution. Following induction of expression, IL-1β is found evenly distributed across the cytosol (Luheshi, Rothwell, et al., 2009). However, unlike IL-1β, IL-1α contains a nuclear localisation sequence (NLS) in the N-terminal pro-piece (Wessendorf et al., 1993) and thus is also found within the nucleus of the cell. NLSs are the best-understood mechanism by which cells transport cargo in and out of the nucleus. Transport through the nuclear envelope is regulated by the karyopherin-β (kapβ) family of transport receptors which target short motifs of basic amino acids (the NLS) for nuclear import (Lange et al., 2007). The NLS on pro-IL-
1α is a highly conserved classical monopartite sequence consisting of KVLKKRRL (human) and KILKKKRL (mouse) at residues 79–86 (Wessendorf et al., 1993). Although the presence of the highly conserved NLS on pro-IL-1α has been known for over 30 years, the precise role the motif plays in IL-1α secretion or signalling remains poorly understood. It has been suggested that the N-terminal pro-piece of IL-1α activates transcription of pro-inflammatory genes (Buryskova et al., 2004; Werman et al., 2004), thus maintaining an overall pro-inflammatory function. However, there is also evidence suggesting that the NLS of pro-IL-1α may be anti-inflammatory in nature. It was observed that pro-IL-1α is actively trafficked to the nucleus to dampen inflammation in apoptotic (Cohen et al., 2010) or necrotic cells (Luheshi, McColl, et al., 2009; Luheshi, Rothwell, et al., 2009).

The mechanisms that regulate nuclear trafficking of pro-IL-1α are uncharacterised. Early research suggested that changing phosphorylation states on crucial lysine residues of the NLS regulates intracellular transport (Kobayashi, Oppenheim, et al., 1990; Sung and Walters, 1993). More recent evidence has also proposed that acetylation regulated by histone deacetylase (HDAC) enzymes and binding to the nuclear shuttling protein HAX-1 (Kawaguchi et al., 2006) positively regulates nuclear redistribution (Cohen et al., 2015).

In addition to nuclear localisation, IL-1α is also found bound to the plasma membrane (Kurt-Jones et al., 1985). At the membrane, IL-1α can bind IL-1R1 on nearby cells and initiate downstream effects such as promoting cellular senescence (Orjalo et al., 2009). However, the biological significance of surface-bound IL-1α is not yet fully understood. The exact mechanism by which IL-1α is transported to the membrane is unclear, however it has been proposed that post-translational modification in the form of myristoylation (Stevenson et al., 1993) and phosphorylation (Beuscher et al., 1988) may mediate surface anchoring via a lectin-binding domain (Brody and Durum, 1989). Further research is required in order to understand the molecular mechanisms governing IL-1α translocation.

1.3.3 Processing

The regulation of IL-1 family cytokines is heavily dependent on post-translational modification, in particular, proteolytic cleavage. IL-1β is produced as an intracellular pro-form and is inactive at IL-1R1 until it undergoes processing to a 17 kDa mature form and is secreted from the cell, a process which is dependent on the formation of a multi-molecular complex called an inflammasome (Martinon et al., 2002).
1.3.3.1 Inflammasomes

Inflammasomes are large intracellular protein complexes that comprise a cytosolic PRR (also known as a ‘sensor molecule’), adapter molecule and the enzyme caspase-1 which cleaves inactive pro-IL-1β to a mature form which is secreted from the cell (Schroder and Tschopp, 2010). The best-characterised inflammasome is known as the NLRP3 inflammasome, so-called because the PRR molecule is nucleotide-binding oligomerisation domain (NOD)-like receptor, pyrin-containing 3 (Figure 1.2).

![Inflammasome structures](image)

**Figure 1.2: Inflammasome activators and structures.** Inflammasomes can form with a variety of different pattern recognition receptors (PRRs). The NLRP3 inflammasome detects ion flux and comprises a leucine rich repeat (LRR) domain, a nucleotide-binding and oligomerization (NACHT) domain and a pyrin domain (PYD). NLRP3 recruits the adapter protein apoptosis-associated speck-like protein containing a CARD (ASC) which comprises a PYD and a caspase activation and recruitment domain (CARD). ASC then bind and recruits caspase-1 (Casp 1). The NLRC4 inflammasome forms as a result of flagellin detection by NAIPs (Nod-like receptor family of apoptosis-inhibitory proteins) and comprises an LRR, NACHT and CARD domain. The NLRP1 inflammasome primarily detects anthrax lethal toxin (LT) and includes a function to find (FIIND) in addition to CARD, LRR, NACHT and PYD domains. The AIM2 inflammasome detects bacterial double-stranded DNA (dsDNA) and comprises a DNA-sensing HIN200 domain linked to a PYD. The Pyrin inflammasome forms as a result of Ras homolog gene family, member A (RhoA) inhibition and comprises a B30.2/SPRY domain (not present in mouse), central coiled-coil domain (CC), a B-box-type zinc finger domain (B-box) and a PYD.
The NLRP3 sensor molecule is made up of three domains: a pyrin death-fold domain (PYD) located at the N-terminus known to interact with homologous domains on the adapter molecule ASC (apoptosis-associated speck-like protein containing a CARD), a central NACHT domain involved in hydrolysis of ATP and protein oligomerisation, and at the C-terminus a LRR sequence which binds to the crucial regulator NIMA Related Kinase 7 (NEK7) (Schmid-Burgk et al., 2015; He et al., 2016; Shi et al., 2016). Following stimulus (see section 1.3.3.2) and formation of the inflammasome complex the adapter protein ASC is recruited to NLPR3 PRR via a PYD-PYD homotypic interaction. ASC is made up of a PYD domain and a CARD domain. Following recruitment, ASC oligomerises into filamentous structures in a prion-like manner via CARD-CARD interactions on the ASC molecules. These events (in addition to a host of post-translational modifications) lead to the formation of a macromolecular platform 1-2 µm in size, the site at which the enzyme caspase-1 is recruited and activated (Franklin et al., 2018).

Caspases are cysteine proteases which cleave a wide variety of protein substrates at a site located after an aspartic acid residue (hence the name c-asp-ase). Mammalian caspases are broadly divided into apoptotic caspases (caspase-2, 3 and 6-10) crucial for initiating apoptotic cell death, and inflammatory caspases (caspase-1, 4, 5, 11, 12) involved in processing of inflammatory cytokines including IL-1β (Man and Kanneganti, 2015). Caspase-1 (previously known as interleukin-converting enzyme, ICE) is synthesised as an inactive 45 kDa zymogen that must undergo autolytic cleavage for catalytic activity. Until recently, the p10 and p20 fragments of caspase-1 were thought to be the active subunits, acting as a tetramer (Denes et al., 2012). It is now understood, however, that the major active form of caspase-1 is the transient p33/p10 species which is further auto-processed into the inactive p10/p20 units (Boucher et al., 2018). In this way, caspase-1 activity is self-limiting.

Although inflammasome-dependent processing of IL-1β is the main focus of this thesis, inflammasome activation also leads to proteolytic processing and activation of related cytokine IL-18 (van de Veerendonk et al., 2011). Unlike IL-1β and IL-1α, IL-18 is constitutively expressed in myeloid cells (Dinarello et al., 1998, 2013). However, IL-18 also shares many similarities with these IL-1-family cytokines. It is produced as an inactive 24 kDa precursor which remains in the cytosol until caspase-1-dependent processing into a mature 18 kDa form which is active at the IL-18 receptor (Gu et al., 1997; Kaplanski, 2018). Notably, IL-18 can also be cleaved in a caspase-1-independent manner such as by neutrophil proteases (Sugawara et al., 2001) or by Fas ligand in a caspase-8-dependent manner (Bossaller et al., 2012). Once cleaved, IL-18 is released from the cell via unconventional secretion mechanisms that are heavily linked to cell death (Daniels and Brough, 2017). Upon binding
to the IL-18 receptor IL-18Rα, and recruitment of co-receptor IL-18Rβ, IL-18 induces a signalling cascade involving TIR domains and MyD88 that is almost identical to that of IL-1R-signalling (Kaplanski, 2018) (Figure 1.1). Another property shared between IL-18 and IL-1αβ is that of extracellular regulation. Similar to the function of IL-1R2 as a decoy receptor (see section 1.2), IL-18 binding protein (IL-18BP) acts to dampen IL-18 signalling (Novick et al., 1999). IL-18BP is constitutively secreted and is present in humans at a 20-fold excess compared to IL-18 (Novick et al., 2001). IL-18BP may act to blunt an overactive inflammatory response (Novick and Dinarello, 2017). Indeed, considering the implications of NLRP3-activation (and resultant caspase-1-dependent IL-18 activation) in sterile disease, far more work should be carried out to understand the role of IL-18 and IL-18BP to investigate potential therapeutics.

1.3.3.2 Inflammasome activation

The mechanisms of inflammasome activation are tightly regulated. As described previously (section 1.3.1), IL-1β must first be transcribed in a ‘priming step’ before inflammasome activation can be effective (Figure 1.1). Priming of the inflammasome involves both transcriptional and post-transcriptional steps. Transcriptional priming of NLRP3 (the production of NLRP3 mRNA) is induced by factors such as by LPS (Bauernfeind et al., 2009) and TNFα (Franchi et al., 2009). Post-transcriptional priming factors have been identified as deubiquitination (Juliana et al., 2012; Lopez-Castejon et al., 2013; Py et al., 2013), ubiquitination (Humphries et al., 2018) and interaction with adaptors TRIF and IRAK1 (Fernandes-Alnemri et al., 2013; Lin et al., 2014).

Once priming has taken place the inflammasome must be activated by ‘signal 2’ (Figure 1.3). Inflammasome activation can be initiated by a diverse range of stimuli (Table 1.1) all of which converge on a handful of non-mutually exclusive downstream events that result on the formation of the NLRP3 complex. The best evidence for a unifying molecular mechanism for NLRP3 activation is altered homeostasis of ion flux from the cell. Early studies suggested that different NLRP3 stimuli all caused K+ efflux (Perregaux and Gabel, 1994). Since then, a crucial role for K+ efflux and inflammasome activation has been further established. Pétrilli et al. (2007) showed that a decrease in K+ concentration can cause inflammasome formation in a cell-free system whilst Muñoz-Planillo et al. (2013) comprehensively demonstrated that K+ efflux is an absolute requirement for NLRP3 activation, acting downstream of almost all known NLRP3 activators. These studies, amongst many others, have demonstrated the crucial role for K+ efflux in inflammasome activation. However, K+ is not the only ion involved in NLRP3 formation; a crucial role for Cl− has also been observed.
Early work suggested that hypotonicity and cell swelling may regulate IL-1β processing (Perregaux et al., 1996). This was later confirmed by Compan et al. (2012) who showed that inflammasome activation can occur as a result of a regulatory volume decrease response (RVD) which involves both K⁺ and Cl⁻ efflux from the cell. It has long been known that non-specific Cl⁻ channel inhibitors can block IL-1β processing (Laliberte et al., 1994; Verhoef et al., 2005; Compan et al., 2012). However, the precise channels involved in regulating this efflux have only recently been identified. Firstly, Daniels et al. (2016) (see chapter Chapter 2:) demonstrated that the volume-regulated anion channel (VRAC), the molecular identity of a crucial subunit of which has been identified as LRRC8A (Voss et al., 2014) or SWELL1 (Qiu et al., 2014), is crucial for NLRP3 inflammasome formation. More recently, two groups have identified intracellular chloride channels the CLICs as key mediators of inflammasome assembly (Domingo-Fernández et al., 2017; Tang et al., 2017). It is clear that the precise interplay between intracellular levels of the two ions K⁺ and Cl⁻ is a crucial trigger of inflammasome formation. However, precisely how this interplay occurs, and the individual roles played by the two ions, remains unknown.
Table 1.1: A selection of major NLRP3 activators and the proposed mechanism of activation (there is often substantial overlap between mechanisms of activation).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Mechanism</th>
<th>References</th>
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<tbody>
<tr>
<td>Aggregated amyloid-β</td>
<td>Lysosomal Stress</td>
<td>Halle et al., 2008</td>
</tr>
<tr>
<td>Alum</td>
<td>Lysosomal Stress</td>
<td>Franchi and Núñez, 2008</td>
</tr>
<tr>
<td>ATP</td>
<td>Ion flux, P2X7</td>
<td>Hogquist et al., 1991; Mariathasan et al., 2006</td>
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<tr>
<td>Candida albicans</td>
<td>Lysosomal Stress</td>
<td>Joly et al., 2009</td>
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<tr>
<td>Cholesterol crystals</td>
<td>Lysosomal Stress</td>
<td>Duewell et al., 2010</td>
</tr>
<tr>
<td>dsDNA</td>
<td>RIPK3/Casp8/TRIF/MLKL/cGAS/STING (human cells)</td>
<td>Kang et al., 2015; Gaidt et al., 2017</td>
</tr>
<tr>
<td>Hypertonicity</td>
<td>ROS</td>
<td>Ip and Medzhitov, 2015</td>
</tr>
<tr>
<td>Hypotonicity</td>
<td>Ion flux</td>
<td>Compan et al., 2012</td>
</tr>
<tr>
<td>Islet amyloid polypeptide</td>
<td>Lysosomal Stress</td>
<td>Masters et al., 2010</td>
</tr>
<tr>
<td>Listeria monocytogenes</td>
<td>Lysosomal Stress</td>
<td>Mariathasan et al., 2006; Kim et al., 2010</td>
</tr>
<tr>
<td>MSU</td>
<td>Lysosomal Stress</td>
<td>Martinon et al., 2006</td>
</tr>
<tr>
<td>Nigericin</td>
<td>Ion flux</td>
<td>Perregaux and Gabel, 1994; Mariathasan et al., 2006</td>
</tr>
<tr>
<td>OxLDL</td>
<td>Ion flux (haemolysins)</td>
<td>Sheedy et al., 2013</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>?</td>
<td>Diamond et al., 2017</td>
</tr>
<tr>
<td>Silica</td>
<td>Lysosomal Stress</td>
<td>Dostert et al., 2008</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>Lysosomal Stress</td>
<td>Luheshi et al., 2012</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>Ion flux (haemolysins)</td>
<td>Mariathasan et al., 2006; Muñoz-Planillo et al., 2009</td>
</tr>
<tr>
<td>Viral mRNA</td>
<td>ROS</td>
<td>Allen et al., 2009</td>
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An additional ionic regulator of NLRP3 formation was suggested to be calcium. Studies using the intracellular Ca\textsuperscript{2+} chelator BAPTA-AM suggested that Ca\textsuperscript{2+} release from intracellular stores may be involved in IL-1\textbeta processing (Brough et al., 2003). Following this, pharmacological inhibition of calcium signalling using the broad-spectrum inhibitor 2-APB also suggested Ca\textsuperscript{2+} signalling may be important for inflammasome activation (Compan et al., 2012; Lee et al., 2012; Murakami et al., 2012). However, the role of Ca\textsuperscript{2+} signalling in NLRP3 activation is controversial. Muñoz-Planillo et al. (2013) suggested that inflammasome activation by calcium is a result of precipitation and activation by lysosomal stress (discussed below) and not direct Ca\textsuperscript{2+} signalling. More recently, two independent groups have dissociated the NLRP3-inhibiting effects of BAPTA-AM and 2-APB from Ca\textsuperscript{2+} signalling. Initially, Katsnelson et al. (2015) suggested that both BAPTA-AM and 2-APB inhibit NLRP3 via mechanisms independent of Ca\textsuperscript{2+} signalling. More recently, we have used a chemical synthesis approach to show that 2-APB can be modulated to inhibit NLRP3 but have no effect on Ca\textsuperscript{2+} signalling (Baldwin et al., 2017) (see chapter Chapter 3:). Interestingly, the 2-APB derivatives synthesised had ~100-fold increased potency on NLRP3 inhibition. These studies suggest that, although Ca\textsuperscript{2+} signalling may play a role in NLRP3 activation, it is not an absolute requirement and can be dispensable.

Although ion flux is emerging as a fundamental pathway by which NLRP3 is activated there are also numerous other mechanisms that may trigger inflammasome formation upstream of this event. There is substantial evidence that many inflammasome-activating stimuli function by inducing frustrated phagocytosis and lysosomal stress (Table 1.1). Following endocytosis, these particulates damage the lysosomal membrane and cause release of the lysosomal cysteine protease cathepsin B (Fujisawa et al., 2006; Chu et al., 2009). Many of the studies implicating lysosomal stress have utilised phagocytosis inhibitor cytochalasin D (Dostert et al., 2008; Masters et al., 2010), cathepsin B inhibitor CA-074-Me (Halle et al., 2008; Hornung et al., 2008; Duewell et al., 2010) or lysosomal deacidifying agent bafilomycin (Masters et al., 2010; Luheshi et al., 2012) to demonstrate the role of cathepsin B and lysosomes in NLRP3 activation. However, macrophages deficient in cathepsin B secrete similar levels of IL-1\textbeta to WT counterparts (Dostert et al., 2009), calling into doubt the specificity of CA-074-Me (Jin and Flavell, 2010). Subsequently, Orlowski et al. (2015) utilised genetic inactivation of a panel of cathepsins to show that there is high redundancy in cathepsin-induced NLRP3 activation and suggested cathepsins are also involved in the priming step of inflammasome activation (see section 1.3.1). Interestingly, the importance of phagocytosis in NLRP3 activation has also been called into doubt. An innovative study by Hari et al. (2014) used an epoxy resin to attach MSU particles to cell culture plate (thus preventing complete phagocytosis) and demonstrated that NLRP3 activation still occurred.
The group also showed that lysosome mobilisation and cathepsin B redistribution were not essential. It seems unlikely particulate matter and lysosomal stress lead to direct activation of NLRP3. In fact, it has been shown that these processes lead to K+ efflux and therefore likely lie upstream of this process in the NLRP3 activation cascade (Muñoz-Planillo et al., 2013). Thus, K+ efflux appears a unifying mechanism for NLRP3 activation. However, there is one major opponent in the competition to be crowned chief regulator of NLRP3 activation, ROS.

Figure 1.3: The key events of NLRP3 inflammasome activation. The NLRP3 inflammasome forms primarily as a result of K+ efflux. This is induced by a variety of factors. Frustrated phagocytosis of particulates such as monosodium urate (MSU), silica, alum, amyloid-β fibrils (Aβ), calcium pyrophosphate dehydrate (CPPD) crystals or bacteria leads to lysosomal stress and K+ efflux via a poorly characterised mechanism involving cathepsins and reactive oxygen species (ROS). Mitochondrial dysfunction also leads to ROS and K+ efflux. Cl− efflux can be induced via osmotic stress through the volume-regulated anion channel (VRAC) leading to inflammasome activation via an uncharacterised mechanism. Ca2+ influx caused by opening of P2X7 by ATP or other mechanisms is linked to inflammasome activation whilst the bacterial toxin nigericin directly leads to K+ efflux via exchange for extracellular H+ ions. K+ efflux leads to formation of the NLRP3 inflammasome comprising NLRP3, ASC and NIMA Related Kinase 7 (NEK7). Activated caspase-1 (Casp1) cleaves pro-IL-1β and gasdermin-D to result in pyroptosis and mature IL-1β secretion.
ROS are classical inflammatory modulators (Mittal et al., 2014) and have been implicated as a possible unifying mechanism for inflammasome activation. ROS are detected in cells stimulated with almost all known inflammasome activators (Martinon, 2010), thus raising obvious queries as to a direct role in inflammasome activation. The ROS model for inflammasome activation has been strongly researched. Dostert et al. (2008) first suggested ROS produced by the enzyme NADPH oxidase to be a unifying theory for NLRP3 activation whilst Cassel et al. (2008) showed that diphenyleneiodonium chloride (DPI), an inhibitor of NADPH oxidase, was able to block silica-induced inflammasome activation in LPS-primed macrophages. Additionally, anti-oxidant N-acetyl-l-cysteine (NAC) has also been shown to inhibit NLRP3 activation (Allen et al., 2009). The direct mechanism for ROS-induced inflammasome activation has been proposed to be through thioredoxin-interacting protein (TXNIP; also known as VDUP1). The theory states that increase in ROS triggers dissociation of TXNIP from constitutive inhibition by oxidoreductase thioredoxin thus freeing the protein to bind to and activate NLRP3 (Zhou et al., 2010).

The physiological source of ROS is suggested to be through mitochondrial stress. Zhou et al. (2011) observed NLRP3 and ASC co-localising with mitochondria under addition of inflammasome activators in a mechanism dependent on the mitochondrial voltage-dependent anion channel 1 (VDAC1). Knockdown of VDAC1 led to abrogation of both mitochondrial ROS production and IL-1β secretion in response to an assortment of inflammasome activators. This could propose ROS production by mitochondria as an essential unifying mechanism for NLRP3 activation.

There is little dispute as to ROS playing a role in inflammasome activation; however, whether ROS production is essential has not been fully accepted. Muñoz-Planillo et al. (2013) questioned whether the concentrations of ROS scavengers used in some of the studies mentioned above were pharmacologically relevant to specific target effects. The authors repeated experiments with lower concentrations known to affect redox state and found no NLRP3 inhibition. Additionally, the role for TXNIP also appears controversial as Masters et al. (2010) found IL-1β secretion from TXNIP−/− macrophages was no different to WT when stimulated with a variety of NLRP3 activators.

In addition to the major proposed molecular mechanisms for inflammasome activation above, research has also been conducted investigating the role of a number of other factors. These include cAMP (Lee et al., 2012), ER stress (Bronner et al., 2015), pH (Edye et al., 2013; Rajamäki et al., 2013), autophagy (Harris et al., 2017) and metabolic stress (Tannahill et al., 2013). It is likely however that all of these events occur upstream of K+ efflux or ROS induction.
Despite hot debates over the search for a unifying mechanism it is perhaps far more likely that inflammasome activation occurs by a number of mechanisms dependent on both temporal and spatial factors. It still remains a major objective of the inflammasome field to fully define these mechanisms.

1.3.3.3 Other inflammasomes

Although NLRP3 remains the best-characterised PRR it is by no means the only sensor capable of forming an inflammasome complex. There are no less than 22 different inflammasome genes in human (34 in mouse), all of which contain a PRR designed to sense DAMPs and/or PAMPs (Lamkanfi and Dixit, 2012). However, only a handful of these have been studied in any depth (Figure 1.2).

Aside from NLRP3, the best studied inflammasomes are nucleotide-binding oligomerisation domain-like receptor, CARD-containing 4 (NLRC4) and absent in melanoma 2 (AIM2). The NLRC4 molecule has a similar structure to NLRP3 but with a CARD domain rather than a PYRIN. Although it is not essential (as the CARD domains on caspase-1 could interact directly with the PRR), ASC is utilised in NLR4 inflammasome formation though CARD-CARD and PYRIN-PYRIN interactions (Latz et al., 2013). The NLRC4 inflammasome is classically activated following detection of bacterial flagellin and type 3 secretion systems (T3SS) (Schroder and Tschopp, 2010). Unlike NLRP3, a direct ligand has been described for NLRC4. The NAIP family of proteins (Vance, 2015) act as adapter proteins between flagellin or parts of the T3SS and are crucial for formation of the inflammasome complex (L. Zhang et al., 2015; Tenthorey et al., 2017). In this way, the NAIPs actually act as the sensors with NLRC4 acting as an adapter. Early work on NLRC4 comprised largely of studies in mice, which possess multiple copies of the NAIP genes. However humans have only a single NAIP protein which is sufficient for detection of cytosolic flagellin and potent activation of the NLRC4 inflammasome (Kortmann et al., 2015). Additionally, gain of function mutations in the NLRC4 gene in humans lead to autoinflammatory diseases such as Infantile Enterocolitis and recurrent Macrophage Activation Syndrome (Canna et al., 2014; Romberg et al., 2014; Moghaddas et al., 2018). Interestingly, these diseases present rather differently to the FMF diseases and can be characterised by chronic elevation of free IL-18 (Duncan and Canna, 2018). Indeed, therapy with IL-18 binding protein can be effective (Novick and Dinarello, 2017).

Some inflammasome PRRs can directly sense ligands. The AIM2 inflammasome utilises a hemopoietic expression, interferon-inducibility, nuclear localization (HIN) domain in the place of NLR which can directly bind dsDNA in the cytosol (Latz et al., 2013). Following
binding, the DNA acts as the oligomerisation platform for the AIM2 inflammasome (Jin et al., 2012). The AIM2 inflammasome is expressed in human cells and can be activated in certain conditions (Zhang et al., 2017). However, recent evidence suggests that DNA transfection into human myeloid cells, a canonical method for activating AIM2, actually leads to NLRP3 activation via the cGAS-STING axis and is independent of AIM2 activation (Gaidt et al., 2017). Further research is required to understand the role of AIM2 in human cells.

The Pyrin inflammasome is formed dependent on the pyrin proteins, coded for by the MEFV gene (mutations in which lead to autoinflammatory disease (Milhavet et al., 2008)). The pyrin protein consists of an N-terminal PYD (not unlike NLRP3), B-box zinc binding domain, a central coiled coil domain and a B30.2/SPRY domain at the C-terminus (de Torre-Minguela et al., 2017). Outside of autoinflammatory activation by gene mutations, the pyrin inflammasome is activated following inhibition of RhoA GTPases by bacteria (Xu et al., 2014) in a mechanism dependent on the release of inhibitory phosphorylation by 14-3-3 proteins (Gao et al., 2016; Park et al., 2016).

NLRP1 was the first PRR described to form an inflammasome (Martinon et al., 2002) and comprises a similar structure to NLRP3 but with the addition of a FIIND and CARD domain at the N-terminus. Studies on patients with rare autoinflammatory skin diseases have shown that activation of NLRP1 is dependent on the autolytic cleavage of the FIIND domain and regulated by an autoinhibitory pyrin domain (Yu et al., 2018). NLRP1 is activated by anthrax lethal toxin however the physiological activator of NLRP1 in humans is yet to be fully characterised. Part of the reason for the lack of understanding of NLRP1 in humans is the existence of multiple differences between human and mouse. Whilst there are three paralogs of the Nlrp1 gene in mice (Nlrp1a, b, c) there is only one in human (Chavarría-Smith and Vance, 2015). Additionally, activators of mouse NLRP1B, such as the lethal factor of anthrax lethal toxin do not necessarily activate human NLRP1 (Moayeri et al., 2012). Although endogenous NLRP1 activators in humans may not be clear the role in autoinflammatory disease is well-defined as multiple mutations in the NLRP1 gene have been characterised that cause overactivation and hyperinflammation (Zhong et al., 2016; Grandemange et al., 2017).

There also exist a number of alternative signalling mechanisms for NLRP3. The non-canonical pathway is reliant on the detection of intracellular LPS by caspase-11 independent of TLR4 (Kayagaki et al., 2011, 2013). Additionally, an ‘alternative’ inflammasome pathway has been proposed involving NLRP3 activation via TLR4-TRIF-RIPK1-FADD-CASP8 independently of K⁺ efflux (Gaidt et al., 2016). These extensive
variations on inflammasome complexes demonstrate the extent of the fine-tuned regulation of caspase-1-dependent IL-1β processing.

1.3.3.4 IL-1α

IL-1α regulation also occurs at the level of proteolytic processing (Figure 1.4). However, the precise mechanisms of IL-1α processing are poorly understood in comparison to IL-1β and inflammasomes.

**Figure 1.4: IL-1α processing and secretion.** Pro-IL-1α is present in the nucleus where is may be involved in gene transcription. Pro-IL-1α is transported in and out of the nucleus via poorly-understood mechanisms involving phosphorylation and acetylation. In the cytosol, pro-IL-1α binds IL-1 receptor 2 (IL-1R2) which prevents processing until NLRP3 inflammasome activation allows caspase-1 (Casp1) mediated cleavage. Pro-IL-1α is processed into mature IL-1α by calpain enzymes in a mechanism dependent on Ca²⁺ influx and by granzyme B or neutrophil elastase. Following processing, IL-1α is secreted from the cell via unconventional mechanisms possibly dependent in gasdermin-D (GSDMD) and membrane rupture. Pro-IL-1α can also leave the cell and is found present as a membrane bound cytokine.
There is evidence that processing is not required for IL-1α activity and that the 31 kDa pro-form of the cytokine is fully active at the IL-1R1 receptor (Mosley et al., 1987; Gross et al., 2012; Kim et al., 2013). However, contrasting research has suggested that, although pro-IL-1α may be active, proteolytic processing can increase biological activity (Afonina et al., 2011; Zheng et al., 2013). Although more research is required to fully understand the contribution of processing to activity, these data suggest that cleavage of pro-IL-1α into its mature form is indeed an important step in the regulation of IL-1.

IL-1α can be processed by many enzymes. However, the best studied of these are those of the calpain family (Kobayashi, Yamamoto, et al., 1990; Carruth et al., 1991; Afonina et al., 2011; Gross et al., 2012; Edye et al., 2013; Zheng et al., 2013; Fang et al., 2017). Calpains, so called due to their similarity to the calcium-related signalling protein calmodulin and the papaya cysteine protease papain, are non-lysosomal cysteine proteases with a diverse repertoire of functions. There are 14 members of the calpain family in humans although by far the best-studied members are μ- and m-calpain (calpains 1 and 2), so called as they require 5–30 micromolar or millimolar Ca^{2+} for activation respectively (Goll et al., 2003). It is currently unknown which of the major members of the calpain family are responsible for IL-1α processing. The calpain system also possesses an endogenous inhibitor calpastatin (Hanna et al., 2008), thus demonstrating further regulation of IL-1α.

As previously mentioned, IL-1α can also be processed by a number of other enzymes. The serine protease granzyme B, released from cytotoxic lymphocytes and natural killer cells, can cleave pro-IL-1α at site Asp103 whilst processing has also been observed dependent on neutrophil elastases and mast cell chymases (Afonina et al., 2011). Although caspase-1 cannot directly cleave IL-1α it is involved in activity, Gross et al. (2012) demonstrated that certain stimuli require caspase-1 and other inflammasome components to induce IL-1α processing and secretion. Further to this, Zheng et al. 2013 discovered the mechanism by which caspase-1 is required for IL-1α processing. Zheng and colleagues demonstrated that IL-1α in macrophages exists bound to the cytosolic IL-1 receptor 2 (IL-1R2) which prevents calpain-mediated cleavage. Caspase-1 activation is required to release IL-1α from IL-1R2 and allow calpain-mediated processing.

1.3.4 Secretion

Following transcription, translation, and post-translational modification in the form of proteolytic processing, IL-1 is secreted from the cell, a process which is poorly understood. Neither IL-1α nor IL-1β possess a leader sequence required for secretion through the ER/Golgi pathway, both are therefore secreted through unconventional mechanisms.
(Daniels and Brough, 2017) (see appendix 1). The secretion of both IL-1α and IL-1β is tightly coupled to cell death.

1.3.4.1 Pyroptosis

Pyroptosis is a form of programmed cell death dependent on the inflammatory caspases 1 and 11 (or 4 and 5) and was initially described in the microbiology field as a form of inflammatory programmed cell death undergone by macrophages infected with salmonella (Cookson and Brennan, 2001). The name pyroptosis is derived from the Greek ‘pyro’, relating to fire or fever, and ‘ptosis’, meaning a falling and was termed as such because pyroptotic cells (unlike apoptotic cells) released inflammatory (fever-inducing) cell contents (Cookson and Brennan, 2001). Pyroptosis has therefore evolved as an innate immune response against intracellular infection. Pyroptotic death not only leads to exclusion of bacteria from the protective intracellular niche (they are immobilised in pore-induced intracellular traps (PITS) (Jorgensen et al., 2016)) but also the induction of a rapid innate immune response by secretion of proinflammatory cytokines. Pyroptosis has thus emerged as the major mechanism by which mature IL-1β is secreted from cells.

Although inflammasome activation, pyroptosis and IL-1β secretion were known to be intrinsically linked the exact process by which IL-1β secretion took place remained a mystery until 2015 when two groups identified the pore-forming protein gasdermin-D (GSDMD) (Kayagaki et al., 2015; Shi et al., 2015). It is now understood that the caspase-mediated N-terminal cleavage product of GSDMD translocates to the plasma membrane and forms 10–14 nm pores in the membrane to induce pyroptosis and IL-1β secretion (Figure 1.3) (He et al., 2015; Ding et al., 2016; Liu et al., 2016). Until recently it was assumed that GSDMD was required for pyroptosis and IL-1β secretion. However, multiple groups have discovered that GSDMD can mediate IL-1β secretion independent of pyroptotic cell death (DiPeso et al., 2017; Evavold et al., 2017; Heilig et al., 2017). There has also been suggestion of a mechanism of IL-1β secretion independent of GSDMD dependent on caspase-8 (Schneider et al., 2017) although this pathway is yet to be fully elucidated.

Gross et al. (2012) reported that pyroptosis also induces IL-1α secretion. The link between GSDMD and IL-1α secretion has been poorly explored. However, NLRP3-activating S. aureus mutants do induce IL-1α secretion in a GSDMD-dependent manner (Evavold et al., 2017). Although most evidence points to the fact that IL-1 is directly secreted through the GSDMD pore, this has not conclusively been shown (Brough et al., 2017). In fact, it remains to be demonstrated exactly how GSDMD pores contribute to the pyroptotic secretion of IL-1α or IL-1β.
1.3.4.2 **Autophagy**

Autophagic cell death is a form of programmed cell death characterised by the intracellular homeostatic mechanism of autophagy. Autophagy is a process by which cells target certain contents for recycling and begins with the formation of a double-membraned autophagosome which can fuse to lysosomes to initiate degradation of the contents (Harris et al., 2017). Autophagy has been implicated in IL-1 regulation/secretion via numerous different mechanisms.

Early studies suggested that inhibition of autophagy leads to accumulation of intracellular ROS and thus activation of NLRP3 by the ROS pathway (Nakahira et al., 2011; Zhou et al., 2011) (see section 1.3.3.2). Harris et al. (2011) also demonstrated that autophagy led to degradation of pro-IL-1β and thus limited signalling in addition to inhibition of NLRP3 via ROS. Additionally, Shi et al. (2012) showed that inflammasome activation induces autophagy and that this process is crucial for degradation of inflammasomes, thus autophagy forms a vital negative feedback loop in IL-1 regulation. However, the role for autophagy as an inhibitor of IL-1 signalling is controversial. Two independent groups have suggested that IL-1β may be secreted in a manner dependent on autophagy, a process which may be a key mechanism by which unconventionally secreted proteins leave the cell (Dupont et al., 2011; M. Zhang et al., 2015). Overall, the role for autophagic death in IL-1β secretion remains unclear.

IL-1α secretion is also linked to autophagy, although the evidence for this is limited. ATG5 mutant mice (which cannot undergo autophagy) exhibit increased IL-1α processing and secretion (Castillo et al., 2012). These data suggest that autophagy is required to provide tonic inhibition of IL-1α and thus is a key regulatory mechanism behind IL-1 signalling.

1.3.4.3 **Necrosis**

IL-1 secretion can also occur following the rapid, un-programmed cell death known as necrosis (Martin, 2016). Cullen et al. (2015) showed that the onset of inflammasome activation and IL-1β secretion correlates with that of necrosis and that the cell death induced by a diverse range of stimuli is independent of NLRP3, ASC or caspase-1. Further studies have also demonstrated that IL-1β secretion occurs immediately following permeabilisation of the cell membrane (Martín-Sánchez et al., 2016). The findings from the study performed by Cullen et al. (2015) are surprising, with the majority of GSDMD studies suggesting that caspase-1 is indeed essential for cell death (Kayagaki et al., 2015; Shi et al., 2015). The exact conditions that contribute to the necrotic release of IL-1β rather than pyroptotic death
are yet to be defined. It is also unclear how GSDMD pores contribute to necrotic IL-1β secretion.

Necrotic cell death is perhaps more closely linked with IL-1α secretion. Concomitant with its diverse cellular expression pattern, nuclear localisation and (albeit weak) activity as a pro-form IL-1α is often termed a ‘prototypical DAMP’ (Martin, 2016; Daniels and Brough, 2017). Thus, similar to other DAMPs such as HMGB1 and IL-33, secretion of IL-1α can be dependent on necrosis. Two independent groups have demonstrated that IL-1α is released during necrosis and contributes to neutrophil infiltration in vivo (Eigenbrod et al., 2008; Cohen et al., 2010) whilst Chen et al. (2007) observed a strong IL-1α-dependent response following intraperitoneal injection of necrotic lysates. It is currently not understood whether the IL-1α released from necrotic cells is the 31 kDa pro or the 17 kDa mature form. In contrast to these studies, it has also been suggested that IL-1α is secreted not from necrotic cells but from host macrophages which detect dying cells (Kono et al., 2010). However, the exact pathway by which necrotic cell lysate induces IL-1α secretion is currently unknown.

1.3.4.4 Necroptosis

Necroptosis is a form of programmed necrosis independent of caspases but dependent on receptor-interacting serine-threonine kinase 3 (RIPK3) and mixed lineage kinase domain-like (MLKL) (Galluzzi et al., 2017). Necroptosis may have evolved as a back-up cell death program to counteract caspase-8 inhibitors employed by microbes to evade apoptotic death (Kaiser et al., 2013) and, as such, can be induced by inhibition of caspases.

Inducing necroptosis leads to the release of numerous inflammatory cytokines including IL-1β (Robinson et al., 2012; Gaidt et al., 2016). However, it has also been reported that inflammasome activation and IL-1β release can occur dependent on RIPK3 and RIPK1 but independent of necroptosis (Vince et al., 2012; Kang et al., 2015). Additionally, England et al. (2014) demonstrated that necroptosis did not induce IL-1β secretion but did induce processing and release of IL-1α from BMDMs. These studies suggest that the type of cell death may play a key role in discriminating between IL-1α and IL-1β secretion. Consistent with its role as an early-stage mediator of inflammation, IL-1α is rapidly secreted primarily as a result of caspase-independent necrotic or necroptotic cell death. IL-1β on the other hand is secreted following programmed pyroptotic death and mediates the later stages of inflammation.
Single-cell studies from multiple labs have shown that caspase-1 activation and IL-1β secretion are intrinsically linked to loss of membrane integrity (Liu et al., 2014; Martín-Sánchez et al., 2016). However, dependent on the cell type and stimulus, IL-1 can also be secreted from live cells.

Multiple groups have demonstrated that human primary monocytes cleave and secrete IL-1β in response to LPS (Viganò et al., 2015; Gaidt et al., 2016) or S. typhimurium (Diamond et al., 2017) in the absence of detectable cell death. Neutrophils are also capable of inflammasome activation independent of cell death. Chen et al. (2014) first demonstrated that activation of the NLRC4 inflammasome in neutrophils induces IL-1β release independent of pyroptosis, while Karmakar et al. (2016) observed the same phenomenon with NLRP3.

There is very little evidence separating IL-1α secretion from cell death. This is consistent with the theory that IL-1α acts primarily as a DAMP, released following loss of membrane integrity (Martin, 2016). There is evidence, however, that IL-1α secretion precedes cell death (Gross et al., 2012) and that secretion can occur at a basal (but very low) level in macrophages (Chen et al., 2007) or aged fibroblasts (Kumar et al., 1992). Interestingly, although IL-1α secretion rarely occurs without cell death, it can be expressed on the membrane and signal through paracrine mechanisms (Orjalo et al., 2009). IL-1α can then be secreted following inflammasome activation in an IL-1β dependent manner (Fettelschoss et al., 2011).

It is clear that IL-1 secretion is tightly regulated by cell death with the cell type, stimulus and timing acting as crucial factors in that regulation. IL-1β can be secreted in a manner dependent or independent of cell death. The physiological explanation for this is not yet known. One theory is that, upon infection, resident sentinel cells (macrophages and dendritic cells) undergo pyroptosis in order to rapidly recruit further cells to the site of injury and expose the invading pathogen to the extracellular environment. Meanwhile, infiltrating neutrophils and monocytes must continue cytokine secretion to propagate the immune response but need not undergo pyroptosis.

IL-1α secretion has a very different pattern to IL-1β. As a classical DAMP/alarmin, the secretion of IL-1α occurs largely as a result of unprogrammed cell death. However, IL-1α is also secreted in pyroptosis with IL-1β. The key to understanding the mechanisms of IL-1α may rely on processing – the early necrotic secretion of IL-1α may be in the unprocessed form to invoke a rapid immune response. Pyroptotic release of cleaved IL-
1α may then occur to amplify this response. Far more research is required to fully understand the underlying regulation of IL-1α secretion.

1.4 IL-1/Inflammasomes in disease

As previously mentioned, dysregulation of IL-1 and inflammasome pathways are heavily implicated in sterile inflammation and non-communicable disease. This section will summarise some of the major diseases in which the pathology is thought to be driven by the IL-1/inflammasome axis.

1.4.1 CAPS and other hereditary diseases

Cryopyrin-associated periodic fever syndromes (CAPS) are a rare family of autosomal-dominant disorders that include familial cold urticaria syndrome (FCAS), Muckle–Wells syndrome (MWS), and neonatal onset multisystemic inflammatory disease (NOMID) (de Torre-Minguela et al., 2017). These syndromes, clinical features of which include fever, chronic pain, fatigue and elevated cytokine and inflammasome component levels in the blood (Baroja-Mazo et al., 2014) all result from gain-of-function mutations in the NLRP3 gene and constitutive inflammasome hyperactivation (Hoffman et al., 2001). CAPS have also been studied in vivo. Mouse studies using genetic knock-in of CAPS mutations revealed further details to the CAPS diseases such as the crucial role for the microbiota (Nakamura et al., 2012), a Th17-dominated neutrophilia (Meng et al., 2009), and apparent redundancy of the adaptive immune system in disease pathogenesis (Brydges et al., 2009). In keeping with clinical and non-clinical research, CAPS-patients respond well to anti-IL-1β therapies such as anakinra (recombinant IL-1Ra) (Hawkins et al., 2004; Goldbach-Mansky et al., 2006) (see section 1.5), demonstrating the non-redundant role played by IL-1 signalling in disease pathology.

Although often only affecting a small number of families, rare hereditary diseases often offer a valuable insight into physiological function. A recent study identified a novel mutation in MEFV, the gene coding for the pyrin inflammasome, leading to a systemic inflammatory disease termed pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND) (Masters et al., 2016). The cause of this disease, a S242R substitution in pyrin, leads to decreased binding of the regulatory protein 14-3-3 and led to constitutive activation of the pyrin inflammasome. This has also recently been observed in a novel mutation E244K (Moghaddas et al., 2017).
1.4.2 Stroke

Stroke is a severe condition caused by dysregulation of blood flow to the brain. There are two main types of stroke; ischaemic (caused when a blockage leads to lack of blood flow) or haemorrhagic (caused by bleeding in the brain). Stroke is a leading cause of disability in the UK and the combination of medical and social care costs approximately £1.7 billion annually (Stroke Association). The only effective therapies for stroke are clot-busting drugs such as rTPA which must be administered within hours of stroke onset and can only be given to treat ischemic strokes. It is now understood that inflammation, and IL-1 plays a role in stroke pathology (Sobowale et al., 2016).

Early evidence of a role for IL-1 in stroke came from rat studies in the 1990s, demonstrating that injection of recombinant IL-1Ra was protective in a middle cerebral artery occlusion (MCAo) model of ischaemic stroke (Relton and Rothwell, 1992; Garcia et al., 1995). Further studies demonstrated that genetic knockout of IL-1α and IL-1β protects against MCAo-induced damage in mice (Boutin et al., 2001), suggesting some level of redundancy and compensation between the IL-1-family cytokines. A recent cross-laboratory study demonstrated that, across five individual centres and eight separate individual studies, IL-1Ra administration can be effective in treating experimental stroke with mice showing improvements in lesion volume, neurological deficits and behavioural outcomes compared to vehicle (Maysami et al., 2016). There is also strong preclinical evidence for a role of IL-1 in haemorrhagic stroke. Rat studies demonstrated that IL-1β neutralisation reduces the histological and inflammatory hallmark of stroke in models of subarachnoid haemorrhage (SAH) (Jedrzejowska-Szypulka et al., 2009; Larysz-Brysz et al., 2012) whilst Greenhalgh et al. (2012) suggested IL-1Ra-mediated protection in SAH models may occur via inhibition of IL-1α signalling. In mice, the caspase-1 inhibitor Ac-YVAD-CMK was protective against neurological deficits induced by perforation of the anterior cerebral artery (Sozen et al., 2009).

Considering the clear role for IL-1 in stroke it could be hypothesised that inflammasomes may also be involved in stroke pathology (Barrington et al., 2017). Indeed, genetic knockout of the adapter protein ASC was protective in the MCAo model in mice (Denes et al., 2015). Surprisingly, this effect was not mediated through the NLRP3 PRR but NLRC4 and AIM2, suggesting for the first time that these inflammasomes may play a role in sterile inflammation. There is also evidence for aberrant inflammasome activation in haemorrhagic stroke. Blood breakdown products such as haem have been shown to activate NLRP3 (Dutra et al., 2014) whilst it is also known that cell swelling, possibly as a result of oedema following haemorrhage, can induce inflammasome activation (Compan et al., 2012).
Following on from extensive preclinical and clinical characterisation of the inflammatory response to stroke, clinical trials have been undertaken to assess the efficacy of IL-1Ra as a therapeutic treatment (see section 1.5.1). Small phase II clinical trials on both ischaemic and haemorrhagic stroke have been carried out, all showing IL-1Ra to be safe and exhibit relevant biological activity in the form of reduced inflammation (Emsley et al., 2005; Singh et al., 2014) and multiple trials are ongoing with primary outcomes to assess reduction in serum IL-6 and secondary outcomes to assess clinical outcomes (ISRCTN74236229, ISRCTN25048895). Together, these preclinical and clinical data suggest that IL-1 is a major driving factor in the pathology of stroke and therefore represents an attractive target for much-needed novel therapeutic strategies.

1.4.3 Alzheimer’s disease

Alzheimer’s disease is a chronic neurodegenerative disorder resulting in neuronal death and classical symptoms of memory loss, confusion, delusions, and aggression. Alzheimer’s currently affects approximately 50 million people globally. However, due to a rapidly aging population that number is set to inflate to over 150 million by 2050 (WHO Dementia Fact Sheet, 2017). There is currently no disease modifying treatment for Alzheimer’s disease. The only approved drugs are cholinesterase inhibitors such as donepezil, which can only modify disease symptoms. Although initially thought to be largely redundant, it is now clear that inflammation/IL-1/inflammasomes play a major role in the developing pathology of AD and may therefore offer a possible target pathway for novel therapeutics (Heneka, 2017; White et al., 2017).

Genome-wide association studies (GWAS) indicate that over half of the genes with which variations flag as a significant risk for AD are related to immune activation (Lambert et al., 2013). Additionally, PET studies using tags recognising the translocator protein TSPO, present on activated microglia, demonstrate that microglial activation is far greater in AD patients compared to healthy age-matched controls and that the degree of activation correlates with AD severity (Cagnin et al., 2001; Yasuno et al., 2012; Zimmer et al., 2014).

There is strong evidence for a role of NLRP3 in AD. One of the pathological hallmarks of AD, misfolded amyloid-β protein can activate the inflammasome in vitro (Halle et al., 2008). Furthermore, Heneka et al. (2013) crossed the APP/PS1 mouse model of Alzheimer’s disease with mice lacking NLRP3. APP/PS1/NLRP3−/− mice not only had reduced inflammation but were also completely protected from AD-associated memory deficits. Interestingly, APP/PS1/NLRP3−/− mice also had reduced plaque burden compared to APP/PS1 counterparts, potentially as a result of increased microglial clearance. This implies
that IL-1 and NLRP3 somehow prevent protective microglial function. Since this seminal paper, multiple groups have demonstrated that either genetic or pharmacological inhibition of inflammasomes could be protective models of AD. Couturier et al. (2016) demonstrated heterozygous ASC knockout mice crossed with 5xFAD model mice were protected from memory deficits and displayed increased astrocyte phagocytic activity. Interestingly there was no difference between ASC+/− and ASC−/− mice suggesting that some inflammasome activation or IL-1β production may be beneficial. Alternatively, ASC may have protective, non-inflammasome functions. Moreover, Daniels et al. (2016) showed that NSAIDs of the fenamate class were NLRP3 inhibitors and were effective at reversing memory deficits and neuroinflammation in the 3xTg mouse model of AD (see chapter Chapter 2:) whilst Dempsey et al. (2017) injected APP/PS1 mice intraperitoneally with the specific NLRP3 inhibitor MCC950 (see section 1.5.3) (Coll et al., 2015) and observed improved neuronal function and neuroinflammation.

There is also evidence for NLRP3 mediating AD in humans. In a recent study, Venegas et al. (2017) showed that ASC specks can be found in the core of amyloid-β plaques in human brains and that these specks can cross-seed Aβ plaques. Importantly, injection of anti-ASC antibodies into brains of APP/PS1 mice reduces Aβ deposition. This study suggests that extracellular ASC (Baroja-Mazo et al., 2014; Franklin et al., 2014) may propagate the spreading and deposition of Aβ plaques and drive AD pathology, therefore implying that NLRP3-mediated pathology of AD in previous models is dependent not on IL-1 but on the ASC speck itself. A valuable follow-up experiment would be to assess differences between IL-1β+/− or IL-1R1−/− mice compared to NLRP3−/− ASC−/− mice in AD models.

Although poorly understood in AD compared to IL-1β, there is some evidence that aberrant IL-1α signalling could be involved in AD pathology. Early studies have suggested that IL-1α polymorphisms may be associated with AD (Nicoll et al., 2000) and that IL-1α expression in microglial cells may correlate with plaque distribution (Sheng et al., 1995). However, polymorphism association is disputed (Serretti et al., 2009) and there has been no major observation of any overexpression of IL-1α mRNA. One possible explanation for this discrepancy would be that IL-1α may be found in its processed form in AD. It is known that AD brains undergo high levels of excitotoxicity and extracellular calcium (Mattson and Chan, 2003). It is also known that calpains are hyper activated in AD brains (Saito et al., 1993; Jin et al., 2015). It is therefore possible that increased calcium concentrations in AD leads to hyper activation of calpain enzymes which process pro-IL1α into more potent mature IL-1α driving disease pathology. However, there is currently no empirical evidence to suggest IL-1α is processed or plays any major role in driving the pathology in AD.
1.5 Targeting IL-1 therapeutically

It is now evident that aberrant IL-1 regulation can be extremely damaging and may drive pathology in a number of diseases. The development of IL-1/inflammasome inhibiting strategies is therefore a major focus in drug development for these diseases (López-Castejón and Pelegrín, 2012; Baldwin et al., 2015). In this section I will outline the ways in which the IL-1 pathway has been targeted both in both clinical and preclinical studies (Figure 1.5).
Figure 1.5: Targeting IL-1 regulation. IL-1 processing/signalling can be targeting at a number of levels. Ion flux can be targeted by Cl⁻ channel-inhibitors such as fenamate non-steroidal anti-inflammatory drugs (NSAIDs), 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) and 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB). High extracellular K⁺ prevents efflux and calcium chelators ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and ethylenediaminetetraacetic acid (EDTA) inhibit Ca²⁺ influx. Oxidised ATP (OxATP), apyrase, Mg²⁺ ions and A74003 block ATP-induced P2X7 signalling whilst glycine and punicaligin appear to insulate cells and prevent membrane rupture. IL-1 processing can also be blocked inside the cell by impairing phagocytic activity with cytochalasin D or bafilomycin A or by blocking cathepsin activity with Ca-074 Me. Anti-oxidants such as diphenyleneiodonium chloride (DPI), N-acetyl-cysteine (NAC) or ascorbate inhibit ROS whilst 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis acetoxyethyl ester (BAPTA-AM) and 2-aminoethoxydiphenyl borate (2-APB) prevent intracellular Ca²⁺-signalling. NLRP3 activation can be blocked via unknown mechanism with compounds such as MCC950, NBC6, CY-09, parthenolide, Bay-11, 3,4-methylenedioxy-β-nitrostyrene (MNS), glyburide and β-hydroxybuturate (which also blocks K⁺ efflux). Modulators of inflammasome post-translational modification such as deubiquitinase inhibitors eyarestatin and b-AP15 and phosphatase inhibitor okadaic acid also prevent NLRP3 activation. Caspase-1 can be inhibited by drugs such as VX-765, YVAD and ritonavir whilst the calpain enzymes can be blocked with ABT-957 or calpain-inhibitor III. Once secreted, IL-1 signalling can be targeted with antibody therapies such as canakinumab (anti-IL-1β) or MABp1 (anti-IL-1α).
1.5.1 Anti-IL-1 therapies

The earliest methods for inhibiting IL-1 signalling came before the discovery of the inflammasome complex. As previously mentioned, anakinra is effective in a number of disease models (Cavalli and Dinarello, 2015). Anakinra, trade name Kineret, is currently licensed to treat RA and is currently in clinical trials to treat a number of diseases including stroke (as mentioned previously), pulmonary arterial hypertension (NCT03057028), traumatic brain injury (TBI) (NCT02997371) and Still’s disease (NCT03265132). Unfortunately, the very property that makes anakinra such a safe therapy (IL-1Ra is already present endogenously) impairs its use long term as it is readily broken down and thus must be injected daily (Granowitz et al., 1992).

In addition to targeting the IL-1 receptor there are also therapies that target the cytokine itself. Rilonacept acts as an IL-1 trap and is able to neutralise IL-1α and IL-1β in the circulation thus preventing binding to IL-1R1 (Moltó and Olivé, 2010). Specific anti-IL-1β therapies are also available. Canakinumab is a fully humanized anti-IL-1β monoclonal antibody and has been approved for the treatment of CAPS (Feist and Burmester, 2010). Canakinumab is also in clinical trials for other inflammatory diseases. Recently, the results of the CANTOS phase III trial showed that IL-1β neutralisation led to 25% reduction in the risk of major adverse cardiovascular events and an incredible 77% reduction in lung cancer mortality in patients enrolled in the trial (Ridker, Everett, et al., 2017; Ridker, MacFadyen, et al., 2017). These results demonstrate that anti-IL-1 therapies could be effective in numerous inflammatory diseases. However, antibody and protein therapies can be plagued by drawbacks such as poor biological availability, expensive formulation and suboptimal administration routes (Chames et al., 2009). Thus, small molecule therapies are in development.

1.5.2 Caspase-1 inhibitors

Upstream of IL-1β secretion is caspase-1, thus a number of drugs have been developed to inhibit this enzyme. The specific inhibitory peptide Ac-YVAD-CHO, used in countless in vitro studies to evaluate involvement of caspase-1 in inflammation, has been used in vivo and shown to be protective in a rat endotoxemia model (Boost et al., 2007). In addition to this, an anti-HIV drug ritonavir has been shown to inhibit caspase-1 (Kast, 2008) and an orally administered pro-drug VX-765 is currently in development to selectively inhibit caspase-1 for treatment of CAPS (Wannamaker et al., 2007).
1.5.3 Inflammasome formation

Further upstream of caspase-1 is the inflammasome complex itself. Direct inhibition of the inflammasome assembly is an attractive target for a number of reasons. Generalised anti-inflammatory drugs have proved ineffective in acute brain injury (Czekajlo and Milbrandt, 2005) suggesting a more specific inhibition of only the most damaging parts of the inflammatory response is required. Caspase-1 inhibition remains a questionable pathway as the enzyme targets hundreds of other proteins and may be heavily involved in membrane repair (Sollberger et al., 2014). Thus, direct inflammasome-binding drugs may selectively dampen the hugely damaging process of pyroptosis in sterile inflammation with a promising side-effect profile.

The NFκB pathway-inhibiting drugs Bay 11-7082 and parthenolide have recently been proposed to block inflammasome formation in a mechanism independent of NFκB (Juliana et al., 2010). The authors show that both drugs abrogate caspase-1 activity and IL-1β release from macrophages when added after the LPS priming step. Interestingly, the authors also found that both compounds were able to inhibit ASC oligomerisation in a mechanism proposed to be through prevention of NLRP3 ATPase activity although whether this is through direct binding remains unknown.

Recent findings have also shown that deubiquitination plays a key role in activation of the NLRP3 inflammasome (Juliana et al., 2012). Consequently it has been found that a panel of deubiquitinase-inhibiting drugs were able to inhibit inflammasome activity by preventing ASC speck formation (Lopez-Castejon et al., 2013). This discovery provides an interesting insight as to the mechanism behind inflammasome activation and may reveal a future potential therapeutic target for inflammasome-dependent disease. However, considering the importance of ubiquitination pathways in physiological cell biology, pharmaceutical pursuit of this avenue may be challenging.

In addition to currently available compounds found to possess anti-inflammasome properties, there has also been activity to synthesize novel, specific inflammasome inhibitors. The cytokine release inhibitory drugs (CRIDs) are small molecule compounds originally developed by Pfizer. One of these compounds, CRID3, has been shown to inhibit both the NLRP3 and AIM2 inflammasomes by preventing ASC speck formation (Coll et al., 2011). CRID3 was recently rebranded as MCC950, a drug with nanomolar potency on inflammasome activation and selective only to NLRP3 (Coll et al., 2015). The molecular target of MCC950 is currently unknown. However, the drug has no effect on K+ efflux suggesting it must bind downstream of this. Target identification of CRID3 found preferential
binding to glutathione S-transferase (GST) Omega (Laliberte et al., 2003) however the precise role of this protein in inflammasome activation is unknown. Recently, a novel chemical was identified that inhibits NLRP3 via direct binding to the NACHT domain. Jiang et al. (2017) used the CFTR inhibitor CY-09 to selectively inhibit NLRP3 via inhibition of ATPase activity.

1.5.4 Modulators of ion transport

Considering the strong position held by the K⁺ efflux theory as a primary mechanism for inflammasome formation it is perhaps unsurprising that a number of drugs interfering with ion transport have been reported to possess inflammasome-inhibitory properties.

Over 20 years ago, the anti-inflammatory agent tenidap was shown to attenuate ATP-induced IL-1β release from macrophages by inhibition of anion transport (Laliberte et al., 1994). Remarkably, the same effect was observed in other anion transport inhibitors DIDS and UK5099 but not in COX-inhibitors naproxen, diclofenac, indomethacin or ibuprofen. Of particular note was the inhibition of IL-1β release by NSAIDs flufenamic and meclofenamic acid, suggesting some form of dual function by these drugs. We have since demonstrated that the fenamate NSAIDs selectively inhibit the NLRP3 inflammasome by blocking Cl⁻ efflux through the ion channel VRAC (Daniels et al., 2016) (see chapter Chapter 2:). Interestingly, there has also been suggestion that MCC950 functions via inhibition of Cl⁻ efflux, although this remains unconfirmed (Jiang et al., 2017).

Another approved drug also recently discovered to possess inflammasome inhibiting properties is the sulphonylurea drug glyburide. Originally approved to treat type 2 diabetes mellitus, glyburide blocks K_\text{ATP} channels to cause depolarisation of pancreatic β-cells and stimulate insulin secretion (Prendergast, 1984). Glyburide has since been accepted as an inhibitor of inflammasome function; however, precisely how this occurs remains unclear as the effects seem independent of interference with P2X7, ATP-binding cassette (ABC) transporter or K_\text{ATP} channel activity (Lamkanfi et al., 2009). In the clinic, glyburide has been observed to possess systemic anti-inflammatory properties and promote survival in sepsis patients (Koh et al., 2011), an effect suggested to signal through inflammasome inhibition.

Recently, the ketone metabolite β-hydroxybuturate (BHB) was identified as a specific NLRP3 inhibitor (Youm et al., 2015). BHB can inhibit K⁺ efflux from cells. However, the exact mechanism of BHB-induced inflammasome-inhibition is unknown.
1.5.5 P2X7 antagonists

A final upstream target for inflammasome inhibition is that of the P2X7 receptor, this particular avenue represents the most progressed in terms of specific pharmacological intervention.

AstraZeneca's AZD9056 has been tested in a number of clinical trials for inflammatory diseases such as rheumatoid arthritis and Crohn's disease and found to be efficacious, although responses were mixed and the drug is no longer being developed (Arulkumaran et al., 2011; Keystone et al., 2012; Eser et al., 2015). In addition to the AZ drug, US pharma giant Pfizer have developed a P2X7 inhibitor named CE 224, 535 (Duplantier et al., 2011), which has also progressed through a number of clinical trials. However, although the drug was well tolerated, no efficacy over placebo was detected for the treatment of RA (Stock et al., 2012). A potential issue with P2X7 targeting may be the high number of polymorphisms present in humans, this could explain why drug development has proved challenging (Arulkumaran et al., 2011).

Although specific anti-inflammasome P2X7 inhibitors are yet to be released there has once again been evidence suggesting anti-inflammaosome properties of a current approved drug that may function via this mechanism. In a recent discovery, Fowler et al. (2014) showed that anti-retroviral nucleoside reverse transcriptase inhibiting drugs (NRTIs) are also capable of inhibiting the NLRP3 inflammasome. This inhibition was only evident in inflammasome activation by ATP and not nigericin or MSU, thus suggesting an effect dependent on P2X7 antagonism.

1.5.6 IL-1α inhibitors

Although anti-IL-1 therapies such as IL-1Ra will block both signalling through both IL-1α and IL-1β, there have also been developed some strategies to specifically inhibit IL-1α. The specific IL-1α neutralising antibody MABp1 (trade name Xilonix) was developed as a possible cancer therapeutic and recently failed in a phase III clinical trial for colorectal cancer (NCT01767857). MABp1 is now in trials for pancreatic cancer (NCT03207724) and has recently completed a phase II trial for Hidradenitis Suppurativa (NCT02643654).

Also relevant to IL-1α-inhibiting strategies are calpain inhibitors. Numerous studies have shown that the calpain inhibitor A-705253, also known as ABT-957, is effective in preclinical models of AD (Nimmrich et al., 2008, 2010; Granic et al., 2010). Another calpain inhibitor, BDA-410, improved working memory in the APP-PS1 mouse (Trinchese et al., 2008).
Although inflammation has not been addressed in discussing the mechanisms involved in the above studies, these effects may be in part mediated through inhibition of IL-1α processing. Additionally, a recent study suggested that the endogenous calpain inhibitor calpastatin may be an effective treatment in kidney inflammation (Hanouna et al., 2017) concomitant with a reduction in IL-1α-mediated inflammation.

Overall, these studies demonstrate how the IL-1/inflammasome pathway can be inhibited by pharmacological intervention and that these strategies are already therapeutically effective in a wide range of diseases. Although not yet in the clinic, it is likely that the plethora of NLRP3-inhibiting drugs currently in development will soon enter trials. This will indeed be the acid test for NLRP3 as a target in non-communicable disease.

1.6 Summary and aims

In summary, the interleukin-1 family of cytokines are critical regulators of sterile inflammation. The two best studied members of the IL-1 family IL-1α and IL-1β are subject to stringent regulation. Regulation of IL-1α and IL-1β occurs at the level of expression, localisation, post-translational modification, secretion and signalling. There is strong evidence to suggest that these regulatory pathways of IL-1 signalling are dysfunctional in disease. Therefore, multiple strategies are being developed to inhibit IL-1 signalling as a way to treat non-communicable disease. However, we currently have an incomplete understanding of the molecular mechanisms regulation of IL-1α and IL-1β.

The studies in this thesis have all been published in peer-reviewed journals and have been cited in this introduction. The overall aim of the research in this thesis is to develop our understanding of IL-1 regulation at a molecular level. These aims will be addressed in three separate published research articles.

1.6.1 Paper 1: Fenamate NSAIDs inhibit the NLRP3 inflammasome and protect against Alzheimer's disease in rodent models

This paper aims to test the fenamate class of NSAIDs as inflammasome inhibitors and characterise the mechanism of action of inhibition. The paper also aimed to assess whether inhibition of NLRP3 could be effective in animal models of Alzheimer’s disease.
1.6.2 Paper 2: Boron-based inhibitors of the NLRP3 inflammasome

Building on paper 1, this paper aims to develop novel, potent inhibitors of NLRP3. Starting from the non-specific and promiscuous chemical 2-APB, the aim was to synthesize and characterise a new class of boron-containing NLRP3 inhibitors and test these in proof-of-concept animal models of inflammatory disease.

1.6.3 Paper 3: CRISPR/Cas9 mediated mutation of mouse IL-1α nuclear localisation sequence abolishes expression

The mechanisms of inflammasome activation and IL-1β regulation are relatively well researched. IL-1α, on the other hand, is poorly understood. In this paper the aim was to address the molecular mechanisms that underlie IL-1α expression. The CRISPR/Cas9 system was used to specifically edit parts of the IL-1α genome in mice and characterised this phenotype.

1.7 Author Contributions

1.7.1 Chapter 2: Fenamate NSAIDs inhibit the NLRP3 inflammasome and protect against Alzheimer's disease in rodent models

Journal: Nature Communications

Status: Published


*These authors contributed equally

Contributions: The original idea for this study was conceived by DB. I designed and performed all the experiments in figures 1 and 2. The electrophysiology experiments in figure 3 were performed by TS and NS whilst the cell experiments were performed by me, CL or SY, both of whom I trained and supervised. The experiments in figure 4 were performed by JRA and SB. I designed and performed the experiments in figure S2 and 3 and designed and supervised experiments by SY in figure S6. Experiments in figure S7 and
8 were carried out by JRA and me. I wrote the manuscript with guidance from DB and handled referee’s comments with JRA & SB.

1.7.2 Chapter 3: Boron-based inhibitors of the NLRP3 inflammasome

*Journal:* Cell Chemical Biology

*Status:* Published


*Contributions:* The original idea for this study was conceived by DB. Molecule synthesis and validation was performed by AB and HH. The biological screening in figures 1 & 2 were performed by HE & NL. I designed and performed the experiments in figure 5Ai, Aii, Aiii, Di, Dii and E. The experiments in figure 6A, Ci, Cii and Ciii were designed and performed by JRA and me. Electrophysiology and calcium imaging experiments in figure 7 were performed by TS and NS. I produced the schematic in figure 7H and performed experiments in figure 7I. I designed and produced the graphical abstract, handled referee’s comments with DB, SF, AGB and JRA and contributed to the writing of the manuscript.

1.7.3 Chapter 4: CRISPR/Cas9 mediated mutation of mouse IL-1α nuclear localisation sequence abolishes expression

*Journal:* Scientific Reports

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Chapter 2: Fenamate NSAIDs inhibit the NLRP3 inflammasome and protect against Alzheimer’s disease in rodent models

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Fenamate NSAIDs inhibit the NLRP3 inflammasome and protect against Alzheimer’s disease in rodent models

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Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenase-1 (COX-1) and COX-2 enzymes. The NLRP3 inflammasome is a multi-protein complex responsible for the processing of the proinflammatory cytokine interleukin-1β and is implicated in many inflammatory diseases. Here we show that several clinically approved and widely used NSAIDs of the fenamate class are effective and selective inhibitors of the NLRP3 inflammasome via inhibition of the volume-regulated anion channel in macrophages, independently of COX enzymes. Flufenamic acid and mefenamic acid are efficacious in NLRP3-dependent rodent models of inflammation in air pouch and peritoneum. We also show therapeutic effects of fenamates using a model of amyloid beta induced memory loss and a transgenic mouse model of Alzheimer’s disease. These data suggest that fenamate NSAIDs could be repurposed as NLRP3 inflammasome inhibitors and Alzheimer’s disease therapeutics.
Since their characterization as COX inhibitors, non-steroidal anti-inflammatory drugs (NSAIDs) have been used to treat a wide variety of diseases with relatively limited side effects. NSAID inhibition of COX prevents the conversion of arachidonic acid to eicosanoids resulting in a reduction in the synthesis of proinflammatory prostaglandins. Evidence indicates a polyvalent effect of NSAIDs with some research suggesting COX-independent activity. One such important action may be to directly limit the production of proinflammatory cytokines.

Many inflammatory diseases are driven by the proinflammatory cytokine interleukin-1β (IL-1β). IL-1β is produced in myeloid cells as an inactive precursor (pro-IL-1β) that requires cleavage by the protease caspase-1 for its activation and secretion. Caspase-1 is also produced as an inactive precursor, which is activated following recruitment to a large multi-protein complex called the inflammasome. Inflammasomes are defined by the presence of a pattern recognition receptor. The best characterized inflammasome-forming pattern recognition receptor, and the most commonly associated with disease is NLRP3 (NLR family, pyrin domain containing 3). In response to pathogen-associated molecular patterns or damage-associated molecular patterns NLRP3 nucleates the oligomerization of ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) molecules into large ‘specks’ that serve as platforms for caspase-1 activation and subsequent release of IL-1β.

The NLRP3 inflammasome is an important contributor to inflammatory diseases, including Alzheimer’s disease, atherosclerosis, metabolic diseases such as type 2 diabetes, and others. Hence, there is substantial interest in the discovery of potentially therapeutic inflammasome inhibitors. One such compound, MCC950, has been identified as a potent NLRP3-selective inhibitor, but is not yet available for clinical use. Fenamate NSAIDs have been shown to inhibit IL-1β secretion from macrophages, although the significance of COX inhibition remains unclear. Here we show that the fenamate class of NSAIDs inhibit the NLRP3 inflammasome via reversible blockade of volume-regulated anion channels (VRAC) in the plasma membrane, and inhibit cognitive impairments in models of Alzheimer’s disease in rodents, thus offering a safe and rapidly translatable option to treat NLRP3-related inflammatory diseases.

**Results**

Fenamate NSAIDs selectively inhibit the NLRP3 inflammasome. Immortalized mouse bone marrow-derived macrophages (iBMDMs) were primed with lipopolysaccharide (LPS; 1 µg ml⁻¹, 2 h), after which the media was replaced with serum-free media. At this point the cells were incubated with a range of NSAIDs (Supplementary Fig. 1) for 15 min before 1 h stimulation with 5 mM ATP to activate the P2X7 receptor and induce NLRP3 inflammasome activation. ELISA analysis of cell supernatants revealed that of the NSAIDs tested the fenamates (N-phenyl-substituted anthranilic acid derivatives such as flufenamic acid, meclofenamic acid, mefenamic acid) were most effective at inhibiting IL-1β release (Fig. 1a). The selective COX-2 inhibitor celecoxib did not inhibit IL-1β release, nor did ibuprofen, even at concentrations supra-maximal for COX inhibition. Western blot analysis of the supernatants also showed that caspase-1-dependent processing of IL-1β was also inhibited by the fenamates (Supplementary Fig. 2). Fenamate NSAIDs had no effect on ATP-induced cell death (Supplementary Fig. 3) suggesting that their effects were specific to IL-1β release, and independent of the stability of ATP. As early studies indicated multiple sites of action for the fenamate NSAIDs, these data reveal the fenamates as inhibitors of IL-1β processing and release and suggest that this effect is independent of COX inhibition. Flufenamic acid, but not ibuprofen, also inhibited IL-1β release induced by the NLRP3 inflammasome activator monosodium urate (MSU) in primary mouse BMDMs (Fig. 1b), suggesting that their effect was not due to direct inhibition of the P2X7 receptor or a direct effect on ATP. To determine whether the fenamates were selective inhibitors of NLRP3-dependent IL-1β release, we tested their effects against other well-characterized inflammasomes in primary BMDMs. NLRC4 inflammasome activation by transfected Salmonella typhimurium flagellin in BMDMs induced IL-1β release, which was not inhibited by flufenamic or mefenamic acid (Fig. 1c). AIM2 inflammasome activation by transfected double-stranded DNA was also unaffected by flufenamic or mefenamic acid (Fig. 1d). These data suggest that the fenamates selectively inhibit the NLRP3 pathway.

![Figure 1](Image)
iBMDMs were transduced with lentivirus to stably express the inflammasome adaptor ASC fused to mCherry. Inflammasome activation in these cells results in the aggregation of ASC into one large ASC speck in the cytoplasm that is readily visible by fluorescence microscopy. ASC–mCherry expressing iBMDMs were treated with LPS (1 μg ml⁻¹, 2 h) and then ATP (5 mM) in serum-free media plus or minus a 15 min pre-incubation with ibuprofen, mefenamic acid or flufenamic acid (all 100 μM). Flufenamic and mefenamic acid both inhibited NLRP3-dependent ASC speck formation whereas ibuprofen had no effect (Fig. 2a). In addition to inhibiting IL-1β release from mouse cells, flufenamic acid also inhibited IL-1β release (Fig. 2b) and production of the active p10 subunit of caspase-1 (Fig. 2c) from nigericin-treated human THP-1 cells. These data show that the fenamates inhibited NLRP3-dependent IL-1β release upstream of ASC speck formation.

Chloride channels regulate the effects of fenamates on NLRP3. To further identify the mechanism of action of fenamates on NLRP3, we sought to determine the reversibility of fenamate inhibition of NLRP3-dependent IL-1β release. Flufenamic acid did not react with protected cysteine in contrast to 3,4-methylene-dioxy-nitrostyrene (MNS; Supplementary Fig. 4), a known inhibitor of NLRP3 via irreversible cysteine modification.¹⁸,¹⁹ Thus the mechanism of fenamate action does not involve irreversible cysteine modification. Following the secretion protocol above, except with three media changes over 15 min to wash out the fenamates flufenamic acid and mefenamic acid, the reversible caspase-1 inhibitor Ac-YVAD-cho, and the irreversible NLRP3 inhibitor MNS, it was revealed that the inhibitory effects of the fenamates on NLRP3-dependent IL-1β release were fully reversible (Fig. 2a). A further experiment, with just one wash, which failed to remove the inhibitor effects of YVAD, completely reversed the effects of flufenamic and mefenamic acid on NLRP3 inflammasome inhibition (Fig. 3b) strongly suggesting that their inhibitory effects were at the plasma membrane. Flufenamic acid is a well-established modulator of ion channels.²⁰ Thus we first-tested the effects of the flufenamic and mefenamic acid on ATP-induced cation currents in LPS-primed iBMDMs using whole-cell patch clamp recordings. Neither flufenamic nor mefenamic acid inhibited ATP-induced cation currents (Fig. 3c–e), nor inhibited ATP-induced increases in intracellular Ca²⁺ or Na⁺ levels (Supplementary Fig. 5). Flufenamic acid is also known to inhibit Cl⁻ currents²⁰ and so we tested the effects of the fenamates on VRAC. VRAC currents measured by whole-cell patch clamp in LPS (1 μg ml⁻¹, 4 h) primed iBMDMs were induced by hypotonicity, also a known activator of the NLRP3 inflammasome.²¹ Both flufenamic and mefenamic acid inhibited VRAC, while non-NLRP3 inhibiting NSAIDs such as ibuprofen and diclofenac had no effect (Fig. 3f–i). In light of these data we tested whether other Cl⁻ channel inhibitors would block NLRP3-dependent IL-1β release. In LPS-primed iBMDMs, a 15 min pre-incubation with either of the Cl⁻ channel inhibitors NPPB (100 μM) or 4,4’-disothiocyanstilbene-2,2’-disulfonic acid disodium salt hydrate (DIDS; 100 μM) completely prevented NLRP3-inflammasome-dependent IL-1β release (Fig. 3k). To further confirm the importance of Cl⁻ currents, and in particular the importance of VRAC, we used the specific VRAC inhibitor DCPIB (10 μM), which also completely inhibited ATP-induced IL-1β release (Fig. 3l). Flufenamic acid and DCPIB also inhibited the regulatory volume decrease induced by hypotonicity in THP-1 cells, which is known to require Cl⁻ efflux²² (Supplementary Fig. 6). These data suggest that the mechanism of action of the fenamate NSAIDs on the NLRP3 inflammasome is via an inhibition of the Cl⁻ channel VRAC.

**Figure 2** | Fenamate NSAIDs inhibit ASC speck formation and caspase-1 activation. (a) iBMDMs stably expressing ASC protein conjugated to mCherry were primed with LPS (1 μg ml⁻¹, 2 h) then pre-treated with selected drug (100 μM, 15 min) before stimulation with ATP (5 mM, 30 min) under live microscopy. Formation of ASC specks (examples indicated by white arrows) was quantified (lower right) and presented as mean % specks counted versus vehicle + s.e.m (n = 4). Scale bars are 20 μm. (b, c) THP-1 cells were primed with LPS (1 μg ml⁻¹, 4 h) and pre-treated with NSAID (200 μM, 15 min) before stimulating with nigericin (50 μM, 1 h). Supernatants were taken and analysed for IL-1β by ELISA (b) and the p10 active subunit of caspase-1 by western blot (c). ELISA data are presented as mean % IL-1β release versus vehicle (DMSO) control + s.e.m (n = 3). *P < 0.05, **P < 0.001 determined by one-sample t-test versus hypothetical value of 100%.
Fenamates inhibit NLRP3 inflammasomes in vivo. We next tested whether the fenamates would have any effect on the NLRP3 inflammasome in vivo. Initially, we used a mouse air pouch model in which MSU-induced IL-1β production is NLRP3 inflammasome dependent. Seven days after the air pouches were raised in the dorsum of C57BL/6 mice they were injected with MSU crystals (3 mg in 1 ml sterile saline) or vehicle with or without flufenamic acid (20 mg kg⁻¹ in sterile saline, 5%...
Cremaphor EL, 5% dimethyl sulfoxide (DMSO) or vehicle. Six hours later pouches were lavaged and IL-1β measured by ELISA. Flufenamic acid significantly inhibited MSU-induced IL-1β production in the pouch (Supplementary Fig. 7). Flow cytometry was performed on lavage samples collected from the air pouch and a specific MSU-dependent recruitment of macrophages/microcytes was observed (Supplementary Fig. 7). Flufenamic acid also inhibited MSU-induced macrophage/microcyte recruitment (Supplementary Fig. 7). We also tested mafenamic acid in a NLRP3-dependent peritoneal model of inflammation. LPS injection into the peritoneum induces low levels of IL-1β that are greatly enhanced with the co-addition of the NLRP3 agonist ATP24-26. In this model the IL-1β production is completely dependent on P2X7 (ref. 25), an established activator of the canonical NLRP3 inflammasome26, and is inhibited by cytokine release inhibiting drugs developed by Pfizer26, which are close analogues and precursors of the NLRP3-selective inflammasome inhibitor MCC950 (ref. 13). Wild-type and NLRP3−/− mice were challenged with LPS (1 μg per mouse) intraperitoneally (i.p.) and IL-1β was measured by ELISA (Supplementary Fig. 8). Peritoneal administration of LPS and ATP induced significant release of IL-1β that was completely blocked by mafenamic acid and by MCC950 (Supplementary Fig. 8). Furthermore, LPS and ATP did not cause IL-1β release from NLRP3−/− mice (Supplementary Fig. 8). These data suggest that fenamates are effective inhibitors of the NLRP3 inflammasome in vivo.

We also tested the effect of fenamates in more disease relevant models. Inflammation contributes to the progression of Alzheimer’s disease27, and on-going research is establishing the NLRP3 inflammasome as central to the development of inflammation, pathology and memory deficits in a mouse model of Alzheimer’s disease28. Amyloid beta (Aβ) is a causative factor in the development of Alzheimer’s disease and is a known activator of the NLRP3 inflammasome28. Mafenamic acid is used routinely in clinical practice and so we tested the effects of mafenamic acid in an in vivo model of Aβ-induced memory loss29. Rats injected intracerebroventricularly with an acute Aβ1−42 injection, or vehicle were treated with daily injections of mafenamic acid (5 mg·kg−1, i.p.) or vehicle for 14 days. Mafenamic acid treatment prevented Aβ1−42 induced memory deficits as measured using the novel object recognition (NOR) test at day 14 (Fig. 4a, Supplementary Fig. 9). This protective effect was sustained up to day 35, 21 days after the mafenamic acid treatment had been terminated (Fig. 4b).

Following the acute Aβ1−42 injection model, we then tested the efficacy of mafenamic acid in 3 × TgAD transgenic mice (a mouse model for Alzheimer’s disease). For this we used a therapeutic administration strategy beginning at an age where pathology and memory deficits have previously been described in the 3 × TgAD model30. At 13–14 months of age management of mafenamic acid by osmotic minipump over 28 days (at 25 mg·kg−1 per day) completely abated memory deficits in the 3 × TgAD mice as measured by NOR (Fig. 4c). The brains of these mice were sectioned and stained for IL-1β and, using Iba1, for microglia. Microglial activation states were scored by morphology as described in the Methods section (Supplementary Fig. 10, modified scale from published literature31,32). In 3 × TgAD mice it has previously been established that amyloid pathology begins to develop in the subiculum at around 12 months of age30. We found that there was substantial microglial activation in the subicula of 3 × TgAD mice, and that the activated/amoeboid microglia expressed IL-1β (Fig. 4d–f, Supplementary Fig. 10). No microglial activation or IL-1β expression was seen in other regions of the brain (Supplementary Fig. 11). Mafenamic acid treatment completely abated Alzheimer’s disease-associated neuroinflammation with levels of microglial activation and IL-1β expression reduced to that of wild-type mice (Fig. 4d–f, Supplementary Fig. 10).

Discussion

The NLRP3 inflammasome is an important contributor to diverse inflammatory diseases including Alzheimer’s disease9, atherosclerosis30, metabolic diseases such as type II diabetes31, and others32. However, despite this, there are currently no clinically available inhibitors of NLRP3. Here we show that fenamate NSAIDs are selective inhibitors of the NLRP3 inflammasome. We also show that the fenamates inhibited the membrane Cl− channel VRAC. Given additional recent research showing the importance of targeting the inflammasome in response to hypotonicity33, we propose that the effects of fenamates on NLRP3 are via inhibition of VRAC. Furthermore we show that inhibiting this pathway was protective and therapeutic in two animal models of Alzheimer’s disease. Treatment with mafenamic acid of an established disease model abated brain inflammation and memory deficits suggesting that inflammation is a druggable target for Alzheimer’s disease.

There has been a significant decline in the number of new therapies being translated to clinic in the last two decades33. However, we now appreciate that the robustness of biological systems depends on the network and there is an appreciation that targeting multiple points in a pathway may be more efficacious than targeting a single node34. The fenamates inhibit COX enzymes35, and as we report here, also inhibit the NLRP3 inflammasome. Therefore the fenamate NSAIDs are attractive candidates for a polyvalent approach to treat inflammatory disease. The mechanism of action of the fenamates in vivo may be as much dependent on VRAC/NLRP3 as it is COX. We have previously reported synergy between inhibitors in inflammatory pathways to cause levels of inhibition far greater than observed singly34. Thus the ability of molecules such as the fenamates to modulate several points in a pathway is likely to confer greater protective effects than molecules that have single targets, thus allowing them to be effective at lower doses.

The selectivity of the fenamates to NLRP3 over other inflammasomes reported here is another advantage since their use would avoid compromising NLRC4 or AIM2 inflammasome-dependent host responses to infection. The current strategies used to target IL-1β in disease rely on biologicals such as the IL-1 receptor antagonist anakinra, or the neutralizing antibody canakinumab36. While these are effective in some inflammatory diseases, they are expensive and may not readily penetrate tissues such as the brain. Thus, used as a monotherapy, or as an adjunct to current therapies, there is real scope to consider fenamate NSAIDs as a frontline treatment for inflammatory disease.

Although there is robust evidence linking NLRP3 to inflammatory disease from animal models (cited above), apart from the cryopyrin-associated periodic syndromes, there is limited evidence in humans. Further characterization of human inflammatory disorders may widen the scope for use of fenamate NSAIDs to target NLRP3 in human disease. Given that some fenamate NSAIDs are already routinely used clinically, and their pharmacokinetic and toxicity profiles are well established37, encouraging results in animal studies could lead rapidly to clinical efficacy trials in inflammatory diseases. Additional epidemiological studies to assess benefits to patients taking...
Figure 4 | Mefenamic acid is protective in rodent models of Alzheimer’s disease through an anti-inflammatory mechanism. (a, b) Female Lister hooded rats (200–230 g) received an acute unilateral intracerebroventricular injection of soluble Aβ1–42 on day 0 (5 nmol in 10 μl) which was followed by 14 days (starting one day before surgery) of i.p. injection of mefenamic acid (5 mg kg⁻¹) or vehicle. Animals were then tested in the novel object recognition task on 14 d (a) and 35 d (b) post surgery. Discrimination index data are presented as mean ± s.e.m (n = 5–10). NS, not significantly different, **P < 0.01 compared with vehicle/vehicle treated animals and ***P < 0.001 compared to Aβ1-42/vehicle group. (c–f) 13–14 month old transgenic mouse model of Alzheimer’s 3×TgAD and wild-type (WT) control were treated with vehicle or 25 mg kg⁻¹ mefenamic acid. (c) On day 18 memory was assessed with the novel object recognition task; discrimination index data are presented as mean ± s.e.m (n = 8–10). ***P < 0.001 compared to vehicle/WT animals and **P < 0.01 compared with vehicle/3×TgAD mice. (d–f) Evaluation of Iba1 and IL-1β expressing microglia within the subicula of 3×TgAD and WT mice following vehicle or mefenamic acid treatment. Microglial activation (d) and IL-1β expression (e) were evaluated and presented as mean ± s.e.m (n = 8–10). ***P < 0.001 compared with vehicle/WT animals and **P < 0.01 compared to vehicle/3×TgAD mice. (f) Representative images of microglial activation and IL-1β co-localization of 3×TgAD and WT subicula following vehicle or mefenamic acid treatment. Scale bars are 15 μm. Statistical analyses performed using two-way ANOVA followed by Sidak corrected post hoc analysis.
Methods

In summary, we have shown that fenamate NSAIDs are potent and selective inhibitors of the NLRP3 inflammasome, which act through inhibition of VCAM, a novel player in NLRP3 inflammasome regulation. Fenamate NSAID inhibition of NLRP3 is rapidly reversible which offers significant clinical benefit. We have characterized their activity both in vitro and in vivo and propose that fenamate NSAIDs can be rapidly repurposed as drugs to target the NLRP3 inflammasome in inflammatory diseases such as Alzheimer’s disease.

Materials

Fenamates (IBMX, ibuprofen, and naproxen) were obtained from Sigma-Aldrich. NLRP3 inflammasome deficient mice were purchased from Jackson Laboratory and were maintained on a C57BL/6 background. Human THP-1 cells were obtained from American Type Culture Collection (ATCC). iBMDMs were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), 10% fetal bovine serum (FBS), penicillin and streptomycin (PenStrep). Cultures were performed in 75 cm² flasks at 37 °C and 5% CO₂.

Cell culture and assays. iBMDMs were obtained from Clara Bryant (Department of Veterinary Medicine, University of Cambridge). iBMDMs were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), 10% fetal bovine serum (FBS), penicillin and streptomycin (PenStrep). Cells were seeded overnight at 5 x 10⁶/ml in 24- or 96-well plates. Cells were plated with LPS then treated with drug or vehicle (DMSO) in serum-free media for 15 min. Following drug incubation, inflammasomes were stimulated by adding ATP (5 mM) for 1 h. Supernatants were collected and analyzed for IL-1β and IL-18 cytokine secretion.

Cell death assays. Cell death was measured by assessing lactate dehydrogenase release using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to manufacturer’s instructions.

ASC speck imaging. Live imaging of ASC speck formation was performed using iBMDMs transfected to stably express ASC conjugated to mCherry protein. Mice (Accession Number NM_023258) was amplified by PCR with primers flanked by gateway recombinase sequence (pENTR-DTOPO/VRAC AGACCG-3′, reverse primer: 5′-GGTCGTGGCTGGATTGCCAAC-3′). A third generation (pENT) intratransfer system was used to express N-terminally mCherry-tagged ASC gene from a constitutively active ubiquitin C promoter. Stably transduced cells were plated overnight at 5 x 10⁶ cells per ml. The following day, cells were primed with LPS (1 µg/ml) for 2 h. iBMDMs were washed and incubated (4 °C) overnight with 4% paraformaldehyde and 0.1% Triton X-100. Fixed cells were washed twice with PBS and then permeabilized with 0.1% Triton X-100 before blocking with 5% milk in phosphate-buffered saline (PBS) for 1 h. Following blocking, cells were incubated with primary antibodies (1:100) in 1% BSA for 2 h. Cells were washed twice with PBS before incubation with Alexa Fluor 488 (Life Technologies) or Alexafluor 555 (Molecular Probes) secondary antibodies. Images were collected at 20x or 40x magnification on a Zeiss Axiovert 200M microscope equipped with a MetaMorph imaging system.

Cytosolic membrane and NMR. Fluorophanic acid or MNS (0.05 mmol) was dissolved in DMSO-d₆ (400 µl) in an NMR tube. N-Acetyl-l-cysteine methyl ester (17.9 mg, 0.10 mmol) solubilized in DMSO-d₆ (100 µl) spectrum was recorded every 10 min after the addition for 2h and then every 1 h until 24h. A Bruker Avance 600 spectrometer was used to record 1H spectra at 300.1 MHz. Chemical shifts are defined in parts per million and referenced against sodium 3-(trimethylsilyl)propionate-2,2,3,3-d₄ (TSP). These studies, and those employing the gCAGG-3'-MDR transfectants, were performed on a 2-stage puffer (Narishige PC-10) from borosilicate glass (Hülsengeh). For all electrophysiological experiments, 10⁶ iBMDMs were seeded in 24-well plates on glass coverslips and primed the next day with 1 µg/ml LPS for 4 h. For electrophysiological recordings of ATP-induced cation currents, patch electrodes were filled with an intracellular solution containing (in mM): KCl, 120; HEPES, 10; EGTA, 1; MgCl₂, 2 (pH 7.3). LPS-primed iBMDMs were superfused with extracellular solution containing (in mM): NaCl, 130; KCl, 5; HEPES, 10; MgCl₂, 2; CaCl₂, 1; glucose, 10 (pH 7.4) The effect of flufenamatic/mefenamic acid was investigated after a stable cation current was induced by application of 5 mM ATP. DMSO was used as vehicle control in these experiments. Leak-subtracted currents were analyzed at ~90 mV and data are presented as mean values ± s.e.m. For volume-regulated Cl⁻ channel currents, patch electrodes were filled with the following intracellular solution (in mM): N-Methyl-D-Glucamine-Chloride (NMG-CI), 120; HEPES, 10; EGTA, 11; CaCl₂, 1; MgCl₂, 2 (pH 7.3). Cells were kept in iso-osmolar extracellular solution containing (in mM): NaCl, 130; KCl, 5; HEPES, 10; glucose, 10; CaCl₂, 2; MgCl₂, 1; n-mannitol, 170 (300 mosmol per kg H₂O). To activate VRAC currents, superfusion was changed to hypo-osmolar extracellular solution containing no n-mannitol (130 mosmol per kg H₂O). To permit a rapid exchange of solutions for drug application, cells were continuously superfused using a four-barrel microperfusion pipette positioned near the recorded cell. Extracellular solutions containing the indicated NSAID were applied after stable activation of Cl⁻ currents, DMSO was used as vehicle control. All recordings were made at a temperature of 23–26 °C. Whole-cell currents were filtered at 3kHz and stored for subsequent analyses. Analysis of leak-subtracted currents at ±90 mV were performed with the program FitMaster (HEKA).

Fluorescence imaging. 10⁶ iBMDMs were seeded one day before experiments on glass coverslips in 24-well plates and primed with LPS (1 µg/ml) for 4 h. Before fluorescence imaging experiments, DsRed recently loaded with 5 µM of the cyan fluorescent dyes in Na²/K⁺ containing extracellular solution at room temperature (20–23 °C) for Ca²⁺ imaging experiments with 3 µM of fura-2-acetoxymethylester (fura-2-AM) for 30 min, for Na⁺ imaging experiments with 10 µM sodium-binding benzofuran-isothiophene acrylnitryl methyl ester (SBFI-AM, both dyes from Molecular Probes) for 60 min. After washing with extracellular solution, glass coverslips were mounted in a chamber on an inverted Olympus IX81 microscope equipped with a x40 water immersion objective (UPlanapo 1.0/30 Olympus Optical, Co.). The fluorescence imaging system consisted of a monochromator, a charge-coupled device (CCD) camera and a Windows 7 based image processing software (Till Photonics). Cells were exposed to alternating 340.5 ± 5 and 380.1 ± 5.7 wavelengths of UV light and emission light was passed through a 400 nm dichroic mirror and a 420 nm long pass emission filter (both Olympus) before image acquisition by the CCD camera. Images were collected...
every 20 s. Cells were continuously superfused with Na\(^+\)-\(\text{K}^+\)-containing extracellular solution using a four-barrel micropipette pipette positioned in close proximity to the viewing field. Ca\(^{2+}\) and Na\(^+\) influx was induced by extracellular solution containing 5 mM ATP in the presence or absence of mefenamic acid. DMSO was used as vehicle control in all experiments. For each individual cell, mean intensity values from background subtracted pictures were determined and ratio \(F_{340}/F_{380}\) calculated, accordingly. Data are presented as mean values ± s.e.m.

**Regulatory volume decrease.** THP-1 cells were adjusted to a density of 1 × 10\(^6\) cells per ml in RPMI media (10% FBS, 1% P/S, 1% Glutamax) and primed with LPS (1 \(\mu\)g ml\(^{-1}\), 4h). A 300 mM isosmotic buffer was prepared consisting of 147 mM NaCl, 10 mM HEPES, 13 mM glucose, 2 mM KCl, 2.5 mM CaCl\(_2\), 1 mM MgCl\(_2\), 0.35 mM Na\(_2\)EDTA 0.74 mM NaH\(_2\)PO\(_4\) as buffer was achieved by diluting the isosmotic buffer 1:4 with sterile water. Cell size and viability measurements were performed on a BD FACScalibur flow cytometer (BD). THP-1 cells were incubated in a 37 °C water bath and a series of measurements were taken for 60 min with 10,000 events recorded each run. Cell swelling was initiated by aspiration of isosmotic buffer followed by addition of hypertonic buffer containing respective drug. Cell volume measurements by forward scatter width were normalized against the average cell volume before hypertonic stimulus.

**Animal experiments.** Animals were maintained under standard laboratory conditions: ambient temperatures of 21 ± 2°C, humidity of 40–50%, 12 h light cycle, and ad libitum access to food and water. All surgeries were performed with the surgeon concealed to the treatment and/or genotype, and all behavioural and histological analyses were performed by a blinded observer. Treatments were randomly allocated. Animal experiments were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and approved by the Home Office and the Local Animal Ethical Review Group, University of Manchester.

**Air pouch inflammation model.** The air pouch model was used to assess the NLRP3 inflammasome response to MSU crystals [27]. On day 0 a subcutaneous air pouch was raised in the dorsum of male C57BL/6 mice (30–35 g) by the injection of 4ml sterile air (filtered through 0.22 µm pore size) with a 25 gauge needle. This was repeated on day 3. On day 7, pouches were injected with 1 ml MSU crystals (3 mg ml\(^{-1}\) in sterile saline, 5% Cremophor EL, 5% DMSO) or vehicle. Following a 6 h incubation, mice were killed by raising the concentration of CO\(_2\) and pouches were lavaged by injecting 4 ml PBS, 1% BSA, 5 mM EDTA. Lavage was collected from each animal, passed through 100 µm cell strainers before analysis by ELISA for IL-1\(\beta\) and/or IL-18 as above or by flow cytometry. For the flow cytometry cells were adjusted to a density of 5 × 10\(^6\) cells per ml in ice-cold PBS, 1% BSA, 5 mM EDTA before plating out on a clear V-bottomed 96-well plate (Thermo Scientific) at 200,000 per well. Cells were stained with Antibodies (Anti-CD4 conjugated to FITC at 1 ng ml\(^{-1}\), anti-Ly6G conjugated to APC at 1 ng ml\(^{-1}\) and anti-F4/80 conjugated to PE at 2.5 ng ml\(^{-1}\), all Biolegend) for 45 min on ice in the dark before washing twice and fixing in paraformaldehyde (1.7–4% in PBS, 20 µl) for 15 min at room temperature. The following day, cells were analysed on a FACScalibur flow cytometer (BD Biosciences) with BD FACSuite software.

**Peritoneal inflammation model.** Randomly allocated wild-type (C57BL/6) and NLRP3 \(\Delta X O\) mice (male, 30–35 g) were dosed i.p. with mefenamic acid (50 mg kg\(^{-1}\)) and the selective NLRP3 inhibitor MCC950 (50 mg kg\(^{-1}\)) or PBS. LPS (1 mg in 0.5 ml PBS) for 4h. Following LPS prime mice were anaesthetised with isoflurane (induced at 3–4% in 3% O\(_2\), 67% N\(_2\)) and maintained at 1–2% whilst kept at 37 °C on a heat blanket) before injection with mefenamic acid, MCC950 or vehicle as above and ATP (0.5 ml, 100 mM PBS) or PBS for 15 min. Peritoneums were then lavaged by an overnight incubation with 2% NDS in PBST and then washed twice and incubated with Alexa-488 (visualising Iba1) and Alexa-594 (visualising IL-1\(\beta\)) conjugated secondary antibodies (Invitrogen) at 1:50 dilution in 2% NDS in PBS for 2h. Sections were then washed (3 × 10 min in PBST and mounted using ProLong Gold Antifade Mountant with DAPI (Thermo Fischer Scientific, Inc., USA). Primary antibodies were used anti-Iba1 (1:1000, Wako Ltd) and anti-IL-1\(\beta\) (1:200, R&D Systems).

**Microscopy and quantification of microglial activation.** Images were collected on an Olympus BX51 upright microscope using a × 40 objective and captured using a Coolscope ES camera. High power field images were taken of the subiculum, CA1 region of the hippocampus and the outer cortex at bregma −2.6 mm and −2.9 mm as shown in Supplementary Fig. 10b. These regions were chosen based on previous publications on pathology progression in 3 × Tg-AD mice [25]. Total microglia were counted and the percentage of IL-1\(\beta\) positive microglia recorded. Microglial morphologies were scored on an activation scale of 0 to 3 based on microglia categories previously described [25]. Scores of 0–3 were considered resting, and 2–3 considered activated (Supplementary Fig. 10b). Each section was treated as a technical replicate, as such scores were averaged for each animal. Counting and scoring were performed by a blinded observer. Example images were collected on a Leica TCS SP5 AOSB upright confocal using a × 63 objective and × 2 confocal zoom. Images were overlaid and stacked using Fiji ImageJ [26].

**Statistical analyses.** Data are presented as mean values ± standard error of the mean (s.e.m.). Statistical analyses performed were one-sample t-tests, one-way analysis of variance (ANOVA) and two-way ANOVA tests with Sidak corrected post hoc. Equal variance and normality were assessed with the Levene’s test and the Shapiro–Wilk test, respectively, and appropriate transformations were applied when necessary. Accepted levels of significance were *P < 0.05, **P < 0.01, ***P < 0.001. Statistical analyses were carried out using GraphPad Prism or SPSS. Images were processed using Fiji ImageJ as described by manual co-rendering with experiments blinded to image identity throughout. Flow cytometry data were analysed and populations quantified using FlowJo V10.
Data availability. The data that support the findings of this study are available from the corresponding author on request.

References

Additional information
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Supplementary Figure 1. Chemical structures of the NSAIDs tested. Non-fenamates are shown in the top row, and fenamates in the row below.
Supplementary Figure 2. The fenamate NSAIDs inhibit processing of IL-1β and caspase-1. (A) iBMDMs were primed with LPS (1 µg ml⁻¹, 2 h) treated with NSAID (100 µM, 15 min) and stimulated with ATP (5 mM, 1 h). IL-1β was detected by western blot. Bands indicate pro-IL-1β at 31 kDa and mature IL-1β at 17 kDa.
Supplementary Figure 3. None of the NSAIDs tested affect ATP-induced cell death. iBMDMs were primed for 2 h with 1 µg ml⁻¹ LPS and pre-treated with (A) celecoxib, (B) ibuprofen, (C) diclofenac, (D) flufenamic acid, (E) meclofenamic acid or (F) mefenamic acid at the indicated concentrations before stimulating with 5 mM ATP for 1 h. Lactate dehydrogenase (LDH) release was evaluated in cell supernatants using a CytoTox assay and data presented as % LDH release compared to ATP alone + s.e.m (n=4). Significance was determined by one-sample t-test vs. hypothetical value of 100 %.
Supplementary Figure 4. Fenamates do not covalently bind cysteine residues. Flufenamic acid or known cysteine modifier MNS (1 eq) and N-acetyl-L-cysteine methyl ester (2 eq) were dissolved in DMSO-<i>d</i><sub>6</sub> (0.5 ml) and kinetically profiled using NMR spectroscopy every 10 min for 2 h and then every 1 h until 24 h.
(A) The $^1$H NMR spectra of flufenamic acid with N-acetyl-L-cysteine methyl ester, annotated peaks are for flufenamic acid which remains unchanged after 24 h. (B) The $^1$H NMR spectra for control MNS shows complete conjugation with N-acetyl-L-cysteine methyl ester after 24 h. * indicates new peaks associated with cysteine modification, with disappearance of H$_1$/H$_2$ doublets of MNS: δ 8.07 and 8.11 (d, $J = 13.5$ Hz, 1H, $\text{C}_8\text{H}_7\text{NO}_2$ x 2). These conjugate peaks include: δ 1.846 (s, 3H, $\text{C}_3\text{H}_3$CO, 1 x diastereoisomer), 1.854 (s, 3H, $\text{C}_3\text{H}_3$CO, 2 x diastereoisomers, 1:1 ratio), 3.617 (s, 3H, $\text{C}_3\text{H}_3$O, 1 x diastereoisomer), 3.630 (s, 3H, $\text{C}_3\text{H}_3$O, 1 x diastereoisomer), 4.66 (t, $J = 8.0$ Hz, 1H, Ph$\text{C}_6\text{H}(\text{S})\text{CH}_2\text{NO}_2$), 5.03-5.12 (m, 2H, Ph$\text{C}_6\text{H}(\text{S})\text{CH}_2\text{NO}_2$), 6.02 (s, 2H, -$\text{OC}_6\text{H}_4$-), 6.87 (br s, 2H, Ph-H$_2$ and Ph-H$_5$), 7.06 (s, 1H, Ph-H$_6$), 8.40 (d, $J = 7.8$ Hz, $\text{NH}$, 1 x diastereoisomer), 8.41 (d, $J = 7.8$ Hz, $\text{NH}$, 1 x diastereoisomer).
Supplementary Figure 5. Fenamates do not affect intracellular Ca\textsuperscript{2+} or Na\textsuperscript{+} concentrations. iBMDMs were primed with 1 µg ml\textsuperscript{-1} LPS for 4 h. (A) Measurements of [Ca\textsuperscript{2+}] were performed in Fura 2 loaded cells. 5 mM ATP caused sustained increases in [Ca\textsuperscript{2+}], which were unaffected by 100 µM mefenamic acid. Summarised Ca\textsuperscript{2+} signals of 46 individual cells as a function of recording time. (B) Steady-state ratiometric Ca\textsuperscript{2+} signals determined before ATP application (control), following application of 5 mM ATP (ATP) and in the presence of ATP and 100 µM mefenamic acid (ATP + mefenamic acid). Data are presented as mean ± s.e.m (260 cells in 8 independent experiments). (C) Measurements of [Na\textsuperscript{+}] were performed in SBFI loaded cells. 5 mM ATP caused sustained increases in [Na\textsuperscript{+}], which were unaffected by 100 µM mefenamic acid. Summarised Na\textsuperscript{+} signals of 42 individual cells as a function of recording time. (D) Steady state ratiometric Na\textsuperscript{+} signals determined before ATP application (control), following application of 5 mM ATP (ATP) and in the presence of ATP and 100 µM mefenamic acid (ATP + mefenamic acid). Data are presented as mean ± s.e.m (277 cells in 8 independent experiments).
Supplementary Figure 6. Flufenamic acid and DCPIB inhibit the regulatory volume decrease (RVD) in THP-1 cells. THP-1 cells were primed with LPS (1 μg ml⁻¹, 4 h). Cells were then incubated in hypo-osmotic buffer (90 mOsm) plus or minus flufenamic acid (200 μM) or DCPIB (20 μM). Cell size was measured by flow cytometry (forward scatter width). Measurements were taken for 60 mins. Cell volume measurements were normalised against the average cell volume prior to hypotonic stimulus. Data are presented as mean ± s.e.m (n=4).
Supplementary Figure 7. Fenamates inhibit MSU-induced F4/80+ monocyte/macrophage infiltration and IL-1β production in an air pouch model of inflammation. (A&B) Subcutaneous air pouches were raised at the dorsum of C57BL/6 mice before injection of MSU (3 mg ml⁻¹, 6 h) with or without flufenamic acid (20 mg kg⁻¹) or vehicle (5 % Cremaphor EL, 5 % DMSO in sterile saline) in sterile PBS. Pouches were lavaged with 4 ml PBS, 1 % BSA, 5 mM EDTA and analyzed for IL-1β levels by ELISA (A) or leukocyte populations by flow cytometry (B). Upon quantification of total cell number it was observed that addition of 20 mg kg⁻¹ flufenamic acid significantly reduced MSU-induced total F4/80+ macrophage infiltration from 1.3 ± 0.61 to 0.14 ± 0.026 million cells (mean ± s.e.m., P<0.01, data not shown). (C-F) Immune cells were analyzed by gating for CD45+ leukocytes (C) then Ly6G+, F4/80- neutrophils or Ly6G-, F4/80+ macrophages/monocytes (D-F). Representative examples are shown of cell populations from mice treated with vehicle then vehicle (D), vehicle then MSU (E) and flufenamic acid then MSU (F). n=4-6. ELISA data are presented as mean IL-1β detected in lavage ± s.e.m. Flow cytometry data are presented as mean % F4/80+, Ly6G- population out of CD45+ population ± s.e.m. *P<0.05 determined by one-way ANOVA and Sidak corrected planned contrast post-hoc test.
Supplementary Figure 8. Mefenamic acid inhibits ATP-induced IL-1 production in a peritoneal model of NLRP3-dependent inflammation. Wild-type (WT) and NLRP3−/− mice were pretreated intraperitoneally (i.p.) with 50 mg kg⁻¹ mefenamic acid, the specific NLRP3 inhibitor MCC950, or vehicle before priming with 1 µg LPS. 4 h following the LPS injection animals were injected with drugs before stimulation with 100 mM ATP i.p. for 15 mins under anesthesia. IL-1β levels in peritoneal lavage (A) and plasma (B), and IL-1α levels in lavage (C) were measured by ELISA. Data are presented as mean IL-1 levels ± s.e.m (n=3-4). ###p<0.001 compared to WT saline control group; *p<0.05, **p<0.01, ***p<0.001 compared to WT ATP-treated groups determined by one-way ANOVA with Sidak’s multiple comparisons.
Supplementary Figure 9. Mefenamic acid reverses Aβ1-42 induced deficits in novel object recognition in vivo. Acute unilateral intracerebroventricular injection of soluble Aβ1-42 on day 0 (5 nmol in 10 μl) was followed by 14 days (starting one day prior to surgery) of i.p. injection of mefenamic acid (5 mg kg⁻¹) or vehicle. Animals were then tested in the NOR task on day 14 and day 35 post-surgery. Exploration of the objects at 14 d (A) and 35 d (B) post-surgery are presented as mean exploration time ± s.e.m (n=5-10 per group). **p<0.01 ***p<0.001 Familiar versus Novel, determined by one-way ANOVA followed by a within group paired student t-tests.
Supplementary Figure 10. (A) Locations of high-power field images depicted on a transverse section of mouse brain stained with cresyl violet. Images were taken as near as possible to the locations indicated at approximately bregma -2.3 mm, -2.6 mm and -2.9 mm. Scale bar is 50 μm. (B) Mean activation score of Iba1 stained microglia within the subicula of 3xTgAD and WT mice following vehicle or mfenamic acid treatment. Data are presented as mean ± s.e.m (n=8-10). Statistical analyses performed using two-way ANOVA followed by Sidak corrected post-hoc analysis. ###p<0.001 compared to vehicle/WT animals and **p<0.01 compared to vehicle/3xTgAD mice. (C) Method of rating the activation state of microglia on a scale of 0-3 and the method of binary identification of activated or resting microglia. Scale bars are 10 μm.
Supplementary Figure 11. There were no significant changes in microglia morphology or IL-1β expression in the CA1 (A-C) or outer cortical (D-F) regions in the triple transgenic Alzheimer's mouse model 3xTgAD. (A&D) Percentage of activated microglia in the CA1 region (A) and outer cortex (D). (B&E) Percentage of IL-1β expressing microglia in the CA1 regions (B) and outer cortex (E). (C&F) Mean microglial activation score of microglia in the CA1 regions (C) and outer cortex (F). Data are presented as mean ± s.e.m (n=8-10). Statistical analyses performed using two-way ANOVA and no significant effects were observed.
Supplementary Figure 12. Uncropped blots appearing in Figure 2C (A) and Supplementary Figure 2 (B).
Chapter 3: Boron-based inhibitors of the NLRP3 inflammasome

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Boron-Based Inhibitors of the NLRP3 Inflammasome

Highlights

- New inhibitors of the NLRP3 inflammasome are described
- NLRP3 inflammasome activation is independent of Ca^{2+}
- These new inflammasome inhibitors are effective in vivo
- The inhibitors described may lead to the development of new drugs

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In Brief

The NLRP3 inflammasome is known to contribute to damaging inflammation during disease. Baldwin et al. describe a new boron-containing series of inflammasome inhibitors, which may lead to the development of new anti-inflammatory molecules and allow further interrogation of inflammatory mechanisms.
Boron-Based Inhibitors of the NLRP3 Inflammasome

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SUMMARY

NLRP3 is a receptor important for host responses to infection, yet is also known to contribute to devastating diseases such as Alzheimer’s disease, diabetes, atherosclerosis, and others, making inhibitors for NLRP3 sought after. One of the inhibitors currently in use is 2-aminoethoxy diphenylborinate (2APB). Unfortunately, in addition to inhibiting NLRP3, 2APB also displays non-selective effects on cellular Ca\(^{2+}\) homeostasis. Here, we use 2APB as a chemical scaffold to build a series of inhibitors, the NBC series, which inhibit the NLRP3 inflammasome in vitro and in vivo without affecting Ca\(^{2+}\) homeostasis. The core chemical insight of this work is that the oxazaborine ring is a critical feature of the NBC series, and the main biological insight the use of NBC inhibitors led to was that NLRP3 inflammasome activation was independent of Ca\(^{2+}\). The NBC compounds represent useful tools to dissect NLRP3 function, and may lead to oxazaborine ring-containing therapeutics.

INTRODUCTION

Inflammation, which contributes to almost all known non-infectious diseases, is triggered by infection or injury sensed by pattern recognition receptors (PRRs) on inflammatory cells. Soluble PRRs have received particular attention due to their ability to form molecular complexes known as inflammasomes, which facilitate the release of inflammatory cytokines such as interleukin-1\(\beta\) (IL-1\(\beta\)), an important aspect of the inflammatory response. Inflammasomes are formed following the activation of cytosolic PRRs, of which the NOD-like receptor (NLR) family, pyrin domain-containing protein 3 (NLRP3), is the best characterized. The NLRP3 inflammasome is formed when NLRP3, described mainly in macrophages and monocytes, senses the presence of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Upon its activation, NLRP3 binds to the adapter protein ASC (apoptosis-associated speck-like protein containing a caspase activation and recruitment domain), which in turn recruits pro-caspase-1 to form an inflammasome complex. This results in the activation of caspase-1, which in turn cleaves pro-forms of the pro-inflammatory cytokines such as IL-1\(\beta\) and IL-18, causing their activation and facilitating their release from the cell (Latz et al., 2013). NLRP3-dependent cytokine release is implicated in the development of several important diseases (McGettrick and O’Neill, 2013; Heneka et al., 2015) and may represent a pharmacological target for the treatment of inflammatory disease (Coll et al., 2015; Daniels et al., 2016). Signaling mechanisms regulating the activation of NLRP3 remain to be fully characterized. One signaling mechanism proposed to regulate the activation of the NLRP3 inflammasome is an increase in intracellular calcium ([Ca\(^{2+}\)]) (Horng, 2014). Many reports suggesting an involvement of Ca\(^{2+}\) in inflammasome activation have used the Ca\(^{2+}\)-signaling inhibitor 2-aminoethoxy diphenylborinate (2APB, 1) (Lee et al., 2012; Murakami et al., 2012; Compan et al., 2012; Rossol et al., 2012).

2APB is a cell-permeable small-molecule inhibitor of Ca\(^{2+}\) homeostasis with multiple targets including inositol 1,4,5-triphosphate (InsP\(_3\))-dependent Ca\(^{2+}\) release, store-operated Ca\(^{2+}\) entry, and potentially also Ca\(^{2+}\) pumps and mitochondria, where effects are described as use-dependent and poorly reversible (Peppiatt et al., 2003). 2APB is also a poorly selective TRP (transient receptor potential) channel blocker (Schafer, 2014). However, recent evidence suggests that the effects of 2APB on inflammasome activation may be independent of an effect on Ca\(^{2+}\) (Katnelson et al., 2015). The utility of 2APB as an inhibitor of NLRP3, however, is limited by its non-selective effects on cellular Ca\(^{2+}\) homeostasis. Our aim was to develop new and potent inflammasome inhibitors based on the scaffold of 2APB but with reduced non-specific effects on Ca\(^{2+}\) homeostasis. We describe NBC6 (and its analogs) as completely new and potent inhibitors of the NLRP3 inflammasome that act independently of Ca\(^{2+}\).
Figure 1. Establishing the Importance of Boron in 2APB for NLRP3 Inflammasome Inhibition

(A–G) Mouse peritoneal macrophages were primed with bacterial endotoxin (lipopolysaccharide [LPS], 1 μg mL⁻¹, 2 hr) and then stimulated with vehicle (0.5% DMSO) or 2APB (75 μM) before stimulation with ATP (5 mM, 20 min) (A), nigericin (20 μM, 15 min) (B), sphingosine (20 μM, 1 hr) (C), monosodium urate crystals (MSU; 250 μg mL⁻¹, 1 hr) (D), calcium pyrophosphate dehydrate crystals (CPPD; 250 μg mL⁻¹, 1 hr) (E), or aluminum hydroxide (Alum; 250 μg mL⁻¹, 1 hr) (F). The half-maximal inhibitory concentration (IC₅₀) for the effects of 2APB on IL-1β release induced by ATP was established using a 3-parameter logistical sigmoidal model (G).

(legend continued on next page)
RESULTS

Inhibitory Effects of 2APB Require Boron

To establish that 2APB was a robust NLRP3 inflammasome inhibitor, mouse peritoneal macrophages were primed with LPS and then stimulated with a range of NLRP3 inflammasome-activating DAMPs. After LPS cells received pre-treatment with 2APB, which was then present for the duration of DAMP stimulation, 2APB inhibited the release of IL-1β in response to NLRP3 inflammasome activators ATP, nigericin, sphingosine, monosodium urate crystals (MSU), calcium pyrophosphate dihydrate crystals (CPPD), or aluminosilicate (Alum) (Figures 1A–1F), consistent with previous work reporting 2APB as an inhibitor of the NLRP3 inflammasome (Lee et al., 2012; Murakami et al., 2012; Compan et al., 2012; Rossol et al., 2012; Katsnelson et al., 2015). To identify the pharmacophore of 2APB responsible for the inhibition of IL-1β processing and release, we screened a small library of 2APB analogs on previously published data investigating the pharmacophore responsible for the effects of 2APB on store-operated Ca2+ entry (Dobrydenova and Blackmore, 2001). The acyclic structure of 2APB (1) is shown in Figure 1H, although in reality the ethanolamine coordinates to the boron (B) atom to give a 5-membered ring cyclic structure. Also shown is the analog diphenylborinic anhydride (DPBA, 2, the dimerized dehydrated form of diphenylborinic acid), diphenyldihydramine (DPH, 3, a carbon analog of acyclic 2APB in which there is no possibility of a closed ring form), 2,2-diphenyltetrahydrofuran (DPTTF, 4, which contains no B), and phenytoin (PHENY, 5, which is similar to 2APB in that it has two phenyl groups attached to a heterocyclic ring, but again does not contain B). To test the effects of these analogs, we primed mouse peritoneal macrophages with LPS and then pre-treated with drug before stimulation with ATP. The drug was also present throughout the ATP stimulation. The effects of the drugs were normalized to ATP-induced IL-1β release in the absence of any drug (Figure 1). The only analog to inhibit IL-1β release, in addition to 2APB, was the B-containing DPBA, with the other analogs having no effect (Figure 1). The half-maximal inhibitory concentration (IC50) for the effects of 2APB on IL-1β release was 67 μM (Figure 1G).

These data suggest the B atom is essential for the inhibitory effects of 2APB on IL-1β release. To further test the requirement for B, we used computational similarity searching using ROCS (rapid overlay of chemical structures) (Grant et al., 1999) and Tanimoto scoring (ShapeTanimoto and ColorTanimoto, for shape and chemical similarity, respectively), to identify diverse commercially available carbon analogs of 2APB for screening. Several of the top ranked hits, selected on shape and pharmacophore match (from a library of ~2 million non-B-containing compounds from the ZINC “LeadsNow” database, zinc.docking.org), were sourced via the repository of the NIH’s National Cancer Institute (NCI) Developmental Therapeutics Program and were screened against ATP-induced IL-1β release using primary mouse bone marrow-derived macrophages (BMDMs). BMDMs were primed with LPS and incubated with vehicle (0.5% DMSO) or molecules (NC1–7, 6–12, Figure 1H) at 40 μM (to allow any enhanced inhibitory activity to be observed) for 15 min before ATP stimulation (5 mM, 1 hr) (A). In all cases supernatants were analyzed by ELISA. Data are presented as mean percentage of IL-1β release versus vehicle (DMSO) control ± SEM (n = 3–9).

Refinement of the Structure-Activity Relationship

Given the apparent dependence on B, we screened a diverse library of commercially available B-containing compounds identified using SciFinder Scholar (called the BC series for boron compound) that shared some features and properties with 2APB (Figure S1). The BC molecules were screened against ATP-induced IL-1β release using primary mouse BMDMs as described above. Cells were primed with LPS and incubated with vehicle or molecules (BC1–24, Figure S1) before ATP stimulation. The effects of the molecules on IL-1β release were normalized to ATP-induced IL-1β release in the absence of any inhibitor (Figure 2A). Through this approach we identified analogs that were orders of magnitude more potent than 2APB at inhibiting IL-1β release (e.g., BC1, IC50 = 67 μM; BC7 (13), IC50 = 1.2 μM; BC23 (14), IC50 = 2.3 μM; Figures 2B and 2C). Our preliminary qualitative structure-activity relationship (SAR) analysis identified the importance of the diarylborinic acid motif and an oxazaborine ring, with conformationally restricted analogs showing enhanced activity (Figure 3). We then modified aspects of our lead BC molecules to improve activity and solubility, in addition to identifying the pharmacophore. Notably we modified the groups at each position of the oxazaborine ring (Figures 3A–3D), with atom numbering of the oxazaborine ring as shown in Figure 3E. A series of dioxo-, oxaz-, and diazaborines (novel boron compounds (BC7–11) based on the structures of BC7/23 were synthesized (for full details see Methods S1). In brief, 1,3-dicarbonyls were reacted with acetonitrile derivatives in the presence of a metal catalyst (Zn(acac)2 or SnCl2) to yield acetylated enaminones, by adapting previously reported methods (Veronese et al., 1986; Singh and Lesher, 1979). These intermediates were readily deacetylated by treating with K2CO3 (Veronese et al., 1986). Subsequent borylation of these enaminones using DPBA afforded the corresponding oxazaborine NBC molecules using a method similar to that previously described (Vasič et al., 1992, Donkóvá et al., 1995) (Figure 3A). The synthesis of oxazaborine NBC18 used similar chemistry, except that the starting material was cyanocacetamide (Figure 3A). Diazaborines were synthesized by directly borylating 1,3-dicarbonyls (Bally et al., 1965) (Figures 3C–3E). Reaction of BC23 with a range of alkyl amines yielded diazaborines (Figure 3D) adapted from Vasič et al. (2012).
Reaction of BC23 with ammonia did not give the expected diazaborine product, and only the dechlorinated compound containing a CHCl group (NBC29) was isolated. cLogP and cLogS calculations were performed for BC7, BC23, NBC1–31, and NBC-EPPS (Figure 3B), and demonstrate that a number of potent NBC molecules (NBC6, 18, 24) have improved physicochemical properties compared with the original lead compounds BC7 and BC23. Furthermore, an experimental LogS value for our lead analog NBC6 was found to be −1.63 in MeOH (0.7 mg mL−1) (data not shown). These data suggest that the NBC molecules are sparingly soluble in aqueous solution.

To screen the NBC series we used the human monocytic THP-1 cell line, since these cells would allow a higher throughput compared with the primary cells used above. Cells were primed with LPS and then treated with vehicle or NBC molecule (NBC1–31) at 10 μM for 15 min before activation of the inflammasome and IL-1β release with nigericin. The NBC molecule was lacking the CCl chelate (NBC8) was the only analog (NBC23) analogs. A 

The effects of the molecules on IL-1β release were measured by ELISA and normalized to ATP-induced IL-1β release in the absence of any inhibitor (A). The chemical structures (i) and half-maximal inhibitory concentration curves (IC50) for BC7 (B) and BC23 (C) are also presented using a 3-parameter logistical sigmoidal model. Data are presented as mean percentage of IL-1β release versus vehicle (DMSO) control ± SEM of at least 3 experiments. *p < 0.05, ***p < 0.001, significant difference from 100% IL-1β release (Holm-Sidak corrected one-sample t test). ***p < 0.001, significant improvement from 2APB treatment (Holm-Sidak corrected post hoc comparison).
**Oxazaborines**

\[ \text{Type A} \]

\[ \text{Type B} \]

**Dioxaborines**

**Diazaborines**

E

Fi

Aniline group increases activity, short aryl attachments favoured

Modulates activity, deacetylation detrimental

CCl₃ substituent essential

NH substituent essential

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chain length of the substituent was not too short ($R_1 = \text{Me}$, NBC12) or too bulky ($R_1 = \text{pyrene}$, NBC22). Full substitution of the primary amide (NBC6) to the $N,N'$-dimethyl tertiary amide derivative (NBC11, 16) lost bioactivity, potentially due to a change in chelation (see X-ray in Figures 4A and 4B). It was interesting to note that secondary amide derivatives containing either phenyl (NBC13) or cyclohexyl (NBC19, 17) substitutions are both active, showing that the ring can be either unsaturated or saturated.

Summary, an oxazaborine scaffold and a CCl$_3$ group on the 4-position was required to inhibit IL-1$\beta$ release (Figure 3E). From the screen of NBC molecules, NBC6 was most potent and more drug-like compared with leads BC7/23, and further analysis revealed increased potency with an IC$_{50}$ of 574 nM (Figure 3F).

X-Ray Crystallography and Computational Modeling of NBCs

When borylating the enaminone intermediates using DPBA, the oxazaborine product could adopt a number of different B chelate structures. For example, NBC6 and NBC11 could chelate to B through the NH of the enamine and either the amide or ketone C=O. To determine the structures of the chelates in the solid phase, crystals of NBC6 and NBC11 were grown in $n$-hexane/toluene (1:1) and X-ray crystallographic analysis was undertaken. The $R$ factors obtained for NBC6 and NBC11 were 4.08% and 2.83%, respectively. The B atom lies out of the ring plane in a boat-envelope conformation in both oxazaborine structures, whereas the other atoms in the heterocycle are planar and are involved in a $\pi$-electron conjugated system (Figure 4A) as previously reported for other oxazaborines (Josefíka et al., 2012; Mikyseka et al., 2017). NBC6 is chelated to B through O/N chelation of the amide C=O and enamine NH (Figure 4A).

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Figure 3. Refinement of the Structure-Activity Relationship

(A) Pathway for oxazaborine syntheses. The method for the synthesis of the oxazaborine compounds are described as types A, B, and C. (i) RCN, Zn(acac)$_2$/SnCl$_4$, dichloromethane/toluene, room temperature to 80°C, 3–16 hr. (ii) K$_2$CO$_3$(sat), EtOH, room temperature, 24 hr. (iii) DPBA, tetrahydrofuran (THF), 50°C, 16 hr. (iv) Cl$_3$CCN, NaOAc, EtOH, room temperature, 16 hr. (A).

(B-D) Table of oxazaborines synthesized with structure type (A, B or C) identified (B). Ad, adamantyl; Cy, cyclohexyl; Pyr, pyrene; Py, pyridinyl; EPPS, 4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid. Also shown in (B) to (D) is the percentage of inhibition of IL-1$\beta$ release from LPS and nigericin-treated THP-1 cells with 10$\mu$M inhibitor and the calculated cLogP and cLogS values for each compound. (C) Pathway for dioxaborine synthesis. (iii) DPBA, THF, 50°C, 16 hr.

**NBC9 was isolated as a by-product during NBC5 synthesis. (D)** Pathway for diazaborine synthesis. (v) RNH$_2$, THF, 50°C, 24 hr.

(E) Summary of SAR analysis of NBCs.

(F) Half-maximal inhibitory concentration curve (IC$_{50}$) for NBC6 (F) is presented using a 3-parameter logistical sigmoidal model (n = 6) (F).

*p < 0.05, **p < 0.01, ***p < 0.001, significant difference from 100% IL-1$\beta$ release (Holm-Sidak corrected one-sample t test), n = 4. ns, not significant.
whereas NBC11 is chelated to B through O/N chelation of the primary amide (NBC8) to an N,N-dimethyl tertiary amide (NBC11) has induced a change in B chelation, which could explain the large difference in observed bioactivity between these two oxazaborines (Figure 3B).

Computational modeling was applied to further investigate the impact of the shape and electronic properties of the NBCs on the bioactivity and observed SAR. All of the compounds were energy optimized at the M06-L/6-31G* level of quantum mechanics (Zhao and Truhlar, 2008). Consistent with the X-ray crystal structures (Figure 4E), computations predicted a highly puckered structure for NBC6, with a mean unsigned ring pucker torsion of 3.44°, compared with values of 3.04° for N and 3.44° for O (Table S1). The average mean unsigned ring pucker torsion was very good: for NBC6, the calculated value was 0.46°, whereas NBC11 the difference is 5.7° (Table S1).

Indeed, the agreement in ring pucker of the 6-membered ring between calculations and the X-ray structures was very good: for NBC6, the mean unsigned pucker was 112.5° (Table S1). Furthermore, the N-B-N angle in these compounds NBC6 and NBC27 is 111.2° for NBC27 and NBC30, respectively, Table S1. This indicates significant deviation from planarity; the greatest puckers for both rings centered at the B atom is on average 0.59°. The average magnitude of the partial atomic charge on carbon C 5 is 0.51 for NBC6 and 0.50 for NBC27, and NBC30 (±0.5 e, Table S1). For oxazaborines, a higher value of q(Ca) appears to be due to the presence of an amido substituent at C6. The correlation of q(C6) and observed activity of the compounds is somewhat modest, with a correlation coefficient r2 of 0.5 (a similar correlation is found for the C2-C2 bond distance); this reflects the influence of other factors, in particular steric constraints on substituents. 3D-QSAR was performed using the topper comparative molecular field analysis (CoMFA) method (Cramer, 2003), based on oxazaborines NBC1–6, 8, 11–20, 22–26, and 28–29 compounds (Table S1).

In summary, the density functional calculations agree well with crystallographically determined geometries and electronic character of the oxazaborine ring that is distinct from the planar, aromatic diazaborine ring, suggesting that these features are responsible for the activity of the oxazaborines. Within the oxazaborine series, there is evidence of a specific steric constraint on substituents at position 6 of the ring.

**Mechanism of Action**

We measured the effects of 2APB, BC7, BC23, and NBC6 on ASC speck formation following ATP stimulation. Immortalized (i)BMDMs transduced with a lentiviral vector to express ASC-mCherry (Daniels et al., 2016) were treated with LPS and then stimulated with ATP for between 30 and 45 min with ASC speck formation measured as described previously (Daniels et al., 2016). 2APB was an effective inhibitor of ASC speck formation, as were BC23 and NBC6 (Figure 4A). Additionally, we showed that 2APB is not a direct inhibitor of caspase-1. Recombinant caspase-1 was incubated with vehicle, YYAD, or 2APB before addition of the fluorogenic substrate Z-YVAD-AFC. Caspase-1 activity was measured 2 hr later. Under these conditions 2APB had no effect on caspase-1 activity while YYAD caused complete inhibition (Figure 5B). We also used a hypotonic THP-1 cell lysis assay to measure the effects of 2APB on caspase-1 activity. 2APB was added to the cells just prior to, or following, lysis in hypotonic buffer. The lysate was incubated with the caspase substrate Z-YVAD-AFC, which in addition to caspase-1 would also be cleaved by caspase-4 and -5, and caspase activity was measured 2 hr later. 2APB had no effect on caspase-1 activity under these conditions, whereas caspase-1 activity was completely inhibited by YYAD or high K+ concentration (Figure 5B).

Further insight into electronic distribution was obtained from Mulliken population analysis: firstly we note that the B atom is predicted to possess a positive partial charge in all of the NBC compounds analyzed (Figures 4C and 4D; Table S1). The charge on the B atom is on average 0.59 e for the 24 O-B-N compounds, 0.60 e for 3 O-B-O compounds, and somewhat less for the 3 N-B-N compounds, with an average value of 0.51 e. This reflects the low Pauling electronegativity of the B atom, reported as 2.04 compared with values of 3.04 for N and 3.44 for O (Allen, 1989). Thus, although we traditionally represent 4-coordinate B atoms with a formal negative charge (e.g., Figure 3), quantum chemical analysis predicts that the B atom in the heterocycle of the NBC compounds possesses a partial positive charge. The magnitude of the partial atomic charge on carbon C 5, q(C 5) is higher for NBC6 and NBC19 (±0.6 e) compared with less active compounds NBC11, NBC27, and NBC30 (±0.5 e, Table S1).

Compounds NBC11, NBC27, and NBC30 (±0.5 e, Table S1). For oxazaborines, a higher value of q(C 5) appears to be due to the presence of an amido substituent at C6. The correlation of q(C 6) and observed activity of the compounds is somewhat modest, with a correlation coefficient r2 of 0.5 (a similar correlation is found for the C2-C2 bond distance); this reflects the influence of other factors, in particular steric constraints on substituents. 3D-QSAR was performed using the topper comparative molecular field analysis (CoMFA) method (Cramer, 2003), based on oxazaborines NBC1–6, 8, 11–20, 22–26, and 28–29 compounds (Table S1).

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Figure 5. NBCs Are Effective NLRP3 Inflammasome Inhibitors

(A) The effects of 2APB, BC7, BC23, and NBC6 on ASC speck formation following ATP stimulation were measured. iBMDMs stably expressing ASC protein conjugated to mCherry were primed with LPS (1 μg mL⁻¹, 2 hr), then pre-treated with selected drug (indicated concentration, 15 min) before stimulation with ATP (legend continued on next page)
in the absence and presence of NBC6 (10 and 30 μM) on NLRP3 inflammasomes in primary wild-type (WT) and NLRP3 KO BMDMs. LPS-primed WT BMDMs were treated with ATP in the absence and presence of NBC6 (10 and 30 μM), the established NLRP3 inhibitor MCC950 (Coll et al., 2015) (30 μM), and the caspase-1 inhibitor YVAD (100 μM). Under these conditions, all inhibitors inhibited the release of IL-1β (Figure 5D). These data suggest that NBC6 can also inhibit NLRP3 via the non-canonical pathway. To determine whether the NBCs were selective inhibitors of NLRP3-dependent IL-1β release, we tested their effects against other well-characterized inflammasomes in primary wild-type (WT) and NLRP3 KO BMDMs. LPS-primed WT BMDMs were treated with ATP in the absence and presence of NBC6 (10 and 30 μM), the established NLRP3 inhibitor MCC950 (Coll et al., 2015) (30 μM), and the caspase-1 inhibitor YVAD (100 μM). Under these conditions, all inhibitors inhibited the release of IL-1β (Figure 5D). These data suggest that NBC6 selectively inhibits NLRP3 at low doses but may also be effective against other inflammasomes at higher doses. To further establish that NBC6 inhibits NLRP3 across cell types, neutrophils were isolated from WT and NLRP3 KO murine bone marrow and primed with LPS followed by nigericin treatment in the presence or absence of 10 μM NBC6. From this we observed complete inhibition of NLRP3-dependent IL-1β release from NLRP3-treated neutrophils (Figure 5F).

We next compared the toxicity of NBC6 with that of MCC950 in kidney (HEK293) and liver (HepG2) cell lines. Neither drug increased cell death (Figure 6C). Together, these data show that the NBCs are effective inhibitors of the NLRP3 inflammasome and can also target NLRP3-dependent inflammasomes in vivo.

[5 mM, 30–45 min] under live microscopy. Formation of ASC specks (examples indicated by white arrows, A [no drug], A [plus NBC6]) were quantified (Aii) and presented as mean percentage of specks counted versus vehicle + SEM (n = 3–6). *p < 0.05, **p < 0.01, ***p < 0.001, significant difference from 100% speck formation (Holm-Sidak corrected one-sample t test, n = 5–6). Scale bars, 20 μm.

(B) Recombinant caspase-1 (10 μg/mL) was incubated with 0.5% DMSO, YVAD (100 μM), or 2APB (75 μM) before addition of the fluorogenic substrate Z-YVAD-AFC. Caspase-1 activity was measured 2 hr later (Bii) (*p < 0.01, significant difference from vehicle control, Holm-Sidak corrected post hoc comparison, n = 4). Hypothetical THP-1 cell lysate assay was also used to measure the effects of 2APB on caspase-1 activity. 2APB (75 μM) was added to the cells just prior to, or following, lysis in hypotonic buffer. The lysate was incubated with 2-YVAD-AFC and caspase-1 activity measured 2 hr later (Biii). YVAD or high K+ concentration were included as controls (Biii) (**p < 0.01, significant difference from relevant lysate vehicle control, Holm-Sidak corrected post hoc comparison, n = 4).

(C) LPS-primed (1 μg/mL, 4 hr) mouse primary BMDMs were treated with NBC6 (10 μM) or vehicle (DMSO) 15 min prior to 1 hr treatment with small-molecule NLRP3 activator imiquimod (70 μM) or DMSO control. Imiquimod significantly induced IL-1β release ("p < 0.01) and this was inhibited by NBC6 treatment ("p < 0.05, Holm-Sidak corrected post hoc comparison, n = 4).

(D) Mouse primary BMDMs were primed with Pam3CSK4 (100 μg/mL, 4 hr) followed by 15 min NBC6 (1 μM), MCC950 (1 μM), or vehicle, then treated with intracellular LPS (2 μg/mL), transfected with Lipofectamine 3000, 24 hr, or Lipofectamine alone ("p < 0.01, significant induction of IL-1β) [Dii] or IL-1α [Diii] release versus Lipofectamine-alone control. *p < 0.01, significant inhibition of IL-1β release, Holm-Sidak corrected post hoc comparison, n = 4–6.

(E) Mouse primary BMDMs were primed with LPS (1 μg/mL, 4 hr) followed by 15 min NBC6 (10 and 30 μM), MCC950 (30 μM), YVAD (100 μM), or vehicle, then treated with canonical NLRP3 activator ATP (5 mM, 1 hr), NLRP3 activator (flagellin, 667 ng/mL), or AIM2 activator (poly[dA:dT], 667 ng/mL), transfected with Lipofectamine 3000 ("p < 0.05, "p < 0.01, ***p < 0.001, significant inhibition of IL-1β release, Holm-Sidak corrected post hoc comparison, n = 3).

(F) Mouse primary bone marrow neutrophils from WT and NLRP3 KO mice (n = 4) were primed with LPS (1 μg/mL, 2 hr), then NBC6 (10 μM) was added 15 min prior to the addition of nigericin (10 μM), which significantly induced IL-1β release ("p < 0.01), which was inhibited by NBC6 treatment ("p < 0.01, Holm-Sidak corrected post hoc comparison). Data are presented as the mean ± SEM.
We recently reported that the fenamates were effective inhibitors of the NLRP3 inflammasome due to inhibition of Cl⁻/C₀ efflux through the volume-regulated anion channel (VRAC) (Daniels et al., 2016). Thus we tested the effects of BC23, BC7, and NBC6 on VRAC. VRAC currents measured by whole-cell patch

![Diagram]

Figure 6. NBCs Are Effective against NLRP3 In Vivo

(A) HEK293 or HepG2 cells were treated with NBC6 (10 μM), MCC950 (10 μM), or DMSO for 4 hr, 8 hr, and 24 hr. Cell death was measured by lactate dehydrogenase release and expressed as percentage lysis control. No significant effects were observed (two-way repeated-measures ANOVA).

(B) LPS-primed (1 μg mL⁻¹, 2 hr) BMDMs were pre-treated with drugs (BC23, NBC6, 30 μM; MNS, 100 μM; YVAD, 100 μM; 2APB, 75 μM) or vehicle (DMSO) in serum-free media for 15 min and washed 3 times, before inflammasome activation was initiated by adding ATP (5 mM) for 1 hr. IL-1β release was measured by ELISA (*p < 0.05, **p < 0.01, ***p < 0.001, significant inhibition of IL-1β release compared with vehicle-ATP control; #p < 0.05, ###p < 0.001, significant effect of washing compared with no-wash drug-ATP control, Holm-Sidak corrected post hoc comparison, n = 5-6).

(C) C57BL/6 and NLRP3 KO mice (n = 6) were injected intraperitoneally with LPS (10 mg kg⁻¹, 3 hr). Separate groups of WT animals receiving LPS were also given a 50 mg kg⁻¹ dose of MCC950 or NBC13. IL-1β in peritoneal lavage (Ci) and plasma (Cii) was measured by ELISA. IL-1α in plasma was measured by ELISA (Ciii). ***p < 0.001, significant difference from saline vehicle control. *p < 0.01, **p < 0.001, significant difference from LPS vehicle group (Holm-Sidak corrected post hoc comparison).

Data are presented as the mean ± SEM.
After establishing that B was essential for inhibition through zomib (Velcade) is the only B-containing drug used clinically. Boron is an unusual element to be present in drug leads; bortezomib (NLRP3 to disease has led to efforts to develop small-molecule and Dixit, 2012). Increasing recognition of the contribution of both of these experimental manipulations may inhibit the inflammasome independently of effects on Ca$^{2+}$ and that the effect on Ca$^{2+}$ for the NBC compounds is not significant.

**DISCUSSION**

The NLRP3 inflammasome contributes to inflammatory diseases and is therefore an important therapeutic target (Lamkanfi and Dixit, 2012). Increasing recognition of the contribution of NLRP3 to disease has led to efforts to develop small-molecule inhibitors (Baldwin et al., 2016). Here we report the development of a unique B-based pharmacophore that inhibits NLRP3-dependent inflammation in both in vitro and in vivo models. Boron is an unusual element to be present in drug leads; bortezomib (Velcade) is the only B-containing drug used clinically. After establishing that B was essential for inhibition through screening of carbon analogs, we synthesized a range of new B-based inhibitors of NLRP3 derived from the early leads 2APB, BC7, and BC33, the most potent compound having an IC$_{50}$ value of 574 nM for the inhibition of release of IL-1$eta$ from THP-1 monocytes. During the preparation of the oxazaborines, for the synthesis of acetylated enamino intermediates, we found that the choice of the metal catalyst was critical. Zr(C$_5$H$_3$)$_4$ was amenable with good electrophiles (e.g., trichloroacetanilide and benzonitrile); however, the stronger catalyst SnCl$_4$ was required with non-electrophilic, weakly activating nitriles (e.g., acetonitrile and tert-butyl acetonitrile). The oxazaborines synthesized herein can be handled easily at room temperature, in contrast to the facile hydrolysis observed for 2APB (Hofer et al., 2013). Molecular modeling calculations agreed well with the X-ray crystal structures of NBC6/11, demonstrating the robustness of predictions using quantum mechanics. This is the first time that biological screening of oxaza-, diaza-, and diazaborines has been reported. The SAR of the 31 NBC molecules revealed interesting key features required for bioactivity, with the oxazaborine ring and CCl$_3$ group being essential pharmacophores for NLRP3 inflammasome inhibition. NLRP3 is composed of three domains: a N-terminal pyrin domain for homotypic interaction with the pyrin domain of the adaptor ASC, a central NACHT domain that binds nucleotides, and a C-terminal leucine-rich repeat domain that senses the PAMPs or DAMPs. The mechanisms regulating the activation of NLRP3 are currently the focus of a major research effort in the field and are still being elucidated. There is very limited evidence for an interaction between PAMP/DAMP and NLRP3; instead these activating stimuli activate a common pathway dependent upon K$^+$ efflux (Muñoz-Planillo et al., 2013). Recently, the protein NEK7 has been identified as an interacting partner of NLRP3 required for its activation (Schmid-Burgk et al., 2016; Shi et al., 2016), and this interaction is also downstream of K$^+$ efflux (Fie et al., 2016). In addition, ubiquitination and deubiquitination are also becoming established as essential steps (Lopez-Castejon et al., 2013; Juliana et al., 2012; Py et al., 2013). There has also been substantial literature to support an involvement of Ca$^{2+}$ in inflammasome activation. Many of the studies reporting Ca$^{2+}$ dependence of NLRP3 activation have been based on, or involved the use of 2APB as an inhibitor of Ca$^{2+}$ signaling (Lee et al., 2012; Murakami et al., 2012; Compan et al., 2012; Rossol et al., 2012), or intracellular Ca$^{2+}$ chelators such as BAPTA-AM (Brough et al., 2003). However, there is now evidence that both of these experimental manipulations may inhibit the inflammasome independently of effects on Ca$^{2+}$ (Katsnelson et al., 2015). Further evidence for an effect independent of Ca$^{2+}$ is provided in Figure 1, and is related to the work of Dobrydneva and Blackmore (2001) on store-operated Ca$^{2+}$ entry. While they showed that DPBA inhibits Ca$^{2+}$ entry (and IL-1$eta$ release presented here), DPTTF, which had no effect on IL-1$eta$ release in our study, inhibited Ca$^{2+}$ entry as effectively as DPBA (Dobrydneva and Blackmore, 2001). Such data further support that the effects of 2APB on IL-1$eta$ processing and release are independent of Ca$^{2+}$. Here we also show that while 2APB effectively inhibits increased [Ca$^{2+}$]$_i$ in response to 100 nM ATP, NBC6 does not, even at concentrations supra-maximal for the inhibition of IL-1$eta$ secretion (Figure 7). Furthermore, addition of 2APB, or NBC6 after the addition of 5 mM ATP, an NLRP3-activating stimulus, did not modify [Ca$^{2+}$]$_i$ (Figure 7). When these data suggest strongly that the effects of 2APB on IL-1$eta$ release are independent of Ca$^{2+}$, and that we have deselected this property in our NBC molecules. This effectively provides a new and unique chemical scaffold for the development of NLRP3-inhibiting drugs that do not have the potentially harmful off-target effects on Ca$^{2+}$ homeostasis.

**SIGNIFICANCE**

Excellent evidence now points toward NLRP3 as an important therapeutic target for multiple major diseases (Guo et al., 2015). There are no drugs available clinically that specifically target NLRP3, although we (Daniels et al., 2016)
Figure 7. Ca\textsuperscript{2+}-Independent Effects of the NBCs

(A) To induce volume-regulated Cl\textsuperscript{-}/C\textsubscript{0} currents (VRAC), LPS-primed (1 \( \mu \)g mL\textsuperscript{-1}, 2 hr) iBMDMs were superfused with hypotonic solution. Representative current traces are shown, which have been measured in the absence (VRAC) or presence of 30 \( \mu \)M NBC6 (Ai), 30 \( \mu \)M BC7 (Aii), or 30 \( \mu \)M BC23 (Aiii).

(B–E) LPS-primed (1 \( \mu \)g mL\textsuperscript{-1}, 2 hr) iBMDMs were kept untreated or were pre-treated for 2 min with 75 \( \mu \)M 2APB or 30 \( \mu \)M NBC6. Subsequently, 100 \( \mu \)M ATP was added to the bath solution. (B–D) Representative Ca\textsuperscript{2+} traces of ATP-stimulated cells in the absence (B, \( n = 12 \)) or presence of 75 \( \mu \)M 2APB (C, \( n = 14 \)), or 30 \( \mu \)M NBC6 (D, \( n = 12 \)). (E) Mean peak Ca\textsuperscript{2+} concentrations determined in cells treated with ATP alone (ATP) or with ATP in the presence of inhibitors. ns, no significant difference; \( ***p < 0.001 \), significant difference from Ca\textsuperscript{2+} signals of ATP-stimulated cells determined in the absence of inhibitors (Holm-Sidak corrected one-sample t test).

(F and G) LPS-primed iBMDMs were also stimulated with 5 mM ATP. Following development of sustained Ca\textsuperscript{2+} increases, 75 \( \mu \)M 2APB or 30 \( \mu \)M NBC6 was added to the ATP-containing solution. Images show representative examples of Ca\textsuperscript{2+} responses following treatment with ATP and the addition of 2APB (\( n = 12 \), F) or the addition of NBC6 (\( n = 12 \), G).

(H and I) LPS-primed iBMDMs were treated with concentrations of the inhibitors maximal for blocking IL-1\( \beta \) release (i.e., 2APB = 75 \( \mu \)M, NBC6 = 30 \( \mu \)M). Inhibitors were added to the iBMDMs 5 min before or 5 min after the addition of ATP (5 mM, 1 hr). (H) With IL-1\( \beta \) release measured by ELISA (I). \( ***p < 0.001 \), significant difference from corresponding vehicle control; ns, no significant effect of ATP administration time.

Data are presented as representative traces from calcium imaging experiments (B–D, F, and G), mean + SEM peak Ca\textsuperscript{2+} concentrations versus treatment with ATP alone (E), or mean + SEM IL-1\( \beta \) release as detected by ELISA (I).
and others (Fowler et al., 2014) have shown that some existing drugs may be repurposed. There is, however, a need for new inhibitors. MCC950 (formerly CRID3 or CP-456,773) is being developed as a potent and selective inhibitor of NLRP3 (Coll et al., 2015). Here we report a new class of molecules based on the oxazaborine ring that will further accelerate the development of NLRP3 inhibitors for use in disease and in generating new fundamental insights.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure, one table, seven schemes, and supplemental text and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2017.08.011.

AUTHOR CONTRIBUTIONS


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We thank Paul MacLellan and Sonya Anabalsi for initial support with organic synthesis. Thanks to Neil O’Hara for assistance with NMR kinetic runs and the Mass Spectroscopy Service in the School of Chemistry, University of Manchester. We thank Dr P.N. Horton and Dr G.J. Tizard of the UK National Crystallography Service, Southampton, for collection of X-ray crystallographic data. We are also grateful to Dr. Amaud Garcon and UMPI for guidance and financial support provided to this project. Preliminary work in this project was supported by a Welcome Trust fellowship (D.B. grant ref. no. 083482/2/57/2). We are also grateful to the SBC Open Innovation Challenge. This work was also supported by the Medical Research Council Confidence in Concept scheme (grant ref. no. MC_PC_13070). A.G.B. is funded by the Division of Pharmacy and Optometry, University of Manchester and the Presidental Doctoral Scholar award. M.J.D.D. is funded by an MRC DTP studentship (MR/K501311/1). C.B.L., J.R.-A., and D.B. are funded by the Alzheimer’s Society (211/AS-PG-2013-2007). We are grateful to Dr. Vishva Dixit (Ganentech) for providing the NLRP3 KO mice and to Prof. Clare Bryant (University of Cambridge) for providing the immortalized BDMMs.

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REFERENCES


### STAR METHODS

#### KEY RESOURCES TABLE

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**Critical Commercial Assays**

| IL-1α ELISA (mouse) | R&D Systems | DY401 |
| IL-1α ELISA (mouse) | R&D Systems | DY400 |
| IL-1α ELISA (human) | R&D Systems | DY201 |
| CytoTox 96® Non-Radioactive Cytotoxicity Assay | Promega | G1780 |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to the Lead Contact, David Brough (David.brough@manchester.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Culture

Primary peritoneal macrophages were prepared as described previously (Le Feuvre et al., 2002). Briefly, peritoneums of male and female C57BL/6 mice (Charles River) were lavaged with 8 ml RPMI 1640 media and cells in the exudate cultured at a density of $1 \times 10^6$ cells ml$^{-1}$ in RPMI media supplemented with 10% fetal bovine serum (FBS), 100 U ml$^{-1}$ penicillin and 100 $\mu$g ml$^{-1}$ streptomycin (PenStrep). Primary bone marrow-derived macrophages (BMDMs) and primary bone marrow neutrophils were prepared by flushing femurs of male and female wild-type C57BL/6 or NLRP3 KO mice. Red cells were then lysed. BMDMs were generated by culturing the resulting bone marrow cells in 70% DMEM (containing 10% FBS, PenStrep) supplemented with 30% L929 mouse fibroblast-conditioned media for 7-10 days. Before experiments, cells were seeded overnight at $1 \times 10^6$ ml$^{-1}$ in 96-well plates. Neutrophils were isolated by density centrifugation of the extracted bone marrow cells in a 64% isotonic Percoll (Sigma-Aldrich) at 1,000xg for 30 min at 4°C. The pellet was then resuspended in RPMI (containing 10% FBS, PenStrep), counted, centrifuged again (2,000xg, 5 min), resuspended at $1 \times 10^6$ ml$^{-1}$, plated in 96-well plates and experimented on immediately. Purity (>90%) and viability (>95%) were determined by Diff-Quik$^\text{TM}$ staining (Cools-Lartigue et al., 2013). THP-1 peripheral blood monocyte-like cells were cultured in RPMI medium supplemented with 10% FBS, PenStrep, 20 mM L-Glutamine and 55 $\mu$M 2-mercaptoethanol. On the day of experiments, cells were seeded overnight at $1 \times 10^6$ ml$^{-1}$ in 96-well plates. Immortalized murine bone marrow-derived macrophages (iBMDMs) (Hornung et al., 2008) and iBMDMs stably expressing ASC conjugated to mCherry protein (Daniels et al., 2016) were cultured in DMEM, 10% FBS, PenStrep. HEK293T kidney cells and HepG2 liver cells were cultured in DMEM, 10% FBS, PenStrep.

In Vivo Peritoneal Inflammation Model

Animals were maintained under standard laboratory conditions: ambient temperatures of 21°C (± 2°C), humidity of 40-50%, 12 h light cycle, ad libitum access to water and standard rodent chow. All procedures were performed blinded to genotype. Treatment
allocations were randomly allocated using True Random Generator software. All animal experiments were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and approved by the Home Office and the local Animal Ethical Review Group, University of Manchester. Male WT C57BL/6 and strain matched NLRP3 KO mice (30 - 35g) were co-administered intraperitoneally (i.p.) with NBC13 (50 mg kg$^{-1}$), MCC950 (50 mg kg$^{-1}$) or vehicle (corn oil), and 10 mg kg$^{-1}$ LPS (from Escherichia coli 0127:B8) or saline control (n=6 per group). Three hours following injection the mice were anesthetized with 3-5% isoflurane, their peritoneums were lavaged with 3 ml of RPMI media and plasma taken by cardiac puncture. Levels of IL-1β in the plasma and lavage and IL-1α in the plasma were analysed by ELISA (Duoset, R&D systems).

**METHOD DETAILS**

**ASC Speck Imaging**
Live imaging of ASC speck formation was performed using IBMDMs transfected to stably express ASC conjugated to mCherry protein (Daniels et al., 2016). Stably transduced cells were plated overnight at 5x10$^5$ cells ml$^{-1}$. The following day, cells were primed with LPS (1 μg ml$^{-1}$, 2 h). 1 h into priming, Hoechst 33342 (2 μM ml$^{-1}$). Immunocytochemistry was added to aid identification of the cells. Following priming, media was changed to DMEM containing 25 mM HEPES pH 7.4 and cells transferred to a BD Pathway Bioimager 855 (BD Biosciences) and imaged at 37°C as described previously (Daniels et al., 2016). Cells were pre-treated with 2APB, BC7, BC23, NBC6, or vehicle for 15 min before imaging.

**Caspase-1 Assays**
The caspase-1 activity of THP-1 cells was determined with the fluorogenic substrate Z-YVAD-AFC (caspase-1 substrate VI, Calbiochem) as previously described (Lopez-Castejon et al., 2013). Briefly, cells were lysed in hypotonic cell lysis buffer (25 mM HEPES, 5 mM EGTA, 5 mM dithiothreitol (DTT), pH 7.5) on ice for 5–10 min and centrifuged to remove the insoluble fraction (12,500 g, 10 min). THP-1 lysates (50 μl) or recombinant caspase-1 (10 U ml$^{-1}$) was incubated with 50 μM YVAD-AFC and 50 μl of reaction buffer (0.2% CHAPS, 0.2 M HEPES, 20% sucrose, 29 mM DTT, pH 7.5) for 2 h. After incubation, the fluorescence of the AFC released from the Z-YVAD-AFC substrate was measured by an increase in fluorescence (excitation 335 nm, emission 460 nm).

**Inflammasome Activation Assays**
Peritoneal macrophages were primed with LPS (1 μg ml$^{-1}$, 2 h) before incubation with inhibitors in serum free media (15 min) followed by stimulation with NLRP3 activators ATP (5 mM, 20 min), mono-sodium urate crystals (MSU, 250 μg ml$^{-1}$, 1 h), calcium pyrophosphate dihydrate crystals (CPPD, 250 μg ml$^{-1}$, 1 h), Aluminium hydroxide crystals (Alum, 250 μg ml$^{-1}$, 1 h), nigericin (20 μM, 15 min), or sphingosine (20 μM, 1 h). THP-1 cells were primed with LPS (1 μg ml$^{-1}$, 4 h) before incubation with inhibitors or vehicle (15 min) in serum-free media followed by stimulation with nigericin (10 μM, 1 h), or ATP (5 mM, 1 h). For AIM2/NLRC4 inflammasome activation primary BMDMs were primed with LPS (1 μg ml$^{-1}$, 4 h). Subsequent to LPS priming, cells were pre-treated with drugs or vehicle (DMSO) in serum-free media for 15 min then stimulated with ATP (5 mM, 1 h), poly(oxidodeoxyadenyl-oxidymidylic) (polyoa:dt) acid sodium salt transfected with Lipofectamine® 3000 (867 ng ml$^{-1}$, 4 h) or flagellin from S. typhimurium (867 ng ml$^{-1}$, 4 h). For K$^+$ efflux-independent NLRP3 activation BMDMs were primed with LPS as above. Subsequent to LPS priming, cells were pre-treated with drugs or vehicle (DMSO, 15 min) in PBS then stimulated with imiquimod (10 μM, 4 h). For non-canonical inflammasome activation cells were primed with Pam3CSK4 (100 ng ml$^{-1}$, 4 h). Subsequent to priming, cells were pre-treated with drugs or vehicle (DMSO, 15 min) in serum-free media then stimulated with LPS transfected with Lipofectamine® 3000 (2 μg ml$^{-1}$, 24 h). Neutrophils were primed with LPS (1 μg ml$^{-1}$, 2 h) in RPMI (containing 10% FBS, PenStrep), drug or vehicle (DMSO) was added 15 min prior to the stimulation with nigericin or vehicle (DMSO) for 1 h. Supernatants were removed and analysed for IL-1β or IL-1α content by ELISA (Duoset, R&D systems) according to manufacturer’s instructions.

**Washout Experiments**
IBMDMs were seeded overnight at 7.5 x 10$^5$ cells ml$^{-1}$ in 24-well plates and primed with LPS (1 μg ml$^{-1}$, 2 h). Subsequent to LPS priming, cells were pre-treated with drugs or vehicle (DMSO, 15 min) in serum-free media and washed 3 times, before inflammasome activation was initiated by adding ATP (5 mM, 1 h).

**Cell Death Experiments**
HEK293T kidney cells and HepG2 liver cells were treated with drug or vehicle (DMSO) for 4, 8 and 24 h in DMEM, 1% FBS, PenStrep. Following treatment, cell death was measured by assessing lactate dehydrogenase release using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) according to manufacturer’s instructions.

**Chemistry Synthesis**
Synthesis, purification and characterisation of NBC molecules are outlined in Methods S1 (Ibrahim et al., 1985; Coenen et al., 1965; Hosoya et al., 2006; Vasil’ev et al., 1994; Siddharan et al., 2010; Clemens and Hyatt, 1985). All chemicals, solvents and deuterated solvents were purchased from Sigma-Aldrich, Alfa-Aesar or Fisher Scientific. $^1$H, $^{13}$C and $^{11}$B($^1$H) NMR spectra were recorded on a Bruker Avance 400 or 300 MHz spectrometer. Chemical shifts (δ) are defined in parts per million (ppm). $^1$H NMR spectra were referenced to tetramethylsilane (TMS, δ=0.0 ppm) or residual undeuterated solvent (CDCl$_3$, δ=7.26 ppm; DMSO-d$_6$, δ=2.50 ppm).
13C NMR spectra were referenced to residual undeuterated solvent as an internal reference. 1H NMR chemical shifts were referenced to external reference BF3·OEt2 (δ=0.00 ppm). ESI and APCI mass spectrometry was carried out on a Waters Acquity UPLC system connected to a Waters SQQD2 mass spectrometer. Accurate mass determination was carried out on a Thermo Exactima Plus EMR Orbitrap Plus LC-MS system. Molecular ion peaks are defined as mass/charge (m/z) ratios. Infrared spectroscopy was recorded on a JASCO FT/IR-4100 spectrophotometer using the Spectra Manager II (JASCO) software package. Microwave irradiation was carried out on a Biotage Initiator Classic microwave using 2-5 ml Biotage glass vials. Analytical thin-layer chromatography (TLC) was performed using silica gel 60 on aluminium sheets coated with F254 indicator. All spots were visualised with KMnO4 or ultraviolet light using a MW Mineralight lamp (254/365) UVGL-58. Flash column chromatography was performed using silica gel with particle size 40-63 µm. Evaporation of solvents was conducted on a Buchi Rotavapor R-200.

**X-ray Crystallography**

X-ray diffraction data were collected at 100 K on the specimen crystals of NBC6 and NBC11 at the National Crystallography Service, Southampton, UK, with MoKα radiation produced by a rotating anode generator. The structures were solved by direct methods with SHELXS and refined with SHELXL, implemented in the WinGX package (Farrugia, 2012), by the full-matrix least-squares technique with anisotropic displacement parameters for the non-hydrogen atoms. Hydrogen atoms attached to carbon were placed in calculated positions and assumed to ride on their attached atom, methyl groups being allowed to rotate. The C12 methyl group of NBC11 showed significant disorder and therefore was assigned by 60% in one position and 40% in another with occupancy factors that refined to 0.59(2): 0.41(2). Positions and isotropic displacement parameters for hydrogen atoms attached to nitrogen atoms were refined freely. Final discrepancy indices R(lobs) and wR2 (all data) were 0.0408, 0.1120 for NBC6 and 0.0283, 0.0801 for NBC11. The highest peaks and deepest holes in a difference electron density map were 0.54, -0.36 and 0.39, -0.25 e Å⁻³ respectively. Cambridge Crystallographic Data Centre CCDC 1563191 (NBC6) and CCDC 1563192 (NBC11) contain the supplementary crystallographic data.

**Chemistry Computational/Modelling**

Initial 3D molecular structures of boron-containing compounds were constructed and then energy minimised using the Tripos force field in SYBYL-X. These geometries were subsequently optimised quantum mechanically with the semi-local M06-L density functional (Zhao and Truhlar, 2006) and the 6-31G* basis set, using the Gaussian 09 electronic structure package (Frisch et al., 2009). These geometries were used as input for structure-activity analysis and for virtual screening. For the latter, the ZINC subset leadsNow was employed, containing 1,943,551 molecules (4/20/12 update). Prior to shape-based screening, multiple conformations of each compound were generated via Omega (Hawkins et al., 2010). Shape-based screening was performed using ROCS (Grant et al., 1998) with Tanimoto scoring via the ShapeTanimoto and ColorTanimoto functions as implemented in OpenEye (Havirits et al., 2007). Topomer CoMFA (Cramer, 2003) was performed using the Sybyl software package, based on NBC6-1, 8, 11-20, 22-26 and 28-29, with R-groups defined at positions 4, 5 and 6 of the oxazaborine ring.

cLogP and cLogS calculations were performed for BC7, BC23, NBC-31 and NBC-EPPS using OSIRIS DataWarrior (version 4.5.2)(Sander et al., 2015).

**Fluorescence Imaging**

One day before experiments, 10⁴ iBMDMs were seeded on glass coverslips in 24-well plates. For Ca²⁺ imaging experiments cells were transferred to the following solution (in mM): NaCl, 130; KCl, 5; HEPES, 10; D-glucose, 10; CaCl₂, 1; MgCl₂, 1 (pH 7.4) and were loaded with 3 µM Fura-2-acetoxymethylester (Fura-2-AM, Molecular Probes, Eugene, USA) for 30 min at RT (20-23 °C). After washing, coverslips were mounted in a chamber on an inverted Olympus IX81 microscope equipped with a water immersion objective 40x UApo/340 (Olympus Optical Co. GmbH, Hamburg, Germany). The fluorescence imaging system consisted of a Polychrome V monochromator, a Hamamatsu Orca 03G camera and the Windows 7 based Live Acquisition software (Till Photonics, München, Germany). Cells were exposed to light of 340±5 and 380±5 nm wavelength every 10 or 20 s in experiments using 100 µM or 5 mM ATP, respectively. Emission light was passed through a 400 nm dichroic mirror and a 420 nm long pass emission filter (both Olympus, Germany) prior to acquisition. Cells were primed with LPS (1 µg/ml, 4 h) and incubated with drug 2APB (75 µM), BC7, BC23, NBC6 (all 30 µM) or vehicle, pre- (2 min) or post- (3 min) ATP stimulation. Data are presented as the ratio of the two background corrected fluorescence intensities. To enable fast drug application, cells were superfused using a four-barrel micro-perfusion pipette positioned in close proximity to the viewing field.

**Electrophysiological Recordings**

One day before experiments, 10⁴ iBMDMs were seeded on glass coverslips in 24-well plates. Membrane currents were measured using the whole-cell configuration of the patch-clamp technique. An EPC 10 patch-clamp amplifier (HEKA, Lambrecht/Pfalz, Germany) was interfaced to a computer for pulse application and data recording using the program PatchMaster (HEKA). Patch electrodes of 3:5 MΩ were fabricated on a two-stage puller (Narishige PC 10, Tokyo, Japan) from borosilicate glass (Hilgenberg, Malsfeld, Germany). For voltage-regulated Cl⁻ current (VRAC) recordings, patch electrodes were filled with the following intracellular solution I1 (in mM): N-Methyl-D-Glucamine-Chloride (NMG-Cl), 120; HEPES, 10; EGTA, 11; CaCl₂, 1; MgCl₂, 2; NaATP, 3 (pH 7.3). Cells were kept in extracellular solution E1 containing (in mM): NMG-Cl, 50; HEPES, 10; D-glucose, 10; CaCl₂, 2; MgCl₂, 1; D-mannitol, 170 (300 mosmol kg⁻¹, pH 7.3). To activate VRAC currents, cells were superfused with hypo-osmolar extracellular solution E2 containing...
(in mM): NMG-Cl, 50; HEPES, 10; D-glucose, 10; CaCl$_2$, 2; MgCl$_2$, 1 (130 mosmol kg$^{-1}$, pH 7.3). All recordings were done at RT (20-23°C). For solution exchange, a four-barrel microperfusion pipette was used. Cells were primed with LPS (1 µg ml$^{-1}$, 4 h) and incubated with drug (2APB (75 µM), BC7, BC23, NBC6 (all 30 µM)) or vehicle, 15 min before stimulation. Whole-cell currents were filtered at 3 kHz and stored for subsequent analyses, which were performed using the program s (HEKA, Lambrecht/Pfalz, Germany).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data are presented as mean values + standard error of the mean (s.e.m). Levels of significance were p<0.05 (*), p<0.01 (**), p<0.001 (***) Statistical analyses were carried out using GraphPad Prism (version 7) or R (version 3.3.0). Percentage control data were analysed with Holm-Sidak corrected one-sample t-tests against the value of 100%. Data with multiple groups were analysed with a one-way ANOVA. Experiments with two independent variables were analysed using two-ANOVA. These analyses were followed by Holm-Sidak corrected post-hoc comparisons. Homoscedasticity and normality of the residuals were evaluated with the Levene’s test and Shapiro Wilks, respectively, and appropriate transformations or corrections were applied where necessary. Dose response curves where fitted using non-linear least squares regression with a 3 parameter logistical sigmoidal model.

**DATA AND SOFTWARE AVAILABILITY**

The crystallographic data for NBC6 and NBC11 is deposited with the Cambridge Crystallographic Data Centre (CCDC) with the deposition numbers CCDC 1563191 (NBC6) and CCDC 1563192 (NBC11).
Chapter 4: CRISPR/Cas9 mediated mutation of mouse IL-1α nuclear localisation sequence abolishes expression

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CRISPR/Cas9 mediated mutation of mouse IL-1α nuclear localisation sequence abolishes expression

Michael J. D. Daniels, Antony D. Adamson, Neil Humphreys & David Brough

Inflammation is a host defense process against infection. Inflammatory mediators include cytokines of the interleukin-1 family, such as IL-1α and IL-1β. Unlike IL-1β, IL-1α carries an N-terminal nuclear localisation sequence (NLS) and is trafficked to the nucleus. The importance of IL-1α nuclear localisation is poorly understood. Here, we used CRISPR/Cas9 to make inactivating mutations to the NLS on the Il1a gene. A colony of NLS mutant mice was successfully generated with precise knock-in mutations to incapacitate NLS function. NLS mutant mice had no gross changes in immunophenotype or inflammatory response but, surprisingly, failed to express IL-1α. We deduced that, in making specific mutations in the Il1a gene, we also mutated a long-non-coding (lnc)RNA in the complementary strand which has cis-regulatory transcriptional control of the Il1a gene itself. The mutations generated in the Il1a gene also result in mutation of the lncRNA sequence and a predicted alteration of its secondary structure, potentially explaining a subsequent failure to function as a transcriptional activator of Il1a expression. Our results serve as a cautionary note that CRISPR-mediated genome editing without full knowledge of genomic context can result in unexpected, yet potentially informative observations.

Inflammation is generally a protective host response to injury and infection. Inflammation is initiated by detection of either ‘pathogen’ or ‘damage’-associated molecular patterns by pattern recognition receptors and subsequent secretion of pro-inflammatory cytokines which amplify the inflammatory response by recruiting immune cells to the site of injury/infection.

The interleukin (IL)-1 family of proteins, the major members of which are IL-1α and IL-1β, are amongst the best-studied pro-inflammatory cytokines. IL-1α and β initiate an inflammatory response by binding IL-1 receptor 1 (IL-1R1) which, following recruitment of IL-1R accessory protein, triggers signalling pathways leading to further pro-inflammatory gene transcription.

Inflammation is not always protective, and both IL-1α and IL-1β are involved in the pathogenesis and progression of numerous non-communicable diseases including stroke, diabetes, atherosclerosis, Alzheimer’s disease, and others. IL-1α and IL-1β are highly regulated. The regulation and secretion of IL-1β, governed by the formation of a multi-molecular complex called an inflammasome, is relatively well researched. However, the mechanisms of IL-1α regulation are poorly understood.

Both IL-1α and IL-1β are synthesised as 31 kDa precursor proteins (pro-IL-1α and pro-IL-1β) in response to a pathogen or damage associated signals, and are secreted from cells via unconventional secretory mechanisms after processing to active 17 kDa forms. IL-1α is also transcriptionally regulated by a long, noncoding RNA (lncRNA) located on the antisense strand of the Il1a gene. lncRNAs are noncoding RNA molecules over 200 nucleotides and have a well-established role in regulation of innate immune mechanisms. lncRNAs therefore provide an additional level of regulation for inflammation upstream of protein translation. AS-IL1α, a lncRNA located on the complementary strand of Il1a itself, is essential for LPS-induced expression of IL-1α.

IL-1 family cytokines are also regulated by subcellular localisation. Once expressed, pro-IL-1β is evenly distributed across the cytosol, whilst pro-IL-1α is both cytosolic and nuclear. Nuclear enrichment of IL-1α is due to the presence of a nuclear localisation sequence (NLS) within the N-terminus pro-piece. NLSs are short motifs
of amino acids that target proteins for active transport through the nuclear envelope by the karyopherin-3 (kap3) family of transport receptors. Classical NLSs comprise short sequences of amino acids characterised by lysine residues and are either monopartite (two stretches separated by a linker region). The NLS of IL-1α is a highly conserved classical monopartite sequence defined as KVLKKRNL in human and KILKKRRNL in mouse.

The importance of nuclear localisation in the regulation of IL-1α is poorly understood. A related IL-1 family member IL-33 requires nuclear localisation for its own sequestration to prevent aberrant damaging inflammation. We have shown previously that NLS function can be abrogated in vivo by mutating a single lysine at position 85 using site-directed mutagenesis and that IL-1α can be retained in the nucleus of necrotic cells suggesting the NLS may serve an anti-inflammatory function by inhibiting release. Similar studies have also been carried out to suggest that nuclear localisation of IL-1α may be important in cell growth, motility, cell death and cytokine secretion. However, in vitro approaches used in the past are hampered by limitations such as overexpression, the use of short constructs rather than full length genes and background expression of endogenous wild type (WT) genes. More accurate approaches are therefore required to assess the role of nuclear localisation of IL-1α.

The ability to target and modify the endogenous version of a gene of interest through gene editing/gene engineering has greatly expanded the range of experimental possibilities and enhanced the accuracy of representative model systems generated. The latest generation of gene editing technology, clustered, regularly interspaced, short palindromic repeat (CRISPR)/Cas9, a re-purposed bacterial adaptive immune system, has rapidly gained popularity owing to its simplicity of application. Targeting of specific genomic regions is achieved through directing a nuclease, Cas9, via a short guide RNA sequence (~110 nt) that is easy to design and generate in the laboratory, the only targeting restriction being the requirement of a protospacer adjacent motif (PAM) site downstream of the guide target site. For the most widely used CRISPR system from Streptococcus pyogenes (SpCas9) this motif is NGG, which is common in most genomes. This restriction is further mitigated by the use of engineered PAM targeting mutants or through the use of CRISPR systems from different bacterial species. CRISPR thus enables us to develop and build more representative biological model systems for experimentation. CRISPR has been demonstrated to work in most genomes targeted, from bacteria to mammals, and has been applied extensively to the generation of transgenic mouse models. In mammalian genomes, once Cas9 binds to target DNA two nuclease domains, RuvC and HNH, facilitate DNA double strand break (DSB) and the resulting DNA repair mechanisms can be exploited to generate desired mutations and modifications. Many DNA repair mechanisms exist, but with respect to CRISPR, Non-Homologous End Joining (NHEJ) and Homology Directed Repair (HDR) are at present most commonly used. NHEJ can result in the insertion or deletion of a few bases of DNA at the target site, known as Indels, which can easily lead to a frameshift of the gene reading frame and knockout of the gene, or the disruption of transcription factor (TF) binding sites. HDR requires the supply of a DNA repair template component, which contains a specific mutation to be generated flanked by appropriately sized homology arms. Precise modification by HDR can be used to make specific point mutations in target genes.

In this study we used CRISPR/Cas9 to target the coding sequence of the IL-1α gene to mutate the NLS in vivo for the first time. However, in doing so we also mutated a lncRNA in the complementary strand resulting in a predicted modification in its structure. Expression of IL-1α was inhibited in the NLS mutant mouse. We propose that this was most likely caused by the corresponding mutation in the IncRNA. Thus, this study demonstrates the potential caveats of CRISPR/Cas9 technology.

**Results**

**The NLS of IL-1α can be successfully mutated in vivo by CRISPR gene editing.** We identified two gRNA target sites in exon 3 of the murine Il1α gene close to the residues to be mutated (Fig. 1A). We designed an 816 bp double stranded DNA repair template with 5′ and 3′ homology to this region (Fig. 1B), designed to modify the K16 NLS motif at position 85/86 to EE. Also included were a further 4 base pair substitutions that function to act as shield mutations (to prevent gRNA/Cas9 re-binding and cutting the repaired region) and generate a unique BseRI restriction site for screening and genotyping purposes. The latest generation of gene editing technology, clustered, regularly interspaced, short palindromic repeat (CRISPR)/Cas9, a re-purposed bacterial adaptive immune system, has rapidly gained popularity owing to its simplicity of application. Targeting of specific genomic regions is achieved through directing a nuclease, Cas9, via a short guide RNA sequence (~110 nt) that is easy to design and generate in the laboratory, the only targeting restriction being the requirement of a protospacer adjacent motif (PAM) site downstream of the guide target site. For the most widely used CRISPR system from Streptococcus pyogenes (SpCas9) this motif is NGG, which is common in most genomes. This restriction is further mitigated by the use of engineered PAM targeting mutants or through the use of CRISPR systems from different bacterial species. CRISPR thus enables us to develop and build more representative biological model systems for experimentation. CRISPR has been demonstrated to work in most genomes targeted, from bacteria to mammals, and has been applied extensively to the generation of transgenic mouse models. In mammalian genomes, once Cas9 binds to target DNA two nuclease domains, RuvC and HNH, facilitate DNA double strand break (DSB) and the resulting DNA repair mechanisms can be exploited to generate desired mutations and modifications. Many DNA repair mechanisms exist, but with respect to CRISPR, Non-Homologous End Joining (NHEJ) and Homology Directed Repair (HDR) are at present most commonly used. NHEJ can result in the insertion or deletion of a few bases of DNA at the target site, known as Indels, which can easily lead to a frameshift of the gene reading frame and knockout of the gene, or the disruption of transcription factor (TF) binding sites. HDR requires the supply of a DNA repair template component, which contains a specific mutation to be generated flanked by appropriately sized homology arms. Precise modification by HDR can be used to make specific point mutations in target genes.

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Cell lysates were analysed for the expression of pro-inflammatory cytokines IL-1β (Fig. 3A,C,E) and IL-6 (Fig. 3B,D,F) by ELISA. There was no difference in IL-1β or IL-6 expression between IL-1αmut/mut or IL-1αmut/+ mice and IL-1α+/+ controls. We next tested whether the cellular release of IL-1β and IL-6 was altered in IL-1αmut/mut mice. IL-6 is secreted in response to LPS stimulation alone \(^{34}\). However, secretion of IL-1β must be induced by both LPS and a second signal which leads to activation of the NLRP3 inflammasome and caspase-1-dependent proteolytic processing from the 31 kDa pro form into a 17 kDa mature form \(^{35}\). BMDMs (Fig. 3G,H), BMCs (Fig. 3I,J) and peritoneal macrophages (Fig. 3K,L) were isolated from IL-1α+/+, IL-1αmut/+ and IL-1αmut/mut mice and stimulated with LPS alone or primed with LPS and then stimulated with ATP or nigericin, and silica to activate the NLRP3 inflammasome. Cells were also stimulated with the Ca\(^{2+}\) ionophore ionomycin, which is known to activate Ca\(^{2+}\)-dependent processing and release of IL-1β \(^{36}\). Supernatants were assessed for IL-6 and IL-1β by ELISA with preferential detection for the mature form of IL-1β. There were no differences between WT and mutants for the release of IL-1β (Fig. 3G,I,K) or IL-6 (Fig. 3H,J,L) across all stimuli. Together, these data show that mutation of the NLS on IL-1α had no effect on either gross immune cell populations or on secretion of classical pro-inflammatory cytokines from multiple immune cell types.

Figure 1. The NLS of IL-1α can be successfully mutated \textit{in vivo} by CRISPR gene editing. Schematic of mouse \textit{Il1a} gene, indicating nuclear localisation sequence (NLS) and CRISPR guide target sites (A). Design of the 816 bp dsDNA repair template, note the point mutations to inactivate NLS and shield mutations to both inactivate CRISPR targeting, and create a unique BseRI restriction site for genotyping purposes (B). Genotyping of founder 53, the 1033 bp PCR product is digested to 421 bp and 612 bp products. Marker is Hyperladder 1 kb (Bioline) (C). Sequence confirmation of mutant allele. Red bases indicate mutations generated by HDR (D).
CRISPR-mediated disruption of the Il1a gene leads to loss of IL-1α expression. We next investigated whether mutation of the NLS of IL-1α had any effect on expression of the IL-1α protein. IL-1α is not normally expressed in monocytes or macrophages and must be induced by a stimulus such as LPS. BMDMs, BMCs and peritoneal macrophages were isolated from IL-1α++/+, IL-1αmut/+ and IL-1αmut/mut mice and stimulated with LPS as described above. Following treatment, lysates and supernatants were measured for IL-1α protein expression by ELISA and western blot. In BMDMs, LPS stimulation induced production of IL-1α in cells isolated from IL-1α+/+ and, to a reduced extent, IL-1αmut/+ mice (Fig. 4A). However, LPS treatment was unable to induce IL-1α production in IL-1αmut/mut mice (Fig. 4A). Finally, IL-1α production in peritoneal macrophages was also measured and once again was attenuated in IL-1αmut/mut and abolished in IL-1αmut/mut cells (Fig. 4C). We next assessed IL-1α secretion from immune cells stimulated with inducers...
of the inflammasome, or with Ca\(^{2+}\) influx as described above. Secretion of IL-1\(\alpha\) was detected in IL-1\(\alpha\)+/+ and, to a reduced extent, IL-1\(\alpha\)mut/+ and, to a reduced extent, IL-1\(\alpha\)mut/mut BMDMs (Fig. 4D), BMCs (Fig. 4E) and peritoneal macrophages (Fig. 4F) stimulated with inflammasome activators ATP or nigericin, ionomycin, and silica to promote cytokine secretion. BMDM supernatants were assessed for IL-1\(\alpha\) (G) and IL-6 (H) as were supernatants taken from BMCs (I) and peritoneal macrophages (J). Data are presented as mean IL-1\(\alpha\) production + s.e.m (n = 4), analysed by two-way ANOVA. There were no significant differences between genotype.

![Figure 3](https://www.nature.com/scientificreports/) Proinflammatory cytokine production/secretion is not impaired in NLS mutant mice. Immune cells were isolated from IL-1\(\alpha\)+/+ or IL-1\(\alpha\)mut/+ or IL-1\(\alpha\)mut/mut mice and treated with LPS (1 \(\mu\)g ml\(^{-1}\), 4 h) before assessing lysates for cytokine production by ELISA. BMDM lysates were assessed for IL-1\(\beta\) (A) and IL-6 (B) as were lysates taken from BMCs (C,D) and peritoneal macrophages (E,F). Immune cells were also stimulated with ATP or nigericin, ionomycin, and silica to promote cytokine secretion. BMDM supernatants were assessed for IL-1\(\alpha\) (G) and IL-6 (H) as were supernatants taken from BMCs (I) and peritoneal macrophages (J). Data are presented as mean IL-1\(\alpha\) production + s.e.m (n = 4), analysed by two-way ANOVA. There were no significant differences between genotype.
IL-1α impairments in IL-1α (Nat) no eExons typically regulate neighbouring genes—not those in which the eExons sit. We have observed that there is no effect on the expression of the neighbouring gene IL-1β (Fig. 3). It is therefore more likely that this effect is due to other factors controlling IL-1α expression.

It has been recently shown that transcription of IL-1α in immune cells is controlled by a natural antisense transcript (NAT)\(^1\). Antisense IL-1α (AS-IL1α) is a long non-coding RNA (IncRNA) encoded on the antisense strand within the Il1α locus. Expression of AS-IL1α is induced by LPS and is required in order to promote IL-1α transcription\(^1\). We hypothesized that the loss of IL-1α expression observed above may be as a result of impaired expression or function of AS-IL1α due to inadvertent genetic perturbation, which in turn prevents induction of IL-1α itself. Thus we measured by qPCR the expression of AS-IL1α in LPS-treated immune cells from IL-1α\(^{+/+}\) or IL-1α\(^{−/−}\) mice compared to IL-1α\(^{+/+}\) littermates and detected no significant difference in expression levels across all three mouse models (Fig. 5A).
Next we investigated potential AS-IL1α lncRNA structural changes generated by the CRISPR induced base mutations. lncRNAs are highly sensitive to structural change and function often relies on secondary structure[2]. We used bioinformatic to computationally model the effect of the mutations made on the AS-IL1α higher-order structure. The RNAmp web server[28] predicts the effect of single nucleotide polymorphisms (SNPs) on local secondary structure based on the RNA folding algorithms used in the ViennaRNA package[29,30]. Computational secondary structure analysis revealed that the SNPs made in the IL-1α sequence led to a significant local structural effect (p = 0.0294) on the AS-IL1α lncRNA in the local region of nucleotides 439–509 (horizontal orange bar indicates area of significant structural change) (Fig. 3B). This region of significant structural change is located around the SNP mutations made (black vertical lines). Graphical display of the mutated sequence indicates that there is a disruption in the formation of loops in the RNA structure (Fig. 3C – areas of structural change highlighted in red (IL-1αmut/mut) vs. green (IL-1α+/-)). This suggests that, although expression of the AS-IL1α lncRNA was not detected by the secondary structure analysis made in the genomic DNA of research secondary structure was predicted to be altered, which may be a potential explanation for the loss of IL-1α expression.

Discussion

We have demonstrated that endogenous, CRISPR-induced point mutations in exon 3 of the Il1a gene results in complete loss of inducible expression in mouse BMDMs, BMCs and peritoneal macrophages. We were unable to detect IL-1α protein or mRNA from this mutant allele, indicating a loss of transcriptional regulation.

A recently discovered regulatory lncRNA has been described, which is critical for Il1a gene activation. Chan et al. demonstrated that AS-IL1α, a lncRNA located on the antisense strand of the Il1a gene, is required for the expression of IL-1α. Indeed, when knocked down by shRNA, LPS-induced IL-1α expression is lost[22]. We show that minor changes in the genetic sequence are predicted to result in major lncRNA structural changes, which may explain the complete abrogation of Il1a gene expression. Such changes in the secondary structure of the lncRNA could be sufficient to inhibit the recruitment of the transcriptional machinery required for expression of IL-1α thus inhibiting its expression (Fig. 3D).

The observation that in cells heterozygote for the NLS mutations there is a 50% reduction in IL-1α protein and mRNA expression compared to WT cells suggests that the AS-IL1α lncRNA is cis-acting, and directly regulates the IL-1α gene on the sense strand. Should the AS-IL1α act in trans we would have expected to have seen partial, if not full, rescue of the mutant allele in heterozygotes. The implications of this discovery are two-fold. Firstly, we show that transcriptional regulation of IL-1α is complex and highly regulated, involving extracellular signaling and intermediate activation of lncRNA regulatory molecules. In mice, immune expression and activity of IL-1α is regulated on multiple levels. Transcription in immune cells can be regulated by NFκB[23], subcellular localization mediated by an N-terminal NLS[24] and activity at the IL-1R receptor is regulated by post-translational modification in the form of processing from a 31 kDa ‘pro’ form to a more biologically active 17 kDa form[25]. Here we suggest that an additional level of regulation, a lncRNA located on the antisense strand[26], may be extremely sensitive to point mutations through loss of normal secondary structure. Such changes within a code demonstrates the complexity of cell biology and potentially opens up a new area of research in understanding and defining the numerous additional levels of regulation that may exist in protein expression.

Loss of function of the AS-IL1α lncRNA is not the only possible explanation for the abolition of IL-1α expression. This effect could also be caused by loss of TF binding to enhancer regions located at the mutated site in exon 3. However, Chan et al. show that shRNA directed specifcally to target AS-IL1α lncRNA expression prevented recruitment of RNA polymerase II to the promoter region of IL-1α at +22 bp downstream of the transcription start site and is thus critical for the recruitment of transcriptional machinery to this gene[22]. In this experiment from Chan et al., the genetic sequence of exon 3 remains unaffected but expression is lost, suggesting that TF recruitment occurs at the promoter regions of Il1a in an AS-IL1α-dependent manner. We thus conclude the most likely explanation for loss of IL-1α expression in this CRISPR modified mouse model is the inadvertent perturbation of the structure of the key IL-1α regulator molecule AS-IL1α.

The results from the study also have far-ranging implications in the field of genome engineering. Previously, mutant forms of genes would be typically introduced to cells in vitro, such as plasmids, viruses or bacterial artificial chromosomes. However, more recently, the ability to directly modify endogenous alleles through gene editing approaches such as ZFNs, TALENs and latterly CRISPR/Cas9 has revolutionized biological research, and potentially medicine. In theory it is now plausible to build a ‘perfect’ representative biological system, where the mutated gene of interest is still controlled by all endogenous regulatory regions and mechanisms. However, this relies on precise knowledge and understanding of the mammalian genome, structure, organisation and gene regulation. In this study our mutations led to an unexpected loss of gene expression. One may envisage a circumstance where such inadvertent effects may not be immediately detected, such as the manipulation of a trans-acting regulatory RNA, cryptic regulatory elements, nested genes and so forth that may have indirect effects on genetic systems. Indeed, non-coding RNAs themselves have been shown in many circumstances to be difficult to target by CRISPR/Cas9, due to their propensity to be found overlapping neighbouring genes[26].

A common method, also employed in this study, is to include ‘shield’ mutations in DNA repair templates for HDR, to prevent sgRNA/Cas9 complexes re-binding and re-cutting repaired DNA. Typically these mutations target the PAM site or seed sequence of the sgRNA target, and are usually silent or synonymous mutations, changing DNA bases but not the amino acid they encode. However, there is a growing literature of examples where this kind of change has resulted in alterations in mRNA splicing, mRNA folding, stability and regulation of translation, and it is becoming clear that codon usage in different species is not random[28]. It is likely that in the near future we will see gene editing strategies employed directly in the clinic, where until now the major concerns have been related to off-target effects (OTEs)[29]. We suggest that, especially in therapy,
Figure 5. NLS mutation does not affect transcription of AS-IL1α lncRNA but may affect secondary structure and function. AS-IL1α RNA isolated from IL-1α+/−, IL-1αmut/+ or IL-1αmut/mut BMDMs treated with LPS (1 µg ml⁻¹, 5 h) was assessed by qPCR. Data are presented as mean AS-IL-1α expression ± s.e.m (n = 4) relative to a reference sample calculated using the standard curve method (A). CRISPR-mediated mutations may affect lncRNA structure and function. RNAsnp analysis showed significant structural change in RNA structure located around the mutations made (mutations indicated by vertical black lines, area of significant change indicated by horizontal orange bar) (B). Graphical display of AS-IL1α secondary structure shows predicted disruption of RNA loops from WT (green) to mutant (red) sequence, p = 0.0294 calculated by RNAplfold algorithm (C). A schematic outlining the proposed mechanism(s) by which the mutation in the Il1a gene leads to loss of IL-1α expression. In the wild-type gene (left) LPS induces transcription of the AS-IL1α which, in turn, promotes recruitment of transcriptional machinery to the promoter region of the Il1a gene and induces production of IL-1α mRNA. Transcription may also be induced by an enhancer region in exon 3 of the DNA. In the mutant gene LPS induces production of the mutant lncRNA which, due to changes in secondary structure, is unable to recruit transcriptional machinery and thus there is no production of IL-1α mRNA (D).
researchers should be cautious about changing the genome with impunity. Some of the newer gene editing strategies developed, such as base editing using deadCas9 or Cas9 nickase in conjunction with cytidine deaminase enzyme that does not induce dsDNA breaks, but instead mediates the direct conversion of bases, may mitigate some of these concerns. CRISPR technology is a powerful weapon in the researcher’s armory, but as we demonstrate, is not without caveats. In this study of the function of IL-1β NLS direct genome engineering has so far proven to be more complicated than expected, and serves as a cautionary notice that any and all implications of genetic modification should be considered. Researchers must be wary of the myriad of unexpected possibilities that may result from making even small changes to the genome.

Materials and Methods

Generation of CRISPR reagents and microinjection. For CRISPR targeting we generated sgRNA for each target site according to Shen et al. Briefly, complementary oligos for the sgRNA target sequences were synthesised (Integrated DNA technologies), annealed and ligated into pUC57-sgRNA expression vector. After sequence confirmation plasmids were mini-prepped, linearised with Dral restriction enzyme and used as template in an in vitro T7 transcription reaction according to manufacturer’s instructions (NEB). Synthesised sgRNA was purified using Ambion Megaclear kit, eluted in injection buffer (10 mM Tris (pH 7.5), 0.1 mM EDTA (pH 8.0), 100 mM NaCl) and concentration determined by nanodrop. Template DNA was synthesised in a vector (Cellectis) and the 816bp dsDNA repair region was excised, gel extracted and PCR cleaned, eluted in injection buffer, and concentration determined by nanodrop. Injection mix (final volume 50µl−1 each) was prepared by combining the two sgRNA (final concentration 50ngµl−1 each) with Cas9 protein (laboﬁna, ﬁnal concentration 100ngµl−1) and incubated at room temperature for 10min, before adding DNA repair template (ﬁnal concentration 10ngµl−1). Standard pronuclear microinjection was performed on B6D2F1 (Envigo) hybrid zygotes which led to 2 founder mice that were then back-crossed to C57BL/6 wild-type mice to assess germline penetrance.

Genotyping and identiﬁcation of positive offspring. We detected offspring harbouring the desired mutation by ampliﬁcation with primers Geno F (CCCCAGCAAGAAAAAGAGG) and Geno R (GACTGAGTCTTCCCTCTGTA) which target outside the HDR template, and digested with BseRI. BseRI digestion gave preliminary indication of a mutant allele, which was conﬁrmed by PCR-Blunt cloning (Invitrogen) and Sanger sequencing. Two founders, animals 53 and 63, were found to have the mutant allele. It should be noted that in both these animals the second allele had also been targeted by CRISPR/Cas9 and undergone NHEJ repair, resulting in a 59bp sgRNA to sgRNA deletion and a +11/+15bp InDel respectively. Animal 53 was used to establish a colony of IL-1β NLS mutant mice after conﬁrmation of germline transmission.

Animals. Animals were maintained under standard laboratory conditions: ambient temperatures of 21°C (±2°C), humidity of 40–50%, 12h light cycle, ad libitum access to water and standard rodent chow. Genotype groups were randomised during the study and experimenters were blinded to genotype during all cell isolation procedures and experiments. All animal experiments were carried out in accordance with the United Kingdom Animals (Scientiﬁc Procedures) Act 1986 and approved by the Home Ofﬁce and the local Animal Ethical Review Group, University of Manchester.

Cell isolations and assays. BMCs. Mouse bone marrow cultures (BMCs) were isolated by flushing marrow from mouse femurs. Red blood cells were lysed by suspending in ACK buffer (Lanza) for 3min at RT. Immediately after isolation cells were seeded at 5 × 10⁶ cells ml−1 in RPMI, 10% FBS, 1% PenStrep, 1% L-glutamine. Cells were treated with LPS (Sigma O26:B6, 1µg ml−1, 3h) then stimulated with nigericin (Sigma, 10µM, 1h), ionomycin (Sigma 10µM, 1h) or silica (US Silica, 300µg ml−1, 3h). Supernatants and lysates were taken and analysed for IL-1β, IL-10 and IL-6 content by ELISA (DuoSet, R&D systems) according to manufacturer’s instructions.

Peritoneal macrophages. Peritoneal macrophages were isolated by peritoneal lavage. Mice were anaesthetized with isoflurane (induced at 3–4% in 33% O₂, 67% NO₂, maintained at 1–2%) and peritoneal cavities lavaged with 4ml RPMI. Peritoneal macrophages were counted and seeded at 1 × 10⁶ cells ml−1 in 96-well plates in Dulbecco’s Modified Eagle’s Medium (DMEM), 10% fetal bovine serum (FBS, Life Technologies), 100 U ml−1 penicillin and 100µg ml−1 streptomycin (PenStrep). Cells were primed with LPS (1µg ml−1, 4h) then stimulated with ATP (Sigma, 5µM, 1h), ionomycin (10µM, 1h) or silica (300µg ml−1, 4h). Supernatants and lysates were taken and analysed for IL-1α, IL-1β and IL-6 content by ELISA (DuoSet, R&D systems) according to manufacturer’s instructions.

BMDMs. Mouse bone marrow-derived macrophages were isolated by flushing marrow from mouse femurs. Red blood cells were lysed by suspending in ACK buffer (Lanza) for 3min at RT. Following lysis, cells were cultured in DMEM, 10% FBS, 1% PenStrep supplemented with 30% L929 mouse ﬁbroblast supernatant conditioned media for 7–10 days. One day prior to experiments, cells were seeded overnight at 1 × 10⁶ cells ml−1 in 96-well plates in DMEM, 10% FBS, 1% PenStrep. Cells were primed with LPS (1µg ml−1, 4h) then stimulated with ATP (5µM, 1h), ionomycin (10µM, 1h) or silica (300µg ml−1, 4h). Supernatants and lysates were taken and analysed for IL-1α, IL-1β and IL-6 content by ELISA (DuoSet, R&D systems) according to manufacturer’s instructions.
Platelets. Platelet cells were isolated from fresh blood as by Cazeneve et al. Briefly, blood was taken via cardiac puncture with acid-citrate-dextrose (ACD) anticoagulant solution at ratio of 1 part ACD to 5 parts blood. Anticoagulated blood was centrifuged at 2300 x g for 45 seconds and the platelet-rich plasma (PRP) taken. PRP was then centrifuged at 2200 x g for 2 min and the platelet-containing pellet lysed with Triton X- solution. Lysates were corrected for total protein using a BCA assay (ThermoFisher) according to manufacturer's instructions and analysed for IL-1α content by ELISA (Duoset, R&D systems).

qPCR. BMDMs were treated with vehicle or LPS (1 μg ml⁻¹, 5 h) lysed in TRIzol Reagent (ThermoFisher) and RNA isolated according to the manufacturer's instructions. RNA (0.6 μg) was converted to cDNA using SuperScript™ III Reverse Transcriptase (ThermoFisher) according to manufacturer's instructions. qPCR was performed using Power SYBR® Green PCR Master Mix (ThermoFisher) in 384-well format using a 7900HT Fast Real-Time PCR System (Applied Biosystems). 16.6 ng cDNA was loaded with 5 pmol primer/well in triplicate. Data were normalized to the expression of the housekeeping gene 18S rRNA. Primers were used were: IL-1α Forward - TCCTCAGTTCAACATCTGTGGTG, IL-1α Reverse - AGAAGATGAGCTGGTGCTCAGTA, AS-IL1α Forward - AGGCCGTGAGCTCATCGTGC, AS-IL1α Reverse - TCCTCAGTTCAACATCTGTGGTG, 18S rRNA Forward - CCACGTTCTATTTTGTTGGT, 18S rRNA Reverse - AGTGGGATCTGTGGTGCTCAGT. Data were analysed using the relative standard curve method.

Western blotting. Western blot analysis was performed on supernatants and lysates for IL-1α and IL-1β. Samples were run on 12% sodium dodecyl sulphate (SDS) polyacrylamide gels. Gels were transferred at 150 V onto nitrocellulose membrane (GE Life Sciences) before exposure using a G:BOX gel doc system. Western blotting was performed using Power SYBR® Green PCR Master Mix (ThermoFisher) in 384-well format using a 7900HT Fast Real-Time PCR System (Applied Biosystems). 16.6 ng cDNA was loaded with 5 pmol primer/well in triplicate. Data were normalized to the expression of the housekeeping gene 18S rRNA. Primers were used were: IL-1α Forward - TCCTCAGTTCAACATCTGTGGTG, IL-1α Reverse - AGAAGATGAGCTGGTGCTCAGTA, AS-IL1α Forward - AGGCCGTGAGCTCATCGTGC, AS-IL1α Reverse - TCCTCAGTTCAACATCTGTGGTG, 18S rRNA Forward - CCACGTTCTATTTTGTTGGT, 18S rRNA Reverse - AGTGGGATCTGTGGTGCTCAGT. Data were analysed using the relative standard curve method.

Flow cytometry. Bone marrow cells were isolated by flushing and lysed with ACK lysis buffer (Lonza). Splenocytes were prepared by homogenising whole spleens and passing through 70 μm cell strainers before ACK lysis. Cells were stained with antibodies consisting panels to detect neutrophil/monocyte populations (CD45 PerCPCy5.5, CD11b eGFP/Biotin, CD16/CD32, CD54, CD64, CD11c (Biotrend)) and T and B-cell populations (CD8 eGFP/Biotin, CD4 (Biotrend), CD19 (Biotrend), TCRβ (Biotrend)) for 25 min on ice in the dark before washing twice and fixing in paraformaldehyde (2% in PBS) for 15 min at RT. Dead cells were excluded by use of a Zombie NIR Live/Dead fixable dye (Biotrend). The following day, flow cytometry was performed using a FACSCanto flow cytometer (BD Biosciences) and analysed with FlowJo software (FlowJo LLC).

RNA structure prediction. The nucleotide sequence of AS-IL1α (Accession - KR095173) was inputted to the RNAmap web server along with the 7 SNPs made (G399U-A441G-C442G-U443A-C444G-U455C-U457C). The ‘mode 2’ algorithm was selected to search for significant structural effects of SNPs on large RNA sequences (>1000 nt) using the local folding method RNAfold.

Statistical analyses. Data are presented as mean values ± standard error of the mean (s.e.m). Levels of significance were P < 0.05 (*), P < 0.01 (**), P < 0.001 (***) Statistical analyses were carried out using GraphPad Prism (version 7). Cytokine production and secretion were analysed with a two-way ANOVA with repeated measures followed by Tukey’s post-hoc comparisons. IL-1α in platelets was measured by a one-way ANOVA with Tukey’s post-hoc comparisons. qPCR data were analysed using a two-way ANOVA with repeated measures followed by Tukey’s post-hoc comparisons. Transformations were applied where necessary. InCNR RNA secondary structure was analysed using the RNAfold algorithm.

Data availability. The data that support the findings of this study are available from the corresponding author on request.

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Author Contributions
M.J.D.D., A.A. and N.H. performed cell and animal experiments. M.J.D.D., A.A., N.H. & D.B. designed and supervised experiments, interpreted data and contributed to writing the manuscript.

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SUPPLEMENTARY INFORMATION

CRISPR/Cas9 mediated mutation of mouse IL-1α nuclear localisation sequence abolishes expression

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Supplementary Figure 1: Full length blots from figure 3. Blots showing IL-1α and β-actin in BMDM lystates from Fig. 3G (A, B) and IL-1α BMDM supernatants from Fig. 3H (C).
Chapter 5: Discussion

5.1 Summary

IL-1 is a potent pro-inflammatory cytokine and is a mediator of sterile inflammation and non-communicable diseases (Dinarello, 1996). The two main pro-inflammatory members of the IL-1 family are IL-1α and IL-1β, both of which signal through the same receptor IL-1R1, stimulation of which leads to a rapid and robust inflammatory response. The IL-1 family cytokines are subject to stringent regulation. The well-researched IL-1β is tightly regulated at the level of post-translational modification – a process whereby the 31 kDa inactive pro form of the cytokine undergoes proteolytic processing to a mature and active form dependent on the formation of a multimeric complex called an inflammasome. IL-1α is poorly understood but is also highly regulated at many levels including that of subcellular localisation where it is actively transported to the nucleus of the cell – the reason for this is not clear. It is crucial that we improve our understanding of the mechanisms of IL-1 regulation in order to treat inflammatory diseases such as those listed in section 1.4.

The work presented in this thesis makes major contributions to the field of IL-1 regulation. First, we showed that the fenamate class of NSAIDs such as mefenamic acid, a drug currently indicated for dysmenorrhea, are selective inhibitors of the NLRP3 inflammasome via a mechanism dependent on blocking the VRAC ion channel (Daniels et al., 2016). This study also demonstrated that pharmacological inhibition of NLRP3 could be effective in rodent models of Alzheimer’s disease (AD) and suggested that mefenamic acid may be repurposed to offer a much-needed treatment for AD. Following on from this work, we developed a novel class of potent, selective inhibitors of the NLRP3 inflammasome (Baldwin et al., 2017). Starting from the compound 2APB, we developed a new class of compounds based on the presence of an oxazaborine ring. After multiple iterations of synthesis and testing we produced NBC6, a compound with no effect on calcium signalling and 100x improved potency on NLRP3 vs. 2APB. We also showed NBC6 was effective in animal models of inflammation. Finally, the CRISPR/Cas9 system was used to evaluate the role of the NLS present at the N-terminus of IL-1α in vivo (Daniels et al., 2017). In doing so, IL-1α transcription was lost leading to the discovery that IL-1α expression may be regulated at the level of secondary structure of the lncRNA AS-IL1α. This study also had important implications for the CRISPR field – demonstrating that care should be taken when modifying the genome. These contributions to the understanding of IL-1 regulation have been summarised in Figure 5.1.
Figure 5.1: Thesis contributions. In the first paper of this thesis, we showed that the fenamate class of NSAIDs, but not other NSAIDs such as ibuprofen and celecoxib, are selective NLRP3 inhibitors and work by reversible blockade of the VRAC chloride channel. In the second study of this thesis, we developed a new class of NLRP3 inhibitors based on the non-selective calcium blocker 2APB. The NBC compounds had increased potency on NLRP3 and no effect on Ca^{2+} signalling thus paving the way for a novel class of boron-containing NLRP3 inhibitors. In the third and final study of this paper we used CRISPR to make a mutant mouse with point mutations in the nuclear localisation sequence of the Il1a gene. The IL-1α<sup>mut/mut</sup> could not produce IL-1α following LPS stimulus. We discovered this phenotype was due to mutation in the secondary structure of the crucial regulatory long non-coding RNA AS-IL1α leading to loss of transcription. These discoveries have increased our knowledge of the regulatory mechanisms of IL-1 signalling.

5.2 General discussion

The early work in this thesis focussed on inhibition of the NLRP3 inflammasome. Aberrant NLRP3 activation has been implicated in a variety of diseases including diabetes (Masters et al., 2010), atherosclerosis (Duewell et al., 2010), haemorrhage (Dutra et al., 2014), AD (Heneka et al., 2013) and many others. However, the majority of evidence for NLRP3 in disease comes from animal studies and, with the exception of the cryopyrin-associated periodic syndromes (CAPS) and pyrin-associated autoinflammation with neutrophilic dermatosis (PAAND) diseases (de Torre-Minguela et al., 2017), there is limited understanding of the role of NLRP3 in human disease.
A recent study gave an important insight into NLRP3 and human disease – specifically, Alzheimer’s disease. Venegas et al. (2017) demonstrated that ASC specks can be found in Aβ plaques and can cross-seed these in AD models. There are currently no disease altering therapies for AD and, with multiple high-profile phase III failures (Hung and Fu, 2017), it is clear that new approaches are required. Complimentary to the promising studies in rodent models (Daniels et al., 2016; Dempsey et al., 2017), Venegas et al. (2017) suggested for the first time in human subjects the potential role of NLRP3 in AD. However, there are currently no available NLRP3 inhibiting drugs.

Repurposing or repositioning of currently available treatments is becoming a major focus in the fight to treat disease. Some estimates state that drug repurposing can cut the cost of drug development from $1-2 billion to $40–80 million and save 5-7 years in getting the compound to market (Papapetropoulos and Szabo, 2018). Drug repurposing success stories include sildenafil (repositioned from an angina treatment to a drug for erectile dysfunction) and thalidomide (repositioned from a morning sickness treatment to a drug for multiple myeloma). Recently, drug repurposing has been a major focus for Alzheimer’s disease as it offers a way to rapidly bring in much-needed novel treatment options. Halliday et al. (2017) performed phenotypic screening assays on a library of 1040 drugs and found that the anti-depressant trazodone was effective at increasing the rate of protein synthesis in disease models and restored memory in a mouse model of frontotemporal dementia.

In the first study of this thesis, we showed that mefenamic acid, a member of the fenamate class of NSAIDs, could inhibit the NLRP3 inflammasome, and was effective in rodent models of AD – suggesting it may be repurposed to treat AD in the future (Daniels et al., 2016).

NSAIDs have long been implicated in Alzheimer’s disease. Numerous observational studies have suggested that NSAID use may offer some protection against Alzheimer’s disease (Etminan et al., 2003; Szekely et al., 2004; Wang et al., 2015). These effects were bolstered by the claim that some NSAIDs were capable of inhibiting the enzyme γ-secretase and reducing amounts of toxic fibrillar Aβ (Weggen et al., 2001; Eriksen et al., 2003). This subset of NSAIDs, which included ibuprofen, were termed selective Aβ_{42}-lowering agents or ‘SALAs’. Unfortunately, subsequent randomised controlled trials (RCTs) failed to prove any beneficial effect of NSAID-use in AD and demonstrated that SALAs were no different to other NSAIDs in AD (Szekely et al., 2008; Jaturapatporn et al., 2012; Miguel-Álvarez et al., 2015; Wang et al., 2015). In our study, we demonstrated that ibuprofen had no effect on the inflammasome. Our inflammasome-inhibiting NSAIDs, the fenamates, have not been tested in detail with regard to protection in AD. This raises the hypothesis that RCTs carried out to
test NSAID-mediated protection against AD were designed using the wrong NSAIDs and that inflammasome inhibiting NSAIDs, the fenamates, should be tested.

Mefenamic acid was an effective inhibitor of NLRP3 at doses of 50-100 μM (Daniels et al., 2016). Following ingestion of a 1 g oral dose, mefenamic acid is rapidly absorbed with a peak plasma concentration of 10 μg/mL (Glazko, 1966). Taking into account the drugs molecular weight of 241 g/mol, this means mefenamic acid is at a concentration of ~43 μM in the plasma, a dose at which it would be effective at inhibiting NLRP3. For our in vivo test, we used an osmotic pump to administer a steady dose of 25 mg/kg/day. It is estimated that this is equivalent to approximately a 12.3x lower dose in humans (calculated due to differences in surface-area to volume ratio) (Nair and Jacob, 2016). Thus, a comparative human dose would be ~2 mg/kg/day which, in a 70 kg human, would be under 150 mg. The standard dose of mefenamic acid for acute pain including dysmenorrhoea is 500 mg three times a day, thus the dose used in our study is in a reasonable range for human comparison.

In addition to the implications with repurposing drugs for AD, this study also made major breakthroughs in target identification for future therapies. We identified VRAC as a crucial regulator of NLRP3, but not AIM2 or NLRC4 inflammasome function (Daniels et al., 2016). As NSAIDs, the primary mechanism of action of fenamates is classically via inhibition of the cyclooxygenase enzymes COX-1 and 2. However, the fenamates actually have relatively poor potency on these enzymes compared with other drugs (Lees et al., 2004). It is known that the fenamate compounds are ion channel inhibitors (Guinamard et al., 2013) and that ion flux is a key component of inflammasome activation (see section 1.3.3.2). However, the molecular identity of the ion channels involved was not known. We identified VRAC as a crucial mediator of Cl− efflux from the cell which leads to NLRP3 activation, paving the way for design and development of novel drugs to inhibit this channel (Daniels et al., 2016). Since our study, another family of Cl− channels have been implicated in NLRP3 activation. The chloride intracellular channels (CLICs) have a diverse set of roles including endosomal trafficking, actin remodelling and pH regulation (Argenzio and Moolenaar, 2016). Two groups have independently identified CLIC function in NLRP3 activation, suggesting that CLICs regulate inflammasome formation downstream of K+ efflux and ROS and that NLRP3 stimuli induce translocation of CLICs to the plasma membrane where they mediate Cl− efflux (Domingo-Fernández et al., 2017; Tang et al., 2017). These studies confirm the importance of Cl− flux in NLRP3 activation and identify a novel target, the CLICs. However, it remains to be confirmed whether CLIC-dependent inflammasome activation is via Cl− efflux through the CLIC channel. Despite its name, ion channel activity of CLICs has been poorly demonstrated and there has even been suggestion that the name be revised (Argenzio and Moolenaar, 2016). Domingo-Fernández et al. (2017) do not study the effect of genetic CLIC
knockdown on Cl⁻ flux whilst (Tang et al., 2017) do so with a poorly validated Cl⁻ dye MQAE and there is no electrophysiological evidence to support their hypothesis. Indeed, while there is no doubt more work must be done to determine the exact role of both the CLICs and VRAC on NLRP3 activation, it seems that Cl⁻ efflux is a crucial mechanism of NLRP3 activation.

Just as the studies in this thesis identify a new ionic regulator of NLRP3 function they also act to condemn another. Ca²⁺ flux has long been considered a key event in formation of the NLRP3 inflammasome and IL-1 regulation (see section 1.3.3.2). We showed that a new class of NLRP3 inhibiting compounds, the NBC series, could be developed from the starting point of the calcium inhibitor 2APB and that these drugs no longer effect Ca²⁺ flux (Baldwin et al., 2017). In agreement with (Katsnelson et al. (2015) this study suggests that the inflammasome inhibiting effects of 2APB and BAPTA-AM are independent of Ca²⁺.

Like the fenamates, NBC6 is selective for the NLRP3 inflammasome over the AIM2 or NLRC4 inflammasomes. However, at higher concentrations, NBC6 does elicit a statistically significant effect over AIM2 and a non-significant effect over NLRC4 (Baldwin et al., 2017). Classically, it is seen as beneficial for a drug to inhibit NLRP3 and not AIM2 or NLRC4 (Coll et al., 2015). This is likely due to the fact that, for most sterile diseases where inflammasomes are implicated, NLRP3 is the key PRR involved. Interestingly this is not the case in ischaemic stroke where it would seem that AIM2 and NLRC4 are involved through as yet poorly understood mechanisms (Denes et al., 2015). Additionally, evolutionary evidence suggests that inflammasomes evolved primarily to defend against opportunistic pathogens and that the majority of vertebrate-adapted pathogens can evade inflammasome-mediated destruction (Maltez and Miao, 2016). The potential redundancy of inflammasomes in protecting against bacterial-mediated disease in humans would make them a promising drug target for sterile disease.

Although potentially a promising therapeutic avenue, the NBC compounds are at a very early stage of development. However, the NBC compounds may also be used as tools in preclinical research. Unlike the fenamates (and 2APB), the NBCs are poorly reversible as shown by washout experiments (Baldwin et al., 2017). This implies that the as yet unknown target may be covalently modified. Additionally, the effect of the NBCs on the AIM2 and NLRC4 inflammasomes at higher concentrations may also distinguish them from the fenamates and MCC950 making them an important part of the toolkit used by biologists to study the function of inflammasomes.
The majority of this thesis is focussed on the well-characterised cytokine IL-1β. However, in chapter 4 we carried out work to characterise the regulation of the poorly understood IL-1α.

Although both IL-1α and IL-1β bind the same receptor, they appear to have different functions (Chen et al., 2007; Freigang et al., 2013)– there is currently no explanation for this. One possible (and perhaps most likely) explanation is that the functions of IL-1α and β differ due to differences in both spatial and temporal expression. Multiple studies have suggested that IL-1α acts as an early-stage alarmin and is released before IL-1β in paradigms such as ischaemic stroke and sterile injury (Chen et al., 2007; Luheshi et al., 2011). The reason for this temporal separation is unknown but may be explained by the differing cellular expression pattern of the two cytokines, with IL-1α more widely expressed in barrier cells such as epithelial cells and IL-1β limited largely to cells of myeloid lineage (see section 1.3.1). It could be the case that IL-1α-expressing cells are more susceptible to ion flux and death (leading to IL-1α processing and secretion) than IL-1β-expressing cells. The initial IL-1α release could then act in a paracrine or autocrine manner to induce IL-1β expression in neighbouring (or infiltrating) cells – a process which would prime them for IL-1β release. Specific cell-type knockout studies should be conducted to test this hypothesis.

Another suggestion as to the reason for differing functions of IL-1α and IL-1β is an additional IL-1R1-independent role of either cytokine. Evolutionary analysis suggests that IL-1α, expressed only in mammals, evolved as a duplication of IL-1β but has been diverging from IL-1β ever since and is now only ~30% homologous at the amino acid level with conserved regions in the N-terminus (contrary to IL-1β which has conserved region in the IL-1R1-binding C-terminus) (Nat. Commun. In Press). The conserved N-terminal region includes a nuclear localisation sequence and an additional highly conserved region of unknown function. This analysis implies that IL-1α has undergone evolutionary pressure to reside in the nucleus of the cell, possibly alluding to an IL-1R1-independent role. Although some suggestions have been made to the identity of this role (see section 1.3.2), there is little consensus. In vivo studies utilising combinations of IL-1α, IL-1β and IL1R1-deficient mice would help elucidate this effect as there should be no difference between IL-1R1−/− mouse and an IL-1β−/− mouse however there may be a difference between an IL-1R1−/− and IL-1α−/− mouse in response to injury.

In order to effectively elucidate the reason for the conserved NLS we used the CRISPR/Cas9 system to make two point mutations in the NLS of IL-1α to neutralise its effects (Daniels et al., 2017). However, these mutations, along with several shield mutations, led to abolished expression of IL-1α in numerous cell types under numerous
stimuli. Bioinformatics studies suggest this effect was due to loss of function in the IncRNA AS-IL1α.

Long non-coding RNAs, transcribed from sequences originally thought to be 'junk DNA', are crucial regulators of biology (Kung et al., 2013). Indeed, recent genome-wide transcriptome studies have revealed thousands of IncRNAs in the human genome (although for the majority of these there is no functional evidence) (Derrien et al., 2012). Long non-coding RNAs can be categorised into long intergenic noncoding RNAs (lincRNAs), intronic IncRNAs and natural antisense transcripts (NATs), the latter of which are particularly common (Werner et al., 2009). Although the majority of annotated IncRNAs have no known function, many characterised IncRNAs have key roles in innate immune function (Atianand et al., 2017). One of those IncRNAs, AS-IL1α, was recently described to be crucial for initiation of IL-1α transcription following stimulus (Chan et al., 2015). In our study, there was no loss of AS-IL1α transcription but there was a significant predicted alteration in the secondary structure – potentially explaining the loss of IL-1α expression (Daniels et al., 2017).

The central dogma of protein production allows redundancy in nucleotide bases. For example the amino acid leucine can be coded by 6 different combinations of nucleotide codons. In this study we used this phenomenon to our advantage to make silent mutations in the Il1a gene to prevent CRISPR/Cas9-mediated cleavage of our edited construct. These are termed 'shield' mutations. Of the seven point mutations made at DNA level we expected only two non-synonymous mutations at the protein level. However, an RNA transcript is affected at the nucleotide level and thus all seven mutations could affect the end product. Long non-coding RNAs are extremely sensitive to structural alterations as they often rely on secondary loop structures to execute their desired effect (Li et al., 2016). We therefore suggest that the change in secondary structure of the IncRNA AS-IL1α may have led to a loss of function and therefore a loss of IL-1α transcription and translation.

These data open up a new level of regulation for IL-1 and many other proteins. What further regulation on functional proteins can be imposed by both the expression and structure of IncRNAs? With the advent of next generation sequencing techniques such as RNASeq we are now able to rapidly assess genomes in their entirety. Where previous focus on this and of GWAS studies in disease has been of protein coding genes, we now must turn our attention to IncRNAs as they may explain disease phenotypes and pave the way for a much-needed novel class of therapeutics.

One approach that could be taken to modify IncRNA function and treat disease would be genetic editing with technologies such as CRISPR. However, as we observed in our study,
complete understanding of the genetic context in which CRISPR is employed is crucial to avoiding unexpected effects. The CRISPR system has revolutionised the field of genetic engineering since its invention in 2012/2013 (Jinek et al., 2012; Cong et al., 2013). Now, CRISPR, which is a repurposed bacterial immune system, has rapidly accelerated in use and has been tested in both human foetuses (Ma et al., 2017) and adults (NCT02793856). Previous studies have suggested that CRISPR can be subject to numerous off-target effects (Fu et al., 2013; Iyer et al., 2015; Schaefer et al., 2017). In our study, we observed an unexpected phenotype from the on-target effect of mutating the \textit{Il1a} gene (Daniels et al., 2017). Therefore, whilst many scientists now strive to minimise off-target effects, here we suggest that it is important to check for unexpected effects that result from correct editing of the targeted gene. Indeed, some of these concerns can be mitigated by newer technologies that allow for direct base-editing thus preventing dsRNA breaks (Komor et al., 2016; Gaudelli et al., 2017). This technique has progressed into human studies (Liang et al., 2017) but more research is required to fully characterise the relative pros and cons of base-editing vs classical CRISPR/Cas9.

5.3 Critical analysis of methodology

Much of the research presented in this thesis followed the paradigm of extensive \textit{in vitro} characterisation followed by testing \textit{in vivo}. In chapter 2 we demonstrated that the fenamate NSAIDs could inhibit NLRP3 and that this inhibition most likely occurred via blockade of the VRAC ion channel. These experiments were performed in murine bone marrow-derived macrophages (BMDMs) and human THP-1 monocytes. Although BMDM and THP-1 cells are perhaps the classic \textit{in vitro} models used to evaluate NLRP3 activation they may not tell a complete story. It is known that primary human monocytes respond in a different manner to BMDMs and THP-1s when activated by certain stimuli (Diamond et al., 2017) and that neutrophils do not undergo pyroptosis upon NLRP3 activation (Karmakar et al., 2016). Additionally, we could have tested the efficacy of the fenamates on NLRP3 in central nervous system (CNS) cells such as microglia and astrocytes. Whilst it is conceivable that the fenamates could be less potent against NLRP3 in the CNS, transcriptomic data suggests that LRRRC8A, the crucial subunit of the VRAC channel, is well-expressed in both astrocytes and microglia (Zhang et al., 2014). Furthermore, we did not rule out that the fenamate-mediated protection from AD-like symptoms \textit{in vivo} was not caused by peripheral effects.
Another criticism of the in vitro screening approach taken is that we did not test whether fenamates could inhibit Aβ-induced NLRP3 activation. However, considering previous studies which have suggested that ion flux occurs downstream of frustrated phagocytosis in the NLRP3 activation pathway (Muñoz-Planillo et al., 2013), we would predict that VRAC-inhibiting drugs would block NLRP3 activation regardless of the phagocytic stimulus. Additionally, the Aβ-induced activation of NLRP3 is not fully confirmed with the majority of studies using supraphysiological concentrations of peptide to activate inflammasomes in vitro (Halle et al., 2008). Overall, NLRP3 activation mechanisms appear largely conserved between different cell types/stimuli suggesting that the in vitro characterisation carried out in chapters 2 and 3 was sufficient to proceed with in vivo models. Indeed, the drugs from these studies were effective in multiple disease models in vivo thus validating the approach taken with in vitro testing.

An additional potential limitation of the in vitro approaches in this thesis is the use of promiscuous pharmacological compounds to identify targets. Criticisms have been levelled at the drugs DIDS and NPPB for non-specific effects on other channels (Verkman and Galietta, 2009) whilst the fenamates themselves have numerous targets including many chloride channels (Guiamard et al., 2013). One way to ameliorate this issue is to use a battery compounds with some non-specific effects and demonstrate that all these compounds lead to inhibition of the target in question. In this way we used numerous ion channel inhibitors NPPB, DIDS and the specific VRAC blocker DCPIB (Decher et al., 2001) to determine the most likely target of the fenamates leading to inflammasome inhibition is VRAC.

The majority of the potential issues in translatable surrounding in vitro screening can be resolved by in vivo validation. In this thesis we used a total of 5 in vivo models of NLRP3-dependent inflammation to demonstrate that our compounds were NLRP3 inhibitors. There are currently in use a number of models of NLRP3-dependent inflammation including (in addition to those used in this thesis: intraperitoneal LPS, intraperitoneal LPS & ATP, air pouch model, rat Aβ oligomer injection, 3xTgAD mouse model) the CAPS mutant mice (Brydges et al., 2009) and the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS) (Sutton et al., 2006). Although there is little doubt these models are a good way of validating NLRP3 inhibitors in vivo (Coll et al., 2015), there are some questions over clinical validity (with the exception of the CAPS mice that harbour the exact mutations seen in human patients).

Previous work has shown that memory deficits that develop in the APP/PS1 mutant mouse are entirely dependent on NLRP3 (Heneka et al., 2013). In this thesis we used a different
mouse model of AD-like symptoms. The triple transgenic (3xTgAD) mouse harbours the APP Swedish, MAPT P301L, and PSEN1 M146V transgenes (all associated with familial AD in humans) (Oddo et al., 2003). Unlike the commonly used APP/PS1 mouse (Radde et al., 2006) the 3xTgAD mice do develop tau inclusions. However, it has been suggested that these inclusions occur as two distinct phenotypes and are not necessarily a good model of AD (Elder et al., 2010). Additionally, a major drawback of almost all transgenic models of AD has been the lack of neuronal loss (Wirths and Bayer, 2010), a problem which remains to be solved. In chapter 2, we also used a rat model of AD symptoms which is dependent on the direct injection of soluble oligomers of Aβ into the brain (Desbène et al., 2012). In this way, similar to the validation of a mechanism in vitro using a battery of drugs, we can improve confidence of a drug in vivo by using a battery of models. AD is a very heterogeneous disease and comes in many shapes and sizes; it would therefore be appropriate to test novel AD drugs in a wide variety of models. Indeed, more work should be done in the future to test these drugs in additional models of neurodegenerative disease.

The vast majority of in vivo AD models employ some level of overexpression or injection of Aβ. However, all the clinical therapeutics that have been developed from these models have failed, many in high-profile phase III dropouts (Hung and Fu, 2017). Additionally, post-mortem studies showing lack of correlation between amyloid burden and cognitive function in monkeys call into question whether amyloid is a major driver of the disease and therefore whether anti-amyloid therapies will be effective (Sloane et al., 1997). Inflammation could explain this discrepancy, as plaques seen in AD may be inflammatory with those seen in healthy aging could be inert. Indeed, a recent study showed that ASC can seed Aβ plaques (Venegas et al., 2017) suggesting inflammasome formation is a crucial step in the progression of AD pathology.

5.4 Future directions

In this thesis, we characterised two novel classes of NLRP3 inhibitors. One of these, the fenamate class of NSAIDs, inhibits NLRP3 via reversible blockade of the VRAC ion channel and is effective in rodent models of AD (Daniels et al., 2016). In order to confirm the role of VRAC in AD it would be pertinent to cross the 3xTgAD mouse (or another genetic model) with a mouse deficient in LRRC8A and assess memory deficits and neuroinflammation. Unfortunately, LRRC8A−/− mice are subject to numerous abnormalities including increased in utero mortality, growth defects, sterility and hind limb weakness (Kumar et al., 2014). Therefore, an inducible knockout approach should be taken with a Cre/lox system
(Danielian et al., 1998). This would also allow for knockout in specific cell types utilising cell-specific Cre promoters allowing for determination if VRAC expression on microglia or infiltrating myeloid cells is more damaging in AD.

Additionally, the SAR approach taken in chapter 3 of this thesis could be used to develop more potent VRAC inhibitors. Thus, in the same way that we increased potency of the boron-containing compounds on NLRP3 and reduced the non-specific effects on Ca\(^{2+}\) signalling (Baldwin et al., 2017) we could increase the potency of the fenamates and decrease non-specific effects on COX, enzymes which are associated with extensive adverse effects (Vonkeman and van de Laar, 2010). Following the development of more potent VRAC inhibitors it would be vital to test these compounds in animal models of NLRP3 dependent inflammation and AD with the addition of a classical NSAID such as ibuprofen for comparison.

The NBC compounds synthesised and characterised in chapter 3 are far more potent NLRP3 inhibitors than the fenamate compounds (Baldwin et al., 2017). However, like numerous NLRP3 inhibiting compounds (Juliana et al., 2010; Coll et al., 2015; Youm et al., 2015), the molecular target remains unknown. Identification of the molecular target would be a valuable body of future work as it could also pose new questions regarding NLRP3 activation.

In chapter 4 of this thesis we used CRISPR/Cas9 to edit the Il1a gene in vivo and knock out the NLS. However, due to the shield mutations used to prevent Cas9 rebinding and cutting the repaired region we also mutated the crucial regulatory lncRNA AS-IL1α, disrupting its structure and possibly preventing IL-1α transcription (Daniels et al., 2017). The CRISPR field is moving at such a rate that, since the design of the sgRNA in this study, multiple new approaches have been taken that would allow for IL-1α NLS mutation without affecting AS-IL1α. The previously discussed base-editing approach (Komor et al., 2016; Gaudelli et al., 2017) could limit gene changes to the sense strand whilst the use of Cas9-nickase enzyme (in contrast to the standard Cas9 nuclease) can induce single-stranded breaks (Ran et al., 2013; Shen et al., 2014) also preserving antisense sequence and therefore lncRNA structure. Considering the high sensitivity of IL-1α to genetic perturbation it would be vital to begin with in vitro testing to validate and confirm NLS KO without effects on AS-IL1α before proceeding with generation of a new mutant mouse.

With hugely promising clinical trial data showing substantial protection from both cardiovascular defects and cancer (Ridker, Everett, et al., 2017; Ridker, MacFadyen, et al., 2017), it is clear that IL-1 signalling is crucial in sterile disease and a viable target for new therapeutics. Overall, this thesis has contributed to the knowledge of the mechanisms
regulating the IL-1-family cytokines IL-1α and IL-1β. However, some major questions of the field remain unanswered. Amongst these crucial points to be addressed are: ‘What are the direct molecular mechanisms that lead to inflammasome formation?’, ‘How important is NLRP3 in human disease?’, and ‘Why do IL-1α and IL-1β bind to the same receptor but perform such clearly different functions in vivo?’ The answers to these questions will greatly aid development of anti-IL-1 therapies, therapies that may hold the key to the treatment and prevention of non-communicable disease.
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Chapter 6: Appendix 1: Unconventional Pathways of Secretion Contribute to Inflammation

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Unconventional Pathways of Secretion Contribute to Inflammation

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Abstract: In the conventional pathway of protein secretion, leader sequence-containing proteins leave the cell following processing through the endoplasmic reticulum (ER) and Golgi body. However, leaderless proteins also enter the extracellular space through mechanisms collectively known as unconventional secretion. Unconventionally secreted proteins often have vital roles in cell and organism function such as inflammation. Amongst the best-studied inflammatory unconventionally secreted proteins are interleukin (IL)-1β, IL-1α, IL-33 and high-mobility group box 1 (HMGB1). In this review we discuss the current understanding of the unconventional secretion of these proteins and highlight future areas of research such as the role of nuclear localisation.

Keywords: unconventional secretion; nuclear localisation; inflammasome; IL-1β; IL-1α; IL-33; HMGB1

1. Introduction

1.1. Unconventional Protein Secretion

The classic dogma of protein secretion involves processing through the endoplasmic reticulum (ER) and Golgi body before secretion from the cell [1]. This process is dependent on the presence of an N-terminal “leader sequence” (also known as a “signal peptide”) which facilitates linking of the ribosome to the ER and translocation of the peptide [2]. This process of conventional secretion forms the most common way by which proteins leave the cell. However, a number of secreted proteins have been discovered which lack leader sequences and thus do not follow this pathway. Classical examples include fibroblast growth factor 2 (FGF2) [3] and the galectins [4]. These proteins follow an unconventional process of secretion through a variety of mechanisms and are often involved in essential processes such as tissue organisation, cell survival and immune regulation [5,6].

FGF2, also known as basic fibroblast growth factor, is a mitogenic factor that acts primarily by binding to high-affinity cell-surface receptors FGFR1-4 [7]. FGF2 lacks a leader sequence and thus is actively secreted by live cells through an unconventional pathway [8]. It is now understood that FGF2 is secreted by a mechanism dependent on interaction with the lipid membrane. Following binding to the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2), FGF2 is able to translocate directly through the cell membrane via formation of a pore dependent on Tec kinase activation [9]. FGF2 is then captured and ‘pulled out’ of the membrane pore by heparan sulphate chains to which it binds with strong affinity thus leading to its non-conventional secretion [10].

The above mechanism of capture by cell surface proteins is also reflected by the unconventional secretion of the galectins. The galectin family of proteins are β-galactose-recognising lectins involved in cell adhesion, promotion of cell-cell interaction, cell growth and apoptosis [11]. It has been known
for over 25 years that galectin-1 (gal-1) can be secreted from the cell despite the lack of an N-terminal leader sequence [12]. However, it is only more recently that the mechanism behind gal-1 secretion is better understood. Gal-1 is secreted from cells by binding to β-galactosidase counter receptors on the cell membrane [13]. Evidence for this lies in the observation that mutants lacking the β-galactoside binding sites and the galectin counter receptors are deficient in gal-1 secretion. Some unconventionally secreted proteins such as IL-1β (discussed further below) utilise microvesicles or exosomes for their unconventional secretion [14]; however, this is not the case with FGF2 or gal-1 [15] which directly cross the plasma membrane.

1.2. Inflammation

Inflammation is our bodies’ response to infection or injury and is generally beneficial, neutralising the pathogen, and promoting repair and recovery. However, inflammation that occurs in the absence of a pathogen, during non-communicable diseases such as Alzheimer’s, diabetes or stroke is damaging and can make the outcome of the disease worse [16]. Inflammation in cases such as this is regarded as sterile and is now recognised as a therapeutic target. Inflammatory responses are regulated in part by cytokines; proteins that are actively secreted from cells which bind specific receptors to initiate inflammation. An inflammatory response can also be induced by factors called damage associated molecular patterns (DAMPs). DAMPs are endogenous molecules stored within cells that are released and invoke an inflammatory response following stress. Cytokines and DAMPs are key regulators of inflammation and can be secreted via unconventional pathways. Here we review four of these inflammatory mediators: IL-1β, IL-1α, IL-33 and HMGB1 and discuss current understanding of the mechanisms of their regulation and secretion.

2. IL-1β

Perhaps the best-studied of these inflammatory factors is the cytokine interleukin-1β (IL-1β). IL-1β is one of 11 members of the IL-1 family and acts as a crucial regulator of sterile inflammation in addition to host responses to infection. Unlike conventionally secreted cytokines such as IL-6, IL-1β does not have a leader sequence required for secretion through the ER/Golgi pathway and is secreted through unconventional means [17]. IL-1β is produced as an inactive 31 kDa pro form by myeloid cells (such as monocytes and macrophages) in response to activation of membrane bound pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) which detect pathogen associated molecular patterns (PAMPs, e.g., bacterial endotoxin), or DAMPs to initiate inflammation. This upregulation of pro-IL-1β expression is known as a priming step and leads to production of cytokine distributed across the cytosol [18].

Pro-IL-1β remains cell-associated until a second stimulus (a further PAMP or DAMP) triggers processing and secretion of 17 kDa mature IL-β which is dependent on the formation of a multi-molecular complex called an inflammasome (Figure 1). Inflammasomes are large (~1 μm) intracellular protein complexes that comprise a cytosolic PRR, adapter molecule and the enzyme caspase-1 which cleaves inactive pro-IL-1β to a mature form which is secreted from the cell [19]. The best characterised inflammasome is known as the NLRP3 inflammasome, so-called because the PRR molecule is nucleotide-binding oligomerisation domain (NOD)-like receptor, pyrin-containing 3. Upon activation by stimuli that induce potassium efflux from the cell [20], NLRP3 recruits the adapter molecule ASC (apoptosis-associated speck-like protein containing a carboxy-terminal CARD) to form a complex. The ASC molecule forms large prion-like filamentous structures known as specks [21] and recruits the 45 kDa zymogen pro-caspase-1 which is cleaved into an active IL-1β-processing form [22]. In addition to this classical activation of NLRP3 there also exists a “non-canonical” inflammasome activation pathway dependent on caspase-11 in mice and caspase-4 and 5 in humans [23].
Interleukin (IL)-1β is produced as an inactive 31 kDa pro-form which, upon immune stimulus and activation of the inflammasome, is cleaved into a mature 17 kDa form and secreted from the cell. IL-1α is localised to the nucleus due to the presence of a nuclear localisation sequence (NLS). Following stimulus IL-1α is transported out of the nucleus, possibly by phosphorylation or acetylation, and either leaves the cell in its bioactive 31 kDa form or is processed to a more potent 17 kDa form by membrane-associated calpains. IL-33 also contains an NLS and thus is stored in the nucleus. It is fully active as a 33 kDa form and processing by caspase-1 leads to production of an inactive protein. High-mobility group box 1 (HMGB1) is stored in the nucleus but, following stimulus, is translocated into secretory lysosomes in the cytosol by Poly(ADP)-ribose polymerase (PARP)-1-dependent acetylation where it can be secreted either actively by live cells or passively due to necrosis.

IL-1β is secreted through an unconventional pathway that is not fully understood. There are multiple suggested modes of IL-1β release. Perhaps the best characterised mode of release is secretion tightly coupled to pyroptotic cell death. Pyroptosis is a form of inflammatory cell death characterised by the concomitant release of IL-1β and IL-18 [24] and can be induced by NLRP3-activating stimuli such as ATP binding to the extracellular ATP sensor P2X7 [25,26]. The activation of the NLRP3 inflammasome relies on association with the serine-threonine kinase NEK7 which forms a complex along with NLRP3, downstream of the crucial potassium efflux step [27,28]. Exactly how closely coupled cell death and IL-1β release are remains a major research question. Early research in macrophages suggested IL-1β was secreted before cell death as measured by release of lactate dehydrogenase [25,29]. More recent evidence, however, has clarified that membrane permeability...
(indicated by uptake of DNA dye propidium iodide) is required for IL-1β secretion in macrophages [30]. In human monocytic THP-1 cells, necrotic cell death is suggested to be required for IL-1β secretion by multiple stimuli [31]. However, primary human monocytes secrete IL-1β without loss of viability [32]. Death-independent inflammasome activation also appears to occur in the case of mouse dendritic cells [33] and neutrophils [34]. These data indicate that IL-1β can be secreted both in an active mechanism independent of cell death but also in a manner that absolutely requires loss of membrane integrity.

The process by which IL-1β is secreted by pyroptotic cells remained a mystery until last year when two groups used genetic screening methods to identify the N-terminal domain of gasdermin-D as a crucial regulator of pyroptosis [35,36]. It has since been discovered that the N-terminal cleavage product of gasdermin-D translocates to the plasma membrane and forms 10–14 nm pores in the membrane to induce pyroptosis [37–39]. In this way IL-1β is secreted through a mechanism not completely dissimilar to FGF2 which allows for IL-1β to directly cross the plasma membrane whilst FGF2 forms a pore required for its own secretion. It is not fully understood why cells undergo pyroptotic death. One recent suggestion is that pyroptotic cells can retain bacteria within their corpses, structures called pore-induced traps (PITs) which are then cleared by secondary phagocytes [40]. This process could provide an evolutionary explanation for pyroptosis.

Human monocytes can secrete IL-1β in endolysosomal vesicles following treatment with ATP or hypotonic conditions [41]. Additionally, IL-1β secretion has also been observed in exosomes following P2X7 receptor stimulation on macrophages [42,43]. IL-1β release is commonly modelled using both “priming” and “activating” steps as described previously. However, inflammasome activation has also been observed with TLR4 stimulus alone in monocytes [44]. Stimulation of human monocytes with TLR4 ligand lipopolysaccharide (LPS) alone elicits ATP release from the cell which then acts back on membrane-P2X7 receptors in an autocrine mechanism to induce inflammasome activation and IL-1β release [45]. A similar form of “one-step” inflammasome activation and IL-1β release has also been observed in a caspase-5 dependent manner in human monocytes which appeared independent of cell death [32].

IL-1β secretion is also strongly associated with autophagy, a fundamental process through which organelles/proteins are “recycled” in a double-membrane vesicle called an autophagosome [46]. The precise role of autophagy in IL-1β secretion is complex. Some studies suggest a tonic inhibition of IL-1β secretion by both degrading pro-IL-1β and by negatively regulating the NLRP3 inflammasome [47]. Conversely, additional research proposes that mature IL-1β is actively packaged into autophagosomal vesicles and secreted following stimulation of autophagy [48,49].

NLRP3-dependent secretion of IL-1β is closely linked to numerous non-communicable diseases including haemorrhagic disorders [50,51], Alzheimer’s disease [52,53], gout [54], diabetes [55] and atherosclerosis [56]. Aberrant inflammasome activity is also involved directly in a family of genetic diseases known as cryopyrin-associated periodic fever syndromes (CAPS) in which gain-of-function mutations lead to spontaneous NLRP3 activation and IL-1β secretion [57].

Due to the crucial involvement of NLRP3 in sterile disease, but the relative lack of importance in host-defence [58] the inflammasome has become a highly attractive target for therapeutic inhibition. There have been multiple compounds shown to inhibit NLRP3-induced IL-1β secretion (reviewed by [59]). The most NLRP3 inhibitor is MCC950, a diarylsulfonylurea-containing compound initially discovered by Pfizer [60] and since shown to be a potent and specific inhibitor of NLRP3 [61]. Currently available drugs can also be repurposed to inhibit NLRP3. Nucleoside reverse transcriptase inhibitors used to treat human immunodeficiency virus (HIV) inhibit P2X7-induced NLRP3 activation in a model of age-related macular degeneration [62], whilst Daniels et al. [63] showed that the fenamate non-steroidal anti-inflammatory drug (NSAID) mefenamic acid is a specific NLRP3 inhibitor and is effective in rodent models of Alzheimer’s disease.
3. IL-1α

IL-1α is also a member of the IL-1 family and is secreted through unconventional pathways. Although a major mediator of inflammation, IL-1α remains relatively poorly researched when compared to IL-1β. IL-1α was discovered along with IL-1β as a pyrogen [64] and is found at the same locus on chromosome 2 in the region q13 to q21 in humans suggesting it occurred as a result of a duplication event [65]. Like IL-1β, IL-1α is involved in a number of disease states including stroke [66], haemorrhage [67], cancer [68], and atherosclerosis [69]. IL-1α also plays a key role in promoting cellular senescence, a state of permanent cell-cycle arrest undergone by cells as a result of ageing or conditions of cell stress [70,71]. IL-1α regulates senescence by promoting the senescence-associated secretory phenotype (SASP), a response characterised by secretion of IL-6 and IL-8 [72].

Unlike IL-1β, whose expression is generally inducible, IL-1α is reported to be constitutively expressed at low levels in various cell types such as fibroblasts [73] and keratinocytes [74]. However, the bulk of the literature has been performed in myeloid cells and suggests that IL-1α expression is inducible and only observed following stimulation [66,75–77]. The constitutive expression of IL-1α is believed to be regulated by the transcription factor Sp1 which binds between −52 and −45 bp at the 5’ end of the IL1A gene [65,78]. Inducible IL-1α expression on the other hand relies primarily on the AP-1 and NF-κB transcription factors [79,80] thus explaining the overwhelming evidence for induction of IL-1α expression following immune stimulus. An additional mechanism regulating IL-1α expression occurs in the form of a long noncoding (lnc)RNA located on the anti-sense strand of the gene [81]. This lncRNA—its emergence induced by immune stimulus—has been shown to be crucial in promoting transcription and expression of IL-1α in murine macrophages.

In addition to a differing expression pattern in terms of cell types and induction, IL-1α can also be distinguished from IL-1β by its sub-cellular distribution. Unlike IL-1β, pro-IL-1α contains a nuclear localisation sequence (NLS) in the N-terminal pro-piece [82] and thus is found within the nucleus of the cell (Figure 1). NLSs are the best understood mechanism by which cells transport cargo in and out of the nucleus. Transport through the nuclear envelope is regulated by the karyopherin-β (kapβ) family of transport receptors which target short motifs of basic amino acids (the NLS) for nuclear import [83]. The NLS on pro-IL-1α is a highly conserved classical monopartite sequence consisting of KVKKRRRL (human) and KILKKRRL (mouse) at residues 79–86 [82]. Although the presence of the highly conserved NLS on pro-IL-1α has been known for over 30 years, the precise role the motif plays in IL-1α secretion or signalling remains poorly understood. It has been suggested that the N-terminal pro-piece of IL-1α activates transcription of pro-inflammatory genes [84,85], thus maintaining an overall pro-inflammatory function. However, there is also evidence suggesting that the NLS of pro-IL-1α may be anti-inflammatory in nature. It was observed that pro-IL-1α is actively trafficked to the nucleus to dampen inflammation in apoptotic [86] or necrotic cells [18,87].

The mechanisms that regulate nuclear trafficking of pro-IL-1α are characterised. Early research suggested that changing phosphorylation states on crucial lysine residues of the NLS regulates intracellular transport [88,89]. More recent evidence has also proposed that acetylation regulated by histone deacetylase (HDAC) enzymes positively regulates nuclear redistribution [90] implying that post-translational modifications may play a crucial role in pro-IL-1α nuclear shuttling. Further research is required in order to fully elucidate the importance of the NLS in IL-1α signalling/release.

IL-1α functions primarily as a pro-inflammatory cytokine by binding IL-1R1 and activating a MyD88-dependent pathway resulting in NF-κB, c-Jun N-terminal kinase (JNK) and p38 signalling cascades similar to IL-1β [91]. Again similar to IL-1β, IL-1α is produced as a 31 kDa pro-form which contains no leader sequence to target it for conventional protein secretion. Unlike IL-1β however, there is evidence that the pro-form of IL-1α is biologically active [92,93], although the physiological significance of this is yet to be fully understood. Some research has suggested that cleavage of pro-IL-1α into a 17 kDa form renders the cytokine far more active at IL-1R1 [94,95]. However, there is also evidence to suggest that the pro and cleaved forms have similar bioactivity [96]. Cleavage of IL-1α
appears to be primarily regulated by calcium-dependent proteases known as calpains [97,98] (Figure 1). This is suggested by evidence showing that both Ca\(^{2+}\)-free conditions and calpain inhibitors prevent IL-1\(\alpha\) processing and release from macrophages [96,99]. However, the specific calpain required for pro-IL-1\(\alpha\) cleavage is not known. The calpain family is made up of 14 distinct members and the best studied are calpain-1 and calpain-2 [100]. These currently stand as the most likely candidates for IL-1\(\alpha\) processing. Calpains classically perform enzymatic cleavage at the inner leaflet of the plasma membrane tethered to phospholipids [101,102]. IL-1\(\alpha\) has been reported to also bind to phospholipids on the inner-membrane of the cell in a Ca\(^{2+}\)-dependent manner [88] suggesting that cleavage may take place following translocation of IL-1\(\alpha\) to the lipid membrane. More recently, calpain activation has been implicated not only in IL-1\(\alpha\) processing but in a number of members of the P2X7-induced secretome including IL-1\(\beta\) [103].

In addition to cleavage of pro-IL-1\(\alpha\) by calpains, the cytotoxic lymphocyte-derived protease granzyme B is also known to cause IL-1\(\alpha\) processing [95]. Functional cleavage by elastase or chymase was also reported in the above study.

The exact mechanism by which IL-1\(\alpha\) leaves the cell is poorly understood although it does appear that IL-1\(\alpha\) release is associated with cell death. Cohen et al. [86] observed IL-1\(\alpha\) secretion from necrotic, but not apoptotic cells. Additionally, the process of necroptosis, a caspase-independent, RIPK-dependent form of programmed necrosis leads to IL-1\(\alpha\) secretion [99]. IL-1\(\alpha\) is also secreted in a caspase-11-dependent manner in cases of non-canonical inflammasome activation [23]. IL-1\(\alpha\) release following non-canonical inflammasome activation, however, appears independent of NLRP3 or potassium ion efflux (unlike IL-1\(\beta\) secretion) [104]. The link between NLRP3 and IL-1\(\alpha\) secretion was also explored by Gross et al. [96] who discovered that, whilst all inflammasome-activating stimuli induced secretion of IL-1\(\alpha\), only ATP, nigericin and candida albicans-induced secretion was NLRP3-dependent, whilst particulate stimuli such as monosodium-urate (MSU) crystals induced IL-1\(\alpha\) secretion independently of NLRP3. In addition to cell-death dependent IL-1\(\alpha\) secretion, IL-1\(\alpha\) can also be expressed on the cell membrane independently of cell death where it plays a crucial role in driving the senescence-associated secretory phenotype (SASP) [71]. However, the mechanism by which this occurs is not understood. There is also some evidence that IL-1\(\alpha\) secretion precedes cell death [96] and that secretion can occur at a basal (but very low) level in macrophages [105] or aged fibroblasts [106].

The biological relevance of IL-1\(\alpha\) nuclear localisation is poorly defined, and nuclear localisation has been reported to both promote and abrogate inflammation. IL-1\(\alpha\) can also be secreted/released from the cell as a cytokine/DAMP. The mechanisms by which IL-1\(\alpha\) is secreted appear complex, varied and poorly understood. It would appear that by being constitutively expressed in non-myeloid cells and active in its pro-form, IL-1\(\alpha\) can act as a DAMP when released under necrotic conditions. However, following induction of expression in immune cells, active processing by calpains and binding to IL-1R1, IL-1\(\alpha\) also functions as a classical cytokine.

4. IL-33

In addition to IL-1\(\alpha\) and IL-1\(\beta\), other members of the IL-1 family have been reported as DAMPs including IL-33 [107]. IL-33 was first discovered as a nuclear protein and initially named NF-HEV (nuclear factor from high endothelial venules) [108]. IL-33 has been implicated in numerous disease states including respiratory disorders such as asthma [109,110] and COPD [111] in addition to arthritis [112,113]. Like IL-1\(\alpha\), IL-33 is constitutively expressed in multiple cell types and is not required to be induced by a priming stimulus [114,115].

The first identification of IL-33 was of a nuclear protein thought to be preferentially expressed in high endothelial venules (HEVs), structures involved in lymphocyte recruitment [108]. Baekkvoold et al. [108] also identified the classical bipartite nuclear localisation sequence on IL-33. In a following study, the same group rediscovered IL-33 as the IL-1 family ligand for the orphan receptor ST2 and observed that the protein associates with heterochromatin and mitotic chromatin in both human and mouse cells [116]. The precise role played by intranuclear IL-33 is not fully understood.
However, it appears that the nuclear localisation is anti-inflammatory. Evidence for this lies in early
discoveries that IL-33 appears to aid chromatin compaction and repress gene expression [116,117].
Additionally, further studies suggested that the N-terminus of IL-33 may dampen NF-κB signalling by
associating with the NF-κB p65 subunit and preventing binding of p65 to target DNA sequences [118].
Perhaps most strikingly, genetic deletion of the classical bipartite nuclear localisation sequence in mice
leads to a lethal IL-33-mediated inflammatory response, suggesting nuclear localisation is vital for
regulating aberrant inflammatory activity [119].

IL-33 elicits extracellular effects by binding to the ST2 receptor [120]. The C-terminus of the protein,
along with the IL-1 receptor accessory protein (IL-1RAcP) forms a complex with the ST2 receptor in
order to initiate a type 2 inflammatory response [121]. Type 2 responses are classically associated
with injury resolution and helminth infection and characterised by secretion of Th2 cytokines such as
IL-4, IL-5, IL-10, and IL-13 [122]. Following formation of the receptor complex, MyD88, IRAK,
IRAK4, and TRAF6 are all recruited to ST2 leading to a downstream activation of NF-κB, JNK and
mitogen-activated protein kinase (MAPK) signalling [120].

Upon the initial discovery of IL-33 it was suggested that, like IL-1β, caspase-1 is required for
processing of a 31 kDa pro-IL-33 to a 20–22 kDa mature, biologically active form [120]. It has since been
clarified, however, that the 20–22 kDa caspase-1 cleavage product in fact corresponds to the N-terminal
domain (which does not bind or activate the ST2 receptor) and that the full-length 31 kDa form of
IL-33 is fully biologically active [123] (Figure 1).

IL-33 is secreted from cells undergoing necrotic death. This includes death as a result of physical
damage in human endothelial cells [123], detergent-based lysis in mouse
macrophages [124] or by parasite-induced necrosis [125]. Cell damage/death is not always required
for IL-33 secretion. Mouse astrocytes treated with recombinant TNFα actively secrete IL-33 without
loss of cellular viability [126] whilst normal human bronchial airway epithelial cells treated with
extract of the common airway allergen Alternaria alternata also secrete IL-33 without compromising cell
integrity [127]. Alternaria-induced IL-33 secretion independent of cell death occurs as a result of a P2Y
receptor-dependent calcium influx which is caused by autocrine-mediated ATP stimulation, similar to
autocrine IL-1β release mentioned previously [45]. In this way, cells can secrete IL-33 in both active
mechanisms dependent on certain stimuli or in passive mechanisms through cell lysis.

The precise mechanisms underpinning IL-33 secretion remain largely undefined. The fact that
IL-33 is constitutively expressed and that it is active as a pro form, much like IL-1α, suggests that it
primarily functions as a classical DAMP. Further research is required to fully elucidate exactly which
conditions result in active secretion of IL-33 (a cytokine role) versus its release as a DAMP during cell
death, and why this occurs.

5. HMGB1

Unconventionally secreted inflammatory modulators do not just come from the IL-1 family.
In fact, the ability to invoke an inflammatory reaction in the absence of classical secretion methods
is highly conserved and found in multiple species across the animal, plant, protozoan and fungus
kingdoms [128]. The best studied example of this is the phylogenetically ancient protein high-mobility
group box 1 (HMGB1).

HMGB1 was initially discovered and characterised as a nuclear protein after it was found to
co-precipitate with chromosomal DNA. Nuclear localisation of HMGB1 occurs due to the presence
of a complex set of localisation signals located both at amino acids 27–43 and 178–184 [129].
Within the nucleus, HMGB1 acts to regulate gene expression. Here it binds loosely to DNA
(distinguishing it from tightly bound histones) and facilitates DNA bending. This process allows
binding of regulatory complexes such as V(D)J recombinases (responsible for generation of the diverse
repertoire of immunoglobulins and T-cell receptors) [130] or nuclear hormone receptors [131].
Indeed, the importance of the nuclear role of HMGB1 is perhaps best illustrated by the fact that gene
knockout is lethal in mice as a result of disruption of gene transcription, induced by the glucocorticoid receptor [132].

Since it was first described in 1976 [133] HMGB1 was known only for its nuclear role described above. However, more than 20 years following this, a crucial additional role for HMGB1 outside of the cell was discovered [134]. HMGB1 is secreted by macrophages in the later stages of sepsis through an unconventional pathway (Figure 1). Moreover, administration of neutralising antibodies to HMGB1 is protective against endotoxin induced lethality. Following this discovery, the cytokine role of HMGB1 has been further confirmed in numerous studies linking to diseases such as sepsis [135], lung disease [136], arthritis [137], stroke [138,139] and haemorrhagic shock [140].

Following its secretion, HMGB1 promotes an inflammatory response. Although no specific HMGB1 receptor has been identified (unlike IL-1β), it would appear that downstream effector functions occur following binding to polygamous receptors such as the receptor for advanced glycation endproducts (RAGE), TLR2 or TLR4. The first receptor reported to confer downstream effects of HMGB1 was RAGE [141,142]. Upon binding RAGE, HMGB1 initiates activation of NF-κB leading to production of classical proinflammatory cytokines [143]. HMGB1 can also initiate pyroptosis in a RAGE-dependent manner [144]. Additionally, RAGE activation also occurs via a membrane bound form of HMGB1, a process shown to be crucial for axonal sprouting and neurite outgrowth in vitro [145]. HMGB1 also binds the membrane PRR TLR4 and can initiate TNFα release from macrophages [146].

HMGB1 does not contain a leader sequence and thus is secreted via unconventional mechanisms independent of the ER/Golgi [147]. Secretion of HMGB1 occurs via both active and passive mechanisms. The first documented secretion of HMGB1 was observed by an active mechanism with relatively delayed kinetics compared to early pro-inflammatory cytokines IL-1β and TNFα [134]. In this pathway HMGB1 release occurs following generation of the bioactive lipid lysophosphatidylcholine (LPC) at the inflammatory site and is mediated by secretory lysosomes [148]. In this regard HMGB1 appears to behave more as a cytokine than a DAMP.

The active secretion of HMGB1 requires exclusion from the nucleus. This occurs due to acetylation of lysine residues, a process which prevents nuclear localisation and thus shifts the equilibrium of HMGB1 intracellular location towards the cytosol [129] (Figure 1). It was also observed that, upon acetylation by acetyltransferases such as P300/CBP-associated factor (PCAF), P300 or CREB-binding protein and nuclear exclusion, HMGB1 accumulates in secretory lysosomes ready for unconventional release from macrophages. Hyperacetylation of lysine residues can also modulate the ability of HMGB1 to bind and/or bend DNA [149]. Hyperacetylation of HMGB1 is not just modulated by increasing activity of acetyltransferases but also by decreasing activity of deacetylase enzymes histone deacetylase (HDAC)1, 4 and 5 [150,151] and of sirtuin-1 [152]. Although the translocation of HMGB1 into the cytosol requires acetylation there are also other factors involved. Poly(ADP)-ribose polymerase (PARP) is required for HMGB1 translocation from the nucleus in response to DNA-alkylating damage but only if HMGB1 is acetylated [153]. Further research has shown that, following stimulation of macrophages with LPS, extracellular signal-regulated kinase (ERK) signalling induced by reactive oxygen species leads to PARP-1 activation which then shifts the acetylation equilibrium of HMGB towards a more acetylated protein leading to translocation from the nucleus and into the cytoplasm (Figure 1) [154]. In addition to acetylation, phosphorylation has also been shown to play a key role in nuclear shuttling of HMGB1 [155] suggesting multiple modes of posttranslational modification may be important for its unconventional secretion. The above described mechanisms indicate how HMGB1 is relieved from nuclear encapsulation and can be actively secreted from live macrophage cells.

HMGB1 is also passively released from necrotic cells. This occurs due to the fact that HMGB1 only binds loosely to chromatin and thus can diffuse into the extracellular space upon loss of membrane integrity [156]. Necrotic release of HMGB1 occurs far more rapidly than the active secretion pathway. HMGB1 is also released during apoptotic cell death [135]. It was previously thought that HMGB1 was
not released by apoptotic cells [156]. However, it has since been confirmed that HMGB1 is released in an “immunologically silent” form following inactivation by oxidation, a mechanism dependent on mitochondrial ROS [157].

HMGB1 is expressed constitutively in multiple cell types and is localised to the nucleus where it plays a major role in promoting gene transcription. In addition to this property, HMGB1 is unconventionally secreted either actively from live cells or passively as a result of necrosis. As a result of this, HMGB1 can induce an inflammatory response independently of its nuclear role and thus acts as a DAMP.

6. Conclusions

The host response to damage or infection relies on secretion/release of a complex combination of inflammatory factors through unconventional pathways. Some factors, such as the prototypical pro-inflammatory cytokine IL-1β, are not expressed within cells until stimulated by an inflammatory stimulus. Conversely, inflammatory mediators such as HMGB1 and IL-33 are constitutively expressed in multiple cell types and are ready for release. IL-1α appears to play both roles as it is suggested to be constitutively expressed in barrier cells such as the epithelium or endothelium but, similar to IL-1β, expression must be induced in myeloid cells.

IL-1α also appears to cross multiple classifications in terms of processing. Whilst the 31 kDa form is active at the IL-1 receptor, calpain-dependent cleavage to a 17 kDa form appears to substantially increase its potency. IL-1β on the other hand is only capable of activating IL-1R following inflammasome and caspase-1 dependent processing to a 17 kDa form. IL-33 is processed to an inactive form by caspase-1 whilst HMBG1 is not modified by processing but activity is heavily regulated by oxidation state.

IL-1β is also unique to the other proteins discussed here in subcellular distribution. IL-1β is distributed evenly across the cytosol whilst IL-1α, IL-33 and HMGB1 are all actively transported to the nucleus. These nuclear factors therefore hold dual functions with both nuclear and secretory roles. An additional dual role that is held by the factors discussed in this review is the property of both active and passive secretion. All four proteins discussed can be passively released upon cell death but also (to varying degrees and in varying cell types/stimuli) actively secreted by live cells. This suggests that there is an additional layer of dual functionality in unconventionally secreted proteins.

The reason for the differing properties amongst unconventionally secreted inflammatory proteins is not fully understood and the list of these proteins is far longer than the four discussed in this review [158–162]. Amongst these other unconventionally secreted inflammatory proteins are the galectins (discussed previously), in particular galectin-3 (gal-3). Gal-3, unlike gal-1 [15], is secreted in a mechanism dependent on microvesicle shedding [163] and is highly expressed in monocytes, macrophages and dendritic cells [164,165]. Gal-3 is a potent proinflammatory immune modulator strongly associated with leukocyte recruitment [166,167], IL-1 production [168] and chemotaxis [169]. An additional inflammatory protein secreted via an unconventional pathway is interleukin (IL)-18. IL-18, initially described as “IFNγ-inducing factor” shares many properties with IL-1β and is secreted concomitantly with IL-1β following inflammasome activation [170] as well as after stimulation with cytokines such as IL-12 [162]. Like IL-1β, IL-18 is produced as a biologically inactive precursor form and is cleaved primarily by caspase-1 [171] but processing can also be dependent on other enzymes such as neutrophil proteinase-3 [172] or triggered by Fas ligand [173] independent of caspase-1. There also exists for IL-18 an endogenous regulatory mechanism called IL-18 binding protein [174] which is constitutively expressed and acts to dampen IL-18 signalling. The secretion of IL-18 is poorly understood, and it has been suggested that pro-IL-18 is released from dying cells and is processed extracellularly [175]. However, more evidence is required to confirm that cell death is absolutely necessary for IL-18 secretion and to uncover the exact mechanism by which IL-18 is released from the cell.
The reason for unconventional protein secretion is not fully understood. It is clear that unconventional secretion is absolutely necessary for function in the case of FGF2 as, when forced through the ER/Golgi pathway by addition of an FGF4 leader sequence, FGF2 failed to bind heparan sulphate due to deleterious posttranslational modifications [176]. This suggests that the unconventional secretory pathway allows cells to secrete proteins without the requirement for posttranslational modification. There have been similar suggestions relating to the secretion of IL-1β as, when forced through the ER/Golgi, IL-1β is N-glycosylated reportedly leading to loss of function [177,178].

It is perhaps more likely, however, that the reason for the unconventional secretion of inflammatory proteins, such as IL-1β, is in order to protect the host when conventional mechanisms are compromised. It has been observed that, under ER stress, cells remain capable of inflammasome activation (and IL-1 secretion) [179], and also that viral infections can dismantle the trans-Golgi network as a way of preventing host responses [180]. Cells with compromised secretion mechanisms may have severely impaired capability to counter infections if it were not for the ability to secrete proinflammatory factors, and thus mount an inflammatory response, through mechanisms independent of the ER/Golgi network.

Unconventional secretion also allows for proteins to hold roles within the cell additional to that of a classic proinflammatory cytokine. One such additional role, a particular focus of this review, is nuclear localisation. The reason for such contrasting roles to be held by the same protein remains largely unknown. One theory is that packaging in the nucleus prevents aberrant release of potentially damaging proinflammatory factors thus acting as a regulatory mechanism much in a similar fashion to IL-1β or IL-18 which require processing for activity. This is perhaps best exemplified by IL-33 which is highly damaging if excluded from the nucleus [119]. However, with factors such as HMGB1 this seems less likely as an active role within the nucleus is well defined. We hypothesise that the inflammatory function of HMGB1 is a more recent evolutionary modification to allow detection of uncontrolled cell death (in which the cell nucleus is ruptured and DNA is released) [181]. In this way, HMGB1 acts as a classical DAMP to alert the host to infection or damage that has compromised the nuclear membrane. In contrast to this, HMGB1 is also secreted through an active mechanism by live cells, implying it also possesses a signalling role independent of accidental cell death.

It would seem that in the case of IL-1α both of these properties are fulfilled as research has suggested that IL-1α is stored in the nucleus as a regulatory mechanism and that IL-1α acts within the nucleus to promote inflammation. Far more extensive research is required to fully elucidate the importance of the nuclear localisation of inflammatory cytokines.

All of the proteins discussed in this review are closely linked to non-communicable disease and thus may offer attractive targets for therapeutic inhibition. However, these factors also hold many other roles so care must be taken to not impair a vital cellular process or host response to infection when targeting. This emphasises the importance of further research to improve the understanding of how IL-1β, IL-1α, IL-33 and HMGB1 signal and are secreted as, in possession of this knowledge, we may be able to modulate specific roles of these factors (for example secretory cytokine role) without affecting homeostatic function (such as the nuclear role). This knowledge will be vital for the design of new therapeutics targeting inflammation.

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Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 7: Appendix 2: Supplementary Methods

7.1 Cell isolations and culture

7.1.1 BMDMs

Primary bone marrow-derived macrophages (BMDMs) were isolated from the femurs of adult mice. Animals were sacrificed by rising concentration of CO\textsubscript{2} with death confirmed by cervical dislocation (a schedule-1 method approved by the Home Office and the local Animal Ethical Review Group, University of Manchester as described in the United Kingdom Animals (Scientific Procedures) Act, 1986) and leg bones dissected. Femurs were separated, cleaned using blue-roll and cut at the pelvic end using a sterile scalpel. Marrow isolation tubes were prepared by placing a 0.6 mL tube inside a 2 mL tube (Starlab) and making a hole in the 0.6 mL tube with a 19-21 gauge needle (BD Biosciences). Finally, 200 µL sterile PBS (Sigma) was added to the small tube. Femurs were placed cut end down inside the marrow isolation tube prepared above and centrifuged by pulsing to max speed. Next, empty bones were disposed of and flushed marrow was resuspended in 1 mL sterile Dulbecco’s Modified Eagle’s Medium with 4500 mg/L glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate (DMEM, Sigma) before passing through a 70 µm cell strainer (Corning). The strainer was rinsed with 5 mL DMEM, the tube centrifuged (1500 x g, 3 mins) before resuspending in 3 mL ACK Lysis buffer (Lonza). Erythrocytes were lysed for 3 mins at RT before the reaction was stopped by addition of 7 mL sterile PBS. Tubes were centrifuged again (1500 x g, 3 mins) and bone marrow cells resuspended in 20 mL DMEM supplemented with 10 % v/v, heat inactivated, foetal bovine serum (FBS, Life Technologies) and penicillin-streptomycin at 100 U/mL penicillin and 100 µg/mL streptomycin (PenStrep, Sigma) and transferred to a T225 flask (Corning). Cells were supplemented with 10 mL conditioned media from L929 mouse fibroblast cells (ATCC) and incubated for 3 days before a media addition of 5 mL L929 media and 10 mL DMEM + FBS + PenStrep. BMDMs were then cultured for a further 4-7 days (total 7-10 days) before use. One day prior to experiments, BMDMs were removed from flasks by scraping, counted using a haemocytometer and resuspended at 1x10\textsuperscript{6} cells/mL before plating out in 24-well or 96-well plates at 5x10\textsuperscript{5} or 1x10\textsuperscript{5} cells/well respectively (Corning). Cells were kept in a humidified incubator at 37°C with 5 % CO\textsubscript{2}.
7.1.2 Immortalised BMDMs

Immortalised BMDMs (iBMDMs) were derived by (Hornung et al., 2008) and obtained from Claire Bryant (Department of Veterinary Medicine, University of Cambridge). These cells have been isolated as with primary BMDMs (see above) and immortalised by lentiviral infection. iBMDMs were cultured in DMEM supplemented with 10% FBS and 100 U/mL/100 µg/mL PenStrep. Upon reaching confluence, cells were passaged by scraping. One day prior to experiments, iBMDMs were removed from flasks (Corning, vented cap), counted using a haemocytometer and resuspended at 5x10^5 cells/mL before plating out in 24-well plates at 2.5x10^5 cells/well (Corning). Cells were kept in a humidified incubator at 37°C with 5% CO₂.

7.1.3 THP-1 cells

THP-1 cells are a human monocyte-like cell line derived from a patient with acute monocytic leukemia (Tsuchiya et al., 1980). THP-1 (ATCC) cells were cultured in RPMI-1640 Medium with sodium bicarbonate (RPMI, Sigma) supplemented with 10% FBS, 100 U/mL/100 µg/mL PenStrep, 20 mM L-Glutamine and 55 µM 2-mercaptoethanol (Life Technologies). On the day of the experiment, THP-1 cells were resuspended at 1x10^6 cells/mL before plating out in 24-well plates at 5x10^5 cells/well.

7.1.4 Peritoneal macrophages

Peritoneal macrophages were isolated from adult mice by peritoneal lavage. Animals were sacrificed by rising concentration of CO₂ with death confirmed by cervical dislocation (a schedule-1 method approved by the Home Office and the local Animal Ethical Review Group, University of Manchester as described in the United Kingdom Animals (Scientific Procedures) Act, 1986) or placed under terminal anaesthesia with isoflurane (induced at 3–4% in 33% O₂, 67% NO₂, maintained at 1–2%) followed by schedule-1. Peritoneums were washed with 70% ethanol to clean fur. Next, a peritoneal injection was made in the centre of the peritoneal cavity of 6 mL RPMI and peritoneums were manually massaged 10 times to dislodge macrophages. Skin was blunt-dissected from the peritoneal sac and a 2-4 mm incision was made in the peritoneum approximately 1 cm distal from the linae alba to release lavage fluid into the pouch made between the peritoneal sac and the skin. Lavage was collected using a 5 mL syringe (BD Biosciences) and strained using a 70 µm strainer (Corning). Cells were counted using a haemocytometer and resuspended at 1x10^6 cells/mL before plating out in 24-well or 96-well plates at 5x10^5 or 1x10^5 cells/well respectively (Corning).
7.1.5 Platelets

Platelet cells were isolated from fresh blood. Animals were placed under terminal anaesthesia with isoflurane (induced at 3–4% in 33 % O$_2$, 67 % NO$_2$, maintained at 1–2 %) and blood taken by cardia puncture with acid-citrate-dextrose (ACD) anticoagulant solution (85 mM trisodium citrate dihydrate, 66.6 mM citric acid monohydrate, and 111 mM anhydrous D(+)glucose) at a ratio of 1 part ACD to 5 parts blood. Anticoagulated blood was centrifuged (2300 x g, 45 s) and the platelet-rich plasma (PRP) taken. PRP was then centrifuged (2200 x g, 2 min) and the platelet-containing pellet lysed with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 % Triton-X).

7.2 Western blotting

Western blot analysis was performed on supernatants and lysates from in vitro cell experiments. Sodium dodecyl sulphate (SDS) polyacrylamide gels were made up (12 % for IL-1β/IL-1α, 15 % for caspase-1) and samples prepared by combining with 5X sample buffer (10 % SDS, 50 % glycerol, 400 mM Tris-HCl pH 6.8, 0.025 % w/v bromophenol blue, 5 % 2-mercaptoethanol) and boiling at 95 °C for 10 min to denature proteins. THP-1 supernatants were concentrated prior to this by centrifugal filtering (Amicon 10K centrifugal filters). To concentrate supernatants, 350 µL thawed supernatant was loaded into centrifugal concentrator tubes and tubes centrifuged (14000 x g, 15 min) before the concentrate was removed (~30 µL) by inverting the filter and further centrifugation (2 min, 14000 x g). Supernatants from iBMDMs and BMDMs were loaded onto gels without further processing.

Samples were loaded alongside a molecular weight marker (All Blue Precision Plus Protein Standards, Bio-Rad) and run at 75 V using a PowerPac Basic (Bio-Rad) for 20 min until consistently moving through the stacking gel then 140 V until samples had run to the bottom of the gel. After running, samples were transferred onto a Whatman Protran nitrocellulose membrane (GE Life Sciences) at 15 V using a Trans-Blot SD semi-dry transfer system (Bio-Rad) for 45 min (mouse IL-1β) or 1 h (human caspase-1). Alternatively, gels were transferred using a Trans-Blot® Turbo™ Transfer System (BioRad) at 25 V for 7 mins (mouse IL-1α).

Once transferred, membranes were blocked in PBS, 0.1 % Tween (PBST), 5 % skimmed milk powder (Marvel) for 1 h at RT, washed 3 x 5 min in PBST and incubated at 4°C overnight in primary antibody (Table 7.1).
Membranes were washed 3 x 5 min in PBST before incubation with secondary antibody in PBST, 5 % milk for 1 h at RT (Table 7.1). Finally, following secondary antibody incubation membranes were again washed and incubated with Amersham ECL Western Blotting Detection Reagent (GE Life Sciences) for 1 min before exposure on photographic film (Scientific Laboratory Supplies) and developing using a JP-33 automatic film processor (JPI) (caspase-1 samples required ~30 min exposure). Alternatively, membranes were imaged using a G:BOX Chemi XX6 (Syngene).

Table 7.1: Western blotting antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Concentration</th>
<th>Diluent</th>
<th>Supplier</th>
<th>Cat#</th>
<th>Chapter</th>
</tr>
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<td>Goat anti-mouse IL-1β</td>
<td>100 ng/mL</td>
<td>PBST 0.1 % BSA</td>
<td>R &amp; D Systems</td>
<td>AF-401-NA</td>
<td>2</td>
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<tr>
<td>Rabbit anti-human caspase-1</td>
<td>666.6 ng/mL</td>
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<td>Santa Cruz</td>
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<td>Goat anti-mouse IL-1α</td>
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<td>Rabbit anti-goat IgG HRP</td>
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<td>Dako</td>
<td>P0449</td>
<td>2&amp;4</td>
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<td>Goat anti-rabbit IgG HRP</td>
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<td>Dako</td>
<td>P0448</td>
<td>2&amp;4</td>
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</tbody>
</table>

7.3 ELISAs

Supernatants collected from cell treatment experiments were evaluated for IL-1α, IL-1β or IL-6 content by enzyme-linked immunosorbent assay (ELISA) (R&D systems, Table 7.2). Nunc Immuno MaxiSorp 96-well plates (VWR) were coated with capture antibody overnight. Cell supernatants were defrosted on ice while the ELISA plate was washed (3 x in PBS 0.05% Tween) and blocked with 1% bovine serum albumin (BSA) in PBS for 1 h. During the blocking stage a 7-point standard curve was prepared with a top standard of 1000 pg/mL recombinant cytokine and 2x dilution increments. This was made in reaction diluent (RD) comprising 0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline (20 mM Trizma base, 150 mM NaCl) pH 7.4. Following blocking, plates were washed as before and 50 μL standards (in duplicate) and samples (diluted in RD if appropriate) loaded. Plates were incubated at RT for 2 h. Following sample incubation, the ELISA plate was once again washed and detection antibody added for 2 h. After the detection incubation step the plate was once again washed and streptavidin-HRP (in RD) was added in the dark for 25 min before a final
wash step and the addition of tetramethylbenzidine (TMB) substrate (BD Biosciences). The reaction was stopped by addition of 1 M H$_2$SO$_4$ (1:1 ratio) and the plate read at 450 nm corrected for baseline at 570 nm using a Synergy HT plate reader (BioTek). Absolute cytokine concentrations were calculated by interpolating from a standard curve fitted by sigmoidal 4PL equation (GraphPad Prism).

Table 7.2: ELISA Kits

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<th>Cat#</th>
<th>Chapter</th>
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<td>R &amp; D Systems</td>
<td>DY401</td>
<td>2, 3&amp;4</td>
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<td>R &amp; D Systems</td>
<td>DY201</td>
<td>3</td>
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<tr>
<td>Mouse IL-1α</td>
<td>R &amp; D Systems</td>
<td>DY400</td>
<td>3&amp;4</td>
</tr>
<tr>
<td>Mouse IL-6</td>
<td>R &amp; D Systems</td>
<td>DY406</td>
<td>4</td>
</tr>
</tbody>
</table>

7.4 Cell death assay

In order to assess levels of cell death/membrane permeability we evaluated supernatants for presence of lactate dehydrogenase (LDH) via a colorimetric substrate assay (CytoTox 96® Non-Radioactive Cytotoxicity kit, Promega). Following cell experiments, 50 μL supernatant was transferred to a clear, flat-bottomed 96-well plate (Corning) and combined with 50 μL LDH substrate. After 15 min, the reaction was stopped with addition of 50 μL stop solution and the plate read at 490 nm using a Synergy HT plate reader (BioTek). Cell death was expressed relative to 100% lysis (calculated using a treatment group in which cells were lysed with 1% Triton-X for 5 mins at 37 °C).

7.5 Protein concentration assay

In order to normalise total protein concentration of platelet lysates a bicinchoninic acid (BCA) assay was performed (Pierce™ BCA Protein Assay Kit, ThermoFisher). A standard curve was prepared of BSA in PBS from 2 mg/mL to 125 μg/mL and samples were diluted as appropriate (to fall within the standard curve). Samples or standards (10 μL) were combined with 200 μL BCA solution (prepared by combining 50 parts solution A with 1 part solution B from the kit) and the plate incubated at 37 °C for 20-30 mins until the standard curve has developed. The plate was then read at 570 nm using a Synergy HT plate reader.
Absolute protein concentrations were calculated by interpolating from a standard curve fitted by third order polynomial equation (GraphPad Prism).

7.6 Quantitative PCR

RNA transcripts were quantified using qPCR. Following cell experiments, supernatants were removed and cell pellets lysed in 1 mL TRizol™ Reagent (ThermoFisher). Next, 0.2 mL chloroform was added to lysates, tubes incubated for 2-3 min (RT) and centrifuged for 15 min (12000 x g, 4 °C). The aqueous phase of the separated mixture containing RNA was transferred to a new tube. RNA was precipitated by addition of 0.5 mL isopropanol to the aqueous phase. Tubes were incubated for 10 min (RT) and centrifuged for 10 min (12000 x g, 4°C). Next, the supernatant was discarded and the RNA pellet washed by resuspending in 75% ethanol, vortexing, and centrifugation for 5 min (7500 × g, 4°C). The washed pellet was air-dried for 5-10 min and solubilised by resuspending in 20 μL RNase-free distilled water before incubation at 55 °C in a heat block for 10 min. Total RNA yield was quantified using a NanoDrop™ spectrophotometer to calculate ratio of absorbance at 260 nm compared to 280 nm, samples outside of the ratio 2-2.2 were not used as they were considered impure.

RNA (0.6 μg) was reverse-transcribed to cDNA using SuperScript™ IV Reverse Transcriptase (ThermoFisher). RNA was mixed with 1 μL random hexamers (ThermoFisher) and topped up to 13 μL with ddH2O. This mixture was heated at 65 °C for 5 min using a Techne TC-PLUS thermal cycler (Techne) thermocycler then combined with 4 μL 5X buffer, 0.5 μL RNaseOUT™ (ThermoFisher), 1 μL 10 mM dNTPs (Bioline), 0.5 μL reverse transcriptase and 1 μL ddH2O and heated to the following settings: 20 °C, 10 min > 50 °C, 60 mins > 85 °C, 10 mins on a Techne TC-PLUS thermal cycler (Techne).

Quantitative PCR was undertaken using SYBR® Green (ThermoFisher). In a MicroAmp™ Optical 384-Well Reaction Plate (ThermoFisher) cDNA was combined with 10 pmol forward and reverse primers (Table 7.3) and 5 uL 2x SYBR® Green mastermix (ThermoFisher). A standard curve was also prepared with known concentrations of cDNA from wild-type mouse BMDMs stimulated with 1 μg/mL lipopolysaccharide (LPS, Sigma) for 4 h. All samples were loaded with at least 3 technical replicates. The qPCR plate was run on a 7900HT Fast Real-Time PCR System (Applied Biosystems) with the following settings: 50 °C, 2 mins > 95 °C, 10 mins > [95 °C, 15 s > 60 °C, 1 min] x 40 cycles > 95 °C, 15 s > 60 °C, 15 s > 95 °C, 15 s. Data were normalized to the expression of the housekeeping gene 18 SrRNA. Data were analysed using the Applied Biosystems software with the relative standard curve method.
7.7 Genotyping

IL-1α NLS mutant mice were genotyped by PCR. Ear snips were digested using REDExtract-N-Amp™ PCR ReadyMix™ (Sigma). Extraction buffer (25 μL) was mixed with tissue preparation buffer (6.25 μL) and added to ear snips. This solution was incubated at RT for 10 min then at 95 ºC for 3 min. Next, 25 μL neutralisation buffer was added to the reaction before diluting DNA 10x for PCR. The PCR reaction was run using Phusion® High-Fidelity DNA Polymerase (New England BioLabs). Diluted DNA (1 μL) was combined with 0.5 μL Phusion, 10 μL Buffer HF, 2 μL 10mM dNTPs (Bioline), 10 pmol forward and reverse primers (Table 7.3) and 34.5 μL ddH₂O and run with the following settings on a Techne TC-PLUS thermal cycler (Techne): 98 ºC, 5 mins > [98 ºC, 20 s > 60 ºC, 20 s > 72 ºC 45 s] x 35 cycles > 72 ºC, 5 min.

Following PCR reaction, DNA was cleaned up using an ISOLATE II PCR Kit (Bioline). DNA samples were loaded onto the columns and centrifuged for 30 s at 11000 x g before discarding flow-through and washing membrane with 700 μL Wash Buffer CW. Finally, membranes were dried and DNA eluted with 30 μL ddH₂O pre-warmed to 65 ºC. Purified DNA was digested with BseRI (New England BioLabs). Full eluate was digested in 40 μL reaction by adding 4 μL Cutsmart buffer, 5 μL ddH₂O and 1 μL BseRI for 4hrs at 37ºC. Following digestion, the reaction mixture was combined with 10 μL 5x DNA loading dye (Bioline) and run on an agarose gel prepared with 2% agarose (Bioline) in Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.3) with ethidium bromide (Sigma) at 80 V. The gel was then imaged under UV light using a G:BOX Chemi XX6 (Syngene).

For IL-1α NLS mutant mice, the WT allele is 866bp but mutant alleles will be digested to 578bp and 288bp.
Table 7.3: Genotyping primer sequences

<table>
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<th>Direction</th>
<th>Sequence</th>
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<td>Forward</td>
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<tr>
<td>Il1a NLS (Geno)</td>
<td>Reverse</td>
<td>AGCCCAAGAGATTAGAGAACA</td>
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<tr>
<td>Il1a (qPCR)</td>
<td>Forward</td>
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<td>Il1a (qPCR)</td>
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<td>AGGCTTGGATTCACTTGAC</td>
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<tr>
<td>18S rRNA (qPCR)</td>
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</table>

7.8 Flow cytometry

Flow cytometry was performed to assess immune cell populations in lavage of mice stimulated with monosodium urate (MSU) crystals (Chapter 2) and in bone marrow and spleen of IL-1α NLS<sup>mut</sup> mice (Chapter 4).

Cells were prepared as follows. Cells were kept on ice at all times apart from erythrocyte lysis steps which were carried out at RT. Air-pouch lavage cells were passed through a 100 μm strainer (Corning), counted using a haemocytometer and adjusted to a density of 5 × 10<sup>6</sup> cells/mL in ice-cold PBS, 1% BSA, 5 mM EDTA (FACS buffer). Cells were plated out (200 μL) on a clear V-bottomed 96-well plate (ThermoFisher). Splenocytes were isolated by dissecting spleens from mice and macerating against a 100 μm cell strainer into a 50 mL tube. Strainers were rinsed with RPMI-1640 before erythrocytes were lysed with ACK buffer as in section 7.1.1. Following lysis splenocytes were resuspended in 500 μL FACS buffer and plated out into U-bottomed 96-well plates (Corning) at 200 μL/well. Bone marrow cells were isolated from mice by dissecting femurs and flushing as in section 7.1.1. Following lysis of erythrocytes as above cells were plated out into U-bottomed 96-well plates (Corning) at 200 μL/well in FACS buffer.
Air-pouch lavage cells were stained with the antibodies denoted in Table 7.4 for 45 min on ice in the dark. Single stains of each antibody were included in order to calculate compensation controls. Cells were then pelleted by centrifugation of the plate (5000 x g, 3 min) washed by resuspending in FACS buffer twice. Cells were fixed in paraformaldehyde 3.7–4.1% in PBS, 200 μL for 15 min at RT then washed again twice as above. The following day, flow cytometry was performed using a FACSVerse flow cytometer (BD Biosciences) with the FACSuite software (BD Biosciences). Following removal of debris and doublets, events were gated as follows for quantification: 'Neutrophils' - CD45+ > Ly6G+, F4/80-; 'Macrophages' CD45+ > Ly6G+, F4/80+.

Splenocyte and bone marrow cells were stained for 25 min on ice for primary antibodies in FACS buffer (Table 7.4), washed as above then stained again for 25 min on ice in secondary antibodies in PBS (Table 7.4). Single stains of each antibody were included in order to calculate compensation controls. Cells were washed as above twice before fixing in paraformaldehyde 2% in PBS, 100 μL for 15 min at RT. Cells were washed as above and left at 4 °C overnight. The following day, flow cytometry was performed using a FACSCanto flow cytometer (BD Biosciences). All data were analysed using FlowJo software (FlowJo LLC). Following removal of debris, doublets and dead cells, events were gated as follows for quantification: 'Neutrophils' - CD45+ > CD11b+ > Ly6C+, Ly6G+; 'Monocytes' - CD45+ > CD11b+ > Ly6C+, Ly6G-; 'B-cells' CD45+ > CD19+, TCRβ-; 'T-cells' CD45+ > CD19+; TCRβ+; 'CD4 T-cells' CD45+ > CD19+, TCRβ+ > CD4+, CD8-; 'CD8 T-cells' CD45+ > CD19+; TCRβ+ > CD4-, CD8+.
Table 7.4: Flow cytometry antibodies

<table>
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7.9 Supplementary References
