UNDERSTANDING HOW INJURED TISSUE COMMUNICATES WITH THE IMMUNE SYSTEM

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

2013

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Understanding how injured tissue communicates with the immune system

Catherine D Savage


Abstract

Inflammation in the absence of infection (sterile inflammation) is a crucial host defence response to tissue injury, but is also considered to contribute to the pathogenesis of many diverse disease states, including stroke. Sterile inflammation is initiated by damage associated molecular patterns (DAMPs) which are endogenous molecules released from necrotic cells or that are modified during disease. The pro-inflammatory cytokines IL-1α and IL-1β are key mediators of inflammation. IL-1β release is controlled by caspase-1 which, in turn, is regulated by the inflammasome. The NOD-1, LRR-, pyrin domain-containing 3 (NLRP3) inflammasome is most typically associated with sterile inflammation and the recognition of DAMPs. Thus, understanding the mechanisms of NLRP3-activating DAMP-induced inflammation may lead to the identification of novel therapeutic targets with which to treat inflammatory diseases. This thesis sought to determine how NLRP3-activating DAMPs affect the pro-inflammatory response of glia, the immune cells of the brain.

Experimental models in vitro typically use a pathogen associated molecular pattern (PAMP) such as LPS to prime cells before observing their response to NLRP3-activating DAMPs. As the brain is protected by the blood brain barrier (BBB), it is unlikely glia would be exposed to PAMP priming. However it remains unclear as to how glia respond to NLRP3-activating DAMPs in the absence of priming, or what the source of endogenous priming is. Therefore, the initial hypothesis was to investigate the pro-inflammatory response of mixed glia in vitro to NLRP3-activating DAMPs in the absence of PAMP priming. It is shown here for the first time that NLRP3-activating DAMPs can initiate an IL-1-NLRP3-independent inflammatory response in mixed glia in the absence of PAMP priming. Moreover, it is shown that the acute phase protein serum amyloid A is elevated in plasma after stroke and may act as an endogenous priming signal to allow IL-1β-dependent inflammation to contribute to the damage after breakdown of the BBB.

Inflammation following acute sterile injury such as stroke is augmented by persisting cell death. It was therefore hypothesised that NLRP3-activating DAMPs released after the initial injury, may initiate a form of programmed cell death that continues to drive inflammation. Using inhibitors of specific types of cell death, it was identified that NLRP3-activating DAMP induced cell death is likely to be necrosis and not programmed cell death.

Further investigation into the biological importance of DAMP-induced IL-1-independent inflammation and the specific contribution of acute phase proteins to brain pathology may aid the identification of new therapeutic targets.
**Declaration**

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

*Catherine D Savage*

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### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3-MA</td>
<td>3-Methyladenine</td>
</tr>
<tr>
<td>AIM2</td>
<td>absence in melanoma-2</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptotic protease activating factor-1</td>
</tr>
<tr>
<td>ASC</td>
<td>apoptosis speck-like protein containing a CARD</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>BAK</td>
<td>Bcl-2 killer</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl-2 associated X protein</td>
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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchorinic acid</td>
</tr>
<tr>
<td>BID</td>
<td>BH-3 interacting-domain death agonist</td>
</tr>
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<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CalpIII</td>
<td>calpain inhibitor III</td>
</tr>
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<td>CARD</td>
<td>caspase activation and recruitment domain</td>
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<td>CBA</td>
<td>cytometric bead array</td>
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<td>C-C motif ligand 5</td>
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<td>CCL5</td>
<td>C-C motif ligand 5</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<td>cIAP</td>
<td>cellular inhibitor of apoptosis</td>
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<td>CO$_2$</td>
<td>carbon dioxide</td>
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<td>CPPD</td>
<td>calcium pyrophosphate dihydrate</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>Ct</td>
<td>cycle threshold</td>
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<td>C-X-C motif ligand 1</td>
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<td>Description</td>
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<td>CYLD</td>
<td>Cylindromatosis</td>
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<td>DAMP</td>
<td>damage associated molecular pattern</td>
</tr>
<tr>
<td>DD</td>
<td>death domain</td>
</tr>
<tr>
<td>dH2O</td>
<td>distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immuno sorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>FADD</td>
<td>Fas-associated death domain protein</td>
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<td>FBS</td>
<td>foetal bovine serum</td>
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<tr>
<td>GOI</td>
<td>gene of interest</td>
</tr>
<tr>
<td>HMGB-1</td>
<td>high mobility group box 1</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
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<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>i.c.v</td>
<td>intracerebroventricularly</td>
</tr>
<tr>
<td>IAP</td>
<td>inhibitor of apoptosis</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemistry</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<td>immunohistochemistry</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>IL-1F</td>
<td>interleukin-1 family</td>
</tr>
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<td>IL-1Ra</td>
<td>interleukin-1 receptor antagonist</td>
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<tr>
<td>IL-1RAcP</td>
<td>interleukin-1 receptor accessory protein</td>
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<td>IL-1RI</td>
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</tr>
<tr>
<td>IL-1RII</td>
<td>interleukin-1 receptor II</td>
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<td>IL-1α</td>
<td>interleukin-1 alpha</td>
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<td>IL-1β</td>
<td>interleukin-1 beta</td>
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<td>INT</td>
<td>tertrazolium salt (2-(p-iodophenyl)-3(p-nitropheryl)-phenyltetrazolium)</td>
</tr>
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<td>IRAK</td>
<td>IL-1R associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
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<tr>
<td>ITAMs</td>
<td>immunoreceptor tyrosine-based activation motifs</td>
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<tr>
<td>IκB</td>
<td>inhibitor of κB</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>KO</td>
<td>knock out</td>
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<tr>
<td>LAL</td>
<td><em>Limulus</em> amebocyte lysate</td>
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<td>LDH</td>
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<td>lipopolysaccharide</td>
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<td>LRR</td>
<td>leucine rich repeat</td>
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<td>MCAo</td>
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<td>MDP</td>
<td>muramyl dipeptide</td>
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<td>MKK</td>
<td>MAPK kinase</td>
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<td>MMLV</td>
<td>Moloney murine leukaemia virus</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<td>MyD88</td>
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<td>poly(IC)</td>
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<tr>
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<td>pyrin domain</td>
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<td>quantitative polymerase chain reaction</td>
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<td>r.t.</td>
<td>room temperature</td>
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<tr>
<td>RAGE</td>
<td>receptor for advanced glycation end products</td>
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<tr>
<td>RD</td>
<td>reagent diluent</td>
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<td>rhIL-1Ra</td>
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<td>RHIM</td>
<td>RIP homotypic interaction motif</td>
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<td>RIP</td>
<td>receptor-interacting serine/threonine-protein kinase</td>
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<td>reverse transcription</td>
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</tr>
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<td>TIRAP</td>
<td>TIR accessory protein</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethylbenzidine</td>
</tr>
<tr>
<td>tMCAo</td>
<td>transient middle cerebral artery occlusion</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF receptor-associated death domain protein</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF associated factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TIR domain-containing adaptor molecule</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing interferon-b</td>
</tr>
<tr>
<td>Ubc</td>
<td>ubiquitin conjugating enzyme</td>
</tr>
<tr>
<td>UEV1A</td>
<td>Ubc E2 variant 1</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked IAP</td>
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Acknowledgements

I would like to thank the BBSRC and the Wellcome Trust for their funding throughout this project.

A huge amount of thanks goes to my supervisor David Brough for the enormous amount of time and support he has given me over the course of my PhD. He has shown endless patience and has always had his door open with encouraging words of wisdom on the other side of it throughout the whole process. I would also like to thank Doug Millar and Nancy Rothwell for their support and guidance throughout.

In particular I would like to mention Adam Denes and thank him for sharing his knowledge and expertise which have guided me throughout, and for his contribution to the in vivo work within this thesis. Furthermore thanks go to Sara Rollinson for being the qPCR guru and having endless answers to my endless list of questions. Finally to Gloria Lopez-Castejon, Hazel Ball, Sylvie Girard and Nadia Luheshi for their teaching, guidance and encouragement throughout.

This process has been a whirlwind of scientific ups and downs that has been fantastic largely due to the brilliant banter of the Brough and Rothwell groups that have shared the ups, kept me up through the downs, and shared my love of lab snow! Particular thanks go to Fiona Britton and Emily Robinson for being level headed from day one and to Holly Summersgill for providing the yin to my yang to keep Dave on his toes.

To all of my friends outside the lab, you know who you are. Thank you for putting up with me and keeping my work-life balance in check!

To my family, for your constant support, belief and encouragement throughout the whole of my education. Thank you for all always being on the end of a phone when I need you; you make me want to aspire to do better. To my Mum, for always driving me on and for her patient proof reading; to my Dad, for his support and for knowing when to crack a much needed (albeit bad) joke; to my Grandma, for her constant reassurance that everything happens for a reason; and to Helen for being my inspirational best friend.

Finally to Iain. Thank you for the coffee machine that has kept me caffeinated throughout writing! Thanks more though for your priceless gifts of belief, reassurance, encouragement, banter and hugs. Your patience knows no bounds for which I shall be eternally grateful.
For my grandparents,

for all the adult conversations we never got to have
1.1. Summary

Inflammation is an important part of host defence responses aiding removal of infection and repair of injured tissue. Dysregulated inflammation can contribute to disease progression through excessive collateral damage to healthy tissue. Inflammation in the absence of infection (sterile inflammation) contributes to acute injury and chronic disease states. The pathogenesis of many neurological conditions, such as stroke, Alzheimer’s disease and epilepsy, is thought to be dictated by the extent of inflammation. Due to the blood brain barrier, the brain is considered a sterile environment. Thus, it is important to have a better understanding of sterile inflammatory mechanisms in glia, the immune cells of the brain, in order to develop therapeutic strategies for neurological disease.

The pro-inflammatory cytokine interleukin (IL)-1 beta (IL-1β) is a key contributor to ischaemic brain injury. Release of mature IL-1β is reliant on the activity of caspase-1 and a molecular platform that controls caspase-1 activity termed ‘the inflammasome’. The NOD-, LRR-, pyrin domain-containing 3 (NLRP3) inflammasome is most typically associated with sterile inflammation. Release of IL-1β via NLRP3 dependent caspase-1 processing is a two-step process relying on the presence of a priming stimulus such as a pathogen associated molecular pattern (PAMP) or a damage associated molecular pattern (DAMP) that induces expression of pro-IL-1β, pro-caspase-1 and NLRP3. A secondary stimulus activates NLRP3 to allow activation of caspase-1 and subsequent processing and release of IL-1β.

This thesis will focus on the currently poorly understood pro-inflammatory responses of mixed glia (microglia and astrocytes) to NLRP3-activating DAMPs in the absence of a priming stimulus.
1.2. Inflammation

Inflammation is an early and non-specific host immune response to infection or injury. It protects the body from pathogenic infection and promotes repair following tissue injury. It is part of the innate immune response that is the immediate defence mechanism of the body. Furthermore it plays a role in shaping the host adaptive immune response in order to return the body to a healthy state and protect against further insult from the same source.

1.2.1. Recognising danger

The 'danger hypothesis' was first proposed in 1994 by Matzinger as a development of Janeway’s self-nonself theory of 1992. The original model described how the body discriminated invading microorganisms from the body’s own cells through recognition of cell surface antigens to initiate an adaptive immune response (Janeway, 1992). Whilst antigen presentation and recognition is indeed an important part of identifying pathogenic invasion and initiating both the innate and adaptive immune response, this theory did not explain the strong immune response generated following sterile tissue injury. Nor could it explain how harmless non-self-microorganisms such as commensal gut microbiota and dietary antigens do not stimulate an immune response. Matzinger’s proposed model instead suggested that the adaptive immune system has evolved to sense danger signals within the body as opposed to self-vs. nonself (Matzinger, 1994, Matzinger, 2002). The innate immune system is now considered to function as a danger sentinel allowing subsequent adaptive immunity in response to infection and injury whilst avoiding collateral damage in the presence of harmless non-self. This section will review how this recognition takes place and what is still unknown.
1.2.1.1. PAMPs and DAMPs

The immune response to pathogenic stimuli is relatively well understood and has evolved to protect the body from viral, bacterial, fungal and parasitic infection. PAMPs are a diverse set of structurally conserved microbial motifs with biochemically distinct features that are recognised by specific pattern recognition receptors (PRRs). Examples are bacterial cell wall components such as gram-positive bacterial peptidoglycan or the gram-negative bacterial endotoxin lipopolysaccharide (LPS); fungal cell wall components such as β-glucan or α-mannan; viral RNAs [mimicked experimentally by the use of polyinosinic:polycytidylic acid – poly(IC)]; bacterial and viral nucleic acids; and the bacterial filament protein flagellin (Janeway, 1992, Newton and Dixit, 2012, Bianchi, 2007).

The inflammatory response to injury in the absence of infection (sterile inflammation) is comparatively poorly understood and is the focus of this thesis. Sterile inflammation occurs in response to tissue trauma, ischaemic-reperfusion injury or chemically induced injury. It is driven by DAMPs which are endogenous molecules released from necrotic cells or that are modified during injury. Like PAMPs, DAMPs are recognised by PRRs.

Both PAMP- and DAMP-induced inflammation involves the up-regulation of pro-inflammatory mediators and recruitment of innate immune cells. The highly cytotoxic nature of pro-inflammatory mediator’s means inflammation is a tightly regulated process, with three major points at which it can be controlled: control of receptors (Dinarello, 2005, Piccinini and Midwood, 2010); regulation of signal transduction downstream of receptors (Newton and Dixit, 2012); control of synthesis and release of pro-inflammatory mediators (Martinon et al., 2009, Netea et al., 2009). In the case of sterile inflammation, dysregulation at any of these points is widely recognised to enhance the pathogenesis of a
diverse range of human disease states. These include atherosclerosis following the inflammatory response to the engulfment of cholesterol crystals by macrophages (Liu et al., 2006, Ruan et al., 2006); gout and pseudogout, inflammatory joint conditions caused by a build-up of monosodium urate (MSU) or calcium pyrophosphate dihydrate (CPPD) crystals, respectively (Dalbeth and Haskard, 2005, Martinon et al., 2006); Alzheimer’s disease in response to β-amyloid plaques in the brain (Weiner and Frenkel, 2006); and ischaemic-reperfusion injury such as stroke in response to necrotic cell death (Allan and Rothwell, 2001, Brough et al., 2011). Thus, a better understanding of what drives sterile inflammation and how to resolve it early during disease pathogenesis is therapeutically very important.

As already illustrated, the term ‘DAMPs’ encompasses a highly heterogeneous population of molecules. They all exist under normal conditions either at low physiological levels or sequestered in cellular compartments from recognition by the immune system. Upon cellular stress and damage, these molecules become immunogenic. Sequestered DAMPs are released after loss of membrane integrity during necrotic cell death. Examples of such DAMPs include the chromatin associated high mobility group box protein (HMGB)-1 (Scaffidi et al., 2002), heat shock proteins (HSPs) (Quintana and Cohen, 2005), and purine metabolites such as adenosine-5’-triphosphate (ATP) (Bours et al., 2006) and MSU (Shi et al., 2003). Other known DAMPs are fragments of the extracellular matrix (ECM) such as biglycan and hyaluronan, that have been proteolytically cleaved by enzymes released from necrotic cells, or proteases released to aid tissue remodelling (Babelova et al., 2009). A more comprehensive list of DAMPs, their cognate receptors and diseases is shown in Table 1-1 though this is by no means all inclusive as the plethora of recognised DAMPs is constantly evolving (Chen and Nunez, 2010, Piccinini and Midwood, 2010).
Table 1-1: DAMPs, their putative receptors, associated pathologies and key references.

Evidence of a sample of the diverse range of DAMPs, the number of receptors that potentially recognise them, and the pathologies with which they are involved.

<table>
<thead>
<tr>
<th>DAMP</th>
<th>Suggested receptors</th>
<th>Associated pathologies</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>P2X7, NLRP3</td>
<td>Cellular injury</td>
<td>(Bours et al., 2006, Iyer et al., 2009, Mariathasan et al., 2006)</td>
</tr>
<tr>
<td>β-amyloid</td>
<td>RAGE, NLRP3, CD36</td>
<td>Alzheimers’s disease</td>
<td>(Halle et al., 2008, Stewart et al., 2010, Weiner and Frenkel, 2006, Yan et al., 1996)</td>
</tr>
<tr>
<td>Biglycan</td>
<td>TLR2, TLR4, P2X4, P2X7, NLRP3</td>
<td>Cellular injury, Atherosclerosis, Rheumatoid Arthritis</td>
<td>(Bahelova et al., 2009, Frey et al., 2013, Piccinini and Midwood, 2010, Schafer et al., 2005)</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>TLR4, CD14, CD91</td>
<td>Cellular injury, Rheumatoid arthritis</td>
<td>(Busu et al., 2001, Hong et al., 2010)</td>
</tr>
<tr>
<td>Cholesterol crystals</td>
<td>NLRP3, CD26</td>
<td>Atherosclerosis</td>
<td>(Duewell et al., 2010, Liu et al., 2006)</td>
</tr>
<tr>
<td>Calcium pyrophosphate dihydrate crystals</td>
<td>NLRP3</td>
<td>Pseudogout</td>
<td>(Martinon et al., 2006)</td>
</tr>
<tr>
<td>DNA</td>
<td>TLR9, AIM2</td>
<td>Cellular injury</td>
<td>(Burckstummer et al., 2009, Hornung et al., 2009)</td>
</tr>
<tr>
<td>Haem</td>
<td>Unknown</td>
<td>Sub-arachnoid haemorrhage</td>
<td>(Greenhalgh et al., 2012)</td>
</tr>
<tr>
<td>Heparin sulphate</td>
<td>TLR4</td>
<td>Cellular injury, Inflammatory bowel disease, Atherosclerosis</td>
<td>(Johnson et al., 2002, Piccinini and Midwood, 2010)</td>
</tr>
<tr>
<td>HMGB1</td>
<td>TLR2, TLR4, TLR9, RAGE, CD24</td>
<td>Cellular injury, Atherosclerosis, Multiple Sclerosis, Rheumatoid arthritis</td>
<td>(Scaffidi et al., 2002, Tian et al., 2007, Yu et al., 2006)</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>TLR2, TLR4, CD44</td>
<td>Cellular injury</td>
<td>(Frey et al., 2013, Schebner et al., 2006, Taylor et al., 2007, Termeer et al., 2002)</td>
</tr>
<tr>
<td>IL-1α</td>
<td>IL-1R</td>
<td>Cellular injury</td>
<td>(Chen et al., 2007, Eigenbrod et al., 2008, Kono et al., 2010b, Luheshi et al., 2011)</td>
</tr>
<tr>
<td>IL-33</td>
<td>IL-1-like receptor ST2</td>
<td>Cellular injury</td>
<td>(Milovanovic et al., 2012, Mousson et al., 2008)</td>
</tr>
<tr>
<td>Lactate</td>
<td>pH detection</td>
<td>Hypoxia, Seizure</td>
<td>(Chesler, 2005, Li and Siesjo, 1997)</td>
</tr>
<tr>
<td>Oxidised-low density lipoprotein (ox-LDL)</td>
<td>TLR4, CD14, LDL receptor</td>
<td>Atherosclerosis, Rheumatoid arthritis</td>
<td>(Liu et al., 2006, Lourida et al., 2007, Miller et al., 2003, Ruan et al., 2006, Sawamura et al., 1997, Xu et al., 2001)</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>NLRP3</td>
<td>Cellular injury, Alzheimers’s disease</td>
<td>(He et al., 2010, Luheshi et al., 2012)</td>
</tr>
<tr>
<td>Tenascin-C</td>
<td>TLR4</td>
<td>Rheumatoid arthritis, Atherosclerosis, Inflammatory bowel disease, Multiple sclerosis</td>
<td>(Midwood et al., 2009, Piccinini and Midwood, 2010)</td>
</tr>
<tr>
<td>Uric acid and MSU crystals</td>
<td>NLRP3</td>
<td>Gout, gouty arthritis</td>
<td>(Chen et al., 2007, Kono et al., 2010a, Martinon et al., 2006, Shi et al., 2003)</td>
</tr>
<tr>
<td>Versican</td>
<td>TLR2</td>
<td>Cellular injury, Atherosclerosis, Multiple sclerosis</td>
<td>(Frey et al., 2013, Kim et al., 2009, Piccinini and Midwood, 2010)</td>
</tr>
</tbody>
</table>
As the list of DAMPs expands, so does the list of receptors that are involved in the sterile inflammatory response. Given the structural diversity of the molecules interacting with the PRRs, the precise mechanism by which DAMPs induce inflammation remains unclear. Ultimately they take one of three specific pathways to initiate and perpetuate the inflammatory response: affecting a PRR e.g. toll-like receptors (TLRs) and NOD-like receptors (NLRs); binding of a non-microbial PRR e.g. receptor for advanced glycation end products (RAGE); or pro-inflammatory cytokine feedback e.g. IL-1α and IL-1β via the IL-1 receptor (IL-1R1)

1.2.1.2. PRRs

PRRs typically fall into two categories: membrane bound PRRs, for example TLRs; and cytosolic PRRs such as NLRs, absent in melanoma-2 (AIM2) receptor and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). PRRs recognise PAMPs or DAMPs either directly or indirectly, initiating signalling cascades that result in an increase in inflammatory mediators and recruitment of leukocytes. Each PRR recognises distinct subsets of PAMPs or DAMPs and requires oligomerisation with cognate receptors and accessory proteins in order for signal transduction to occur (Piccinini and Midwood, 2010).

TLRs

The most extensively researched group of PRRs is the TLR family. There are ten known members of the TLR family in humans (TLR1-10) and twelve in mice (TLR1-9 and 11-13) (O'Neill, 2006). They are type I transmembrane proteins expressed on cells of the innate immune system, and contain domains of leucine-rich repeats (LRRs) that recognise PAMPs or DAMPs either extracellularly (TLR1, 2, 4, 5, 6 and 11) or in endolysosomal compartments (TLR3, 7, 8, 9 and 10) (Bowie and O'Neill, 2000). All TLRs also contain a highly conserved cytoplasmic toll/IL-1-receptor (TIR) domain that enables them to engage
cytoplasmic signalling proteins (Bowie and O'Neill, 2000, Radons et al., 2003). This domain is also found on IL-1R1, as discussed later.

Upon contact with a PAMP or DAMP the TIR domain in all TLRs except TLR3, engages myeloid differentiation primary response gene (88) (MyD88) either directly via the TLR TIR domain interacting with the MyD88 TIR domain, or indirectly via the TIR accessory protein (TIRAP) (Figure 1-1) (Lin et al., 2010). As well as a TIR domain MyD88 also contains a death domain (DD), which associates with the DD of IL-1R associated kinase 4 (IRAK4) (Li et al., 2002). Upon this association IRAK4 autophosphorylates and its DD binds the DD’s of related kinases IRAK1 and IRAK2 (Figure 1-1) (Kawagoe et al., 2008, Lin et al., 2010). Mice deficient in MyD88 and IRAK4 are susceptible to a wide range of pathogenic infections, whilst humans with MyD88 or IRAK4 deficiency are predisposed to some bacterial infections in infancy and early childhood. This indicates IRAK4 is necessary for normal TLR signal transduction and initiation of the immune response (Picard et al., 2003, von Bernuth et al., 2012).

The MyD88-IRAK complex oligomerises with E3 ubiquitin ligases and E2 ubiquitin-conjugating enzymes (Ubc’s) to form the TLR signalling complex (Figure 1-1). The E3 ubiquitin ligases include tumour necrosis factor (TNF)-associated factor 6 (TRAF6) and cellular inhibitor of apoptosis (cIAP) -1 and -2; and the E2 Ubc’s include Ubc13 and Ubc E2 variant 1 (UEV1A) (Tseng et al., 2010). TRAF6 in particular is essential for the polyubiquitination of downstream molecules that are required for signal transduction (Deng et al., 2000, Lomaga et al., 1999). TRAF6 can form polyubiquitin chains on itself, cIAP1, cIAP2, IRAK1 and IRAK2 (Figure 1-1) (Skaug et al., 2009, Tseng et al., 2010).
Figure 1-1: The TLR signalling complex
Upon PAMP recognition by the LRR region of the TLR, MyD88 engages the TLR via its TIR domain or TIRAP. The MyD88 DD associates with IRAK4, which autophosphorylates itself and IRAK1 and 2 allowing association of its DD with IRAK1 and 2. The MyD88-IRAK complex then oligomerises with the E3 ubiquitin ligases TRAF6, cIAP1 and 2; and the E2 Ubc’s Ubc13 and UEV1A. TRAF6 polyubiquitination of the complex allows subsequent recruitment and activation of downstream signalling pathways. solid line – phosphorylation; punctate line – ubiquitination; PAMP – pathogen associated molecular pattern; LRR – leucine rich repeat; TIR – toll/IL-1 receptor; TIRAP – TIR accessory protein; MyD88 - myeloid differentiation primary response gene; DD – death domain; IRAK - IL-1R associated kinase; TRAF6 - TNF-associated factor 6; cIAP - cellular inhibitor of apoptosis; Ubc13 – ubiquitin conjugating enzyme 13; UEV1A – Ubc E2 variant 1A
These polyubiquitin chains recruit TGF-β-activated protein kinase-binding protein 2 and 3 (TAB2 and TAB3) in complex with TGF-β-activated protein kinase 1 (TAK1) (Figure 1-2A) (Kanayama et al., 2004, Wang et al., 2001). TAK1 is a member of the mitogen activated protein kinase kinase kinase family and is essential for the activation of the MAPKs p38α and c-Jun N-terminal kinase (JNK) (Sato et al., 2005, Shim et al., 2005). Upon encountering TRAF6 and Ubc13 polyubiquitin chains, the TAK1 complex translocates into the cytosol where it autophosphorylates, resulting in its activation (Figure 1-2B) (Tseng et al., 2010, Xia et al., 2009).

Active TAK1 phosphorylates MAPK kinase (MKK) 4 and 7 (Figure 1-2C) that, in turn, phosphorylate the MAPK JNK (Derijard et al., 1995, Finch et al., 2001). Upon activation JNK translocates to the nucleus where it regulates the activity of activator protein-1 (Figure 1-2D). MKK 3 and 6 have both been shown to associate with TRAF6 in response to TLR4 stimulation with LPS (Wan et al., 2009) and it is likely they are also regulated by TAK1 activity (Figure 1-2C). Once phosphorylated these activate the MAPK p38α, which also translocates to the nucleus and controls the activation of the transcription factors CREB and c/EBPβ (Figure 1-2D) (Derijard et al., 1995, Raingeaud et al., 1996).

AP-1, CREB and c/EBPβ all affect the expression of inflammatory genes such as the cytokines IL6, IL8, IL10, IL1α, IL1β, Tnfa; the chemokines Cxcl1 and Cxcl2; and the adhesion molecules Icam1 and Vcam1; to mention but a few (Das et al., 2009, Holzberg et al., 2003, Kang et al., 2008, Kim et al., 2008, Krause et al., 1998, Wolter et al., 2008).
Figure 1-2: Activation of the MAPKs JNK and p38α

(A) Polyubiquitination of the TLR signalling complex recruits TAB2 and TAB3 in complex with TAK1. (B) The TAK1 complex translocates to the cytosol where it is activated by autophosphorylation. (C) Active TAK1 phosphorylates MKK6 and 3, and MKK 4 and 7 which, in turn, phosphorylate p38α and JNK respectively. (D) Phosphorylated p38α and JNK translocate to the nucleus and affect changes in inflammatory gene expression. solid line – phosphorylation; broken line – translocation

TAB2/3 - TGF-β-activated protein kinase-binding protein 2/3; TAK1 - TGF-β-activated protein kinase 1; MAPK – mitogen activated protein kinase; MKK – MAPK kinase; JNK - c-Jun N-terminal kinase; P - phosphate
TAK1 is required for activation of the nuclear transcription factor κB (NFκB), however this process also requires inhibitor of κB (IKB) kinase (IKK) activity (Israel, 2010). Activation of NFκB is under the control of several inhibitory modulators, all of which are reliant on polyubiquitination events within the cell. NFκB subunits are sequestered in the cytosol under normal conditions by IκB proteins that mask their nuclear localisation signals (Jacobs and Harrison, 1998). There are multiple proteins in the IκB family capable of inhibiting NFκB, however the best studied is IκBα. IκBα is regulated by the IKK complex that is made up of IKKα, IKKβ and the NFκB essential modulator (NEMO) (Laplantine et al., 2009, Rothwarf et al., 1998, Zandi and Karin, 1999, Zandi et al., 1997). Activity of the IKK complex is controlled by polyubiquitination of NEMO by E2 ubiquitinating ligases that are part of the TRAF6-TLR signalling complex (Figure 1-3A) (Kanayama et al., 2004, Rahighi et al., 2009, Tokunaga et al., 2009). IKKα/β activation leads to phosphorylation of IκBα targeting it for polyubiquitination by E3 ubiquitin ligases and subsequently, proteasomal degradation (Figure 1-3B) (Kanarek et al., 2010, Kanarek and Ben-Neriah, 2012). IκBα thereby releases its inhibition of NFκB subunits, which translocate to the nucleus and drive pro-inflammatory gene expression (Figure 1-3C) (Newton and Dixit, 2012). The IKK complex also phosphorylates the p50 NFκB1 transcription factor pre-cursor, p105. As a consequence, polyubiquitination and degradation releases the p50 subunit to translocate to the nucleus where it drives inflammatory gene expression either directly or indirectly via the CREB/ATF transcription factor family (Banerjee and Gerondakis, 2007).
Figure 1-3: Activation of NFκB subunits

(A) E2 ubiquitin ligases of the TLR/TRAF6 complex polyubiquitinate NEMO. This allows activation of the IKKα/β complex which phosphorylates IκBα. (B) Phosphorylated IκBα is targeted by E3 ubiquitin ligases and is subsequently degraded by the lysosome. (C) Lysosomal degradation of IκBα releases the NFκB subunits p50 and p65, which translocate to the nucleus and affect changes in inflammatory gene expression. solid line – phosphorylation; punctate line – ubiquitination broken line – translocation/degradation; NEMO - NFκB essential modulator; IKK – IκB kinase; IκBα – inhibitor of κB α; E3 – E3 ubiquitin ligase; P - phosphate
Whilst NFκB influences pro-inflammatory gene expression, it also induces gene expression to provide negative regulatory feedback. This is done via expression of Nfkbia the gene that encodes IκBα protein thus restoring inhibition of NFκB subunits (Sun et al., 2005). An alternative form of negative feedback is provided by the expression of Tnfaip3 that encodes the deubiquitinating enzyme A20. A20 counters ubiquitination by TRAF6 and cIAPs thereby switching off the effect of TLR signalling (Vereecke et al., 2011).

TLR3 is the only TLR not to employ a MyD88-dependent signalling pathway in response to binding double stranded RNA (dsRNA). TLR3 recognises dsRNA within the endosomal compartment and engages the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent signalling pathway via TIR domain-containing adaptor molecule (TRAM) (Figure 1-4A). This pathway can also be engaged by internalised TLR4 receptors responding to relevant PAMPs or DAMPs. TRIF binds the receptor-interacting serine/threonine-protein kinase (RIP) 1 via a RIP homotypic interaction motif (RHIM) (Cusson-Hermance et al., 2005) and is subsequently ubiquitinated by the E3 ubiquitin ligase Peli1 (Chang et al., 2009) (Figure 1-4B). This ubiquitination may result in recruitment and activation of TAK1 and IKK pathways as described above (Newton and Dixit, 2012) (Figure 1-4C). TRIF also engages the type I interferon (IFN) pathway via TRAF3 which polyubiquitinates itself to activate the serine/threonine protein kinase TANK-binding kinase 1(TBK1) (Figure 1-4D). TBK1 in turn phosphorylates the transcription factor interferon regulatory factor (IRF) 3 (Figure 1-4E), which translocates to the nucleus to initiate transcription of IFNα, IFNβ and other IFN-induced genes such as the chemokine CCL5 (Hiscott et al., 1999, Takeuchi and Akira, 2010).
Figure 1-4: TRIF-dependent signalling

(A) Endosomal TLR3 or internalised TLR4 engages TRIF via its cytosolic TRAM domain. (B) TRIF recruits forms a complex with RIP1 which is ubiquitinated by the E3 ubiquitin ligase Peli1. (C) Ubiquitination by Peli1 may result in recruitment and activation of TAK1 and IKK as described above (Figure 1-2 and Figure 1-3 respectively). (D) TRIF also engages TRAF3 causing it to self-polyubiquitinate, activating TBK1. (E) Active TBK1 phosphorylates IRF3 which translocates to the nucleus and affects changes in IFN-inducible gene expression. **solid line** – phosphorylation; **punctate line** – ubiquitination **broken line** – translocation; TRAM – TIR domain containing adaptor molecule; TRIF – TIR-domain-containing adapter-inducing interferon-β; RIP1 – receptor-interacting serine/threonine-protein kinase; Peli1 – E3 ubiquitin ligase; TRAF3 - TNF-associated factor 3; TBK1 – TANK-binding kinase 1; IRF3 – interferon regulatory factor 3
**NLRs**

NLRs are a family of cytosolic PRRs. Key members of this family include nucleotide-binding oligomerisation domain containing protein (NOD) 1, NOD2, NLRP1, NLRP3 and NLR family caspase activation and recruitment domain (CARD)-containing protein 4 (NLRC4). Common to all NLRs is either a CARD or a pyrin domain (PYD) at the N-terminus; a central nucleotide-binding domain (NACHT); and ligand binding LRRs at the C-terminus (Schroder and Tschopp, 2010).

**NOD1 and NOD2**

NOD1 and NOD2 initiate NFκB and MAPK pathways in response to the bacterial peptidoglycans (PGNs) γ-D-glutamyl-meso-dia-minopimelic acid and muramyl dipeptide (MDP), respectively (Fukata et al., 2009). Upon PGN recognition, the CARD domain of both receptors interacts with a homotypic CARD domain on RIP2 (Bertin et al., 1999, Kobayashi et al., 2002). This kinase then interacts with NEMO, converging on the initiation of the NFκB pathway as described above (Inohara et al., 1999, Ogura et al., 2001). Despite converging on the same signalling pathway, this has a distinct initiation process to TLR signalling as it can occur independently of MyD88 and TRAF6 (Park et al., 2007). Alternatively, NOD1 and NOD2 activate the MAPKs p38 and JNK, although the molecular mechanism through which this occurs is not so well understood (Inohara et al., 2005).

**The inflammasomes**

The NLRs NLRP1, NLRP3 and NLRC4 all respond to distinct PAMPs or DAMPs by forming part of a multimolecular caspase-1-activating platform called the inflammasome, of which the NLR is the receptor component. Other components recruited to the inflammasome upon ligand recognition are the adaptor protein apoptosis speck-like
protein containing a CARD (ASC), and the aspartate-specific cysteine protease zymogen pro-caspase-1. Pro-caspase-1 contains a CARD that either directly interacts with the CARD of the NLR, or with the CARD of ASC that binds the NLR via its PYD (Figure 1-5) (Mariathasan et al., 2006, Schroder and Tschopp, 2010). Though not an NLR, the IFN-induced AIM2 receptor is also considered to form an inflammasome that specifically detects cytoplasmic deoxyribonucleic acid (DNA) following infection or tissue injury. It does so by binding the DNA via a p200 domain, subsequently engaging ASC via its PYD (Figure 1-5) (Hornung et al., 2009, Hornung and Latz, 2010b).

The inflammasome is integral to controlling caspase-1 activity. Caspase-1 is a pro-inflammatory cysteine protease whose activity is required for processing and secretion of the pro-inflammatory cytokines IL-1β and IL-18. As will be explained in due course, these cytokines have potent pro-inflammatory characteristics. Therefore caspase-1, like all caspases, is produced as an inactive zymogen to increase the degree to which it is regulated (Martinon et al., 2002). Dysregulated inflammasome activity has been associated with human inflammatory disease states. For example the group of autoinflammatory diseases known as cryopyrin associated periodic syndrome that includes Muckle-Wells syndrome and familial cold autoinflammatory syndrome are associated with elevated IL-1β levels that are linked to NLRP3 mutations and over activity (Agostini et al., 2004, Brydges et al., 2009, Meng et al., 2009). The inflammasome is therefore thought to be central to the shaping of the inflammatory response (Schroder and Tschopp, 2010).
Figure 1-5: Components of the inflammasomes

(A) NLRP3 binds ASC via its PYD. ASC subsequently binds pro-caspase-1 via respective CARD domains allowing removal of the pro-caspase-1 CARD domain and formation of active caspase-1. (B) NLRP1 binds pro-caspase-1 directly via its CARD domain. (C) NLRC4 also binds pro-caspase-1 directly via its CARD domain. (D) Like NLRP3, AIM2 binds ASC via its PYD. ASC subsequently binds and activates pro-caspase-1 via respective CARD domains. PYD – pyrin domain; NACHT – nucleotide binding and oligomerisation domain; LRR – leucine rich repeats; CARD – caspase recruitment domain; FIIND – domain with function to find; p200 – DNA binding domain. Adapted from Schroder and Tschopp (2010).
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NLRP3

The NLRP3 inflammasome can sense whole pathogens such as the fungi *Candida albicans*, the bacteria *Listeria monocytogenes*, and influenza virus, to name but a few (Gross et al., 2009, Joly et al., 2009, Kim et al., 2010, Thomas et al., 2009). However, NLRP3 has emerged as having a key role in the sensing of cytosolic stress and is considered the key receptor for sterile inflammation (Cassel and Sutterwala, 2010) as illustrated by the number of DAMPs with which it is thought to interact (Table 1-1). It is also the most extensively studied of the inflammasomes. The mechanism by which NLRP3 is activated is highly debated. Given the diverse range of agonists that can activate this receptor, it is likely that NLRP3 recognises a common cellular signal instigated by each agonist as opposed to the agonist itself. There are 3 distinct and yet not necessarily exclusive models accepted as pathways through which signalling can converge on NLRP3.

The first is via efflux of potassium (K⁺) ions. This occurs in response to DAMPs such as ATP. In the example of ATP, the purinergic receptor P2X ligand-gated ion channel 7 (P2X7) is engaged resulting in opening of ion channels and subsequent K⁺ efflux. Inhibition of K⁺ efflux abolishes NLRP3 activation (Eisenbarth et al., 2008, Dostert et al., 2008) thus, it is proposed that NLRP3 is a sensor of low intracellular K⁺, however the mechanism by which this happens is unknown (Petrilli et al., 2007).

Activation of P2X7 also leads to the formation of a pannexin-1 hemi-channel, the inhibition of which abrogates mature IL-1β release from human and mouse macrophages (Pelegrin and Surprenant, 2006). It is considered that this pore formation allows NLRP3 agonists into the cytosol to act directly on the NLRP3 receptor (Kanneganti et al., 2007); however this is controversial due to the diversity of NLRP3 agonists and the unknown entity of how they interact with NLRP3.
The second mechanism is via phagocytosed particles such as MSU or CPPD (Martinon et al., 2006). Once internalised, these cause lysosomal rupture releasing the content of the lysosome into the cytosol. The NLRP3 inflammasome may sense these lysosomal components and become activated (Hornung et al., 2008). Cathepsin B is one such protease released after lysosomal destabilisation that can activate the NLRP3 inflammasome in response to β-amyloid uptake (Halle et al., 2008). However it is unlikely that Cathepsin B is solely responsible for IL-1β release as inhibitors of Cathepsin B only partially impair NLRP3 activation (Hornung et al., 2008), and Cathepsin B deficient macrophages have normal IL-1β release in response to some particulate agonists such as MSU and alum (Dostert et al., 2009). Thus investigation into mechanisms of NLRP3 activation by lysosomal destabilisation requires further work.

The third mechanism postulated is via reactive oxygen species (ROS). ROS are highly reactive oxygen containing molecules such as hydrogen peroxide and superoxide anions that are generated by cellular enzymes such as NADPH oxidase; or as a metabolic by-product by the mitochondria (Droge, 2002). They play an important role in cellular physiology, but are also part of an evolutionarily conserved innate immune mechanism. NADPH-oxidase dependent ROS generation is responsible for anti-microbial responses in plants (Bolwell, 1999, Ma and Berkowitz, 2007) and mammals (Bogdan et al., 2000, Niethammer et al., 2009). In mammals NADPH-oxidases generate an oxidative burst directed at invading pathogens (Bogdan et al., 2000). Furthermore, ROS release from necrotic cells generates a gradient that directs leukocyte recruitment to the site of injury (Niethammer et al., 2009).

NLRP3 agonists generate the production of ROS, and the use of antioxidants inhibits the activation of NLRP3 (Zhou et al., 2010). Thus ROS are proposed as a mechanism of NLRP3 activation. ROS can be produced as a result of incomplete
phagocytosis, an alternative model for particulate stimulation of NLRP3 to the lysosomal destabilisation model described above. In this model, targeting ROS production by inhibiting NADPH oxidase attenuates NLRP3 activity in response to asbestos and silica (Dostert et al., 2008, Cassel et al., 2008). However, non-particulate DAMPs are also capable of instigating ROS driven NLRP3 activity. For example, ATP induces ROS-dependent NLRP3 activation via phosphoinositide 3-kinase activity and use of NADPH oxidase inhibitors to block ROS generation supresses’ this activity (Cruz et al., 2007, Hewinson et al., 2008). The ROS mechanism may also be inter-regulated with K\(^+\) efflux as this can trigger ROS production (Petrilli et al., 2007, Fay et al., 2006); however further work is required to better understand the interaction of these two mechanisms.

As already mentioned, mitochondria are a source of physiological ROS generated as a by-product of oxidative phosphorylation. Aberrant mitochondrial ROS production induces NLRP3 activity (Zhou et al., 2011). Furthermore, mitochondrial DNA that is associated with the inner mitochondrial membrane initiates NLRP3 activation when released into the cytoplasm after mitochondrial disruption (Nakahira et al., 2011, Zhou et al., 2011). Again, however, the precise mechanism by which NLRP3 is activated is not clear. A recent study has demonstrated for the first time, that oxidised DNA interacts directly with NLRP3 to activate the NLRP3 inflammasome (Shimada et al., 2012). This may be a central mechanism on which all of the above converge. The addition of extracellular K\(^+\) abolished mitochondrial disruption; therefore it can be considered this is dependent on K\(^+\) efflux. Furthermore, though NLRP3 bound both oxidised and non-oxidised DNA directly, only oxidised DNA specifically activated the NLRP3 inflammasome, whilst non-oxidised DNA activated the AIM2 inflammasome (Shimada et al., 2012). As mitochondrial disruption occurs as a feature of apoptosis (section 1.3.1), it
remains to be seen whether this process can happen in the absence of apoptosis. However it is the first mechanism shown to directly engage NLRP3.

Other mechanisms continue to emerge as having regulatory control over NLRP3, for example ubiquitination and microRNA-223 have both been shown to negatively regulate NLRP3 activity (Haneklaus et al., 2013). The key point to highlight is that though it is known NLRP3 is important in inflammation and particularly in sterile inflammation, the mechanism by which it is controlled remains far from fully understood.

**NLRP1 and NLRC4 (Ipaf)**

Compared to NLRP3 relatively little is known about NLRP1 and NLRC4 (otherwise known as Ipaf). Both are involved in the recognition of pathogenic stimuli. As this thesis focuses primarily on sterile inflammation, the role of NLRP1 and NLRC4 in innate immunity to pathogenic infection are reviewed here in brief, but are more extensively reviewed elsewhere (Martinon et al., 2009, Sutterwala and Flavell, 2009, Franchi et al., 2012).

NLRP1 is structurally different from other inflammasomes in that it contains both a CARD and a PYD (Figure 1-5B). Due to this it does not require ASC in order to regulate caspase-1; however the presence of ASC augments the effect when NLRP1 is stimulated with MDP in the human monocyte cell line Thp-1 (Faustin et al., 2007). Besides capase-1, NLRP1 also interacts with inflammatory caspase-5 (Martinon et al., 2002). NLRP1 has been identified as the receptor for lethal anthrax toxin (Boyden and Dietrich, 2006, Terra et al., 2010) but also senses the PGN component MDP when in complex with NOD2 (Hsu et al., 2008).

NLRC4 is important in the detection of intracellular bacterial pathogens such as *Salmonella enterica serovar Typhimurium, Legionella pneumophila* and *Pseudomonas aeruginosa* (Amer et al., 2006, Franchi et al., 2006, Franchi et al., 2007). It does so by
recognising a conserved region on the C-terminal of bacterial flagella, or on proteins structurally homologous to this region that are found in the host cell secretory system such as PrgJ (Franchi et al., 2006, Lightfield et al., 2008, Miao et al., 2006). NLRC4 interacts with flagella or PrgJ-like proteins via distinct NAIP proteins, another family of NLR receptors (Kofoed and Vance, 2011). This interaction may occur via the LRR domain of NAIP family members allowing conformational change of NAIP and subsequent NLRC4 activation, however this is still speculative and further work into this mechanism is required (Franchi et al., 2012). NLRC4 activation results in isolation and eradication of the invading micro-organism. Whilst IL-1β and IL-18 are processed and released as a product of NLRC4 activation and subsequent caspase-1 activity (Raupach et al., 2006), they are not thought to be the main effector mechanism of this immune response. Rather, NLRC4 activation initiates a form of caspase-1 dependent necrosis known as pyroptosis (Miao et al., 2010) and inhibits intracellular bacterial replication (Akhter et al., 2009, Archer et al., 2010). Interestingly in the case of *Legionella pneumophila* the inhibition of bacterial replication is dependent on neuronal apoptosis inhibitory protein 5 interaction but not necessarily caspase-1 activity, illustrating caspase-1 is not the sole target of the inflammasome (Lamkanfi et al., 2007).

**AIM2**

Intracellular DNA is known to trigger an innate immune response (Muruve et al., 2008, Vilaysane and Muruve, 2009). A number of studies have shown the p200 family member AIM2 as the receptor for cytoplasmic DNA (Burckstummer et al., 2009, Fernandes-Alnemri et al., 2009, Hornung et al., 2009) and this has been extensively reviewed (Hornung and Latz, 2010b, Krieg, 2009, Schroder et al., 2009). All members of the p200 family share a section of 200 conserved amino acids referred to as the p200 domain (Choubey et al., 2010). Other p200 proteins have roles in cell growth and
differentiation (Asefa et al., 2004). The p200 domain of AIM2 binds cytoplasmic DNA and initiates an innate immune response by forming an inflammasome complex, initiating processing and release of IL-1β and caspase-1 dependent cell death. (Fernandes-Alnemri et al., 2009, Burckstummer et al., 2009). It does so via a PYD domain that is required for homotypic protein interaction with the PYD of ASC, and subsequent caspase-1 recruitment and activation (Figure 1-5D) (Burckstummer et al., 2009, Fernandes-Alnemri et al., 2009, Hornung et al., 2009).

Whilst AIM2 is important in the recognition of intracellular microbial DNA, it is particularly important to consider its role in the recognition of host DNA. Cellular stress that leads to mitochondrial disruption releases host mitochondrial DNA into the cytoplasm. This engages the AIM2 inflammasome (Shimada et al., 2012) indicating AIM2 may be an additional receptor involved in sterile inflammation. Given the evidence that NLRP3 responds to oxidised DNA released after mitochondrial disruption (Shimada et al., 2012), and the broad spectrum of sterile agonists to which NLRP3 responds (section 1.2.1.2 – NLRP3 and Table 1-1), it is unlikely that targeting the AIM2 receptor alone would necessarily be therapeutically beneficial. However very little is currently known about the role of AIM2 in sterile inflammation therefore further work is required to determine how significant a contribution it makes to the sterile inflammatory response.

**RLRs**

RLRs are another family of cytosolic PRRs made up of RIG-I, melanoma differentiation associated gene 5 and laboratory of genetics and physiology 2 (Kawai and Akira, 2009). These are RNA helicases that detect viral infection through the recognition of single stranded or double stranded viral RNA. The presence of dsRNA is a hallmark of viral replication, and its detection co-ordinates a type I IFN anti-viral response thereby preventing viral development within the cell (Yoneyama et al., 2004, Kato et al., 2005,
Kato et al., 2006). RNA is detected by the repressor domain at the C-terminal of RLRs (Cui et al., 2008). RLRs also contain a central DExD/H helicase domain with an ATP-binding motif, and tandem CARD domains which interact with corresponding CARD domains on downstream targets in order to initiate a type I IFN response and transcription of inflammatory cytokines (Yoneyama et al., 2004, Takahasi et al., 2008). RLRs also potentially interact with ASC to induce caspase-1 activation and IL-1β processing in response to viral RNA (Poeck et al., 2010). The role of RLRs in host defence against viral infection is more extensively reviewed elsewhere (Yoneyama and Fujita, 2008, Kawai and Akira, 2009, Kawai and Akira, 2006, Rehwinkel and Reis e Sousa, 2010).

1.2.1.3. Non-microbial PRRs

RAGE is a receptor involved in the initiation of innate immunity that recognises endogenous molecules generated or released during cellular stress or damage. As suggested by the receptor name, RAGE detects advanced glycation end products under normal conditions or during pathological states involving oxidative stress (Li et al., 2012a). However, RAGE is also known to detect DAMPs such as HMGB1, S100 proteins and β-amyloid (Hofmann et al., 1999, Tian et al., 2007, Yan et al., 1996). Therefore RAGE is considered to be a non-PRR based mechanism for detecting sterile inflammation in disease states such as atherosclerosis, rheumatoid arthritis, diabetes, cancer and Alzheimer’s (Sims et al., 2010). The part played by RAGE in driving sterile inflammation is still largely unknown and it is possible that its key role is to augment the inflammatory response. It is ubiquitously expressed at low levels (Brett et al., 1993), however the RAGE promoter has NFkB and specificity protein 1 transcription factor binding sites which allow it to be rapidly upregulated in response to pro-inflammatory mediators (Li and Schmidt, 1997, Schmidt et al., 2001). It is a type I transmembrane protein with three extracellular immunoglobulin-like domains, C1, V and C2 (Dattilo et al., 2007). It signals via its
cytosolic C-terminal, however the precise mechanism underlying this signalling requires further clarification. A putative binding site for extracellular signal related kinases -1 and -2 has been demonstrated (Ishihara et al., 2003), however due to the contaminating elements that are found in most RAGE agonist preparations, the signalling pathways that ensue to upregulate pro-inflammatory cytokine expression remain unclear.

1.2.1.4. Pro-inflammatory cytokine recognition

The cytokines IL-1α and IL-33 are constitutively expressed and sequestered in endo-, epi- and mesothelial cells throughout the body. Intracellularly they can both act as DNA binding nuclear factors (Carriere et al., 2007, Werman et al., 2004). Evidence suggests both may act as early indicators of tissue damage (Cayrol and Girard, 2009, Haraldsen et al., 2009, Luheshi et al., 2011), yet relatively little is known about them compared to the related cytokine IL-1β. Necrotic cell death may lead to the aberrant release of these cytokines into the extracellular space alerting the innate immune system to tissue damage (Mousson et al., 2008, Eigenbrod et al., 2008). Both are produced as precursors, but unlike IL-1β and IL-18, the precursors are biologically active and initiate inflammation.

Pro-IL-1α acts via IL-1R1, as detailed in section 1.2.2.4. When released by necrotic dendritic cells and mesothelial cells, pro-IL-1α induces an increase in the chemokine C-X-C motif ligand (CXCL)-1 which results in the rapid recruitment of neutrophils to the injured tissue (Chen et al., 2007, Eigenbrod et al., 2008). This pro-inflammatory effect is specific to necrosis as pro-IL-1α is sequestered in apoptotic cells (Cohen et al., 2010). However, not all necrotic cells have this effect as necrotic macrophages do not induce neutrophil recruitment, indicating the actions of pro-IL-1α may be dependent upon the type of cell from which it is released (Kono et al., 2010b).
Pro-IL-33 acts via the IL-1-like receptor ST2. Two splice variants of ST2 mRNA results in the transmembrane receptor, ST2L, and a soluble decoy receptor sST2 (Bergers et al., 1994, Oshikawa et al., 2002). ST2 requires recruitment of the IL-1R accessory protein (IL-1RAcP) in order to transduce a signal (Schmitz et al., 2005). The IL-33/ST2 axis then transduces pro-inflammatory signalling in the same manner as IL-1R1 via the MyD88-IRAK4-IRAK1/2 pathway as detailed below in section 1.2.2.4 (Lin et al., 2010, Moulin et al., 2007). Pro-IL-33 is active when released from necrotic cells, however inactivated when processed by caspases during apoptosis (Cayrol and Girard, 2009). Active IL-33 induces release of cytokines associated with the initiation of the adaptive immune response and therefore acts as a bridge between innate and adaptive immune systems (Schmitz et al., 2005).

Whilst pro-IL-1α and pro-IL-33 have been shown to initiate pro-inflammatory responses, as indicated above they have some dual functionality depending on the environment in which they are released and the cells they are released from (Luheshi et al., 2009, Milovanovic et al., 2012). It appears likely they act to alert the immune system to cellular damage and initiate the healing response, however much more work is needed to fully understand the role of these cytokines in inflammation.

1.2.2. Propagating inflammation

As illustrated above, recognition of danger results in signalling converging on similar pathways that affect pro-inflammatory gene transcription. PAMPs and/or DAMPs are recognised by local innate immune cells residing in tissues such as mast cells, fibroblasts and macrophages; circulating leukocytes such as neutrophils and monocytes; and vascular cells such as endothelial cells (Newton and Dixit, 2012, Chen and Nunez, 2010). Mast cells produce histamine and prostaglandins that act on vascular smooth muscle causing vasodilation. This increases blood flow to the area thereby increasing the
number of circulating leukocytes brought to the site (Newton and Dixit, 2012).

Macrophages and mast cells increase their transcription of chemokines such as IL-8, C-C motif ligand 5 (CCL5), CCL2, CXCL-1 and CXCL-2, among others (Eigenbrod et al., 2008, Kumai et al., 2004, Shichita et al., 2012, Terao et al., 2008, Terao et al., 2009). These are responsible for the chemotactic attraction of circulating leukocytes to the affected site. Macrophages and endothelial cells increase ROS production that can direct oxidative bursts at invading microorganisms (Bogdan et al., 2000) and contribute to IL-1β processing (section 1.2.1.2 – NLRP3) (Cassel et al., 2008, Cruz et al., 2007, Dostert et al., 2008, Hewinson et al., 2008). Furthermore, migrating leukocytes follow ROS gradients to the affected site (Niethammer et al., 2009). In addition to responding to the initial stimulus, local cells react to each other’s pro-inflammatory responses. For example, endothelial cells respond to the release of cytokines and chemokine’s by upregulating vascular adhesion molecules important in the adhesion and transmigration of neutrophils from the blood stream into the tissue (Wang et al., 1995, Muller, 2003, Zarbock et al., 2011). Fibroblasts respond to the increase in pro-inflammatory mediators by proliferating and producing proteases, collagenases and more prostaglandins. The proteases and collagenases are involved in further tissue destruction and tissue remodelling resulting in excessive fibrosis and formation of scar tissue (Chen and Nunez, 2010, Martin and Leibovich, 2005).

Besides the responses described above and perhaps more importantly, local innate immune cells propagate inflammation by increasing transcription of key pro-inflammatory cytokines including IL-6, TNFα and IL-1β.

Interleukin-6 is a pleiotropic cytokine rapidly upregulated and released by innate immune cells and has both pro- and anti-inflammatory actions. It is able to diffuse across endothelial barriers and therefore escapes into the circulation and, among other things, is a
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major regulator of the acute phase response (Schultz and Arnold, 1990). Acute phase proteins such as C-reactive protein (CRP) and serum amyloid A (SAA) are synthesised in response to IL-6 (Uhlar and Whitehead, 1999, Castell et al., 1989, Jensen and Whitehead, 1998, Schultz and Arnold, 1990). These proteins are mainly synthesised in the liver and released into the plasma (Schultz and Arnold, 1990), however they can be synthesised in other cells such as macrophages, endothelial cells and smooth muscle cells (Urieli-Shoval et al., 1998). Acute phase proteins increase up to 1000-fold in inflammatory conditions and are therefore frequently used as biomarkers of inflammation (Morrow et al., 1998, Morrow et al., 2000). A build-up of SAA can lead to amyloidosis (Uhlar and Whitehead, 1999), and there is recent evidence that it may affect expression and release of proinflammatory cytokines from human monocytes (Furlaneto and Campa, 2000, Niemi et al., 2011), however whether acute phase proteins have a direct role in the pathogenesis of inflammation or are just by-products that can be used as biomarkers remains unclear.

TNFα is another pleiotropic cytokine that is expressed in rapid response to tissue injury and can have either a cell survival or a cell death effect depending upon its environment (Baxter et al., 1999, Heller and Kronke, 1994). It acts via TNF receptor 1 or 2 (TNFR1 or TNFR2) each of which engages different signalling pathways with different outcomes (Peschon et al., 1998). TNFR1 activates NFκB and p38 MAPK pathways whereas TNFR2 targets JNK pathways specifically (Jupp et al., 2001). Both receptors can mediate caspase dependent apoptosis or necroptosis, although TNFR1 does so more potently (Vandenabeele et al., 1995). This perhaps is due to the fact it is more ubiquitous in nature than TNFR2, which is expressed more specifically on endothelial and haemopoietic cells (Vandenabeele et al., 1995). Mechanisms of TNF-mediated caspase-dependent cell death are discussed in due course and are reviewed more extensively elsewhere (Newton and Dixit, 2012, Peschon et al., 1998, Aggarwal, 2003). Due to these
multiple functions, the exact contribution of TNFα to inflammation is a subject of great debate. For example, in a mouse model of stroke, targeting TNFα using TNFRI knock-out mice or anti-TNFα antibodies has contradictory effects, the former being neurotoxic and the latter neuroprotective (Hallenbeck, 2002).

IL-1β is a potent pro-inflammatory cytokine central to the process of both PAMP and DAMP driven inflammation and is the key cytokine of interest in this thesis. IL-1 has been described as the proto-typical pro-inflammatory cytokine (Dinarello, 1998). IL-1 levels increase during inflammatory states and it is known to regulate pro-inflammatory gene expression, having diverse effects including upregulating cytokine expression, acute phase proteins and adhesion molecules important in the process of neutrophil recruitment and thus the production of subsequent inflammatory mediators (Wang et al., 1995, Dinarello, 1996). IL-1 is associated with the pathogenesis of many sterile chronic inflammatory disease states in the periphery such as juvenile onset arthritis, chronic obstructive pulmonary disorder, atherosclerosis, obesity and diabetes (Galea et al., 1996, Chung, 2001, Church et al., 2008, Duewell et al., 2010, Dinarello, 2011, Vandanmagsar et al., 2011).

1.2.2.1. The IL-1 family of cytokines

The best characterised members of the IL-1 family are the agonists IL-1α (IL-1F1) and IL-1β (IL-1F2) and IL-18 (IL-1F4) (Barksby et al., 2007, Dinarello, 2009). Other IL-1 family ligands have been identified through sequence homology, including IL-33 (IL-1F11) and its cognate receptor ST2, as mentioned above (Milovanovic et al., 2012). IL-18 acts on the IL-18 receptor and, much like IL-33, is involved in bridging the gap between the innate and the adaptive immune responses stimulating T helper cell proliferation as well as neutrophil infiltration (Gracie et al., 2003).
The IL-1 family includes IL-1RI and IL-1RIAcP essential for IL-1\( \alpha \) and \( \beta \) signalling (Boutin et al., 2003). Signalling via IL-1RI is described in more detail in due course. There is also a decoy receptor, IL-1RII that has a truncated intracellular domain that cannot associate with IL-1RAcP to transduce signalling when the receptor is bound (Colotta et al., 1993). This forms part of the self-regulation within the IL-1 family that ensures tight control of the pro-inflammatory actions of IL-1\( \alpha \) and \( \beta \). Another example of IL-1 family self-regulation is the naturally occurring IL-1 receptor antagonist (IL-1Ra) (IL-1F3), which directly competes with IL-1\( \alpha \) and \( \beta \) for binding of IL-1RI (Dinarello, 2009). There is also IL-18 binding protein that binds and neutralises IL-18 activity (Novick et al., 1999), and non-IL-1 specific regulators of inflammation IL-F5 and IL-F7 that can mediate anti-inflammatory activity and down-regulate proinflammatory mediators such as TNF\( \alpha \), IL-6 and CXCL2 (Costelloe et al., 2008, Sharma et al., 2008).

With regards to IL-1, this thesis focuses predominantly on the induction of IL-1\( \beta \) and IL-1\( \alpha \) during sterile inflammation in the brain. Constitutive IL-1 expression in the brain is very low (Vitkovic et al., 2000), but is significantly elevated in neurodegenerative disease states (Allan et al., 2005, Brough et al., 2011). It has been associated with the pathogenesis of neuroinflammatory conditions including Creutzfeldt-Jakob disease, Parkinson’s disease, Alzheimer’s disease, epilepsy and stroke (Blum-Degen et al., 1995, Lodick and Rothwell, 1996, Van Everbroeck et al., 2002, Allan et al., 2005, Simi et al., 2007). Intracerebroventricular (i.c.v) injection of IL-1 after reperfusion of rats undergoing an experimental model of stroke (middle cerebral artery occlusion – MCAo), exacerbates inflammation in the brain measured by oedema, infarct size and neutrophil infiltration (Yamasaki et al., 1995). Furthermore, mice that are double KO for IL-1\( \alpha \) and \( \beta \) are protected after MCAo compared to WT mice (Boutin et al., 2001).
Blocking IL-1 is therapeutically beneficial. The most extensively investigated mechanism for this has been using the naturally occurring antagonist IL-1Ra administered either i.c.v or peripherally in animal models of brain injury (Relton and Rothwell, 1992, Garcia et al., 1995, Mulcahy et al., 2003, Greenhalgh et al., 2012, Loddick and Rothwell, 1996). Recombinant human IL-1Ra (rhIL-1Ra) has also been used in phase II clinical trials for treatment of acute stroke patients. When administered within 6 hours of the onset of stroke symptoms, inflammatory biomarkers were reduced and the patient’s clinical outcome after 3 months was ameliorated in patients receiving rhIL-1Ra compared to those receiving placebo (Emsley et al., 2005).

Targeting caspase-1, required for the release of active IL-1β, is also neuroprotective. Caspase-1 deficient mice have significantly reduced ischaemia-induced damage (Schielke et al., 1998). Furthermore, use of caspase-1 inhibitors is neuroprotective after ischaemic conditions in the brain (Loddick et al., 1996, Hara et al., 1997, Li et al., 2000, Rabuffetti et al., 2000, Ross et al., 2007, Wu et al., 2010). However, the role of caspase-1 in cell death may not be purely dependent on IL-1β (Denes et al., 2011b) as discussed below.

The importance of IL-1 in sterile inflammation after acute brain injury is clear. However, the use of IL-1Ra has its limitations as a therapeutic solution. In animal models, IL-1Ra is only effective when administered up to 3 hours after MCAo (Mulcahy et al., 2003). Moreover, there are limitations to administering it peripherally in that is seen only to enter the brain parenchyma in areas of blood brain barrier (BBB) breakdown (Greenhalgh et al., 2010). This may not be an issue as it is reaching affected tissue in the case of cerebral ischaemia. However this may limit use of IL-1Ra in other models of neuroinflammation.
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It is possible anti-IL-1β therapies target the cerebrospinal fluid and periphery as opposed to parenchymal levels of IL-1β meaning BBB penetration is not an issue (Denes et al., 2011c). The relevance of systemic IL-1β to neuroinflammation is supported by evidence that administration of systemic IL-1β exacerbates neuronal damage and BBB breakdown in response to MCAo (McColl et al., 2007). Research also indicates that in co-morbid models such as obese or atherosclerotic rodents, systemic IL-1 is elevated and increases inflammatory markers within the brain (Drake et al., 2011). Thus though the contribution of IL-1 to neurodegeneration is apparent, the mechanism by which it achieves this is complex and still not fully understood, making further research in this area imperative.

1.2.2.2. **IL-1β production**

IL-1α and β are produced as 31kDa precursors, pro-IL-1α and pro-IL-1β, in response to activation of signalling pathways by pro-inflammatory stimuli as described above. As already mentioned, pro-IL-1α is constitutively expressed at low levels and biologically active and therefore does not need processing to have an effect via IL-1RI. It may play a role in driving acute phase inflammation via IL-1RI following tissue necrosis (Eigenbrod et al., 2008, Chen et al., 2007, Dinarello, 2010). However recent evidence shows processing of pro-IL-1α by proteases released during inflammation enhances its biological potency indicating IL-1α has a role in inflammation beyond the initial alerting to danger (Afonina et al., 2011).

Pro-IL-1β is inactive and requires proteolytic cleavage by the cysteine protease caspase-1 in order to release mature, active 17kDa IL-1β. The release of active IL-1β is therefore considered a two-step process requiring independent stimuli (Hornung and Latz, 2010a, Lopez-Castejon and Brough, 2011).
STEP I: PRIMING

The first step is known as ‘priming’ and involves NF-κB dependent induction of pro-IL-1β expression and expression of the inflammasome receptor components (Hornung and Latz, 2010a).

Typically in vitro, cells are primed using a microbial product such as the bacterial endotoxin LPS. This acts via TLR4 to induce NF-κB dependent expression of pro-IL-1β, NLRP3 and other inflammasome receptors (Bauernfeind et al., 2009, Marina-Garcia et al., 2008). In vivo priming is not required to get an IL-1β response to DAMPs (Scaffidi et al., 2002, Kono et al., 2010a). It is unlikely there are high levels of LPS present in the body, particularly in the brain, suggesting there must be a sterile endogenous priming stimulus.

Evidence suggests TLR2 and 4 acting DAMPs such as HSPs and HMGB-1 are able to stimulate an IL-1β response in vitro and in vivo (Vabulas et al., 2001, Yu et al., 2006). TLR2 and 4 have also been shown to play a role in hyaluronan and soluble proteoglycan component driven inflammatory responses (Babelova et al., 2009, Jiang et al., 2005, Scheibner et al., 2006, Schaefer et al., 2005). TLR2 and 4 are also implicated in inflammation associated with atherosclerosis, obesity and insulin resistance (Mullick et al., 2005, Michelsen et al., 2004, Shi et al., 2006). Furthermore TLR4 KO mice have demonstrable neuroprotection after MCAo (Hyakkoku et al., 2010). Thus it is possible that the endogenous priming component is a TLR-acting DAMP.

However, in a model of peritoneal inflammation, TLR2 and TLR4 double KOs show only a slightly reduced inflammatory response (Chen et al., 2007). The functional relevance of TLRs in sensing endogenous priming therefore remains unclear. NF-κB acting cytokines such as TNFα are also able to induce IL-1β responses under sterile conditions (Franchi et al., 2009), and the acute phase protein SAA is suggested to induce IL-1β expression in neutrophils and macrophages (Furlaneto and Campa, 2000, Niemi et
Further study is still needed to elucidate the source and mechanism of endogenous priming especially within the protected structure of the brain.

**STEP 2: PROCESSING**

As already mentioned, in order for mature IL-1β to be released it must be processed by caspase-1. Given active caspases drive and control both inflammation and cell death, they are also produced as inactive pre-cursors whose activity is tightly regulated by the previously mentioned inflammasomes (section 1.2.1.2 – The inflammasomes). The second stimulus prompts the formation of the inflammasome allowing pro-caspase-1 to be cleaved thereby allowing IL-1β processing (Martinon et al., 2002, Brough and Rothwell, 2007, Schroder and Tschopp, 2010).

**1.2.2.3. IL-1β release**

IL-1β is not released from cells via the classical secretory pathway of the endoplasmic reticulum (ER) and golgi apparatus. Classical secretion involves cotranslational delivery of proteins and requires a signal peptide on the N-terminus of the nascent protein as it emerges (Cross et al., 2009, Rothman, 1994). This binds the ER and translocates the protein through the ER lumen and golgi apparatus. During this process the protein is folded and undergoes post-translational modifications before golgi derived vesicles fuse with the plasma membrane and release the finished protein from the cell (Jackson, 2009). IL-1β lacks the signal peptide sequence that targets proteins for conventional secretion and is therefore said to follow a non-conventional secretory route (Auron et al., 1984, Rubartelli et al., 1990). Diverse proposals have been made for how release occurs, yet no unified concept has been concluded. These mechanisms are reviewed more extensively elsewhere and summarised in brief below (Eder, 2009, Lopez-Castejon and Brough, 2011).
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The first mechanism involves exocytosis of IL-1β containing lysosomes. This allows a small amount of IL-1β that is targeted to lysosomes for degradation during autophagy, to be rescued from its fate and released from the cell (Andrei et al., 1999, Harris et al., 2011). The next mechanism proposed has been termed protective release and involves IL-1β release in microvesicles shed from the plasma membrane (Bianco et al., 2009, Bianco et al., 2005, MacKenzie et al., 2001); or in released exosomes (Qu et al., 2007). In the case of microvesicle shedding, IL-1β is thought to be released extracellularly when the vesicle encounters ATP or another IL-1RI expressing cell (MacKenzie et al., 2001, Pizzirani et al., 2007). As ATP is a DAMP released from necrotic cells, this method could be very relevant in the context of sterile inflammation. Exosomal release is postulated to protect IL-1β from rapid degradation in the plasma allowing it to reach targets distal to its source (Kudo et al., 1990, Lopez-Castejon and Brough, 2011). The final suggested mechanism is that IL-1β is released in an event that is terminal for the cell. That is, pro-IL-1β is released after cell lysis and processed extracellularly (Stehlik, 2009); or through pores formed in the plasma membrane during pyroptosis (Fink and Cookson, 2006). Pyroptosis is crucial to host defence against intracellular microorganisms, but also occurs in response to sterile stimuli (section 1.3.3) (Bergsbaken and Cookson, 2007, Bergsbaken et al., 2009). IL-1β release is not the main purpose of pyroptosis; however pore-formation that occurs during this process may provide a passage through which IL-1β can escape (Lopez-Castejon and Brough, 2011).

It was recently suggested that these release mechanisms may not be mutually exclusive and may exist on a continuum dependent on the extracellular requirements of IL-1β (Lopez-Castejon and Brough, 2011). However, this remains a dynamic area of IL-1β research with much yet to be understood.
1.2.2.4. **IL-1 signalling**

Once IL-1α and β are released they rapidly affect the mRNA expression of hundreds of genes in multiple cell types including monocytes, macrophages, endothelial cells and fibroblasts (Holzberg et al., 2003, Jeong et al., 2004, Jura et al., 2008, Vincenti and Brinckerhoff, 2001). They do so via IL-1RI, a member of the Toll/IL-1 receptor family. Unlike TLRs, IL-1RI is expressed ubiquitously on multiple cell types throughout the body (Weber et al., 2010). This helps to amplify initial danger signals recognised by TLRs, into full systemic inflammatory responses. Although IL-1RI specifically binds IL-1α and β, it can also bind IL-18 and IL-33, though with lower affinity (Arend et al., 2008).

The mechanism of IL-1RI signalling is very similar to that described for TLRs (section 1.2.1.2 – TLRs). Upon IL-1 binding, IL-1RI undergoes a conformational change that allows the recruitment of IL-1RAcP to the receptor. This recruitment is a requirement for signal transduction (Casadio et al., 2001, Wesche et al., 1997). Like TLRs, IL-1RI has a cytosolic TIR domain that engages the same receptor complex made up of MyD88, IRAK4, IRAK1/2 and TRAF6. Signalling induces JNK, p38 MAPK or NFκB signalling resulting in the stabilisation of mRNA and regulation of gene expression (section 1.2.1.2 - TLRs) (Weber et al., 2010).

1.2.3. **Consequences of inflammation**

Due to the ubiquitous nature of IL-1RI and the many IL-1 responsive genes, IL-1 signalling rapidly augments inflammation (Jura et al., 2008, Weber et al., 2010). Of particular note is that IL-1β leads to a rapid upregulation of adhesion molecules on endothelial cells (Wang et al., 1995). These are responsible for the recruitment of circulating neutrophils and monocytes, and their extravasation from the blood stream into the affected tissue (Woodfin et al., 2009, Ley et al., 2007, Schmidt et al., 2011). Besides engulfing infected and damaged tissue, infiltrating leukocytes produce a second wave of
inflammatory mediators including more cytokines, chemokines, ROS and proteases such as cathepsin B and matrix metalloproteinases (MMPs). These mediators can be cytotoxic and tissue is degraded indiscriminately thereby causing collateral damage to surrounding healthy tissue (Allen et al., 2012, Chen and Nunez, 2010). This loss of function of otherwise healthy tissue can have devastating consequences in situations such as the brain after cerebral ischaemia. Thus, it is imperative to learn how to control this process in order to limit this collateral damage.

1.3. **Cell death and sterile inflammation**

As already discussed, necrotic cell death occurring after extreme damage to a cell such as during conditions of trauma or ischaemia typically constitutes loss of cell membrane integrity and subsequent release of the usually sequestered contents of the cells into the extracellular space. Further cell death usually occurs during the inflammatory process that follows; however this is not necessarily necrotic cell death. Rather, it may be a form of programmed cell death that occurs in response to the DAMPs released from the initial injury. Thus understanding the mechanisms of DAMP-induced cell death is important. This next section discusses well-researched mechanisms of cell death and their potential relevance to sterile inflammation.

1.3.1. **Apoptosis**

Apoptosis is an evolutionarily conserved form of programmed cell death that functions to get rid of excess or potentially harmful cells. It is integral to development and maintaining homeostasis. With regards to the immune system, apoptosis is essential for the safe disposal of unwanted phagocytosed matter, whether it is of pathogenic origin or cellular debris.
Apoptosis occurs under the control of caspases and the Bcl-2 family of proteins. Caspases fall into three phylogenetically distinct sub-categories: inflammatory; cell death initiator; cell death effector. Inflammatory caspases include caspase-1, -4 and -5 that, as already discussed, control the processing and release of the pro-inflammatory cytokines IL-1β and IL-18 (Martinon and Tschopp, 2007). Cell death initiator caspases are caspase-2, -8, -9 and -10. These are activated upstream of cell death effector caspases and control the activation of effector caspases. Cell death effector caspases are caspase-3, -6 and -7 (Hengartner, 2000). When activated, these instigate processes that ultimately result in apoptosis.

1.3.1.1. **Effector caspases**

Although caspase action is very specific, effector caspases act on many substrates within the cell. Some of these may be innocent bystanders, but some are integral to the process of apoptosis (Nicholson, 1999, Thornberry et al., 1997). Caspase-3 activates endonucleases that cut genomic DNA between nucleosomes rendering it non-functional (Wyllie, 1980). Nuclear lamins are cleaved resulting in nuclear shrinking and budding characteristic of apoptosis (Buendia et al., 1999). There is loss of the cytoskeletal structure (Kothakota et al., 1997), and p21 activated kinases are cleaved and involved in the dismantling of the cytoskeletal structure and blebbing of the cell that is seen (Rudel and Bokoch, 1997). Due to these widespread effects, the cell produces caspases as inactive zymogens and their activation is very tightly controlled.

1.3.1.2. **Activating caspases**

Two pathways have been described for the mechanism of activating cell death caspases: the extrinsic and the intrinsic (Figure 1-6). This allows for signals either from outside or inside the cell, respectively, to converge on a pathway that condemns the cell to
its fate. The extrinsic pathway relies on signalling via death receptors such as the Fas receptor (Hengartner, 2000, Siegel and Fleisher, 1999). Caspase-8 is key to death receptor signalling (Krammer, 2000). The intracellular domains of death receptors interact with the adaptor molecule Fas-associated death domain protein (FADD). This adaptor molecule pulls pro-caspase-8 molecules together allowing them to oligomerise with each other by proximity, resulting in activation of caspase-8 (Muzio et al., 1998, Siegel, 2006).

The Bcl-2 family is a group of proteins encoded by the Bcl2 gene that contains pro- and anti-apoptotic members, the control and balance of which is regulated by caspases and ultimately determines a cells fate. The Bcl-2 family has been extensively reviewed elsewhere (Antonsson and Martinou, 2000, Kaufmann et al., 2012, Strasser, 2005). Whilst active caspase-8 directly cleaves and activates effector caspase-3, it also cleaves pro-apoptotic BH-3 interacting-domain death agonist (BID), a member of the Bcl-2 family, thereby feeding into the intrinsic apoptosis loop (Figure 1-6A). Truncated BID (tBID) inhibits the anti-apoptotic Bcl-2 and Bcl-XL which usually suppress the action of Bcl-2 associated X protein (BAX) and/or Bcl-2 killer (BAK) on the outer mitochondrial membrane (Figure 1-6Bi) (Li et al., 1998). This inhibition of the anti-apoptotic Bcl-2 family members results in BAX and BAK activity leading to outer mitochondrial membrane permeabilisation and subsequent release of sequestered pro-apoptotic factors such as cytochrome c and second mitochondria activator of caspases (SMAC) (Figure 1-6C) (Gross et al., 1999, Siegel, 2006, Loeffler and Kroemer, 2000). At this point, the intrinsic apoptotic pathway converges with the extrinsic death receptor pathway as BAX also senses intracellular DNA damage allowing mitochondrial damage to follow (Rich et al., 2000) (Figure 1-6Bii).

Pro-apoptotic factors released from the mitochondrial intermembrane space act in two different ways. Factors such as SMAC block the action of inhibitors of apoptosis
(IAPs). IAPs are a family of structurally related endogenous inhibitors of cell death that inhibit caspases either directly or indirectly. X-linked IAP (XIAP) is one of the best characterised of the family and inhibits caspase-3, -7 and -9 by direct binding (Kaufmann et al., 2012, Eckelman et al., 2006, Srinivasula et al., 2001). cIAP -1 and -2 act upstream of mitochondria regulating receptor-mediated signalling pathways via interaction with TNF-receptor associated factors -1 and -2. Release of mitochondrial factors such as SMAC results in auto-ubiquitination of IAPs, targeting them for degradation and thereby results in disinhibiting pro-apoptotic caspases (Figure 1-6D) (Du et al., 2000, Verhagen et al., 2000, Varfolomeev et al., 2007).

Cytochrome c is another pro-apoptotic factor released from the mitochondrial membrane. Whilst the role of cytochrome c in cell respiration is very well established, it also has an integral role in the activation of initiator caspase-9. Oligomerisation of pro-caspase-9 with cytochrome c and apoptotic protease activating factor-1 (APAF-1) forms a structure termed the apoptosome. This essentially activates caspase-9 allowing it to cleave and activate effector caspase-3 (Figure 1-6E) (Li et al., 1997, Zou et al., 1997, Cain et al., 1999, Caroppi et al., 2009).

The activation of caspase-8 and -9 are just two well-researched examples of how effector caspases can be activated. However, they demonstrate the high level of regulatory mechanisms the cell has in place to ensure amplification of the initial death signal and that the cell is committed to its fate.

1.3.1.3. Inhibiting apoptosis

Inhibition of caspases can slow the progress of apoptosis and has been shown to rescue cells (Robertson et al., 2000, Earnshaw et al., 1999). Moreover, caspase-9 and -3 KO mice have defective apoptosis (Zheng et al., 1999). Therefore caspases present themselves as a possible therapeutic target, not only due to their inflammatory association
but also because of their intrinsic links with cell death. Furthermore, although apoptosis has been considered inherently non-inflammatory, there is evidence linking oxidised mitochondrial DNA to the activation of NLRP3 indicating it may have a role in inflammation (Shimada et al., 2012). However, despite caspase inhibition reducing apoptotic cell death, it does not completely wipe it out indicating there are other forms of programmed cell death that must be considered (Kitanaka and Kuchino, 1999).
Figure 1-6: Extrinsic and intrinsic pathways in apoptosis

(A) Intracellular domain of death receptors such as TNFR interacts with FADD which pulls pro-caspase-8 molecules in to oligomerise. Active caspase-8 can cleave pro-caspase-3 initiating apoptosis. Alternatively caspase-8 cleaves BID. (Bi) tBID inhibits Bcl-2 and Bcl-X<sub>L</sub> thereby disinhibiting mitochondria-associated BAX and BAK. (Bi) Intracellular DNA damage is also sensed by BAX. (C) Active BAX and BAK permeabilise the outer mitochondrial membrane allowing release of pro-apoptotic factors including SMAC and cyt<sub>c</sub>. (D) Release of SMAC causes autoubiquitination and subsequent lysosomal degradation of IAPs. IAP degradation disinhibits pro-caspase3 activation resulting in apoptosis. It also disinhibits caspase-9. (E) Released cyt<sub>c</sub> oligomerises with APAF1 and caspase-9 to form the apoptosome. This activates caspase-9 which can cleave pro-caspase-3 resulting in apoptosis. **punctate line** – ubiquitination; **broken line** – degradation; **TNFR** – TNF receptor; **FADD** - Fas-associated death domain protein; **BID** - BH-3 interacting-domain death agonist; **tBID** – truncated BID; **BAX** – Bcl-2 associated X protein; **BAK** – Bcl-2 killer; **SMAC** - second mitochondria activator of caspases; **IAPs** – inhibitors of apoptosis; **cytc** - cytochrome c; **APAF1** - apoptotic protease activating factor-1.
1.3.2. Necroptosis

The most recently defined form of programmed cell death, necroptosis is a “backup” pathway to death-receptor mediated apoptosis. It only occurs when caspase-8 is blocked and therefore the death-receptor apoptotic pathway cannot be initiated (Holler et al., 2000, Fiers et al., 1995). Necroptosis is dependent on the formation of the necrosome, a cytosolic complex formed by the phosphorylated kinases, RIP 1 and RIP3 (Cho et al., 2009).

1.3.2.1. Mechanism

As with apoptosis, there are multiple initiators of necroptosis that converge on death receptors. The best characterised of these with regards to necroptosis is TNFR1. Depending on circumstances, activation of TNFR1 results in either cell survival or cell death. Activation of TNFR1 releases the silencer of death domain from the intracellular terminal, allowing the formation of complex I (Andera, 2009). Complex I consists of TNFR1, TNF receptor-associated death domain protein (TRADD), FADD, and RIP1 ubiquitinated by E3 ligases such as TRAF 2 and 5, and cIAPs-1 and -2 (Vercammen et al., 1998, Wertz and Dixit, 2008). In its ubiquitinated state, RIP1 activates the IKK complex via NEMO (Ea et al., 2006). IKK and NEMO activate the NF-κB pathway resulting in transcription of pro-survival genes (Baldwin, 2012). Deubiquitination of RIP1 inhibits NK-κB activation and drives the cell down the cell-death pathway (Kovalenko et al., 2003, Urbanik et al., 2011). Thus the ubiquitination state of RIP1 in complex I determines the cells fate.

Cylindromatosis (CYLD) and A20 are two proteins capable of deubiquitinating RIP1 (Kovalenko et al., 2003, Wertz et al., 2004), though others may exist. Deubiquitinating RIP1 releases complex I into the cytosol whereby it recruits caspase-8 and RIP3 to form complex II. Functional caspase-8 cleaves RIP1 and RIP3 thereby
preventing them from functioning (Kim et al., 2000, Feng et al., 2007), and triggers apoptosis via the caspase cascade as described in section 1.3.1. However, inhibition of caspase-8 allows RIP1 and RIP3 to be phosphorylated and form a complex termed the “necrosome” (Holler et al., 2000, Declercq et al., 2009). RIP1 and RIP3 interact via RHIMs and RIP1 phosphorylates RIP3 (Li et al., 2012b). The interaction between RIP1 and RIP3 and the subsequent phosphorylation is essential for formation of the necrosome (Cho et al., 2009, He et al., 2009b, Cho et al., 2011).

After formation of the necrosome, necroptosis ensues however the method by which this occurs remains unclear. In spite of the differing of initiation, the execution of necroptosis has all of the characteristics of necrosis with increased cell volume, oxidative burst, mitochondrial membrane hyperpolarisation, and eventual loss of lysosomal and plasma membrane integrity. As the necrosome does not interact directly with cellular organelles (He et al., 2009b), it is likely that it works upstream of processes that result in cell death such as generation of ROS, NO and effects on metabolic pathways (Vanden Berghe et al., 2010, Wu et al., 2012).

### 1.3.2.2. Necroptosis as a Therapeutic Target

Work on utilising necroptosis as a therapeutic target is still very much in its infancy but shows some promise. Necroptosis contributes to cell death in models of ischaemic-reperfusion injury, neuronal excitotoxicity, and myocardial infarction (Rosenbaum et al., 2010, Smith et al., 2007, Li et al., 2008). Necrostatins, particularly necrostatin-1, target the necrosome (Degterev et al., 2008) and show therapeutic potential (Degterev et al., 2005, Wang et al., 2007, Zheng et al., 2008). Necrostatin-1 specifically blocks recruitment of RIP1 to complex II thereby preventing its interaction with RIP3 and necrosome formation (Wu et al., 2012). Whilst there are conceivable benefits to targeting necroptosis, there
remains so much unknown about this cell death pathway and how it interacts with other cell death and regulatory mechanisms that must first be better understood.

1.3.3. Pyroptosis

Pyroptosis is a more recently defined form of programmed cell death and is caspase-1 dependent. It was initially considered to be caspase-1-dependent apoptosis (Zhou et al., 2000, Chen et al., 1996), however this conflicted with evidence that apoptosis is not affected in caspase-1 KO cells (Kuida et al., 1995). Pyroptosis has since been defined as having a distinct mechanism and different outcomes to apoptosis, the key one being that pyroptosis is characteristically pro-inflammatory (Bergsbaken and Cookson, 2007).

1.3.3.1. Mechanism of cell death

Pyroptosis is essentially a form of programmed cell necrosis in that the ultimate result is loss of plasma membrane integrity. It is cellular suicide ensuring invading pathogens are destroyed along with the cell (Bergsbaken and Cookson, 2007). Pyroptosis may also play a role in sterile inflammation, however its relevance is still unclear and needs further investigation (Bergsbaken et al., 2009).

Due to regulation of caspase-1 activity by the inflammasome (section 1.2.1.2, The inflammasomes), for pyroptosis to occur priming is required. This is likely achieved by intracellular recognition of microbial invasion (Martinon and Tschopp, 2007). Active caspase-1 mediates pore formation in the plasma membrane of cells. This results in a loss of normal ionic gradients subsequently increasing osmotic pressure and water uptake. The cell swells and eventually lyses under the pressure releasing its cellular content (Fink and Cookson, 2006). This process can be inhibited non-specifically by blocking ion flux with glycine thereby controlling changes in osmotic pressure (Fink and Cookson, 2006); or specifically by blocking caspase-1 activity (Bergsbaken et al., 2009). Due to swelling,
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Pyroptotic cells are much larger in size than those undergoing apoptosis (Sun et al., 2005, Fink and Cookson, 2006). Pyroptotic cells also retain both their nuclear and mitochondrial membrane integrity distinguishing them from apoptotic cells (Cervantes et al., 2008, Bergsbaken and Cookson, 2007).

There is DNA damage during pyroptosis, but it is different to that observed during apoptosis and is thought to be reliant on caspase-1 dependent nuclease activity (Fink and Cookson, 2006, Enari et al., 1998). The precise mechanism for this however is unknown. Whilst DNA damage is characteristic of pyroptosis it is not an essential part of the mechanism, as inhibition of the nuclease activity does not prevent pyroptosis (Fink and Cookson, 2006).

Caspase-1 is thought to mediate over 100 other processes within the cytoplasm, including cytoskeletal and metabolic processes (Denes et al., 2011b). However, up till now the focus of caspase-1 research has been on its role in pro-inflammatory cytokine processing thus, more work on alternative caspase-1 targets is required.

1.3.3.2. Inhibiting pyroptosis

As pyroptosis is inherently a protective mechanism against invading microorganisms, the use of caspase-1 inhibitors to prevent pyroptosis is questionable. However, due to the role of caspase-1 in inflammation, caspase-1 inhibitors have already been used in many models in order to investigate the caspase-1 substrates IL-1β and IL-18 in disease pathogenesis. Though IL-1β is a clear therapeutic target (section 1.2.2.1), several models indicate interfering with the actions of caspase-1 to be more effective than the use of IL-1Ra further indicating the multifaceted nature of caspase-1 function (Schielke et al., 1998, Ona et al., 1999, Sarkar et al., 2006, Denes et al., 2011b). In terms of sterile inflammation, much more work is required on the relevance of pyroptosis to sterile inflammation before this could be taken forward as a therapeutic target.
1.3.4. **Autophagy**

Autophagy is not a mechanism of cell death as such, although excessive autophagy can result in cell death (Kroemer and Levine, 2008). However, autophagy is still a very important cell regulatory mechanism to consider in relation to inflammation. In the quiescent cell, autophagy functions at a constant basal level to maintain a cell’s homeostasis by sequestering, degrading and recycling damaged organelles and mis-folded proteins (Ding and Yin, 2008). It regulates energy and nutrient homeostasis and plays an essential role in tissue development (Yang and Klionsky, 2010). Autophagy also plays an important role in clearing inflammasome components and cytokines in order to regulate inflammation (Harris et al., 2011, Shi et al., 2012). For this reason, autophagy presents an attractive therapeutic target for inflammatory diseases. However, due to the diverse and complex array of processes with which it is involved, manipulating autophagy for therapeutic benefit requires a better understanding in order to achieve a balance by which to control inflammation (Jones et al., 2013).

1.3.4.1. **Mechanism**

Autophagy involves the formation of an autophagosome, an isolating double membrane around the cytosolic constituent targeted for degradation. The autophagosome fuses with a lysosome forming an autolysosome where degradation of the content takes place.

In its simplest form, autophagosome formation is reliant on the mammalian target of rapamycin (mTOR) (Kim et al., 2011, Jones et al., 2013). There are many regulatory triggers that affect mTOR such as metabolic stress (Egan et al., 2011, Sag et al., 2008), regulation by anti-inflammatory cytokines such as IL-10 (Park et al., 2011), or recognition of PAMPs or DAMPs (Delgado et al., 2008, Xu et al., 2007). Either way, release of mTOR inhibition facilitates interaction of unc-51-like kinase 1 (ULK1) and AMP kinase
(Mizushima, 2010, Lee et al., 2010). These translocate to the endoplasmic reticulum where they recruit the type III phophatidylinositol-3-kinase VPS34 in complex with beclin-1 (Simonsen et al., 2004, Filimonenko et al., 2010). This complex allows development of the autophagosome.

1.3.4.2. \textit{Autophagy and inflammation}

As already mentioned, autophagy is a regulatory mechanism for controlling the transcription, processing and secretion of cytokines, particularly those of the IL-1 family. It can do this by degrading the endogenous stimulus that triggers inflammasome formation in the first place (Nakahira et al., 2011, Zhou et al., 2011). Alternatively, autophagy can target IL-1 or inflammasome components directly for degradation (Harris et al., 2011, Shi et al., 2012). As pro-inflammatory stimuli acting via the NLRP3 inflammasome also activate autophagy, it is considered that this is yet another important regulatory step in controlling the inflammatory response (Shi et al., 2012).

Due to this, autophagy is implicated in a role in autoimmune disorders and atherosclerosis (Zhou and Zhang, 2012, Razani et al., 2012). Autophagy-enhancing drugs such as rapamycin, an inhibitor of mTOR, have proven effective at reducing tissue damage in models of rheumatoid arthritis (Cejka et al., 2010, Laragione and Gulko, 2010). Whilst this is encouraging, caution must be taken when interpreting these results as the effects of rapamycin are not specific to mTOR and also have a more generalised immunosuppressive effect. Thus in order to understand how autophagy could be used as a therapeutic tool, more work is needed to extract the specific contribution of autophagy to suppression of inflammation in these models.
1.4. Sterile inflammation and cell death in the brain

The focus of this thesis is sterile inflammation and cell death in the brain, specifically concentrating on the role of glia in response to NLRP3-activating DAMPs using cerebral ischaemia as an in vivo model acute brain injury. Glial cells are the non-neuronal population of central nervous system cells and are mainly constituted of astrocytes, microglia and oligodendrocytes. With regards to inflammation after sterile injury, microglia are the predominant source of inflammatory mediators (Vezzani et al., 1999, Mabuchi et al., 2000, Lee et al., 2000, Denes et al., 2007). However, neurones, astrocytes and oligodendrocytes are all capable of producing pro-inflammatory cytokines (Pearson et al., 1999, De Simoni et al., 2000) and neurotoxic mediators (Thornton et al., 2006, Fogal et al., 2007, Thornton et al., 2008). Thus in considering the effect of pro-inflammatory stimuli within the brain, it is important to consider the combined effect on different cell types, as they may be synergistic or antagonistic to one another.

1.4.1. Microglia

Microglia are considered the resident macrophage of the CNS (Hanisch and Kettenmann, 2007) and are the main immune effector cells. They constitute approximately 20% of the total glial population and in their “resting” state they have a ramified morphology with long fine processes that constantly survey the surrounding environment (Nimmerjahn et al., 2005, Wake et al., 2009). Microglia are not uniformly distributed throughout the brain, however it has been suggested that the speed at which their processes move may allow them to survey the entire parenchyma within a few hours (Nimmerjahn et al., 2005). Small numbers of microglia take on a phagocytic phenotype in which they clear apoptotic bodies during development.

Microglial activation occurs rapidly in response to perturbations within the CNS. In models of cerebral ischaemia in rats, it has been shown that microglial activation can
occur within a matter of hours and precedes breakdown of the blood brain barrier which typically occurs around 24h after the initial ischaemia (Lynch et al., 2004, Stoll et al., 1998, Yang and Rosenberg, 2011). During activation, microglia undergo morphological changes whereby they display decreased ramification, and proliferate and migrate to the site of injury. Activation occurs on a continuum with microglia also becoming more amoeboid in shape and becoming phagocytic. This allows cellular debris as a result of the injury to be cleared but can also accompany synaptic degeneration (Town et al., 2005, Gehrmann et al., 1995).

As the macrophages of the CNS, activated microglia are responsible for the initiation and propagation of the inflammatory response within the brain. They release and respond to a plethora of inflammatory mediators including IL-1, IL-6, TNF-α, IFNγ. These in turn affect astrocyte activation, expression of cell adhesion molecules and the recruitment of peripheral immune cells (Hanisch and Kettenmann, 2007, Badoer, 2010). Active microglia also produce large amounts of nitric oxide (NO), ROS and proteases all of which can exert neurotoxic effects. Microglial derived NO in particular has been shown to be important in neuronal cell death (Boje and Arora, 1992, Chao et al., 1992). Furthermore it has been demonstrated many times in vitro that activated microglia are neurotoxic when grown in co-culture with neurones (Bal-Price et al., 2002, Brown and Bal-Price, 2003, Ma et al., 2002).

Whilst active microglia have a predominantly pro-inflammatory role in the CNS, they can also produce neurotrophic factors such as brain-derived neurotrophic factor which can aid neuronal plasticity. Furthermore targeted ablation of proliferating microglia within the CNS has been shown to worsen infarct volume after cerebral ischaemia (Lalancette-Hebert et al., 2007). This indicates microglia have an important neuroprotective role as well as their role in inflammation that contributes to neurodegeneration.
1.4.2. Astrocytes

Astrocytes are the most abundant glial cell type and are found throughout the white and the grey matter. There are two main subtypes: fibrous and protoplasmic. Astrocytes have several physiological functions including regulating the supply nutrients to neurones, regulating homeostasis of the extracellular milieu, and providing a mechanical support network to the endothelial cells that make up the BBB (Sofroniew and Vinters, 2010).

Astrocytes have long processes that make contact with all other cell types in the CNS. A key example of this is where they terminate in contact with endothelial cells that make up the BBB. Along with pericytes and perivascular microglia, these astrocyte end feet form an integral part of the BBB and mediate its permeability (Kaur and Ling, 2008, Abbott, 2002).

Under physiological conditions astrocytes express and release growth factors and neurotrophins that aid in the migration, growth and differentiation of both neuronal and glial pre-cursors (Sofroniew and Vinters, 2010). Moreover, whilst they express and release a number of neurotrophic factors, astrocytes also control the homeostasis of the parenchyma via the removal of neurotransmitters, the uptake of free $K^+$ in the extracellular space (Chen and Swanson, 2003) and the regulation of extracellular pH (Deitmer, 1995). There is also an increasing body of evidence that astrocytes can communicate with other astrocytes or with neurons via regulated increases in intracellular $Ca^{2+}$ concentrations (Nedergaard et al., 2003, Volterra and Meldolesi, 2005).

Astrocytes proliferate in response to CNS injury, however this astrogliosis is more delayed than the microglial response, occurring around 24h after neuronal injury (Eddleston and Mucke, 1993). Astrogliosis is controlled by cytokines, many of which are microglia-derived. It is widely accepted that an important function of reactive astrocytes is the formation of the glial scar. This is a physical barrier of fibrillary astrocytes that
separates the damaged tissue from the healthy tissue, and is characteristically seen around the infarcted tissue a few days after cerebral ischaemia. The glial scar is considered a double-edged sword in that it contains the damaged necrotic tissue and prevents this area from spreading any further. However, reactive astrocytes release proteoglycans that have been shown to inhibit axonal growth thus preventing regeneration within the infarct (Pekny and Nilsson, 2005, Silver and Miller, 2004).

Reactive astrocytes can release trophic factors such as nerve growth factor, basic fibroblast growth factor, transforming growth factor β, and brain-derived neurotrophic factor, among others (Trendelenburg and Dirnagl, 2005). However, much like microglia, astrocytes can also release a plethora of neurotoxic mediators including cytokines, chemokines and MMPs which, as already discussed, can have detrimental effects within the CNS (John et al., 2005). Ultimately therefore, the contribution of astrocytes to the propagation of CNS inflammation is hard to define as it is dependent on the balance of neurotrophic vs neurotoxic mediators.

1.4.3. Oligodendrocytes

Oligodendrocytes make up approximately 5-8% of the glial population and surround neuronal axons, supporting the neurones and aiding nerve transmission. These cells have the least to do with immune function in the brain, although there is some evidence that they divide and accumulate at the site of injury. Here they have been suggested to both contribute to the formation of the glial scar, and aid in remyelination of damaged axons (Chen et al., 2002, Frost et al., 2003).

1.4.4. Sterile inflammation and cell death in brain pathology

As has already been described, sterile inflammation contributes to acute injury and chronic disease. In the case of the CNS, inflammation is known to contribute to the progression of acute injury such as cerebral ischaemia and chronic diseases such as
Alzheimer’s disease, Parkinson’s disease and multiple sclerosis. It is known that these disorders are associated with abnormal cytokine expression profiles and that there is large amount of cell death in the brain that contributes to loss of function in each disorder, and also propagates further inflammation. As has already been discussed, activated astrocytes and microglia are both capable of producing neurotoxic factors that contribute to cell death. It is less well understood how the original cell death that occurs as a result of the injury initiates inflammation prior to disruption of the BBB. In this thesis, cerebral ischaemia has been used as a model of sterile inflammation of the brain in order to investigate these mechanisms.

Cerebral ischaemia is one of the major causes of death worldwide and is the leading cause of disability. There are very limited treatment options for patients and as such it remains a major health and economic burden (Denes et al., 2011c). Cerebral ischaemia occurs when there is a disruption in cerebral blood flow meaning that metabolic needs of the brain cells are greater than the blood supply of glucose and oxygen to that region. This can be caused by partial or full occlusion of any of the blood vessels that serve the brain. The disruption in cerebral blood flow results in a depletion of extracellular ATP levels and the subsequent failure of the Na⁺/K⁺ ATPase pump which usually maintains resting membrane potential. Neuronal depolarisation leads to the release of large amounts of glutamate which stimulates N-methyl-D-aspartate receptors on neighbouring neurones and results in major excitotoxicity in the ischaemic core (Lipton and Rosenberg, 1994).

The necrotic cell death within the ischaemic core is irreversible and the tissue cannot be salvaged. However the surrounding tissue, the penumbra, can potentially be saved if the inflammation is brought under control in time. Thus it is important to understand how this inflammation and cell death is being driven in order to find ways in which to intervene without disrupting the beneficial effects of inflammation. Lessons from
controlling sterile inflammation during cerebral ischaemia can potentially then be applied across the aforementioned conditions in which sterile inflammation contributes to disease progression.

1.5. Conclusion and aims

Sterile inflammation contributes to the pathology of many devastating neurodegenerative diseases; cerebral ischaemia being a prime example. DAMPs released from necrotic cells at the core of the injury drive inflammation by acting on PRRs on glia, the immune cells of the brain. IL-1β contributes to the progression of sterile inflammation and is a therapeutic target in stroke. IL-1β release is controlled by caspase-1 activity, which is controlled by an intracellular multi-molecular platform termed ‘the inflammasome’. The NLRP3 inflammasome is perceived to be integral to driving sterile inflammation, the recognition of DAMPs and the subsequent IL-1β response. Extensive in vitro studies show that cell must be primed to achieve an IL-1β response, but the endogenous source of this priming in vivo is not clear. The pro-inflammatory effects of NLRP3-activating DAMPs on unprimed glia are unknown. Furthermore the mechanisms by which glia are primed in vivo in order to orchestrate an IL-1β-dependent response remain elusive.

Cells have an array of mechanisms to control cell death and, in theory, protect the host. Some of these mechanisms have been described in this chapter, however many of the examples given from research have been using pathogenic stimuli to determine the cell death mechanisms. Cell death in response to DAMPs contributes to the progression of sterile inflammation, but the mechanism of cell death induced by specific DAMPs is relatively unclear. Understanding this mechanism may prove to be fundamental to controlling sterile inflammation.
The aims of this thesis were therefore to:

1) Determine if NLRP3-activating DAMPs were pro-inflammatory on mixed glia in the absence of a prior priming stimulus

2) Identify potential endogenous priming stimuli that could drive IL-1 expression in glia after cerebral ischaemia

3) Understand the mechanisms of NLRP3-activating DAMP-induced cell death in mixed glia
2.1. Animals

Primary murine mixed glial cultures were prepared from C57BL/6J mice (Harlan Laboratories, UK) or NLRP3 KO mice (Genentech, USA) where specified. Plasma was collected and tissues were homogenised from C57BL/6J or IL-1α/β double KO mice. Animals were kept in ventilated cages in a 12 h light/dark cycle at 21 ± 1°C at the University of Manchester. All experiments were performed under the University of Manchester project license number (40/3076) in accordance with the Animals (Scientific Procedures) Act (1986).

2.2. MCAo, perfusion, tissue homogenisation, immunohistochemistry and cytometric bead array

All in vivo surgery and subsequent ex vivo analysis (Figure 3-11, section 3.3.2.1) was kindly carried out by Dr Adam Denes formerly of the University of Manchester, as described by Savage et al. (2012).

2.2.1. Cytometric bead array (CBA)

In brief, the CBA was used to examine IL-1α, IL-1β, IL-6 and CXCL1 protein levels in homogenised liver and brain samples from WT or IL-1α/β double KO animals after 60 min MCAo and 24h reperfusion. CBA analysis is essentially a high throughput ELISA (see section 2.6) allowing for the measurement of multiple analytes from one sample. Antibody coated fluorescently labelled beads for each analyte are quantified on a BD LSR II flow cytometer (BD Bioscience, UK). CBA Flex Sets for each analyte in question were used according to the manufacturer’s instructions (BD Biosciences, UK). All CBA analysis was carried out by Dr Adam Denes.
2.3. Cell culture

2.3.1. Primary murine mixed glial cultures

Murine mixed glial cells from post-natal day 1-4 C57BL/6J mice were cultured as described previously (Pinteaux et al., 2002). Briefly, mice pups were decapitated and the heads pinned to a wax-coated petri dish. A sagittal incision from brain stem to nose was made through the skin and then the skull and the cortical hemispheres were dissected out. The meninges and blood vessels were removed by rolling the brains on sterile filter paper, and the brains were stored in 10mL glial maintenance media Dulbecco’s modified Eagles medium (DMEM; Sigma, UK) + 10% Foetal bovine serum (FBS; Invitrogen, UK), 1% penicillin-streptomycin cocktail (P/S; Invitrogen, UK). Tissue was triturated using first a 10mL and subsequently a 5mL stripette, then centrifuged for 10 min at 155 g. The pellet was re-suspended in the appropriate volume of glial maintenance media (12ml/brain) and seeded into uncoated tissue culture plates (Corning®, UK) at a density of 1 brain/48cm². Mixed glial cultures were grown at 37°C in a humidified incubator (5% CO₂, 95% air) for 10-20 days. Media was changed after day in vitro (DIV)5 and every three/four days thereafter. Cells reached confluence around DIV14 and were not maintained beyond DIV20.

2.3.2. J774 murine macrophage cell line

J774 macrophages cells were reconstituted and kept in maintenance media (DMEM + 10% FBS, 1% P/S) until confluent. To passage the cells, media was aspirated and cells were washed in phosphate buffered saline (PBS; Sigma, UK) to remove any leftover serum. Cells were resuspended in trypsin for 2 min at 37°C (Sigma, UK). Maintenance media was added to the cell suspension to neutralise the trypsin, and cells were counted and pelleted at 300g for 5 min to remove the trypsin. Cells were resuspended in
maintenance media, seeded at $5 \times 10^5$ cells/mL in 24-well plates and cultured overnight (O/N) in a humidified incubator (5% CO$_2$, 95% air). On day 2 cells were treated as detailed below (section 2.5.1) for use as quantitative polymerase chain reaction (qPCR) standards.

2.4. Cell treatment

Mixed glia underwent a full media change prior to treatment. All assays were completed in serum-containing maintenance media unless otherwise stated.

2.4.1. Measuring the effect of DAMPs or PAMPs

To investigate the effect of DAMPs or PAMPs on mixed glia, cells were treated for 4 or 24 h with vehicle (distilled sterile water or sterile PBS; Sigma, UK); the DAMPs ATP (Sigma, UK), MSU (InvivoGen, UK), CPPD (InvivoGen, UK); or the PAMPs LPS (Sigma, UK) or poly(IC) (Sigma, UK) at the concentrations specified in each chapter. Respective effects in mixed glia were compared to untreated cells. No effect was observed in response to vehicle controls.

2.4.2. Mechanisms of priming mixed glia

To investigate mechanisms of priming mixed glia, cells were incubated for 24 h with vehicle (PBS or 0.1% bovine serum albumin (BSA; Sigma, UK) in dH$_2$O), LPS or SAA (PeproTech) at the concentrations indicated in section 3.2. Subsequently, without a media change, cells were treated for 1 h with vehicle or ATP (5mM) to induce IL-1$\alpha$ and $\beta$ processing and release.

2.4.3. Inhibiting DAMP-induced cell death

To investigate mechanisms of DAMP-induced cell death, mixed glia were pre-incubated with inhibitors or their respective vehicles as indicated in Table 2-1 for 5 min.
Subsequently, without a media change, cells were incubated with vehicle, ATP (5mM), MSU (250μg/ml), or sphingosine (20mM) for 24 h.

<table>
<thead>
<tr>
<th>Target</th>
<th>Drug</th>
<th>Conc.</th>
<th>Vehicle</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cathepsin B</strong></td>
<td>(L-3-trans-(Propylcarbamoyl)oxirane-2-Carbonyl)-L-Isoleucyl-L-Proline Methyl Ester (Ca-074Me)</td>
<td>50μM</td>
<td>DMSO</td>
<td>Calbiochem®, UK</td>
</tr>
<tr>
<td><strong>Caspase-1</strong></td>
<td>Ac-YVAD-CHO (YVAD)</td>
<td>100μM</td>
<td>DMSO</td>
<td>Calbiochem®, UK</td>
</tr>
<tr>
<td><strong>Caspases</strong></td>
<td>z-VAD-FMK (ZVAD)</td>
<td>50μM</td>
<td>DMSO</td>
<td>Calbiochem®, UK</td>
</tr>
<tr>
<td><strong>Calpain</strong></td>
<td>Calpain inhibitor III (CalpIII)</td>
<td>50μM</td>
<td>DMSO</td>
<td>Calbiochem®, UK</td>
</tr>
<tr>
<td><strong>Phosphoinositol-3 kinase (PI3K)</strong></td>
<td>3-Methyladenine (3-MA)</td>
<td>5mM</td>
<td>dH2O</td>
<td>Sigma, UK</td>
</tr>
<tr>
<td><strong>RIP1 kinase</strong></td>
<td>Necrostatin-1 (Nec-1)</td>
<td>100μM</td>
<td>DMSO</td>
<td>Sigma, UK</td>
</tr>
<tr>
<td><strong>Spleen tyrosine kinase (Syk)</strong></td>
<td>Piceatannol (Pic)</td>
<td>50μM</td>
<td>DMSO</td>
<td>Sigma, UK</td>
</tr>
</tbody>
</table>

*Table 2-1: Inhibitors of cell death and their respective vehicles*
Details of inhibitors, concentrations and respective vehicles used for investigating mechanisms of NLRP3-activating DAMP-induced cell death and inflammation

2.5. **qPCR**

All qPCR work was completed using ribonuclease (RNase) free filter tips, RNase free plastics and equipment cleaned with sodium dodecyl sulphate (SDS; Sigma, UK) and 70% v/v RNase free ethanol (Fisher Scientific, UK).

2.5.1. **Generation of standards and controls**

Standard curves were generated using RNA from the macrophage cell line J774 treated for 24 h with LPS (1μg/ml) and IFNγ (20ng/ml; Sigma, UK). RNA for negative and positive controls was from untreated or 24 h LPS treated J774 cells, respectively. RNA was extracted and used as detailed below (sections 2.5.2 - 2.5.4).
2.5.2. RNA extraction

After treatment (section 2.4.1), RNA was extracted using the TRIzol® method from mixed glia cultured in 6-well plates. Briefly, cells were washed 3x in warm media to remove any cell debris and incubated in 1mL TRIzol® (Invitrogen, UK) for 5 min at room temperature (r.t.) to allow for dissociation of nucleoprotein complexes. Subsequently, samples were transferred from the tissue culture plate to eppendorfs containing 200µL chloroform (Fisher Scientific, UK) and shaken vigorously for 15 s prior to 3 min incubation at r.t. Samples were then centrifuged for 20 min at 12,000g, 4°C. This step allowed for phase separation of the sample. The clear aqueous phase on the top contains RNA, whilst the organic phenol-chloroform phase that separates to the bottom contains protein. The white interphase in the middle contains DNA. RNA from the aqueous phase was collected and precipitated in 1mL isopropanol (Fisher Scientific, UK) for 15 min at r.t. The RNA was pelleted by centrifuging at 12,000g for 15 min at 4°C. The RNA pellet was then washed in 70% and 100% ethanol (Fisher Scientific, UK) sequentially, and allowed to dry for 1 h at r.t. The final RNA pellet was resuspended in 20µL RNase-free H2O (Sigma, UK) and the amount of RNA collected was quantified using a nanodrop 1000 spectrophotometer and nanodrop-1000 software (v3.1.0; Nanodrop technologies, USA). The purity of the RNA was deemed usable if the A260/280 ratio was ≥1.85. RNA was stored at -80°C until use.

2.5.3. Reverse Transcription (RT)

RNA was converted to complementary DNA (cDNA) using Moloney murine leukaemia virus (MMLV) reverse transcriptase (Invitrogen, UK). 1µg of RNA was mixed with 1µL of oligo-(dT)12-18 primer (oligo(dT); Invitrogen, UK) and made up to a final volume of 11µL in RNase-free H2O. The volume required for 1µg RNA was calculated
using the equation below, where the concentration of RNA was as read from the nanodrop-1000 (section 2.5.2).

\[
\text{volume} = \frac{\text{mass}}{\text{concentration}}
\]

Samples were incubated at 70°C for 10 min to allow for denaturing of the RNA secondary structure. Samples were quenched on ice and 9µL RT mastermix was added (Table 2-2). Samples were then incubated at 37°C for 1 h to allow for cDNA synthesis. The reaction was inactivated by heating the sample at 70°C for 15 min. cDNA was stored at -80°C or diluted in 80µL RNase free H₂O if being used immediately.

<table>
<thead>
<tr>
<th>Method</th>
<th>Reagent</th>
<th>Volume (µL) per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse Transcription</td>
<td>5 x reaction buffer (Invitrogen, UK)</td>
<td>4µL</td>
</tr>
<tr>
<td></td>
<td>0.1M Dithiothreitol (DTT; Invitrogen, UK)</td>
<td>2µL</td>
</tr>
<tr>
<td></td>
<td>2'-deoxynucleoside 5’triphosphate mix (dNTPs; Invitrogen, UK)</td>
<td>1µL</td>
</tr>
<tr>
<td></td>
<td>RNaseOUT™ recombinant ribonuclease inhibitor (RNaseOUT; Invitrogen, UK)</td>
<td>1µL</td>
</tr>
<tr>
<td></td>
<td>MMLV reverse transcriptase</td>
<td>1µL</td>
</tr>
<tr>
<td>qPCR</td>
<td>Power SYBR® Green PCR mastermix (SYBRGreen; Applied Biosystems, UK)</td>
<td>5µL</td>
</tr>
<tr>
<td></td>
<td>RNase free H₂O</td>
<td>1µL</td>
</tr>
<tr>
<td></td>
<td>Gene specific QuantiTech primer (Qiagen, UK)</td>
<td>1µL</td>
</tr>
</tbody>
</table>

*Table 2-2: Components of master mixes for reverse transcription and qPCR*

Details of elements required to make mastermix for RT and qPCR reactions

2.5.4. qPCR

7µL qPCR mastermix (Table 2-2) was added per well of a 384-well plate. The mastermix contained validated primer sets specific for the genes of interest (GOIs): IL-1β, IL-1α, caspase-1, NLRP3, ASC, iNOS, TNFα, IL-6 and CXCL1; and for the housekeeping genes GAPDH and sDHA (QuantiTech primer assays, Qiagen). The standard curve was
comprised of a 10-fold dilution series with a top standard of 75ng total cDNA loaded. Standards were loaded in triplicate. Samples were diluted to a concentration of 16.67ng/µL, and 3µL was loaded in triplicate across the plate such that each well contained 50ng cDNA. A set of no template controls (NTCs) were run for each primer set to check for primer dimerisation and contamination.

CDNA was run on a thermal cycler (Applied Biosystems, UK) using SDS v2.3 (Applied Biosystems, UK). The heat cycle for the qPCR reaction is depicted in Figure 2-1. In brief, samples were heated to 50°C for 2 min to allow for amplification of the SYBRGreen dye. Samples were then heated for 10 min at 95°C to activate the polymerase within the SYBR® Green mastermix. Samples were denatured at 95°C for 15 s and then cooled to 60°C for 1 min. The denaturing step breaks the hydrogen bonds between the RNA and the DNA, allowing for primers specific to the GOI to anneal and the polymerase to subsequently elongate the DNA when the samples are cooled to 60°C. This cycle is repeated 40 times allowing exponential amplification of the GOI, until the PCR products run out. SYBRGreen dye binds specifically to double stranded DNA, therefore as the GOI is amplified, so the fluorescence increases. The greater the starting amount of the GOI, the faster the fluorescence increases. The fluorescence is read at the end of each cycle. Once fluorescence rises above background levels it is said to have reached the cycle threshold (Ct). The cycle number at which fluorescence crosses this threshold is recorded and relative quantification of the GOI in each sample can be calculated from the standard curve.

Here, the Ct was set manually to achieve a slope efficiency of >99%. For each primer set a single product on melt curve analysis was established demonstrating absence of genomic DNA. The housekeeping genes GAPDH and SDHA did not vary with treatments. Data were normalised to expression levels of the housekeeping gene SDHA to
allow for variation in the quality of RNA used. Data is expressed relative to basal levels of the GOI in untreated mixed glia.
Figure 2-1: qPCR heat cycle and phases
Following activation of SYBR Green and polymerase, RNA and cDNA are denatured at 95°C for 15s (a). Samples are cooled to 60°C for 1 min to allow annealing (b) of primer and DNA elongation (c). SYBR Green fluoresces when bound to double stranded DNA (d). This amplification cycle is repeated 40 times (e). In doing so, fluorescence increases exponentially with gene amplification until the PCR product runs out. GOI is normalised to housekeeping genes and quantified relative to basal expression in untreated control.
2.6. **Enzyme-linked immune sorbent assay (ELISA)**

Following treatment (section 2.4.1), supernatants were harvested, and cells were lysed in 100µL lysis buffer (PBS + 1% Triton-X100; Sigma, UK) with protease inhibitors (Calbiochem, UK). Mixed glial IL-1β and IL-1α expression was measured from lysates; and IL-1β, IL-1α, IL-6 and CXCL1 release was measured from supernatants by ELISA using the DuoSet® ELISA development system (R&D, UK). 96-well immuno maxisorp plates (Sigma, UK) were coated overnight at 4°C with the relevant capture antibody diluted in PBS. The plates were then washed 4x in wash buffer (PBS + 0.1% Tween; Sigma, UK). Plates were blocked for 1 h at r.t. in reagent diluent (RD) (PBS + 1% BSA) to prevent any non-specific binding of the sample to the capture antibody. Plates were washed again 4x in wash buffer. Standard curves consisting of 2-fold serial dilutions from 4ng/mL – 3.9pg/mL were loaded with the samples onto the plates and incubated for 2 h at r.t. Plates were washed 4x in wash buffer and incubated for 1h at r.t. in respective detection antibodies diluted in RD. Plates were once again washed 4x in wash buffer and then incubated for 30 min at r.t. in HRP-conjugated streptavidin (R&D, UK) diluted in RD. The streptavidin binds to the detection antibody and amplifies its signal. Plates were washed 4x in wash buffer and incubated for a further 20 min at r.t. in substrate reagent (R&D, UK) made up of a 1:1 mix of substrate A (H₂O₂) and substrate B (tetramethylbenzidine, TMB) in the dark. The HRP catalyses the oxidation of TMB, and the reduction of H₂O₂ to water. The resulting oxidised TMB is blue and is proportionate to the amount of analyte present in the sample. The reaction was then stopped with 50µL/well 1M sulphuric acid which turns the blue oxidised TMB yellow. The intensity of this yellow product correlates with the amount of analyte present in the sample and was read at 490nm and 570nm using a spectrophotometer (Northstar Scientific Ltd, UK). Optical imperfections of the plate were accounted for by taking a final corrected optical
density of 490nm-570nm. Optical densities were plotted against the standard curve to allow quantification and analysis of protein levels in the samples using GraphPad Prism (GraphPad, UK). Plasma SAA levels were quantified using the PHASE™ murine SAA assay (TriDelta Development Ltd, UK) according to the manufacturer’s instructions.

2.7. Bicinchoninic acid (BCA) protein assay

A 2-fold dilution series of BSA from 800µg/mL to 12.5µg/mL was loaded in a 96-well plate. Cell lysates or tissue homogenates were diluted 1:5, 1:10 or 1:20 in PBS and 10µL was loaded into the plate in triplicate. 200µL BCA reagent (ThermoScientific, UK) was added per well and the plate was incubated at 60°C for 30 min. Copper in the reagent is reduced by protein in the sample. The bicinchoninic acid in the reagent chelates with the reduced copper ions to form a purple product the optical density of which can be read on a spectrophotometer at 570nm. Optical densities for each sample were averaged and protein concentrations were read from the standard curve.

2.8. Western Blotting

Samples analysed for cathepsin B were treated in serum free media. Those for IL-1α and IL-1β remained in serum containing media. After treatment (section 2.4) whole cell lysates (section 2.6) or supernatants were mixed 5:1 with 5x sample buffer (10% w/v SDS, 50% v/v glycerol, 400mM Tris-HCl (pH 6.8), 0.025% w/v bromophenol blue, 5% v/v β-mercaptoethanol, in water). The sample buffer contained β-mercaptoethanol which helps to denature the protein structure in the sample by cleaving disulphide bonds. Samples were heated to 100°C for 5 min to help denature the proteins. Samples were then separated on 10-12% SDS-polyacrylamide gels alongside a molecular weight protein ladder, and transferred onto nitrocellulose membrane using a semi-dry transfer system. Membranes were blocked in 5% milk made up in wash buffer (PBS + 0.01% Tween) for 1
h at r.t., washed 3x in wash buffer and then incubated in the relevant primary antibody (Table 2-3) O/N at 4°C. Membranes were washed 15x over 15 min in wash buffer, and incubated in the relevant secondary antibody (Table 2-3) 1:1000 in 5% milk for 1 h at r.t. Membranes were washed 15x over 15 min and then incubated in emission chemiluminescent (ECL) reagent (Amersham, UK) for 1 min. The HRP conjugated to the secondary antibody catalyses the breakdown of the ECL substrate and the luminescence released is captured on photographic paper and developed. Images were captured using Northern Eclipse software (Northern Eclipse, UK).

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Source</th>
<th>Conc.</th>
<th>Diluted in</th>
<th>HRP-conjugated Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-mouse IL-1β</td>
<td>R&amp;D, UK</td>
<td>1:1000</td>
<td>5% milk</td>
<td>Rabbit anti-goat</td>
</tr>
<tr>
<td>Goat anti-mouse IL-1α</td>
<td>R&amp;D, UK</td>
<td>1:1000</td>
<td>5% milk</td>
<td>Rabbit anti-goat</td>
</tr>
<tr>
<td>Goat anti-mouse Cathepsin B</td>
<td>R&amp;D, UK</td>
<td>1:1000</td>
<td>5% milk</td>
<td>Rabbit anti-goat</td>
</tr>
</tbody>
</table>

Table 2-3: Western blotting antibodies
Details of western blot antibodies used throughout thesis, concentrations, diluents and respective HRP-conjugated secondary’s

2.9. Flow Cytometric analysis

Following treatment (section 2.4.1), mixed glia were gently resuspended in 500µL PBS + 0.5mM ethylenediaminetetraacetic acid (EDTA; Sigma, UK). Cell were pelleted at 400g for 10 min to remove the EDTA, and resuspended in 500µL glial maintenance media (section 2.3.1). 200µL of each sample was plated into a 96 round-bottomed-well plate. Cells were pelleted in the plate at 400g for 3 min at 4°C. Media was removed and cells were resuspended in 30µL anti-mouse CD16/CD32 block (eBioscience, UK) diluted 1:400 in FACS buffer (0.1% BSA, 0.05% sodium azide in PBS). Cells were incubated in block for 30 min at 4°C to prevent any non-specific antibody binding. FACS buffer was added
to each well and the plate was spun again at 400g for 3 min at 4°C. Block and FACS buffer were removed and cells were resuspended in 30µL fluorescent-conjugated antibody mastermix (Table 2-4) made up in FACS buffer. Cells were left in primary antibody for 1 h at 4°C in the dark. FACS buffer was added to each well and cells were pelleted at 400g for 3 min at 4°C. Cells were washed in FACS buffer and repelleted. FACS buffer was removed and cells were resuspended in fix (4% paraformaldehyde (PFA) in FACS buffer; Sigma, UK). The surface expression of these markers was analysed using a CyAn advanced flow cytometer (Beckman Coulter, USA) and Summit v4.3 software (Dako, UK). Microglia were gated based on co-expression of CD11b and CD45 and data is expressed as a percentage of these cells.

<table>
<thead>
<tr>
<th>Cell surface protein</th>
<th>Antibody</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>FITC-conjugated anti-CD11b</td>
<td>1:200</td>
</tr>
<tr>
<td>CD45</td>
<td>PerCP-Cy5.5-conjugated anti-CD45</td>
<td>1:500</td>
</tr>
<tr>
<td>CD11c</td>
<td>PE-conjugated anti-CD11c</td>
<td>1:100</td>
</tr>
<tr>
<td>MHCII</td>
<td>APC-conjugated anti-MHCII</td>
<td>1:200</td>
</tr>
</tbody>
</table>

*Table 2-4: Flow cytometry antibodies*
Details of flow cytometry antibodies used, concentrations and respective fluorescent conjugates. All antibodies were from BD bioscience (UK).

2.10. Gelatin gel zymography

Released gelatinase activity was measured by gelatin-substrate zymography as previously described (Kleiner and Stetler-Stevenson, 1994). The assay works on the same electrophoretic basis as a western blot however unlike western blotting, the samples are not denatured, thus ensuring that the enzyme retains its native state and thereby it’s enzymatic activity. After treatment in serum-free media (section 2.4.1) samples were diluted 5:1 in sample buffer without β-mercaptoethanol and run on an SDS-polyacrylamide gel containing gelatin alongside molecular weight markers. The SDS was removed from the
gel by washing in PBS containing 2.5% Triton X-100. Enzymes were renatured in activity buffer (50mM Tris-HCl pH 7.5, 5mM CaCl2, 5μM ZnCl2, 0.02% NaN3) for 96 h at 37°C. This allowed for enzymatic digestion of the gelatin within the gel. Gels were then stained in 0.5% Coomassie Brilliant Blue R-250 (Sigma, UK) in 40% methanol and 10% acetic acid (Fisher Scientific, UK) for 1 h at r.t. The coomassie stains the gelatin. Upon destaining in 10% acetic acid, 10% methanol at r.t., clear bands appear wherever the enzyme has digested the gelatin. Gels were imaged using Northern Eclipse software.

**2.11. LDH Assay**

Cell death was measured using the CytoTox96® NonRadioactive Cytotoxicity Assay (Promega, UK) which measures levels of lactate dehydrogenase in conditioned media. LDH is a cytosolic enzyme released upon cell lysis. The LDH assay is a 30 min coupled enzyme assay in which released LDH catalyses the conversion of tertrazolium salt (2-(p-iodophenyl)-3(p-nitrophenyl)-phenyltetrazolium; INT) to a formazan product which is red via the chemical reactions outlined below:

\[
\begin{align*}
\text{NAD}^+ + \text{lactate} & \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH} \\
\text{NADH} + \text{INT} & \xrightarrow{\text{Diaphorase}} \text{NAD}^+ + \text{formazan (red)}
\end{align*}
\]

The enzyme reaction is then stopped using 1M acetic acid, and colorimetric absorbance is read at 490nm using a spectrophotometer. The amount of colour produced is proportional to the amount of LDH in the conditioned media thereby giving an indication as to the extent of cell lysis.

50μL conditioned media was transferred to a 96-well plate and incubated at r.t. with 50μL reconstituted LDH reagent. After 30 min, 50μL stop solution (1M acetic acid) was added and absorbance was measured. Total cell death was calculated using media
from an untreated well in which cells were lysed in 9% v/v Triton X-100. Percentage cell
death was calculated as a proportion of this total cell death. Serum levels and phenol red in
the culture media interfere with this reaction giving high background therefore media alone
was always run as a blank to account for this.

2.12. *Limulus* amebocyte lysate (LAL) assay

The LAL assay was used to test for endotoxin levels in the DAMPs used as stimuli
throughout. This was to account for the fact that many of them are produced
recombinantly, and therefore potential responses could be due to endotoxin contamination
within the preparation rather than the DAMP itself. LAL is the aqueous extract from the
red blood cells of the horseshoe crab. It clots when it reacts with bacterial endotoxin. The
amount of endotoxin within a substrate therefore correlates with the speed at which the
LAL clots.

The assay was performed according to the manufacturer’s instructions. Briefly, a
10-fold serial dilution from 10EU/mL to 0.01EU/mL was made from control standard
endotoxin (Associates of Cape Cod Inc., UK). Samples were diluted 1:10 and 1:100.
100µL/well standard and sample was loaded into an endotoxin free plate (Associates of
Cape Cod Inc., UK). 100µL/well LAL substrate (Associates of Cape Cod Inc., UK) was
added and absorbance was read at 20s intervals over 1 h 30 min at 405nm in an incubating
plate reader set at 37°C. The time taken to reach an optical density of 0.5 was recorded,
and bacterial endotoxin levels were calculated from this against the standard curve.

Table 2-5 indicates the levels of endotoxin detected in each preparation and the
relative concentration to which the cells will therefore have been exposed.
Table 2-5: Endotoxin levels
Endotoxin concentration (EU/ml) in stock vehicle and DAMPs used to treat mixed glia throughout thesis. Relative concentration to which cells are subjected is calculated based on dilution of vehicle or DAMP. Results for sphingosine are not shown as were below detection limit of the assay.

<table>
<thead>
<tr>
<th></th>
<th>Total conc. measured (EU/mL)</th>
<th>Conc. applied <em>in vitro</em> (EU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vehicle (ATP)</td>
<td>&lt;0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ATP</td>
<td>0.183</td>
<td>0.00183</td>
</tr>
<tr>
<td>vehicle (MSU/CPPD)</td>
<td>&lt;1</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>MSU</td>
<td>&lt;1</td>
<td>&lt;0.04</td>
</tr>
<tr>
<td>CPPD</td>
<td>&lt;1</td>
<td>&lt;0.04</td>
</tr>
</tbody>
</table>

2.13. Immunocytochemistry (ICC)

Untreated cells grown on 13-mm coverslips (Scientific Laboratory supplies Ltd, UK) were fixed in ice cold PBS containing 4% PFA, 4% sucrose (Sigma, UK) O/N at 4°C, and subsequently washed 3x in PBS and stored in PBS at 4°C for up to 4 days. Cells were permeabilised in 0.1% v/v Triton-X100 (Sigma, UK) and auto-fluorescence from endogenous peroxidase activity in the cells was quenched using ammonium chloride (NH₄Cl; Sigma, UK). Cells were washed 3x in PBS and non-specific hydrophobic interactions were blocked using 5% normal donkey serum (NDS; Jackson ImmunoResearch, UK) for 1 h at r.t. Primary antibodies were diluted in NDS as detailed in Table 2-6 and left on the cells for 1 h at r.t. Subsequently, cells were washed 3x in PBS and fluorochrome-labelled donkey secondary antibodies were diluted in 1% BSA as detailed in Table 2-6. Cells were incubated in the corresponding secondary antibody (Table 2-6) for 1 h at r.t in the dark so that the fluorescence was not quenched by the light. Cells were washed again 3x in PBS and then 3x in water. At this point the coverslips were removed from the tissue culture plate, dried by capillary action and mounted onto slides using Pro-long® Gold mounting medium containing 4’,6-diamidino-2-phenylindole.
(DAPI) nuclei stain (Invitrogen, UK). Mounting medium was allowed to set in the dark at r.t., and cells were then stored at 4°C until use. Cells were imaged on an Olympus BX51 microscope (Olympus, UK), and images were captured and processed using a CoolSnap ES camera (Photometrics, UK) and MetaVue software (Nikon, UK).

<table>
<thead>
<tr>
<th>Target</th>
<th>Primary antibody</th>
<th>Source</th>
<th>Conc.</th>
<th>Fluorescent conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astrocytes</td>
<td>Glial fibrillary acidic protein (GFAP)</td>
<td>Abcam, UK</td>
<td>1:200</td>
<td>Alexa 488</td>
</tr>
<tr>
<td>Microglia</td>
<td>Iba1</td>
<td>Wako, USA</td>
<td>1:100</td>
<td>Alexa 594</td>
</tr>
</tbody>
</table>

Table 2-6: ICC antibodies
Details of antibodies used for immunocytochemical characterisation of mixed glial cultures and the fluorescent conjugates used with their respective secondary’s

2.14. Statistical analysis

Unless otherwise stated, for two groups paired t-tests (two-tailed) were used. For three or more groups non-parametric Kruskal-Wallis or two-way analysis of variance (ANOVA) and Dunn’s post-hoc multiple- or paired comparison tests were used. All data are expressed as mean ± standard deviation (SD). ***P<0.001, **P<0.01, *P<0.05.
Chapter 3: Characterising the inflammatory response of mixed glia to NLRP3-activating DAMPs
3.1. Introduction

Inflammation is an important response to injury that promotes healing and repair. However, dysregulated inflammation often leads to worsened disease progression and tissue injury. The IL-1 cytokine family are key inflammatory mediators that play a major role in damaging inflammatory processes during sterile disease (Dinarello, 2011), and contain two well-characterised pro-inflammatory isoforms – IL-1α and IL-1β. The role of IL-1 in sterile disease is illustrated in the case of acute brain injury such as cerebral ischaemia, where both IL-1α and IL-1β are established as major contributors to worsened outcome (Brough et al., 2011, Luheshi et al., 2011). Both isoforms are expressed after injury or disease predominantly by macrophages or, in the case of the CNS, by microglia as 31kDa precursors, pro-IL-1α or pro-IL-1β (Denes et al., 2008, Luheshi et al., 2011). Whereas pro-IL-1α is biologically active without needing to be cleaved to its mature 17kDa form (Mosley et al., 1987), pro-IL-1β is more tightly regulated and must be cleaved by the protease caspase-1 in order to be active and thereby exert its effects (section 1.2.2). Caspase-1 is also produced as an inactive precursor, pro-caspase-1. Its activity is tightly regulated by a cytosolic multi-molecular platform known as the inflammasome, which contains a cytosolic PRR (Schroder and Tschopp, 2010). Inflammasome complexes are distinguished by their PRR and that most implicated in sterile injury is the NLRP3 inflammasome complex (section 1.2.1.2 – NLRP3) (Cassel and Sutterwala, 2010). Recognition of an inflammatory stress signal by these cytosolic PRRs allows cleavage of active caspase-1 and subsequently processing and release of mature active IL-1β.

Release of active IL-1β is considered a two-step process requiring a ‘priming’ stimulus to induce an increase in expression of pro-IL-1β and inflammasome components, and a subsequent stimulus to induce processing and release of active IL-1β (Hornung and Latz, 2010a, Lopez-Castejon and Brough, 2011). Stimuli that can induce each of these
steps are categorised into either PAMPs or DAMPs. These are recognised by membrane bound PRRs such as the TLR family, or cytosolic receptors such as the NLR family. A single stimulus is frequently inefficient at inducing both steps required for the release of active IL-1β (section 1.2.1) (Chen and Nunez, 2010, Takeuchi and Akira, 2010).

During sterile injury, DAMPs are released in abundance from necrotic cells. The effect of these DAMPs is frequently studied *in vitro* on cells that have been primed with LPS. However in the case of acute sterile injury where there is rapid localised cell death, DAMPs are likely act on cells that have not been subjected to a prior priming stimulus. Whilst microglia, the resident macrophages of the brain, have been shown to respond similarly to macrophages when challenged with PAMPs and DAMPs (Brough et al., 2002, Halle et al., 2008), microglia are unique from macrophages in that they originate from the yolk sac and are self-renewing throughout life (Ginhoux et al., 2010). In acute brain injury such as cerebral ischaemia, the inflammatory response of glia may be affected by local DAMPs released from necrotic tissue. Furthermore, circulating inflammatory mediators such as the acute phase proteins SAA and CRP that are known to be elevated in the periphery during inflammatory conditions (Niemi et al., 2011, Ather et al., 2011, Migita et al., 2012), and have the capacity to prime cells of the peripheral immune system (Niemi et al., 2011, McColl et al., 2007), may exert a priming effect on glia after breakdown of the BBB occurs.

This chapter focuses specifically on three well-established NLRP3-inflammasome activating DAMPs, ATP, MSU and CPPD crystals. These DAMPs are known to exert IL-1-independent inflammatory effects outside of the CNS. ATP induces an increase in IL-6 release in fibroblasts (Solini et al., 1999) and mast cells (Bulanova et al., 2005), and has been shown to induce release of the active cathepsins from macrophages (Lopez-Castejon et al., 2010). MSU and CPPD crystals stimulate IL-6 production in monocytes (Guerne et
al., 1989) and osteoblast like cells (Bouchard et al., 2002) in the absence of any priming stimulus. MSU is also known to influence adaptive immune responses independently of the NLRP3-inflammasome (Kool et al., 2011), potentially via the Syk pathway (Ng et al., 2008).

Acute brain injury, such as cerebral ischaemia, is associated with a marked central inflammatory response and yet, there is little known about the effect of key mediators of sterile inflammation, NLRP3-inflammasome activating DAMPs, on glia in the absence of a PAMP priming stimulus in vitro. Moreover, an efficient endogenous priming stimulus in the CNS is yet to be established. This chapter therefore sought to determine the following:

1) The pro-inflammatory effect of NLRP3-inflammasome activating DAMPs on glia, the immune cells of the CNS, in the absence of pathogenic priming

2) The ability of a potentially relevant endogenous priming stimulus to prime glia in order to respond to NLRP3-activation
3.2. **Methods**

Experiments were carried out as specified in chapter 2. Specifics for this experimental chapter are outlined below.

### 3.2.1. Measuring the pro-inflammatory effects of NLRP3-activating DAMPs on glia in the absence of priming

Cultures of mixed glia were isolated from murine post-natal day 1-4 pups and maintained until confluent (around 14-18 days). For unprimed experiments, cells were treated with one of the NLRP3 inflammasome-activating DAMPs selected, ATP (5mM), MSU (250μg/mL) or CPPD (250μg/mL); or with one of the two PAMPs selected, LPS (1μg/mL) or poly(IC) (50μg/mL). Indicated concentrations were as initially applied, however integrity of the stimulus was not measured at the end time point, therefore it cannot be assumed that this concentration was maintained throughout. LPS acts via TLR4 and poly(IC) acts via TLR3 to induce cell priming therefore both of these PAMPs were considered positive controls for pro-inflammatory responses. Untreated or vehicle treated cells were used as the negative control. After either 4h or 24h, supernatants were collected to measure protein release, and cells were either lysed for mRNA extraction using TRIzol® (4h treatment groups), or for protein extraction using a triton based lysis buffer (4h and 24h treatment groups).

Changes in mRNA levels of inflammasome components and pro-inflammatory mediators were measured by qPCR from 4h treated samples. Protein expression and release of pro-inflammatory mediators was measured at both 4h and 24h by ELISA, western blot and gelatin gel zymography. Samples analysed for cathepsin B release or MMP activity were assayed in serum-free experimental media. Cells were also resuspended in EDTA after treatment and labelled for flow cytometric analysis of cell surface marker expression.
3.2.2. *In vivo* measurement of IL-1 contribution to inflammatory response after experimental stroke

*In vivo* work on characterisation of the inflammatory response after stroke was in WT C57BL/6J and IL-1α/β double KO mice subjected to 60 min MCAo followed by 24h reperfusion. Immunohistochemistry was used to detect microglial activation, and pro-inflammatory mediators were measured by CBA analysis. Surgery, immunohistochemistry and CBA analysis was kindly conducted by Dr Adam Denes formerly of the University of Manchester.

3.2.3. Measuring the priming capacity of SAA

For priming experiments plasma or homogenised liver or brain samples from naïve, sham and stroked WT mice were used. Stroked mice were subjected to 60 min MCAo followed by 4h reperfusion. The plasma and homogenised liver or brain samples were analysed by ELISA and Western blot for SAA levels. SAA was applied to mixed glial cultures (0.03-3μg/mL) for 24h and IL-1 expression was measured in the lysates by ELISA. LPS (1μg/mL) and poly(IC) (50μg/mL) were used in parallel for 24h as positive priming controls. Endotoxin contamination in the SAA was controlled for by treatment with proteinase K (50μg/ml for 1h) and subsequent heat treatment (5min at 90°C) before application to the cells. This denatured the SAA, thus any remaining response is attributable to endotoxin contamination.

Additionally, to analyse IL-1 processing and release, LPS and poly(IC) primed cells were treated for a further 1h with ATP (5mM), MSU (250μg/mL) or CPPD (250μg/mL), and SAA primed cells were treated for an extra 1h with ATP (5mM). Processing of released IL-1 to its mature form was confirmed by western blot. Finally, in order to better understand the variation in mixed glial responses, cell composition was quantified by immunohistochemistry.
3.2.4. Statistical analysis

Unless otherwise stated, data is pooled from a minimum of 3 experiments and statistical significance is measured using a non-parametric Kruskal-Wallis test with Dunn’s paired comparison post-hoc. All data are expressed as mean ± SD. ***P<0.001, **P<0.01, *P<0.05.
3.3. **Results**

3.3.1. **Pro-inflammatory effects of NLRP3 inflammasome-activating DAMPs in mixed glial cultures**

3.3.1.1. *Effect of NLRP3 inflammasome-activating DAMPs on glial priming*

As already discussed, NLRP3 is proposed as a sensor of sterile inflammation (Cassel and Sutterwala, 2010), but the pro-inflammatory properties of NLRP3-activating DAMPs in the absence of PAMP priming is poorly understood. Therefore the pro-inflammatory effects of three of the best characterised NLRP3-activating DAMPs were investigated in the absence of PAMP priming. Due to the involvement of NLRP3 as an inflammasome responsible in part for IL-1β processing and release, initially the effect of ATP, MSU and CPPD on cell priming was investigated.

Mixed glial cultures were exposed to a DAMP for 4h, after which mRNA was collected and analysed for expression of markers of pro-inflammatory cell priming by qPCR. These markers were IL-1β, IL-1α, caspase-1, NLRP3 and ASC. A small but nonetheless significant increase was observed in the expression of IL-1β and IL-1α after exposure to CPPD (Figure 3-1A and Figure 3-1B). No significant change was observed in caspase-1or NLRP3 expression (Figure 3-1C and Figure 3-1D), however a significant decrease in ASC was observed on exposure to CPPD (Figure 3-1E).

In order to put these changes into context against a more established and well understood pro-inflammatory response, mixed glial cultures were treated separately with either of two PAMPs: LPS or poly(IC). LPS induced a significant increase in IL-1β and IL-1α (Figure 3-2A and Figure 3-2B). Poly(IC) did not induce a significant increase in either IL-1β or α mRNA expression when compared to LPS, however the increase in expression was roughly 100-1000 fold that seen after exposure to CPPD. LPS and
poly(IC) both induced a significant increase in caspase-1 (Figure 3-2C). LPS induced a significant increase in NLRP3 mRNA expression (Figure 3-2D), but no change in ASC (Figure 3-2E). Poly(IC) did not induce a significant change in NLRP3 expression compared to LPS (Figure 3-2D) but induced a significant decrease in ASC expression (Figure 3-2E).

**Figure 3-1: Effect of DAMPs on expression of mRNA associated with cell priming** mRNA levels of genes associated with cell priming were measured by qPCR after 4h exposure of mixed glial cultures to the NLRP3inflammasome-activating DAMPs ATP (5mM), MSU or CPPD crystals (both at 250μg/mL). Data were normalised to expression levels of the housekeeping gene SDHA across all treatments and fold change is expressed relative to basal mRNA levels in untreated mixed glia. Genes analysed: IL-1β (A), IL-1α (B), caspase-1 (C), NLRP3 (D) and ASC (E). Data are pooled from at least 5 separate experiments and are expressed as mean ± SD. *P < 0.05 vs. untreated.
Figure 3-2: Effect of PAMPs on expression of mRNA associated with cell priming

mRNA levels of genes associated with cell priming were measured by qPCR after 4h exposure of mixed glial cultures to the TLR4 agonist LPS (1μg/mL), or the TLR3 agonist poly(IC) (50μg/mL). Data were normalised to expression levels of the housekeeping gene SDHA across all treatments and fold change is expressed relative to basal mRNA levels in untreated mixed glia. Genes analysed: IL-1β (A), IL-1α (B), caspase-1 (C), NLRP3 (D) and ASC (E). Data are pooled from at least 5 separate experiments and are expressed as mean ± SD. *P < 0.05, **P < 0.01 vs. untreated.
To observe if changes seen at mRNA level were translated through to protein expression, mixed glia were exposed for 24h to each DAMP, and IL-1β and IL-1α protein levels were measured in the cell lysates by ELISA. No increase was observed in IL-1β or IL-1α protein after 24h (Figure 3-3A and Figure 3-3B).

Once again, as a positive control, the PAMPs LPS and poly(IC) were used. LPS induced a significant increase in IL-1β and IL-1α, whilst poly(IC) induced a significant increase in IL-1β and a trend towards an increase in IL-1α (Figure 3-4A and Figure 3-4B, respectively).
Protein levels of IL-1β (A) and IL-1α (B) were measured by protein specific ELISA after 24h exposure of mixed glial cultures to the NLRP3 inflammasome-activating DAMPs ATP (5mM), MSU or CPPD crystals (both at 250μg/mL). Data are pooled from at least 5 separate experiments and are expressed as mean ± SD.

Protein levels of IL-1β (A) and IL-1α (B) were measured by protein specific ELISA after 24h exposure of mixed glial cultures to the TLR4 agonist LPS (1μg/mL), or the TLR3 agonist poly(IC) (50μg/mL). Data are pooled from at least 5 separate experiments and are expressed as mean ± SD. *P < 0.05, **P < 0.001 vs. untreated.
3.3.1.2. **Effect of NLRP3 inflammasome-activating DAMPs on non-priming related inflammatory responses**

As these NLRP3-activating DAMPs did not have a notable effect on the priming state of mixed glia, changes in mRNA expression of iNOS, TNFα, IL-6 and CXCL1 were measured by qPCR to determine their effect on a more general pro-inflammatory response. No significant response was seen in iNOS or TNFα to any of the DAMPs except CPPD which induced a significant increase in iNOS (Figure 3-5A and Figure 3-5B). CPPD also induced a significant increase in IL-6 and CXCL1 (Figure 3-5C and Figure 3-5D). ATP and MSU did not induce a significant increase in either of these genes; however there was a much larger trend toward an increase in these than in any other pro-inflammatory gene measured.

Once again, the response of these pro-inflammatory genes to the PAMPs LPS and poly(IC) was measured as a positive control. LPS induced a significant increase in all four pro-inflammatory genes. Compared to LPS, the response to poly(IC) was not a significant increase, although there was a trend toward an increase across all four genes (Figure 3-6).
mRNA levels of genes associated with a pro-inflammatory response were measured by qPCR after 4h exposure of mixed glial cultures to the NLRP3-inflammasome-activating DAMPs ATP (5mM), MSU or CPPD crystals (both at 250μg/mL). Data were normalised to expression levels of the housekeeping gene SDHA across all treatments and fold change is expressed relative to basal mRNA levels in untreated mixed glia. Genes analysed: iNOS (A), TNFα (B), IL-6 (C) and CXCL1 (D). Data are pooled from at least 5 separate experiments and are expressed as mean ± SD. *P < 0.05, **P < 0.01 vs. untreated.
Figure 3-6: Effect of PAMPs on expression of mRNA associated with a pro-inflammatory response

mRNA levels of genes associated with a pro-inflammatory response were measured by qPCR after 4h exposure of mixed glial cultures to the TLR4 agonist LPS (1μg/mL), or the TLR3 agonist poly(IC) (50μg/mL). Data were normalised to expression levels of the housekeeping gene SDHA across all treatments and fold change is expressed relative to basal mRNA levels in untreated mixed glia. Genes analysed: iNOS (A), TNFα (B), IL-6 (C) and CXCL1 (D). Data are pooled from at least 5 separate experiments and are expressed as mean ± SD. **P < 0.01 vs. untreated.
The largest trends in DAMP-induced mRNA change were in IL-6 and CXCL1. These have been reported to be upregulated in response to focal cerebral ischaemia, a model of sterile injury in the brain in which cells local to the injury are subjected to DAMPs released from dying cells in the core of the injury (Chapman et al., 2009, Denes et al., 2010a). Therefore, to observe whether these trends at mRNA level translated through to protein level, IL-6 and CXCL1 released from DAMP-treated mixed glia was measured in the supernatant of cultured cells 4h and 24h after treatment by ELISA. ATP, MSU and CPPD all induced a significant increase in released IL-6 and CXCL1 4h after treatment (Figure 3-7Ai and Figure 3-7Aii). After 24h, MSU and CPPD induced a significant increase in IL-6 and CXCL1 release (Figure 3-7Bi and Figure 3-7Bii).

LPS and poly(IC) both induced a significant increase in IL-6 after 4h and 24 h (Figure 3-8Ai and Figure 3-8Bi). LPS also induced a significant increase in CXCL1 after 4h and 24h, whilst poly(IC) induced a trend toward an increase in CXCL1 at both time points (Figure 3-8Aii and Figure 3-8Bii).
**Figure 3-7: IL-6 and CXCL1 release from mixed glia 4h and 24h after DAMP treatment**

4h (A) and 24h (B) after exposure of mixed glial cultures to the NLRP3 inflammasome-activating DAMPs ATP (5mM), MSU or CPPD crystals (both at 250μg/mL), protein levels of IL-6 (i) and CXCL1 (ii) were measured by protein specific ELISA. Data are pooled from at least 5 separate experiments and are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 vs. untreated.
Figure 3-8: IL-6 and CXCL1 release from mixed glia 4h and 24h after PAMP treatment 4h (A) and 24h (B) after exposure of mixed glial cultures to the TLR4 agonist LPS (1μg/mL), or the TLR3 agonist poly(IC) (50μg/mL), protein levels of IL-6 (i) and CXCL1 (ii) were measured by protein specific ELISA. Data are pooled from at least 5 separate experiments and are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 vs. untreated.
Effect of NLRP3 inflammasome-activating DAMPs on microglial phenotype

Data thus far indicates the DAMPs tested induce a pro-inflammatory cytokine response albeit limited in comparison to the robust response elicited by the PAMPs tested. Other pro-inflammatory changes induced by exposure to DAMPs were subsequently investigated.

It is reported that MHC class II and CD11c, cell surface markers that indicate dendritic cell phenotype, are upregulated on microglia after cerebral ischaemia, (Felger et al., 2009). Therefore the response of MHC class II and CD11c on microglia from mixed glial cultures exposed to ATP, MSU or CPPD for 24h was measured by flow cytometry.

Neither MHC class II or CD11c were upregulated in response to any of the DAMPs tested (Figure 3-9Ai and Figure 3-9Bi). In contrast, LPS and poly(IC) induced robust significant increases in both cell surface markers (Figure 3-9Aii and Figure 3-9Bii).
Figure 3-9: DAMPs do not induce an upregulation of cell surface markers MHC class II and CD11c on microglia

Expression of cell surface markers associated with microglial activation was quantified by flow cytometry after 24h exposure of mixed glial cultures to the NLRP3 inflammasome-activating DAMPs ATP (5mM), MSU or CPPD crystals (both at 250μg/mL) (i); or the PAMPs LPS (1μg/mL) or poly(IC) (50μg/mL) (ii). Cell surface markers analysed were MHC class II (A) and CD11c (B). Co-expression of CD11b and CD45 was used to select microglia and data are expressed as a percentage of these cells. Representative dot plots showing the microglial population in the presence of either ATP or LPS are shown (iii). Data are pooled from at least 5 separate experiments and are expressed as mean ± SD. *P < 0.05, ***P < 0.001 vs. untreated.
3.3.1.4. *Effect of NLRP3-inflammasome activating DAMPs on mixed glial protease production*

During neuroinflammatory states such as cerebral ischaemia proteases such as Cathepsin B and gelatinases such as MMP9 contribute to inflammation and breakdown of the BBB (Benchoua et al., 2004, Candelario-Jalil et al., 2009). As previously discussed, this may contribute to worsened outcome, as breakdown of the BBB exposes the CNS to inflammatory events occurring within the periphery (Denes et al., 2010b).

Culturing mixed glia for 24h with ATP, MSU or CPPD induced release of the mature single chain form (28-30kDa) of Cathepsin B. This response was comparable to that after culturing mixed glia with the PAMP LPS (Figure 3-10A). Gelatin gel zymography for gelatinase activity showed that mixed glia cultured with ATP for 24h release a huge amount of active MMP9 (83kDa) consistent with previous literature that P2X7 activation induces active MMP9 release from monocytes (Gu and Wiley, 2006). This was far greater than the amount induced by the PAMP LPS (Figure 3-10B). MSU and CPPD did not induce release of active MMP9. Pro-MMP2 (72kDa) was constitutively released across all treatments including the negative control, and was processed to mature MMP2 by ATP (Figure 3-10B).
Figure 3-10: DAMPs induce release of active proteases from cultured mixed glia
Mixed glia were cultured for 24h in serum free media with the NLRP3 inflammasome activating DAMPs ATP (5mM), MSU or CPPD crystals (both at 250μg/mL); or the PAMPs LPS (1μg/mL) or poly(IC) (50μg/mL). Release of Cathepsin B (28-30kDa) (A) was measured by Western blot. All samples were loaded neat. Release of active MMP9 (83kDa) (B) was measured by gelatin gel zymography. All samples were loaded neat except the ATP treated sample which was also loaded 1:3 (+*). Positive control for MMP9 was conditioned media from transmigrated neutrophils kindly donated by Dr Charlotte Allen. Blots and gel are representative of at least five experiments.
3.3.2. The role of priming in the inflammatory response of mixed glia

3.3.2.1. The contribution of IL-1 to the inflammatory response in vivo

Thus far it has been demonstrated that NLRP3 inflammasome-activating DAMPs can induce an inflammatory response from mixed glia in the absence of PAMP priming, however cannot elicit an IL-1 response. However, it is known that IL-1 plays a part in driving the inflammatory response to sterile injury. As already discussed, interventions using IL-1Ra are neuroprotective in models of acute cerebral ischaemia. Furthermore, caspase-1 KO mice show reduced lesion volume in the ipsilateral hemisphere after cerebral ischaemia. To observe the contribution of IL-1 to the DAMP induced inflammatory response to sterile injury, IL-6 and CXCL1 cytokine levels were measured in ipsilateral and contralateral hemispheres of WT and IL-1α/β double KO mice subjected to transient middle cerebral artery occlusion (tMCAo), an experimental model of cerebral ischaemia.

After 60 min MCAo and 24h reperfusion, there was a large lesion in the ipsilateral hemisphere of the WT mice. Glia within this area will have been exposed to DAMPs released by dead cells in the core of the infarct. To indicate this, microglial activation was quantified by immunohistochemistry. Microglia in the striatum and the cortex of the ipsilateral hemisphere were significantly activated compared to the contralateral hemisphere as observed by immunohistochemical staining (Figure 3-11A). IL-6 and CXCL1 levels quantified by CBA increased in the ipsilateral hemisphere independently of IL-1, as shown in the IL-1α/β double KO mice. However the presence of IL-1 significantly increased the IL-6 and CXCL1 response (Figure 3-11B and Figure 3-11C). Whether this was due to the increased damage likely associated with the presence of IL-1 is unknown.
Surgery, immunohistochemistry and CBA analysis was kindly conducted by Dr Adam Denes formerly of the University of Manchester. Cortical cell death occurs after transient middle cerebral artery occlusion (tMCAo) (insert Aii). There is a significant increase in microglial activation in this region of the ipsilateral hemisphere compared to the same region in the contralateral hemisphere (Ai) as measured morphologically by immunofluorescent co-staining for Iba1 (red) and CD45 (green) in WT animals (Aii, Aiii). Damage in the IL-1α/β -/- animals was not quantified. Protein levels in vivo were measured by protein specific ELISA for IL-6 (B) and CXCL1 (C) in WT and IL-1α/β double KO mice subjected to 60 min tMCAo followed by 24h reperfusion. IL-6 and CXCL1 both increase significantly in the ipsilateral cortex of WT mice and this increase is significantly attenuated in IL-1α/β double KO mice. Data are from a minimum of three independent experiments and are expressed as mean ± SD. Increase in microglial activation is analysed using two-way ANOVA followed by a Bonferroni post hoc analysis ***P < 0.001. Cytokine data is analysed using paired T-test for comparison of hemisphere and unpaired T-test used for comparison of genotype with Bonferroni correction applied post hoc. *P < 0.025.
3.3.2.2. The acute phase protein serum amyloid A is capable of priming mixed glia in a PAMP-like manner in vitro

Given the in vivo evidence that IL-1 augments the inflammatory response to sterile injury, it follows that in the absence of PAMP priming there are endogenous priming mechanisms that enable IL-1 to contribute to the inflammatory response following a sterile injury in vivo. It is highly unlikely that this priming stimulus is LPS, yet typically in vitro it is LPS that is used to prime cells. Although many DAMPs are reported to prime inflammatory responses via PRRs of the TLR family, the priming mechanism in the brain is as yet unknown.

Serum amyloid A (SAA) is an acute-phase protein that is upregulated 1000-fold during infection and inflammation (Niemi et al., 2011). Consistent with previous literature (McColl et al., 2007), after 60 min MCAo and 4h reperfusion, SAA is upregulated in the liver (Figure 3-12A) and significantly increases in the plasma (P < 0.05) (Figure 3-12B). SAA also increases in the liver 4h after sham surgery and shows a trend towards an increase in the plasma (Figure 3-12A and Figure 3-12B). No increase in SAA was seen in the ipsilateral cortex of the brain 4h after tMCAo or sham surgery (data not shown).
Figure 3-12: The acute phase protein SAA is upregulated in the liver and plasma 4h after tMCAo
Levels of the protein SAA from WT mice subjected to 60 min tMCAo followed by 4h reperfusion were analysed. SAA in the liver was measured by Western blot (12kDa) (A). SAA in the plasma was analysed by SAA specific ELISA (B). Sham surgery was used as a control for surgical effect. Data are from a minimum of three independent experiments and are expressed as mean ± SD. *P < 0.05 vs. naïve mice.
Chapter 3: Characterising the inflammatory response of mixed glia to NLRP3-activating DAMPs

As previously mentioned, early breakdown of the BBB after cerebral ischaemia allows circulating inflammatory mediators to penetrate into the brain. SAA induces IL-1β expression and NLRP3 dependent responses in macrophages, monocytes and synovial fibroblasts (Niemi et al., 2011, Ather et al., 2011, Migita et al., 2012), but its effects on glia are unknown. Culturing mixed glia for 24h with varying doses of SAA induced a dose dependent increase in IL-1β (Figure 3-13A) and IL-1α (Figure 3-13B). This response was comparable to that induced by 24h culture with the PAMPs LPS and poly(IC) (Figure 3-13C). Heat treatment and protein degradation with proteinase K demonstrated the response was dependent on SAA and not due to endotoxin contamination within the protein preparation (Figure 3-13D).

As discussed thus far, processing and release of IL-1β is a two-step process. Following priming, a subsequent stimulus is required. 1h stimulation with the NLRP3 inflammasome-activating DAMPs used initially in this chapter induced processing and release of mature IL-1β (Figure 3-14A) and mature IL-1α (Figure 3-14B) from PAMP primed mixed glia. To ensure SAA fully primed mixed glia in the same manner as PAMPs, cells primed with SAA for 24h were stimulated for a further 1h with 5mM ATP. This stimulated a significant increase in processing and release of mature IL-1β into the supernatant as measured by ELISA and confirmed by western blot (Figure 3-15).
**Figure 3-13: SAA induces a dose dependent increase in IL-1β and IL-1α comparable to that induced by PAMPs**

Protein levels of IL-1β (A) and IL-1α (B) were measured by protein specific ELISA after 24h exposure of mixed glial cultures to decreasing concentrations of the acute phase protein SAA (3-0.03μg/mL). Levels are comparable to those elicited by 24h exposure to the PAMPs LPS and poly(IC) (C). To ensure IL-1β response was due to SAA and not endotoxin contamination, J774 cells were exposed to 24h SAA (3μg/mL) with or without proteinase K (50μg/mL) and heat treatment (5 min, 90°C) (D). Data are pooled from at least 3 separate experiments and are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle.
Figure 3-14: NLRP3-inflammasome activating DAMPs induce processing and release of mature IL-1\(\beta\) and IL-1\(\alpha\) from PAMP primed mixed glia

Released levels of IL-1\(\beta\) \(\text{(Ai)}\) and IL-1\(\alpha\) \(\text{(B)}\) were measured by protein specific ELISA from supernatants of mixed glial cultures exposed for 1h to the NLRP3 inflammasome-activating DAMPs ATP (5mM), MSU or CPPD crystals (both at 250μg/mL) with or without a 24h priming stimulus of either of the PAMPs LPS or poly(IC). Processing of pro-IL1\(\beta\) (31kDa) to mature IL-1\(\beta\) (17kDa) in PAMP + DAMP treated cultures was analysed by western blot \(\text{(Ai)}\). Data are pooled from at least 3 separate experiments and are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 vs. vehicle.
Figure 3-15: SAA primed mixed glia process and release mature IL-1β on encountering a second DAMP stimulus

Released levels of IL-1β were quantified by protein specific ELISA in supernatants from mixed glial cultures exposed to 24h priming with SAA (3μg/mL) and a subsequent 1h with ATP (5mM) (A). Processing from pro- (31kDa) to mature (17kDa) IL-1β was analysed by western blot (B). Data are pooled from at least 3 separate experiments and are expressed as mean ± SD. *P < 0.05 vs. vehicle.

3.3.3. Effect of mixed glial culture composition on inflammatory response

To account for the variability in the responses seen across all treatments in mixed glial cultures, the cells were characterised by immunocytochemistry and their composition was quantified (Figure 3-16). Between cultures there was a lot of variation in the number of microglia relative to the number of astrocytes. This variation is represented in the table (Figure 3-16). This variation explains the variability of the inflammatory responses measured as slight variation in the number of microglia, can massively affect the pro-inflammatory output of mixed glia (Losciuto et al., 2012).
Figure 3-16: Diversity of mixed glial composition between cultures
Three separate mixed glial cultures from separate C57/BL6J litters were characterised using immunofluorescence staining for GFAP (astrocytes, green), and Iba1 (microglia, red). Astrocytes were quantified as an average percentage of DAPI (blue) stained cells containing GFAP across 3 separate areas from 3 coverslips of each culture. Microglia were quantified as an average of the remaining percentage of DAPI containing cells. Scale bar represents 50μm.
3.4. Discussion

In this chapter, it has been illustrated that NLRP3-inflammasome activating DAMPs induce an IL-1 independent pro-inflammatory response from murine mixed glia in the absence of a priming stimulus. Relative endotoxin levels within the DAMPs were measured prior to use and pro-inflammatory responses are not thought to be attributable to endotoxin contamination (Table 2-5, section 2.12). It has also been shown that the acute phase protein SAA can prime cells ready to process and release mature IL-1α and β in a manner similar to that of the PAMPs LPS and poly(IC), and therefore may act as an endogenous priming stimulus following breakdown of the BBB during sterile injury to the CNS.

3.4.1. NLRP3-inflammasome activating DAMPs are pro-inflammatory in the absence of priming

As reviewed in section 1.2, inflammation is recognised as a major contributor to the worsening of acute brain injury. DAMPs are released in abundance from necrotic cells at the core of the injury and are thought to mediate local inflammatory responses. These inflammatory responses have been reported with regards to formation of the NLRP3 inflammasome, caspase-1 activation and subsequent IL-1β processing and release (Cassel and Sutterwala, 2010). Moreover, use of IL-1Ra as an intervention targeting IL-1 has shown to be protective in experimental models of stroke (Brough et al., 2011), and has shown promise as a treatment in clinical trials (Emsley et al., 2005) making the study of DAMP induced IL-1 release very relevant to acute brain injury. However, as a cell requires an initial priming step to induce the expression of NLRP3 and pro-IL-1β in the first place, the study of the pro-inflammatory actions of these DAMPs is frequently carried out in cells primed with the PAMP LPS (Bauernfeind et al., 2009, Hornung and Latz, 2010a). Here, the effects of key mediators of sterile inflammation, NLRP3-inflammasome
activating DAMPs, on the inflammatory cells of the CNS, glia, in the absence of PAMP priming, are reported for the first time. Work was carried out on cultures of mixed glia comprised mainly of astrocytes and microglia (Figure 3-16) (Pinteaux et al., 2002). Both are a source of inflammatory cytokines *in vitro* (Brough et al., 2002, Bianco et al., 2009) therefore from these experiments it was not possible to determine the primary source of inflammatory mediators, and further experiments with purified cell cultures would be needed to test this.

Due to the key role played by both IL-1β and IL-1α during inflammation (section 1.2.2), experiments initially looked at the effect of three well characterised NLRP3 inflammasome-activating DAMPs (ATP, MSU and CPPD) on mRNA levels of both pro-inflammatory cytokines (IL-1β and IL-1α) and the key inflammasome components associated with their processing and release (caspase-1, NLRP3 and ASC). Consistent with previous findings in macrophages, any minor effects on mRNA levels (Figure 3-1) did not translate through to an effect on IL-1 protein expression as no increase in IL-1β or α was observed after 24h incubation with any of the DAMPs (Figure 3-3). This was in complete contrast to the TLR acting PAMP positive controls used: LPS and poly(IC) (Figure 3-2 and Figure 3-4).

Differing greatly from this lack of effect on IL-1 and the priming system, all three NLRP inflammasome-activating DAMPs induced IL-6 and CXCL1 release from glia (Figure 3-7). Increases in the pro-inflammatory mediator’s iNOS and TNFα were observed at mRNA level; however the most robust increases were in IL-6 and CXCL1 (Figure 3-5). IL-6 is well known as an acute phase pro-inflammatory cytokine and CXCL1 is a pro-inflammatory chemokine involved in recruitment of neutrophils across the BBB after cerebral ischaemia. Both increase in the periphery and the brain after focal ischaemic injury and are thereby associated with worsened outcome (Chapman et al., 2009, Denes et
al., 2010a). These data show that DAMPs can induce pro-inflammatory responses in the absence of PAMP priming, albeit less robust than those induced by stereotypical PAMPs (Figure 3-6 and Figure 3-8). The mechanism for this response is not known and none of the ligands tested are reported to be TLR agonists. However all three DAMPs induced cell death within the culture after 24h therefore it is possible that this contributes to IL-6 and CXCL1 production.

3.4.2. NLRP3 inflammasome-activating DAMPs induce the release of active pro-inflammatory proteases in the absence of priming

All three DAMPs tested also induced release of Cathepsin B from cultured glia in the absence of priming (Figure 3-10). ATP induced Cathepsin B has been shown to be a pro-inflammatory protease capable of extracellular matrix degradation (Lopez-Castejon et al., 2010) and inhibition of Cathepsin B in vivo is neuroprotective in experimental stroke (Benchoua et al., 2004). Evidence that other NLRP3-activating DAMPs also induce Cathepsin B is indicative of a common DAMP induced pro-inflammatory mechanism that may contribute to degradation of BBB integrity.

ATP induced a massive release of active gelatinase activity, also in the absence of priming (Figure 3-10). This was not induced by the other two DAMPs or by the stereotypical PAMP LPS suggesting it is an ATP specific response. Due to known actions of active MMP9, the relevance of this data in vivo could be two-fold. Like Cathepsin B, MMP9 contributes to the degradation of the BBB (McColl et al., 2008). MMP9 is also known to be neurotoxic in neuroinflammatory models in vitro (Thornton et al., 2008).

The ability of NLRP3 inflammasome-activating DAMPs to induce protease activity in the absence of priming could play a pivotal role during the acute phase response in the increased production of acute phase inflammatory reactants, leukocyte recruitment, BBB breakdown and the subsequent influx of peripheral inflammatory mediators.
3.4.3. SAA may act as an endogenous priming mechanism allowing DAMP-induced IL-1 release

The priming independent pro-inflammatory response of increased IL-6 and CXCL1 is also seen in vivo in IL-1α/β double KO mice after experimental cerebral ischaemia (Figure 3-11). Despite this response in the absence of IL-1β and α, Figure 3-11 indicates IL-1 significantly increases the pro-inflammatory response after stroke. The list of DAMPs capable of priming cells has expanded rapidly over the last decade and includes complement receptor proteins (Ramaglia et al., 2012), S100b protein (Kim et al., 2004), HSPs (Flohé et al., 2003), high mobility group box protein-1 (Park et al., 2003, Park et al., 2004) and ECM molecules such as biglycan (Babelova et al., 2009) to name but a few reviewed more thoroughly in section 1.2.1.1.

Despite this progress, an efficient endogenous priming mechanism that enables an IL-1 response in the brain remains unclear. In experimental stroke at early time points (4h post tMCAo), IL-1α protein expression in the brain precedes IL-1β, and IL-1β increases at later time points (Luheshi et al., 2011). With this in mind, it is plausible that a substantial source of priming stimuli is from the periphery following breakdown of the BBB. As discussed above, DAMPs stimulate the release of active proteases which can contribute to this BBB breakdown in the absence of priming, opening the gates to priming stimuli from the periphery, thus allowing for the IL-1 response to contribute to inflammation. Patients with chronic systemic inflammatory conditions such as atherosclerosis, obesity and diabetes are considered ‘at risk’ patients for stroke (Drake et al., 2011). There are a number of possible plasma derived candidates elevated in these conditions that could act as priming stimuli. For example, minimally oxidised LDL associated with type II diabetes acts to enhance IL-1β expression (Masters et al., 2010) and could act to prime glia at the lesion site following BBB disruption.
The acute phase protein SAA is elevated across a diverse range of co-morbid inflammatory conditions (O’Hara et al., 2000, Schulte et al., 2008, Niemi et al., 2011). It is a TLR2 and 4 agonist (Cheng et al., 2008, He et al., 2009a, Niemi et al., 2011) and in the periphery primes NLRP3-dependent responses in macrophages (Ather et al., 2011, Niemi et al., 2011) and synovial fibroblasts (Migita et al., 2012). This chapter shows, consistent with previous literature (McColl et al., 2007), SAA increases in the periphery early (4h) after surgical procedures (Figure 3-12A and B). Moreover, for the first time it shows SAA induces IL-1β and α expression in a dose dependent manner in vitro in mixed glia (Figure 3-13A and B). This response is comparable to that induced by the stereotypical PAMPs LPS and poly(IC) (Figure 3-13C). Furthermore, SAA primed glia release mature IL-β (Figure 3-15), but only on encountering a secondary stimulus as is characteristic of PAMP primed glia (Figure 3-14).

In order to fully ascertain the contribution of SAA to inflammation after sterile injury in the brain, further work would be required intervening with the production or action of SAA. However, as SAA is not the only plasma derived mediator that could contribute to cell priming after BBB disruption, it is unlikely that this would be a good candidate as a therapeutic target. Nonetheless, this data provides insight into how the IL-1 system may be primed and therefore able to contribute to the progression of inflammation after sterile injury.

In conclusion, NLRP3-activating DAMPs induce an IL-1β-independent pro-inflammatory response in glia in the absence of a priming stimulus. IL-1 significantly augments this response in vivo indicating sterile endogenous priming mechanisms contribute to inflammation during acute brain injury. The acute phase protein SAA primes
mixed glia in vitro in a manner similar to LPS and thus may prime glia during stroke after breakdown of the BBB.
Chapter 4: Investigating mechanisms of NLRP3-activating DAMP-induced inflammation & cell death
4.1. Introduction

Necrotic cell death after trauma such as ischaemia-reperfusion injury in the brain (stroke) supplies an initial release of DAMPs that stimulate the inflammatory response and is considered irreversible. This core of necrotic cell death is surrounded by a penumbra in which a second wave of cell death ensues in response to the necrotic inflammation and results in further loss of function, a greater inflammatory response and worsened outcome (Ferrer and Planas, 2003). Reducing inflammation and inhibiting cell death in this region saves precious tissue, thus isolating the predominant mechanism of this second wave of cell death could be therapeutically important.

Evidence associates various forms of programmed cell death with damage in the ischaemic penumbra. Caspase-1 is heavily implicated in cell death after cerebral ischaemia outside of the contribution of IL-1β processing. Whilst IL-1Ra is neuroprotective, this is only the case if it is administered within 3hr of tMCAo (Mulcahy et al., 2003). At this point, IL-1α but not IL-1β is elevated in the parenchyma indicating the protective nature of IL-1Ra may not be via direct inhibition of parenchymal IL-1β rather, IL-1α or peripheral IL-1β (Luheshi et al., 2011). This is further supported by evidence that IL-1β single KO mice are not protected after tMCAo, but that IL-1α/β double KO mice are (Boutin et al., 2001). Caspase-1 inhibition in a rat model of cerebral ischaemia is also protective at 3h, but not at 6h indicating inhibition of caspase-1 may confer protection via a non-IL-1β based target (Denes et al., 2011b, Ross et al., 2007). Caspase-1 activity is integral to the process of pyroptosis in relation to protection of the host against invading microbes (Bergsbaken and Cookson, 2007). The importance of caspase-1 in cerebral ischaemia thereby infers pyroptosis could also play a role in sterile inflammation.

Caspase-1 has also been implicated in apoptosis (Denes et al., 2011b). Moreover inhibition of mitochondrial membrane permeabilisation that precedes apoptosis has been
shown to be neuroprotective in several different models signifying apoptosis could be important in penumbral cell death (Galluzzi et al., 2009). Caspase-independent necroptosis may also be important to consider. Inhibition of RIP1 kinase prevents necrosome formation and subsequent necroptosis and therefore could be considered protective in sterile inflammation (Smith et al., 2007). Indeed inhibition of necroptosis reduces infarct size after transient MCAo in mice (Degterev et al., 2005) via specific inhibition of RIP1 kinase (Degterev et al., 2008). However conversely, enabling necroptosis via caspase inhibition may also be protective by allowing selective cell death of activated microglia (Fricker et al., 2013). Similarly autophagy can be manipulated either to induce protection or cell death (Kroemer and Levine, 2008, Papadakis et al., 2013).

Thus understanding the mechanisms of cell death induced by DAMPs is incredibly important. Controlling the interplay of cell signalling between each mechanism in response to DAMPs could allow therapeutic manipulation of the outcome within the penumbra.

I have shown that NLRP3-acting DAMPs can induce IL-1-independent inflammation in mixed glia. I have also shown that it is likely glia are exposed to an endogenous priming stimulus that allows NLRP3-acting DAMPs to induce an IL-1 response. This chapter investigates the mechanism of cell death induced by NLRP3-activating DAMPs in order to understand how this may propagate inflammation and whether there is a particular mechanism that might provide a likely therapeutic target.

4.2. Methods

Experiments were carried out as specified in chapter 2. Specifics for this experimental chapter are outlined below.
4.2.1. Measuring the pro-inflammatory effects of NLRP3-activating DAMPs on glia in the absence of NLRP3 and priming

Cultures of mixed glia were isolated from murine post-natal day 1-4 pups from mice with NLRP3 KO phenotype and maintained until confluent (around 14-18 days). Cells were treated with ATP (5mM), MSU (250μg/mL) or CPPD (250μg/mL). Indicated concentrations were as initially applied, however integrity of the stimulus was not measured at the end time point, therefore it cannot be assumed that this concentration was maintained throughout. Cells from pups with a WT phenotype were used as a positive control; untreated cells were used as the negative control. After 24h, supernatants were collected to measure LDH levels for cell death, and protein release by ELISA. To confirm the NLRP3 KO phenotype, cells were treated for 24 h with LPS (1μg/ml) and subsequently for 1 h with ATP (5mM). Supernatants were then analysed by western blot for mature IL-1β.

4.2.2. Measuring the effect of cathepsin B inhibition on NLRP3-activating DAMP-induced inflammation

Mixed glia with a WT phenotype from C57BL6/J mice were cultured as described above. Cells were pre-incubated for 5 min with the cathepsin B inhibitor Ca-074Me (50μM), and subsequently, without media change, treated for 24 h with ATP (5mM), MSU (250μg/ml) or CPPD (250μg/ml). Supernatants were collected for analysis of LDH levels for cell death and protein release by ELISA. Effect of inhibition was analysed compared to inhibitor vehicle plus respective DAMP.

4.2.3. Measuring the role of known programmed cell death pathways in NLRP3-activating DAMP-induced inflammation

Mixed glia with a WT phenotype from C57BL6/J mice were cultured as described above. For experiments in the absence of priming, cells were pre-incubated for 5 min with
one of the following inhibitors: YVAD (100μM), ZVAD (50μM), necrostatin-1 (100μM) or calpain inhibitor III (50μM); and subsequently, without media change, treated for 24 h with ATP (5mM), MSU (250μg/ml) or sphingosine (20mM). For experiments in the presence of priming, glia were initially incubated in LPS (1μg/ml) for 24 h prior to exposure to inhibitors and DAMPs. Supernatants were collected for analysis of LDH levels for cell death and protein release by ELISA and western blot. Effect of inhibition was analysed compared to inhibitor vehicle plus respective DAMP.

4.2.4. Measuring the role of alternative signalling pathways in NLRP3-activating DAMP-induced inflammation

Mixed glia with a WT phenotype from C57BL6/J mice were cultured as described above. For experiments in the absence of priming, cells were pre-incubated for 5 min with one of the following inhibitors: piceatannol (50μM), 3-MA (5mM); and subsequently, without media change, treated for 24 h with ATP (5mM), MSU (250μg/ml) or sphingosine (20mM). For experiments in the presence of priming, glia were initially incubated in LPS (1μg/ml) for 24 h prior to exposure to inhibitors and DAMPs. Supernatants were collected for analysis of LDH levels for cell death and protein release by ELISA and western blot. Effect of inhibition was analysed compared to inhibitor vehicle plus respective DAMP.

4.2.5. Statistical analysis

Unless otherwise stated, data is pooled from a minimum of 3 experiments and statistical significance is measured using a non-parametric Kruskal-Wallis test with Dunn’s paired comparison post-hoc. All data are expressed as mean ± SD. ***P<0.001, **P<0.01, *P<0.05.
4.3. Results

4.3.1. Effect of NLRP3 inflammasome-activating DAMPs on mixed glia in the absence of NLRP3 and priming

Prolonged cell death beyond that caused by the initial injury is common in sterile inflammation, and yet the mechanism of this cell death remains elusive. As cell death drives further inflammation and therefore further cell death, understanding the mechanism of cell death could help reveal potential therapeutic targets. As demonstrated in section 3.3.1, NLRP3 inflammasome-activating DAMPs can initiate restricted inflammation independent of priming and IL-1. During this analysis, it was observed that 24h exposure to DAMPs in the absence of a priming stimulus induced glial cell death (<40% as measured by LDH release) (Figure 4-1B). However it was not clear if the inflammatory response observed was due to this cell death, or independent and perhaps causative of the cell death.

To determine the mechanism of NLRP3 inflammasome-activating DAMP-induced cell death, initially the importance of NLRP3 was observed. Mixed glial cultures isolated from either WT or NLRP3 KO mice were stimulated for 24h with ATP, MSU or CPPD after which the supernatants were collected and analysed for LDH to measure cell death. IL-6 and CXCL1 release were also analysed as a read-out for DAMP induced inflammation (section 3.3.1).

The NLRP3 KO phenotype was confirmed by analysing the supernatants of mixed glia from WT and NLRP3 KO mice for mature IL-1β after 24h exposure to LPS and a subsequent 1h exposure to ATP (Figure 4-1A). No decrease in cell death was observed in mixed glia from NLRP3 KO mice compared with those from WT mice in response to ATP, MSU or CPPD (Figure 4-1B). No significant decrease in IL-6 and CXCL1 was observed.
in the supernatants of mixed glia from NLRP3 KO mice compared to those from WT mice (Figure 4-1C and Figure 4-1D).

![Figure 4-1: Effect of NLRP3 KO on NLRP3 inflammasome-activating DAMP-induced cell death and cytokine production](image)

NLRP3 KO phenotype was confirmed by western blot for mature IL-1β in primary mixed glia after 24h LPS (1μg/ml) priming and 1h subsequent stimulation with ATP (5mM) (A). Blot is representative of 3 separate experiments. LDH levels from the supernatants of primary mixed glia from WT (■) and NLRP3 KO (□) mice subjected to 24h ATP (5mM), MSU (250μg/ml) or CPPD (250μg/ml) were analysed using the CytoTox96® Nonradioactive Cytotoxicity Assay for cell death (B). IL-6 (C) and CXCL1 (D) release was analysed by ELISA from the same supernatants. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
4.3.2. Effect of cathepsin B inhibition on NLRP3 inflammasome-activating DAMP-induced cell death and cytokine production

Among the pro-inflammatory mediators produced by mixed glia in response to NLRP3 inflammasome-activating DAMPs in the absence of priming, was the pro-inflammatory lysosomal cysteine protease Cathepsin B (section 3.3.1.4). It was therefore considered that the pro-inflammatory mediators released after the initial stimulus may dictate subsequent cell death in an autocrine manner. Cathepsin B has been implicated in the progressive pathogenesis of cerebral ischaemia and is suggested as a therapeutic target (Benchoua et al., 2004, Seyfried et al., 2001, Yamashima, 2000).

Mixed glia from WT mice were pre-incubated with the selective cathepsin B inhibitor Ca-074Me for 15 minutes, and subsequently exposed for 24h to ATP, MSU or CPPD. Supernatants were collected and analysed for LDH, IL-6 and CXCL1 release. The cathepsin B inhibitor by itself did not induce an increase in cell death, IL-6 or CXCL1 (Figure 4-2A). Inhibition of cathepsin B had no effect on DAMP-induced cell death (Figure 4-2A) or IL-6 release (Figure 4-2B). Inhibition of cathepsin B also failed to significantly impact ATP, MSU and CPPD induced CXCL1 release although variability of the data may have masked an effect (Figure 4-2C).
Figure 4-2: Effect of cathepsin B inhibition on NLRP3-activating DAMP-induced inflammation

To assess the effect of cathepsin B on DAMP-induced cell death, ATP, MSU and CPPD-induced cell death was assessed by LDH release from mixed glia in the absence and presence of the cathepsin B inhibitor Ca-074Me (50μM) (A). To assess the contribution of cathepsin B to DAMP-induced inflammation, IL-6 (B) and CXCL1 (C) release were measured by ELISA in response to ATP, MSU and CPPD in the absence and presence of Ca-074Me. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
4.3.3. Investigating the role of programmed cell death in NLRP3-activating DAMP-induced inflammation in the absence and presence of priming

As DAMP-induced cell death did not appear to be related to NLRP3 function, or a downstream effect of the cathepsin B response initiated by the DAMPs, the involvement of caspases and calpains and the initiation of programmed cell death was considered. As discussed in the introduction to this chapter, different forms of programmed cell death have been implicated in the pathogenesis of various sterile diseases.

This section considers mechanisms of cell death in both unprimed and primed mixed glial cultures. Whilst it is necessary to better understand how pro-inflammatory mediators are produced in the absence of a priming stimulus, it is likely that an endogenous priming mechanism exists (section 3.3.2). Many programmed cell death mechanisms rely on the expression and activation of molecules that are regulated by priming. For example, the activation of caspase-1 relies on cell priming (section 1.2) and is integral to pyroptotic cell death (section 1.3.3). Furthermore, it is shown here that NLRP3 expression is significantly enhanced in primed cells (Figure 3-2), thus in primed conditions the contribution of NLRP3-activating DAMPs to cell death may be more apparent. Therefore investigating cell death mechanisms in primed mixed glia is equally as important.

Although each of the DAMPs used here are known activators of NLRP3, it is important to consider their mechanisms of action may involve signalling pathways independent of NLRP3. As seen above, NLRP3 KOs are not protected from cell death induced by ATP, MSU or CPPD; lending further conviction to the theory that NLRP3-independent mechanisms may be involved. Up to this point, the pro-inflammatory characteristics of CPPD have been very similar to those of MSU and both act in a similar manner with the crystal structure being engulfed resulting in rupture of the phago-lysosomal membrane within the cell much like other engulfed particulate matter (Chen et
al., 2007, Duewell et al., 2010, Halle et al., 2008, Hornung et al., 2008). This results in NLRP3 activation and IL-1β processing and release in primed cells (section 3.3.2.2). To widen the variety of mechanisms employed by the NLRP3-activating DAMPs being studied, the endogenous lipid mediator sphingosine was used as an NLRP3 inflammasome-activating DAMP instead of CPPD for the next section of work. Sphingosine is a soluble endogenous molecule, the level of which increases intracellularly in pathological situations (He et al., 2010, Rodriguez-Lafrasse and Vanier, 1999). It is a more recently characterised DAMP that regulates IL-1β processing and release via NLRP3, the mechanism of which is dependent on dephosphorylation of a serine-threonine residue by the phosphatases PP1 or PP2A (Luheshi et al., 2012). Like MSU, it can also act via lysosomal destabilisation, however unlike known particulate lysosomal DAMPs, its actions can be independent of cathepsin release (Luheshi et al., 2012). Sphingosine can hence be considered here as a model of non-particulate lysosomal destabilisation, allowing the mechanism of cell death and cytokine production induced by different NLRP3-activating DAMPs to be compared. Furthermore, the actions of sphingosine during disease beyond the effect it has on IL-1β processing are yet to be determined, thus it was of interest to compare these to the actions of the well-established NLRP3-activating DAMPs ATP and MSU.
4.3.3.1. Targeting caspase-dependent cell death in unprimed and primed mixed glia

Initially due to the evidence linking caspase-1 to cerebral ischaemia (section 4.1), the contribution of caspase-1 mediated pyroptosis to DAMP-induced cell death was assessed. YVAD specifically inhibits caspase-1 dependent pyroptosis in mouse macrophages in response to bacterial infection (Bergsbaken and Cookson, 2007). Caspase-1 activation mediates pore-formation in the plasma membrane of pyroptotic cells and subsequent ion flux and cell lysis (Fink and Cookson, 2006). Thus, inhibition of cell death by YVAD is quantifiable by measuring the release of LDH from the cells.

In unprimed cells, YVAD alone did not affect cell death, IL-6 or CXCL1 release compared to its vehicle (Figure 4-3A). Moreover, YVAD did not have any effect on cell death in response to any of the DAMPs (Figure 4-3A). YVAD did not significantly reduce IL-6 or CXCL1 release vs. vehicle treatment in response to ATP or MSU (Figure 4-3B and Figure 4-3C, respectively). Interestingly, sphingosine did not induce an increase in the release of either IL-6 or CXCL1 from mixed glia (Figure 4-3B and Figure 4-3C).
Figure 4-3: Effect of caspase-1 inhibition on NLRP3-activating DAMP-induced inflammation in unprimed cells

To assess the effect of caspase-1 activity on DAMP-induced cell death, ATP (5mM), MSU (250μg/ml) and sphingosine (20mM)-induced cell death was assessed after 24h by LDH release from mixed glia in the absence and presence of the caspase-1 inhibitor YVAD (100μM) (A). To assess the contribution of caspase-1 activity to DAMP-induced inflammation, IL-6 (B) and CXCL1 (C) release were measured by ELISA from the same supernatants. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
To observe the effect of priming on cell death, cells were incubated for 24h with LPS and subsequently pre-treated for 15min with YVAD before 24h exposure to ATP, MSU or sphingosine. Again, the presence of YVAD had no effect on cell death induced by ATP, MSU or sphingosine (Figure 4-4A). To ensure YVAD was pharmacologically active, IL-1β processing and release was assessed by ELISA and confirmed by western blot. Due to the integral involvement of IL-1α in the acute phase of sterile inflammation (Cohen et al., 2010, Eigenbrod et al., 2008, Luheshi et al., 2011), IL-1α processing and release was also assessed by ELISA and confirmed by western blot. YVAD successfully inhibited the processing and release of IL-1β. Due to variability of the data this inhibition was not statistically significant as measured by ELISA, except in the case of sphingosine induced IL-1β processing and release (P < 0.05) (Figure 4-4Bi). However confirmation by western blot showed a distinct reduction in the release of mature 17kDa IL-1β (Figure 4-4Bii). YVAD had no effect on the processing and release of IL-1α (Figure 4-4Ci and ii).
To assess the effect of caspase-1 activity on DAMP-induced inflammation in primed cells, mixed glia were incubated for 24h with LPS (1μg/ml) and subsequently for 24h with ATP (5mM), MSU (250μg/ml) or sphingosine (20mM). Cell death was assessed by LDH release from mixed glia in the absence and presence of the caspase-1 inhibitor YVAD (100μM) (A). IL-1β (B) and IL-1α (C) release were measured by ELISA (i) and western blot (ii) from the same supernatants. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
Other members of the caspase family besides caspase-1 are activated by oxidative stress and are also associated with cell death in neurodegenerative processes (Hortelano et al., 1997, Leist et al., 1997). These caspases form signalling cascades that are both activators and effectors of apoptosis (section 1.3.1). Thus pan caspase inhibition effectively prevents apoptosis and reduces cell death (Earnshaw et al., 1999, Robertson et al., 2000). Here, the contribution of apoptosis was assessed in both unprimed and primed cells using the pan caspase inhibitor ZVAD. Though apoptosis does not initially result in the large increase in LDH observed with necrotic cell death, it is possible this occurs later during secondary necrosis, a process of cell membrane disruption following apoptosis (Krysko et al., 2006, Vanden Berghe et al., 2010). Thus if apoptosis were the central mechanism of cell death, inhibition of it could still be observed via LDH measurement.

In unprimed mixed glia, ZVAD alone did not induce cell death compared to its vehicle; however it did appear to induce a negligible amount of IL-6 and CXCL1 release (Figure 4-5). ZVAD had no effect on cell death in mixed glia exposed to ATP (Figure 4-5A). Sphingosine and MSU did not appear to induce high levels of cell death and this was not affected by the presence of ZVAD (Figure 4-5A). ZVAD did not affect IL-6 release stimulated by ATP or MSU and the IL-6 release in response to sphingosine remained negligible regardless of the presence of ZVAD (Figure 4-5B). ZVAD had no effect on ATP stimulated CXCL1 release (Figure 4-5C). CXCL1 release was potentiated in MSU and sphingosine treated cells that had been exposed to ZVAD. This was more notable in the sphingosine induced expression as sphingosine alone does not induce CXCL1 release (Figure 4-3C and Figure 4-5C), though due to the variability of the data, this was not a significant increase over DAMP-induced CXCL1 release (Figure 4-5C).
Figure 4-5: Effect of pan caspase inhibition on NLRP3-activating DAMP-induced inflammation in unprimed cells

To assess the effect of general caspase activity on DAMP-induced cell death, ATP (5mM), MSU (250μg/ml) and sphingosine (20mM)-induced cell death was assessed after 24h by LDH release from mixed glia in the absence and presence of the caspase-1 inhibitor ZVAD (50μM) (A). To assess the contribution of general caspase activity to DAMP-induced inflammation, IL-6 (B) and CXCL1 (C) release were measured by ELISA from the same supernatants. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
To observe the effect of priming on cell death, cells were incubated for 24h with LPS and subsequently pre-treated for 15min with ZVAD before 24h exposure to ATP, MSU and sphingosine. ZVAD had no effect on cell death induced by ATP, MSU or sphingosine (Figure 4-6A). ZVAD successfully inhibited the processing and release of IL-1β (Figure 4-6Bi). This effect was not significant when measured by ELISA but, once again, western blot confirmed distinct inhibition of IL-1β processing and release by ZVAD (Figure 4-6Bii). IL-1α processing and release was not affected by ZVAD as measured by ELISA and confirmed by western blot (Figure 4-6Ci and ii).
To assess the effect of general caspase activity on DAMP-induced inflammation in primed cells, mixed glia were incubated for 24h with LPS (1μg/ml) and subsequently for 24h with ATP (5mM), MSU (250μg/ml) or sphingosine (20mM). Cell death was assessed by LDH release from mixed glia in the absence and presence of the caspase-1 inhibitor ZVAD (50μM) (A). IL-1β (B) and IL-1α (C) release were measured by ELISA (i) and western blot (ii) from the same supernatants. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
4.3.3.2. Targeting caspase-independent cell death

Necroptosis is a caspase-independent form of programmed cell death that occurs when caspase-8 mediated apoptosis is blocked (Fiers et al., 1995, Holler et al., 2000). As cells were not protected by caspase inhibition (Figure 4-3 - Figure 4-6), it was considered whether the DAMPs act on RIP kinases to induce necroptosis. The necroptosis inhibitor necrostatin-1 has been suggested as a novel therapeutic agent in cerebral ischaemia and other sterile inflammatory conditions (Degterev et al., 2005, Smith et al., 2007). It specifically inhibits RIP1 kinase activity thus preventing its association with RIP3 kinase preventing the formation of the necrosome and thereby preventing necroptosis (Degterev et al., 2008, Wu et al., 2012).

In unprimed mixed glia, necrostatin-1 alone did not induce an increase in mixed glial cell death and had no effect on cell death induced by ATP, MSU or sphingosine (Figure 4-7A). Moreover, necrostatin-1 had no effect on IL-6 and CXCL1 production in response to ATP and MSU (Figure 4-7B and Figure 4-7C). As before, there was no IL-6 or CXCL1 release in response to sphingosine alone. However in the presence of necrostatin-1, sphingosine induced IL-6 and CXCL1 release, though care must be taken with the interpretation of this data due to the variability (Figure 4-7B and Figure 4-7C).

Inhibition of necroptosis in LPS primed cells had no effect on DAMP-induced cell death (Figure 4-8A), or on DAMP-induced processing and release of IL-1β (Figure 4-8Bi and ii) or IL-1α (Figure 4-8Ci and ii).
To assess the contribution of necroptosis to DAMP-induced cell death, ATP (5mM), MSU (250μg/ml) and sphingosine (20mM)-induced cell death was assessed after 24h by LDH release from unprimed cells in the absence and presence of the RIP1 kinase inhibitor necrostatin-1 (nec-1) (100μM) (A). To assess the contribution of necroptosis to DAMP-induced inflammation, IL-6 (B) and CXCL1 (C) release were measured by ELISA from the same supernatants. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
To assess the contribution of necroptosis to DAMP-induced inflammation in primed cells, mixed glia were incubated for 24h with LPS (1μg/ml) and subsequently for 24h with ATP (5mM), MSU (250μg/ml) or sphingosine (20mM). Cell death was assessed by LDH release from mixed glia in the absence and presence of the RIP1 kinase inhibitor necrostatin-1 (nec-1) (100μM) (A). IL-1β (B) and IL-1α (C) release were measured by ELISA (i) and western blot (ii) from the same supernatants. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
4.3.3.3. Targeting calpain-driven cell death

Calpains are a family of calcium (Ca\(^{2+}\))-dependent cytoplasmic cysteine proteases that are active at neutral pH. There are 15 known isoforms, several of which are tissue specific, but the most well-characterised are the ubiquitous \(\mu\)-calpain (or calpain I) and m-calpain (or calpain II) (Goll et al., 2003). Within the brain \(\mu\)-calpain is expressed predominantly in the dendrites and cell bodies of neurones, whilst m-calpain is found localised in neuronal axons and glia (Nixon, 1986, Onizuka et al., 1995). Much like cathepsins and caspases, calpains are integral to both normal cellular physiology and cell death (Yamashima, 2000). Under physiological conditions calpains respond to nM fluxes in Ca\(^{2+}\) homeostasis, and are regulated by the endogenous protein inhibitor calpastatin (Vaisid et al., 2007, Vosler et al., 2008). Among the many substrates on which calpains act are cell cycle-related proteins and cytoskeletal components. Within the brain it is therefore considered that calpains are important in the neuronal remodelling that underlies synaptic plasticity and memory formation (Chan and Mattson, 1999). They also have an integral role during embryonic development (Dutt et al., 2006, Arthur et al., 2000). During excitotoxic conditions such as cerebral ischaemia cytosolic Ca\(^{2+}\) concentration can rise as high as 10\(\mu\)M leading to overactivation of calpains (Hyrc et al., 1997, White et al., 2000, Verkhratsky, 2007). Calpain hyperactivity plays a key role in both necrotic and apoptotic cell death acting via caspase activation or lysosomal rupture and subsequent release of lysosomal proteases (Chan and Mattson, 1999, Malagelada et al., 2005, Yamashima et al., 2003). As calpastatin, the calpain inhibitor, is another substrate of calpain, the natural inhibition of calpain is reduced as the Ca\(^{2+}\) concentration increases (Porn-Ares et al., 1998).

Calpain activity associated with neurodegeneration has been suggested as a therapeutic target for rescuing cells in the post-ischaemic penumbra (Bevers and Neumar,
2008, Vosler et al., 2008). Due to this and the relationship of calpain with release of lysosomal proteases such as cathepsin B (Chan and Mattson, 1999), inhibition of calpain was considered as a mechanism of reducing DAMP-induced cell death.

Incubating unprimed mixed glia with calpain inhibitor III (CalpIII) alone for 24h did not induce a significant increase in cell death (Figure 4-9A). There was no reduction in cell death in response to ATP, MSU or sphingosine in the presence of CalpIII (Figure 4-9A). Calpain inhibition did not significantly affect IL-6 and CXCL1 release in response to ATP and MSU (Figure 4-9B and Figure 4-9C, respectively). Consistent with previous results, sphingosine did not induce any increase in IL-6 or CXCL1 (Figure 4-9B and Figure 4-9C, respectively).
Chapter 4: Investigating mechanisms of NLRP3-activating DAMP-induced inflammation & cell death

**Figure 4-9: Effect of calpain inhibition on NLRP3-activating DAMP-induced inflammation in unprimed cells**

To assess the contribution of calpain to DAMP-induced cell death, ATP (5mM), MSU (250μg/ml) and sphingosine (20mM)-induced cell death was assessed after 24h by LDH release from mixed glia in the absence and presence of calpain inhibitor III (CalpIII) (50μM) (A). To assess the contribution of calpain to DAMP-induced inflammation, IL-6 (B) and CXCL1 (C) release were measured by ELISA from the same supernatants. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
To determine if cell priming has an effect on the involvement of calpain in cell death, CalpIII was applied to LPS primed cells 15 min prior to 24 h incubation with each DAMP. CalpIII had no effect on DAMP-induced cell death in LPS primed mixed glia (Figure 4-10A). Furthermore it had no effect on ATP induced IL-1β processing (Figure 4-10Bi and ii). MSU and sphingosine did not induce as much expression and release of IL-1β in the presence of CalpIII as noticeable by western blot (Figure 4-10Bii).

Pro-IL-1α is a substrate of calpain (Kobayashi et al., 1990); therefore mature IL-1α processing and release was measured to indicate the efficacy of the CalpIII inhibitor. ELISA did not show a reduction of IL-1α processing and release in response to all three DAMPs, due to the non-specific nature of the ELISA for pro- vs. mature IL-1α (Figure 4-10Cii). However western blot confirmed CalpIII activity with a distinct reduction in 17kDa calpain-dependent mature IL-1α (Figure 4-10Cii).
To assess the contribution of calpain to DAMP-induced inflammation in primed cells, mixed glia were incubated for 24h with LPS (1μg/ml) and subsequently for 24h with ATP (5mM), MSU (250μg/ml) or sphingosine (20mM). Cell death was assessed by LDH release from mixed glia in the absence and presence of calpain inhibitor III (CalpIII) (50μM) (A). IL-1β (B) and IL-1α (C) release were measured by ELISA (i) and western blot (ii) from the same supernatants. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
4.3.4. Investigating alternative signalling pathways involved in NLRP3-activating DAMP-induced inflammation in the absence and presence of priming

As there was no cytoprotective effect observed through targeting specific forms of programmed cell death, mechanisms of cell activation were considered. MSU crystals have been shown to interact with the cell membrane in a non-receptor specific manner to activate dendritic cells via the Syk pathway in the absence of TLR/PAMP priming (Ng et al., 2008). Additionally, the precise mechanism through which MSU and sphingosine induce NLRP3 activation in primed cells remains unclear. As Syk is also known to activate the NLRP3 inflammasome in response to fungal and malarial infection (Mocsai et al., 2010, Gross et al., 2009, Shio et al., 2009), it was considered whether Syk represents an alternative mechanism to lysosomal destabilisation, by which sterile stimuli induce IL-1β processing in primed cells. Thus the relevance of Syk to the inflammatory response to DAMPs observed in the absence and presence of priming was observed using the Syk inhibitor piceatannol.

Unprimed mixed glia were pre-incubated in piceatannol for 15 minutes and then stimulated with ATP, MSU and sphingosine for 24h. Piceatannol alone had no effect on cell death, IL-6 or CXCL1 release (Figure 4-11). Furthermore it did not reduce DAMP induced cell death, though MSU and sphingosine induced cell death was negligible to start with (Figure 4-11A). This loss of cell death may be due to loss of activity within the DAMP preparation, however IL-1β processing and release was unaffected (Figure 4-12Bi), so this is unlikely. IL-6 and CXCL1 production induced by ATP was not affected by the presence of piceatannol (Figure 4-11B and Figure 4-11C, respectively). In the presence of piceatannol, MSU did not induce IL-6 or CXCL1 release (Figure 4-11B and Figure 4-11C, respectively). This result however requires clarification as the significance of it may be masked by the variability of the MSU alone data to which it is compared. Consistent with
the data so far, sphingosine alone did not induce an increase in IL-6 or CXCL1, and the presence of piceatannol did not affect this (Figure 4-11B and Figure 4-11C, respectively).

In LPS primed mixed glia, piceatannol had no effect on DAMP induced cell death, though once again MSU and sphingosine induced cell death was minimal (Figure 4-12A). Piceatannol had no effect on ATP induced IL-1β (Figure 4-12Bi and ii) or IL-1α (Figure 4-12Ci and ii) processing and release. There was no significant effect of MSU or sphingosine on the processing of IL-1β and IL-1α in the presence of piceatannol (Figure 4-12Bi and Figure 4-12Ci), and western blot suggested there was a reduction in the 17kDa form of both in response to piceatannol (Figure 4-12Bii and Figure 4-12Cii, respectively), however this requires further work to confirm.
To assess the contribution of Syk to DAMP-induced cell death, ATP (5mM), MSU (250μg/ml) and sphingosine (20mM)-induced cell death was assessed after 24h by LDH release from mixed glia in the absence and presence of the Syk inhibitor piceatannol (50μM) (A). To assess the contribution of Syk to DAMP-induced inflammation, IL-6 (B) and CXCL1 (C) release were measured by ELISA from the same supernatants. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
To assess the contribution of Syk to DAMP-induced inflammation in primed cells, mixed glia were incubated for 24h with LPS (1μg/ml) and subsequently for 24h with ATP (5mM), MSU (250μg/ml) or sphingosine (20mM). Cell death was assessed by LDH release from mixed glia in the absence and presence of the Syk inhibitor piceatannol (Pic) (50μM) (A). IL-1β (B) and IL-1α (C) release were measured by ELISA (i) and western blot (ii) from the same supernatants. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
Considering the potential effects of Syk inhibition on MSU and sphingosine induced cell activity, other cell regulatory mechanisms were considered. Autophagy is a major cell regulatory mechanism, the function of which under normal physiological conditions is to preserve cellular integrity (Ding and Yin, 2008). It aids the clearance of inflammasome components and intracellular cytokines thereby regulating inflammation (Harris et al., 2011, Shi et al., 2012). However whilst there is evidence autophagy is important in controlling inflammation, autophagy is also known to contribute to neutrophil activation and IL-1β release in response to the DAMP MSU (Mitroulis et al., 2011). Furthermore, cells undergoing autophagic cell death have been shown to be immunogenic when phagocytosed by macrophages (Ayna et al., 2012, Petrovski et al., 2011). Thus it is intriguing as to whether autophagy is involved in the DAMP induced inflammation observed in this study, and whether its involvement influences the inflammation either by enhancing or inhibiting it.

To investigate the contribution of autophagy to NLRP3-activating DAMP-induced inflammation, unprimed or primed mixed glia were pre-incubated with the type III PI3K inhibitor 3-MA for 15 minutes before 24h stimulation with respective DAMPs.

In unprimed mixed glia, 3-MA alone had no effect on cell death compared to its vehicle (Figure 4-13A). 3-MA did not affect cell death in response to ATP, MSU or sphingosine, though cell death in response to MSU and sphingosine was low to start with (Figure 4-13A). 3-MA had no effect on ATP induced IL-6 and CXCL1 release. 3-MA did appear to inhibit MSU induced IL-6 and CXCL1 release although there was too much variability in the data for this to reach statistical significance. Consistent with previous findings, sphingosine did not induce IL-6 or CXCL1 release and 3-MA had no effect on this (Figure 4-13B and Figure 4-13C, respectively).
In LPS primed mixed glia, 3-MA had no effect on cell death in response to ATP, MSU or sphingosine (Figure 4-14A). 3-MA had no significant effect on IL-1β or IL-1α processing in response to any of the DAMPs as measured by ELISA (Figure 4-14B and Figure 4-14C, respectively). That being said, when used in conjunction with 3-MA, MSU did not induce IL-1β or α processing and release as expected, as measured by ELISA (Figure 4-14B and Figure 4-14C, respectively).
Figure 4-13: Effect of autophagy inhibition on NLRP3-activating DAMP-induced inflammation in unprimed cells

To assess the contribution of autophagy to DAMP-induced cell death, ATP (5mM), MSU (250μg/ml) and sphingosine (20mM)-induced cell death was assessed after 24h by LDH release from mixed glia in the absence and presence of the autophagy inhibitor 3-MA (5mM) (A). To assess the contribution of autophagy to DAMP-induced inflammation, IL-6 (B) and CXCL1 (C) release were measured by ELISA from the same supernatants. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
To assess the contribution of autophagy to DAMP-induced inflammation in primed cells, mixed glia were incubated for 24h with LPS (1μg/ml) and subsequently for 24h with ATP (5mM), MSU (250μg/ml) or sphingosine (20mM). Cell death was assessed by LDH release from mixed glia in the absence and presence of the autophagy inhibitor 3-MA (5mM) (A). IL-1β (B) and IL-1α (C) release were measured by ELISA from the same supernatants. Data are pooled from at least 3 separate experiments and are expressed as mean ± SD.
4.4. Discussion

Work in this chapter has shown that NLRP3-activating DAMPs can act independently of NLRP3 to induce inflammation and cell death in mixed glia. This is most likely necrotic cell death as inhibitors of different forms of programmed cell death - apoptosis, pyroptosis and necroptosis – had no effect on cell death in primed or unprimed glia. Furthermore it has emphasised that whilst different DAMPs may converge on the same inflammatory outcomes, the signalling pathways and mechanisms by which these are achieved do not necessarily converge too. Therefore therapeutic measures should target the downstream outcomes of initial necrotic cell death as opposed to the original initiation of cell death and inflammation.

4.4.1. NLRP3-activating DAMPs induce NLRP3 independent inflammation

In chapter 3 it was shown that NLRP3-activating DAMPs are capable of inducing an inflammatory response in the absence of a priming stimulus. Though key pathways in the inflammatory response to necrotic cells have been isolated (Chen et al., 2007), the specific mechanism of the response of glial cells to NLRP3-activating DAMPs has never been looked at. Furthermore it was observed that each of the DAMPs used induced an increase in cell death as measured by LDH release (Figure 4-1B). As discussed in section 1.3, programmed cell death has been associated with sterile inflammatory disorders, thus a better understanding of the mechanism by which this occurs is fundamental to the discovery of therapeutic targets (Chen and Nunez, 2010).

As each of the DAMPs used in chapter 3 are known to act on NLRP3, and given the importance of NLRP3 in the inflammatory response to sterile injury (section 1.2.1.2 – NLRP3), experiments initially observed the dependence of some of the inflammatory responses seen in chapter 3, and the cell death observed, on the NLRP3 receptor. Mixed glia from NLRP3 KO mice were used and responses compared to glia from WT mice. The
phenotype of the NLRP3 KO was confirmed by western blot of LPS and ATP-treated supernatants which showed there was no IL-1β processing (Figure 4-1A). Cells from NLRP3 KO mice had comparable cell death to DAMPs as WT cells (Figure 4-1B). IL-6 and CXCL1 responses were also comparable (Figure 4-1C and Figure 4-1D, respectively). This implies that the inflammation observed in chapter 3 occurs via NLRP3-independent signalling pathways.

4.4.2. NLRP3-activating DAMP-induced inflammation is not due to programmed cell death

To determine if the IL-6/CXCL1 response observed was due to DAMP-induced cell death, different mechanisms of programmed cell death were inhibited using well-established inhibitors. As both cell death and an inflammatory response were observed in unprimed glia subjected to NLRP3-activating DAMPs, experiments were initially carried out in unprimed glia in order to try to delineate the inflammatory mechanism employed by the DAMPs acting alone. However, as demonstrated in section 3.3.2, it is likely that endogenous priming factors would contribute to the acute phase of inflammation. Priming is crucial to the activation of molecules involved in cell death mechanisms for example, priming is required for caspase-1 activation which is integral in mediating pyroptosis. Finally it has been shown in the literature that the activation state of microglia may be important in influencing cell death outcomes (Fricker et al., 2013). Thus experiments were also carried out on LPS primed mixed glia. At this point, the NLRP3-activating DAMP sphingosine was used instead of CPPD in order to observe mechanisms employed by a non-particulate lysosomal destabilising DAMP (section 4.3.3).

Owing to the strong association between caspase-1 and inflammation in cerebral ischaemia (Ross et al., 2007, Denes et al., 2011b), pyroptosis or caspase-1-dependent apoptosis was targeted initially using the caspase-1 inhibitor YVAD. As a highly
conserved form of programmed cell death previously associated with cell death in the penumbra during cerebral ischaemia (Robertson et al., 2000), apoptosis was also further considered using the pan caspase inhibitor ZVAD.

Neither YVAD (Figure 4-3A) nor ZVAD (Figure 4-5A) were cytoprotective in unprimed mixed glial cultures exposed to ATP, MSU or sphingosine for 24h. Priming for 24h with LPS prior to DAMP treatment made no difference to this (Figure 4-4A and Figure 4-6A, respectively). Thus the NLRP3-acting DAMP-induced cell death observed here is not caspase-dependent apoptosis or pyroptosis regardless of the primed state of the cell.

IL-6 and CXCL1 release were not significantly affected by caspase-1 inhibition in unprimed mixed glia (Figure 4-3B and Figure 4-3C, respectively) consistent with the theory that these may be cell death induced responses. However, contrary to this theory, the basal cell death to DAMPs was much lower during the ZVAD experiments (Figure 4-5A), yet the basal IL-6 and CXCL1 responses were the same (Figure 4-5B and C, respectively), indicating perhaps this is not a cell death-driven process. ZVAD had no effect on ATP induced IL-6 and CXCL1 but induced a trend toward an increase in MSU and sphingosine induced CXCL1 release (Figure 4-5C). This indicates ZVAD may potentiate the inflammatory actions of lysosomal acting DAMPs. This is consistent with the effect of cathepsin B inhibition which induced a trend toward a decrease in CXCL1 release (Figure 4-2C) and data that lysosomal proteases can regulate chemokine production (Hasan et al., 2006). However in order to verify this more work would be required to confirm if the effects seen using ZVAD and cathepsin B inhibition are significant, and also to link caspase activity and cathepsin activity by observing if ZVAD has an effect on cathepsin B production. Following data showing an increase in active Cathepsin B release from NLRP3-activating DAMP-treated glia (section 3.3.1.4), the role of cathepsin B in
DAMP-induced cell death observed here was considered, but cathepsin B inhibition did not reduce DAMP induced cell death (Figure 4-2A). It is also important to consider that cathepsin B is not the only lysosomal cathepsin released that affects cell death (Repnik et al., 2012) and therefore inhibition of other lysosomal proteases should be investigated for future work.

Interestingly, sphingosine consistently did not induce any IL-6 or CXCL1 production throughout all experiments in this section of work. The activity of the concentration being used was confirmed by the presence of IL-1β processing in LPS primed cells (Figure 4-4B, Figure 4-6B, Figure 4-8B, Figure 4-10B, Figure 4-12B and Figure 4-14B). Thus lysosomal destabilisation alone may be insufficient to induce an IL-6 and CXCL1 based inflammatory response.

The activity of YVAD and ZVAD was confirmed by the suppression of IL-1β processing and release from DAMP-treated LPS primed cells (Figure 4-4B and Figure 4-6B, respectively). IL-1α processing and release was also measured due to its relevance to the acute phase of sterile inflammation (Luheshi et al., 2011). Neither YVAD nor ZVAD significantly affected IL-1α processing and release (Figure 4-4C) consistent with previous literature that IL-1α processing can be a caspase-1-dependent process that occurs independent of caspase catalytic activity (Gross et al., 2012).

Necroptosis is a more recently defined form of programmed necrotic cell death controlled by RIP kinases in response to apoptotic stimuli when caspase-8 mediated apoptosis is inhibited (section 1.3.2) (Holler et al., 2000, Vercammen et al., 1998). The necroptosis inhibitor necrostatin-1 specifically binds RIP1 kinase and prevents necrosome formation and subsequent necroptosis (Degterev et al., 2008). It is suggested that in the case of delayed cell death in cerebral ischaemia, conditions are not optimal for the activation of apoptosis in all cells and that necrostatin-1 may have a protective effect.
(Degterev et al., 2005, Smith et al., 2007). However more recently it was shown that
inducing necroptosis specifically in activated microglia may be protective to neurones
grown in co-culture (Fricker et al., 2013). Thus necroptosis presents a form of cell death
that could equally be involved in the progression of penumbral cell death or indeed the
prevention of neuronal cell death.

Caspase inhibition did not offer cytoprotection in mixed glial cultures used here.
Thus, in line with previous studies in which apoptotic inhibition did not prevent
programmed cell death (Oppenheimer et al., 2001, West et al., 2006), it was considered
whether inhibition of necroptosis may be protective. Given the afore-mentioned research
into the neuroprotective nature of necroptosis specifically in activated microglia (Fricker
et al., 2013), the importance of testing necroptosis inhibition in both unprimed and primed
conditions is apparent.

Necrostatin-1 was not protective in unprimed or primed cells (Figure 4-7A and
Figure 4-8A). This is not considered to be due to the caspase activity given the evidence
that pan caspase inhibition does not increase cell death (Figure 4-5A and Figure 4-6A) as
would be expected were ATP, MSU or sphingosine capable of inducing necroptosis.
Furthermore it is not thought that RIP kinases are responsible ATP or MSU induced
inflammation as IL-6 and CXCL1 release in unprimed mixed glia, and IL-1β and IL-1α
release in LPS-primed mixed glia were not affected by the presence of necrostatin-1.
However necrostatin-1 and sphingosine may have a synergistic effect allowing the
production and release of IL-6 and CXCL1 (Figure 4-7B and Figure 4-7C). Typically thus
far, sphingosine alone does not induce IL-6 or CXCL1 release, thus this synergy would be
interesting to study further as it may hold a clue as to sphingosine’s mechanism of action.
However, the significance of this effect is masked by the variability of the data. Thus, this
would need repeating to confirm the effect and whether it is worth investigating further.
Having determined that ATP, MSU and sphingosine-induced inflammation is unlikely to be related to apoptosis, pyroptosis or necroptosis, it was considered what other proteases may be active during sterile inflammation that could also affect cell death. Caspase and cathepsin B inhibition did not have any protective effect (Figure 4-2A - Figure 4-6A), thus attention was turned to the calpain family of proteases. Calpains are involved in the processing and release of mature IL-1α which, as already discussed, may have an integral role in the early stages of acute inflammation (Luheshi et al., 2011). Moreover calpains increase in activity during excitotoxic conditions such as cerebral ischaemia (Yamashima, 2000). With this increase in activity it is considered that calpains drive both apoptotic and necrotic cell death (Chan and Mattson, 1999, Malagelada et al., 2005, Yamashima et al., 2003).

Calpain inhibition in this case did not reduce cell death induced by ATP, MSU or sphingosine in unprimed or primed cells indicating that DAMPs are not inducing cell death via calpain activation (Figure 4-9A and Figure 4-10A). Calpain inhibition did not affect IL-6 or CXCL1 production from ATP, MSU and sphingosine treated unprimed cells (Figure 4-9B and Figure 4-9C). This was not due to calpain not being effective as IL-1α processing and release was inhibited as shown by western blot for IL-1α (Figure 4-10Cii). Three distinct bands of interest are shown on this western blot. The 31kDa pro-IL-1α band was not affected by the presence of calpain. A 17kDa shows whether or not pro-IL-1α was cleaved to mature IL-1α and the presence of calpain inhibitors decreases the size of this band showing inhibition of IL-1α processing. This is a clearer distinction of inhibition of processing than can be seen on the ELISA as, unlike the IL-1β ELISA, the IL-1α ELISA is not specific to mature IL-1α and thus also picks up pro-IL-1α released by dying cells (Figure 4-10Ci). A 19kDa band shows non-specific protease cleavage of IL-1α in the
supernatants. This was affected by calpain presumably because in the presence of calpain inhibition there is more pro-IL-1α available for alternate processing.

IL-1β processing was not affected by calpain inhibition (Figure 4-10B). As calpains can cause lysosomal rupture and release of lysosomal proteases, given more time, it would be interesting to look at whether calpain affects lysosomal cathepsin B release and whether this may be an IL-1β-independent mechanism of inflammation.

4.4.3. Alternative signalling pathways in NLRP3-activating DAMP-induced inflammation

Having established that the NLRP3-activating DAMP-induced inflammation is not reliant on any form of programmed cell death, alternative signalling pathways were considered. Both the Syk pathway and autophagy have been implicated in the control of IL-1β (Harris et al., 2011, Mitroulis et al., 2011, Mocsai et al., 2010, Shi et al., 2012). MSU induces a potent inflammatory response independent of NLRP3 signalling and potentially dependent on the Syk pathway in order to activate dendritic cells (Ng et al., 2008). Moreover autophagy is important in the clearance of cell debris after necroptotic cell death in the ischaemic penumbra, though induction of autophagy is not reliant solely on necroptosis and thus could be regulated independently of necroptosis by circulating DAMPs (Degterev et al., 2005).

It was observed here that inhibition of Syk using piceatannol and autophagy using 3-MA, had no effect on cell death in unprimed or primed cells (Figure 4-11A - Figure 4-14A). Interestingly, MSU did not induce an increase in IL-6 or CXCL1 in the presence of both piceatannol and 3-MA (Figure 4-11B and C; Figure 4-13B and C). Requirement of Syk in autophagic regulation of fungal infection in macrophages has recently been suggested (Ma et al., 2012), thus despite the variability of the data masking this result from
being a significant inhibition it would be very interesting to investigate further in the context of converging Syk and autophagic signalling pathways.

MSU and sphingosine did not induce the same extent of IL-1β and IL-1α processing in the presence of piceatannol. Again the significance of this effect is masked by the variability of the data and would thus need further investigation (Figure 4-12Bi and Ci), however western blot shows a decrease in the mature 17kDa form of both cytokines confirming the likelihood of the effect (Figure 4-12Bi and Ci). A similar trend in IL-1β and IL-1α processing is seen in response to MSU in the presence of 3-MA lending further strength to the idea of observing convergence of these two pathways. However first, this decrease in IL-1β and IL-1α processing would need confirming by western blot.

Furthermore, though 3-MA is widely used in the literature as an inhibitor of autophagy (Mitroulis et al., 2010), it is important to consider that its effects are specific to type III PI3K activation and therefore may affect other pathways. Use of the mTOR inhibitor rapamycin which is known to induce autophagy (Jones et al., 2013) could be used in future to see the converse effect and thus confirm the specificity of any effect as being autophagic.

Piceatannol and 3-MA had no effect on ATP induced inflammation in unprimed or primed cells (Figure 4-11 - Figure 4-14), and 3-MA did not effect sphingosine induced IL-1 processing as measured by ELISA (Figure 4-14B and C). Thus the convergence of Syk and autophagy signalling may only apply to particulate lysosomal DAMPs. It would be interesting to observe the effects of piceatannol on the abundance of light chain 3 phosphatidylethanolamine, a marker of autophagic activity (Tanida et al., 2008), in response to MSU, to see if loss of Syk signalling effects autophagosome formation and MSU induced inflammation.
To conclude, NLRP3-activating DAMP-induced inflammation does not appear to be NLRP3-dependent and it is likely that any cell death caused during this inflammation is necrotic cell death and not programmed cell death. Of great interest now is the alternative pathways through which these DAMPs could be acting and the relevance of Syk signalling and autophagy in MSU-induced inflammation.
5.1. Introduction

Inflammation in the absence of infection (sterile inflammation) is a major contributor to the pathogenesis of many acute and degenerative disease conditions. In the brain, collateral damage caused by chronic inflammation can have devastating consequences. Cerebral ischaemia is a sterile inflammatory condition of the brain. It is one of the major causes of death worldwide and the leading cause of disability. As such, better treatment after cerebral ischaemia could have major health and economic implications (Denes et al., 2011c). In order to improve treatment of cerebral ischaemia, it is important to better understand the molecular mechanisms that play a role in driving inflammatory damage. Thus understanding how injured tissue communicates with the immune system to drive an inflammatory response and cause further damage in the brain is fundamental.

During the primary injury DAMPs released from necrotic tissue initiate an inflammatory response from glia, the immune cells of the brain. The pro-inflammatory family of cytokines IL-1 contribute to this response and studies targeting IL-1α and IL-1β show promise in reducing injury after experimental stroke in rodents (section 1.2.2.1). IL-1β release is controlled by caspase-1 activity which in itself is controlled by an intracellular multi-molecular platform termed ‘the inflammasome’. Of the known inflammasomes, NLRP3 is thought to recognise the presence of many known DAMPs. In order to initiate an IL-1 response, extensive studies show that cells must be primed. In vitro this is typically achieved using the PAMP LPS, however, in vivo the endogenous priming stimulus remains unclear. Some NLRP3-activating DAMPs have also been shown to act independently of NLRP3 to initiate a non-IL-1-driven inflammatory response in peripheral macrophages in the absence of any priming (Bouchard et al., 2002, Bulanova et al., 2005, Guerne et al., 1989, Kool et al., 2011, Lopez-Castejon et al., 2010, Ng et al., 2008, Solini...
et al., 1999). The effects of NLRP3-activating DAMPs on unprimed glia have never been investigated and yet may be an important and underestimated component of the inflammatory response.

This thesis sought to determine the following:

1) The pro-inflammatory effects of NLRP3-activating DAMPs on glia in the absence of priming
2) Potential endogenous priming mechanisms
3) Mechanisms of cell death associated with NLRP3-activating DAMP-induced inflammation

The key findings of this thesis are summarised in Figure 5-1 in which I have tried to illustrate the potential interactions and mechanisms that may occur in an ischaemic brain. NLRP3-activating DAMPs initiate a restricted IL-1 independent inflammatory response in glia in the absence of priming (section 3.3.1). What appears to be necrotic cell death caused by NLRP3-activating DAMPs may increase the abundance of DAMPs and could cause a positive inflammatory feedback via viable cells (section 4.3.3) (Figure 5-1B). Cytokines and chemokines released as part of the pro-inflammatory response could act on glia to further the glial inflammatory response and initiate a peripheral inflammatory response (Figure 5-1C) whilst proteases released may act to damage the BBB (Figure 5-1D). Circulating mediators increase in the periphery and may infiltrate the BBB to prime glia (section 3.3.2) (Figure 5-1E), or may prime circulating peripheral leukocytes allowing for a peripheral IL-1 response (Figure 5-1F). Neither cell death nor the inflammation induced here seem to be dependent on NLRP3 (section 4.3.1).

Data presented in this thesis has been discussed in detail at the end of each results chapter. Here, the relevance of this data will be discussed in context of how it aids our understanding of how NLRP3-activating DAMPs contribute to inflammation in the brain.
Figure 5-1: Hypothetical model of pro-inflammatory effects of NLRP3-activating DAMPs on mixed glia

Injury in the brain results in release of DAMPs from necrotic cells (A). NLRP3-activating DAMPs cause further necrotic cell death which contributes to the persisting presence of DAMPs that continue to drive inflammation (B). NLRP3-activating DAMPs also act on unprimed glia inducing the release of the acute phase cytokine IL-6, the neutrophil chemoattractant CXCL1, and proteases such as cathepsin B and MMP9. Glial derived cytokines have a positive feedback effect on themselves (Ci) and also dissipate into the periphery (Cii). This may cause a peripheral inflammatory response. Proteases may cause BBB breakdown (D). Acute phase proteins increase rapidly in the circulation and may infiltrate the brain parenchyma via areas of BBB breakdown (E). On doing so, they have the potential to prime glia allowing the release of IL-1 in response to circulating DAMPs. Circulating acute phase proteins may prime peripheral leukocytes allowing an increase in circulating IL-1β (F).
5.2. **NLRP3-activating DAMPs initiate a restricted inflammatory response in the absence of priming**

Inflammation is recognised to contribute to the worsening of acute brain injury, and inhibition of IL-1 with its antagonist IL-1Ra is protective in experimental models of stroke (Brough et al., 2011) and has shown promise in clinical trials (Emsley et al., 2005). DAMPs are present in abundance after ischaemic injury and may mediate local inflammatory responses. DAMPs are most widely reported for their actions on the NLRP3 inflammasome, the formation of which leads to the activation of caspase-1 and subsequent cleavage and release of active IL-1β (Cassel and Sutterwala, 2010). *In vitro* this process requires the use of a priming stimulus to increase IL-1β and NLRP3 expression (Bauernfeind et al., 2009, Hornung and Latz, 2010a). Typically a PAMP such as LPS is used to achieve this. *In vivo* it is unlikely a PAMP would be present in the brain to prime cells, thus the response of glia to NLRP3-activating DAMPs in the absence of PAMP priming is equally important to understand.

This thesis contains the first study of the effect of NLRP3-activating DAMPs on glia in the absence of a priming stimulus. Despite having no effect on the expression of IL-1α or β or the cell surface phenotype of glia, the DAMPs ATP, MSU and CPPD all induced an increase in the release of the cytokine IL-6, the chemokine CXCL1, and the protease cathepsin B (section 3.3.1). The increase in IL-6 and CXCL1 occur independent of NLRP3 (Figure 4-1, section 4.3). Additionally ATP induced a substantial increase in the release of active MMP9 (Figure 3-10, section 3.3). These findings suggest NLRP3-activating DAMPs can also induce an IL-1 independent inflammatory response from glia in the absence of a priming stimulus.

IL-6 and CXCL1 are known to be downstream mediators of IL-1 induced inflammation (Calkins et al., 2002, Miller et al., 1997). It has previously been shown that
administration of systemic IL-1β acts synergistically with MCAo to induce a significant increase in circulating cytokines and chemokines, in particular in chemokines associated with neutrophil mobilisation such as CXCL1 (McColl et al., 2007). It is also known that administration of IL-1Ra in acute stroke patients attenuates circulating levels of IL-6, CXCL1 and the acute phase reactant CRP (Emsley et al., 2005). Furthermore it is shown here that the presence of IL-1 augments the IL6 and CXCL1 response after MCAo (Figure 3-11, section 3.3.2). However, to date there is little evidence of the presence of IL-1β at the protein level in the brain early enough to influence ischaemic brain damage (Luheshi et al., 2011), and the mechanism by which plasma levels of IL-1β increase is not known. This work suggests DAMPs released by necrotic cells within the brain induce low levels of IL-6 and CXCL1 and it can be hypothesised that they could cross the BBB to initiate a peripheral immune response.

IL-6 in the periphery increases the expression and release of acute phase proteins from the liver (Castell et al., 1989, Jensen and Whitehead, 1998, Schultz and Arnold, 1990). Among these acute phase proteins is serum amyloid A which increases significantly in the plasma immediately after a stroke (McColl et al., 2007). Work here shows SAA can prime glia for a NLRP3-dependent IL-1β response (Figure 3-13, section 3.3.2), and this may also be true for circulating leukocytes, though this remains to be tested. Thus IL-6 released from the brain may be involved in the priming of peripheral leukocytes for an IL-1β response (Figure 5-1Cii).

CXCL1 is a major chemokine that specifically mobilises and directs neutrophil migration (Lira et al., 1994). As neutrophils transmigrate across the cerebral endothelium, they develop a neurotoxic phenotype (Allen et al., 2012). Activated neutrophils produce more cytokines, chemokines, proteases and reactive oxygen species that can further contribute to lesion volume and neurological deficit (McColl et al., 2007, Wright et al.,
Active MMP9 is also associated with stroke and neutrophil extravasation into the parenchyma. It does so by contributing to activation of endothelial cells and breakdown of the BBB (Candelario-Jalil et al., 2009, Denes et al., 2010b, Wang et al., 2007). As well as allowing neutrophils into the parenchyma, this breakdown also allows inflammatory mediators to enter, and further leakage of inflammatory mediators and DAMPs from the parenchyma into the periphery. Therefore low level CXCL1 release from glia in response to DAMPs and ATP-induced increase in active MMP9 as seen here may initiate a neutrophil response in the periphery and contribute to degradation of the BBB (Figure 5-1Cii and D). It could also be hypothesised that this BBB breakdown allows circulating acute phase proteins such as SAA to access the brain thereby providing a priming stimulus for glia at the site of inflammation, and subsequent IL-1β release upon encountering circulating NLRP3-activating DAMPs (Figure 3-15, section 3.3.2; Figure 5-1E).

Cathepsin B is one of several lysosomal proteases that is active cytosolically and promotes cell degradation (Boya et al., 2003). It has been associated with the activation of pro-inflammatory caspases in the early stages of cerebral ischaemia in mice, and cathepsin B specific inhibition is protective in rats after experimental stroke (Benchoua et al., 2004, Seyfried et al., 2001). Cathepsin B activity has also been shown to be integral to activation of the NLRP3 inflammasome in macrophages and monocytes (Niemi et al., 2011). Its strong association with NLRP3 and caspase activation indicate cathepsin B to be upstream of IL-1β activity in the brain. Furthermore the data here supports this evidence in that ATP, MSU and CPPD all induced an increase in cathepsin B yet not IL-1β in the absence of priming (Figure 3-10, section 3.3.1). This could present cathepsin B as an attractive therapeutic target for reducing the eventual effects of IL-1β in the brain. However, it is unlikely cathepsin B is solely responsible for the IL-1β response as cathepsin B inhibitors
only partially impair NLRP3 activation and some NLRP3 agonists are still able to trigger IL-1β release in cathepsin B deficient macrophages (Dostert et al., 2009, Hornung et al., 2008). It is just one of many cathepsins involved in inflammation thus it may be that they work in concert with one another to drive inflammation (Repnik et al., 2012). Data shown here indicates cathepsin B inhibition is not protective against NLRP3-activating DAMP-induced cell death (Figure 4-2, section 4.3.2) further supporting the theory that cathepsin B is not solely responsible for NLRP3-activating DAMP-induced effects. Thus the importance of cathepsin B during cerebral ischaemia requires further investigation.

Further work to test the hypotheses discussed above is required and discussed in due course (section 5.7). However, this work indicates that NLRP3-activating DAMP-induced responses in unprimed glia could play an integral role in the initiation of inflammation during sterile injury in the brain and that may be relevant to other inflammatory conditions.

5.3. The role of priming in acute brain injury

Despite the ability of NLRP3-activating DAMPs to initiate a NLRP3 and IL-1 independent inflammatory response, IL-1 still has an integral role in the progression of inflammation after acute brain injury as shown here (Figure 3-11, section 3.3.2) and in the literature (Brough et al., 2011, Emsley et al., 2005). Priming is not only essential for the expression of pro-IL-1β, but also for the expression of NLRP3 (Anderson et al., 2008, Bauernfeind et al., 2009). As already mentioned above, it is plausible with breakdown of the BBB that circulating acute phase proteins such as SAA could access the parenchyma, and it is shown here that SAA is capable of priming glia for an IL-1β response in the same manner as LPS (Figure 3-13 and Figure 3-15, section 3.3.2). Whilst further investigation into the temporal profile of SAA production after stroke and its role in priming of glia in
vivo may be of interest, it must be considered that this is not the only acute phase protein of interest, nor the only endogenous inflammatory mediator with priming capacity.

CRP is very similar to SAA in that it is an acute phase protein that increases from trace amounts up to 1000-fold following inflammatory insults such as cerebral ischaemia (Schultz and Arnold, 1990). The kinetics of CRP production are closely related to the inflammatory state of the body and as such it is frequently used clinically in humans as an indicator of inflammatory distress (Van Leeuwen and Van Rijswijk, 1994). Much like SAA, CRP synthesis in the liver is influenced primarily by IL-6, though other cytokines can also induce its synthesis. The effects of increased CRP are multifaceted and primarily revolve around the induction of innate host defence mechanisms (Schultz and Arnold, 1990). Thus it would not be surprising if CRP were to have similar effects on glia should it cross the blood brain barrier, and it may also be another protein responsible for priming of peripheral leukocytes.

Acute phase proteins are not the only potential candidates for cell priming. In the brain after necrotic cell death, there is a plethora of DAMPs that have the potential to prime cells. Though we have seen here that some DAMPs that act specifically on NLRP3 cannot prime cells, many can act via TLRs and thus may affect signalling mechanisms known to control pro-inflammatory gene expression (section 1.2.1.2). HMGB1 has been well-researched in the literature. It is pro-inflammatory when released from necrotic but not apoptotic cells (Scaffidi et al., 2002). There is an increase in NFκB activity and pro-inflammatory gene expression, including IL-1β, after exposure of human monocytes, macrophages and neutrophils to HMGB1 (Andersson et al., 2000, Park et al., 2003). Initially research showed this was dependent on RAGE activating NFκB pathways via redox-dependent mechanisms (Sims et al., 2010, Tian et al., 2007, Lander et al., 1997). However, TLR2 and 4 have also been shown to be important in HMGB1 activation of
macrophages (Park et al., 2004, Yu et al., 2006). The magnitude and kinetics of the pro-inflammatory cytokine response to HMGB1 has been compared to the stereotypical priming stimulus LPS, with HMGB1 being suggested as a key candidate for endogenous priming. Despite this evidence, HMGB1 is yet to be demonstrated as a clear candidate for priming of cells in the brain after cellular necrosis.

Much like HMGB1, the S100 family of DAMPs can also act via RAGE to promote inflammation (Hofmann et al., 1999). Specifically, comparisons have been drawn between S100b and acute phase proteins (Sen and Belli, 2007). Basal S100b has a physiological role in the brain acting as a neurotrophic factor and aiding neurone survival. However, S100b levels rapidly increase in pathological conditions and may contribute to neuroinflammation (Griffin et al., 1995, Mrak and Griffinbc, 2001, Van Eldik and Wainwright, 2003). The effect of S100b on glia in the absence of priming was tested here and did not induce IL-1β or IL-1α (data not shown). Thus although evidence from the literature may seem promising, on initial observation S100b does not appear to have endogenous priming properties that mimic LPS.

These are but a few of the potential endogenous priming stimuli. However, given the number of them and the number of putative receptors on which each can act, it is unlikely that specifically inhibiting any single one of these or their receptor would be of therapeutic benefit. However, the work done here shows for the first time that peripheral inflammatory mediators have the capacity to prime glia should they access the brain and thus demonstrates that even in the absence of systemic infection, the systemic inflammatory state could influence inflammatory events in the brain. This supports evidence that patients with systemic inflammation are more susceptible to worsened outcome after stroke (McColl et al., 2007, Meisel et al., 2005). Far less is known about the role of systemic inflammation in the progression of CNS disease, however it has become
an area of increasing interest (Murray et al., 2013, Denes et al., 2011a, Denes et al., 2010b) and this work further highlights its importance. Future therapeutic strategies should perhaps consider targeting systemic inflammation, but more work is needed to confirm this and is discussed further in section 5.7.

5.4. NLRP3-activating DAMP-initiated cell death is necrotic

Prolonged cell death after initial injury is a hallmark of sterile inflammation and not only contributes to sustained inflammation, but also to a devastating loss of function. Thus understanding the mechanism of cell death within the ischaemic penumbra could prove therapeutically important. Whilst cell death in the core is widely accepted to be necrotic, several theories have emerged in the literature as to mechanisms of programmed cell death that follow within the penumbra.

Initial evidence suggested a role for apoptosis in penumbral cell death. Apoptosis occurs in isolated neurones in the brain under physiological conditions as part of synaptic plasticity and development, and is non-inflammatory (Becker and Bonni, 2004, Chan and Mattson, 1999). Apoptotic neurones are engulfed by neighbouring microglia before they undergo secondary necrosis and become inflammatory. However, neurones in the ischaemic cortex undergo death characteristic of apoptosis (Linnik et al., 1993), and caspase-3, -8 and -9, key effectors of apoptosis, are upregulated in the penumbra after stroke along with proinflammatory caspase-1 (Ferrer and Planas, 2003). Overexpression of anti-apoptotic Bcl-2 displays reduced neuronal damage after ischaemic injury (Kitagawa et al., 1998, Linnik et al., 1995), and the use of caspase inhibitors within 6h of reperfusion significantly reduced infarct size (Endres et al., 1998, Hara et al., 1997).

However, conflicting evidence as to the efficacy of inhibiting apoptosis after cerebral ischaemia (Oppenheim et al., 2001, West et al., 2006), combined with evidence of new forms of programmed necrotic cell death (Rosenbaum et al., 2010, Wu et al., 2012,
Kitanaka and Kuchino, 1999) led to investigation into alternative methods of programmed cell death within the penumbra. Necroptosis is RIP1 kinase dependent cell death that has the characteristics of necrosis and occurs when caspase-dependent cell death is blocked (Cho et al., 2009, Holler et al., 2000). It has also been implicated in penumbral cell death after stroke, with inhibition of RIP1 kinase with necrostatin-1 conferring protection after ischaemic brain injury (Degterev et al., 2005).

As already mentioned, caspase-1 is upregulated in the penumbra after stroke. Though caspase-1 is mostly associated with the processing and release of IL-1β during cerebral ischaemia, it plays an integral role mediating over 100 other processes including pyroptosis, a host defence mechanism against invading pathogens (Bergsbaken et al., 2009, Denes et al., 2011b). As caspase-1 KO animals show neuroprotection after cerebral ischaemia compared to the WT phenotype, it is plausible pyroptosis could have a role host defence during ischaemic cell death in the penumbra. However this is yet to be reported in the literature and the work here does not support this hypothesis with regards to NLRP-activating DAMP induced cell death (section 4.3.3).

It is now accepted that a variety of different mechanisms result in cell death and damage in the penumbra after stroke (Zille et al., 2012). The data observed here indicates that NLRP3-activating DAMP-induced cell death is necrotic cell death and does not occur via any of the mechanisms of programmed cell death discussed above (section 4.3.3). This indicates that while pro-inflammatory mediators released as a consequence of exposure to DAMPs may go on to trigger programmed cell death within the penumbra as suggested in the literature; it is unlikely this programmed cell death is induced directly by these DAMPs regardless of the primed state of the cells on which they act. Thus, much like it would be hard to target one particular DAMP to prevent the release of pro-inflammatory mediators,
it is unlikely that the cell death seen here is preventable. Rather, it is the consequences of that cell death that should be the focus of future research.

5.5. **Syk and PI3K in particulate NLRP3 activation**

Syk is a tyrosine kinase that is recruited to the plasma membrane and activated in response to the engagement of cell surface receptors containing cytoplasmic immunoreceptor tyrosine-based activation motifs (ITAMs). These include the classical immunoreceptors, T cell receptors, B cell receptors and Fc receptors, all of which are involved in the adaptive immune response (Mocsai et al., 2010). The Syk signalling pathway is not, however, isolated to cells involved in adaptive immunity. Syk can mediate diverse biological functions, but of particular relevance here is the role of Syk in NLRP3 activation (Mocsai et al., 2010). Syk is integral to IL-1β expression and NLRP3 activation in anti-fungal immunity (Gross et al., 2009). Moreover, NLRP3 is activated in a Syk-dependent manner in response to malarial hemozoin. Malarial hemozoin is the crystalline by-product of haemoglobin digestion produced during malarial infection. This Syk-dependent hemozoin activation of NLRP3 may involve the release of uric acid (Shio et al., 2009, Griffith et al., 2009). Thus although the majority of the literature has focused on Syk in terms of infectious immunity, it is plausible that Syk also has a role in sterile inflammation.

Syk is integral to the phagocytosis of particulate matter, as is PI3K, a family of kinases activated downstream of Syk (Crowley et al., 1997, Greenberg and Grinstein, 2002). This has been demonstrated using MSU crystals. There is some evidence that MSU binds the CD16/CD32 complex leading to neutrophil activation (Barabe et al., 1998). However, receptor recognition is not necessary as recruitment of signalling molecules containing ITAMs to where the particle comes into contact with the cell can also occur (Ng
et al., 2008). Syk is recruited to the ITAMs and subsequently phosphorylated by Src family kinases, generating PI3K mediated phagocytosis of the particle (Shi et al., 2010).

Limited literature shows both Syk and PI3K are integral to MSU-activation of the NLRP3-inflammasome (Desaulniers et al., 2001). The work in this thesis shows for the first time that Syk and PI3K have a role in NLRP3 activation in response to MSU in glia. Though further work is needed to confirm these results, in the presence of piceatannol (Syk inhibitor) or 3-MA (PI3K inhibitor), MSU did not induce IL-1β release in LPS primed glia (Figure 4-12 and Figure 4-14, section 4.3.4). This supports evidence for the role of Syk and PI3K in MSU activation of NLRP3, though it is likely Syk and PI3K are responsible for the phagocytosis of MSU crystals and thus all their subsequent cell activating effects. It can also be seen here that this response is specific to the particulate DAMP MSU, as the same effect is not observed in response to sphingosine, a non-particulate lysosomal DAMP (Figure 4-12 and Figure 4-14, section 4.3.4). This further supports the theory that Syk and PI3K are responsible for MSU phagocytosis. Together with the literature, this provides strong evidence that Syk and PI3K should be considered further with regards to sterile particulate activation of NLRP3 and the IL-1β inflammatory response.

5.6. Experimental design

One of the key features of experimental design that must be considered throughout this piece of work is the use of primary mixed glial cultures and the variability of the data that comes with them. As can be seen by the characterisation of the cultures (Figure 3-16, section 3.3.3), the ratio of microglia to astrocytes varied to a high degree between cultures. It is known that even the smallest variation in percentage of microglia can have a huge effect on the response seen from the culture (Losciuto et al., 2012). This is reflected in the variability of the data throughout leading to the loss of statistical significance in some potentially very interesting results (Figure 4-12 and Figure 4-14, section 4.3.4). To correct
for this, the ‘n’ number could be increased to observe the significance of these effects. However, the use of primary mixed glial cultures also provided other limitations to the study. The amount of RNA that could be collected for qPCR measurements was hindered by using mixed glial cultures thereby restricting the number of genes that could be observed in each experimental set. The use of a mix of microglia and astrocytes also means that the effects seen cannot be attributed to a specific cell type, and any compensatory effects that the cells have on one another is unknown. The literature indicates that astrocytes may have a dampening effect on microglial inflammation (Guo and Bhat, 2006, Singh et al., 2011) thus, if the two cell types were separated, it would enable the individual cell type responses to be isolated.

To compensate for this it is possible to use either cell lines or purified cell cultures to investigate the response of microglia and astrocytes to NLRP3-activating DAMPs separately. It is possible to purify primary astrocytes and microglial cells by shaking microglial cells from the culture (Giulian and Baker, 1986). As the astrocytes form a confluent layer within the culture plate (Figure 3-16, section 3.3.3), they are more abundant than the microglia that are obtained using this technique. For this reason it may be better to use the murine microglial cell line, BV-2, when characterising the response specifically of microglia. This is a well-established cell line (Blasi et al., 1990) that provides a confluent layer of microglia and therefore would yield better RNA and protein levels. Astrocyte cell lines are less commonly used throughout the literature. Though the use of cell lines would likely increase reproducibility of the data and therefore decrease the variation, there is a preference to use primary cells where possible as they are more representative of the in vivo model. Moreover, though using a mixed glial culture limits the determination of specific glial subset contribution to the response to DAMPs, it is
perhaps a more representative model of the in vivo state where both cell types would be present.

Apparent from the work done in this thesis is the suggestion that priming plays a big role in the inflammatory response of glia after acute brain injury. It is shown that IL-1 contributes to the inflammatory response in the brain after MCAo (Figure 3-11, section 3.3.2), and that NLRP3-activating DAMPs are unable to elicit this response in the absence of a priming stimulus (Figure 3-3, section 3.3.1). Whilst potential alternative mediators that could provide this endogenous priming mechanism in the brain after injury have been discussed, it should also be considered that this may simply be a disparity between in vitro and in vivo models and that the primed state of the glia is lost during the cell culture process. This must be carefully considered in the future planning of experiments into endogenous priming mechanisms.

5.7. Future directions

While this thesis sheds some light on the role of NLRP3-activating DAMPs in the initiation of inflammation during acute brain injury, further investigation is required in order to establish the biological importance of these effects in host defence responses to sterile injury.

5.7.1. Establishing the contribution of NLRP3-activating DAMP-induced inflammation on BBB breakdown and leukocyte recruitment

Previous work on breakdown of the BBB has focused on glial and neuronal recognition of infection and subsequent activation of the endothelium using the TLR4 agonist LPS. This endothelial cell activation is measured by the increase in ICAM and VCAM expression and neutrophil transmigration (Leow-Dyke et al., 2012, Sumi et al., 2010). However there is less work investigating the mechanism by which DAMPs
communicate with the endothelium to promote inflammation after acute brain injury. Evidence here indicates NLRP3-activating DAMPs can act on glia to induce the release of inflammatory mediators that have been shown throughout the literature to promote endothelial cell activation, breakdown of the BBB and neutrophil transmigration into the parenchyma (Candelario-Jalil et al., 2009, Denes et al., 2010b, Wang et al., 2007, Lira et al., 1994, McColl et al., 2007). Neutrophils are activated as they transmigrate and subsequently exert cytotoxic effects within the parenchyma (Allen et al., 2012, Wright et al., 2010). The release of these mediators in response to NLRP3-activating DAMPs could therefore be integral to the initiation of subsequent inflammatory responses.

Preliminary work shows that the DAMPs ATP, MSU and CPPD do not activate mouse brain endothelial cells, as observed by ICAM and VCAM expression after 4h stimulation (data not shown). Furthermore after 24h treatment, neuronal cultures had no IL-6 or CXCL1 response to ATP, no IL-6 response to MSU or CPPD, and at least a 10-fold smaller CXCL1 response to MSU and CPPD (data not shown). The cathepsin and MMP9 responses have not yet been tested, however this initial data indicates glia are required to augment the inflammatory response to NLRP3-activating DAMPs.

Therefore, the hypothesis could be tested that unprimed glia release mediators in response to NLRP3-activating DAMPs, that in turn activate the endothelium and promote neutrophil transmigration. This could be investigated using glial and endothelial cell cocultures in which the endothelial cells are cultured in transwell inserts (Sumi et al., 2010) to observe their response to glial activity induced by NLRP3-activating DAMPs. Of particular interest would be the response to ATP due to the large MMP9 response observed during this thesis (Figure 3-10, section 3.3.1.4). Observation of endothelial cell activation would dictate the value of subsequent investigation into the effect of this glial inflammatory response on neutrophil transmigration using an in vitro neutrophil trans-
endothelial migration assay as seen previously (Leow-Dyke et al., 2012). Given the strong association of neutrophil transmigration with cytotoxic consequences (Wright et al., 2010), these may help to determine the importance of the IL-1β-independent inflammation observed in this thesis (section 3.3.1).

5.7.2. Establishing the contribution of peripheral inflammatory mediators to acute inflammation in the brain

Preliminary data here indicates that peripheral inflammatory mediators could affect inflammation in the brain after acute brain injury (section 3.3.2.2). This is supported by an increasing wealth of research into the contribution of peripheral inflammation to acute CNS injury. Systemic infection is associated with an increased risk of stroke (Grau et al., 1995, Smeeth et al., 2004) and patients presenting with infection have a worsened clinical outcome after stroke (Palasik et al., 2005, Perry, 2004). On top of this, non-infectious peripheral inflammation caused by co-morbidities such as atherosclerosis, diabetes and obesity has been shown to have an effect of the inflammatory state of the brain making patients more susceptible to stroke (Drake et al., 2011, Murray et al., 2013).

As shown by Drake et al. (2011), comorbidities increase inflammation in the brain prior to acute brain injury. This can be seen by increased endothelial cell activation in the microvessels supplying blood to the brain and increased microglial activation within the brain. It is known that accompanying systemic inflammation is the increase in acute phase proteins, in particular SAA and CRP which can increase up to 1000-fold (Schultz and Arnold, 1990) and on accessing the brain SAA has the potential to prime glia (section 3.3.2.2). Therefore the hypothesis could be tested that acute phase proteins upregulated during systemic inflammation can induce a “primed” brain pathology. In vivo studies looking at the effect of systemic SAA and/or CRP injection on brain inflammatory markers would indicate whether or not these acute phase proteins can access the brain and play a
role in the pathology of brain inflammation; or whether they are simply acute phase by-products of other inflammatory mediators present during systemic inflammation. Assessing the contribution of acute phase proteins in this way, may help progress our understanding of the mechanisms by which systemic inflammation contributes to inflammation in the CNS.

5.7.3. Establishing the role of the inflammasome in cerebral ischaemia

It is known that targeting caspase-1 or IL-1α and β confers protection after experimental stroke (section 1.2.2.1) (Boutin et al., 2001, Emsley et al., 2005, Loddick et al., 1996, Loddick and Rothwell, 1996, Mulcahy et al., 2003, Rabuffetti et al., 2000, Schielke et al., 1998) and that the inflammasome is integral to caspase-1 activation and IL-1β processing and release (section 1.2.2.2) (Schroder and Tschopp, 2010). However the differential role of the inflammasome in cerebral ischaemia has not been investigated and may provide important insights into the mechanisms of IL-1-driven inflammation in the brain after stroke. Though NLRP3 is established as the major receptor for sterile inflammation (Cassel and Sutterwala, 2010), it is yet to be shown the extent to which it contributes directly to damage in the brain during cerebral ischaemia. We have shown here that some of the pro-inflammatory effects of NLRP3-activating DAMPs are NLRP3-independent (Figure 4-1, section 4.3.1) and that, consistent with the literature, priming is required in order for NLRP3 to contribute to inflammation (section 3.3.2) (Hornung and Latz, 2010a). However, as discussed, it is still unclear as to what the source of this endogenous priming is.

The AIM2 inflammasome is known to recognise cytoplasmic DNA (Schroder et al., 2009) however its role in sterile inflammation is yet to be well-established. A common component required for activation of both of these inflammasomes is ASC. Thus, to distinguish the role of the inflammasome to damage in the brain and loss of function after
cerebral ischaemia, mice with an ASC KO phenotype could be compared to mice with WT phenotype after experimental stoke. This would indicate if ASC-dependent inflammasomes were important in damage during cerebral ischaemia. Depending on the results from this, mice with either an NLRP3 KO or AIM2 KO phenotype could also be used. Investigation into the role of NLRP3 in cerebral ischaemia would also help to clarify the importance of the results shown in this thesis as it would highlight the importance of NLRP3-independent pro-inflammatory responses to NLRP3-activating DAMPs, and thus the importance of investigating the pro-inflammatory effects of these DAMPs in a NLRP3-free environment.

5.8. Summary

Sterile inflammation contributes to worsened outcome after acute brain injury such as stroke and IL-1 is known to be a key contributor to this inflammation. This thesis provides support for the hypothesis that NLRP3-activating DAMPs released from necrotic cells after acute brain injury contribute to this inflammation. It shows that these DAMPs have IL-1 independent inflammatory effects in the absence of priming. In addition it shows that IL-1 augments this inflammation in primed systems and that systemic serum amyloid A may act as an endogenous priming molecule. Investigation into the biological importance of these IL-1 independent inflammatory effects and the specific contribution of acute phase proteins to inflammatory brain pathology, could further our understanding of how acute brain injury and systemic inflammation coalesce to augment the inflammatory response and thus aid the identification of novel therapeutic targets.
Publications

Papers


Abstracts


Savage, C.D., Denes, A., Lopez-Castejon, G., Brough, D. Understanding how injured tissue communicates with the immune system. Poster presentation, Life Sciences (2011)

Savage, C.D., Denes, A., Lopez-Castejon, G., Brough, D. Understanding how injured tissue communicates with the immune system. Poster presentation, NRI showcase (2011)

Savage, C.D., Denes, A., Lopez-Castejon, G., Brough, D. Understanding how injured tissue communicates with the immune system. Poster presentation, International Cytokine Society on the IL-1 family of cytokines (2011)

Savage, C.D., Denes, A., Lopez-Castejon, G., Brough, D. Understanding how injured tissue communicates with the immune system. Poster presentation, Manchester Neuroscience Symposium (2011)


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