Effects of oxidative stress on expression and activation of the NLRP3 inflammasome in primary human monocytes

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Abstract

Interleukin-1β (IL-1β) is a proinflammatory cytokine and an important component of innate immunity. However, overproduction of IL-1β is implicated in a number of autoinflammatory diseases. The discovery of the nucleotide binding oligomerisation domain (NOD)-like receptor containing a pyrin domain 3 (NLRP3) inflammasome provided crucial insight into how IL-1β processing is regulated, but despite decades of research the exact mechanisms by which the inflammasome can be activated are not fully understood. One hypothesis is that increases in reactive oxygen species (ROS), as occurs during oxidative stress, leads to activation of the inflammasome but the available data are contradictory. The association between inflammation and oxidative stress is well-known and it is possible that the NLRP3 inflammasome could function as an important mechanistic link between ROS, oxidative stress and inflammatory disease.

The aim of this study was to investigate the function of ROS in NLRP3 activation in primary human monocytes – an innate immune cell that extravasates into inflamed tissues and secretes IL-1β, thus playing a significant role in innate immunity, inflammation and disease.

ROS production was measured in primary human monocytes that were exposed to monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD) crystals together with toll-like receptor 2 (TLR2) ligand Pam3Csk4. Both type of crystal induced NLRP3-dependent IL-1β secretion, but only MSU induced significant increases in ROS, suggesting that the production of ROS is not necessary for inflammasome activation. These findings were subsequently confirmed using small molecule antioxidants and antioxidant enzymes which inhibited ROS production but had no effect on MSU-induced IL-1β secretion.

The effects of more subtle changes in redox homeostasis on the activation of NLRP3 were then assessed. Uric acid is one of the major antioxidants in the blood and when present at high concentrations is the greatest risk factor for the development of gout – an inflammatory disease driven by MSU-induced IL-1β secretion. Uric acid had no effect on activation of the inflammasome nor did it influence gene expression of inflammasome components in human monocytes. Although differences were observed in antioxidant capacity in sera and monocytes from individuals with gout, chronic kidney disease and rheumatoid arthritis, there were no correlations between intra- or extracellular redox state and IL-1β secretion from monocytes, suggesting that inflammasome activation is redox-independent.
The results of this work demonstrate that activation of NLRP3 in primary human monocytes is not affected by ROS and thus the inflammasome is unlikely to be redox-regulated in these cells. This is an important finding for understanding the complex relationship between inflammation and oxidative stress, and indicates that this relationship is not always causal in nature in human monocytic cells.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>βMe</td>
<td>Beta mercaptoethanol</td>
</tr>
<tr>
<td>4-HNE</td>
<td>4-hydroxynonenal</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>8-hydroxydeoxyguanosine</td>
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<tr>
<td>AIM2</td>
<td>Absent in melanoma</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>AP-1</td>
<td>Activating protein 1</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APE1/Ref1</td>
<td>AP endonuclease 1/redox factor 1</td>
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<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing a caspase recruitment domain</td>
</tr>
<tr>
<td>ASK</td>
<td>Apoptosis signal regulated kinase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>B2M</td>
<td>Beta-2 Microglobulin</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow derived macrophage</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>CAPS</td>
<td>Cryopyrin-associated periodic syndromes</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation antigen</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic granulomatous disease</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
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<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
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<tr>
<td>CPPD</td>
<td>Calcium pyrophosphate dehydrate</td>
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<td>CREB</td>
<td>Cyclic adenosine monophosphate response element-binding protein</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CTB</td>
<td>CellTitre Blue™</td>
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<tr>
<td>CV</td>
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<tr>
<td>Cyclo-dA</td>
<td>8,5’-cyclo-2’-deoxyadenosine</td>
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<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
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<tr>
<td>DCF</td>
<td>Dichlorofluorescin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>DCFDA</td>
<td>Dichlorofluorescin diacetate</td>
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<tr>
<td>DMARD</td>
<td>Disease-modifying antirheumatic drug</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>DPI</td>
<td>Diphenyliodonium</td>
</tr>
<tr>
<td>dsDNA</td>
<td>Double stranded DNA</td>
</tr>
<tr>
<td>ECSOD</td>
<td>Extracellular superoxide dismutase</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinases</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FCAS</td>
<td>Familial cold auto-inflammatory syndrome</td>
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<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
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<tr>
<td>GOI</td>
<td>Gene of interest</td>
</tr>
<tr>
<td>Gpx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione reduced</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione oxidised</td>
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<tr>
<td>H2O2</td>
<td>Hydrogen peroxide</td>
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<tr>
<td>HBD</td>
<td>Heparin binding domain</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
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<td>HGMB1</td>
<td>High mobility group box 1</td>
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<tr>
<td>HPRT1</td>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intracellular cell adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin-1 receptor</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor associated kinase</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus activated kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun amino (N)-terminal kinases</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LAL</td>
<td>Limulus amebocyte lysate</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoproteins</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>LRR</td>
<td>Leucine rich repeat</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MSU</td>
<td>Monosodium urate</td>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>MWS</td>
<td>Muckle-Wells syndrome</td>
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<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response 88</td>
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<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
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<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphatase</td>
</tr>
<tr>
<td>NaS</td>
<td>Sodium selenite</td>
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<tr>
<td>NBT</td>
<td>Nitroblue Tetrazolium</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide binding oligomerisation domain like receptor</td>
</tr>
<tr>
<td>NLRA</td>
<td>NLR containing an acidic domain</td>
</tr>
<tr>
<td>NLRC</td>
<td>NLR containing a CARD</td>
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<tr>
<td>NLRP</td>
<td>NLR containing a pyrin domain</td>
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<tr>
<td>NLRX</td>
<td>NLR with no homology to the N-terminal domain of any other NLR subfamily</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide binding oligomerisation domain</td>
</tr>
<tr>
<td>NOMID</td>
<td>Neonatal-onset multisystem inflammatory disorder</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2-related factor</td>
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<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
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<tr>
<td>Pam3</td>
<td>Pam3Csk4</td>
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<tr>
<td>PAMP</td>
<td>Pattern-associated molecular pattern</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-T</td>
<td>PBS-Tween</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
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<td>PGH2</td>
<td>Prostaglandin H2</td>
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<td>PGK1</td>
<td>Phosphoglycerate kinase 1</td>
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<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
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<td>PPIA</td>
<td>Peptidylprolyl isomerase A</td>
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<td>PRR</td>
<td>Pattern recognition receptor</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>Prx</td>
<td>Peroxiredoxin</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin domain</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>Rac2</td>
<td>Ras-related C3 botulinum toxin substrate 2</td>
</tr>
<tr>
<td>RG</td>
<td>Reference gene</td>
</tr>
<tr>
<td>RIG</td>
<td>Retinoic acid-inducible gene</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RIPK</td>
<td>Receptor-interacting protein kinase</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-I-like receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPL32</td>
<td>Ribosomal protein L32</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rosewell Park memorial institute medium</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SECIS</td>
<td>Selenocysteine insertion sequence</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TAC</td>
<td>Total antioxidant capacity</td>
</tr>
<tr>
<td>TAK</td>
<td>Transforming growth factor beta-activated kinase</td>
</tr>
<tr>
<td>TBHP</td>
<td>Tetra-Butyl hydroperoxide</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR adaptor proteins</td>
</tr>
<tr>
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<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<tr>
<td>TNFR</td>
<td>TNF receptor</td>
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<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
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<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
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<tr>
<td>TRIF</td>
<td>TIR-domain-containing adaptor-inducing IFN-β (TRIF)</td>
</tr>
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<td>TRX</td>
<td>Thioredoxin</td>
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<tr>
<td>TrxR</td>
<td>Thioredoxin reductase</td>
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<tr>
<td>TT</td>
<td>Transient transfection</td>
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<tr>
<td>v/v</td>
<td>Volume per volume</td>
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<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>XO</td>
<td>Xanthine oxidase</td>
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Declaration

I declare that research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree

Signed

Ben Alberts

28/02/2019
Chapter 1 - Introduction
1.1 The innate immune system

Innate immunity is the first line of defence against pathogens. It encompasses physical and anatomical barriers, myeloid and lymphoid immune cells, and humoral components. Physical barriers are comprised of non-haematopoietic cells and include epithelial cells of the skin and the mucosal membranes which protect body cavities from invading pathogens (Turvey and Broide 2010). When physical barriers are breached, innate immune cells of haematopoietic origin recognise and eliminate the invading pathogen. Recognition of molecules that are common to pathogens but absent in the host by innate immune cells is the first step in activating the immune system. The recognition of these molecules results in the initiation of two innate immune responses: phagocytosis and inflammation. These responses are rapid, occurring very soon after recognition of foreign molecules by immune cells (Brubaker et al. 2015).

1.1.1 Innate immune cells

The innate immune system comprises a range of myeloid and lymphoid cells which are derived from haematopoietic stem cells. Many of these leukocytes are phagocytic cells which recognise, engulf and kill foreign bodies via multiple cytotoxic mechanisms. Phagocytes, including monocytes, neutrophils and macrophages, all phagocytose and eliminate the threat using a range of intracellular mechanisms, such as acid hydrolysis or generation of highly toxic reactive oxygen species (ROS) during the respiratory burst (discussed below) (Turvey and Broide 2010; Koenderman et al. 2014).

Monocytes constitute around 10-15% of the leukocyte population (Geissmann et al. 2010) and can be grouped into three subsets according to expression of membrane receptor cluster of differentiation antigen 14 (CD14) which is a lipopolysaccharide (LPS) co-receptor, and CD16 which is an Fc receptor. Classical monocytes have high expression of CD14 and no CD16 (CD14++CD16-), non-classical monocytes express low levels of CD14 and high levels of CD16 (CD14+CD16++), and the intermediate monocyte phenotype has high expression of CD14 and low expression of CD16 (CD14++CD16+) (Ziegler-Heitbrock et al. 2010). Classical monocytes constitute around 90% of the circulating monocyte population and express high levels of chemokine receptors which mediate monocyte extravasation when ligated by a chemokine (Sandblad et al. 2015). Their main function is to recognise and phagocytose particles bearing damage associated molecular patterns (DAMPs) and pathogen associated molecular patterns (PAMPs), and are the main producers of pro-inflammatory cytokines out of the three monocyte subsets (Boyette et al. 2017). Intermediate monocytes express lower levels of chemokine receptors, and cytokine...
secretion from these cells lies in between that of classical and non-classical monocytes (Boyette et al. 2017). Non-classical monocytes are found in the endothelium and respond to DNA and RNA particles, but are weakest producers of cytokines out of the three subsets (Cros et al. 2010). During innate immune activation, monocytes are attracted to the area of infection by chemokines, undergoing extravasation through the endothelial wall. Monocyte recruitment during infection is rapid, occurring within one hour of infection (Italiani and Boraschi 2014). Monocytes are a fundamental cell-type in the immune response, being one of the first cells targeted to the affected area where they orchestrate further immune responses.

Macrophages play an important role in maintaining tissue homeostasis aiding in both the removal of harmful stimuli as well as tissue repair. They are also antigen presenting cells, which present antigenic peptides from pathogens on cell surface proteins such as major histocompatibility complexes (MHCs). These antigen containing MHCs are then recognised by innate and adaptive immune cells, resulting in further activation of the immune response (Blum et al. 2013). Macrophages reside in all tissues and are classified into two subsets: the classically activated M1 macrophages and the alternatively activated M2 macrophages (Mills et al. 2000). The differentiation of macrophages into these subsets is mediated by signalling molecules such as cytokines and growth factors, which induce intracellular signalling pathways that facilitate differentiation towards the M1 or M2 phenotype (Labonte et al. 2014). M1 macrophages are induced during host invasion by pathogens, becoming activated in response to PAMPs, DAMPs, and inflammatory cytokines. The recognition and subsequent phagocytosis of the pathogen or harmful molecule results in the release of proinflammatory cytokines which further enhance immune responses. M2 macrophages are generally considered anti-inflammatory and mediate wound healing, tissue fibrosis and angiogenesis by secreting anti-inflammatory cytokines as well as growth and survival factors (Fujiwara and Kobayashi 2005; Martinez and Gordon 2014; Hirayama et al. 2017). Macrophages play an important role in both the exacerbation of immune responses as well as mediating immune resolution and repair, making them a fundamental cell-type in the innate immune response.

1.1.2 Pattern recognition receptors
A fundamental function of innate immunity is to recognise threats to the host, which can include pathogens from microbial origin, as well as non-pathogenic molecules released during tissue damage (Janeway and Medzhitov 2002). Recognition of these molecules is facilitated by germline-encoded pattern recognition receptors (PRRs), which recognise a number of evolutionary conserved sequences that are unique to microbes. PRRs detect
PAMPs such as LPS found on the outer membrane of gram-negative bacteria, bacterial flagellin, and nucleic acids such as RNA unique to bacteria or viruses. They also detect molecules associated with tissue damage or cell stress, detecting DAMPs such as uric acid, adenosine triphosphate (ATP) and high mobility group box 1 (HGMB1) protein (Takeuchi and Akira 2010). Numerous classes of PRR families have been identified, including toll-like receptors (TLRs), C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and nucleotide binding oligomerisation domain (NOD)-like receptors (NLRs) (Brubaker et al. 2015). The PRRs provide recognition of a range of different threats in distinct compartments of the cell, providing all round protection.

TLRs are the most abundant PRR and are expressed in all leukocytes. The human TLR family consists of 10 members, with some localised to the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10) and others present intracellularly (TLR3, TLR7, TLR8, and TLR9). The cell-surface TLRs largely recognise microbial membrane components, including LPS on the surface of gram-negative bacteria by TLR4 (Hirschfeld et al. 2000), flagellin by TLR5 (Hayashi et al. 2001), diacyl lipopeptides originating from mycoplasma by TLR6 (Kawasaki and Kawai 2014), and influenza A viruses by TLR10 (Lee et al. 2014). TLR2 forms heterodimers with TLR1 or TLR6, and recognises a range of PAMPs originating from various microbes including gram-negative bacteria and fungi such as peptidoglycans and lipoteichoic acid (Oliveira-Nascimento et al. 2012). Intracellular TLRs recognised PAMPs and DAMPs that enter the cell, being activated by bacterial and virus derived nucleic acids, such as double-stranded RNA in the case of TLR3 (Botos et al. 2009), viral single stranded RNA by TLR7 and TLR8 (Mancuso et al. 2009) and non-methylated DNA motifs by TLR9 (Kumagai et al. 2008).

In addition to the TLRs, NLRs are PRRs which reside in the cytosol and protect the host following internalisation of a microbe. NOD1 and NOD2 are two prototypical members of the NLR family and detect components of bacterial cell walls. Following activation, NODs associate with receptor-interacting protein kinase (RIPK)-2 to initiate activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways (see section 1.2.1). In addition to the NODs, some NLRs contain a pyrin domain at their N-terminus and are components of multimeric complexes called inflammasomes, which become activated upon recognition of DAMPs or PAMPs (Kim et al. 2016).

The inflammasomes
The term ‘inflammasome’ was coined in 2002 to describe a group of high molecular weight complexes which form in the cytosol of stimulated immune cells (Martinon et al. 2002). The
NLR family is characterised by the presence of a central nucleotide-binding and oligomerisation (NACHT) domain and a C-terminal leucine rich repeat (LRR). The members of the NLR family are distinguished according to the effector domains at the N-terminus: NLR containing an acidic domain, NLRA; NLR containing a baculoviral inhibitory repeat domain, NLRPB; NLR containing a caspase recruitment domain (CARD), NLRC; NLR containing a pyrin domain (PYD), NLRP; and NLR with no homology to the N-terminal domain of any other NLR subfamily (NLRX) (Ting et al. 2008). Inflammasome assembly is driven by recognition of PAMPs or DAMPs resulting in activation of the inflammasome sensor molecule, which recruits an adaptor protein known as apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC). ASC is comprised of a PYD and CARD, which link the inflammasome sensor molecule to pro-caspase-1 (Latz et al. 2013). Caspase-1 is part of a family of cysteine proteases which play an important role in inflammation and programmed cell death (Sollberger et al. 2014). It is transcribed as an inactive precursor, pro-caspase-1, that is autoproteolytically cleaved following recruitment into the inflammasome complex. Cleavage of pro-caspase-1 results in the release of mature caspase-1 into the cytosol of the cell, allowing it to proteolytically cleave the proinflammatory cytokines pro-IL-1β and pro-IL-18 (Broz and Dixit 2016).

Since the initial description of the inflammasome, an array of inflammasome sensor molecules have been described, with 22 human NLRs having been identified (Bryant and Fitzgerald 2009; Amin et al. 2017). Of the inflammasomes, NLRP1, NLRC4, NLRP3 and Absent in Melonoma 2 (AIM2) are most well-characterised. Like the TLRs, the NLRs are activated by a range of specific stimuli. NLRP1 was the first described and recognises microbial stimuli, being activated by anthrax lethal toxin from *Bacillus anthracis* (Martinon et al. 2002), as well as muramyl dipeptide, a bacterial peptidoglycan (Faustin et al. 2007; Chavarria-Smith and Vance 2015). The NLRC4 inflammasome provides host defence against a range of microbes and microbial molecules, including *Salmonella typhimurium* (Mariathasan et al. 2004) and multiple components of bacterial type III secretion systems (Miao et al. 2010). AIM2 was the first non-NLR inflammasome described and is a nucleic acid sensor which forms an inflammasome in response to double-stranded DNA (dsDNA) from both bacterial and viral origin (Fernandes-Alnemri et al. 2010; Rathinam et al. 2010). The NLRP3 inflammasome is the most well-characterised inflammasome to date and is activated by a range of structurally diverse PAMPs and DAMPs (Schroder and Tschopp 2010). The mechanisms involved in NLRP3 activation are a particular focus of this study and will be discussed in detail in section 1.4.
1.1.3 Cytokines

Activation of PRRs results in secretion of a range of soluble proteins called cytokines that play a fundamental role in host defence. Cytokines are synthesised and secreted by nearly all nucleated cells, including monocytes, macrophages, neutrophils, B cells and T cells (Akdis et al. 2016). They have a range of immune functions, including being pro- and anti-inflammatory, activating adaptive immune responses, and can also function as growth factors mediating cell proliferation and differentiation. Upon their release from activated cells, cytokines orchestrate immune responses by activating cytokine receptors on local cells and triggering intracellular signalling pathways (discussed below). Cytokines can act in an autocrine manner, acting on the cell that secreted them, or a paracrine manner, acting on nearby cells, and in some cases can exert endocrine effects acting on distant cells, although they generally function locally in the area of secretion (Zhang and An 2007; Turner et al. 2014). Cytokines are indispensable components of innate immunity and many different families of cytokines exist, including the interleukins and TNF.

Interleukins (ILs) are cytokines that induce signal transduction pathways involved in both pro- and anti-inflammatory responses. They were first described in 1977 following the discovery of IL-1 and there are now more than 60 cytokines designated as interleukins which are assigned to different IL families based on sequence homology and function (Dinarello et al. 1977; Akdis et al. 2016). The IL-1 family was first described as a human leukocytic pyrogen that was comprised of 2 major proteins; IL-1α and IL-1β (Dinarello et al. 1977). The human IL-1 family is now comprised of 11 members of which seven are pro-inflammatory (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ), three are antagonists for IL-1 cytokines which limit the effect of the cytokines (IL-1 receptor (IL-1R) antagonist (IL-1Ra), IL-36Ra and IL-38) and one is anti-inflammatory (IL-37) (Weber et al. 2010). IL-1Rs can be found on the membrane of most immune cells, including monocytes, neutrophils, T lymphocytes and B lymphocytes, and receptor ligation results in activation of the NF-κB and MAPK signalling cascades (discussed below), which promote inflammatory responses (Turner et al. 2014). IL-1 cytokines are produced by an array of immune cells and play an essential role in inflammation and immunity via activation of these intracellular signalling pathways.

TNF (also known as TNFα) is a pro-inflammatory cytokine first described in 1975 and is a central component of immunity and inflammation (Carswell et al. 1975). Secreted from activated macrophages, monocytes, natural killer (NK) cells and fibroblasts among others, TNF is initially produced as a transmembrane precursor which requires cleavage into its active form by the metalloprotease TNF converting enzyme (Mohan et al. 2002). TNF ligates
TNF receptors 1 and 2 resulting in downstream activation of NF-κB and MAPKs and mediating immune responses against bacterial, viral and parasitic infections (Sedger and McDermott 2014). In addition to its role in host defence, TNF has also been described to play an important homeostatic role, aiding in tissue regeneration, macrophage desensitisation and suppression of adaptive immune system during autoinflammatory disease (Kalliolias and Ivashkiv 2016).

A multitude of other cytokines also exist including: growth factors such as G-colony stimulating factors G-CSF, GM-CSF and M-CSF which initiate the generation of granulocytes and monocytes; chemokines which direct leukocytes to the area affected; and interferons which play a role in antiviral responses (Turner et al. 2014). Together, cytokines orchestrate immune responses by promoting inflammation and also coordinate immune resolution and tissue repair.

1.1.4 Reactive oxygen species (ROS)
In addition to PRRs and cytokines, ROS also play an important role in innate immunity. ROS is a collective term used to describe radical and non-radical oxygen species formed by the partial reduction of oxygen (Ray et al. 2012). ROS are produced continuously by living organisms as a by-product of normal aerobic cellular metabolism. They are extremely toxic at high concentrations where they cause damage to DNA, RNA, proteins and lipids. Innate immune phagocytes, including neutrophils, monocytes and macrophages, utilise this toxicity during phagocytosis, producing ROS during the respiratory burst to aid the removal of engulfed substance. However, it is now clear that ROS also play important roles in immune responses in addition to directly killing pathogens, by mediating the activation of PRRs, inducing cell signalling mechanisms and regulating the function of proteins (Yang et al. 2013), making them an import component of innate immunity.

ROS can be categorised into free radical and non-radical species. Free radicals contain an unpaired electron in their atomic outer shell making them unstable and highly reactive, reacting readily with electron donors and acceptors (Cheeseman and Slater 1993). Various species of free radical exist, including the superoxide anion (O$_2$•⁻), hydroxyl radical (OH•), peroxyl radical (ROO•) and alkoxyl radical (RO•). Non-radicals do not contain an unpaired electron and are generally considered more stable than free radicals yet can still be highly toxic. There are also various species of non-radical including hydrogen peroxide (H$_2$O$_2$), singlet molecular oxygen (¹O$_2$), hypochlorous acid (HOCl) and peroxynitrite (OONO⁻) (Sies et al. 2017). The superoxide anion is the primary ROS generated in cells and is a precursor to numerous secondary ROS (Fig. 1.1). Superoxide is formed by the monovalent reduction
of oxygen in the respiratory electron transport chain during aerobic metabolism (Turrens 2003). Superoxide can then be reduced spontaneously or in a process catalysed by superoxide dismutase (SOD) enzymes which results in the formation of H$_2$O$_2$, which in turn can be partially reduced to form the hydroxyl radical via the Fenton reaction (Fukai and Ushio-Fukai 2011). Secondary ROS can also form via the cross-reaction of two oxidants. Superoxide and H$_2$O$_2$, for example, can react to form hydroxyl radicals. Superoxide also reacts with nitric oxide (NO$^-$), a type of reactive nitrogen species, to form peroxynitrite, another highly reactive ROS (Beckman and Koppenol 1996).

The major site for superoxide production is the mitochondrial electron transport chain, with around 1-2% of mitochondrial oxygen consumption resulting in the production of superoxide (Boveris and Chance 1973). During cellular metabolism, the electron transport chain transports electrons from donors to electron acceptors, creating an electrochemical proton gradient that drives the synthesis of ATP (Murphy 2009). This process is not 100% efficient and superoxide can leak from the electron transport chain at two major sites; complex I (NADH dehydrogenase) and complex III (ubiquinone cytochrome c reductase) (Phaniendra et al. 2015). Superoxide is not the only ROS produced in the mitochondria. Mitochondria also contain a form of nitric oxide synthase, which generates nitric oxide, which as mentioned can cross-react with superoxide to form peroxynitrite (Giulivi et al. 1998; Radi et al. 2002). The ROS produced during metabolism need to be maintained at low concentrations to prevent damage to cellular components (discussed in section 1.3.1).

ROS can also be generated by various enzymatic complexes found in different cell types. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a membrane-bound enzyme found in phagocytic cells such as monocytes, macrophages and neutrophils (Cross and Segal 2004). NADPH oxidase is comprised of gp91$^{phox}$, p22$^{phox}$, p40$^{phox}$, p67$^{phox}$, p47$^{phox}$ and Ras-related C3 botulinum toxin substrate 2 (rac2) subunits (Sumimoto et al. 2005). In resting cells, NADPH oxidase exists in an unassembled state with subunits present in both the cell membrane and cytosol (Panday et al. 2015). Upon activation of the cell, the cytosolic subunits translocate to the membrane and assemble the NADPH oxidase complex, which transfers electrons from NADPH to oxygen resulting in the formation of both superoxide and H$_2$O$_2$ (Turrens and Boveris 1980). It is this NADPH oxidase-generated ROS that are utilised by immune cells against invading bacteria and pathogens during the respiratory burst (Segal et al. 2012). The importance of this respiratory burst in the immune response is emphasised by chronic granulomatous disease (CGD) in which there is a mutation in one of the four genes encoding the NADPH oxidase subunits. As a result of this mutation, CGD patients are susceptible to severe bacterial and fungal infections due to the
inability of phagocytes to mount the respiratory burst response against these pathogens (Heyworth et al. 2003).
Figure 1.1 Superoxide acts as a precursor to many other ROS. Following its generation during mitochondrial respiration, superoxide can react with numerous metabolites to form other highly reactive ROS. Cl\textsuperscript{-}, chloride ion; e\textsuperscript{-}, electron; Fe\textsuperscript{2+}, ferrous ion; Gpx, glutathione peroxidase; GSH, glutathione; GSSG, oxidised glutathione; H\textsuperscript{+}, hydrogen proton; NO, nitric oxide; NO\textsubscript{2}\textsuperscript{-}, nitrite; SOD, superoxide dismutase. Schematic adapted from Krumova and Cosa (2016).
1.2 Mechanisms of innate immunity

1.2.1 PRR activation and downstream signalling

PRR recognition of PAMPs and DAMPs results in the activation of intracellular inflammatory signalling pathways which induce the transcription of pro-inflammatory molecules, including cytokines and chemokines. For example, ligation of the TLRs triggers a conserved signalling cascade that results in activation of NF-κB and interferon (IFN)-regulatory factor transcription factors (Kawasaki and Kawai 2014). This process is mediated by various adaptor molecules including myeloid differentiation primary response 88 (MyD88), TIR-domain-containing adaptor-inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM), which function downstream of TLR activation and result in activation of NF-κB and MAPK signalling pathways. Following TLR ligation, MyD88 forms a complex with interleukin-1 receptor-associated kinase (IRAK) family members, which subsequently results in IRAK1 release. IRAK1 in turn associates with TNF receptor associated factor 6 (TRAF6) which promotes polyubiquitination of both TRAF6 itself, and transforming growth factor beta-activated kinase 1 (TAK1). TAK1 activates two different pathways which lead to activation of the NF-κB pathway and the MAPK pathway (Oliveira-Nascimento et al. 2012). An example of this downstream signalling is depicted in figure 1.2.

NF-κB is an inducible transcription factor that induces expression of numerous genes involved in inflammation. There are five members of the NF-κB family, consisting of p50, p52, RelA (also known as p65), RelB, and c-Rel (Oechtinghaus and Ghosh 2009). In resting cells, NF-κB proteins are sequestered in the cytoplasm by a family of inhibitory proteins called IκB, the most studied of which is IκBα. These proteins inhibit NF-κB activity by blocking its nuclear localisation signal, stopping NF-κB from entering the nucleus (Jacobs and Harrison 1998). NF-κB activation requires the inducible degradation of IκBα via site-specific phosphorylation by IκB kinase (IKK) complexes. IKK is itself composed of two catalytic subunits, IKKa and IKKβ, and a regulatory subunit named NF-κB essential modulator (NEMO), otherwise known as IKKγ (Liu et al. 2017). Following its activation downstream of a PRR, IKK phosphorylates IκBα, resulting in proteasomal degradation of IκBα. The release of NF-κB from IκBα exposes its nuclear translocation signal allowing the transcription factor to translocate into the nucleus where it binds to regulatory DNA sequences known as κB sites and initiates gene transcription (Wan and Lenardo 2010). NF-κB is a central regulator of inflammation, and its translocation to the nucleus results in the transcription of numerous inflammatory genes including: cytokines such as interleukin (IL)-1β, IL-6, TNF and IP-10; cell adhesion molecules including intracellular adhesion molecule
ICAM-1 and P-selectin; stress response genes such as angiotensin II and COX-2; as well as regulators of apoptosis and growth factors (Pahl 1999).

In addition to activating NF-κB, PRR activation also results in activation of the MAPK signalling cascade. The activation of MAPK causes a three-kinase cascade with comprises MAPK kinase kinase (MAPKKK) which activates a MAPK kinase (MAPKK or MEK) which in turn phosphorylates one of the MAPKs. MAPKs have been characterised into three defined subgroups: extracellular signal-regulated kinases (ERKs) consisting of ERK-1 and -2, the c-Jun N terminal kinases (JNKs) including the JNK-1, -2 and -3 isoforms, and the p38 MAPKs which include p38α, p38β, p38γ and p38δ isoforms (Cargnello and Roux 2011). ERK is largely activated by growth factors, such as epidermal growth factor (EGF) and its activation results in transcription of several factors genes involved in proliferation, cell survival and differentiation (McCain 2013). JNK and p38α are activated downstream of TLR2 and TLR4 ligation. p38α is required for activation of cyclic adenosine monophosphate response element-binding protein (CREB) and c/EBPβ transcription factors, resulting in induction of several genes encoding for chemokines, cytokines and cell adhesion (Schieven 2009). Conversely, JNK regulates the activity of transcription factor activating protein 1 (AP-1), resulting in expression of pro-inflammatory cytokine TNF (Ip and Davis 1998).

The activation of NF-κB and MAPK signalling pathways downstream of PRR activation leads to the transcription of an array of molecules which mediate immune responses, initiating inflammation via the secretion of pro-inflammatory cytokines, as well as causing phagocytosis of the threat.
Upon dimerisation with TLR1 or TLR6, TLR2 engages with TIR adaptor protein (TIRAP) and myeloid differentiation primary response 88 (MyD88) which stimulates downstream signalling via the interleukin-1 receptor-associated kinases (IRAKs), TNF receptor associated factors (TRAFs) and transforming growth factor beta-activated kinases (TAKs). This subsequently leads to activation of mitogen-activated protein kinases (MAPKs), c-Jun N terminal kinases (JNK) and p38, as well as the IκB kinases (IKKs). This results in downstream activation of the transcription factors CREB, activator protein 1 (AP1), and nuclear factor-κB (NF-κB) which translocate into the nucleus and induce the transcription of pro-inflammatory cytokines. TAB, TAK binding protein. Schematic adapted from O'Neill et al. (2013).
1.2.2 Phagocytosis
Recognition of PAMPs or DAMPs is often rapidly followed by internalisation of the particle or pathogen by phagocytic cells. Phagocytosis begins with the formation of the phagosome which develops when the cell membrane surrounds the pathogen or molecule and fuses behind it, bringing the pathogen, contained within the phagosome, into the cytoplasm. The phagosome subsequently undergoes a series of maturation sequences before fusing with lysosomes to form phagolysosomes (Xu and Ren 2015). Lysosomes deliver hydrolytic and bactericidal components such as lysosyme and acid hydrolases into the phagosome which cause the removal of the engulfed substance by degrading bacterial cell walls and proteins. In addition, the phagocytes also initiate the activation of NADPH oxidase on the membrane of phagosomes which result in the rapid formation of superoxide, HOCl, H$_2$O$_2$ and nitric oxide during the respiratory burst. The entry of these ROS into the phagosome further aids in the removal of the pathogen via reacting with the proteins, DNA and lipids found on the pathogen (Slauch 2011). Phagocytosis is an important mechanism which facilitates the removal of PAMP or DAMP containing substances and also causes the secretion of cytokines from the phagocyte, initiating inflammation.

1.2.3 Inflammation
Inflammation is an essential component of the innate immune response against infection and can also occur in response to changes in tissue homeostasis during injury (Xiao 2017). Inflammation performs various important functions facilitating the migration of immune cells into the affected area which mediate destruction of the source of disturbance, removal of any damaged tissue, and restoration of tissue homeostasis (Medzhitov 2008).

The classical symptoms of inflammation were described as early as 30BC by Celsus and include redness, pain, swelling and heat (Tracy 2006). The response is tightly regulated involving a cascade of immunological and physiological processes orchestrated by cytokines. As discussed, the initial recognition of infection or tissue damage is mediated by tissue resident macrophages, dendritic cells and mast cells, leading to the production of inflammatory mediators. An rapid effect of the secretion of cytokines and chemokines is the vasodilation of blood vessels, allowing plasma proteins and leukocytes, such as neutrophils and monocytes, to enter the area of infection or injury (Pober and Sessa 2014). Cells which migrate to the site intensify the inflammatory response, secreting a wide array of pro-inflammatory molecules (Ashley et al. 2012). Following the successful removal of the pathogen, inflammatory responses enter a resolution and repair phase governed largely by M2 macrophages. This resolution and repair phase involves the secretion of anti-inflammatory cytokines, which block immune cell migration and promote tissue remodelling.
(Sherwood and Toliver-Kinsky 2004; Barton 2008). The resolution of inflammation is critical for limiting any collateral damage caused by inflammation and for restoring tissue homeostasis. If inflammation is not resolved, damage can be caused to host tissue.

In addition to host defence against invading pathogens, inflammation can also occur in the absence of microbial infection. This type of inflammation, known as sterile inflammation, comprises many of the mechanisms involved in microbial induced inflammation, including leukocyte recruitment and the production of pro-inflammatory cytokines and chemokines (Rock et al. 2010). In contrast to microbial induced inflammation, sterile inflammation is initiated by endogenous stimuli which are not always harmful to the host. The most common causes of sterile inflammation are physical trauma and ischemia, during which DAMPs, that are normally maintained intracellularly and thus hidden from the immune system, are released into the extracellular environment where they are recognised by leukocytes (Barton 2008). DAMPs which initiate sterile inflammation include high-mobility group box 1 (HMGB1), heat shock proteins (HSPs), and purine metabolites such as ATP and uric acid among others (Ohashi et al. 2000; Mollen et al. 2006; Tsung et al. 2007). The release of intracellularly stored pro-inflammatory cytokines from necrotic or apoptotic cells can also induce sterile inflammation (Mollen et al. 2006). Sterile inflammation highlights the importance of the innate immune response, not only in providing defence against non-self (i.e. pathogens), but also sensing host-derived danger signals of cellular stress or injury. This inflammatory response to host-derived danger signals actually plays a fundamental role in the resolution and repair of tissues after non-pathogenic injury. IL-1β is a key modulator of sterile inflammation and IL-1β is governed by the inflammasome complexes discussed earlier, of which NLRP3 is the main sensor of sterile stimuli (Chen and Nuñez 2010). The processing of IL-1β by NLRP3 is the main focus of this study and will be discussed in depth in later sections.

Inflammation is therefore an essential element of innate immunity, orchestrating responses to infection and tissue damage. However, dysregulation of inflammation can be detrimental to health and uncontrolled inflammation can become chronic, giving rise to a number of chronic inflammatory diseases including neurodegenerative diseases, such as Alzheimer’s disease and Parkinson’s disease (Amor et al. 2014), cardiovascular diseases (Lopez-Candales et al. 2017), metabolic diseases such as diabetes and gout (Busso and So 2010), and autoimmune diseases such as RA (McInnes and Schett 2011). Chronic inflammation occurs when the inflammatory response becomes prolonged, lasting for months or even years at a time (Schett and Neurath 2018). There are various mechanisms by which inflammation can become chronic, including: failure to eliminate the stimulus of
inflammation as occurs when an infectious agent is resistant to the immune responses; when the immune system becomes sensitised to an ordinary component of the body; and following exposure to low levels of irritants that are not eliminated by immune responses (Straub and Schradin 2016; Schett and Neurath 2018). The dysregulation of the inflammatory response results in continual recruitment of inflammatory cells and secretion of pro-inflammatory cytokines which exacerbate immune responses in a vicious cycle (Tisoncik et al. 2012). The precise mechanisms which give rise to chronic inflammation are not fully understood, but improving our understanding of these events could provide potential therapeutic targets. Cytokines certainly play a role in exacerbating inflammation and targeting specific cytokines in the treatment of inflammatory disease is now an established strategy (Garth et al. 2018; Hausmann 2018). This strategy has developed since the successful use of anti-TNF drugs for the treatment of RA, which was shown to have dramatic anti-inflammatory effects in the 1990s (Williams et al. 1992; Elliott et al. 1994). Evidence of oxidative stress has been reported in many chronic inflammatory diseases, including Alzheimer’s disease (Schrag et al. 2013), diabetes (Keane et al. 2015) and RA (Sarban et al. 2005). Given its involvement in innate immune responses, oxidative stress could provide an important mechanism by which chronic inflammation develops. However, despite the associations between oxidative stress and these diseases, defining an exact causal role is difficult because ROS are both induced by inflammation and also play a role in inducing inflammation.
1.3 Redox regulation of inflammation

Under normal conditions, ROS are maintained at physiologically relevant concentrations by a range of antioxidant systems. The equilibrium between ROS production and removal is known as redox (reduction and oxidation) homeostasis and disruption of this balance can arise from either a reduction in antioxidant capacity or increased generation of ROS beyond the available antioxidant capacity, ultimately resulting in oxidative stress (Ursini et al. 2016). Oxidative stress occurs when the imbalance in redox homeostasis favours the increase in oxidant concentration (Betteridge 2000). The original view of ROS and antioxidants was that ROS were toxic and antioxidant enzymes provided protection against the negative effects of these oxidants. However, it is now appreciated that ROS also play an important role in cell signalling and survival, an understanding which led to the concept of redox regulation as opposed to oxidative stress. Oxidative stress occurs when ROS concentrations are elevated and can result in irreversible damage to cellular components which can ultimately lead to cell death (see section 1.3.2). Redox regulation, on the other hand, involves reversible modifications which play a key role in cell signalling by regulating the activity of proteins, enzymes and signalling molecules (Ghezzi 2005).

ROS can cause both reversible and irreversible modifications to proteins by directly oxidising amino acid residues on the protein. These modifications provide posttranslational regulation of protein function and can inhibit or activate proteins. The oxidation of amino acids can result in conformational changes to the structure of the protein which can have a range of effects, including activating or suppressing the protein. Several oxidative modifications exist, including the nitration of the amino acid tyrosine (Radi 2013), carbonylation of amino acid side chains (Suzuki et al. 2010) and the oxidation of thiols on the amino acid cysteine (Ghezzi et al. 2005). Carbonylation and nitration are both irreversible modifications, whereas thiol oxidation is considered reversible. There are two major reversible thiol modifications which can occur: protein glutathionylation, which involves the formation of disulphide bonds between a cysteine found in a protein and a cysteine found in glutathione (Ghezzi 2005), and disulphide bond formation, which involves the formation of inter- or intra-protein disulphide bonds between two cysteine residues (Cai and Yan 2013). These modifications can cause changes to protein structure resulting in stabilisation or inhibition of the protein, and can even protect the protein from irreversible modifications (Ahmad et al. 2017). The reversibility of these oxidative modifications is mediated by components of the antioxidant system (discussed below), including the glutaredoxins and thioredoxins, which act as reducing agents that reduce oxidised thiols and thus reverse the oxidative modification (Ghezzi 2005). Reversible oxidation can act as
a molecular switch which can regulate protein function. The oxidative modification of signalling molecules and transcription factors is one mechanism by which ROS can influence inflammatory responses.

Oxidative protein modification is a regulatory mechanism which can alter the function of various components of inflammation. ROS can influence cell signalling pathways such as the MAPK cell signalling pathway, which as discussed is a cell signalling pathway involved in inflammation. This occurs via alterations to protein-protein interactions upstream of MAPK. For example, apoptosis signal-regulated kinase 1 (ASK1) is a MAPKKK that regulates JNK and p38 MAPK signalling. Under resting conditions, ASK1 is sequestered by the antioxidant Trx. The oxidation of two cysteine residues in the redox centre of Trx leads to its dissociation from ASK-1, which subsequently activates the signalling molecule and initiates downstream signalling which can ultimately result in the transcription of inflammatory cytokines (Fujino et al. 2007).

ROS can also activate redox-sensitive transcription factors. The crosstalk between ROS and transcription factor NF-κB is now well established, although the relationship is complex. NF-κB can be both activated and inhibited by ROS, with the activity of the transcription factor determined by its redox status in the cytosol and nucleus (Morgan and Liu 2011). For example, oxidation of NF-κB subunits p50 and p65 in the nucleus strongly inhibits their DNA binding capacity, reducing NF-κB-induced gene transcription. This inhibition of p50 and p65 is a reversible oxidative modification, with nuclear redox regulator AP endonuclease 1/redox factor 1 (APE1/Ref-1) reducing the oxidised subunits and restoring their ability to bind DNA (Ando et al. 2008). Conversely, ROS can activate NF-κB by inducing the formation of disulphide bonds in the upstream NF-κB modulator NEMO which leads to activation of IKK complexes (Morgan and Liu 2011). Furthermore, ROS are involved in mediating the activation of NF-κB by cytokines such as IL-1β and TNF modulating the downstream signalling events from the their receptors (Gloire et al. 2006). The complexity of the relationship between NF-κB and ROS is amplified by the fact that NF-κB can lead to the transcription of various pro-oxidants, such as cyclooxygenase-1 which converts arachidonic acid to prostagland H₂ in a process that involves the generation of superoxide (Marnett et al. 1999), as well as various antioxidants including SOD enzymes and thioredoxins (Djavaheri-Mergny et al. 2004).

Through the activation of these signalling pathways and transcription factors, ROS can induce inflammatory responses by inducing the transcription of inflammatory cytokines. They can also regulate cell adhesion molecules (CAMs) which are essential for leukocyte
extravasation from the circulation during inflammatory responses. CAMs including intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM)-1 and E-selectin are all regulated by oxidative stress at the transcriptional level in endothelial cells. H$_2$O$_2$, for example, increases transcription of ICAM-1 in human umbilical vein endothelial cells (HUVECs), which can be reversed by addition of antioxidants such as catalase (Lo et al. 1993). The reversible modification of proteins involved in immune regulation requires ROS at physiologically appropriate levels. When ROS concentration is increased, oxidative stress can occur, resulting in modifications of cellular components that can be detrimental to cells and human health.

### 1.3.1 Antioxidant defences

An antioxidant is defined as a substance which significantly delays, prevents or removes oxidative damage to a target molecule (Halliwell 2007). ROS are maintained at physiologically relevant levels by a multifaceted network of antioxidant systems, which exist both intracellularly and extracellularly, and can be divided into 2 distinct categories: enzymatic antioxidants, including the SODs, glutathione peroxidases (Gpxs), Trxs, peroxiredoxins (Prxs) and catalase; and non-enzymatic antioxidants such as ascorbic acid, vitamin E and uric acid (Birben et al. 2012).

Intracellular SOD enzymes provide the first line of defence against superoxide anions, catalysing its dismutation to H$_2$O$_2$. Three isoforms of SOD exist: cytosolic Cu/ZnSOD (SOD1), mitochondrial MnSOD (SOD2) and extracellular SOD (ECSOD). SODs are oxidoreductase enzymes that catalyse dismutation of superoxide into H$_2$O$_2$ through alternate reduction and reoxidation of the catalytic metal (copper (Cu) or manganese (Mn)) atom at their active site (Fig. 1.3 A). In addition to scavenging superoxide, SODs also block the cross-reaction of superoxide with other ROS, including NO and thus reduce the production of the highly reactive peroxynitrite (Fukai and Ushio-Fukai 2011).

Formation of H$_2$O$_2$ via SOD activity can also be destructive to cellular components and thus cells possess multiple antioxidant enzymes that catalyse reduction of H$_2$O$_2$. The Gpx family of enzymes are selenocysteine-containing proteins that reduce H$_2$O$_2$ to water. Eight Gpx enzymes have been described in mammals, localised in different compartments within the body: Gpx1 in the cell cytosol and mitochondria, Gpx2 in the intestinal epithelium, Gpx3 in extracellular spaces and Gpx4 associated with membranes to provide protection from oxidative challenge. Gpx5 is located in the epididymis, Gpx6 has only been found in humans and Gpx7 and Gpx8 are both located in the endoplasmic reticulum (Brigelius-Flohé and Maiorino 2013). Gpxs 1-4 and 6 are selenoproteins, containing a selenocysteine in their
catalytic core. Gpx activity involves a three-step mechanism whereby the selenol (Se\textsuperscript{-}) becomes oxidised to selenic acid (SeOH) in the presence of H\textsubscript{2}O\textsubscript{2}. Glutathione (GSH) then catalyses the conversion of SeOH to selenadisulfide (Se-SG), producing water. The second GSH reduces the Se-SG back to Se\textsuperscript{-} by thiol disulphide exchange, producing oxidised GSH (GSSG) and a regenerated Gpx enzyme (Fig. 1.3 B).

Catalase is an intracellular antioxidant enzyme localising mainly in peroxisomes and is also found in the cytosol (Sepasi Tehrani and Moosavi-Movahedi 2018). Catalase reduces H\textsubscript{2}O\textsubscript{2} to water and molecular oxygen using the heme iron at its core as an electron donor (Fig. 1.3 C). Like Gpx3 and catalase, the Prxs also reduce H\textsubscript{2}O\textsubscript{2} becoming oxidised as a result (Fig. 1.3 E). Finally, Trx is an oxidoreductase that reduces disulphide bonds or oxidised thiols in proteins using NADPH as an electron donor and Trx reductase (TrxR) as an enzyme to recycle Trx to its reduced form (Fig. 1.3 D) (Lu and Holmgren 2014). Together, the antioxidant enzymes orchestrate the reduction of superoxide and H\textsubscript{2}O\textsubscript{2} to water. This maintains ROS at low levels by blocking the formation of secondary ROS and protecting against oxidative damage that occurs when ROS concentrations are elevated.

In addition to the antioxidant enzymes, there are also non-enzymatic antioxidants that play important roles in redox homeostasis. GSH is the most abundant antioxidant in all cell compartments. GSH carries an active thiol group in the form of a cysteine residue that can be oxidised by H\textsubscript{2}O\textsubscript{2} resulting in the formation of GSSG. GSSG in turn is reduced back into GSH by GSH reductase, which uses NADPH as an electron donor. The GSH/GSSG ratio is commonly used as a determinant of oxidative stress. GSH donates its electron to H\textsubscript{2}O\textsubscript{2} to reduce it to H\textsubscript{2}O and O\textsubscript{2}. GSH also functions as a co-factor for a range of antioxidant enzymes, including the Gpxs (Fig. 1.3) (Lushchak 2012). Finally, vitamins E (\alpha-Tocopherol) and C (ascorbic acid) are also non-enzymatic antioxidants. Vitamin C scavenges oxygen free radicals and is involved in regenerating vitamin E. Vitamin E is found in the cell membrane and donates its electron to the peroxyl radical protecting against lipid peroxidation (Birben et al. 2012).

Together, these antioxidant systems cooperate to maintain oxidants at low concentrations where they function as cell signalling molecules. Any dysfunction of the antioxidant systems can be detrimental, with loss of redox homeostasis resulting in oxidative stress.
Figure 1.3 Catalytic cycles of antioxidant enzymes. (A) Superoxide dismutase (SOD) enzyme activity involves the reduction and reoxidation of the catalytic metal at its active site. Two superoxide anions ($O_2^{-}$) are removed, resulting in the production of hydrogen peroxide ($H_2O_2$) and molecular oxygen ($O_2$). (B) Glutathione peroxidase (Gpx) activity involves a mechanism where selenol (Se^-) becomes oxidised to selenic acid (SeOH) by $H_2O_2$. Glutathione (GSH) then catalyses reduction of SeOH to selenadisulfide (Se-SG) where a second GSH reduces the Se-SG back to Se^- by thiol disulphide exchange. (C) The ferric ion (Fe$^{3+}$) at the core of catalase is oxidised by $H_2O_2$ to an iron intermediate (compound I), which is reduced back to its reduced state by a second $H_2O_2$ molecule. (D) The cysteine at peroxiredoxins (Prx) active core is oxidised by $H_2O_2$ to form sulfenic acid, which is reduced back to cysteine by thioredoxin (Trx). (E) Trx enzymes reduce oxidised proteins to become oxidised themselves. The electrons are then accepted by NADPH in a process catalysed by thioredoxin reductase (TrxR). $H_2O$, water; $OH^-$, hydroxyl radical; NADP+; nicotinamide adenine dinucleotide phosphate; NADPH; reduced NADP+. (Collet and Messens 2010; Fukai and Ushio-Fukai 2011; Brigelius-Flohé and Maiorino 2013; Wu and Reddy 2014; Glorieux and Calderon 2017).
1.3.2 Oxidative stress

Oxidative stress occurs as a result of a disturbance in redox homeostasis that favours the elevation of ROS. This can result from either overproduction of ROS or as a result of reduced antioxidant defences and can be detrimental to the health of the cell, causing irreversible damage to DNA, proteins and lipids (Betteridge 2000).

DNA can be damaged by ROS in several ways. DNA modifications occur via base degradation, single- or double-stranded DNA breaks, purine or pyrimidine modifications, point mutations, deletions or translocations. The promoter region of genes contain regions rich in guanine (G) and cytosine (C) and these GC-rich regions are particularly sensitive to oxidative modification (Cadet and Wagner 2013). The oxidation of the C-8 region of guanine by OH•, for example, results in the formation of 8-hydroxydeoxyguanosine (8-OHdG) which is a major product of oxidative DNA damage that can result in mutations to DNA and can also block the transcription of genes by stopping the binding of transcription factors to DNA (Valavanidis et al. 2009). Additionally, oxidation of adenine leads to formation of 8,5’-cyclo-2’-deoxyadenosine (cyclo-dA) which can also inhibit transcription (Cadet and Wagner 2013; Guerrero et al. 2013). Oxidative DNA damage can result in DNA mutation which is particularly pertinent to carcinogenesis and has also been documented in numerous non-cancerous diseases, such as type II diabetes mellitus, RA, Alzheimer’s disease and Parkinson’s disease (Evans et al. 2004).

As discussed, protein modifications by ROS play an important role in regulation of the innate immune system by altering transcription factor activity and also influencing intracellular signalling pathways. During oxidative stress, ROS can cause the inhibition of proteins by oxidising protein amino acids and by protein fragmentation. H₂O₂ at elevated concentrations, can induce irreversible modifications by oxidising thiolate anions to sulfinic (SO₂H) or sulfonic (SO₃H) species (Schieber and Chandel 2014). Oxidised proteins can also accumulate within cells and can give rise to numerous pathologies, including Alzheimer’s disease and Parkinson’s disease. Misfolded proteins, for example, aggregate when they cannot be removed resulting in the formation of protein aggregates which can initiate inflammation and disease (Dunlop et al. 2009). Oxidative damage can therefore facilitate inflammatory pathologies by inhibiting proteins or causing them to be degraded or become accumulated.

In addition to protein and DNA damage, oxidative stress can cause damage to lipids via lipid peroxidation. This occurs when ROS oxidise lipids containing carbon-carbon double bonds, a process mitigated mainly by hydroxyl radicals. When the lipid peroxidation rate
increases, repair mechanisms can be overwhelmed and cell damage occurs resulting in the initiation of apoptotic and necrotic signalling pathways. Furthermore, lipid peroxidation results in the formation of lipid hydroperoxides including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (Ayala et al. 2014). MDA is highly reactive and its reaction with amino acids residues on other molecules can lead to the formation of protein or DNA adducts. MDA adducts in turn promote intra- or inter-molecule cross-linking which can alter the conformation and the function of the biomolecule. For example, MDA-modified low density lipoproteins (LDLs) have been documented in atherosclerotic plaques and these MDA-modifications have been shown to contribute to the inflammatory response in this pathology by increasing the binding of complement to the LDL (Holvoet et al. 1995; Ayala et al. 2014). 4-HNE is also highly reactive and at low concentrations can regulate transcription factors such as AP-1 and NF-κB. When at elevated concentrations 4-HNE, like MDA, can result in DNA and protein adducts which ultimately result in irreversible cell damage and initiation of programmed cell death or necrosis (Ayala et al. 2014). Lipid peroxidation products, particularly MDA and 4-HNE, are commonly used as biomarkers for disease, having been shown to be increased in various pathologies, including Alzheimer’s disease and cancer (Skoumalova and Hort 2012; Zhong and Yin 2015).

Since the first description of oxidative stress in the 1970 (Paniker et al. 1970), a vast amount of literature has been published associating oxidative stress with the pathophysiology with a range of inflammatory diseases (Pizzino et al. 2017). The ability for ROS to modify proteins, DNA and lipids can ultimately result in cellular senescence, the induction of inflammation and the formation or exacerbation of pathological conditions.

1.3.3 Oxidative stress and inflammatory diseases
The relationship between oxidative stress and inflammatory disease is complex but the two states are closely associated with oxidative stress both inducing inflammation and conversely inflammation inducing ROS production. As discussed, ROS play an important role in innate immunity being utilised in the respiratory burst response against phagocytosed particles, as well as regulating cell signalling. Oxidative stress involves the dysregulation of redox homeostasis, resulting in elevated levels of ROS which cause cellular damage and are commonly associated with disease (Biswas 2016). However, the exact relationship between inflammation and oxidative stress is complex and it is difficult to fully ascertain whether ROS has a causative role or are generated as a by-product of inflammation. An association does not always mean a causative effect and thus it remains that ROS could be produced but not play a particular role in the disease (Ghezzi et al. 2017). Nonetheless,
ROS are known to induce the activation of various inflammatory transcription factors, including NF-κB, inducing the secretion of pro-inflammatory cytokines. This ability for ROS to directly influence inflammatory signalling pathways is a potential mechanism by which oxidative stress can drive pathology (Schett and Neurath 2018). Improving the understanding of the relationship between oxidative stress and inflammation could provide important insight into future therapeutic strategies for a range of chronic inflammatory diseases.

RA is a chronic autoimmune inflammatory disease which results in progressive destruction of articular cartilage and bone, and affects approximately 1% of the world’s population (Gibofsky 2014). The joint damage that characterises RA is the end result of complex autoimmune and inflammatory processes that involve both innate and adaptive immunity. The exact cause of RA remains unknown, but disease pathogenesis can involve a combination of genetic susceptibility and environmental triggers which subsequently result in loss of self-tolerance of proteins that contain citrulline residues. This loss of self-tolerance causes leukocyte infiltration into the synovium, resulting in synovitis and overproduction of pro-inflammatory cytokines which are central to the pathophysiology of disease (McInnes and Schett 2011). The disease is driven by TNF and IL-6, which induce further cytokine production and chemokine expression, driving leukocyte infiltration and also mediate systemic effects such as cognitive dysfunction and anaemia. Current treatment strategies are aimed at inhibiting these cytokines using TNF inhibitors such as etanercept and adalimumab, and IL-6 receptor inhibitors such as tocilizumab, which are commonly used alongside the disease-modifying antirheumatic drug (DMARD) methotrexate. This combination therapy has improved the treatment of RA with around 30-60% of RA patients entering clinical remission when methotrexate is used in combination with anti-TNF or anti-IL-6 drugs (Kaneko 2013; Machado et al. 2013; Tanaka 2016).

In addition to being driven by TNF and IL-6, oxidative stress has been suggested to play a causative role in the pathogenesis of RA (Quinonez-Flores et al. 2016). For example, a reduction in antioxidant capacity has been documented in RA, hinting at a causal link of oxidative stress in disease progression. Sarban et al. (2005) examined the expression of antioxidant enzymes and total antioxidant capacity in RA patients, osteoarthritic patients and healthy controls, finding reduced Gpx3, catalase and total antioxidant capacity in the blood of RA patients. This was confirmed in another study which documented reduced levels of Gpx and SOD in the plasma of RA patients compared to healthy controls (Seven et al. 2008) and increased levels of H$_2$O$_2$, superoxide and oxidative stress markers, including MDA, have also been shown in blood from RA patients (Quinonez-Flores et al. 2016). These
findings suggest that oxidative stress may have a causal role in the development of RA, providing a potential mechanism to target for therapy.

Oxidative stress is also associated with many other diseases. For example, it is known to play a pivotal role in the progression of chronic kidney disease (CKD). ROS play an important role in the regulation of kidney function, which makes the kidney vulnerable to redox imbalances and oxidative stress (Daenen et al. 2018). The presence of ROS can affect renal blood flow and sodium retention, and cause the onset of inflammation (Nistala et al. 2008). Oxidative stress has been associated with the deterioration of renal function, with increased levels of oxidative stress makers and decreased levels of SOD and GSH antioxidants in patients with renal failure (Kotur-Stevuljevic et al. 2013). These findings do not necessarily indicate a causal effect of oxidative stress in CKD pathophysiology, however several studies have shown that adjusting redox homeostasis can lead to improvements in renal injury in animal models (Nishiyama et al. 2004; Nakamura et al. 2011) therefore suggesting that oxidative stress may mediate renal dysfunction.

The association between oxidative stress and inflammatory disease has resulted in a long-standing interest in using antioxidants as treatment. Gene therapy involves the delivery of genes to cells, most commonly using viral vectors that are efficiently taken up by the cell. Once within the cell, the vector integrates with the genome and allows the continuous production of the functioning protein, allowing lifetime expression of the protein. This strategy is particularly aimed at the treatment of diseases driven by reduced function of a particular protein (Gonçalves and Paiva 2017). This strategy has been tested in many animal models to treat arthritis using antioxidant enzymes. For example, ECSOD and catalase genes, transfected into rat synoviocytes and injected into the knee joints of mice with antigen-induced arthritis, caused a significant reduction in joint swelling after 6-13 days, highlighting the potential use of ECSOD and catalase gene therapy as an anti-inflammatory strategy (Dai et al. 2003). In addition, adenovirus mediated gene transfer of ECSOD was shown to reduce arthritis severity and macrophage infiltration in mice with collagen-induced arthritis (Kelkka et al. 2012). The successful treatment of inflammatory disease with gene therapy in mouse models strengthen the idea of a causative role of oxidative stress in disease and suggest that improving antioxidant capacity can have anti-inflammatory and thus therapeutic effects.

The success of antioxidant gene therapy in animal models has resulted in clinical studies examining the use of small molecule antioxidants, such as N-acetyl-L-cysteine (NAC), vitamins E and C, and selenium containing supplements as therapy. A meta-analysis
summarised the outcome of published studies investigating the efficacy of vitamin D as a therapeutic antioxidant and demonstrated a 24% lower risk of developing RA in patients with higher intake of vitamin D than a group of patients who took low levels of the vitamin (Song et al. 2012). Clinical trials have also tested the use of small molecule antioxidants in the treatment of chronic obstructive pulmonary disease (COPD), an inflammatory lung disease. Eight randomised control trials, involving a total of 2,214 patients, demonstrated that oral supplementation with NAC significantly reduced the risk of further COPD exacerbations (Sutherland et al. 2006). However, despite showing some success, the majority of clinical trials in humans have not shown a benefit of antioxidant supplementation (Bjelakovic et al. 2015), with some even showing a negative effect on the health of volunteers given vitamin E as a potential treatment for prostate cancer (Klein et al. 2011).

Despite the mounting evidence that oxidative stress is involved in inflammatory disease, it remains difficult to determine the exact relationship between oxidative stress and inflammation. The two certainly appear to closely associated; each being able to potentiate the other. The ability for ROS to regulate cell signalling pathways and other components of the inflammatory response could be a mechanism by which ROS induces the onset of inflammation and disease. Defining the exact mechanisms by which oxidative stress may drive inflammatory disease is desirable because of the potential use of antioxidants as a therapeutic strategy. One particular component of the inflammatory response which has recently been suggested to be redox-regulated is the NLRP3 inflammasome which processes the proinflammatory cytokine IL-1β and has been implicated in a range of chronic inflammatory diseases (Mangan et al. 2018).
1.4 Role of oxidative stress in secretion of IL-1β

IL-1β is proinflammatory cytokine that plays a key role in the development of acute and chronic inflammatory diseases, as well as autoimmune disorders. IL-1β overproduction occurs in various diseases, including RA, osteoarthritis, neuropathic pain, multiple sclerosis and Alzheimer’s disease (Ren and Torres 2009). Gout is an inflammatory arthritis driven by IL-1β, which is produced in response to uric acid crystals. Gout is characterised by bouts of acute and painful inflammation, which can become chronic if not resolved (Terkeltaub et al. 2009). The successful use of IL-1Ra and IL-1β targeting antibodies for the treatment of numerous inflammatory diseases emphasises the importance of IL-1β in the pathophysiology of acute, chronic and autoimmune inflammatory diseases. Anakinra, for example, is a recombinant form of IL-1Ra which blocks the ligation of the IL-1 receptor (IL-1R) by IL-1β and has been used in the treatment of gout (Ottaviani et al. 2013), and systemic-onset juvenile idiopathic arthritis (Pascual et al. 2005). Rilonacept is a monoclonal antibody which binds IL-1β, preventing its interaction with the IL-1R and has been shown to have success in treatment of patients with cyropyrin-associated periodic syndromes (CAPS) (Hoffman et al. 2012) and gout (Terkeltaub et al. 2009). Finally, canakinumab is a IL-1β neutralising antibody which has been tested for treatment of CAPS, type I diabetes, chronic obstructive pulmonary disease and cardiovascular diseases (Dinarello et al. 2012). Improving the understanding of IL-1β processing and secretion may provide important insight into mechanisms which could be targeted for future therapeutic strategies.

IL-1β is produced following the ligation of numerous PRRs and is transcribed as an inactive precursor, pro-IL-1β. An additional regulatory step is required for secretion of the cytokine. The elucidation of the inflammasome complexes (discussed in section 1.2.2.4) provided vital insight into the mechanisms involved in IL-1β processing and secretion. Of the inflammasomes identified, NLRP3 is the most studied. There is also increasing evidence suggesting that NLRP3 is itself sensitive to changes in redox homeostasis, and thus NLRP3 could provide an important link between redox homeostasis, oxidative stress and inflammatory disease.

1.4.1 Structure and formation of the NLRP3 inflammasome complex
The NLRP3 inflammasome is a tripartite structure consisting of NLRP3, ASC and pro-caspase-1. The NLRP3 protein consists of an N-terminal PYD and a C-terminal CARD which flank a central nucleotide binding domain. ASC links NLRP3 and pro-caspase-1 via their PYD and CARD domains (Fig. 1.4 A). A two-step nucleation-polymerisation mechanism for inflammasome formation has been described. Firstly, NLRP3 nucleates the
PYD filaments of ASC, resulting in the interaction with the PYD domain from NLRP3. Secondly, ASC nucleates the CARD filaments of pro-caspase-1 recruiting it into the inflammasome (Lu et al. 2014). The aggregation of pro-caspase-1 into the inflammasome complex greatly increases its local concentration and initiates proximity-induced autoproteolytic cleavage of pro-caspase-1 (Lu and Wu 2015).

When activated, the inflammasome complex forms a large complex of around 700 kilodalton (kDa) in the cell cytosol (Martinon et al. 2004). Multiple models for the shape of the NLRP3 inflammasome have been hypothesised. The most commonly used in schematic representations is the spoke-wheel model where caspase-1 constitutes the centre of wheel with peripheral NLRP3 proteins (Fig. 1.4 B). A branching tree model has also been hypothesised where NLRP3 makes up the base of an ASC trunk, and caspase-1 associates as branches coming off the trunk (Bae and Park 2011; Elliott and Sutterwala 2015). Interestingly, Man et al. (2014) imaged the ASC speck formed upon inflammasome activation using confocal microscopy and demonstrated an external ASC layer surrounding the NLR proteins, which in turn surround the effector caspases. This study demonstrated that multiple NLRs can form within the same ASC speck, showing NLRP3 and NLRC4, both activated by Salmonella enterica Serovar Typhimurium (S Typhimurium), to position together within the same macromolecular complex. This structure, with ASC at the periphery of the complex, allows dynamic recruitment and easy movement of speck constituents allowing the inflammasome to remain functionally active, with NLRP3 joining the speck at a later time point than NLRC4 (Man et al. 2014) (Fig. 1.4 C).

The NLRP3 inflammasome is activated by a wide array of structurally diverse NLRP3 activators including viral RNA, components of microbial cell walls, nucleic acid, pore-forming toxins, crystalline structures like silica and asbestos, as well as endogenous danger signals such as ATP, serum amyloid A and uric acid crystals (table 1.1). The diversity of these PAMPs and DAMPs indicate that NLRP3 is not likely to be activated by direct interaction with these stimuli, instead NLRP3 is proposed to sense a common cellular event such as dysregulation of cellular homeostasis (Liston and Masters 2017). Canonical NLRP3 activation is now widely accepted to involve two distinct steps. First, an initial priming signal is provided which licences NLRP3 activation by inducing transcription of genes encoding both the NLRP3 protein and pro-IL-1β (Bauernfeind et al. 2009). A second signal, provided by one of the activators is then required to induce formation of the NLRP3 inflammasome complex. The exact mechanism by which NLRP3 is indirectly activated by its many stimuli remains elusive, but three intracellular signalling events have been proposed: lysosomal degradation, potassium efflux, and generation of ROS.
Figure 1.4 Oligomerisation of the NLRP3 Inflammasome. (A) The NLRP3 inflammasome consists of three separate proteins: the NLRP3 adaptor protein, apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC)-1 and pro-caspase-1. Upon inflammasome activation, the inactive NLRP3 adaptor protein associates with ASC through PYD-PYD interactions. Pro-caspase-1 is subsequently recruited into the inflammasome structure via CARD-CARD interactions. The increased localised concentration of pro-caspase-1 results in its autoproteolytic cleavage, resulting the release of active caspase-1. (B) The traditional spoked-wheel model for inflammasome structure where NLRP3 surrounds the central ASC speck which links NLRP3 to pro-caspase-1. (C) Another hypothesised structure for NLRP3 is the layered speck where ASC speck surrounds molecules of NLRP3 and pro-caspase-1. CARD, caspase recruitment domain; LRR, leucine rich repeat; NLRP3, nucleotide binding oligomerisation domain (NOD)-like receptor (NLR) containing a pyrin domain 3; PYD, pyrin domain. Schematic adapted from Elliot & Sutterwala (2015) and Man et al. (2014).
<table>
<thead>
<tr>
<th>Sterile activators</th>
<th>Associated disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyloid-β Plaques</td>
<td>Alzheimer's Disease</td>
<td>Halle et al. (2008)</td>
</tr>
<tr>
<td>Cholesterol crystals</td>
<td>Atherosclerosis</td>
<td>Duewell et al. (2010)</td>
</tr>
<tr>
<td>Calcium pyrophosphate dihydrate (CPPD) Crystals</td>
<td>Pseudogout</td>
<td>Martinon et al. (2006)</td>
</tr>
<tr>
<td>Extracellular ATP</td>
<td>Tissue damage</td>
<td>Ferrari et al. (2006)</td>
</tr>
<tr>
<td>Islet amyloid polypeptide</td>
<td>Type 2 diabetes</td>
<td>Masters et al. (2010)</td>
</tr>
<tr>
<td>Monosodium urate (MSU) Crystals</td>
<td>Gout</td>
<td>Martinon et al. (2006)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Environmental activators</th>
<th>Associated disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminium salt crystals</td>
<td>N/A</td>
<td>Hornung et al. (2008)</td>
</tr>
<tr>
<td>Asbestos</td>
<td>Asbestosis</td>
<td>Dostert et al. (2008)</td>
</tr>
<tr>
<td>Silica</td>
<td>Silicosis</td>
<td>Cassel et al. (2008)</td>
</tr>
<tr>
<td>UV radiation</td>
<td>Sunburn</td>
<td>Feldmeyer et al. (2007)</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>Viral activators</th>
<th>Associated disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Herpes simplex virus 1</em></td>
<td>Herpes</td>
<td>Johnson et al. (2013)</td>
</tr>
<tr>
<td><em>Influenza A</em></td>
<td>Influenza</td>
<td>Ichinohe et al. (2010)</td>
</tr>
<tr>
<td><em>Human rhinovirus</em></td>
<td>Rhinovirus</td>
<td>Triantafilou et al. (2013)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bacterial activators</th>
<th>Associated disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>Listeriosis</td>
<td>Kim et al. (2010)</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>Tuberculosis</td>
<td>Dorhoi et al. (2012)</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>Acute rheumatic fever, scarlet fever, pharyngitis</td>
<td>Harder et al. (2009)</td>
</tr>
</tbody>
</table>

Table 1.1 Non-exhaustive list of NLRP3 activators and their associated diseases.
The formation of the NLRP3 inflammasome ultimately results in the activation of caspase-1, which in turn catalyses the cleavage of pro-IL-1β and pro-IL-18 in to their active forms which are secreted from the cell. However, unlike other cytokines, members of the IL-1 family lack a signal sequence and are therefore not secreted via conventional mechanisms involving the endoplasmic reticulum and Golgi-compartment (Rubartelli et al. 1990). Numerous mechanisms by which mature IL-1β release occurs have been postulated over the years including transport across cell membranes via specific membrane receptors, and via release in vesicles following microvesicle shedding (Lopez-Castejon and Brough 2011). Despite evidence both for and against these mechanisms for IL-1β release, neither of these models has been widely accepted. Recently, another mechanism for IL-1β release has been described involving a form of programmed necrosis called pyroptosis, which occurs downstream of NLRP3 activation (Shi et al. 2015). Pyroptosis is mediated by gasdermin-D which is processed by caspase-1 into a N- and C-terminal fragment. The N-terminal fragment targets the plasma membrane where it forms oligomeric pores which mediate the release of cytosolic proteins into the extracellular environment. The formation of these oligomeric pores also reduces the cells ability to maintain osmotic balance and consequently results in the lysis of the cell (Sborgi et al. 2016). These events have been shown to occur in response to a range of NLRP3 activating stimuli in murine BMDMs (Shi et al. 2015). Therefore, in addition to its role in the processing of pro-IL-1β into its active form, caspase-1 also plays a fundamental role in the release of the cytokine from cells by activating gasdermin-D and initiating pyroptosis.

1.4.2 Priming of the NLRP3 inflammasome

Canonical activation of the NLRP3 inflammasome requires two signals to achieve activation. The requirement for an initial priming signal represents an important regulatory checkpoint for limiting NLRP3 activation. The necessity for this priming signal is emphasised in mouse bone marrow derived macrophages (BMDMs) which do not form the inflammasome complex in the absence of a priming stimulus, even when exposed to known NLRP3 stimuli (Bauernfeind et al. 2009; Juliana et al. 2012). Priming of the inflammasome largely occurs via transcriptional regulation, increasing the transcription of both NLRP3 and pro-IL-1β, which under resting conditions are insufficient for activation of the inflammasome (Bauernfeind et al. 2009). Priming does not influence the expression of ASC or pro-caspase-1, which are constitutively expressed in resting human monocytes (Ghonime et al. 2014) and murine BMDMs (Bauernfeind et al. 2009).

Priming stimuli include activators of NF-κB, such as ligands for TLRs, TNFRs and IL-1Rs (Franchi et al. 2009). The requirement for NF-κB activation in NLRP3 priming was
demonstrated using the NF-κB inhibitor Bay11-7082, which reduced NLRP3 mRNA expression in LPS stimulated mouse BMDMs (Bauernfeind et al. 2009). NF-κB activation during NLRP3 priming occurs downstream of the MyD88 and TRIF signalling pathways which have been shown to compensate for one another. Macrophages deficient in only MyD88 or TRIF respond normally to stimulation with LPS and ATP, whereas macrophages deficient in both MyD88 and TRIF fail to form NLRP3 in response to stimulation (Bauernfeind et al. 2009). Interestingly, MyD88 and TRIF have different effects on inflammasome priming. MyD88 signals via IRAK1 and IRAK4 and is responsible for NF-κB-dependant transcriptional priming. TRIF signalling, on the other hand, mediates inflammasome priming independent of transcription (Fernandes-Alnemri et al. 2013; Lin et al. 2014).

This transcription-independent priming mechanism allows the rapid activation of the inflammasome. The mechanism was first described in mouse macrophages which form NLRP3 inflammasome complexes within ten minutes of LPS stimulation, well in advance of NLRP3 mRNA expression which requires two hours to be upregulated (Juliana et al. 2012). A pool of NLRP3 is now known to exist cytosolically and is sequestered by ubiquitin. TLR priming by LPS results in the deubiquitination of the NLRP3 and activation of the inflammasome. Blocking deubiquitination using deubiquitinating enzyme inhibitors prevents LPS induced deubiquitination of cytosolic NLRP3 (Juliana et al. 2012) and consequently inhibits NLRP3-dependent IL-1β secretion in peritoneal macrophages (Py et al. 2013). BRCC3 was identified as a regulator of NLRP3 ubiquitination using a deubiquitinase screen, and transfection of macrophages with BRCC3 small interfering RNA (siRNA) reduces caspase-1 activation and subsequent IL-1β secretion (Py et al. 2013). This non-transcriptional regulation of NLRP3 provides an important mechanism for the rapid activation of the inflammasome in response to NLRP3 activators. Inflammasome activation is maintained by transcriptional regulatory mechanisms which are required to increase availability of inflammasome components and pro-IL-1β.

NLRP3 priming by microbial stimuli is well defined and microbial products, such as LPS, are commonly used to prime the inflammasome in vitro. However, sterile inflammatory diseases, such as atherosclerosis and gout, involve NLRP3 activation in the absence of an infection meaning that other non-microbial molecules must be able to prime the inflammasome. Various sterile priming mechanisms have now been suggested. As discussed, ROS are known to influence activation of NF-κB and a role for ROS in NLRP3 priming has been described, with inhibition of ROS significantly reducing NLRP3 inflammasome gene expression in LPS stimulated mouse macrophages (Bauernfeind et al.
Hypoxia, which is known to occur during atherosclerosis, has also been shown to increase the expression of NLRP3 in human macrophages (Folco et al. 2014). Furthermore, many other changes to cellular homeostasis are known to prime the inflammasome including products of lipid metabolism such as palmitate, and alterations to glycolysis (Masters et al. 2010; Wen et al. 2011; Patel et al. 2017). These alterations licence the activation of the inflammasome in immune cells upon the recognition of sterile NLRP3 activators.

1.4.3 Oligomerisation of the NLRP3 inflammasome
As mentioned, the formation of the NLRP3 inflammasome is induced by a wide range of structurally diverse stimuli. The diversity of the NLRP3 activators has led to the general consensus that the inflammasome is not directly activated by its stimulants, instead acting as a sensor for changes in cellular homeostasis (Horvath et al. 2011). In support of this, there is little evidence for a direct interaction between the NLRP3 inflammasome and its activators (Schroder et al. 2010), although ATP has been demonstrated to directly bind to purified recombinant NLRP3 from THP-1 cells suggesting that in some cases a direct interaction may occur (Duncan et al. 2007). The exact pathway linking the inflammasome to its activators remains to be fully defined, but three mechanisms are commonly suggested to play a role: ionic flux, lysosomal destabilisation and the generation of ROS (depicted in figure 1.5).
Figure 1.5 Canonical activation of the NLRP3 inflammasome. (A) An initial priming signal activates NF-κB, which translocates to the nucleus and induces transcription of pro-IL-1β and NLRP3. (B) Immune cell recognition of various agonists results in a multitude of intracellular effects, including: lysosomal rupture, mitochondrial dysfunction, ROS production and ionic flux. (C) These events result in the oligomerisation of the NLRP3 inflammasome via an as of yet unknown mechanism. (D) Recruitment of pro-caspase-1 into the inflammasome complex results in its pro-autoproteolytic cleavage and release into the cytosol. Caspase-1 catalyses the cleavage of pro-IL-18 and pro-IL-1β into their active forms, which are subsequently secreted from the cell and induce or exacerbate an inflammatory response. ATP, adenosine triphosphate; Ca^{2+}, calcium ion; K^+, potassium ion; MAVS, mitochondrial antiviral signalling; NEK7, Never In Mitosis A-Related Kinase 7; TLR, toll-like receptor; TNFR, TNF receptor; Trx, thioredoxin; TXNIP, thioredoxin interacting protein; Ub, ubiquitin. Adapted from Jo et al. (2016).
**Ion fluxes in NLRP3 activation**

In resting cells, cytosolic potassium (K⁺) is maintained at a high concentration relative to the extracellular environment. This is controlled by transmembrane sodium (Na⁺)/K⁺ ion channels which pump Na⁺ into the cell whilst pumping K⁺ out (Kaplan 2002). K⁺ efflux was originally linked with NLRP3 activation after the observation that increasing extracellular K⁺ concentration ameliorated NLRP3-dependent IL-1β secretion in vitro (Pétrilli et al. 2007). Since, a decrease in intracellular K⁺ concentration has been shown in murine BMDMs in response to many NLRP3 stimuli, including bacterial pore-forming toxins, nigericin, ATP and particulate matter (Munoz-Planillo et al. 2013). Potassium efflux can occur via many different mechanisms. For example, ATP binds the P2X7 receptor, a cationic purinergic receptor through which K⁺ is removed from the cell. Nigericin, conversely, is proposed to activate K⁺/H⁺ ionophores which exchange K⁺ for H⁺ across the cell membrane (Katsnelson et al. 2015).

Despite having been shown to occur in response to many stimuli, the exact mechanism by which potassium efflux induces inflammasome formation remains unknown. Recently, a role for Never In Mitosis A-Related Kinase 7 (NEK7) has been described using a triple-tagged NLRP3 protein to identify proteins that interact directly with the inflammasome (He et al. 2016). The triple-tagged NLRP3 was reconstituted in NLRP3⁻/⁻ BMDMs and cells were treated with LPS and ATP to induce activation of the inflammasome. Liquid chromatography was then used to search for interacting partners and highlighted NEK7 as a major interacting partner with NLRP3, which was confirmed using immunoprecipitation. Interestingly, the NLRP3-NEK7 interaction is inhibited by increasing extracellular potassium concentration providing a potential mechanism which links K⁺ efflux to the inflammasome (He et al. 2016). However, a potassium efflux-independent mechanism for NLRP3 activation has also been demonstrated, showing that imiquimod, a small molecule ligand for TLR7, activates the inflammasome in mouse bone marrow derived dendritic cells (BMDCs) even when potassium efflux was blocked. Instead, the study showed that imiquimod induced a robust increase in ROS, which in turn resulted in NEK7 activation and NLRP3 activation (Gross et al. 2016), suggesting that NEK7 may actually be involved in mediating ROS-induced inflammasome activation.

In addition to potassium efflux, a role for calcium ions (Ca²⁺) in NLRP3 activation has also been demonstrated. This was shown following inhibition of ultra violet B (UVB)-induced NLRP3 activation and IL-1β secretion in keratinocytes using the Ca²⁺ chelator BAPTA-AM (Feldmeyer et al. 2007). Calcium influx is also required for NLRP3 activation by extracellular ATP, nigericin and monosodium urate (MSU) crystals in murine BMDMs (Murakami et al.
Crystalline NLRP3 activators induce cell swelling and Ca\textsuperscript{2+} influx via the transient receptor potential (TRP) channels TRPV2, TRPM2 and TRPM7 (Zhong et al. 2013), the blocking of which reduces IL-1\textbeta secretion in THP-1 cells (Compan et al. 2012). The exact mechanism by which Ca\textsuperscript{2+} influx activates the inflammasome is unknown but is suggested to involve the production of mitochondrial ROS (mtROS) and release of mitochondrial DNA (mtDNA), which induce NLRP3 activation (Hornung 2014). Questions remain, however, about the necessity for Ca\textsuperscript{2+} flux in NLRP3 activation. Nigericin and ATP induced efflux of Ca\textsuperscript{2+} is not necessary for NLRP3 activation in BMDMs and BMDCs, actually occurring after the activation of the inflammasome (Katsnelson et al. 2015). Many studies documenting Ca\textsuperscript{2+} dependent activation of the inflammasome have used the chemical inhibitor 2-aminoethoxy diphenylborinate (2APB), which inhibits inositol 1,4,5-triphosphate receptor (IP\textsubscript{3}R) and increases Ca\textsuperscript{2+} release from the endoplasmic reticulum. A more recent study, however, has refuted these findings, demonstrating that 2APB has off-target effects, inhibiting the inflammasome independently of calcium mobilisation, therefore suggesting that inflammasome activation occurs independently of calcium flux (Baldwin et al. 2017).

The intracellular concentrations of chloride (Cl\textsuperscript{-}), Na\textsuperscript{+} and zinc (Zn\textsuperscript{2+}) have also been shown to fluctuate during NLRP3 signalling (Gong et al. 2018). The mechanisms by which ionic fluctuation activates the inflammasome remain unclear. Mitochondrial dysfunction downstream of potassium and calcium flux is thought to be a fundamental signal for NLRP3 activation (Murakami et al. 2012). Overall, the role for cationic flux in NLRP3 activation has been studied in some detail, but the mechanism linking fluctuations in ion concentration to the inflammasome remain to be fully defined. It is plausible that these ion fluxes occur upstream of the common signal for NLRP3 activation, such as mitochondrial damage or ROS generation.

**Lysosomal destabilisation**

Lysosomes are membrane-bound organelles responsible for breaking down proteins, polysaccharides, and complex lipids (Luzio et al. 2007). As discussed earlier (section 1.2.2), phagocytosis involves the uptake of an extracellular molecule into the cell and the formation of the phagolysosome (Xu and Ren 2015). The main function of phagolysosome is the degradation of the phagocytosed particle using an armoury of acid hydrolases, such as lipases and proteases, as well as fluctuations in lysosomal pH and ion homeostasis (Haas 2007). Many inflammasome agonists, such as MSU crystals, calcium pyrophosphate dehydrate (CPPD) crystals and amyloid-beta plaque fibrils, are phagocytosed by leukocytes during the immune response and activate the NLRP3 inflammasome (Halle et al. 2008; Hornung et al. 2008). The activation of NLRP3 by these stimuli has been shown to correlate
with the disruption of lysosome integrity and lysosome membrane permeabilisation (LMP) which consequently results in release of lysosome contents into the cytosol of the cell (Hornung and Latz 2010).

A number of studies have provided evidence for lysosome rupture-induced NLRP3 activation and IL-1β secretion. Chemically inducing lysosomes destabilisation using Leu-Leu-OMe (LLME) induces secretion of IL-1β in mouse macrophages in an NLRP3 dependent manner (Hornung et al. 2008). The release of lysosomal proteases, such as cathepsins, into the cytosol are believed to mediate the activation of the inflammasome. Cathepsins are serine proteases which degrade proteins within lysosomes (Turk et al. 2012). Cathepsin B has been increasingly linked to lysosomal-induced NLRP3 activation, having been shown to leak into the cytosol following stimulation with silica and cholesterol crystals in human PBMCs (Hornung et al. 2008; Duewell et al. 2010). Inhibition of cathepsin B, using chemical inhibitor Ca074Me, reduces amyloid-β plaque induced IL-1β secretion in murine microglial cells (Halle et al. 2008). Furthermore, a role for other cathepsins in NLRP3 activation has also been suggested. Lima et al. (2013) stimulated a panel of cathepsin-deficient macrophages with LLME and showed that cathepsin C knockout prevented IL-1β secretion, whereas knockout of B, L and S did not. In contrast, another study showed that peritoneal macrophages deficient in cathepsins B, L, S or C displayed no difference in IL-1β secretion in response to silica compared to wild-type macrophages instead showing that reduction of single cathepsin had little effect on NLRP3 activation, but the knockout or inhibition of multiple inhibited IL-1β secretion in murine macrophages (Orlowski et al. 2015).

Although there is evidence for a role of lysosomal rupture and cathepsin release in inflammasome activation, the exact mechanisms leading to NLRP3 activation following lysosomal destabilisation remain unknown. Activation of the stress-responsive kinases JNK has been shown to follow lysosome rupture in THP-1 macrophages. Interestingly, a role for calcium in this process has also been described, highlighting a potential link between lysosomal rupture, calcium flux and NLRP3 activation (Okada et al. 2014). Furthermore, an increase in mtROS production has also been shown to follow the release of cathepsin B in adenovirus type 5 stimulated THP-1 macrophages, proposing that oxidative stress may mediate activation of NLRP3 downstream of lysosome destabilisation (McGuire et al. 2011). Conversely, lysosome degradation has been shown to actually occur after mtROS-induced activation of the inflammasome in murine BMDMs (Heid et al. 2013), suggesting that lysosomal destabilisation is dispensable for NLRP3 activation. These findings, together with the fact that not all NLRP3 activators are phagocytosed, suggests that lysosomal destabilisation is not required in NLRP3 activation; rather, the evidence suggests it is likely
to cause a change in ROS levels, which in turn could induce the activation of the inflammasome. Frustrated phagocytosis occurs when phagocytes fail to fully engulf large extracellular molecules and results in lysosomal destabilisation. Interestingly, frustrated phagocytosis has also been shown to induce the generation of ROS (Padmore et al. 2017). Perhaps then it is the increase in ROS following frustrated phagocytosis and lysosomal destabilisation which mediates the activation of the NLRP3 inflammasome by particulate stimuli.

**ROS and activation of the NLRP3 inflammasome**

Since the first description of NLRP3 (Martinon et al. 2002) numerous studies have demonstrated the importance of ROS in its activation. However, the exact role of ROS remains a matter of debate, with numerous other studies demonstrating NLRP3 activation to occur independently of ROS (table 1.2).

A role for ROS in NLRP3 activation stemmed from early findings that inhibition of NADPH oxidase enzymes using the chemical NADPH oxidase inhibitor diphenyleneiodonium (DPI) reduces ATP-induced IL-1β secretion in LPS-primed alveolar macrophages (Cruz et al. 2007). The inhibition of IL-1β secretion by DPI was replicated in ATP-stimulated primary human monocytes (Hewinson et al. 2008), and the importance of NADPH oxidase induced ROS was quickly confirmed by knocking down NADPH oxidase p22phox subunit in THP-1 cells which did not secrete IL-1β in response to MSU crystals or asbestos (Dostert et al. 2008). Together, these studies presented strong evidence suggesting that NADPH oxidase induced ROS production was a critical mechanism by which NLRP3 activators induce formation of the inflammasome. However, this has since been disputed using mononuclear cells from patients with CGD, an immunodeficiency disease caused by defects in phagocyte NADPH oxidase subunits. The secretion of IL-1β from mononuclear cells from CGD patients in response to MSU crystals and silica is unaffected compared to healthy donors, despite the loss of NADPH oxidase induced ROS (Meissner et al. 2010). This finding was confirmed in two more studies using PBMCs from patients with CGD which showed normal production of IL-1β in an NLRP3 dependent manner (Van Bruggen et al. 2010; van de Veerdonk et al. 2010). Interestingly, despite demonstrating that NADPH oxidase induced ROS is not involved in NLRP3 activation, (Van Bruggen et al. 2010) still demonstrated that ROS inhibition using DPI and NAC reduced IL-1β secretion in healthy LPS primed PBMCs stimulated with MSU crystals. This highlighted the potential role of ROS from other intracellular sources in the activation of the inflammasome.
<table>
<thead>
<tr>
<th>Effect on NLRP3</th>
<th>Cell type</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation</td>
<td>Alveolar macrophages</td>
<td>Treatment of cells with ATP induces ROS. Inhibition of NADPH-oxidase derived ROS by DPI reduced ATP-induced caspase-1 activation and IL-1β production</td>
<td>Cruz et al. (2007)</td>
</tr>
<tr>
<td>Activation</td>
<td>THP-1 cells</td>
<td>Knockdown of p22phox subunit of NADPH-oxidase suppressed IL-1β release in THP-1 cells in response to asbestos and MSU</td>
<td>Dostert et al. (2008)</td>
</tr>
<tr>
<td>Activation</td>
<td>THP-1 cells Human monocytes</td>
<td>Extracellular ATP causes NADPH-oxidase induced oxidation and IL-1β secretion in THP-1 cells. Treatment with NAC and DPI inhibited caspase-1 processing in response to extracellular ATP</td>
<td>Hewinson et al. (2008)</td>
</tr>
<tr>
<td>Activation</td>
<td>THP-1 cells</td>
<td>Inhibition of mitochondrial complex I induced ROS production correlating with NLRP3 activation. Inhibition of mitochondrial respiration abrogated IL-1β production and caspase-1 activation</td>
<td>Zhou et al. (2010b)</td>
</tr>
<tr>
<td>Activation</td>
<td>Alveolar macrophages</td>
<td>Inhibition of miROS by SS-31 dose-dependently inhibited cyclic stretch induced caspase-1 activation and IL-1β production</td>
<td>Wu et al. (2013)</td>
</tr>
<tr>
<td>Activation</td>
<td>Murine BMDMs HEK293T cells</td>
<td>Mitochondrial dysfunction is linked to NLRP3 activation and mitochondrial DNA is required for NLRP3 inflammasome-mediated IL-1β secretion</td>
<td>Shimada et al. (2012)</td>
</tr>
<tr>
<td>Activation</td>
<td>HEK293T cells THP-1 cells</td>
<td>Redox regulated TXNIP binds directly to NLRP3 in response to MSU exposure. siRNA blocking of TXNIP expression abrogated caspase-1 activation and IL-1β secretion</td>
<td>Zhou et al. (2010a)</td>
</tr>
<tr>
<td>Activation</td>
<td>Human PBMCs</td>
<td>IL-1β secretion is ROS-dependent but in an NADPH-oxidase-independent manner. Cells from p22phox- and NOX2-deficient patients had normal IL-1β secretion</td>
<td>Van Bruggen et al. (2010)</td>
</tr>
<tr>
<td>No role</td>
<td>Human monocytes</td>
<td>IL-1β secretion is normal in mononuclear cells from patients with chronic granulomatous disease (CGD), a disease characterised by defective NADPH-oxidase enzymes</td>
<td>Meissner et al. (2010)</td>
</tr>
<tr>
<td>No role</td>
<td>Human monocytes</td>
<td>NLRP3 can be activated in a ROS-independent manner in monocytes from patients with CGD. Inhibition of ROS with DPI did not decrease caspase-1 activation or IL-1β secretion in these cells</td>
<td>van de Veerdonk et al. (2010)</td>
</tr>
<tr>
<td>No role</td>
<td>Murine macrophages</td>
<td>Elevated ROS in SOD1-deficient mouse macrophages inhibited caspase-1 activation. This could be reversed dose-dependently with SOD-mimetics which increased ATP-induced IL-1β secretion</td>
<td>Meissner et al. (2008)</td>
</tr>
<tr>
<td>No role</td>
<td>Murine BMDMs</td>
<td>Nrf2, a redox sensitive transcription factor for a range of antioxidant genes, is essential for inflammasome activation and IL-1β secretion in response to cholesterol</td>
<td>Freigang et al. (2011)</td>
</tr>
<tr>
<td>No role</td>
<td>Murine BMDMs</td>
<td>IL-1β secretion in TXNIP-deficient macrophages secreted normal levels of IL-1β compared to wild-type macrophages in response to MSU, ATP and islet amyloid polypeptide</td>
<td>Masters et al. (2010)</td>
</tr>
<tr>
<td>No role</td>
<td>Murine macrophages</td>
<td>Inhibition of ROS by DPI and NAC inhibited NLRP3 priming but not activation. Only had an effect when cells were precultured with ROS inhibitors prior to activation</td>
<td>Bauernfeind et al. (2011)</td>
</tr>
</tbody>
</table>

Table 1.2 Contradictory roles of ROS in activation of the NLRP3 inflammasome. Human monocytes denotes the use of primary human monocytes. Murine macrophages relates to a C57BL6/J mouse macrophage cell line.
The mitochondria have since emerged as a key source of ROS during the activation of the inflammasome. Mitochondrial ROS generation, induced by inhibiting mitochondrial complex I using rotenone, closely correlates with IL-1β secretion in THP-1 monocytes (Zhou et al. 2010b). Furthermore, the downregulation of voltage dependent anion channels, which are ultimately required for mtROS production inhibits caspase-1 activation and IL-1β secretion in THP-1 cells (Zhou et al. 2010b). A role for mtROS in NLRP3 activation has also been shown in alveolar macrophages where the inhibition of mtROS using the mitochondria-targeted antioxidant SS-31 reduced caspase-1 activation and IL-1β secretion (Wu et al. 2013), and more recently in primary human monocytes where mitochondrial targeted antioxidant mitoTEMP was shown to reduce IL-1β secretion in response to ugonin U (Chen et al. 2017).

Interestingly, mitochondrial dysfunction, which involves the production of mtROS, has been shown to both induce and be induced by potassium flux and lysosomal instability, providing a potential link between these two mechanisms and NLRP3 activation (Shimada et al. 2012). Oxidised mitochondrial DNA, released during mitochondrial dysfunction, has been shown to directly interact with the inflammasome (Nakahira et al. 2011; Heid et al. 2013). This was shown in HEK293T cells co-transfected with an NLRP3-Flag construct and purified mitochondrial DNA labelled with bromodeoxyuridine in which mitochondrial DNA was demonstrated to directly bind with NLRP3 adaptor proteins prior to inflammasome formation (Shimada et al. 2012). Finally, and in further support of a role for mitochondria and mtROS in the activation of the inflammasome, NLRP3 and ASC have been shown to localise to the mitochondria during NLRP3 activation, in a process mediated by the redox sensitive mitochondrial antiviral signalling (MAVS) protein (Subramanian et al. 2013; Elliott et al. 2018). However the requirement for MAVS varies according to the NLRP3 agonist with nigericin being dependent on MAVS whereas crystalline structures less so (Subramanian et al. 2013), suggesting that inflammasome localisation to mitochondria is not a common mechanism shared by all stimuli. Other studies have suggested that MAVS only plays a role in NLRP3 activation in response to RNA viruses, not sterile activators (Allam et al. 2014; Franchi et al. 2014). More recently NLRP3 adaptor proteins have been shown to actually recruit to the dispersed trans-golgi network via interaction with the negatively charged phospholipid phosphatidylinositol-4-phosphate, where NLRP3 forms small complexes prior to recruitment into ASC-specks and subsequent downstream signalling (Chen and Chen 2018). This suggests that NLRP3 components do not localise to the mitochondria, challenging the findings of past studies.
In addition to mtROS, a role for ROS produced from other sources has also been described. Superoxide produced by xanthine oxidase has been shown to activate the inflammasome in THP-1 cells activated by TLR7 and TLR8 ligand R848, in a process which can be inhibited using xanthine oxidase inhibitor allopurinol (Nicholas et al. 2011). The role of xanthine oxidase in NLRP3 activation has also been characterised in murine BMDMs and PMA primed THP-1 cells, with the knockdown of XO or treatment of cells with allopurinol significantly reducing IL-1β secretion in response to MSU crystals in these cells (Ives et al. 2015). The author of this study demonstrated that xanthine oxidase can induce the generation of mtROS via the PI3K-AKT-mechanistic target of rapamycin (mTOR) pathway, perhaps suggesting that xanthine oxidase ROS generation occurred upstream of mitochondrial dysfunction (Ives et al. 2015). Of course, the inflammasome may well be a sensor of ROS regardless of the source. If this was the case though it is interesting that NADPH oxidase induced ROS is not required for inflammasome activation.

Despite the mounting evidence for a role of ROS, some studies question the importance of oxidants in inflammasome activation. The inhibition of ROS using DPI or NAC has been shown to significantly reduce caspase-1 activation in LPS and nigericin stimulated BMDMs. However, this reduction only occurred when the antioxidants were added prior to LPS stimulation, not when added after an initial LPS preincubation suggesting that ROS are only required for NLRP3 priming (Bauernfeind et al. 2011). This was also shown in one of the studies using cells from CGD patients, in which DPI inhibited transcription of proinflammatory cytokines but not caspase-1 activation (van de Veerdonk et al. 2010). There is also evidence for NLRP3 activators to induce inflammasome oligomerisation even in the presence of ROS inhibitors. For example, linezolid, an oxazolidinone class of antibiotic, induces caspase-1 activation and IL-1β secretion in murine macrophages even in the presence of ROS inhibitors NAC, DPI and Mito-TEMPO (Iyer et al. 2013), suggesting that NLRP3 inflammasome activation is independent of changes to ROS. A further factor which questions the importance of ROS in NLRP3 activation is the inability of chemically induced oxidative stress to activate the inflammasome. This has been demonstrated in murine BMDMs stimulated with rotenone, antimycin A or H$_2$O$_2$ (Munoz-Planillo et al. 2013) and in human monocytes stimulated with H$_2$O$_2$ even in the presence of a priming signal (Carta et al. 2011). These findings all suggest that inflammasome activation can occur independently of oxidative stress, demonstrating that ROS may not be a required signal for inflammasome activation.
How exactly NLRP3 senses the changes in ROS also remains elusive but redox-sensitive proteins which interact with the inflammasome have been identified. Zhou et al. (2010a) described the association of NLRP3 with the redox-sensitive thioredoxin interaction protein (TXNIP), an association that was required for downstream inflammasome activation. Under basal redox conditions, TXNIP binds the intracellular antioxidant Trx blocking its reductase activity (Yoshihara et al. 2013). High concentrations of H₂O₂ causes the disassociation of TXNIP from Trx, allowing TXNIP to associate with NLRP3 in HEK293T cells. In fact, the downregulation of TXNIP expression using siRNA abrogates NLRP3 dependent IL-1β secretion in MSU and R837 stimulated THP-1 macrophages (Zhou et al. 2010a). The description of TXNIP as a redox-sensitive interacting partner for the NLRP3 inflammasome provided a potential mechanism by which ROS could activate the inflammasome. However, the importance of TXNIP has since been challenged by data showing that TXNIP knockout incompletely impairs caspase-1, suggesting that other unknown factors may also be able to activate the inflammasome in parallel to ROS-induced TXNIP (Abais et al. 2014). Furthermore, Masters et al. (2010) showed that TXNIP mRNA expression was in fact reduced in LPS-stimulated BMDMs and that TXNIP-deficient BMDMs secreted IL-1β when stimulated with MSU or ATP. Interestingly, a more recent study has even described a protective effect of TXNIP demonstrating that TXNIP deficiency exacerbated IL-1β secretion in murine Kupffer cells stimulated with palmitic acid (He et al. 2017). Thus, the role of TXNIP in NLRP3 activation remains to be completely elucidated but it overall does not appear to be a fundamental mechanism by which the inflammasome is activated.

To add to the confusion surrounding ROS and NLRP3 activation, a role for antioxidant enzymes in NLRP3 activation has also been suggested. Meissner et al. (2008) demonstrated a requirement for SOD1 in the processing of the inflammasome with SOD1-deficient macrophages secreting less IL-1β than WT macrophages. Interestingly, superoxide was shown to mediate reversible glutathionylation of two cysteine residues on capsase-1, inhibiting its activity and subsequent secretion of IL-1β (Meissner et al. 2008). In confirmation of these findings, copper chelation using tetrathiomolybdate reduced NLRP3-dependent caspase-1 activation by inhibiting SOD1 activity in human macrophages (Deigendesch et al. 2018). The inhibition of SOD would of course have effects on the cells redox environment, so perhaps the inflammasome becomes activated in response to these changes.

Other antioxidants have also been implicated in NLRP3 activation. Nuclear factor E2-related factor (Nrf2) is a redox-sensitive transcription factor which induces the transcription of glutathione, thioredoxin and other antioxidants such as NADPH dehydrogenase [quinone]
1 and heme (Hennig et al. 2018). Nrf2 is essential for cholesterol crystal induced NLRP3 activation in murine BMDCs and BMDMs, with Nrf2-deficient cells secreting reduced IL-1β compared to Nrf2-expressing controls (Freigang et al. 2011), a response confirmed in LPS stimulated BMDMs (Zhao et al. 2014) and MSU stimulated THP-1 cells (Jhang et al. 2015). Furthermore, soluble uric acid, a potent superoxide scavenger and an important antioxidant in human plasma (Ames et al. 1981), has recently been shown to prime NLRP3. PBMCs pre-treated with soluble uric acid secrete greater levels of IL-1β when subsequently stimulated with LPS and MSU crystals by decreasing the availability of IL-1Ra (Crisan et al. 2015). Uric acid was later shown to activate the Akt-PRAS40 pathway which inhibited autophagy and enhanced NLRP3 priming (Crisan et al. 2017). Interestingly, uric acid has also been shown to activate the NLRP3 inflammasome in murine BMDMs in a ROS-dependent manner, with NAC inhibiting IL-1β secretion in response to uric acid (Braga et al. 2017). Uric acid has been shown to act as a pro-oxidant in certain settings (Sautin and Johnson 2008), and it is therefore conceivable that the effects of soluble uric acid in NLRP3 activation were in fact mediated by increased ROS. Hyperuricemia is the single greatest risk factor for the development of gout which as mentioned is driven by IL-1β secretion (Busso and So 2010). It would be interesting to further investigate what role uric acid may have on the activation of the inflammasome in human monocytes, which are exposed to serum uric acid prior to migrating into the peripheral joints during a gouty arthritic attack.

The majority of studies have demonstrated an important role for ROS in NLRP3 activation, but there are studies contradicting these findings. Thus, the exact link between ROS and NLRP3 remains elusive. A potential theory for the involvement of both antioxidant enzymes in NLRP3 signalling is the idea of a biphasic redox response. The biphasic response is a unified theory that suggests a role for both ROS and antioxidants in inflammasome activation. Firstly, PAMPs ligate TLRs and induce a rapid increase in ROS production. This upregulation in ROS is closely followed by an increase in antioxidant defence systems in an attempt to maintain redox homeostasis (Tassi et al. 2009). The importance of the antioxidant response in IL-1β secretion was demonstrated by selectively inhibiting Trx and SOD1 using siRNA in human monocytes, which caused a 40% and 70% reduction in IL-1β secretion, respectively, following stimulation with IFN-γ and zymosan (Tassi et al. 2009). This theory suggests that redox modulation is the important factor in IL-1β processing and provides a potential explanation for the evidence both for and against the involvement of ROS in NLRP3 activation.
1.4.4 Cell-type specific activation of NLRP3

It is clear from the literature that the exact role of ROS, antioxidants and oxidative stress in activation of the NLRP3 inflammasome remains controversial with many contradictory studies. Possible reasons for these discrepancies could be due to differences in signalling pathways in different cell types as well as different species.

Various different types of immune cell have been used to study the activation of NLRP3 in vitro, including monocytes, macrophages, dendritic cells and kupffer cells. Differential expression of NLRP3 mRNA has been documented between different haematopoietic cells, with NLRP3 being primarily expressed in dendritic cells and monocytes, whilst expressed at lower levels in macrophages, with even lower expression in T cells, B cells and NK cells (Guarda et al. 2011). Human monocytes have also been shown to secrete significantly greater quantities of IL-1β in response to NLRP3 activators, such as MSU crystals, than macrophages and dendritic cells (Netea 2009; Pazar et al. 2011). Furthermore, it is now known that monocytes secrete low levels of IL-1β in the presence of TLR ligands alone, not requiring a secondary signal, whereas macrophages require two distinct signals for NLRP3-dependent IL-1β secretion (Netea 2009; Gaidt et al. 2016). As previously discussed, this has been attributed to monocytes having constitutively activated caspase-1 (Netea 2009).

An alternative pathway for TLR4-induced IL-1β secretion which engages the NLRP3 inflammasome in the absence of pryoptosome formation has also been shown to exist in monocytes but not macrophages (Gaidt et al. 2016). Thus, the activation of NLRP3 is likely to occur via different mechanisms depending on the cell types and care should be taken to consider this when evaluating results from macrophages, monocytes, and dendritic cells in the study of the inflammasome.

A further consideration is the use of cell lines in evaluating how NLRP3 is activated. Cell lines are widely used in research and offer several advantages over the use of primary cells. Cell lines are a pure population of cells that provide more consistent and reproducible results than primary cells (Kaur and Dufour 2012). They are cost effective, easy to culture and can be used without ethical approval. However, cell lines also have a number of limitations. Most have been transformed using an oncogene or oncoprotein, which can result in phenotypic changes which may alter inflammasome signalling (Maqsood et al. 2013). The human monocytic THP-1 cell line is widely used in the study of NLRP3 activation but various limitations with THP-1 cells have been described. Firstly, they are far less responsive to LPS stimulation than primary human monocytes, secreting reduced levels of IL-1β, a phenomenon thought to result from their reduced expression of CD14 (Bosshart and Heinzelmann 2016). There are also known differences in the redox state of primary
human monocytes and THP-1 cells. THP-1 cells have up-regulated antioxidant systems which quickly buffer any ROS produced upon TLR stimulation, whereas antioxidant systems in primary cells are expressed at lower levels with a much more delicate balance between pro- and anti-oxidant systems allowing efficient redox remodelling upon stimulation (Carta et al. 2011). This difference in redox homeostasis could clearly lead to discrepancies in findings between THP-1 cells and human cells.

Furthermore, species differences in how NLRP3 is activated, particularly when mouse cells are used to study activation of the NLRP3 inflammasome. Murine models provide an invaluable resource in biomedical research, allowing in vivo studies as well as the opportunity to generate knockout mice for examination of the effect of specific proteins in cell biology. Mice are generally considered to mirror human biology relatively well, and whole genome sequencing of mouse and humans has revealed a small number (around 300) of genes that are unique to mice or humans (Mouse Genome Sequencing et al. 2002; Mestas and Hughes 2004). However, the composition of immune cells in the blood, TLR expression, leukocyte defensin availability, as well as differences in cytokine availability have all been shown to differ between humans and mice (Rehli 2002; Davis 2008; Khanna and Burrows 2011; Bailey et al. 2013; Perlman 2016).

In the context of the NLRP3 inflammasome, differences exist between mouse and human cells which advocate that care should be taken when interpreting data from different species. As discussed, human monocytes secrete IL-1β secretion in response to TLR ligation alone (Netea 2009; Gaidt et al. 2016), whereas mouse cells have classically been shown to require two distinct signals (Wang et al. 2013). This difference has been suggested to result from the increased sensitivity of human cells, which secrete more IL-1β when stimulated with NLRP3 activators than mouse cells. Mouse cells have actually been shown to secrete IL-1β in response to LPS alone when LPS is used at a much higher dose (Wang et al. 2013), suggesting that the different results seen in human and mouse cells could relate to differences in sensitivity. The increased LPS-induced inflammasome activation in human cells compared to mouse cells could also relate to the autocrine function of ATP, which is released from cells and activates the P2X7 receptor on human monocytes which is a known signalling pathway involved in NLRP3 activation (Gombault et al. 2012). Interestingly, P2X7 is up-regulated in LPS treated human macrophages but not in mouse macrophages under the same conditions, suggesting that mice are less likely to secrete IL-1β due to the autocrine effect of ATP (Schroder et al. 2012). Finally, there is an alternative mechanism for NLRP3 activation which occurs when TLR4 is ligated by LPS in human monocytes. This pathway results in NLRP3 formation which is independent of pyroptosome
formation and potassium efflux (Gaidt et al. 2016) in a TLR4-dependent mechanism that signals via the TRIF-RIPK-FADD pathway resulting in caspase-8 activity which subsequently activates the NLRP3 inflammasome via cleavage of an unknown substrate (Gaidt et al. 2016; Gaidt and Hornung 2017). This pathway was demonstrated to occur in human monocytes but not in murine monocytes suggesting that signals involved in inflammasome activation may well be species-dependent.

The activation of the NLRP3 inflammasome can therefore differ according to cell type used, the use of primary cells or cell lines, and between different species. These differences may explain some of the discrepancies observed in the mechanisms involved in the activation of the inflammasome, particularly the role of ROS in this system.

1.4.5 Pathological conditions associated with the NLRP3 inflammasome

The NLRP3 inflammasome has been associated with the development of many diseases. Improving understanding of the mechanisms involved in the activation of the NLRP3 inflammasome could therefore highlight potential pathways to target for and provide therapeutic options for the treatment of these pathologies.

Cryopyrin-associated periodic syndromes

Cryopyrin-associated periodic syndromes (CAPS) are a group of autoinflammatory diseases caused by gain-of-function mutations in the NLRP3 gene. These mutations in the cause hyperactivity of the inflammasome, resulting in unregulated release of IL-1β (Kubota and Koike 2010). CAPS consists of three conditions driven by overactive NLRP3: familial cold auto-inflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS) and neonatal-onset multisystem inflammatory disorder (NOMID). In fact, the NLRP3 gene was first discovered following the direct sequencing of genomic DNA from patients with FCAS (Hoffman et al. 2001). CAPS are characterised by neutrophilia and systemic inflammation driven by neutrophils in the skin, joints, muscles and cerebrospinal fluid (Broderick et al. 2015). CAPS patients present with elevated levels of many inflammatory markers such as C-reactive protein, serum amyloid A, as well as increased IL-1β and IL-18. In vitro studies of CAPS have also shown increased basal release of IL-1β in monocytes and macrophages in the absence of a stimulus (Agostini et al. 2004). The current treatment options for CAPS are all focused on blocking the action of IL-1β. These include Anakinra, a recombinant IL-1R agonist, Rilonacept, a decoy IL-1 receptor which captures and sequesters IL1β, and Canakinumab, a recombinant monoclonal antibody which blocks the interaction of IL-1β with its receptors (Landmann and Walker 2017).
Crystallopathies

Gout is a crystallopathy driven by IL-1β which is secreted following NLRP3 activation in response to MSU crystals (Choi et al. 2005; Martinon et al. 2006). MSU crystals form during periods of elevated serum uric acid concentration. Uric acid is one of the final products of purine nucleotide metabolism (Maiuolo et al. 2016). In non-primates, uric acid is metabolised by the enzyme uricase into the soluble compound allantoin, allowing plasma uric acid concentration to remain low. Humans, however, have lost functional uricase in what is hypothesised to have been of evolutionary benefit because of uric acid’s antioxidant properties (Ames et al. 1981). Humans are there at increased risk of elevated serum uric acid concentrations, especially during periods of increased dietary purine intake. Uric acid is normally maintained at physiological levels via excretion through the kidneys and thus a reduction in renal function can also cause increased serum uric acid concentrations (Alvarez-Lario and Macarron-Vicente 2010). Precipitation of uric acid into MSU crystals occurs when uric acid concentration is above 6.8mg/dL, at which point uric acid becomes supersaturated and the risk of crystallisation increases (Richette and Bardin 2010). Precipitation of uric acid into MSU crystals is also influenced by reduced external temperature which can lower the solubility point of uric acid (Loeb 1972) or by a reduction in pH which increases calcium availability which can induces nucleation of MSU crystals (Wilcox and Khalaf 1975; Martillo et al. 2014).

Gout is characterised by bouts of inflammation which are usually self-limiting, resolving within a few days. Chronic gout occurs when inflammatory attacks become recurrent and is characterised by chronic synovitis, damage to bones and cartilage as well as tophi formation (Ragab et al. 2017). The current treatment of gout includes the use of non-steroidal inflammatory drugs (NSAIDs), steroids and colchicine. Colchicine disrupts the assembly of microtubules and supresses MSU-induced NLRP3 activation (Leung et al. 2015). The exact mechanism by which colchicine blocks NLRP3 formation is not fully understood, but could relate to disruption of transport of NLRP3 inflammasome and subsequent assembly into active inflammasomes (Leung et al. 2015). IL-1β inhibitors have been shown to successfully treat gout, with canakinumab, for example, reducing the risk of new inflammation by 62% versus other treatments (Schlesinger et al. 2012; Ottaviani et al. 2013). Finally, uric acid lowering therapy is used in some cases, particularly in patients with recurring attacks. The main classes of urate lowering therapy are the xanthine oxidase inhibitors, such as allopurinol and febuxostat, which decrease production of uric acid (Becker et al. 2005), or uricosuric agents, such as sulfinpyrazone and benz bromarone, which increase renal excretion of uric acid (Burns and Wortmann 2012).
Pseudogout clinically resembles gout but is caused by crystals of CPPD rather than MSU. CPPD crystals form during extracellular synthesis of pyrophosphate where inorganic pyrophosphate is generated from extracellular ATP which is subsequently metabolised to pyrophosphate by enzymes such as ectonucleotide pyrophosphatase 1 which have nucleoside triphosphate pyrophosphohydrolase activity (Costello et al. 2011). Much like gout, pseudogout is characterised by bouts of inflammation in the peripheral joints. Examination of crystal structure is required for a definitive diagnosis of gout or pseudogout as MSU crystals are needle-shaped and have a strong negative birefringence, while CPPD crystals are rhomboidal and have a weak positive birefringence (Ivorra et al. 1999; Filippou and Frediani 2012). Like MSU crystals, CPPD crystals can activate the NLRP3 inflammasome resulting in IL-1β induced inflammation which, in contrast to gout, can last for weeks to months (Martinon et al. 2006; Rosenthal and Ryan 2016). As such, pseudogout management is similar to that of gout and is aimed at reducing inflammation using glucocorticoids, colchicine and NSAIDs. The use of IL-1 targeted therapy for the treatment of pseudogout has also been reported, with some studies demonstrating successful treatment of pseudogout with anakinra (McGonagle et al. 2008; Molto et al. 2012).

In addition to its known involvement in CAPS and crystallopathies, the activation of NLRP3 has also been associated with numerous other diseases, including RA (Choulaki et al. 2015), Alzheimer’s disease (Yin et al. 2018) and atherosclerosis (Paramel Varghese et al. 2016), although an exact causative role is yet to be fully defined.

The NLRP3 inflammasome is therefore an important intracellular complex which regulates immune responses and inflammation, but is also involved in the pathophysiology of numerous inflammatory conditions. The NLRP3 inflammasome could also provide an important mechanistic link between oxidative stress and inflammation and improving understanding of the role of ROS in activation of the inflammasome may provide insight into future therapeutic strategies.
1.5 Project aims

Understanding of the NLRP3 inflammasome since its description in 2002 has improved dramatically, but questions still remain about how it is activated in response to such a diverse range of stimuli. ROS and oxidative stress provide potential mechanisms for NLRP3 activation, and there is substantial literature demonstrating their importance in inflammasome signalling. The aim of this project is to understand the role of ROS and oxidative stress in activation of NLRP3 in human monocytes. These cells were chosen for study given their central role in orchestrating inflammatory responses in many chronic inflammatory diseases. These cells are also easily accessible from peripheral human blood allowing the study of primary human cells and increasing the relevance of the results obtained. The hypothesis of this study was that increases in ROS and oxidative stress could activate the NLRP3 inflammasome in these cells. To test this hypothesis three key research aims were identified:

1. Determine how necessary and sufficient ROS are for activating the NLRP3 inflammasome. This will be addressed in two ways. Firstly, ROS levels during activation of the NLRP3 inflammasome will be measured to assess whether IL-1β secretion in response to crystal stimuli is coupled with increases in ROS in human monocytes (chapter 3). Secondly, antioxidants will be used to reduce ROS and oxidative stress in human monocytes during NLRP3 activation to investigate whether removal of ROS would affect activation of the inflammasome (chapter 4).

2. Soluble uric acid is a major antioxidant in the blood and can influence priming and activation of the inflammasome in human PBMCs and murine BMDMs. The effect of high concentrations of uric acid on activation of NLRP3 in human monocytes will be assessed to determine whether extracellular redox state could influence activation of NLRP3 (chapter 5).

3. Oxidative stress and changes in redox status are associated with inflammation in many diseases. To investigate whether changes in redox status could influence the subsequent activation of the NLRP3 inflammasome, monocytes will be isolated from peripheral blood from people with gout, rheumatoid arthritis or chronic kidney disease. These conditions are all associated with increases in oxidative stress and production of IL-1β and thus provide unique opportunities to determine the relationship between expression and activation of NLRP3 and redox status (chapter 6).
Chapter 2 - Materials and methods
2.1 Equipment

<table>
<thead>
<tr>
<th>Product</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>Accublock digital dry bath</td>
<td>Labnet</td>
</tr>
<tr>
<td>Agilent 2100 bioanalyser</td>
<td>Agilent</td>
</tr>
<tr>
<td>CKX41 inverted microscope</td>
<td>Olympus</td>
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<tr>
<td>Epson perfection, V350 PHOTO scanner</td>
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<tr>
<td>Film processor: SRX-101A Developer</td>
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<td>G-BOX Syngene agarose gel imager</td>
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<td>Hypercassette</td>
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<td>BD Biosciences</td>
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<td>Microfuge R centrifuge</td>
<td>Beckman Coulter</td>
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<tr>
<td>Mini centrifuge: Pico 17 Heraeus 24 x 1.5/2.0mL rotor</td>
<td>Thermo Scientific</td>
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<td>Neubauer Improved Haemocytometer</td>
<td>Marienfeld Superior</td>
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<td>Odyssey Fc Western blot imaging system</td>
<td>LI-COR</td>
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<td>Plater reader: Synergy HT</td>
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<td>Plate washer: ELx50</td>
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<td>Platform rocker STR6</td>
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<td>PowerPac Basic</td>
<td>Biorad</td>
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<td>qPCR machine: Stratagene MX3000P</td>
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<tr>
<td>FiveCompact pH meter</td>
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<tr>
<td>Veriti 96-well thermocycler</td>
<td>Applied Biosystems</td>
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<tr>
<td>Water bath: SUB Aqua 18 Plus</td>
<td>Grant</td>
</tr>
</tbody>
</table>

Table 2.1. List of equipment used throughout study
2.2 Buffers and media

General buffers
Dulbecco’s phosphate buffered saline (DPBS): 0.2g KCl, 0.2g KH$_2$PO$_4$, 8.0g NaCl, 1.15g Na$_2$HPO$_4$. Tissue culture grade without phenol red (Sigma)
Phosphate buffered saline (PBS) 10X: 80g NaCl, 2g KCl, 14.4g Na$_2$HPO$_4$, 2.4g KH$_2$PO$_4$, made up to 1 litre in distilled water, pH 7.4
PBS-Tween (PBS-T): 1X PBS supplemented with 0.01-0.1% (v/v) Tween-20
Tris buffered saline (TBS): 20mM Tris-HCl, 137mM NaCl, 10mM EDTA, made up to 1 litre in distilled water, pH 7.4

Protein purification
ECSOD dialysis buffer: 20mM Tris, 10mM Ethylenediaminetetraacetic acid (EDTA), pH 8.0
Gpx3 dialysis buffer: 1X PBS

Enzyme linked immunosorbent assay (ELISA)
ELISA blocking buffer: 1% or 2% (w/v) bovine serum albumin (BSA) in 1X PBS
ELISA coating buffer: 50mM sodium carbonate (Na$_2$CO$_3$), 50mM sodium bicarbonate (NaHCO$_3$), 15mM sodium azide (NaN$_3$), pH 9.6
ELISA reagent diluent: 0.5% or 1% BSA in 1X PBS
ELISA stop solution: 6% (v/v) Sulfuric acid (H$_2$SO$_4$) in distilled water
ELISA wash buffer: 1X PBS + 0.01% (v/v) Tween-20

Western blot
Blocking buffer: 5% (w/v) milk in PBS-T
Running gel buffer (4X): 1.5M Tris HCl in 1 litre distilled water, pH 8.8
Running buffer (1X): 25mM Tris, 14.413g glycine, 0.1% SDS in 1 litre distilled water, pH 8.3
Stacking gel buffer (4X): 500mM Tris HCl in 1 litre distilled water, pH 6.8
SDS-PAGE sample buffer (6X): 375mM Tris-HCl; 1.2% SDS; 60% glycerol, 0.006% Bromophenolblue (BPB), in distilled water; pH 6.8
Transfer buffer (1X): 3.027g Tris base, 14.41g glycine, 200mL methanol in 1 litre distilled water
Wash buffer: PBS-T (0.05% (v/v)

Cell preparation buffers
MACs CD14 microbeads buffer: Dulbecco’s modified PBS (DPBS) pH 7.2, 0.5% BSA, 2mM EDTA
NP-40 cell lysis buffer (10X): 10% NP-40, 1.5M NaCl, 500mM Tris-HCl, pH 8

Cell viability
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) 10X Buffer: 5mg of MTT in 1mL of tissue culture grade PBS
**MTT stop solution**: 10% (w/v) SDS in 0.01M HCl in distilled water

Cell culture
Dulbecco’s modified eagle medium (DMEM) containing 25mM HEPES and 4.5g/l glucose
Foetal bovine serum (FBS) (Sigma) added to cell culture media at 5-10% (v/v) following 30 minutes at 56ºC to inactivate components of the complement system and other inhibitors of cell growth.
Opti-MEM™ I reduced serum medium (Optimem) containing L-Glutamine and HEPES. Without phenol red (Gibco - 11058021)
Penicillin (5000U/mL) and streptomycin (5000µg/mL) (Pen/Strep) (Gibco) were added to all cell culture media at a final concentration of 1% (v/v)
Roswell Park memorial institute medium (RPMI) containing 2mM L-glutamine
### 2.3 Reagents

<table>
<thead>
<tr>
<th>Ligand name</th>
<th>Purpose</th>
<th>Working concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
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<td>NLRP3 activator</td>
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<td>Invivogen</td>
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<td>Sigma</td>
</tr>
<tr>
<td>Catalase</td>
<td>H$_2$O$_2$ scavenger</td>
<td>10-500 U/mL</td>
<td>Sigma</td>
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<tr>
<td>Hydrogen peroxide (H$_2$O$_2$)</td>
<td>Oxidant</td>
<td>0.25-10mM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Luria Bertani (LB) Broth</td>
<td><em>E. coli</em> growth</td>
<td>2% (w/v)</td>
<td>FisherScientific</td>
</tr>
<tr>
<td>Monosodium urate (MSU) crystals</td>
<td>NLRP3 activator</td>
<td>10-20mg/dL</td>
<td>Invivogen</td>
</tr>
<tr>
<td>Menadione sodium bisulfate</td>
<td>ROS inducer</td>
<td>5-20µM</td>
<td>Sigma</td>
</tr>
<tr>
<td>MCC950</td>
<td>NLRP3 inhibitor</td>
<td>750nM</td>
<td>Invivogen</td>
</tr>
<tr>
<td>N-Acetyl cysteine (NAC)</td>
<td>Small molecule ROS inhibitor</td>
<td>5-10mM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Pam$_3$Csk$_4$ (Pam3)</td>
<td>TLR1/2 ligand</td>
<td>100ng/mL</td>
<td>Enzo Life Sciences</td>
</tr>
<tr>
<td>Paraquat</td>
<td>ROS inducer</td>
<td>50-500µM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Qiazol</td>
<td>Cell lysis for RNA extraction</td>
<td>N/A</td>
<td>Qiagen</td>
</tr>
<tr>
<td>QUANTI-Blue</td>
<td>Quantification of NF-κB inducible SEAP reporter</td>
<td>N/A</td>
<td>Invivogen</td>
</tr>
<tr>
<td>KPL 3,3’,5,5’-Tetramethylbenzidine (TMB)</td>
<td>Peroxidase substrate</td>
<td>N/A</td>
<td>Sera Care</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Superoxide scavenger</td>
<td>500 U/mL</td>
<td>Sigma</td>
</tr>
<tr>
<td>Uric acid</td>
<td>N/A</td>
<td>10-40mg/dL</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

Table 2.2 List of reagents used throughout study
2.4 Antibodies

Enzyme linked immunosorbent assay antibodies

<table>
<thead>
<tr>
<th>Target</th>
<th>Capture antibody [working conc.]</th>
<th>Detection antibody [working conc.]</th>
<th>Standard [working conc.]</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>IL-1β-IL-1F2 [2µg/mL] (R&amp;D Systems #MAB601)</td>
<td>IL-1β/IL-1F2 biotinylated antibody [0.4µg/mL] (R&amp;D systems #BAF201)</td>
<td>Recombinant human IL-1β [10ng/mL-0.0137ng/mL] (Peprotech #200-01B)</td>
</tr>
<tr>
<td>TNFα (kit)</td>
<td>TNFα capture [4µg/mL] (R&amp;D DuoSet DY210)</td>
<td>TNFα detection [50µg/mL]</td>
<td>TNFα standard [1-0.0156ng/mL]</td>
</tr>
<tr>
<td>Gpx3 (kit)</td>
<td>Plate pre-coated with primary antibody (AdipoGen AG-45A-0020YEK-KI01)</td>
<td>GPx3 biotinylated antibody diluted 1:1000 (AdipoGen)</td>
<td>Human recombinant GPx3 Standard [32-0.5ng/mL] (AdipoGen)</td>
</tr>
</tbody>
</table>

Table 2.3 Antibody pairings for sandwich ELISAs
Western blot antibodies

<table>
<thead>
<tr>
<th>Target</th>
<th>Capture Antibody [dilution] (Supplier)</th>
<th>Detection Antibody [dilution] (Supplier)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gpx3</td>
<td>αGpx3 [diluted 1/400 in 5% milk] (R&amp;D #AF4199)</td>
<td>αGoat [diluted 1/20,000 in 5% milk] (Sigma A8919)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>αGoat IgG-CFL 680† [diluted 1/20,000 in 5% milk] (Santa Cruz Biotechnology sc-516245)</td>
</tr>
<tr>
<td>Gpx3</td>
<td>αV5 [diluted 1/5000 in 5% milk] (Invitrogen #R96025)</td>
<td>αMouse [diluted 1/5000 in 5% milk] (Enzo Life sciences ADI-SAB-100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>αM-IgGxBP-CFL 680† [diluted 1/5000 in 5% milk] (Santa Cruz Biotechnology sc-516180)</td>
</tr>
<tr>
<td>ECSOD</td>
<td>αECSOD [diluted 1/5000 in 5% milk] (R&amp;D #MAB34201)</td>
<td>αMouse [diluted 1/5000 in 5% milk] (Enzo Life sciences ADI-SAB-100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>αM-IgGxBP-CFL 680† [diluted 1/5000 in 5% milk] (Santa Cruz Biotechnology sc-516180)</td>
</tr>
</tbody>
</table>

Table 2.4 Antibody pairings for Western blotting. †Fluorescently labelled antibody. Milk (Marvel dried skimmed) was dissolved in PBS-T at 5% (w/v)

Flow cytometry antibodies

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Fluorophore</th>
<th>Isotype</th>
<th>Quantity per test (µg/10⁶ cells)</th>
<th>Catalogue number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>Pacific blue</td>
<td>Mouse IgG1κ</td>
<td>1.25</td>
<td>#300417</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD14</td>
<td>APC-eFlour 780</td>
<td>Mouse IgG1κ</td>
<td>0.5</td>
<td>#47-0149-42</td>
<td>eBiosciences (ThermoFisher)</td>
</tr>
<tr>
<td>CD16</td>
<td>e-Flour 450</td>
<td>Mouse IgG1κ</td>
<td>0.25</td>
<td>#48-0168-42</td>
<td>eBiosciences (ThermoFisher)</td>
</tr>
<tr>
<td>CD19</td>
<td>Pacific blue</td>
<td>Mouse IgG1κ</td>
<td>1.25</td>
<td>#302232</td>
<td>Biolegend</td>
</tr>
</tbody>
</table>

Table 2.5 Antibodies used for flow cytometry
2.5 Cell line tissue culture and stimulation

Cells were frozen in suspension in FBS + 10% (v/v) DMSO (Sigma-Aldrich, Gillingham, UK). Cell stocks kept in liquid nitrogen were rapidly thawed by swirling in a 37°C water bath and then added to complete cell culture media.

2.5.1 Cell lines

Cell lines were passaged 2-3 times per week as required and are listed in table 2.6

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Species / cell type</th>
<th>Culture medium</th>
<th>Specific supplements</th>
<th>Antibiotic selection</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV2</td>
<td>Murine microglial</td>
<td>DMEM</td>
<td>10% FBS, Pen/Strep</td>
<td>N/A</td>
<td>Adherent</td>
</tr>
<tr>
<td>CHO-S</td>
<td>Chinese hamster ovary</td>
<td>Freestyle CHO medium</td>
<td>L-glutatime (1mM)</td>
<td>N/A</td>
<td>Suspension. Grown with constant rotation</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney</td>
<td>DMEM</td>
<td>10% FBS, Pen/Strep</td>
<td>N/A</td>
<td>Adherent. Contains SV40 large T-antigen</td>
</tr>
<tr>
<td>HEK293T ECSOD</td>
<td>Human embryonic kidney</td>
<td>DMEM</td>
<td>10% FBS, Pen/Strep</td>
<td>100µg/mL Zeocin</td>
<td>Adherent. HEK293T with stably transfected ECSOD cDNA.</td>
</tr>
<tr>
<td>THP-1</td>
<td>Human monocyte</td>
<td>RPMI</td>
<td>10% FBS, Pen/Strep</td>
<td>N/A</td>
<td>Suspension</td>
</tr>
<tr>
<td>THP1-Blue</td>
<td>Human monocyte</td>
<td>RPMI</td>
<td>10% FBS, Pen/Strep</td>
<td>100µg/mL Zeocin</td>
<td>Suspension. THP-1 with stably integrated NF-κB-inducible SEAP reporter</td>
</tr>
</tbody>
</table>

Table 2.6 Cell lines used in this study. For future reference ‘complete’ media is considered as media supplemented with 10% FBS and 1% (v/v) Pen/strep.

**BV2 cells**

BV2 cells are a murine microglial cell line generated by the infection of primary microglial cell cultures with a v-raf/v-myc oncogene carrying retrovirus (J2). The cells were produced in the laboratory of Blasi et al. (1990). BV2 cells were a kind gift from Dr Egle Solito (Queen Mary, University of London).
BV2 cells were used for optimisation of ROS assays. Cells were cultured in 75cm³ flasks and maintained in complete DMEM at a density of around 1.0-1.5 x 10⁶ cells/mL. Cells were allowed to reach 70-80% confluence before being split for maintenance or used for experiments.

**CHO-S cells**
Chinese hamster ovary (CHO) cells are a mammalian epithelial cell line derived from the ovary of the Chinese hamster and immortalised by Dr Theodore Puck in the late 1950s. The CHO-S cell line is commercially available and widely used in biological and medical research for the production of recombinant proteins. The freestyle CHO-S cell line used in this study were derived from the CHO-S cell line and are adapted to suspension culture. The cells were purchased from Invitrogen.

In this study, CHO-S cells were used for the production of recombinant GPx3. CHO-S cells were maintained at between 0.2-2.0 x 10⁶ cells/mL in Freestyle CHO media. During culture, cells were constantly rotated at between 125-150 rpm on an orbital shaker at 37°C and 8% CO₂. Cells were allowed to reach required density (~2x10⁶ cells/mL) and then either split for expansion or transfected.

**HEK293T and HEK293T-ECSOD**
HEK293 cells were generated in 1973 by transformation of human embryonic kidney (HEK) cells using sheared adenovirus 5 DNA in Alex Van der Eb’s laboratory (Graham et al. 1977). The HEK293T cells used in this study were created following the stable transfection of the HEK293 cell with a temperature-sensitive mutant of the SV40 large T-antigen, which becomes inactive at 40°C. Like the CHO-S cell line, HEK293T cells are easily transfected with DNA and the SV40 large T antigen enables the replication of the plasmid within the cell, thus increasing the yield of recombinant protein.

HEK293T cells were previously stably transfected with cDNA encoding for human ECSOD and were a kind gift from Professor Yuti Chernajovsky (Queen Mary, University of London). Both cell lines were maintained at 0.5-1.0 x 10⁶ cells/mL in 150cm³ flasks in 20mL of complete DMEM. Cells were cultured twice weekly to prevent over confluence. HEK293T-ECSOD cells were supplemented with 150µg/mL zeocin to ensure the selection of cells transfected with the ECSOD plasmid. Cells were grown until around 80% confluence and then either split to continue growth or used to express desired proteins.
**THP-1 and THP-1 Blue cells**

THP-1 cells are a human monocyte cell line derived from an acute monocytic leukemia patient in 1980 in Keiya Tada’s laboratory (Tsuchiya et al. 1980). The THP-1 and THP1-Blue cells a kind gift from Professor Yuti Chernajovsky (Queen Mary, University of London).

THP-1 Blue cells were derived from the human THP-1 monocyte cell line with a stable integration of an NF-κB-inducible SEAP reporter construct. Cells were maintained in suspension between 0.5-1.0 x 10^6 cells/mL in complete RPMI and split twice weekly to prevent over confluence. Cells were grown until confluent and then either split for maintenance or used in an experiment. THP1-Blue cells were supplemented with 100µg/mL zeocin to ensure selection of cells transfected with the NF-κB-inducible SEAP reporter construct.

**2.5.2 Cell line stimulations**

**BV2 cell stimulations**

BV2 cells were seeded at 1x10^5 cells/mL and incubated at 37ºC for 24 hours to allow adherence. Following adherence media was replaced with 100µL of complete DMEM or Optimem containing stimulants for 2-24 hours depending on experimental protocol.

**HEK293T cell stimulations**

HEK293T cells were used for expression of recombinant proteins. See section 2.13

**THP-1 and THP1-Blue cell stimulations**

THP-1 and THP1-Blue cells were seeded at 0.5-1.6x10^5 cells/well in 96-well plates depending on the experiment. Stimulants at 2X concentration were added to the wells to result in a final volume of 50-100µL in each well and cells incubated for 6-18 hours at 37ºC, 5% CO₂. Following stimulation, cells were spun at 300xg for 10 minutes at room temperature to ensure cells were at the bottom of the well and 10-60µL supernatant was collected for experimental analyses.

For some initial experiments, THP-1 cells were treated with 20nM phorbol 12-myristate 13-acetate (PMA) for 24 hours to initiate adherence and monocyte differentiation towards a macrophage-like phenotype. Following 24 hours, media was removed, cells were washed once with complete RPMI and stimulants added to cells for a further 18 hours.
2.6 Ethical approval and recruitment of patients

Ethical approval
Local R&D approval was obtained from Brighton and Sussex University Hospital (BSUH). The National Research Ethics Service (NRES) committee North West – Lancaster approved the study under the project reference 15/NS/0083. Human tissue was handled under the Brighton and Sussex Medical School HTA tissue licence (HTA #12561).

Recruitment of study participants and patient characteristics
All patient samples were collected by a qualified physician or a dedicated research nurse/research practitioner at the Clinical Research Investigation Unit (CIRU) from Brighton and Sussex University Hospitals (BSUH) either during their visit to the clinic or on a later date if more convenient for the patient. Patients from A&E, acute medical units or hospital wards were also recruited. Patients received a patient information sheet and consent form providing details about the study. All those involved in recruitment and collection of samples were GCP trained.

Samples were anonymised prior to collection and transportation to the laboratory at Brighton and Sussex Medical School. Relevant clinical parameters were recorded by a member of CIRU on an anonymised clinical proforma. Patient characteristics are provided in appendices 1 and 2.

Gout samples were collected from any patient who had previously been diagnosed as having this crystalopathy. Patients were recruited from rheumatology clinics at the Royal Sussex County Hospital (RSCH) or were invited to participate in the study by letter from local GP surgeries.

CKD patients were recruited from haemodialysis clinics at the Sussex Kidney Unit at the RSCH. All patients were undertaking tri-weekly sessions of haemodialysis. Blood was taken prior to entering the dialysis machine.

RA patients were recruited from rheumatology clinics at RSCH. A database containing information for these patients was scanned weekly and potential individuals for recruitment were identified.
Healthy samples were collected from volunteers that were registered on the RSCH’s donor list. Individuals who had taken any form of treatment in the week prior to collection were excluded.

A sample size calculation was run via G*Power using IL-1β secretion data from monocytes isolated from healthy controls versus RA patients (data generously provided by Dr Sandra Sacre). The G Power output demonstrated that a sample size of 34 patients per group was required to give a power of 90%. A statistician (Dr Patricia de Winter, University of London, personal communication) was also contacted for advice regarding this calculation.
2.7 Processing of whole blood

2.7.1 Isolation of monocytes from single donor plateletpheresis residues
Single donor plateletpheresis residues were purchased from the National Blood Service and eluted into 50mL falcon tubes. The blood was diluted to 40mL with unsupplemented RPMI and 20mL layered on top of 16mL Lympholyte-H. The blood-lympholyte mix was centrifuged at 905xg for 30 minutes at room temperature with no braking. PBMCs were extracted from the lymphocyte/plasma interphase layer and washed three times in 50mL unsupplemented RPMI at 300xg for 10 minutes at room temperature. Following the third wash, cells were resuspended in 25mL of unsupplemented RPMI. All 25mL was layered on top of 16mL of isosmotic Percoll solution, made up of 0.8mL 1.5M NaCl, 7.2mL Percoll (Sigma-Aldrich, Gillingham, UK) and 8mL of PBS citrate (Invitrogen, Paisley, UK). The blood-Percoll mix was spun at 905xg for 15 minutes without braking. Monocytes were extracted from the Percoll/RPMI interphase layer, and washed three times with 50mL unsupplemented RPMI at 300xg for 10 minutes (Repnik et al. 2003). Cells were then resuspended in 50mL of RPMI containing 5% FBS and counted by haemocytometer. Cells were frozen at 20x10^6 cells/mL in FBS + 10% (v/v) DMSO in 0.5mL aliquots for future use.

For future reference, monocytes isolated from single donor plateletpheresis residues will be referred to as ‘primary human monocytes’.

2.7.2 Isolation of monocytes from patient blood
Whole blood was collected in 10mL BD Vacutainers spray coated with K2EDTA (Becton Dickinson, Plymouth, UK). Around 30mL of blood was obtained, pooled and made up to 40mL with unsupplemented RPMI. 20mL of blood was slowly layered on to 16mL Lympholyte-H cell separation media (Cederlane, Burlington, Canada) and centrifuged at 905xg for 30 minutes without braking. PBMCs at the lymphocyte/plasma interphase were extracted and washed three times in 50mL of unsupplemented RPMI. Cells were centrifuged at 300xg for 10 minutes at room temperature. Each donor usually yielded around 30-40x10^6 PBMCs, as counted by haemocytometer.

Following the final wash, PBMCs were resuspended in 80μL of MACs buffer per 10^7 cells and transferred to a 1.5mL Eppendorf. 20μL of positive selection anti-human CD14 conjugated microbeads (Miltenyi Biotec, Aubern, CA, USA) per 10^7 cells was added. PBMCs were incubated in the presence of CD14 beads for 15 minutes at 4°C with end over end rotation. Cells were then washed once in MACs buffer, spun at 300xg for 10 minutes and resuspended in 0.5mL MACs buffer. The cells were passed through a MACs magnetic
separation (MS) column within a magnetic field. The column was washed three times with 0.5mL MACs buffer to remove unbound cells. The column was then removed from the magnetic field and CD14 positive monocytes were flushed out in 1mL RPMI supplemented with 5% FBS using a plunger. Lymphocytes, i.e. cells unbound to the column, were collected, spun at 300xg for 10 minutes and resuspended in 1mL Qiazol (QIAGEN, Germantown, MD, Germany) and stored at -20ºC for future use. Monocytes eluted from the MS column were counted using a haemocytometer. This process usually yielded around 10-15% of PBMC count. Monocytes were stimulated for 18 hours in RPMI + 5% FBS with different combinations of Pam3 (100ng/mL), MSU crystals (10-20mg/dL) and CPPD crystals (2.5-5mg/dL). With the remaining monocytes, 1.0-2.0 x10^6 cells were separated, spun down and resuspended in 0.5mL Qiazo for future qPCR experiments. Any unused cells were stored in 0.2-0.5mL FBS + 10% DMSO in liquid nitrogen until future use.

For future reference, monocytes isolated from patient blood will be referred to as ‘patient monocytes’ and disease specified where applicable.

2.7.3 Isolation of serum from patient blood
In addition to the 30mL whole blood collected in K2EDTA coated vacutainers, 10mL of blood in silica coated vacutainers was also obtained. Blood was allowed to clot for a minimum of one hour at room temperature and then centrifuged at 1300xg for 10 minutes. Serum was collected and aliquoted into 0.5mL aliquots, taking care not to disturb the cell pellet, before being stored at -80ºC for future use.

2.7.4 Flow cytometry
Flow cytometry was used to confirm successful isolation of monocytes via each method. Samples were thawed into RPMI + 5% FBS and spun at 300xg for 10 minutes. Cells were then washed three times in DPBS supplemented with 5% FBS. Cells were counted and resuspended to 5x10^6 cells/mL following the final wash step. 5x10^5 cells were then transferred to round bottom polystyrene tubes and 2.5µL of antibodies (table 2.5) were added to respective tubes. Cells were incubated with antibodies for 20 minutes at 4ºC before being washed twice with PBS + 5% FBS. Cells were finally fixed with 200µL of 1% (w/v) paraformaldehyde and 10,000 events were collected by the flow cytometer (LSR II – BD Biosciences). Stained cells were compared to unstained control cells. Two gating strategies were applied to account for viable cells and also to ensure that only singlet cells were applied to the LSR II whilst discounting doublets. A Fluorescence Minus One Control (FMO) method was applied using control cells and cells stained for CD14 or CD16 only, allowing gates to be set for each population.
2.8 Stimulation of primary human monocytes and patient monocytes

Primary human monocytes
Primary human monocytes from single donor plateletpheresis residues were thawed and washed twice in RPMI with 5% FBS prior to seeding. Cells were seeded in 96-well cell culture plates (Nunc, Thermo Fischer Scientific, Massachusetts, USA) at a density of 0.5-3.0x10^5 cells/well depending on experiment. For most stimulation experiments cells were seeded at 3.0x10^5 cells/well. For experiments in chapter 4 using antioxidants, cells were seeded at 0.5x10^5 cells/well for ROS measurements and 2.0x10^5 cells/well for stimulations to allow matched experiments. After seeding, cells were incubated for 2 hours at 37ºC, 5% CO₂ to allow adherence. Following this, cell media and any non-adherent cells were removed and fresh RPMI (5% FBS) or OPTI-Mem containing stimulants was added. Cells were stimulated for 6-18 hours depending on experimental design before collection of cell supernatants. Cell conditioned media were stored at -20ºC.

Patient monocytes
Patient monocytes were stimulated in 384-well tissue culture plates (Corning Inc, Corning, NY, USA). Cells were seeded immediately after isolation at a concentration of 4x10^4 cells per well in 25µL in RPMI containing 5% FBS. Stimulants at 2X concentration were added to the wells to result in a final volume of 50µL in each well. Cells were stimulated with a mixture of Pam3 (100ng/mL), MSU crystals (10mg/dL) or CPPD crystals (2.5mg/dL). Each experimental condition was repeated with 3-6 technical replicates depending on cell yield. Supernatants were collected after 18 hours of stimulation and stored at -20ºC for future ELISA analysis. Cellular viability was routinely assessed by MTT analysis (see section 2.9).
2.9 Cell viability assays

Various cell viability assays were utilised to assess the health of cells following stimulation. Cell viability was routinely assessed directly after collection of conditioned media from cells.

MTT viability assay

Tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was dissolved at 5mg/mL in DPBS to form a 10X stock and stored at 4ºC, protected from light until future use. On the day of use, the 5mg/mL stock was diluted to 0.5mg/mL in culture medium and 25µL (384-well) or 100µL (96-well) added directly to cells and incubated at 37ºC, 5% CO₂ for at least 4 hours. The MTT assay is based on the reduction of yellow tetrazolium salts into purple formazan crystals by metabolically active cells using the dehydrogenase enzymes and NADH as a cofactor (Mosmann 1983). Formazan production is therefore proportional to metabolism and is used as an indirect measure of viability. The purple formazan crystals were solubilised by addition of a stop solution made up of 10% SDS in 0.01M HCl and incubated for at least 4 hours to allow crystals to dissolve. Dissolved crystals result in a dark purple solution which can be quantified by measuring absorbance at 590nm using Synergy HT plate reader.

CellTitre-Glo™ Viability Assay

The CellTitre-Glo™ (CTG) viability assay is a more sensitive measure of cell viability and as such was used in some experiments where toxicity of compounds was unknown. This allowed optimisation of the concentrations of stimulants determining a concentration at which it should be used. The CTG assay is also a measure of metabolism and similar to the MTT assay provides an indirect measure of viability. Addition of CTG reagent to cells results in cell-lysis and release of adenosine triphosphate (ATP) from the cell. The luciferin in the CTG reagent is consequently mono-oxygenated to oxyluciferin, a process catalysed by the presence of Mg²⁺, ATP and molecular oxygen, resulting in light production which can be quantified by measuring luminescence using Synergy HT plate reader.

In this study, 100µL CTG reagent were added to cells in wells of a 96-well plate and incubated at room temperature for 20 minutes protected from light to allow the luminescent signal to stabilise. Following this, 40-60µL of cell lysate was transferred to a white opaque plate and chemiluminescent signal measured.

CellTitre Blue™ viability assay

A CellTitre Blue™ (CTB) viability assay (Promega) was used to determine viability of non-adherent THP-1 monocytes. Following stimulations, cell culture plates were spun at 300xg
for 10 minutes to pellet cells and 40-60μl supernatant removed for future use. The volume in each well was made up to 90μL using complete culture medium and then 18μL/well of CTB reagent added per well. The contents of wells were mixed by gentle tapping of the plate before incubation for two hours at 37°C, 5% CO₂. During this incubation period, metabolically active cells reduce the resazurin compound in CTB to fluorescent resorufin. Fluorescence was measured (ex530/em590nm) using Synergy HT plate reader.
2.10 Enzyme-linked immunosorbent assays (ELISA)

**Human IL-1β ELISA**

Cytokine concentration in cell conditioned media were measured by ELISA. Nunc-immuno MaxiSorb™ flat bottom 96-well plates (Thermo-Scientific) or 384-well plates (SantaCruz Biotechnology) high binding ELISA plates were coated with 50µL or 20µL capture antibody in ELISA coating buffer respectively and incubated overnight at 4°C. Plates were then washed three times with ELISA wash buffer (PBS, 0.01% (v/v) Tween-20) using a BioTek ELx50 plate washer (BioTek, Winooski, VT, USA). ELISA plates were blocked with 100µL (96-well) or 50µL (384-well) 2% (w/v) BSA in PBS for one hour at room temperature on a plate rocker. Plates were then washed three times with ELISA wash buffer. Recombinant protein standards or samples (table 2.3) were diluted in ELISA reagent diluent (0.5% BSA) and 50µL (96-well) or 20µL (384-well) were added to wells in triplicate for two hours at room temperature. Plates were then washed three times with ELISA wash buffer and 50µL or 20µL biotin-conjugated detection antibody, diluted in ELISA reagent diluent, was added to wells and plates incubated for further 1-2 hours at room temperature on a rocker. Plates were again washed three times before addition of 50µL or 20µL streptavidin-horseradish peroxidase (HRP) conjugate (R&D systems, Minneapolis, USA), diluted as specified by the supplier in ELISA reagent diluent. Plates were incubated for 1 hour at room temperature protected from light. Wells were then washed six times with ELISA wash buffer to ensure complete removal of unbound streptavidin-HRP conjugate. Colour development was performed using 20µL or 50µL of Tetramethylbenzidine (TMB) microwell peroxidase substrate solution (Insight Biotechnology, Middlesex, UK). Once colour had developed sufficiently, the reaction was halted by addition of 50µL or 20µL ELISA stop solution (6% (v/v) H₂SO₄ in distilled water). Optical densities were measured at 450nm using Synergy HT plate reader.

Samples were sometimes diluted up to 10 times prior to addition to ELISA plate. Sample dilution was completed in ELISA diluent (0.5% BSA).

**Human TNFα ELISA**

Nunc-immuno MaxiSorb™ flat bottom 96-well plates high binding ELISA plates were coated with 50µL capture antibody in ELISA coating buffer respectively and incubated overnight at 4°C. Plates were then washed three times with ELISA wash buffer. ELISA plates were blocked with 200µL 1% (w/v) BSA in PBS for 1 hour at room temperature on a plate rocker. Plates were washed three times with ELISA wash buffer. Recombinant protein standards (table 2.3) or samples were diluted in ELISA reagent diluent (1% BSA) and 50µL added to
wells in triplicate for 2 hours at room temperature. Plates were then washed three times with ELISA wash buffer and 50µL biotin-conjugated detection antibody, diluted in ELISA reagent diluent, was added to wells and plates incubated for further 2 hours at room temperature on rocker. Plates were again washed three times before addition of 50µL streptavidin- HRP conjugate (R&D systems, Minneapolis, USA), diluted as specified by the supplier in ELISA reagent diluent. Plates were incubated for 30 minutes at room temperature protected from light. Wells were then washed six times with ELISA wash buffer to ensure complete removal of unbound streptavidin-HRP conjugate. Colour development was performed using 50µL of TMB microwell peroxidase substrate solution. Once colour had developed sufficiently, the reaction was halted by addition of 50µL or 20µL ELISA stop solution (6% (v/v) H₂SO₄ in distilled water). Optical densities were measured at 450nm using Synergy HT plate reader.

Samples were often diluted up to 10 times prior to addition to ELISA plate. Sample dilution was completed in ELISA diluent (1% BSA).

**Human glutathione peroxidase 3 ELISA**

A human glutathione peroxidase 3 (Gpx3) ELISA assay kit was purchased (AdipoGen) and used to determine concentration of recombinant Gpx3 following transfections. The ELISA was processed according to the manufacturer instructions. Briefly, standards and solutions (100µL) were added to pre-coated ELISA plates for 1 hour at 37°C. Plates were then washed 3 times with ELISA wash buffer and detection antibody (100µL) added for 1 hour at 37°C. Plates were again washed 3 times and streptavidin-HRP (100µL) added to plates for 1 hour at 37°C. Plates were then washed 5 times with ELISA wash buffer and 100µL substrate TMB added for 20 minutes at room temperature protected from light. Once colour had developed sufficiently, the reaction was halted by addition of 100µL ELISA stop solution. Optical densities were measured at 450nm using Synergy HT plate reader.
2.11 Quantitative polymerase chain reaction (qPCR)

Quantitative polymerase chain reaction (qPCR) was used to examine the expression of messenger RNA (mRNA) of various genes of interest (GOI) in human monocytes.

Sample preparation and RNA extraction
For monocytes isolated from patient blood, 0.5-2.0 x10^6 cells were used for qPCR. Following isolation from blood, cells were centrifuged at 300xg for 10 minutes and resuspended in 0.5mL QIAzol lysis reagent and mixed until completely lysed. QIAzol stocks were stored at -20ºC until further use.

RNeasy mini kits (Qiagen, UK) were used for RNA extraction from patient monocytes. Firstly, 100µL chloroform was added to samples and mixed manually by vigorous shaking for 15 seconds. The qiazol:chloroform mix was then centrifuged for 15 minutes at 12,000xg at 4ºC. This centrifugation allowed the separation into two phases; an upper aqueous phase containing RNA and a lower organic phase containing denatured proteins. The upper aqueous phase was collected and mixed with 1.5 volumes of 100% ethanol and solution transferred to an RNeasy mini spin column. The RNeasy column was centrifuged at 8000xg at room temperature to allow the ethanol:RNA mix to pass through. This was followed by an on-column DNase wipe-out step, which efficiently removes genomic DNA, and subsequent washes with buffers provided. Following these washes RNA was eluted by addition of RNase-free water and centrifugation of the column (8000xg for 1 min).

Primary human monocytes from single donor plateletphoresis residues were added to wells in a 24-well plate at a density of 1.0-2.0x10^6 cells and allowed to adhere for 2 hours. Non-adherent cells and cell media were removed and replaced with 0.5mL media containing required stimulants. Samples were incubated for required amount of time and cell supernatants collected and centrifuged to account for cells that had lost adherence during the incubation. Non-adherent and adherent cells were washed twice with 0.5mL PBS, lysed in 0.4mL QIAzol and then combined to produce a 0.8mL QIAzol aliquot and stored at -20ºC until future use.

THP-1 monocytes were added to wells in a 6 well-plate at a density of 1.5x10^6 cells at a volume of 0.5mL. Stimulants at 2X concentrations were added to wells to create a final well volume of 1.0mL. Cells were incubated for the specified time. Culture media were collected, washed with PBS following centrifugation and lysed in QIAzol (0.25mL). Wells were also
washed and lysed in QIAzol (0.25mL) to collect cells that had adhered during stimulation. QIAzol solutions were then combined and stored at -20°C until future use.

For primary human monocytes and THP-1 cells, RNA was extracted without the use of RNeasy columns because of greater availability of RNA. Chloroform was added to samples and mixed manually by vigorous shaking for 15 seconds and samples incubated on ice for 2-3 minutes. The samples were then spun at 12,000xg for 15 minutes at 4°C to induce the separation of phases. The top aqueous phase was collected, mixed with an equal volume of ice-cold isopropanol and stored at 4°C overnight to precipitate the RNA. Glycogen (50µg/mL) was added to samples from primary human monocytes to aid RNA precipitation. Samples were then centrifuged at 12,000xg for 30 minutes at 4°C and the RNA precipitated pellet washed in 75% (v/v) ethanol for 5 minutes on ice. The samples were then centrifuged at 7500xg for 15 minutes at 4°C. The supernatant was removed, and the RNA pellet left at room temperature until completely dry. RNA was resuspended in 20µL RNase-free H2O and incubated at 60°C for 10 minutes to aid RNA solubilisation.

RNA purity (ratio 260/280) and concentration were measured using a Nanodrop (ThermoScientific).

RNA integrity was assessed using an Agilent 2100 Bioanalyser. 65µL of Agilent RNA 6000 Nano gel was mixed with 1µL of RNA 6000 Nano dye, vortexed and centrifuged at 13,000xg for 10 minutes at room temperature. An Agilent RNA 6000 Nano chip was added to a priming station and 9µL of the gel-dye mix added to the Nano chip. The chip was pressurised using a syringe for 30 seconds to allow for the gel to spread through the chip. Following which, 9µL of the gel-dye mix was added to each of the two remaining ‘G’ marked wells and then 5µL of RNA 6000 Nano marker added to each sample and ladder well single well. RNA was denatured for 2 minutes at 70°C and then 1µL of RNA added to each well. An RNA ladder was also added and the chip vortexed for 60 seconds. The chip was added to the Agilent 2100 Bioanalyser and total eukaryote RNA measured. An RNA integrity number (RIN) was obtained for each RNA by calculating the ratio of 18S and 28S RNA (Schroeder et al. 2006).

RNA integrity was examined in 10% of the RNA used throughout the study. RNA isolated using the RNeasy spin columns achieved a RIN of 7 or more, with the majority obtaining a RIN of 8-9. RNA isolated without using RNeasy columns achieved a RIN of 6.5-9.2.
Reverse transcription
qPCR requires complementary DNA (cDNA) as a template and RNA was therefore reverse transcribed prior to analysis by qPCR. A two-step reverse transcription was employed using the QuantiTect reverse transcription kit (Qiagen, UK). Following RNA quantification, 250ng of RNA was transferred to 0.2mL PCR tubes and made up to 6µL with RNase-free water. In some samples RNA yield did not allow 250ng RNA to be used for reverse transcription. For these samples, a total of 6µL of RNA was transferred to 0.2mL PCR tubes. 1µL of gDNA wipe-out buffer was added and samples heated at 42ºC for 2 minutes and then 4ºC for 2 minutes using a Veriti 96-well thermocycler (Applied Biosystems). Immediately after, 3µL of reverse transcription master mix from the QuantiTect reverse transcription kit, comprising of reverse transcriptase buffer, an RT primer mix and reverse transcriptase, was added to the PCR tubes and samples were incubated for 15 minutes at 42ºC, 3 minutes at 95ºC and then returned to 4ºC. The reverse transcriptase buffer contains deoxyribose nucleoside triphosphatases which form the building blocks of DNA. The RT primer mix consisted of a blend of oligo-dT and random primers, which allows high cDNA yields from all regions of RNA transcripts. The QuantiTect Reverse Transcriptase kit combines a blend of Omniscript and Sensiscript reverse transcriptases which have a high affinity for RNA and are capable of catalysing cDNA synthesis from a wide range of RNA concentrations. The cDNA was stored at -80ºC until future use. Prior to qPCR, cDNA was diluted 1:10 in 5µg/mL of transfer RNA to reduce adherence of the cDNA to plastic tubes.

qPCR
Absolute quantification was used to calculate exact copy number of the genes analysed in each experimental sample. For absolute qPCR a standard curve of known copy number for each reference gene (RG) or gene of interest (GOI) was added to each qPCR plate alongside the experimental samples. Assays and standards were designed and purchased from qStandard (London, UK), who provided melt curves showing single peak amplification for each RG and GOI. Experimental samples were normalised to the expression of RGs for that sample. All standards were serially diluted 1:10 in 0.5µg/mL of tRNA, creating a standard curve ranging from 10^1-10^7 copy numbers.

Copy numbers for GOIs and RGs were determined by qPCR using the QuantiFast SYBR green PCR kit and run on a Stratagene Mx3000 thermocycler (Agilent Technologies, UK) or Rotor-Gene thermocycler (Qiagen, Germany) under the following thermocycling program; 40 cycles of 95ºC for 15 seconds and 60ºC for 30 seconds with an initial cycle of 95ºC for 15 minutes.
Reference genes and gene of interests used, and their primer sequences are described in tables 2.7 and 2.8, respectively.

Following thermocycling, the copy number for each GOI and RG was calculated by interpolation from standard curves on the same plate. Copy numbers for GOIs were normalised using a normalisation factor calculated in GeNorm using copy numbers calculated from reference gene standard curves for $B2M$ and $RPL32$. $B2M$ and $RPL32$ were used as the expression of these genes were shown to be stable in the experimental conditions used.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Beta-2 Microglobulin (<em>B2M</em>)</td>
<td>5’-ctctctttttggcctggag-3’</td>
<td>5’-accagacatagcaatcag-3’</td>
</tr>
<tr>
<td>Human Hypoxanthine phosphoribosyltransferase 1 (<em>HPRT1</em>)</td>
<td>5’-ggaaagaatgtcttgatttggaag-3’</td>
<td>5’-gggtcttttcaccagcaagc-3’</td>
</tr>
<tr>
<td>Human peptidylprolyl isomerase A (<em>PPIA</em>)</td>
<td>5’-cgaggaaacgtctacttagc-3’</td>
<td>5’-caccctgacacataaacctg-3’</td>
</tr>
<tr>
<td>Human phosphoglycerate kinase 1 (<em>PGK1</em>)</td>
<td>5’-cctcggaccgaatcacc-3’</td>
<td>5’-agcagcctaatcctcctgt-3’</td>
</tr>
<tr>
<td>Human ribosomal protein L32 (<em>RPL32</em>)</td>
<td>5’-catctctttctggcatcat-3’</td>
<td>5’-acccttgtgtaatgcctct-3’</td>
</tr>
</tbody>
</table>

Table 2.7 List of qPCR reference genes tested and their primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Caspase-1 (<em>CASP1</em>)</td>
<td>5’-atgcctgtcctgtgatgtgg-3’</td>
<td>5’-ctttactttctgtgccacaga-3’</td>
</tr>
<tr>
<td>Human Interleukin-1β (<em>IL1B</em>)</td>
<td>5’-gtaatgacaaaataatcctgtggccttg-3’</td>
<td>5’-tttggaatctacactcttcagc-3’</td>
</tr>
<tr>
<td>Human NLRP3 (<em>NLRP3</em>)</td>
<td>5’-gagatgagccgaaggtgggtct-3’</td>
<td>5’-gctcttacgttacttctgactct-3’</td>
</tr>
<tr>
<td>Human ASC (<em>PYCARD</em>)</td>
<td>5’-gctaacgtgtcgcgtcgacat-3’</td>
<td>5’-ccactcaacgttggtgacct-3’</td>
</tr>
<tr>
<td>Human Superoxide Dismutase 2 (<em>SOD2</em>)</td>
<td>5’-aagtaccaggagggtggtgg-3’</td>
<td>5’-cgtcagttctcttaaacctgtgc-3’</td>
</tr>
<tr>
<td>Human Thioredoxin Reductase 1 (<em>TXNRD1</em>)</td>
<td>5’-tatggggcaatttattgtgctctcaca-3’</td>
<td>5’-gctccaacaaccaggggtcttac-3’</td>
</tr>
</tbody>
</table>

Table 2.8 List of qPCR genes of interest tested and their primer sequences
2.12 Measurement of reactive oxygen species

Various methods for measuring ROS were tested. ROS assays were first developed in cell lines and then transferred into primary human monocytes and further optimised if necessary.

2.12.1 2',7'-dichlorofluorescin diacetate (DCFDA) Assay

A DCFDA assay (Abcam, Cambridge, UK) was used to measure intracellular ROS. Cells were seeded at 5x10^4 cells/well in complete RPMI. Following adherence, cell growth medium was removed and cells were stained with 5-20µM DCFDA in Optimem for 30 minutes at 37°C. After 30 minutes, cells were washed once with Optimem and 100µL of Optimem containing stimulants were added per well. Cells were incubated for a further 6 hours before fluorescence was measured (ex485nm/em585nm) using Synergy HT plate reader. During staining, DCFDA enters the cell and becomes deacetylated by cellular esterases to a non-fluorescent compound. This non-fluorescent compound becomes oxidised by ROS into 2',7'–dichlorofluorescein (DCF) which is highly fluorescent.

2.12.2 Nitroblue Tetrazolium (NBT) assay

A Nitroblue Tetrazolium (NBT) assay was used as a measure of intracellular superoxide anion production, according to published protocols (Falasca et al. 1993; Freeman et al. 1999). Cells were seeded in 96-well plates and allowed to adhere if necessary. Growth media was replaced with Optimem containing stimulants and 10-25µg/mL NBT. Cells were incubated for a further 2-6 hours at 37°C to allow reduction of NBT, resulting in the formation of formazan crystals. Following stimulation, cells were washed with 100% methanol to ensure fixation and then wells were air dried for 10 minutes. Formazan crystals were dissolved in a mixture of 1-part 2M potassium hydroxide (KOH) to 1.167 parts DMSO. Plates were gently shaken for 10 minutes at room temperature to aid dissolving of crystals and absorbance at 690nm was measured using Synergy HT plate reader.

2.12.3 ROS-Glo™

A ROS-Glo assay kit (Promega) was used to measure hydrogen peroxide generation in cell stimulations (Kelts et al. 2015). Cells were seeded at 5x10^4 cells/well (cell density determined following optimisation experiments) and allowed to adhere if necessary. Cells were then treated for 2-6 hours with stimulants in Optimem with the addition of a H_2O_2 substrate (working conc. 25µM). Measuring H_2O_2 has various benefits. Firstly, it has the longest half-life of all ROS. In addition, various other ROS are converted to H_2O_2 and thus can be used as a general measure of fluctuations to ROS level. During culture with cells,
the H$_2$O$_2$ substrate added alongside stimulants reacts with H$_2$O$_2$ to generate a luciferin precursor. With the addition of ROS-Glo detection reagent, composed of Ultra-Glo recombinant luciferase and D-cysteine, the luciferin precursor is converted to luciferin by the D-cysteine and the luciferin in turn reacts with the Ultra-Glo recombinant luciferase to generate a luminescent signal that is proportional to H$_2$O$_2$ concentration. The luciferin detection mix was added for 20 minutes and then transferred to a white plate for measurement of luminescence using Synergy HT plate reader.
2.13 Expression and purification of recombinant proteins

2.13.1 Expression of recombinant glutathione peroxidase 3 (Gpx3)

Gpx3 bacterial inoculation and plasmid purification

The gene coding for human Gpx3 was previously cloned in the plasmid pcDNA4 with C-terminal V5 and His-tags. Cloning was performed by Dr L Mullen (BSMS). *Escherichia coli* (*E. coli*) transformed with pcDNA4-Gpx3 was streaked on agar plates containing carbenicillin (200µg/mL). After overnight incubation at 37°C, a single colony was picked and cultured in 200mL of Luria Bertani (LB) broth with 200µg/mL carbenicillin. Following overnight growth, the broth was decanted into 50mL falcons and the *E. coli* pelleted by centrifugation at 3000xg for 20 minutes. Pellets were frozen at -80°C until required.

Extraction of plasmid DNA was completed using a DNA Endo-free Maxiprep kit (Qiagen, UK) according to manufacturer’s instructions. Bacterial pellets were lysed and clarified. Plasmid DNA was isolated by applying cleared lysates to ion-exchange resin and elution using the buffer supplied. DNA was precipitated with isopropanol and DNA pellets were solubilised in endotoxin-free dH2O.

DNA yield and quality (A260/280 ratio) was determined using a NanoDrop spectrophotometer.

Expression of recombinant Gpx3

HEK293Ts were plated at 2x10^6 cells per 10cm^2 plate in complete DMEM and incubated for 24 hours to allow adherence. After 24 hours, 9mL of DMEM containing 161nM sodium selenite (Na₂SeO₃) (final concentration 145nm) was added to the cells, and then supplemented with 1mL transfection mix. Transfection mixes were prepared 30 minutes prior to use and contained DNA and polyethyleneimine (PEI) at a ratio of 1:3 in Optimem. Incubation prior to use allowed complex formation between DNA and PEI. Cells were incubated with transfection mix for 24 hours at 37°C, 5% CO₂, after which cells were washed with serum-free DMEM and incubated in serum-free DMEM containing 145nm sodium selenite (NaS) and 50ng/mL TNFα for 48 hours. TNFα was added to drive expression of Gpx3. After 48 hours, supernatants containing recombinant Gpx3 were collected, centrifuged at 300xg for 10 minutes to remove cell debris, and stored at -80°C until future use.
Recombinant Gpx3 expression was also completed in CHO-S cells. CHO-S cells were grown to 2x10^6 cells/mL in 5 litre vented and baffled shaker flasks. On the day prior to transfection, CHO-S cells were split 1:2 into two flasks to achieve 300mL of 1x10^6 cells/mL and allowed to grow for 24 hours at 37°C, 8% CO_2 under constant rotation. During this 20-hour incubation cell numbers doubled, achieving a confluence of 2x10^6 cells/mL. Transfection mixes containing DNA and PEI at a ratio of 1:3 in Optimem were prepared 30 minutes prior to use and 5mL of transfection mix containing 600µg of plasmid DNA was added per flask. Finally, flasks were supplemented with NaS (145nM) and then incubated for 72 hours. After 72 hours, supernatants containing recombinant Gpx3 were collected, centrifuged at 300xg for 10 minutes to remove cell debris, and stored at -80°C until future use.

**Stable transfection of HEK293T cells with Gpx3**

**Plasmid digestion and purification**

For the stable transfection of HEK293T cells, the Gpx3-pcDNA4 plasmid was initially linearised using the restriction enzyme *PvuI* (New England Biolabs, Ipswich, UK), which cleaved the plasmid within the ampicillin resistance region. For the digestion, 10µg of Gpx3-pcDNA4 was combined with 10µL of 10X NEBuffer (1M NaCl, 500mM Tris-HCl, 100mM MgCl_2, 1mg/mL BSA, pH 7.9) and 2µL of *PvuI* and the volume was made up to 100µL with ddH_2O. The solution was mixed and incubated at 37°C overnight to allow digestion of the plasmid.

The linearisation of the plasmid was assessed using a ethidium bromide (EtBr) agarose gel made up of 0.8% agarose, 0.5% TAE buffer (24.2% Tris base, 5.7% acetic acid and 50mM sodium-EDTA). The agarose solution was heated and then poured into a mould and allowed to solidify, once almost completely solidified EtBr was added (0.01%). Samples were combined with 5µL of SDS-PAGE loading buffer (see section 2.14), added to the gel and gels were subjected to electrophoresis at 100millivolts (mV) for 2 hours. Following electrophoresis, the gel was transferred to a G-Box Syngene fluorescent gel imager (Syngene) and imaged by UV.

The linearised plasmid were purified using a QIAprep miniprep kit (Qiagen) according to the manufacturer’s instructions. Briefly, 100µL of the linearised plasmid was mixed with 500µL buffer P1 and then 50mM sodium acetate added. The sample was added to a QIAquick column and centrifuged at 17,000xg for 1 minute. The column was washed once with 750µL
buffer PE and then eluted with 30µL dH₂O. Four lots of elution were combined to achieve a cDNA concentration of 333ng/µL.

HEK293T stable transfection and selection
HEK293T cells were plated at 2x10⁶ cells/mL in 10cm³ plates in complete DMEM and incubated at 37°C, 5% CO₂ for 24 hours. Transfection mixes were prepared 30 minutes prior to use and contained DNA and PEI at a ratio of 1:3 in Optimem. Incubation prior to use allowed complex formation between DNA and PEI. Cells were incubated with transfection mix for 48 hours at 37°C, 5% CO₂, after which the medium was replaced with serum-free culture medium containing 400µg/mL zeocin and 145nM NaS. The addition of zeocin allowed selection of cells with incorporation of the plasmid DNA. Colonies were selected, isolated by placing a greased ring around them, and trypsinised before being transferred to a well of a 6 well plate. A total of 12 clones were selected. Clones were continuously selected with zeocin (250-500µg/mL to ensure selection of cells containing the plasmid.

2.13.2 Purification of recombinant Gpx3

Dialysis
Cell conditioned media containing recombinant Gpx3 were dialysed against 15-20 litres of PBS. Dialysis membranes with a molecular weight cut-off of 10kDa was used to remove salts and phenol red from the cell conditioned media.

Affinity purification
Gpx3 was cloned with a C-terminal V5+His-tags, enabling purification using nickel affinity chromatography with HIS GraviTrap TALON columns (GE Healthcare, Buckinghamshire, UK). Columns were equilibrated with 10 column volumes (CV) of sodium phosphate binding buffer (50mM sodium phosphate pH 8.0, 300mM NaCl, 0.01% Tween-20). Dialysed supernatants were filtered and degassed and then applied to the column at a rate of 1mL/minute. After applying dialysed supernatants, columns were washed twice with 10mL of sodium phosphate binding buffer to remove any loosely bound contaminants, followed by washes containing increasing concentrations of imidazole (20mM and 50mM) to remove loosely bound protein. Finally, protein was eluted in 10 CVs of elution buffer (300mM imidazole). The flow-through, i.e. solution passed through column, for each washing step was collected and analysed by Western blot to examine protein elution.
Buffer exchange and concentration
Eluted fractions of purified recombinant Gpx3 were concentrated to 2.5mL using Vivaspin-20 centrifugal concentrators (Satorius, AG, Göttingen, Germany) and protein was then applied to PD-10 desalting columns (GE Healthcare). PD-10 desalting columns were used to remove salts (imidazole) and were equilibrated with 5x5mL of DPBS prior to addition of protein. Protein was allowed to enter the column bed and then eluted by addition of 3.5mL of DPBS. The 3.5mL elution was then further concentrated using a fresh Vivaspin-20 centrifugal concentrator to around 0.3-0.5mL, aliquoted into small volumes and stored at -80°C until future use.

Recombinant Gpx3 protein quantification
Protein concentration for Gpx3 was determined by ELISA as described in section 2.10.

Recombinant Gpx3 enzyme activity assay
Gpx3 activity was assessed using a HT Glutathione Peroxidase assay kit (Trevigen, Gaithesbury, MD). Samples were diluted up to 100 times in 1X assay buffer prior to addition to plate and measured in duplicate. For each reaction, 1X assay buffer (140µL) and 10X reaction mix (20µL) were added per well. Next, 20µL of the positive control (glutathione peroxidase), negative control (1X assay buffer) and pre-diluted samples were added. The assay was initiated by the addition of 20µL of cumene hydroperoxide to each well. Plates were immediately transferred to BioTek Synergy HT microplate reader, prewarmed to 25°C, and absorbance was measured at 340nm every minute for 10 minutes. Activity was determined using slopes from standards and samples, then corrected for dilution factors.

The HT glutathione peroxidase assay relies on the oxidation of glutathione (GSH) to its oxidised form (GSSG). Glutathione reductase then reduces oxidised glutathione back to GSH form. This reaction uses NADPH as an electron donor, resulting in its oxidation to NADP⁺. The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340nm and the rate of decrease in A₃₄₀ was directly proportional to the glutathione peroxidase activity in the sample.
2.13.3 Expression of recombinant extracellular superoxide dismutase (ECSOD)

ECSOD was produced using HEK293T cells which were stably transfected with cDNA coding for human ECSOD. Cells were cultured until 80% confluent in complete DMEM supplemented with 150µg/mL Zeocin. Cells were then washed in serum-free DMEM and incubated for 72-96 hours. Supernatants were collected, centrifuged at 300xg for 10 minutes to remove cell debris, and stored at -80°C until future use.

2.13.4 Purification of recombinant ECSOD

Dialysis
Supernatants containing recombinant proteins were dialysed against at 15-20 litres Tris-EDTA pH 7.4 ECSOD. Dialysis membrane with a molecular weight cut-off of 10kDa allowed removal of salts and phenol red from cell conditioned media solution, being replaced with respective buffers prior to affinity purification.

Affinity purification
ECSOD contains a C-terminal heparin binding domain (HBD), allowing its purification using heparin-Sepharose columns. 1mL heparin-Sepharose columns were purchased (GE healthcare, Buckinghamshire, UK) and equilibrated at 4°C with Tris-EDTA (20mM Tris, 10mM EDTA) using a peristaltic pump pumping buffer through at a rate of 1mL/min for 10-15 minutes. Dialysed protein solutions were filtered and degassed for 15-30 minutes, and then applied to column at 1mL/min using a peristaltic pump, taking care that no air bubbles entered column. Washing and elution of protein was performed using an ÄKTA Protein Purification fast protein liquid chromatography (FPLC) system (GE Healthcare, Buckinghamshire, UK) or peristaltic pump. Following loading of the protein, the column was washed with 10 mL Tris-EDTA to remove loosely bound contaminants, before 10mL washes with Tris-EDTA containing increasing concentrations of NaCl (100mM, 175mM, 250mM, 375mM, 500mM). Protein was eluted at higher concentrations of salt, with a majority being eluted in the final two steps, allowing for collection of 10mL pure ECSOD protein in Tris-EDTA NaCl buffer. All flow-through was collected in fresh 15mL falcon tubes and analysed by Western blot. Purification using the AKTA provided the benefit of real-time analysis of protein elution by measurement of UV absorbance (280nm) at each step and a peak in UV absorbance demonstrating the elution of protein.

Buffer exchange and concentration
Eluted fractions of purified recombinant ECSOD were concentrated and desalted as previously described for recombinant Gpx3 (section 2.13.2)
Recombinant ECSOD protein quantification

A human ECSOD ELISA kit was initially tested for quantification of recombinant ECSOD concentration. However, a standard curve could not be created from the standard provided and was therefore deemed unusable. Instead, protein concentration of ECSOD was determined by Pierce BCA protein assay (Fisher Scientific, UK). Protein purity was determined to be very high in 500mM NaCl elution following Coomassie staining of polyacrylamide gels (see section 2.14). Although a generic measure of protein, the purity of ECSOD as confirmed by Coomassie meant that the BCA assay would measure mostly recombinant BCA. For the BCA assay, samples were diluted up to 50 times in DPBS and added to 96-well plate in a final volume of 25μL. Standards made up of BSA diluted in DPBS ranging from 2mg/mL through 0.0078mg/mL were made prior to use and added to plate at final volume of 25μL. Reactions were initiated by addition 200μL of a working reagent according to the manufacturer instructions. Plates were incubated for 30 minutes at 37ºC before absorbance was read at 570nm. A standard curve was plotted and protein concentration was interpolated and dilution accounted for.

Recombinant ECSOD enzyme activity assay

ECSOD activity was assessed using a HT Superoxide Dismutase assay kit (Trevigen, Gaithersbury, MD). Samples were diluted up to 100 times in 1X SOD buffer prior to use. Standards were prepared in 1X SOD buffer ranging from 10-0.1 units activity. Samples and standards were added to wells in 25μL and combined with 150μL of master mix containing 15μL 10X SOD buffer, 5μL WST-1 reagent, 5μL xanthine oxidase and 125μL dH2O. The reaction was initiated by addition of 25μL 1X xanthine solution and plate immediately transferred to plate reader prewarmed to 25ºC. Absorbance was measured at 450nm every minute for 10 minutes.

The HT Superoxide dismutase assay relies on the conversion of xanthine to uric acid and H2O2 by xanthine oxidase. This reaction results in the formation of superoxide radical ions, which in turn result in the conversion of WST-1 to WST-1 formazan. WST-1 formazan can be measured at 450nm. The presence of highly active SOD results in the reduction of superoxide ion concentrations and thus inhibits the conversion of WST-1 to WST-1 formazan. Thus, the inhibition of WST-1 formazan formation is directly proportional to SOD activity. Activity of recombinant ECSOD was calculated from the change in absorbance at 450nm and calculation of recombinant ECSOD required to inhibit 50% of WST-1 formazan formation.
2.13.5 Testing of antioxidant proteins on monocytes

THP-1 cells or primary human monocytes were exposed to antioxidant enzymes in the presence of NLRP3 stimuli to investigate the effect of inhibiting ROS in activation of the inflammasome. All stimulations containing antioxidants were completed in serum-free Optimem to reduce any effect of serum on antioxidant activity.

THP-1 monocytes were seeded at 0.5x10^5-1.6x10^5 cells/well in 20-25µL of Optimem depending on experimental design. Antioxidant enzymes at 2X concentrations were added on top to increase well volume to 40-50µL. Cells were preincubated in each antioxidant for 1 hour at 37°C, 5% CO₂. After the 1-hour incubation, stimulants at 2X or 5X, depending on experimental design, were added to wells and antioxidant concentration was maintained. Cells were stimulated for 6-18 hours before measurement of ROS, NF-κB or cytokine secretion.

Primary human monocytes were seeded at 0.5x10^5-2.0x10^5 cells/well in RPMI supplemented with 5% FBS and allowed to adhere for 2 hours. After 2 hours, any non-adherent cells were removed along with cell culture media. Cells were washed once and antioxidants in Optimem were added in 40-50µL depending on experimental design. Cells were preincubated in antioxidants for 1 hour. After preincubation, stimulants at 2X concentrations were added to wells. Media was not replaced in order to save antioxidant enzymes and limit washing of primary human monocytes. Primary human monocytes were stimulated for 6-18 hours before measurement of ROS, NF-κB or cytokine secretion.

For parallel ROS-Glo and stimulation experiments, cells were seeded at 0.5x10^5 cells/well for ROS measurement and 1.6x10^5 (THP-1 monocytes) or 2.0x10^5 cells/well (primary human monocytes) for stimulations. Cell viability was assessed for both plates.

Recombinant antioxidant enzymes

Recombinant ECSOD was used at a working concentration of 10-500 U/mL. Recombinant Gpx3 was used at a working concentration of 1-2.5 U/mL.

Catalase

Commercial catalase was purchased from Sigma at an activity of 2000U/mg. On the day of use, 4mg of catalase was weighed out and diluted in 1mL of Optimem and then sterile filtered to give a stock of 8000U/mL. This was then further diluted to required working concentrations; 25-500U/mL.
**N-Acetyl cysteine**

N-Acetylcysteine was purchased from Sigma. On the day of use 82mg was weighed out and dissolved in 940µL of DPBS. This stock solution was very acidic and as such needed to be adjusted to pH 7.4 using 50-54µL of 10M NaOH. The pH was checked using pH strips and then volume made up to 1000µL before sterile filtering. This yielded a stock solution of 500mM NAC which was diluted to a working concentration of 5-10mM.

**2.13.6 Endotoxin testing of recombinant proteins**

**Limulus amebocyte lysate gel clot assay**

Recombinant proteins were tested for endotoxin levels using a Limulus Amebocyte Lysate (LAL) assay (Lonza). This is an extremely sensitive indicator of endotoxin presence within biological samples. The presence of gram-negative bacterial endotoxin catalyses the activation of a proenzyme in the LAL. This results in the formation of enzyme coagulase which in turn hydrolyses bonds within the clotting protein coagulogen, forming a gelatinous clot and indicating the presence of endotoxin. An endotoxin standard was tested and the assay was able to detect as low as 0.125EU/mL of endotoxin. Recombinant proteins were diluted to working concentrations or 10-fold greater and 250µL transferred to each lysate vial. Samples were incubated at 37ºC for 1 hour to allow the reaction to occur. Vials were then removed and gently inverted through 180ºC. A positive result was characterised by formation of a firm gel that remained in place upon rotation of the vial. A negative result was indicated by the absence of a clot. Lysates that showed increased turbidity or viscosity but did not clot were considered negative results.

**Endotoxin removal**

Endotoxin contamination of recombinant antioxidant proteins were removed using a Proteus NoEndo™ micro (µ) Spin column kit according to the manufacturer’s instructions. Briefly, 200µL of NoEndo™ resin slurry was added to a batch incubation chamber and washed by centrifugation at 13,000xg for 20 seconds. The NoEndo™ resin was washed twice with 600µL DPBS at 13,000xg for 20 seconds to remove ethanol from the slurry mix. Up to 600µL of samples were then added to the batch incubation chamber and incubated for 2-6 hours at room temperature. Samples were vortexed for 15 seconds every 15 minutes to ensure mixing of the NoEndo™ resin with the sample. After the incubation time, samples were eluted by centrifugation at 13,000xg for 20-60 seconds. Sample protein concentration was tested by BCA afterwards and stock activity adjusted. Samples were also retested for endotoxin by LAL assay before use in cell stimulations.
2.14 Western blotting

Recombinant proteins were analysed by Western blot throughout the process of recombinant protein production to determine the quantity and quality of the proteins throughout the purification procedure.

Sample preparation
90µL SDS-PAGE buffer (6X) was combined with 10µL of the reducing agent beta mercaptoethanol (βMe) prior to sample preparation. Under these conditions the protein becomes denatured and unfolds allowing better separation on SDS-PAGE gels. SDS also creates a negative coat across the denatured proteins, allowing them to run evenly through the gel. 30µL of samples were combined with 5µL SDS-PAGE buffer + βME solution and samples were heated for 5 minutes at 100ºC.

For some samples, protein required concentration prior to addition to the SDS-PAGE gels. On the day before use, 0.5mL of protein containing solution was mixed with 1.5mL of ice-cold acetone. The acetone-protein mix was vortexted to mix thoroughly and stored overnight at -20ºC to allow protein precipitation. The following day, samples were spun at 17,000xg for 10 minutes. The supernatant was subsequently removed and protein pellets air dried for at least 30 minutes at room temperature. Once completely dry, the protein was resuspended in 35µL of SDS-PAGE buffer containing βME, as described above, and added directly to SDS-PAGE gels.

Preparation of acrylamide gels
Polyacrylamide gels were poured the day before use and stored at 4ºC. Gels were made in two parts. Firstly, the running gel (table 2.9) was poured and allowed to polymerise at room temperature for at least 30 minutes. Secondly, the stacking gel (table 2.9) was poured on top of the polymerised running gel. A comb was added to the stacking gel to form wells for protein loading. The stacking gel was allowed to polymerise at room temperature for 15 minutes and the gel was then left at 4ºC overnight.
<table>
<thead>
<tr>
<th>Running gel (12%)</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5mL 4X running gel buffer</td>
<td>1.25mL 4X stacking gel buffer</td>
</tr>
<tr>
<td>100µL 10% SDS</td>
<td>50µL 10% SDS</td>
</tr>
<tr>
<td>4mL acrylamide/bis</td>
<td>835µL acrylamide/bis</td>
</tr>
<tr>
<td>100µL 10% ammonium persulphate solution (APS)</td>
<td>75µL 10% APS</td>
</tr>
<tr>
<td>3.4mL distilled water</td>
<td>2.86mL distilled water</td>
</tr>
</tbody>
</table>

**Table 2.9 Preparation of Western blot acrylamide gels**

**Electrophoresis**

Once polymerised, the gel was loaded with samples. For each gel, a lane was loaded with 5µL of PageRuler Plus Prestained molecular weight ladder (ThermoFisher Scientific). The samples were then loaded to the gel in 5-35µL volumes. The gel was submerged in 1X running buffer (see section 2.2) and subjected to electrophoresis at 150V for 1-1.5 hours.

**Transfer to nitrocellulose membrane**

Following electrophoresis proteins in the gel were transferred to nitrocellulose membrane by electroblotting. A sandwich was made which surrounded the gel and nitrocellulose membrane with filter paper and sponges. The sandwich was immersed in 1X Western blot transfer buffer (see section 2.2) and an ice pack added to maintain a low temperature. Electroblotting was run at 400mA for 90 minutes at 4ºC.

**Blocking, antibody incubation and luminescent detection**

Nitrocellulose membranes were blocked for at least one hour in 5% milk in PBS-T (0.1% Tween-20) and then probed with primary antibody (table 2.4) overnight at 4ºC. The following day, membranes were washed 3 times in PBS-T for 15 minutes. Secondary antibodies, conjugated with HRP, were added to the membrane for one hour at room temperature with constant agitation. Blots were then washed 3 times with PBS-T.

Some blots were developed by chemiluminescence using photographic film. For these samples, blots were exposed to enhanced chemiluminescence made with 1:1 (v/v) luminol and hydrogen peroxide (H₂O₂) for 2 minutes with constant mixing. The blots were then moved to an x-ray film cassette and exposed in the dark against autoradiography film for required amount of time for bands to appear. The film catches the light emitted by the HRP and thus highlights the presence of a band when protein is present. Films were processed using a Konica SRX101A processor (Konica Minolta, UK).
Some blots used fluorescently labelled secondary antibodies (table 2.4) and were developed using a LiCor Odyssey Fc machine. Following exposure to secondary antibody, membranes were washed three times with PBS-T and then added to the LiCor Odyssey Fc which allows fluorescent detection at 700 and 800 channels.

**Coomassie brilliant blue staining**
Protein purity was assessed using Coomassie Brilliant Blue staining. Following electrophoresis of the polyacrylamide gels, gels were collected, washed once with ddH$_2$O for 1 hour and then stained with Simple Blue Safe Stain (Invitrogen, CA, USA) for at least 2 hours at room temperature under constant agitation. Gels were then washed for at least one hour in ddH$_2$O to remove background staining and gels were scanned (Epson perfection, V350 PHOTO) for analysis.
2.15 Experiments with soluble uric acid

Preparation of soluble uric acid
Uric acid (Sigma) was dissolved in cell culture medium (RPMI + 5-10% FBS or OptiMEM) at a concentration of 60-100mg/dL on the day of use. Incubation at 37°C for 0.5-1 hours with occasional mixing was necessary to achieve fully solubility. Solutions of uric acid were sterile filtered (0.22µM) and diluted to required concentrations. When maintained at 37°C, as for cell stimulations, no precipitation of the uric acid was seen by eye or under the microscope (Fig. 2.1 A). Precipitation of the soluble uric acid only occurred when the solution was left at room temperature for 24-48 hours (Fig. 2.1 B).

Figure 2.1 Soluble uric acid remains in solution in vitro. (A) No precipitation of soluble uric acid was observed when uric acid was maintained at 37°C. (B) Precipitation of uric acid was only seen when solutions were left at room temperature for 24-48 hours. Crystals were added to cell culture following precipitation to provide crystal visualisation.

Preincubation of monocytes in soluble uric acid
For some experiments, monocytes were exposed to soluble uric acid (30mg/dL) for 24-48 hours prior to stimulation. For these experiments, THP-1 monocytes were allowed to reach confluence (~1x10⁶ cells/mL in 20 mL) and flasks were split in half between two new flasks (10mL in to each). The volume was topped up to 20 mL with 10mL of 60mg/dL soluble uric acid made up in complete RPMI. On the day of stimulation, cells were collected, centrifuged to remove old media and resuspended in fresh complete RPMI or OptiMEM containing fresh soluble uric acid at the required concentration. Primary human monocytes were exposed to soluble uric acid (30mg/dL) for up to 16 hours prior to stimulation. Cells were plated at required density in 96-well plates, allowed to adhere and then RPMI (5% FBS) containing uric acid (30mg/dL) added. After preincubation, the media was replaced with RPMI (5% FBS) or OptiMEM containing soluble uric acid at the required concentration.
2.16 Measurement of NF-κB activation

NF-κB activation was measured using THP1-Blue monocytes (section 2.5) which secrete an NF-κB inducible SEAP reporter following NF-κB activation. The alkaline phosphatase (AP) in SEAP was detected using QUANTI-Blue™ colorimetric enzyme (Invivogen) which, in the AP changes QUANTI-Blue colour from pink to purple/blue.

THP1-Blue monocytes were stimulated for 2-18 hours in 96-well plates and then plates were centrifuged (10 min, 300xg) to move cells to bottom of the well. 10µL of cell-conditioned media was transferred to 190µL of pre-warmed QUANTI-Blue (37°C). Plates were incubated for 1 hour at 37°C to allow colour change to intensify and absorbance measured at 630nm using Synergy HT plate reader.
2.17 Analysis of patient serum

Serum was isolated from patient whole blood as described in section 2.7.3

**Serum total protein concentration**

Total concentrations of protein in patient serum was measured using a bicinchoninic acid (BCA) assay (Pierce). Samples were diluted 1:250 in DPBS prior to addition to 96-well plate. This dilution was determined by initial optimisation experiments. Protein concentration was interpolated from a standard curve made up of BSA ranging from 0.0156 to 2 mg/mL. Diluted samples and standards (25µL) were added to 96-well plate and reaction initiated by addition of 200µL working reagent according to manufacturer's instructions. Plates were incubated at 37°C for 30 minutes hidden from light. After which absorbance was read at 570nm using Synergy HT plate reader.

**Serum uric acid measurement**

An Amplex Red Uric Acid / Uricase Assay Kit (ThermoFisher, Paisley, UK) was used for quantifying patient serum uric acid concentrations. A standard curve of uric acid was prepared in 1X reaction buffer provided ranging from 0.78-50µM uric acid. Serum was diluted 1:25 in 1X reaction buffer and 50µL of samples and standards were added to a 96-well plate. A working solution of 100µM Amplex red, 0.4U/mL HRP and 0.4U/mL uricase was made up in 1X reaction buffer and reactions initiated by addition of 50µL of this mix per well. Samples were incubated for 30 minutes at 37°C protected from light. Fluorescence was measured at excitation and emission wavelengths of 530 and 590nm, respectively, using Synergy HT plate reader. Uric acid concentrations in samples were interpolated from the standard curve and then converted from µM to mg/dL by dividing µM values by 59.484.

This assay relies on the conversion of uric acid to hydrogen peroxide, allantoin and carbon dioxide, a process catalysed by the enzyme uricase. The H₂O₂ in the presence of HRP reacts with the Amplex red reagent, resulting in the generation of the red-fluorescent product resorufin.

**Serum Gpx3 activity**

Gpx3 activity in human serum was measured using the Gpx3 activity assay as described in section 2.13.2. Samples were diluted 1:5 in 1X assay buffer. A limited number of samples were assayed at any given time to ensure rapid addition of cumene hydroperoxide. Fresh standards were used for each set of samples. Gpx3 activity was determined as rate of A450
reduction per minute and then normalised to overall protein concentration in serum measured by BCA assay.

**Serum ECSOD activity**

ECSOD activity in human serum was measured using the Gpx3 activity assay as described in section 2.13.4. Samples were assayed without dilution following an initial dilution titration. ECSOD activity was determined as percentage inhibition of WST-1 reagent formation and then normalised to overall protein concentration in serum measured by BCA assay.

**Serum total antioxidant capacity**

The total antioxidant capacity (TAC) in patient serum was measured using a commercially available Antioxidant Assay Kit (Abnova). This assay is based on the reduction of Cu\(^{2+}\) to Cu\(^{+}\) when exposed to an antioxidant. The Cu\(^{+}\) then forms a coloured complex with a dye reagent, which can be quantified by measuring absorbance at 570nm. Trolox ranging from 4.68-1000µM was created in distilled water and used to generate a standard curve. Samples were assayed without prior dilution. Samples and standards were added to wells of a 96-well plate in 20µL volumes and reactions initiated by the addition of 100µL of working reagent per well. Plates were incubated at room temperature for 10 minutes and then the absorbance at 570nm measured using Synergy HT plate reader. TAC was expressed as µM of Trolox equivalents and then normalised to total protein concentration in serum measured by BCA assay.

For some experiments, intracellular TAC was measured. Cells were thawed and washed once with PBS before centrifugation (300xg for 10 minutes) and resuspension in 100µL ice-cold NP-40 cell lysis buffer. Samples were maintained on ice for 30 minutes and vortex at 10-minute intervals. Samples were spun at 1500xg for 5 minutes and lysate collected and stored at -80°C until the day of use. TAC was measured as above and normalised to protein content measured by BCA.
2.18 Statistical analyses

Each data set was tested for normality using a Shapiro-Wilk test. If the data were normally distributed a One-way analysis of variance (ANOVA) followed by a Tukey’s multiple comparison test or a t-test were used for analysis. If the data were not normally distributed then significance was determined using a non-parametric Kruskal-Wallis with Dunn's multiple comparison post-hoc test or using a Mann-Whitney U test. For some data sets, significance was determined using multiple t-tests corrected using a Holm-Sidak multiple comparison post-hoc test. All correlations were analysed using a non-parametric Spearman's correlation test. The level of significance was set at $p<0.05$ for all tests. All analyses were conducted using GraphPad Prism version 7 (GraphPad software, San Diego, CA). For some optimisation experiments data is presented as technical replicates within an experiment, and therefore statistical analysis was not completed. Technical replicates are shown as mean ± standard deviation. Biological replicates are shown as mean ± standard error of the mean. Dr Patricia de Winter (University of London, personal communication) was contacted for advice regarding statistics (de Winter and Cahusac 2014).
Chapter 3 - The role of reactive oxygen species in activating the NLRP3 inflammasome
3.1 Introduction

The NLRP3 inflammasome has been extensively studied since its discovery in 2002 due to its involvement in a range of diseases (Mangan et al. 2018). Despite all this research various important questions remain. Inflammasome activation is a two-step process requiring two distinct signals (discussed in more detail in section 1.4). An initial ‘priming’ signal regulates the inflammasome on a transcriptional and translational level via NF-κB activation, increasing NLRP3 and pro-IL-1β protein levels (Bauernfeind et al. 2009). A second signal provided by an NLRP3 activator is subsequently required to initiate activation of the NLRP3 inflammasome, ultimately resulting in its oligomerisation and the cleavage of caspase-1 into its active form. Numerous pathogen-associated factors incite priming of the inflammasome via activation of NF-κB, often occurring downstream of TLR ligation (Xing et al. 2017). Lipopolysaccharide (LPS) and Pam3Csk4 (Pam3) are two of the most commonly used pathogen-associated molecular patterns (PAMPs) for investigating NLRP3 signalling in vitro. LPS and Pam3, ligands for TLR4 and TLR2 respectively, induce the transcription of pro-IL-1β and NLRP3 via NF-κB signalling (Snodgrass et al. 2013; Ghonime et al. 2014). The priming signal provides an important regulatory checkpoint in NLRP3 activation, and its importance is signified by the lack of response to known NLRP3 stimulants in the absence of a priming stimuli (Sutterwala et al. 2014).

A wide range of structurally diverse stimuli have been shown to be inflammasome inducers, including bacterial, viral and fungal pathogens, pore-forming toxins and sterile crystals (Martinon et al. 2006; Cassel and Sutterwala 2010; Lamkanfi and Dixit 2011) (Table 1.1). In contrast to NLRP3 priming, mechanisms involved in NLRP3 activation are less well defined. Three main events (see also section 1.4) have been shown to occur upstream of NLRP3 activation in response to several of its stimuli: ionic flux, lysosomal degradation and the production of ROS. Potassium (K+) efflux has emerged as a common signal in NLRP3 activation with studies demonstrating amelioration of NLRP3-dependent IL-1β secretion following inhibition of K+ efflux (Pétrilli et al. 2007). Since then, potassium efflux has been shown to occur in response to many of the known NLRP3 stimuli, yet mechanisms upstream and downstream remain elusive (Munoz-Planillo et al. 2013). Lysosomal destabilisation and the release of cathepsin B into the cytosol is a mechanism thought to link particulate matter, such as MSU crystals, to NLRP3 activation. Cathepsin B release from damaged lysosomes has been shown to activate the inflammasome and chemical inhibition of this cysteine protease has been shown to attenuate NLRP3 activation (Halle et al. 2008; Hornung et al. 2008). However, cathepsin-B-deficient murine macrophages have shown normal inflammasome activation in response to particulate matter, bringing its role in NLRP3
activation into doubt (Dostert et al. 2009). Despite evidence for both of these pathways, the
mechanism by which they result in inflammasome activation remains unknown.

The relationship between ROS and inflammation is complex, yet the two are closely related
with ROS being able to induce inflammatory mechanisms and inflammation causing an
increase in ROS production. Investigations into the role of ROS in NLRP3 activation has
gained much interest in the field with many hypothesising that ROS is the common signal
linking the inflammasome to many of its stimuli. Indeed, various studies provide evidence
for a fundamental role for ROS during activation of the NLRP3 inflammasome (Hewinson
et al. 2008; Zhou et al. 2010b). However, many other studies also demonstrate a
dispensable role for ROS in NLRP3 activation or even demonstrate a potential role for
antioxidant systems in the process (Meissner et al. 2008; Masters et al. 2010; Bauernfeind
et al. 2011). A particular example of the contrasting role of ROS in NLRP3 activation is
highlighted by the evidence both for and against NOX-dependent ROS in inflammasome
activation. Numerous early studies demonstrated a role for NOX-dependent ROS
generation in NLRP3 activation by ATP, silica and asbestos, showing that blocking NOX
activity attenuated IL-1β secretion downstream of NLRP3 activation (Cruz et al. 2007;
Dostert et al. 2008). The role of NOX-dependent ROS was quickly challenged, however,
with studies showing normal NLRP3 activation in PBMCs from patients with CGD, a disease
characterised by dysfunction in NOX enzyme activity (Van Bruggen et al. 2010; van de
Veerdonk et al. 2010). Evidently, the exact role of ROS in NLRP3 activation remains
uncertain, but could provide a mechanism that links the inflammasome to its many
structurally diverse stimuli.

The contrasting findings regarding NLRP3 activation mean that the exact mechanism
remains to be fully elucidated. There are a few potential reasons for these conflicting results.
Firstly, it seems possible that NLRP3 activation is cell-type specific so the use of a range of
cell lines, human primary cells and mouse cells in published work makes interpretation of
results more difficult. For example, numerous redox differences have been noted between
human monocyte cell lines and primary human monocytes (Carta et al. 2011). There are
also differences in the behaviour of immune cells from mouse and human origin. Mouse
cells require the canonical two-step mechanism for NLRP3 activation with cells being
dependent on an initial priming signal prior to an NLRP3 activating signal. In human
monocytes however, a priming signal alone is able to induce low levels of IL-1β secretion
via autocrine signalling or via the monocytes constitutively active caspase 1 (Netea 2009;
Wang et al. 2013). A further complication in the investigation of ROS in NLRP3 activation
is the difficulty of measuring ROS in vitro and the use of non-specific ROS inhibitors. DPI,
for example, is a chemical inhibitor of ROS used in several studies which concluded that increased ROS production plays a fundamental role in NLRP3 activation (Cruz et al. 2007; Bauernfeind et al. 2011). However, DPI is not specific and exerts numerous off-target effects which make interpretation of results difficult. For example, DPI has been shown to block calcium channels which have also been suggested to play a role in activation of the inflammasome (Tazzeo et al. 2009). Therefore, the role of ROS in NLRP3 activation remains unknown and requires further investigation.

The aim of this chapter was to establish an in vitro model for activating the NLRP3 inflammasome in human monocytes and to use this to investigate changes to ROS levels in these cells during stimulation. The THP-1 cell line were initially used for optimisation of experiments. Cell lines are pure populations of cells and therefore provide more consistent and reproducible results than primary human cells, which can be inherently different from one another (Kaur and Dufour 2012). Furthermore, THP-1 cells have been used to investigate the NLRP3 inflammasome in many studies and therefore these cells provide a useful opportunity to compare results found here to other studies. Once fully optimised the experiments were then carried out in primary human monocytes. Three assays for measuring ROS were selected, the widely used DCFDA assay as well as two other ROS measurements strategies: Nitroblue Tetrazolium (NBT) and ROS-Glo™. By measuring changes in ROS levels in human monocytes during exposure to NLRP3 priming (Pam3) and activating (MSU and CPPD crystals) stimuli we aimed to examine the importance and necessity of ROS in activation of the inflammasome, particularly in primary human monocytes.
3.2 Use of an in vitro model for investigating NLRP3 activation

An in vitro model was designed to activate the NLRP3 inflammasome via the canonical pathway and IL-1β secretion used as an indirect measure of NLRP3 activation. For initial experiments, THP-1 cells were plated and exposed to phorbol 12-myristate 13-acetate (PMA) (20nM) for 24 hours to initiate adherence and differentiation towards a macrophage-like phenotype. Cells were then washed once with RPMI, preincubated for 4 hours with Pam3 (100ng/mL) and stimulated with MSU or CPPD crystals for 18 hours.

Crystals were titrated to obtain optimal concentrations. MSU crystals at high concentrations (>15mg/dL) resulted in ~25% reduction in cell viability (Fig. 3.1 A). Similarly, CPPD crystals at concentrations greater than 2.5mg/dL also caused cell toxicity (Fig. 3.1 B). IL-1β secretion was measured in cell culture supernatants where cell death was minimal. In agreement with canonical activation of the inflammasome, addition of crystals alone did not result in IL-1β secretion (Fig. 3.1 C-D). The addition of Pam3 alone resulted in a small increase in IL-1β secretion which was substantially increased with the addition of crystalline stimuli. MSU at 5mg/dL did not have an additive affect over Pam3-induced IL-1β, but 10mg/dL induced a robust increase in IL-1β secretion when added alongside Pam3 (Fig. 3.1 C). This was not a result of cell death as viability at this concentration was comparable to unstimulated and Pam3 stimulated controls (Fig. 3.1 A). CPPD crystals at 1mg/dL induced a small increase in IL-1β secretion which was dramatically increased in cells stimulated with 2.5mg/dL CPPD. This massive increase is likely related to the reduction in cell viability seen at this concentration, particularly compared to Pam3 controls (Fig. 3.1 B). MSU and CPPD were used at 10mg/dL and 2.5mg/dL respectively for future experiments. Finally, to test whether maintaining Pam3 concentration during crystal stimulation would alter IL-1β secretion, cells were preincubated for 4 hours and then stimulated for 18 hours with crystals alone or crystals added alongside Pam3 in fresh cell media. The removal of Pam3 made no difference to MSU or CPPD crystal-induced IL-1β secretion (Fig. 3.1 E).

In early experiments, THP-1 cells were treated with PMA which caused the cells to undergo monocyte differentiation and acquire phenotypical characteristics which closely resemble human macrophages (Park et al. 2007). This protocol was used to induce adherence so that experimentally they could be treated in a similar manner to primary human monocytes, which naturally adhere to tissue culture plastic. However, most experiments were completed using THP-1 cells not treated with PMA to model monocyte responses and optimise experiments prior to testing in primary human monocytes (section 3.3). For clarity, THP-1 cells not treated with PMA will be referred to as THP-1 monocytes.
Figure 3.1 MSU and CPPD crystal optimisations in PMA-treated THP-1 cells. PMA-treated THP-1 cells were preincubated with Pam3 for 4 hours and then stimulated for 18 hours with increasing concentrations of (A&C) MSU or (B&D) CPPD crystals. (E) PMA-treated THP-1 cells were preincubated for 4 hours in Pam3 and then stimulated for 18 hours with crystals alone or with crystals added alongside Pam3. IL-1β secretion was measured by ELISA and viability was assessed using an MTT assay. Data represent means for three technical replicates ± standard deviation (SD).
3.3 Inhibition of NLRP3 inflammasome activation

To confirm that MSU and CPPD crystal-induced IL-1β secretion could be used as an indirect measure of NLRP3 activation, THP-1 monocytes were stimulated in the presence or absence of MCC950. MCC950 is a diarylsulfonyleurea-containing compound which inhibits NLRP3 activation by inhibiting ASC oligomerisation (Coll et al. 2015).

For these experiments, Pam3 and crystal stimuli were added at the same time to limit media removal steps which could alter the cell number of non-adherent cells. A robust increase in IL-1β secretion was seen when crystals were added alongside Pam3 compared to Pam3 alone (Fig. 3.2 A). This technique of adding Pam3 and crystals at the same time was used for all further experiments to limit media removal for THP-1 monocytes. MCC950 was initially titrated from 300-5000nM to test optimal concentrations in the system and successfully inhibited MSU+Pam3-induced IL-1β secretion by >75% (Fig. 3.2 A). MCC950 was reconstituted in DMSO and thus very low concentrations of DMSO were present in cell stimulations (maximum 0.025% v/v). Vehicle controls were added in experiments to represent the same concentration of DMSO as the highest concentration of MCC950 used. Cell viability analysis by CTB assay confirmed that MCC950, DMSO and crystals did not cause cell death at any concentration (Fig. 3.2 B). MCC950 at 750nm was determined as the optimal concentration for all future cell stimulations with higher concentrations having no additive inhibitory effect. This concentration was also selected to limit the concentration of DMSO (vehicle) added to final cell stimulations.

THP-1 monocytes were exposed to Pam3 ± MSU (10-20mg/dL) for 18 hours alongside MCC950 (750nm). As already demonstrated, MCC950 significantly inhibited MSU-induced IL-1β secretion by 80-90% (Fig. 3.2 C), without affecting cell viability (Fig. 3.2 D). Of note, MCC950 also reduced Pam3-induced IL-1β secretion by around 60%, but this did not reach statistical significance. A similar result was seen in response to CPPD+Pam3-induced IL-1β secretion, with MCC950 inhibiting 80-90% of IL-1β in cell supernatants without causing cell toxicity (Fig. 3.2 E-F). These results confirmed the activation of NLRP3 under these experimental conditions.
Figure 3.2 NLRP3 activation by MSU and CPPD crystals in THP-1 monocytes. (A-B) THP-1 monocytes were stimulated for 18 hours with Pam3 (100ng/mL) ± MSU (10mg/dL) crystals alongside increasing concentrations of MCC950. IL-1β secretion was determined by ELISA and viability by CTB assay. (C-D) MCC950 inhibition of MSU-induced IL-1β secretion in the absence of cell death. (E-F) MCC950 inhibition of CPPD-induced IL-1β secretion in the absence of cell death. (A-B & E-F) Data represents means of three technical replicates ± SD. (C-D) Data represents means from three independent experiments ± standard error of the mean (SEM). Significance was determined by multiple t-tests with Holm-Sidak’s multiple comparison correction test (***,P<0.0001 vs. vehicle control).
3.4 NLRP3 inflammasome activation in primary human monocytes

Primary human monocytes were isolated from single donor plateletpheresis residues as described in section 2.7. Flow cytometry was used to validate the purity of monocytes isolated and used for experiments. Two separate gating strategies were applied. Firstly, cells were sorted by size according to their forward and side scatter profiles. A gate was added to select lymphocyte and monocyte populations according to size and exclude non-viable cells (Fig. 3.3 A). Secondly, a gate was applied to ensure that only singlet cells were included in analysis, with doublets being omitted (Fig. 3.3 B). The Percoll density gradient separation method yielded >70% CD14+CD16- classical monocytes and a small population (~2%) of CD14+CD16+ monocytes (Fig. 3.3 C-D). Flow cytometry was repeated for another two primary human monocyte donors. For these donors, monocyte yield was lower than the original sample at around 45-50% CD14+CD16- and 5-10% CD14+CD16+. To account for T lymphocyte and B lymphocyte populations, samples were subsequently stained with CD3 and CD19 antibodies, respectively. The first additional donor had a T cell population of 7.7% and a B cell population of 4.7%, whereas the second had a T cell population of 17.8% and a B cell population of 5.4%. Together, it was concluded that the population of monocytes was 70-80% pure, with lymphocytes composing less than 30% of the population. Furthermore, primary human monocytes but not lymphocytes adhere to tissue culture plastic further ensuring a high purity of monocytes in experiments.

Primary human monocytes were exposed to MSU crystals for 18 hours and IL-1β secretion measured by ELISA (Fig. 3.4 A). Pam3 induced a small but insignificant increase in IL-1β secretion, which was greatly increased by the addition of MSU crystals alongside the TLR2 ligand. MSU crystals did not cause cell death, as confirmed by a Cell-Titre Glo™ (CTG) assay (Fig. 3.4 B). As seen in THP-1 stimulations, MSU crystals alone had no effect on IL-1β secretion, confirming the necessity for Pam3 as a priming signal. Of note, the difference in secretion between MSU at 10mg/dL and 20mg/dL was much less in primary human monocytes compared to THP-1 monocytes. Therefore, MSU at 10mg/dL was used for the majority of future experiments to limit any effect on cell viability.

To confirm that MSU-induced IL-1β secretion was NLRP3-dependant, primary human monocytes were stimulated with Pam3 and MSU alongside MCC950 (750nm). MCC950 inhibited both Pam3- and MSU+Pam3-induced IL-1β secretion by 75-80% confirming the activation of the inflammasome (Fig. 3.4 C). Data was normalised to Pam3 to account for variability in IL-1β secretion between donors. Viability was checked using an MTT assay and confirmed that neither MCC950 nor its vehicle (DMSO) caused cell death (Fig. 3.4 D).
Figure 3.3 Purity of monocytes isolated by Percoll density gradient. (A) Representative data showing the flow cytometric gating strategy. Cells were identified by size according to their forward and side scatter profiles. A broad gate was applied to select viable mononuclear cells and lymphocytes. (B) A second gate was applied to account for single cell populations whilst discounting doublets. (C) Q1 represents CD14+CD16- cells, Q2 represents CD14+CD16+ cells, Q3 represents CD14-CD16- cells and Q4 represents CD14-CD16+ cells. (D) Final population statistics were calculated using Diva software.
Primary human monocytes were stimulated with MSU (10-20mg/dL) ± Pam3 (100ng/mL) for 18 hours. (A) IL-1β secretion was measured as an indirect measure of NLRP3 activation. (B) The effect of crystals on cell viability was determined by CTG assay. (C) NLRP3 activation was confirmed using MCC950 (750nM) and (D) viability confirmed by MTT assay. Data represent means from (A-B) 6 individual donors or (C-D) 3 individual donors ± SEM. Significance was determined by One-way ANOVA with Tukey’s post-hoc test (A) or multiple t-tests with Holm-Sidak’s multiple comparison correction (C) (*P<0.05, **P<0.01, ****P<0.0001).
3.5 Optimisation and validation of absolute real time quantitative PCR

Absolute qPCR was used to examine gene expression of inflammasome components in THP-1 and primary human monocytes throughout this study. Four reference genes (HPRT1, B2M, RPL32 and PGK1) were initially chosen and analysed for their stability of expression in monocytes under the experimental conditions used. Both B2M and PGK1 stability had previously been demonstrated in primary human monocytes stimulated for 3 hours with LPS (Piehler et al. 2010). Absolute qPCR involves the addition of a standard curve of the gene being measured on each plate. Each standard curve was made up of known copy numbers of the genes, ranging from $10^7$ - $10^1$ copies. Copy numbers for samples were then interpolated from this curve and normalised to copy numbers for reference genes (RGs). Representative standard curve plots for each RG are shown in figure 3.5. Standard curves for gene of interests (GOIs); pro-caspase-1 (CASP1), pro-interleukin-1β (IL1B), NLRP3 and ASC (PYCARD), were prepared and run on every plate alongside cDNA from experimental samples (Fig. 3.6). The Stratagene 3000 qPCR machine amplified all GOIs well (efficiency between 90-100%) apart from PYCARD which had poor efficiency and did not amplify past $10^3$ copies. As such, some samples were sent away for PYCARD analyses using a Rotorgene qPCR machine, which successfully amplified the complete PYCARD standard curve with high efficiency to ensure more precise results (Fig. 3.6 E).

The stability of expression of the reference genes used was analysed using geNorm (Vandesompele et al. 2002). The stability of the reference genes was initially tested in cDNA from unstimulated human monocytes isolated from 24 separate donors. GeNorm analyses identified PGK1 and HPRT1 as the least stable with both scoring M values of >1.0 and these two GOIs were therefore discounted for further use. In these original 24 donors, B2M and RPL32 scored an M score of 0.544 when used in combination, just above the desired <0.5 threshold. To further probe the stability of B2M and RPL32, expression of these RGs was analysed in monocytes from an additional 23 donors, increasing the number of samples to 47. For the 47 samples, B2M and RPL32 had an M score of 0.497 confirming their stability when used in combination and were therefore used for all future qPCR analyses.

Additional testing was performed to examine the stability of B2M and RPL32 in monocytes that were stimulated with the ligands used throughout the study. THP-1 monocytes were stimulated for 3 hours with Pam3 ± MSU (10mg/mL), CPPD (2.5mg/dL) or soluble uric acid (30mg/dL). RNA was extracted, reverse transcribed and copy numbers for B2M and RPL32 were obtained via qPCR. Copy numbers for both RGs achieved an M score of 0.381 when
used in combination in these cells even when stimulated, confirming their stability for use after *in vitro* stimulation.

The majority of qPCR in this study was run on a Stratagene 3000 qPCR machine, the reliability of which was checked throughout by calculating qPCR efficiency using the standard curves added to each qPCR plate. PCR efficiency denotes a doubling of cDNA copy number during each cycle and should ideally be maintained between 90-110% for all experiments. Additionally, to check the reproducibility of our reverse transcription and qPCR reactions, 10% of the RNA was randomly selected for a second reverse transcription reaction. *B2M* and *RPL32* were measured in these samples again and cycle threshold (Ct) values for each was compared to the original samples. All but one of the re-reverse-transcribed samples gave similar Ct values to those of the original samples, demonstrating the reproducibility of the reverse transcription process (Fig. 3.7 A-B). One sample (cDNA identifier 23) did have a significantly different Ct value. Reasons for this are unknown but could relate to poor reverse transcription or failure to add certain components of the reverse transcription mix to this particular sample.
Figure 3.5 Representative qPCR standard curves for reference genes. Serial dilutions of known gene copy numbers were generated for four reference genes; (A) B2M, (B) HPRT1, PGK1 (C) and RPL32 (D). Data represents means from three technical replicates ± SD.
Figure 3.6 Representative qPCR standard curves for genes of interest. Serial dilutions of known copy numbers for gene of interests were used to create standard curves for each gene. Data represents means from three technical replicates ± SD.
Figure 3.7 Validation of reverse transcription. Reliability and reproducibility of qPCR was validated by re-reverse transcribing RNA and then re-running the qPCR analyses for (A) B2M and (B) RPL32. Data points represent means from three technical replicates ± SD.
3.6 Gene expression of inflammasome components in response to stimulants

A time course was initially completed to assess optimal time points for the induction of key inflammasome components in response to Pam3 and MSU crystals. RNA was isolated from THP-1 monocytes following stimulation for 1, 3 or 6 hours with Pam3 (100ng/mL) or MSU crystals (10mg/dL). As expected *IL1B* was greatly increased in the presence of Pam3 peaking at a 182-fold increase in expression at 3 hours before dropping to an 89-fold increase at 6 hours (Fig. 3.8 B). *CASP1, NLRP3* and *PYCARD* expression did fluctuate according to both the time point and the stimulant but overall it was clear that the expression of these GOIs was not induced by Pam3 or MSU stimulation. Control cells for each of these genes were the most unstable, with *CASP1* being increased at 1 hour, whereas *NLRP3* was reduced at this time point in unstimulated cells (Fig. 3.8). A three-hour stimulation was identified as the optimal time point to see the largest difference in *IL1B* expression and used for future experiments. *IL1B* expression was of particular interest here because of its known increase in expression following TLR ligation in the literature (Bauernfeind et al. 2009).

THP-1 monocytes were consequently stimulated for 3 hours with Pam3 alone or alongside MSU crystals (10mg/dL) to examine whether NLRP3 activation could affect expression of NLRP3 inflammasome components in addition to the effect of priming alone (Fig. 3.9). As seen previously, expression of *IL1B* was significantly induced by Pam3 stimulation (Fig. 3.9 B). This expression was unaffected by NLRP3 activation following the addition of MSU crystals alongside Pam3. Interestingly, there was a trend for Pam3 stimulation to reduce expression of *CASP1, NLRP3* and *PYCARD* although only *CASP1* was significantly reduced in MSU+Pam3 stimulated cells compared to MSU alone (Fig. 3.9 A). In all cases, the addition of MSU crystals alongside Pam3 had no effect compared to stimulation with Pam3 alone nor did crystals in the absence of Pam3 have any effect compared with unstimulated cells.
Figure 3.8 Time course of inflammasome component expression. THP-1 monocytes were stimulated for 1, 3 or 6 hours with RPMI (solid circle), Pam3 (100ng/mL – white square), MSU (10mg/dL – white circle). Copy numbers for (A) CASP-1, (B) IL1B, (C) NLRP3 and (D) PYCARD were assessed at each time point by qPCR after normalisation to expression of B2M and RPL32. Data points represent means from three technical measures of cDNA obtained from a single well of a 6 well plate ± SD.
Figure 3.9 Expression of inflammasome components in response to Pam3 and MSU crystals. THP-1 monocytes were stimulated for 3 hours with Pam3 (100ng/mL) ± MSU crystals (10mg/dL). Expression of CASP1 (A), IL1B (B), NLRP3 (C) and PYCARD (D) was analysed by qPCR. Copy numbers were normalised to B2M and RPL32. Bars represent means from three independent experiments ± SEM. Significance was determined by one-way ANOVA with Tukey's post-hoc test (*P<0.05, **P<0.01, ***P<0.001).
3.7 Measuring reactive oxygen species in cell lines and primary human monocytes

The role of ROS in NLRP3 activation has long been disputed with many studies showing contrasting results (section 1.4.3). As described in section 1.5.4, these contrasting results are likely a consequence of the use of different cell types, cell lines or species. Here, cell lines were used to optimise ROS measurement \textit{in vitro}. Following these optimisations, all ROS measuring assays were applied to primary human monocytes with the overall aim of investigating the effect of Pam3 and MSU or CPPD crystals on ROS generation – i.e. did activation of the NLRP3 inflammasome occur in parallel with an increase in ROS? Three methods for measuring ROS levels were identified and tested; DCFDA assay, NBT assay, and ROS-Glo assay.

3.7.1 Optimising oxidant stimulant concentrations

Three pro-oxidant stimuli; menadione, paraquat and H$_2$O$_2$, were used in various experimental conditions as inducers of ROS. Oxidant-inducing stimuli are often highly toxic in nature and it was therefore important to carefully optimise \textit{in vitro} concentrations prior to use. To do this, cells were exposed to increasing concentrations of the stimulant for up to 18 hours and viability was measured using CTG or MTT assay.

Menadione is a quinone that can function as a precursor in the synthesis of Vitamin K. This compound generates intracellular ROS production at various sites through redox cycling (Hassan and Fridovich 1979; Loor et al. 2010). Menadione concentration had previously been optimised in THP-1 cells by Dr L Mullen (Brighton and Sussex Medical School). A test experiment was run whereby PMA-treated THP-1 cells were stimulated for 18 hours with menadione (10-20µM). THP-1 cells were treated with PMA to initiate adherence so that experimentally they could be treated the same way as primary human cells, which adhere to tissue culture plastic. Menadione caused minimal cell toxicity under these conditions either alone or when added alongside Pam3 (Fig. 3.10 A). Of note, these experiments were completed prior to the decision to only use THP-1 monocytes without PMA treatment. Nonetheless, the data obtained provided useful insights into the ability to measure ROS \textit{in vitro}.

Primary human monocytes were next seeded at various cell densities and exposed to menadione (5-40µM) for 18 hours. Cell viability was assessed by CTG assay. A 10-20% reduction in cell viability was seen at 5µM menadione and this was further reduced (~40%) at 15µM menadione (Fig. 3.10 B). Therefore, a maximum concentration of 5µM was used for subsequent experiments in primary human monocytes.
H$_2$O$_2$ is a highly reactive ROS and is widely used as an inducer of oxidative stress and apoptosis in vitro (Singh et al. 2007). Primary human monocytes were exposed to increasing concentrations of H$_2$O$_2$ (0.25-10mM) for 18 hours. Cell viability, measured by CTG, was reduced at very low concentrations, with around 10-20% reduction in viability by 1mM H$_2$O$_2$. Cell viability was greatly reduced by 5mM H$_2$O$_2$ with a 60-80% reduction and almost complete cell death was seen at 7.5mM H$_2$O$_2$ (Fig. 3.10 C). Therefore, a maximum concentration of 1mM was used for subsequent experiments in primary human monocytes.

Paraquat is a redox-active viologen and is widely used as a potent oxidative stress inducer (Miller et al. 2007). Paraquat was used for optimisation of NBT assays in BV2 cells and was initially titrated to determine optimal concentration. NBT was initially tested using the BV2 cell line as this assay had previously been used in these cells by our group. BV2 cells were seeded at various cell densities and stimulated with paraquat (100-500µM) for 4 or 24 hours (Fig. 3.10 D and E, respectively). After 4-hour exposure, no reduction in cell viability was observed at any cell densities showing 100% viability when compared to unstimulated cells. Paraquat toxicity occurred at 250µM for 24-hour exposure, decreasing BV2 viability by >50% in 1-10x10$^4$ cells/well. Paraquat was used at a concentration of 50-100µM for future experiments to ensure cell toxicity did not occur.

These experiments are comprised of triplicate technical replicates and as such a statistical test could not be completed. Nonetheless, the results obtained here were used as the basis for which concentrations for each oxidant were used in subsequent experiments.
Figure 3.10 Optimisation of oxidant concentrations. Optimal concentrations for oxidant stimulants were determined by cell viability analysis prior to use in ROS measurement assays. (A) PMA-treated THP-1 monocytes were stimulated for 18 hours with menadione (10-20µM) and viability checked by MTT assay. (B) Primary human monocytes treated for 18 hours with menadione at increasing concentrations (5-40µM) and viability checked by CTG assay. (C) Primary human monocytes treated for 18 hours with H$_2$O$_2$ at increasing concentrations (0.25-10mM) and viability checked by CTG assay. BV2 cells were stimulated for 4 (D) or 24 (E) hours with increasing concentrations of paraquat (100-500µM) and viability checked by MTT. Data points represent means from three technical replicates ± SD.
3.7.2 Dichlorodihydrofluoresceindiacetate (DCFDA) assay

DCFDA was initially tested in PMA-treated THP-1 cells. Cells were seeded at 5x10⁴ cells per well of a 96-well plate and after 24 hours PMA treatment were washed and stained with DCFDA (1-10µM) for 45 minutes. Following the 45-minute staining period, media was replaced with fresh media containing stimulants for 6 hours. Fluorescence was quantified as a measure of DCFDA to DCF conversion giving an indirect measure of ROS generation in the cells. Increasing DCFDA concentration from 1µM to 5µM resulted in a robust increase in fluorescence intensity, demonstrating increased diffusion and oxidation of the compound within cells (Fig. 3.11 A). Menadione and tetra-Butyl hydroperoxide (TBHP), a chemical inducer of H₂O₂ provided with the DCFDA assay, were used as positive controls. However, in this system neither positive control had any effect on DCF fluorescence. To examine whether this lack of effect was due to the concentration of positive control used, PMA-treated THP-1 cells were pre-stained with DCFDA (5µM) and stimulated with increasing concentrations of positive controls for 6 hours. Menadione caused a slight dose-dependent increase in DCF fluorescence, whereas TBHP had no effect (Fig. 3.11 B).

DCFDA was then tested in primary human monocytes. Monocytes from two donors were seeded at 2.5x10⁴ cells per well in 384-well plates and stimulated with 10-50µM DCFDA for 30 minutes. DCFDA was removed and cells were stimulated for 6 hours with TBHP and menadione. As expected, increased DCFDA concentration resulted in increased DCF fluorescence. However, exposure to ROS-inducing stimulants menadione (2.5-5µM) and TBHP (5µM) had no effect on DCF fluorescence in this system (Fig. 3.11 C). Overall DCFDA did not yield consistent results, nor did the addition of positive controls cause an increase in ROS generation. Therefore, the use of this assay was halted.
Figure 3.11 Optimisation of DCFDA assay. (A) DCFDA concentration was titrated in PMA-treated THP-1 cells and 5µM determined as optimal concentration. (B) Oxidative stress inducers menadione and TBHP were titrated in PMA-treated THP-1 cells following DCFDA (5µM) staining. DCF fluorescence (ex485/em528) was determined after 6-hour exposure to stimulants. (C) Primary human monocytes from two donors were exposed to 10-50µM DCFDA and then stimulated for 6 hours with TBHP and menadione. Data represent means from three technical replicates ± SD.
3.7.3 Nitroblue tetrazolium (NBT) assay

NBT is a yellow powder made up of two tetrazole moieties and can be used to detect generation of ROS, specifically the superoxide anion. Upon superoxide generation, the yellow NBT becomes reduced and forms a blue formazan crystal which can consequently be dissolved with DMSO and potassium hydroxide (Freeman and King 1972; Choi et al. 2006).

NBT was initially tested using the BV2 cell line. BV2 cells were used as this assay had previously been tested in these cells by our group. NBT concentration was initially titrated from 0-50µg/mL at various cell densities. Cells were stimulated for 4 hours, after which NBT crystals were fixed with methanol and consequently dissolved with a mixture of DMSO and KOH. The highest parquat-induced increase in ROS generation was demonstrated in higher cell densities with 2.5µg/mL NBT, showing a 4-fold increase in superoxide after 4 hours with 100µM parquat at 5x10^4 cells/well in a 96-well plate (Fig. 3.12 A).

The protocol, having worked well in BV2 cells, was then tested in PMA-treated THP-1 cells. THP-1 cells were seeded at 5-15x10^4 cells/well in a 96-well plate and stimulated for 4 hours with parquat (50-100µM) in the presence of NBT (10-50µg/mL). The response in these cells was greatly reduced, with lower overall fluorescence and little effect of parquat on NBT reduction (Fig. 3.12 B). Of course this could relate to the use of higher cell numbers for the THP-1 cells, however when comparing the 5x10^5 cells/well between BV2 and THP-1 cells, there is a clear loss of effect in the THP-1 cells.

To investigate whether NLRP3 activation may involve superoxide generation, PMA-treated THP-1 cells and primary human monocytes were treated for 2-6 hours with Pam3 ± MSU or CPPD crystals in the presence of 25µg/mL NBT. After two hours, neither stimulant had any effect on superoxide generation. At 4 hours MSU and CPPD crystals induced a small (1.5-2 fold) increase in superoxide generation (Fig. 3.13 A-B). The results of the NBT assay in primary human monocytes varied between donors. After 2 hours, MSU and CPPD crystals induced a small increase in NBT fluorescence. This effect was not seen after 6 hours stimulation with the crystals (Fig. 3.13 C-D). It is important to note that NBT absorbance was much less in THP-1 cells and primary human monocytes compared to BV2 cells with the gain needing to be increased to get an increased fluorescence reading. The results were therefore considered less reliable in these cell systems compared to BV2 cells. This, in addition to the lack of effect of positive control parquat on THP-1 cell ROS meant that the use of the NBT assay was not pursued.
Figure 3.12 Optimisation of NBT assay. (A) BV2 cells were seeded at 1-5x10⁴ cells/well in a 96-well plate and stimulated 4 hours with Paraquat (50-100µM) in the presence of NBT (10-50µg/mL). (B) PMA-treated THP-1 cells were seeded at 5-15x10⁴ cells /well in a 96-well plate and stimulated for 4 hours with Paraquat (50-100µM) in presence of NBT (10-50µg/mL). ROS generation was determined by measuring absorbance at 630nm. Data represent means from three technical replicates ± SD.
Figure 3.13 Measurement of ROS in human monocytes using NBT assay. PMA-treated THP-1 cells were seeded at 15x10^4 cells/well and stimulated for 2 hours (A) or 4 hours (B) with Pam3 ± MSU or CPPD in the presence of 25µg/mL NBT. Primary human monocytes from two donors were seeded at 30x10^4 cells/well and stimulated for 2 hours (C) or 6 hours (D) with Pam3 ± MSU or CPPD in the presence of 25µg/mL NBT. ROS generation was determined by measuring absorbance at 630nm. Data represent means from three technical replicates ± SD.
3.7.4 ROS-Glo assay

A luminescence-based H₂O₂ measurement assay called ROS-Glo™ was also tested. The ROS-Glo assay provides a H₂O₂ substrate which, upon reaction with H₂O₂, is converted to a luciferin precursor. The luciferin precursor is further reduced to luciferin through reaction with a ROS-Glo detection solution and can be measured spectrophotometrically.

ROS-Glo was initially optimised in PMA-treated THP-1 cells. Cells were seeded at low densities (5x10⁴ cells/well in a 96-well plate), as suggested by the manufacturer, and stimulated for 6 hours - the longest time period recommended by the manufacturer. An initial test was run whereby H₂O₂ generation was measured in cell supernatants and in lysed cells from the same well (Fig. 3.14 A). Lysis was initiated by the ROS-Glo detection reagent added following cell stimulation. Both techniques provided near identical results, with a marked and dose-dependent increase in ROS in response to menadione. The non-lytic protocol was used for all future experiments in adherent cells to allow multiplexing with cell viability measurements. The dose-dependent increase in ROS in response to menadione translated well in THP-1 monocytes increasing ROS levels 3-fold compared to unstimulated cells (Fig. 3.14 B). Due to the non-adherent nature of these cells, H₂O₂ substrate oxidation was measured in cell lysates. To further validate the use of ROS-Glo in measuring ROS generation in vitro THP-1 monocytes were stimulated with menadione alongside increasing concentrations of H₂O₂ reducing antioxidant enzyme catalase. Catalase successfully reduced ROS-generation in a dose-dependent manner in both unstimulated and menadione treated cells, inhibiting ROS-generation up to 80% at its highest concentration (Fig. 3.14 C).

With the protocol working in response to oxidative stress inducer menadione, PMA-treated THP-1 cells were subsequently stimulated for 6 hours with Pam3 ± MSU and CPPD crystals. This experiment was a direct measure of whether NLRP3 activation is coupled with an increase in ROS generation, thus providing insight into the importance of ROS during inflammasome activation. Pam3 and MSU induced a small increase in ROS-generation, with MSU and Pam3 added together showing 1.5-fold higher ROS levels than unstimulated controls (Fig. 3.14 D). Interestingly, no effect of Pam3 nor MSU was seen in THP-1 monocytes, again stimulated for 6 hours (Fig. 3.14 E).
Figure 3.14 Optimisation of ROS-Glo assay. (A) PMA-treated THP-1 cells and (B) THP1-Blue monocytes were exposed to menadione (10-20µM) for 6 hours. (C) THP-1 monocytes were stimulated with menadione alongside increasing concentrations of catalase for 6 hours. (D) PMA-treated THP-1 cells were stimulated with Pam3 ± MSU or CPPD for 6 hours. (E) THP-1 monocytes were stimulated for 6 hours with Pam3 ± MSU. All stimulations included a ROS-Glo H2O2 substrate which, upon reaction with H2O2 is reduced to luciferin and detected by measuring luminescence. (A-D) Data represent means from three technical replicates ± SD. (E) Data represent means from three independent experiments ± SEM. Significance was determined by One-way ANOVA with Tukey’s post-hoc test and no significant differences were found (E).
After successful experiments in THP-1 cells using menadione as a positive control, the ROS-Glo assay was optimised for use in primary human monocytes. Cells were seeded at 2.5-30x10⁴ cells/well and stimulated for 6 hours with menadione at concentrations determined in previous experiments (Fig. 3.10 B). ROS-Glo luminescence showed the largest increase at 5x10⁴ cells but also showed similar results for 10x10⁴ and 20x10⁴ cells/well (Fig. 3.15 A). Interestingly, wells containing 30x10⁵ cells/well showed no increase in ROS-generation. In future experiments ROS-Glo analyses were completed at a cell density of 5x10⁴ cells/well, matching conditions used for THP-1 cells.

A time course analysis of ROS-generation in response to menadione and MSU was completed to determine optimal timings for stimulation (Fig. 3.15 B). Menadione induced only a marginal increase in ROS whereas MSU crystals induced a marked increase at most time points. ROS-generation showed a sharp increase between 0.5-2-hour stimulation time points and then plateaued showing smaller increases in ROS between 2 and 6 hours. MSU caused a 2-fold increase in ROS levels at 2 hours and 6 hours. Cells were consequently stimulated for 2 or 6 hours for all future experiments. This experiment was completed using triplicate technical repeats and thus statistical analysis could not be completed, however, results were used to determine optimal timings for future experiments.

In primary human monocytes, MSU crystals induced a significant increase in ROS generation when added alongside Pam3. Cells were stimulated for 2 hours based on results from the previous time course experiment. MSU crystals induced a 1.5-fold increase in ROS generation when added alone, but this did not reach statistical significance (p=0.08). A significant increase in ROS-generation was seen when MSU crystals were added alongside Pam3, compared to Pam3 alone (Fig. 3.15 C). Interestingly, CPPD did not affect ROS-generation in either primary human monocytes or THP-1 cells suggesting that ROS-generation may differ according to stimulant.
Figure 3.15 Measurement of ROS in human monocytes using ROS-Glo assay. (A) Primary human monocytes were seeded at varying densities and stimulated for 6 hours with menadione. (B) Primary human monocytes seeded at $5 \times 10^4$ cells/well were stimulated for 0.5-6 hours with menadione and MSU crystals (10mg/dL). (C) Primary human monocytes were stimulated for 2 hours. ROS-generation was measured by detection of oxidised H$_2$O$_2$ substrate by luminescence. (A-B) Data represent means from triplicate measures from a single donor ± SD. (C) Data represent means from 6 individual donors ± SEM. Significance was determined by one-way ANOVA with Tukey’s post-hoc test (***P<0.001).
3.8 Stimulation of primary human monocytes with oxidants does not induce NLRP3 activation

ROS measurement by ROS-Glo showed increases in ROS generation in response to MSU crystals but not in response to CPPD crystals in primary human monocytes. To further probe the role of ROS in NLRP3 signalling, primary human monocytes were stimulated for 18 hours with Pam3 ± menadione or H$_2$O$_2$. These oxidants induce oxidative stress, and were used to examine whether chemically induced ROS generation would result in NLRP3 activation and consequent IL-1β secretion.

As seen previously, Pam3 induced a small increase in IL-1β secretion, although inter-donor IL-1β secretion varied substantially. Overall H$_2$O$_2$ stimulation had no effect on IL-1β secretion and there was no consistent effect on Pam3-induced IL-1β either (Fig. 3.16 A-B). Viability analyses confirmed that H$_2$O$_2$ at this concentration did not cause cell death in these cells (Fig. 3.16 C). Likewise, menadione had no significant effect on IL-1β secretion when added alone or when added alongside Pam3 (Fig. 3.16 D-E). Again, MTT viability analysis confirmed that menadione did not cause cell death when used at this concentration (Fig. 3.16 F). Data were plotted as individual values for IL-1β secretion to highlight the lack of consistent effect seen with the addition of both oxidants. Overall, oxidant stimulation had no influence on the secretion of IL-1β under these experimental conditions.
Figure 3.16 Oxidants do not activate the NLRP3 inflammasome. (A) Primary human monocytes were stimulated for 18 hours with H\textsubscript{2}O\textsubscript{2} alone or (B) alongside Pam3. (C) Cell viability was assessed by MTT assay. (D) Primary human monocytes were stimulated for 18 hours with menadione alone or (E) alongside Pam3. (F) Cell viability was assessed by MTT assay. IL-1β secretion was determined by ELISA. Data represent means of triplicate measurements for each donor (A, B, D, E). Data represent means of 4 (C) or 5 (F) individual donors ± SEM. Significance was determined using a Mann-Whitney U test which pooled the means of each donor for each condition and comparing it to the pooled means for unstimulated controls.
3.9 Discussion

The aim of this chapter was to develop an *in vitro* model for investigating the role of ROS in NLRP3 inflammasome activation. Protocols were optimised to activate the NLRP3 inflammasome in THP-1 cells and primary human monocytes and used to examine whether ROS generation occurs during priming and activation of the inflammasome. To further probe the role of ROS, cells were also stimulated with oxidants and IL-1β was measured to determine if artificial induction of ROS generation could induce NLRP3 oligomerisation.

Canonical NLRP3 activation requires two signals; an initial priming signal, followed by a secondary activation signal (Pellegrini et al. 2017). Two known crystal activators of the inflammasome were selected for use in this study, MSU and CPPD crystals. These crystals drive the pathophysiology of gout and pseudogout, respectively, which are two inflammatory diseases driven by IL-1β secretion occurring downstream of NLRP3 activation (Choi et al. 2005; Martinon et al. 2006). Our results were consistent with canonical NLRP3 activation with cells not secreting IL-1β following exposure to crystals alone, but secreting robust concentrations of IL-1β when added alongside the TLR2 ligand Pam3. The activation of the NLRP3 inflammasome was confirmed using MCC950 (Coll et al. 2015), showing that NLRP3 was consistently activated under these experimental conditions.

Interestingly, Pam3 alone induced low levels of NLRP3-dependent IL-1β secretion in both THP-1 cells and primary human monocytes. This phenomenon has also been noted elsewhere and is thought to relate to constitutively active caspase-1 in monocytes which allows IL-1β secretion in response to TLR-2 and TLR-4 ligation to occur independently of NLRP3 oligomerisation (Netea et al. 2009). However, there was a trend MCC950 to reduce Pam3-induced IL-1β suggesting that Pam3 was able to activate the NLRP3 inflammasome. ATP is a known NLRP3 agonist acting via the purinergic receptor P2X7, and its release has been demonstrated to activate NLRP3 if a microbial signal is present (Piccini et al. 2008; Gombault et al. 2012). Therefore, a potential explanation for this Pam3-induced activation of the inflammasome is that ATP released from the activated monocyte could have activated the inflammasome in an autocrine manner. Another potential explanation is the alternative NLRP3 signalling pathway, which has been described in human monocytes. In this pathway, TLR4 activation by LPS was shown to induce NLRP3 activation independently of pyroptosome formation and potassium flux. However, this pathway is thought to be specific to TLR4 signalling, not occurring downstream of TLR2 ligation (Gaidt et al. 2016). Our use of TLR2 ligand Pam3 thus rules out the involvement of the alternative inflammasome pathway here, suggesting the likely cause of the Pam3 induced IL-1β secretion is an
autocrine effect of ATP. This could be confirmed in a future experiment using oxidised ATP, which irreversibly inhibits the P2X7 receptor, to determine whether released ATP is indeed driving the release of IL-1β from primary human monocytes stimulated with Pam3 alone (Netea et al. 2009). Nonetheless, the addition of crystal stimuli alongside Pam3 consistently induced a robust increase in NLRP3-depedent IL-1β secretion in both THP-1 and primary human monocytes.

Gene expression of inflammasome components was also assessed and Pam3 induced an increase in IL1B expression, confirming that the TLR2 ligand was able to prime the inflammasome. Interestingly, Pam3 stimulation did not increase expression of NLRP3. This is in contrast to published data showing that NF-κB activation by LPS increases expression of NLRP3 mRNA in murine macrophages (Bauernfeind et al. 2009). A rapid mechanism for the non-transcriptional activation of the inflammasome is known where NLRP3 proteins in the cytosol become de-ubiquitinated following TLR ligation, allowing the release of IL-1β in the absence of NLRP3 transcription (Juliana et al. 2012; Py et al. 2013). This contrasting result could therefore be a timing issue as perhaps NLRP3 transcription is not required during short term stimulations. However, a 5-fold increase in NLRP3 expression has been documented in primary human monocytes exposed to LPS for 3 hours compared to unstimulated monocytes (Awad et al. 2017). The exact reason why NLRP3 transcription was not increased here remains unknown, but measuring NLRP3 protein concentrations over this time period may provide further insight into what is happening in response to Pam3. Of note, the study by Awad et al. (2017) did show a significant reduction in ASC expression in LPS stimulated monocytes, which is in agreement with our data showing a trend for reduced PYCARD expression in Pam3-stimulated THP-1 cells compared to unstimulated controls. To our knowledge, this is one of the only studies to examine the absolute copy numbers of NLRP3 inflammasome components in THP-1 cells, with the majority of studies showing relative expression. It is particularly interesting to note the copy numbers of inflammasome components in resting monocytes, with gene expression of the transcriptionally regulated NLRP3 and IL1B being much lower compared to the non-transcriptionally regulated CASP1 and PYCARD genes demonstrating the necessity for a priming signal for NLRP3 activation.

An interesting finding of this chapter which should be noted is the lack of cell death caused by NLRP3 activating stimuli in both THP-1 and primary human monocytes. This opposes the newly established mechanism for IL-1β release which involves caspase-1 induced activation of gasdermin-D which in turn leads to the formation of oligomeric pores which facilitate the release of cytosolic proteins into the extracellular environment. The formation
of these oligomeric pores also affects the cells ability to maintain osmotic balance, resulting in cell lysis - an effect which has been confirmed in murine BMDMs in response to a range of NLRP3 activating stimuli (Shi et al. 2015). The exact reason for not observing cell death under the experiment conditions used here is unknown, but it should be noted that the metabolism-based cell viability assays used throughout this study do not provide a measure of cell lysis. Instead a lactate dehydrogenase assay should be used in future experiments to assess whether membrane pore formation is occurring in response to MSU and CPPD crystals in primary human monocytes. Interestingly, a recent study demonstrated gasdermin-D-dependent IL-1β release in the absence of cell lysis in BMDCs, suggesting that low level gasdermin-D pore formation could precede cell lysis under certain experiment conditions (Heilig et al. 2018). Furthermore, another study from the same group found that gasdermin-D pores can also be repaired in murine BMDMs via the recruitment of endosomal sorting complexes required for transport (ESCRT) demonstrating that NLRP3 activation does not always result in cell death (Ruhl et al. 2018). It is therefore plausible that the lack of cell death observed here could be a result of this, with low level IL-1β release occurring in a gasdermin-D-dependent fashion in the absence of cell death. Further studies will be necessary to confirm this under the experimental conditions used throughout this study but could provide useful insight into the mechanism of IL-1β release from primary human monocytes.

The measurement of ROS in vitro proved particularly difficult. DCFDA is a fluorescence-based assay widely used to measure ROS in living cells (Munoz-Planillo et al. 2013). However, DCFDA is not without its limitations. It is used as a measure of H₂O₂ despite not reacting directly with it. Additionally, the intermediate DCF radical formed following the deacetylation of DCFDA readily reacts with oxygen to form superoxide radicals which can lead to artificial amplification of the reported ROS levels (Kalyanaraman et al. 2012). Here, DCFDA did not work showing no increase in DCF fluorescence even when cells were stimulated with positive controls. Conversely, NBT showed promising results in BV2 cells with large increases in ROS in response to positive control paraquat. The inability to translate these results to THP-1 cells potentially highlights differences in redox homeostasis between these cell types. The ROS-Glo™ assay provided the most consistent measurement of ROS and was therefore selected for further use. The assay is a measure of H₂O₂ which freely diffuses across cell membranes. Measuring H₂O₂ provides many advantages over measuring other ROS, including the fact that it has the longest half-life of ROS in cultured cells. Furthermore, various other ROS are converted to H₂O₂ in cells, meaning that changes in H₂O₂ can reflect an overall change in ROS levels (Wittmann et al.)
Therefore, any change in ROS-Glo luminescence was considered as a change in overall ROS levels rather than a change in H$_2$O$_2$ specifically.

A role for ROS in NLRP3 priming has recently been established, with ROS inhibitors N-acetyl cysteine (NAC) and diphenyliodonium (DPI) inhibiting NLRP3 priming by LPS in murine macrophages (Bauernfeind et al. 2011). An increase in ROS following TLR2 ligation by Pam3 was not observed under the experimental conditions used here suggesting that ROS were not involved in inflammasome priming. It is possible, however, that the levels of ROS induced by Pam3 are subtle and were therefore not detected in this system. The inhibition of ROS using antioxidant enzymes and chemical ROS inhibitors may provide further insight into the importance of ROS in Pam3 induced priming.

MSU crystals induced a significant increase in ROS generation in primary human monocytes suggesting that ROS may play a role in NLRP3 activation. In confirmation of this finding, an increase in ROS by MSU crystals has been shown elsewhere in primary human synoviocytes (Zamudio-Cuevas et al. 2016) and human monocytes (Zhou et al. 2010a). Interestingly, THP-1 cells and primary human monocytes demonstrated varying responses to MSU crystals, with MSU causing an increase in ROS in primary human monocytes but not in THP-1 cells. These findings may be explained by the known differences in redox status in resting primary human monocytes compared to THP-1 cells (Carta et al. 2011). In resting primary human monocytes, ROS production is low and redox homeostasis can therefore be maintained by low levels of antioxidant systems. In contrast, THP-1 cells express elevated levels of antioxidants such as GSH, thioredoxin and cysteine (Carta et al. 2011). This may help explain the lack of ROS in response to MSU in THP-1 cells compared to primary human monocytes with the MSU-induced ROS being rapidly removed by the elevated antioxidant systems in THP-1 cells. This also provides an explanation for the tolerance of much higher concentrations of menadione in THP-1 cells compared to primary human monocytes (Carta et al. 2011). Lower concentrations of menadione had to be used in primary human monocyte stimulations to avoid cell toxicity, explaining why a lack of ROS generation was seen when primary monocytes in response to the oxidant.

The difference in MSU crystal-induced ROS generation between THP-1 cells and primary human monocytes questions the importance of ROS in the activation of the inflammasome. In THP-1 monocytes MSU-induced IL-1β secretion occurred in the absence of a measurable increase in ROS. The increased antioxidant levels in THPs likely means that any MSU-induced ROS was rapidly dealt with, suggesting that ROS are not required for NLRP3 activation in response to MSU-crystals in these cells. This is also supported by the fact that
CPPD had no effect on ROS generation in either cell type, despite inducing a robust increase in NLRP3-dependent IL-1β secretion. Together, these findings suggest that ROS increases may occur as a by-product of MSU crystal exposure as opposed to being a necessary signalling mechanism for NLRP3 activation. This difference in redox-response could relate to the different structures of MSU and CPPD crystals. MSU crystals are thin needle-shaped structures with pointed ends (Paul et al. 1983), are larger than CPPD crystals, and can often be seen protruding from phagocyte in vitro (Higgins 2016). CPPD crystals on the other hand are rhomboidal and are easily phagocytosed by leukocytes (Ivorra et al. 1999). The differences in the structure of these stimuli may mean that MSU and CPPD crystals have alternative effects on immune cells, with one inducing NLRP3 activation via ROS increases and the other not, similar to that seen in response to hydroxyapatite crystals of different sizes (Lebre et al. 2017). Overall, the results obtained from THP-1 and primary human monocytes suggest that NLRP3 activation is not dependent on ROS-generation in response to crystalline stimuli.

The ability for ROS to activate the inflammasome was subsequently examined in primary human monocytes by stimulating them with H₂O₂ and menadione added alone or alongside Pam3. H₂O₂ has previously been demonstrated to cause a dose-dependent increase in IL-1β secretion when added alongside LPS in THP-1 cells (Zhou et al. 2010a; Carta et al. 2011). In primary human monocytes, however, neither oxidant caused an increase in IL-1β secretion suggesting that ROS are not able to induce the inflammasome in these cells. However, it is important to note that the low concentrations of menadione used had not induced an increase in ROS in primary human monocytes in previous experiments. It would be interesting to see whether increasing the concentration would cause IL-1β release, but this is likely to cause cell death making result interpretation difficult. In confirmation of the results presented here, Carta et al. (2011) did not see an increase in IL-1β secretion when primary human monocytes were exposed to H₂O₂ alongside LPS even when H₂O₂ concentration was increased to 5mM. This again highlights the varying redox states between the two cell systems and advocates that ROS are not required for activation of the inflammasome in primary human monocytes.

In conclusion, a reproducible method for activating the NLRP3 inflammasome was optimised in THP-1 cells and primary human monocytes. This method enabled analysis of gene expression and ROS generation in THP-1 cells and primary human monocytes. MSU crystals induced a sharp increase in ROS generation in primary human monocytes suggesting its involvement in NLRP3 activation. However, a lack of ROS generation in response to Pam3 and CPPD crystals, coupled with the ability of both stimuli to induce
NLRP3-dependent IL-1β secretion brings the importance of ROS in NLRP3 activation into question. Moreover, increasing ROS artificially in primary human monocytes did not result in increased IL-1β production. To further validate that ROS are not required for NLRP3 activation, the evaluation of the wider role of oxidative stress and inhibition of ROS using antioxidant enzymes and chemical inhibitors is necessary. The scavenging of ROS will provide insight into whether even small changes in the level of ROS, as would be seen during normal cell signalling, are necessary for NLRP3 activation, and if so one would hypothesise that the removal of ROS would stop NLRP3 formation. Examination of intracellular antioxidant enzyme expression during NLRP3 priming and activation may also provide insight into how changes in redox state might affect the inflammasome. ROS elevation is closely coupled with an increase in the expression of antioxidants in cells and it would be of interest to examine whether Pam3, which did not cause a measurable increase in ROS, may alter the expression of these genes.
Chapter 4 - Inhibition of ROS generation and NLRP3 activation in human monocytes
4.1 Introduction

The production of ROS is an important component of the innate immune response and ROS are known to play an integral role in the inflammatory response (discussed in section 1.2.5.4). ROS have long been associated with the activation of the NLRP3 inflammasome, yet there are also many studies that question this role of ROS (section 1.4.3). Under normal physiological conditions ROS act as signalling molecules which can potentiate the inflammatory response via activation of redox-sensitive transcription factors, induction of pro-inflammatory cytokines and activation of inflammasomes (Mittal et al. 2014). The results of the experiments presented in chapter 3 suggested that ROS generation was not necessary for NLRP3 inflammasome activation, with CPPD crystals inducing a robust increase in IL-1β secretion in the absence of a measurable increase in ROS.

The inhibition of ROS using inhibitors such as N-Acetyl cysteine (NAC) and diphenyleneiodonium (DPI) is a useful technique which allows investigation into the importance of ROS in cellular mechanisms. DPI, which inhibits the production of ROS by inhibiting the ROS-generating NADPH oxidase enzymes, has been shown to inhibit NLRP3 priming and activation in bone-marrow derived macrophages (BMDMs) as well as primary human and THP-1 monocytes (Bauernfeind et al. 2011; Carta et al. 2011). However, DPI has numerous off-target effects and can even induce oxidative stress in certain conditions (Riganti et al. 2004). It has also been shown to inhibit calcium channels which have been implicated in the activation of the inflammasome (Tazzeo et al. 2009). NAC is a thiol that easily diffuses into cells, where it becomes deacetylated to form a precursor for GSH, the major intracellular antioxidant, and is often used as an inhibitor of ROS (Zafarullah et al. 2003; Sun 2010). NAC is widely used for the in vitro study of oxidative stress and has indeed been reported to inhibit NLRP3-dependent IL-1β secretion in LPS challenged bovine monocytes (Liu et al. 2015) and THP-1 cells (Pazar et al. 2011; Zhou et al. 2015; Wang et al. 2017). There are few studies examining the effect of NAC on NLRP3 activation in primary human monocytes, however one study treated primary human monocytes with NAC but saw no effect on LPS-induced IL-1β secretion (Lavieri et al. 2014). It might therefore be interesting to examine the effect of NAC on pam3- and MSU-induced IL-1β secretion in primary human monocytes to investigate of the importance of ROS in inflammasome activation in these cells.

Besides small molecule antioxidants, an array of antioxidant enzymes also exist which function to maintain ROS at physiologically relevant levels (Birben et al. 2012). Various families of antioxidant enzymes exist in different environments throughout the body, residing
both intracellularly and extracellularly. As discussed previously (section 1.3.1) the superoxide dismutase enzymes (SODs) are an antioxidant family which scavenge superoxide, catalysing its dismutation to \( \text{H}_2\text{O}_2 \) via the alternate reduction and re-oxidation of the catalytic metal at their active site (Fig. 1.3 A). Three SOD isoforms exist, localising to different environments; cytosolic Cu/ZnSOD (SOD1), mitochondrial MnSOD (SOD2) and extracellular SOD (SOD3 – referred to as ECSOD from here on) (Fukai and Ushio-Fukai 2011). Although important for scavenging superoxide, the dismutation of \( \text{O}_2^- \) by SOD enzymes results in the production of \( \text{H}_2\text{O}_2 \), another ROS which plays a role in cell signalling and oxidative stress (Wittmann et al. 2012). Therefore, numerous antioxidant enzymes exist to reduce \( \text{H}_2\text{O}_2 \) to water and oxygen (section 1.3.1). For example, the glutathione peroxidase (Gpxs) are antioxidant enzymes which catalyse the reduction of \( \text{H}_2\text{O}_2 \) via the reduction and oxidation of a selenocysteine at their catalytic core (Johansson et al. 2005; Brigelius-Flohé and Maiorino 2013) (Fig. 1.3 B).

Antioxidant enzymes are able to constantly cycle between oxidised and reduced states, allowing continuous activity when in the presence of oxidants or reducing substrates. For example, the oxidised Gpx selenocysteine is continuously reduced back to its original state by GSH, reactivating Gpx and allowing further reduction of \( \text{H}_2\text{O}_2 \) (Brigelius-Flohé and Maiorino 2013). In contrast to small molecule antioxidants, each molecule of enzyme is able to catalyse the conversion of large quantities of substrate simultaneously so that they are highly efficient at reducing ROS. This might make them useful for \textit{in vitro} examination of the role of ROS in particular pathways. An important consideration for experimental use of exogenous antioxidant enzymes to reduce ROS is the native environment of the enzyme i.e. whether it is intra- or extracellular as this is likely to influence the catalytic activity. For experiments involving exogenous addition of antioxidant enzymes to cells, extracellular enzymes should be chosen such as Gpx3 or ECSOD, both of which are found in extracellular spaces \textit{in vivo}.

The aim of this chapter is to further investigate the importance of ROS during NLRP3 activation. Previous findings (chapter 3) suggested that ROS were not required for NLRP3 activation, with NLRP3 activators inducing IL-1\( \beta \) secretion in the absence of a measurable increased in ROS. To confirm whether ROS are dispensable for activation of NLRP3, ROS concentrations in monocyctic cells will be inhibited using either small molecule antioxidant NAC or the antioxidant enzymes Gpx3 or ECSOD during stimulation of the cells with NLRP3 activators. Priming and activation of NLRP3 will be investigated in response to these antioxidant treatments to fully determine the requirement for ROS in these signalling pathways.
4.2 Inhibition of ROS using N-Acetyl Cysteine in primary human monocytes

Primary human monocytes were stimulated with Pam3 ± MSU crystals in the presence or absence of NAC at 5 and 10mM (Parmentier et al. 2000). For these experiments, cells were exposed to NAC for one hour prior to a six hour stimulation with Pam3 and MSU crystals. NAC was also added alongside the stimulants for the 6-hour stimulation. A 6-hour time point was chosen following initial optimisation experiments in chapter 3. ROS measurement timings were increased from 2 hours to allow for sufficient cytokine production in parallel stimulations. NAC strongly reduced ROS levels, reducing both basal and MSU crystal-induced ROS increases by up to 90% (Fig. 4.1 A). Interestingly, NAC caused a 75% reduction in Pam3-induced IL-1β secretion but had no effect on MSU+Pam3-induced IL-1β secretion (Fig. 4.1 B). TNFα secretion was also measured as an indirect measure of NF-κB activation. NAC inhibited around 50% of TNFα in response to Pam3 but had no effect on TNFα when MSU was present (Fig. 4.1 C). For this experiment, cytokine secretion data was normalised to Pam3 to account for the variability in cytokine secretion seen between donors.

ROS play an integral role in normal cell function by acting as secondary messengers. Consequently, complete removal of ROS can be detrimental to cell viability. Viability was routinely assessed after each stimulation using MTT or CTG analysis. NAC caused little cell toxicity in primary human monocytes at the concentrations used, as demonstrated by representative CTG data from two donors (Fig. 4.1 D).
Figure 4.1 NAC inhibits ROS in primary human monocytes. Primary human monocytes were preincubated in cell media or NAC for 1 hour prior to a 6 hour stimulation with Pam3 (100ng/mL) ± MSU crystals (10mg/dL). (A) ROS generation was assessed using a ROS-Glo™ assay. (B) IL-1β secretion and (C) TNFα secretion were measured by ELISA. (D) CTG data from 2 donors representative of viability data for all 6 donors. (A-C) Data represent means from 6 individual donors ± SEM or (D) triplicate measures from each donor ± SD. Significance was determined by One-way ANOVA with Tukey’s post-hoc test (A) or Kruskal-Wallis analysis with Dunn’s post-hoc test (B-C) after an initial Shapiro-Wilk normality test (**P<0.01, ***P<0.001, ****P<0.0001).
4.3 Expression and purification of recombinant Gpx3

4.3.1 Transient transfection of HEK293T cells

In addition to NAC, two extracellular recombinant enzymes, Gpx3 and ECSOD, were used for these experiments. Although recombinant antioxidant enzymes such as Gpx and SOD are commercially available to purchase, their specific extracellular variants are not readily available. Therefore, Gpx3 and ECSOD were expressed and purified using mammalian cell lines (HEK293Ts and CHO-S cells) which provide a cost-effective method for the rapid production of recombinant proteins (Wurm and Bernard 1999). A benefit of using mammalian cell lines for recombinant protein expression is the ability for proteins to undergo the correct post-translational modifications, such as glycosylation and phosphorylation, required for protein folding and activity (Jenkins 2007). Both Gpx3 and ECSOD require post-translational modification, with Gpx3 requiring post-translational glycosylation (Song et al. 2014) and ECSOD requiring post-translational N-glycosylation and disulphide bond formation (Edlund et al. 1992; Peterson et al. 2008; Ota et al. 2017).

Gpx3 was previously cloned into the mammalian expression plasmid pcDNA4 (Invitrogen) by Dr L. Mullen (Brighton and Sussex Medical School). The Gpx3-pcDNA-4 plasmid contained the gene encoding Gpx3 flanked by a CMV promoter and a 3’ selenocysteine insertion sequence (SECIS). The plasmid also contained genes encoding resistance to antibiotics Ampicillin and Zeocin allowing efficient selection in E. coli and eukaryote cells respectively. In addition, there were also sequences for His- and V5-tags inserted at the 3’ end of the Gpx3 sequence allowing for its purification using nickel-based TALON columns and enabling detection by Western blot using anti-V5 antibodies. The sequence of the cloned gene was confirmed by Sanger sequencing prior to use.

HEK293T cells were transiently transfected with the Gpx3-pcDNA-4 in the presence or absence of sodium selenite (NaS). Transfected cells cultured without NaS did not produce any recombinant Gpx3, confirming the necessity to include NaS in culture medium in order for the cells to secrete Gpx3 (Fig. 4.2 A). The presence of Gpx3 in cell-conditioned media was routinely assessed by Western blot following each transient transfection. Monomeric Gpx3 has a molecular weight of ~23kDa, but the addition of the v5-epitope and his-tag increases this to 28kDa (Ottaviano et al. 2009). The presence of a band at around 28kDa confirmed the presence of recombinant Gpx3 in the cell-conditioned media (Fig. 4.2 B).

After sufficient quantities of cell-conditioned media containing Gpx3 had been collected (~0.5L) recombinant Gpx3 was purified by affinity chromatography using cobalt-based
chromatography resin. Western blot analysis of the eluted fractions confirmed the successful elution of Gpx3 using 50mM and 300mM of imidazole (Fig. 4.2 C). Coomassie staining of acrylamide gels stains all proteins on the gel blue allowing interpretation of the purity of the sample. The Coomassie stain confirmed a very high purity of Gpx3 in the fraction eluted with 300mM imidazole (Fig. 4.2 D). Both the 50mM and 300mM eluate were subsequently concentrated and protein concentration assessed by ELISA, confirming a protein yield of around 2mg/mL. Enzymatic activity was assessed using a kinetic activity assay and was very low at around 30U/mL for the 300mM eluate. Overall, production of Gpx3 via transient transfection produced around 2.3 mg of purified Gpx3 per litre of cell-conditioned media. The activity level, being so low, meant that very high quantities of recombinant protein would have to be produced and therefore it was decided to attempt other methods to improve the efficiency of production of recombinant Gpx3.
Figure 4.2 Expression and purification of recombinant Gpx3 from HEK293T cells. (A) HEK293T cells were transiently transfected in the presence or absence of selenocysteine (NaS) and Gpx3 production in cell-conditioned media was assessed by Western blot. (B) The presence of recombinant Gpx3 in cell-conditioned media was assessed by Western blot after each transient transfection (TT). (C) Recombinant Gpx3 was purified via immobilised metal-affinity chromatography using Cobalt-based columns and eluted using increasing concentrations of imidazole. Gpx3 content of each elution fraction was assessed by Western blot. All Western blot analyses were performed under reducing conditions using polyclonal anti-V5 or anti-Gpx3 antibodies. (D) The purity of the final batches of fully purified and concentrated Gpx3 was evaluated by Coomassie staining of the agarose gel. The black arrows indicate the position of recombinant Gpx3.
4.3.2 Transient transfection of CHO-S cells

Chinese hamster ovary (CHO) cells are a routinely used cell line for production of recombinant eukaryotic proteins because of their post-translational modifications and efficient expression. Here, CHO-S cells were used for transfections and allowed collection of circa 1.2L of cell-conditioned media per transfection (Dumont et al. 2016).

CHO-S cells were transfected and cultured for 24-120 hours to determine the optimal time following transfection for protein collection. The cells were also transfected in the presence or absence of NaS to determine the importance of its addition in this system. The yield of recombinant protein was very low and the protein required concentration using acetone before detection on a Western blot (Fig. 4.3 A). This was in contrast to cell-conditioned media from HEK293T cells, in which recombinant Gpx3 could be visualised without concentration of protein on Western blots (Fig. 4.2 A-B). Protein yield peaked at 72 hours after transfection. The addition of NaS did not affect production of the protein as indicated by bands representing Gpx3 of comparable size on the Western blot (Fig. 4.3 A). The molecular weight of Gpx3 produced by CHO-S cells was greater than Gpx3 produced using HEK293Ts cells. This is likely caused by increased glycosylation in the CHO-S cell system. A Gpx3 activity assay showed that cells transfected alongside NaS produced Gpx3 with greater activity and therefore NaS was supplemented to all future transfections (Fig. 4.3 B).

The ratio of cDNA to polyethyleneimine (PEI) was also optimised. PEI is a cationic polymer which condenses DNA into positively charged particles which bind to anionic cell surfaces and are endocytosed, efficiently delivering the cDNA into the cytosol of the host cell (Baker et al. 1997). CHO-S cells were transfected for 72 and 96 hours with cDNA added to PEI at ratios of 1:1 (25µg cDNA to 25µL PEI) and 1:3 (25µg cDNA to 75µL PEI). Regardless of the cDNA:PEI ratio, production of Gpx3 was low and again the protein required concentration before a clear band could be detected by Western blot. Levels of expression were unaffected by cDNA to PEI ratio (Fig. 4.3 C) and a ratio of 1 to 3 was used for future transfections.

After the initial optimisations, approximately 1.2 litres of cell-conditioned media were collected from a single transfection and was purified via immobilised metal affinity chromatography. The concentration of protein after purification and concentration was extremely low, yielding around 47.5µg Gpx3 per litre, almost 50 times less than that produced via transient transfection of HEK293T cells. Therefore, CHO-S cells were no longer used for Gpx3 transfections.
Figure 4.3 Expression of recombinant Gpx3 in CHO-S cells. (A) CHO-S cells were transiently transfected for 24–120 hours with Gpx3-pcDNA-4 ± sodium selenite (NaS). Expression of Gpx3 in cell-conditioned media was concentrated and assessed by Western blot. (B) The enzymatic activity of recombinant Gpx3 from the 72 hour transfection was determined by Gpx3 activity assay. (C) CHO-S cells were transfected for 72 or 96 hours with Gpx3-pcDNA added alongside PEI at a ratio of 1 to 1 (1:1) or 1 to 3 (1:3), and protein expression in cell-conditioned media assessed by Western blot following concentration of the protein. All Western blot analyses were run under reducing conditions using a polyclonal anti-v5 antibody. The black arrows indicate the position of recombinant Gpx3. (B) Data represent means from three technical replicates ± SD.
4.3.3 Stable expression of Gpx3 in HEK293T cells

In an attempt to simplify production and increase the overall yield of recombinant Gpx3, HEK 293T cells were stably transfected with cDNA for Gpx3. Stably transfected cells are generated by integration of the plasmid DNA into the genome of the cell. This allows the cells to constantly express the protein of interest, even after replication, allowing for increased scalability and yield (Kim and Eberwine 2010).

Although both circular and linear plasmids have shown success, it is generally accepted that a linear plasmid improves the chances of obtaining a stably transfected cell line. The linearisation of the plasmid DNA also allows cleavage of the plasmid at a desired region, reducing the risk of random breakpoints and increasing the chance of producing an intact protein (Stuchbury and Munch 2010). The Gpx3-pcDNA-4 plasmid was digested using the restriction enzyme \( Pvu \), which cleaved DNA at a region within the ampicillin resistance gene. Circular plasmids form two conformations (supercoiled and relaxed) resulting in the formation of two separate bands on agarose gels following electrophoresis. Linear plasmids, on the other hand, form a single band. Successful linearisation of the Gpx3 plasmid was confirmed by the appearance of a single band on the agarose gel (Fig. 4.4 A).

HEK293T cells were transfected with both linear and circular forms of the plasmid. Following transfection, cells with successful integration of the plasmid were selected using Zeocin at 250-400µg/mL with single zeocin-resistant clones selected for further culturing by ring-cloning. In addition to these ring cloned populations, a polyclonal population of cells was produced containing cells that were not ring cloned but were growing in media supplemented with zeocin. The polyclonal cells successfully expressed recombinant Gpx3, as confirmed by Western blot (Fig. 4.4 B). The ring-cloned cells expressed low levels of Gpx3, with only very faint bands visible on the Western blot. Interestingly, the clones where protein expression could be seen on a Western blot (clones 2, 5 and 7) were all transfected with the linear plasmid, whereas clones 3 and 6 were transfected with the uncut plasmid and did not express any recombinant Gpx3 (Fig. 4.4 C). The production of recombinant Gpx3 from the selected clones was very low. Therefore, long-term selection of the clones would have been required to select for cells with stable transfection and to improve the expression of Gpx3. The expression of Gpx3 was halted at this point and samples from initial HEK293T transient transfections used for \textit{in vitro} experimentation.
Figure 4.4 Stable expression of Gpx3 in HEK293T cells. (A) Successful linearisation of Gpx3-pcDNA4 was confirmed using agarose gel electrophoresis. Expression of recombinant Gpx3 from the polyclonal cell line (B) and individual clones (C) was assessed by Western blot performed under reducing conditions using a polyclonal anti-V5 antibody. The black arrows indicate the position of recombinant Gpx3.
4.4 Expression and purification of ECSOD from stably expressing HEK293T cells

Recombinant ECSOD was produced using a stably transfected HEK293T-ECSOD cell line. After collection of approximately 1 litre of cell-conditioned media containing recombinant ECSOD, the protein was purified using fast-protein liquid chromatography (FPLC). ECSOD contains a C-terminal heparin binding domain (HBD), which enables its binding to the extracellular matrix in vivo (Sandström et al. 1992), and also allowed the purification of the protein using heparin-sepharose columns. ECSOD was eluted from heparin-sepharose columns using increasing concentrations of NaCl (100-500mM). UV absorbance (280nM) of each elution step was monitored. The presence of a distinct peak in UV absorbance occurred for each elution fraction, with the biggest peak occurring in the final fraction (500mM NaCl) (Fig. 4.5 A).

Fractions (F) 4 and 5 were concentrated and the protein content assessed by Western blot. Purified ECSOD is expected to form two bands under nonreducing conditions, comprised of a dimer at 60kDa and a monomer at 30kDa. Under reducing conditions the expected band for ECSOD is expected to form at around 30kDa (Enghild et al. 1999). A large band at ~30kDa was present in both fractions confirming the presence of recombinant ECSOD. The majority of protein was eluted in the final elution step (Fig. 4.5 B). Moreover, fraction 5 had high purity with a large band present at the correct molecular weight following Coomassie staining of the agarose gel (Fig. 4.5 C).

After purification and concentration, protein concentration of fraction 5 was measured by BCA assay. The 1.2 litres of cell-conditioned media, purified and concentrated to 0.875mL, yielded a final concentration of 1.99mg of ECSOD per mL with an activity of 177,000 U/mL, as assessed using an activity assay. This production of highly active ECSOD meant that a single batch of purified supernatants could be used for all experiments.
Figure 4.5 Purification of recombinant ECSOD. (A) Recombinant ECSOD was purified via fast-protein liquid chromatography using an AKTA chromatography system. UV absorbance (solid) was assessed during each fraction (F1-5) wash to confirm protein elution. The total volume following each wash step is signified by the dotted line. (B) Protein content and (C) purity of fractions 4 and 5 was assessed by Western blot and Coomassie staining, respectively. The black arrows indicate the position of recombinant ECSOD.
4.5 Gpx3 inhibits ROS generation in primary human monocytes

Expression and purification of recombinant Gpx3 proved difficult. Nonetheless, enough protein was produced via transient transfections to allow for a single proof experiment to assess whether the protein was able to reduce ROS \textit{in vitro}. Primary human monocytes were preincubated for an hour with Gpx3 (1 or 2.5 U/mL) prior to a 6 hour stimulation with Pam3+MSU crystals alone. During the 6-hour stimulation, Gpx3 was added alongside the stimulants to maintain the concentration used for preincubation. MSU crystals, as previously demonstrated, induced an almost 3-fold increase in ROS production which was inhibited up to 40% by Gpx3 (Fig. 4.6 A). However, Gpx3 proved very toxic causing a large reduction in cell viability even at these low concentrations (Fig. 4.6 B).
Figure 4.6 Recombinant Gpx3 reduces ROS-generation but is toxic in primary human monocytes. Primary human monocytes from a single donor were incubated in Gpx3 for one hour prior to a six hour stimulation with Pam3 (100ng/mL) and MSU crystals (10mg/dL). Gpx3 concentration was maintained during the 6 hour stimulation. (A) ROS generation was assessed using ROS-Glo assay. (B) Viability was determined following stimulation using CTG assay. Data represent means of three technical replicates from a single donor ± SD.
4.6 Catalase inhibits ROS but does not affect NF-κB activation in THP-1 monocytes

Due to difficulties in producing sufficient quantities of recombinant Gpx3 using HEK293T and CHO-S cell lines, a commercially available catalase was used. Catalase, like Gpx3, is an antioxidant enzyme which catalyses the reduction of H₂O₂ in humans. Unlike Gpx3, catalase is found intracellularly and attached to cell membranes. The successful reduction of ROS using catalase had previously been demonstrated in THP-1 monocytes as validation for the ROS-Glo assay (Fig. 3.14). Catalase at 25 and 50 U/mL inhibited around 40-50% of the menadione-induced ROS generated in THP-1 monocytes (Fig. 3.14). The complete removal of ROS can be detrimental to the health of the cell, and a decision was therefore made to use catalase at these concentrations in an attempt to limit reductions in cell viability.

The importance of ROS in NF-κB activation was assessed using THP1-Blue monocytes which are transfected with an NF-κB inducible SEAP reporter. When NF-κB is activated in these cells, the SEAP reporter is secreted and can be detected using the colorimetric enzyme QUANTI-Blue™ and quantified by measuring absorbance (section 2.17). THP1-Blue monocytes were preincubated in catalase for an hour prior to a six hour stimulation with Pam3, MSU crystals and menadione added alongside the antioxidant enzyme. Menadione was included as a positive control to ensure that the ROS assay was working, and also to examine whether the addition of an oxidant would activate NF-κB in this system. Experiments investigating ROS generation and NF-κB activation were completed in parallel using the same batch of cells, allowing linked analyses of the effect of reducing ROS on the activation of inflammatory responses.

Catalase caused a 20-40% inhibition of ROS-generation (Fig. 4.7 A). As previously seen (chapter 3), Pam3 and MSU crystals did not induce ROS generation in the THP-1 monocytes. TLR-2 ligation by Pam3 resulted in a significant increase in NF-κB activation (Fig. 4.7 B). MSU crystals did not induce activation of NF-κB when added alone or alongside Pam3. Despite the reductions in ROS by catalase, NF-κB activation was unaffected by addition of the antioxidant enzyme suggesting that H₂O₂ does not influence activation of the transcription factor (Fig. 4.7 B). Menadione was added as a positive control for the ROS-Glo assay, but also allowed further examination of the role of ROS on NF-κB activation. Menadione caused a significant increase in generation of ROS (Fig. 4.7 A) but had no effect on NF-κB activation (Fig. 4.7 B), suggesting that chemical induction of ROS does not activate the transcription factor. Catalase did not have any adverse effect on cell viability at the concentrations used (Fig. 4.7 C).
Figure 4.7 ROS inhibition by catalase does not affect NF-κB activation in THP1-blue monocytes. THP1-blue monocytes were preincubated for 1 hour in catalase prior to a 6 hour stimulation with Pam3 (100ng/mL), MSU crystals (10mg/dL) and menadione (20mM). (A) ROS generation was assessed by ROS-Glo. (B) NF-κB activation was determined via QUANTIblue reaction with NF-κB inducible SEAP in cell-conditioned media. (C) Cell viability was measured using CTG. Data represent means from 3 individual experiments ± SEM (A-B) or means three technical replicates ± SD representative of the three individual experiments (C). Significance was determined by one-way ANOVA with Tukey’s post-hoc test (*P<0.05, **P<0.01, ****P<0.0001).
4.7 Effect of catalase on ROS and cytokine secretion from stimulated primary human monocytes

Primary human monocytes were subsequently stimulated in the presence or absence of catalase to determine whether removal of H$_2$O$_2$ may alter cytokine secretion. Primary human monocytes isolated from two donors were initially stimulated with Pam3 ± MSU crystals added alongside catalase at 25 and 50 U/mL, matching the concentrations used in THP-1 cells. This experiment was completed to test the concentration of catalase required for ROS inhibition in primary human monocytes. MSU crystals induced a 1.5-2.0 fold increase in ROS-generation and catalase caused a small inhibition in ROS at these concentrations (Fig. 4.8 A). These effects on ROS occurred in the absence of cell death confirmed by CTG analysis (Fig. 4.8 B). This experiment was only completed in two donors because of the limited inhibition of ROS by catalase. The confirmation that catalase did not cause cell death at 50U/mL indicated that it might be safe to increase levels of catalase in these cells for future experiments.

Catalase concentration was subsequently increased to 100U/mL in an attempt to increase the inhibition of ROS production in primary human monocytes. Catalase at 100 U/mL caused a 40-50% reduction in ROS-generation and significantly inhibited MSU-induced ROS levels reducing them to that seen in unstimulated controls (Fig. 4.9 A). Despite the reductions in ROS, catalase did not reduce the secretion of IL-1β (Fig. 4.9 B) or TNFα (Fig. 4.9 C) in primary human monocytes. In fact, there was a clear trend for the antioxidant enzyme to cause an increase in cytokine secretion, although not all of these increases reached statistical significance. In primary human monocyte experiments, TNFα secretion was quantified as an indirect measure of NF-κB activation. Catalase caused a significant increase in Pam3-induced TNFα secretion suggesting that catalase may be influencing the NF-κB pathway (Fig. 4.9 C). A viability measurement confirmed that these increases in cytokine secretion were not a result of catalase-induced toxicity (Fig. 4.9 D).
Figure 4.8 ROS inhibition by catalase in primary human monocytes. Primary human monocytes from two separate donors were stimulated for 2 hours with Pam3 (100ng/mL) ± MSU crystals (10mg/dL) added alongside catalase. (A) ROS-generation was assessed using a ROS-Glo assay. (B) Cell viability was determined after stimulation by CTG analysis. Data represent means from triplicate measurements for each donor ± SD.
Figure 4.9 Catalase inhibits ROS-generation and increases cytokine production in primary human monocytes. Primary human monocytes were incubated for 1 hour in catalase prior to a 6 hour stimulation with Pam3 (100ng/mL) ± MSU crystals (10mg/dL). During this stimulation, catalase concentration was maintained. (A) ROS-generation was measured using ROS-Glo assay. (B) IL-1β and (C) TNFα secretion were quantified in cell-conditioned media by ELISA. (D) Viability was assessed by CTG. Data represent means from 4 individual donors ± SEM (A-C) or triplicate measurements from a single donor representative of viability results seen for all 4 donors ± SD (D). Significance was determined by one-way ANOVA with Tukey’s post-hoc test (*P<0.05, **P<0.01, ****P<0.0001).
4.8 ECSOD reduces ROS in THP-1 monocytes

The effect of recombinant ECSOD on ROS-generation and cell viability was initially tested in THP-1 monocytes to determine if the antioxidant was capable for reducing ROS and to assess whether it would cause toxicity. THP-1 monocytes were stimulated for 6 hours with increasing concentrations of ECSOD, added alone or alongside positive control menadione (20mM). ECSOD caused a dose-dependent inhibition in menadione-induced ROS generation, inhibiting around 50% of ROS when added at 50U/mL and up to 80% when added at 500U/mL (Fig. 4.10 A). Cell viability was assessed in a parallel cell plate and confirmed that ECSOD did not cause any cell death at the concentrations used (Fig. 4.10 B).
Figure 4.10 Recombinant ECSOD inhibits ROS in THP-1 monocytes. (A) THP-1 monocytes were stimulated for 6 hours with increasing concentrations of ECOSD added alongside menadione (20mM). ROS-generation was measured using a ROS-Glo assay. (B) Viability was assessed by CTG analysis. Data represent means of three technical replicates ± SD.
4.9 ECSOD induces inflammatory responses in THP-1 monocytes

THP-1 monocytes were stimulated for 18 hours in the presence of ECSOD and cytokine secretion measured. ROS-generation had previously been measured after 6 hours, however stimulations were increased to 18 hours to allow sufficient time for cytokine production and secretion in these cells. Surprisingly, there was a strong trend for recombinant ECSOD to cause a dose-dependent increase in IL-1β secretion. In fact, ECSOD at 250U/mL significantly increased MSU+Pam3-induced IL-1β secretion compared to cells stimulated with MSU+Pam3 in the absence of the antioxidant (Fig. 4.11 A). ECSOD did not cause a significant increase in TNFα secretion, but again there was a definite trend for increased TNFα production (Fig. 4.11 B). Viability analysis showed a small reduction in viability when cells were exposed to recombinant ECSOD at 100-250 U/mL (Fig. 4.11 C), which could explain the increased cytokine production. The slight reduction in cell viability seen in this experiment compared to the previous experiment is likely a result of the increased stimulation time.

At this point a decision was made to reduce stimulation times to 6 hours. This was to allow linked analysis of ROS generation alongside measurement of NF-κB activation and cytokine secretion. These timings also matched the catalase experiments shown previously.

NF-κB activation was subsequently measured using THP1-blue cells stimulated with Pam3 and MSU added alongside ECSOD for 6 hours. For this experiment, recombinant ECSOD was used at 25 and 50 U/mL. These concentrations had previously been shown to reduce around 50% of menadione induced ROS (Fig. 4.10). By reducing the concentration to 25 and 50U/mL, it was hoped that less of the precious protein would be used therefore allowing extra future experimentation. At 25U/mL ECSOD inhibited menadione induced ROS production by around 50% but had less effect on ROS levels in unstimulated cells and cells stimulated with Pam3 ± MSU crystals (Fig. 4.12 A). Interestingly, ECSOD induced a very robust increase in NF-κB activation inducing similar levels of NF-κB to Pam3 when added at 50 U/mL (Fig. 4.12 B) without causing cell death (Fig. 4.12 C).

The activation of NF-κB by ECSOD, as well as the increased cytokine secretion caused by both ECSOD and catalase bought into question the cleanliness of these antioxidant enzymes. These increases in immune response were unexpected and pointed at potential endotoxin contamination. Therefore, experimental use of the proteins was stopped until the endotoxin content of the proteins had been assessed.
Figure 4.11 Recombinant ECSOD induces cytokine secretion in THP-1 monocytes. THP-1 monocytes were preincubated for 1 hour in ECSOD and then stimulated for 18 hours with Pam3 (100ng/mL) ± MSU crystals (10mg/dL). ECSOD concentration was maintained during the 18 hour stimulation. (A) IL-1β and (B) TNFα secretion was determined by ELISA. (C) Cell viability was assessed by CTG analysis. (A-B) Data represent means from 3 individual experiments ± SEM or (C) means of three technical replicates ± SD. Significance was determined by one-way ANOVA with Tukey's post-hoc test (****P<0.0001).
Figure 4.12 Recombinant ECSOD increases NF-κB activation in THP-1 monocytes.

THP-1 blue cells preincubated for 1 hour in ECSOD prior to 6 hour stimulation with Pam3 (100ng/mL), MSU crystals (10mg/dL) and menadione (20mM). ECSOD concentration was maintained during the 6 hour stimulation. (A) ROS-generation was determined by ROS-Glo assay. (B) NF-κB activity was assessed by measuring SEAP by QUANT-Blue assay. (C) Viability was assessed by MTT assay. Data represent means of three technical replicates ± SD.
4.10 Contamination of antioxidant enzymes with TLR ligands

Recombinant ECSOD and catalase both induced cytokine secretion in human monocytes. ECSOD also induced a robust increase in NF-κB activation in THP1-blue monocytes. These results raised the question of whether the antioxidant enzymes used were contaminated with endotoxin and that the effects on IL-1β, TNFα and NF-κB were an artefact of this contamination rather than a true effect of the enzymes themselves. The main reason for the use of mammalian cell lines for expression of recombinant proteins here was in order to avoid endotoxin contamination commonly seen in proteins produced using *E. coli* (Mamat et al. 2015). Endotoxin (otherwise known as LPS) is a large molecule found in the outer membrane of Gram-negative bacteria, which can be found in tap water, air and on skin (Gorbet and Sefton 2005). LPS is commonly used in research to induce activation of TLR4 and causes secretion of both IL-1β and TNFα *in vitro* (Schwarz et al. 2014). In addition to the use of mammalian cells for protein expression, other precautionary steps were taken to avoid endotoxin contamination, including the use of endotoxin-free water and PBS for protein resuspension.

To determine whether the antioxidant enzymes were contaminated with endotoxin, samples were tested by limulus amebocyte lysate (LAL) assay. The LAL assay is an extremely sensitive measure of endotoxin contamination and remains the gold standard for detection of low level endotoxins in biological samples (Blechova and Pivodova 2001). The LAL kit allowed detection of 0.125 EU/mL endotoxin or greater, as confirmed following a titration of endotoxin standards (table 4.1).

Catalase and ECSOD were diluted to 500 and 5000 U/mL in endotoxin-free water, and added to LAL. These concentrations represented the highest working concentration used in experiments as well as 10-fold higher concentration. Recombinant ECSOD caused a clot at 5000 U/mL but not at 500 U/mL suggesting that endotoxin contamination was present but at relatively low levels (<1.25 EU/mL). Catalase, on the other hand, caused clot formation at both 500 and 5000 U/mL. Gpx3 was also added to a LAL test at 10U/mL (4 times working concentration) and formed a solid clot(table 4.2).

Overall, the LAL assay confirmed the presence of endotoxin in the recombinant proteins. This contamination invalidated the data obtained thus far as any effect of catalase and ECSOD on cytokine secretion was most likely caused by the endotoxin.
<table>
<thead>
<tr>
<th>Standards</th>
<th>Gel formed?</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125 EU/mL</td>
<td>Yes</td>
</tr>
<tr>
<td>0.100 EU/mL</td>
<td>No</td>
</tr>
<tr>
<td>0.060 EU/mL</td>
<td>No</td>
</tr>
<tr>
<td>0.030 EU/mL</td>
<td>No</td>
</tr>
<tr>
<td>Tissue-grade water</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 4.1 Sensitivity of the Limulus Amebocyte Lysate (LAL) assay.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Concentration</th>
<th>Gel formed?</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECSOD</td>
<td>5000 U/mL</td>
<td>Yes</td>
</tr>
<tr>
<td>ECSOD</td>
<td>500 U/mL</td>
<td>No</td>
</tr>
<tr>
<td>Catalase</td>
<td>5000 U/mL</td>
<td>Yes</td>
</tr>
<tr>
<td>Catalase</td>
<td>500 U/mL</td>
<td>Yes</td>
</tr>
<tr>
<td>Gpx3</td>
<td>10 U/mL</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 4.2 Endotoxin testing of ECSOD, Gpx3 and catalase. All three enzymes were positive for LPS, forming a solid gel clot when added to LAL. ECSOD contamination was low with less than 0.125 EU/mL endotoxin at the highest concentration added to cells in previous experiments (500 U/mL).
4.11 Stimulation of THP-1 and primary human monocytes with endotoxin

To determine at what concentration endotoxin may activate inflammatory pathways in THP1-blue monocytes and primary human monocytes, cells were stimulated with endotoxin at the range of concentrations tested in the LAL assay. For reference, 1 EU/mL endotoxin equates to 100-200 pg endotoxin/mL. Here, 1EU/mL was considered as 100pg/mL for all calculations.

THP1-Blue cells were stimulated for 18 hours with endotoxin and NF-κB activation measured. Cells were stimulated for extended periods to ensure that any effect of endotoxin would be observed and to match the longest stimulation time point used thus far in THP-1 cells. Endotoxin at 0.0625-0.125EU/mL had no effect on NF-κB activation during this time period. Small increases in NF-κB activation were observed at 0.5EU/mL endotoxin with a large increase at 5EU/mL (Fig. 4.13 A).

To examine what effect endotoxin would have on primary human monocytes, cells were stimulated for 6 hours with endotoxin to mimic experiments using antioxidant enzymes in this chapter. Endotoxin was used at a range of concentrations that covered the concentrations measured using the LAL assay. There was minimal IL-1β secretion from cells exposed to 0.0625-0.125EU/mL endotoxin. At 0.5EU/mL there was a small increase in IL-1β secretion, and this was dramatically increased in cells exposed to endotoxin at 1.0 and 5.0EU/mL (Fig. 4.13 B). Likewise, TNFα secretion was unaffected by endotoxin at 0.0625-0.125EU/mL with a small induction of TNFα secretion at 0.25EU/mL. This was increased dose-dependently with endotoxin at 1.0-5.0EU/mL inducing a robust secretion of TNFα in primary human monocytes (Fig. 4.13 C), confirming activation of NF-κB. Endotoxin at a concentration of 0.125 EU/mL (signified by arrows in figure 4.13) had little effect on the activation of NF-κB or cytokine secretion.

These data suggested that proteins which did not cause a gel clot on the LAL assay, i.e. had less than 0.125EU/mL endotoxin contamination, could be used for in vitro experimentation without having an effect on immune responses. Interestingly, the effect of ECSOD used at 500U/mL, which was negative on the LAL assay, was a true effect and not necessarily caused by the contamination. Despite this, it was deemed important to try and remove the endotoxin from the antioxidants before future use in vitro.
Figure 4.13 Endotoxin activates NF-κB and induces cytokine secretion. (A) THP1-Blue monocytes were stimulated for 18 hours with a range of endotoxin concentrations and NF-κB activation determined using QUANTI-Blue analysis of NF-κB-inducible SEAP reporter in cell-conditioned media. Primary human monocytes were stimulated for 6 hours with a range of endotoxin concentrations. (B) IL-1β and (C) TNFα secretion was assessed by ELISA. (D) Viability of primary human monocytes after endotoxin stimulation was measured using a CTG assay and normalised to unstimulated controls. The arrows specify the lowest concentration of endotoxin that could be detected using the LAL assay kit. Data represent means of three technical replicates ± SD.
4.12 Removal of endotoxin from antioxidant enzymes

Contamination of catalase and ECSOD with endotoxin made it difficult to interpret the data produced thus far. Subsequently, preparations of catalase and ECSOD were subjected to endotoxin removal protocols in an attempt to remove the contaminating endotoxin.

Protein concentration following endotoxin removal was measured by BCA assay to assess whether any protein was lost during this procedure (table 4.3). There were large losses of 65.9 and 89.1% for catalase and ECSOD respectively. The stock concentrations for each enzyme were adjusted accordingly under the assumption that activity had not been affected. This was to ensure the correct final concentrations were used during the stimulations.

The antioxidant enzymes were then reanalysed for endotoxin content using the LAL assay. Catalase and ECSOD were diluted to 125-500 U/mL to test the highest concentration that could be used without causing clot formation. Both proteins caused clot formation at 500 U/mL but not at 250 U/mL (table 4.4). Endotoxin at 0.125EU/mL had no effect on NF-κB activation or cytokine secretion in monocytes (Fig. 4.13) and therefore proteins with less than 0.125EU/mL were considered endotoxin-free and useable for experimentation. The LAL tests were repeated to ensure validity of the results and confirmed that both proteins were endotoxin-free at 250U/mL (<0.125 EU/mL). In order to achieve an endotoxin concentration of <0.125 EU/mL, catalase required repeated treatment with the endotoxin removal resin. Recombinant ECSOD, on the other hand, was only treated once due to the amount of protein lost following this single wash step.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Conc. prior to treatment</th>
<th>Conc. after treatment</th>
<th>Activity prior to treatment</th>
<th>Activity after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>10 mg/mL</td>
<td>3.41 mg/mL</td>
<td>20,000 U/mL</td>
<td>6,820 U/mL</td>
</tr>
<tr>
<td>ECSOD</td>
<td>1.993 mg/mL</td>
<td>0.217 mg/mL</td>
<td>177,000 U/mL</td>
<td>19,271 U/mL</td>
</tr>
</tbody>
</table>

**Table 4.3 Protein concentrations and activities following endotoxin removal.** Catalase and ECSOD were treated with endotoxin removal resin. Protein concentration was measured by BCA assay and enzymatic activity accounted for accordingly.

<table>
<thead>
<tr>
<th>Standard / sample</th>
<th>Concentration</th>
<th>Gel formed?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>&lt;0.005 EU/mL</td>
<td>No</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>0.25 EU/mL</td>
<td>Yes</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>0.125 EU/mL</td>
<td>Yes</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>0.0625 EU/mL</td>
<td>No</td>
</tr>
<tr>
<td>ECSOD</td>
<td>500 U/mL</td>
<td>Yes</td>
</tr>
<tr>
<td>ECSOD</td>
<td>250 U/mL</td>
<td>No</td>
</tr>
<tr>
<td>ECSOD</td>
<td>125 U/mL</td>
<td>No</td>
</tr>
<tr>
<td>Catalase</td>
<td>500 U/mL</td>
<td>Yes</td>
</tr>
<tr>
<td>Catalase</td>
<td>250 U/mL</td>
<td>No</td>
</tr>
<tr>
<td>Catalase</td>
<td>125 U/mL</td>
<td>No</td>
</tr>
</tbody>
</table>

**Table 4.4 Endotoxin content of ECSOD and catalase after treatment with endotoxin removal resin.** Both ECSOD and catalase formed a clot at 500U/mL demonstrating endotoxin contamination at this concentration. However, endotoxin was undetectable (<0.125EU/mL) when samples were diluted to 250U/mL.
4.13 Stimulation of primary human monocytes with endotoxin-free antioxidants

Primary human monocytes were preincubated for 1 hour with endotoxin-free catalase or ECSOD and then stimulated for 6 hours with Pam3 ± MSU crystals. Antioxidant enzymes were added alongside stimulants for the 6 hour stimulations. Both ECSOD and catalase were used at a concentration of 250U/mL. This was selected as the highest concentration that was considered endotoxin-free by the LAL assay (i.e. containing less than 0.125EU/mL endotoxin). The concentrations were also based on the viability obtained from previous primary human monocytes showing no cell toxicity when stimulants were used at 100U/mL. By increasing concentrations to 250U/mL it was hoped that greater levels of ROS inhibition would be observed, thus providing the best opportunity to examine the importance of ROS in the immune response of these cells.

As seen consistently throughout this study, MSU crystals added alongside Pam3 induced a significant increase in ROS generation. Catalase was more effective at reducing ROS, reducing levels in unstimulated and Pam3 stimulated cells by around 70%, whereas ECSOD had no effect on the ROS-levels in unstimulated or Pam3 stimulated cells. Both catalase and ECSOD significantly inhibited MSU-induced ROS generation, both bringing ROS down to levels comparable with unstimulated cells (Fig. 4.14 A).

Despite the inhibition of ROS, catalase had no effect on IL-1β secretion, with cells secreting comparable levels of IL-1β when stimulated alongside the antioxidant enzyme. In contrast, ECSOD actually increased IL-1β secretion, causing significant increases in Pam3- and MSU+Pam3-induced IL-1β secretion in the primary human monocytes (Fig. 4.14 B).

TNFα secretion, used as an indirect measure of NF-κB activation, was unaffected by the antioxidant enzymes, with neither catalase or ECSOD changing the levels of TNFα secretion compared to cells stimulated in the absence of antioxidants. However, it is important to note that there was definitely a trend for ECSOD induced secretion of TNFα, with cells stimulated with ECSOD alone secreting similar levels of TNFα to cells stimulated with Pam3, although this did not reach statistical significance (Fig. 4.14 C).

No cell death was observed in response to the stimulants (Fig. 4.14 D), confirming the effects on cytokine secretion were not due to toxicity. The viability data presented is from the stimulation data and is representative of the ROS-Glo viability data.
Figure 4.14 Endotoxin-free antioxidants reduce ROS generation but have different effects on cytokine secretion in primary human monocytes. Primary human monocytes were seeded in parallel for measurement of ROS and cytokine secretion. Monocytes were preincubated for 1 hour in media (black bars), catalase (250U/mL – grey bars) or ECSOD (250U/mL – white bars) and then stimulated for 6 hours with Pam3 (100ng/mL) ± MSU crystals (10mg/dL). Catalase and ECSOD were present in the 6 hour stimulations. (A) ROS generation was measured by ROS-Glo assay. (B) IL-1β and (C) TNFα secretion were quantified by ELISA. (D) Viability was assessed by CTG. Viability data is from stimulations, and are representative of data from viability obtained after ROS-Glo. Data represents means from 3 individual donors ± SEM. Significance was determined by one-way ANOVA with Tukey's post-hoc test (*P<0.05, **P<0.01, ***P<0.001).
4.14 Discussion

In this study, the small molecule antioxidant NAC and recombinant antioxidant enzymes, catalase and ECSOD, were used to examine the effect of inhibiting ROS on the priming and activation of the NLRP3 inflammasome. All three of these antioxidants caused a significant inhibition in MSU-induced ROS, with NAC causing the largest reductions in ROS in primary human monocytes. Interestingly, and in confirmation of findings presented in this thesis so far, the removal of ROS had no influence on MSU-induced IL-1β secretion, demonstrating that ROS are not required for the activation of the NLRP3 inflammasome in primary human monocytes.

NAC was the most potent antioxidant, almost completely inhibiting ROS in primary human monocytes. Interestingly this ROS removal decreased Pam3-induced IL-1β by 75% and TNF by 50% secretion, although this did not reach statistical significance. This might suggest a role for ROS in NLRP3 priming but not activation. Consistent with these data, NAC has previously been shown to inhibit LPS induced IL-1β secretion in THP-1 cells (Pazar et al. 2011; Zhou et al. 2015). It is interesting however, that a measurable increase in ROS generation has not been observed in response to Pam3 in this study. Triggering of a single TLR has been shown to induce a self-limiting redox response in primary human monocytes which results in the slow release of IL-1β (Tassi et al. 2009; Lavieri et al. 2014). This is a likely explanation for not observing an increase in ROS when Pam3 is added and suggests that the levels of ROS involved in NF-κB activation and NLRP3 priming are subtle and dealt with rapidly by the cell, therefore going undetected in ROS measurements. Although an increase in ROS was not measured in response to Pam3, NAC significantly reduced ROS levels in cells during this stimulation and this inhibition of ROS only affected Pam3, but not Pam3+MSU-induced cytokine secretion. Pam3 is known to induce TNFα secretion via activation of NF-κB (Brandt et al. 2013) and therefore TNFα secretion was used as an indirect measure of NF-κB activation in primary human monocytes throughout this study. NAC did reduce TNFα secretion by around 50% in Pam3 stimulated primary human monocytes suggesting that ROS may mediate activation NF-κB and thus NLRP3 priming in these cells.

Overall, the data obtained using NAC suggest that ROS are only required for priming of the NLRP3 inflammasome, having no effect on IL-1β secretion in response to MSU crystals. Indeed, a requirement for ROS in NLRP3 priming but not activation has been shown by others, with ROS inhibition using DPI only inhibiting IL-1β secretion in murine BMDMs when DPI was added prior to LPS preincubation, but not affecting cytokine secretion when added.
after the initial LPS preincubation (Bauernfeind et al. 2011). However, if Pam3-induced NF-κB activation was inhibited, as was suggested by the reductions in IL-1β and TNFα secretion in monocytes here, one would also expect a reduction in NLRP3 activation as well because of the downstream implications on pro-IL-1β and NLRP3 expression. This was not the case here with MSU+Pam3-stimulated monocytes secreting high levels of IL-1β even after an initial preincubation with NAC. This is perhaps explained by the only partial inhibition of Pam3-induced TNFα secretion, meaning that NF-κB activation still occurred to some extent and this could have been enough to licence activation of the inflammasome. It would interesting to measure NF-κB activation and nuclear translocation in primary human monocytes to examine what effect ROS inhibition would have on the activation of the transcription factor. It would also be interesting to examine what effect NAC might have on expression of *IL1B* and *NLRP3* in Pam3-stimulated primary human monocytes to examine whether inflammasome priming is inhibited by the removal of ROS.

An interesting finding of this study is the increase in cytokine secretion from primary human monocytes induced by recombinant ECSOD even after endotoxin removal. The use of recombinant ECSOD in the study of NLRP3 activation is limited but one study has demonstrated an inhibitory effect of ECSOD on *in vivo* NLRP3 inflammasome expression in a mouse model, as well as an inhibitory effect on cytokine secretion from human epidermal cells *in vitro* (Nguyen et al. 2018). In contrast to, and in support of the findings presented here, another study has documented elevated IL-1β secretion in LPS primed primary human monocytes when stimulated with ATP and recombinant ECSOD (100U/mL) in combination, despite ECSOD reducing ATP induced ROS generation (Hewinson et al. 2008). Interestingly, a role for SOD enzymes, particularly SOD1, in NLRP3 activation has been described and in fact SOD enzymes have been shown to play an important role in modulating the inflammatory response. Murine macrophages deficient in SOD1 produce elevated concentrations of superoxide, which actually inhibits caspase-1 activation by reversible oxidation of two redox sensitive cysteine residues, resulting in an almost complete inhibition of LPS-induced IL-1β secretion from these macrophages (Meissner et al. 2008). Additionally, the inhibition of SOD1 by copper chelation has more recently been shown to reduce NLRP3 activation (Deigendesch et al. 2018), confirming that SOD is required for NLRP3 activation. However, these studies do not investigate what effect overexpression of the enzyme would have on inflammatory responses. Much further investigation is required to understand the increased cytokine secretion caused by ECSOD. These increases could also still be indicative of endotoxin contamination despite the efforts made to remove it from the purified protein or the presence of another non-LPS contaminant.
The addition of the antioxidants to the extracellular space raises the question of what effect the proteins would have had on ROS within the cell, especially for catalase which is an intracellular antioxidant. There is growing evidence of interplay between intra- and extracellular redox systems. For example, human adipocytes exposed to a more oxidised extracellular redox state have demonstrated increased levels of intracellular ROS production as well as increased membrane lipid peroxidation (Jones et al. 2016). Both superoxide and H$_2$O$_2$ have been shown to cross cell membranes via chloride channel-3 and aquaporin channels, respectively, and elicit intracellular signalling (Hawkins et al. 2007). H$_2$O$_2$ also freely diffuses across cellular membranes (Antunes and Cadenas 2000). Extracellular redox state can be greatly influenced by intracellular redox state, with cells releasing radicals as messenger molecules for cell-cell and other paracrine signalling (Banerjee 2012). It is therefore highly likely that the addition of antioxidant enzymes outside of cells will affect the redox state within cells. The differing effects of catalase and ECSOD on cytokine secretion in primary human monocytes could be linked to their different targets, with catalase reducing H$_2$O$_2$ and ECSOD superoxide, and the ability of these targets to exit the cell. Interestingly, there is evidence for recombinant ECSOD uptake by cells, showing that the heparin binding domain of the enzyme aids in its internalisation by HEK293T cells and translocation to the nucleus (Ookawara et al. 2002; Kim et al. 2017). This might provide a mechanism for ECSOD entry into the cell where it might induce inflammatory responses. However, further investigation is required to confirm if recombinant ECSOD is able to enter human monocytes and if so what effect it would have on their immune response.

The contamination of recombinant proteins is a common problem with proteins produced in E. coli (Mamat et al. 2015). Here, mammalian cell lines were used for the production of recombinant enzymes in a conscious effort to avoid this type of contamination and therefore, the contamination of the antioxidants in this study came as a surprise. The exact origin of this contamination remains unknown but endotoxin contamination of cell culture media, MSU crystals and menadione can be ruled out by their consistent lack of effect on NF-$\kappa$B activation in these experiments. One possibility for the contamination of the recombinant proteins is that the buffers used in the protein purification process contained low amounts of endotoxin that were then concentrated by elution from chromatography columns in small volumes. The contamination of catalase was also surprising, but one study has examined the level of contamination in catalase purchased from different suppliers and showed elevated endotoxin levels (1ng per 100,000 units of protein) in 4 out of 5 samples Gordon (1986). Overall, the presence of endotoxin makes the interpretation of early experiments difficult because of the potential effect on the immune response of the cells.
To rectify this, samples were exposed to endotoxin removal resin. The removal of endotoxin from biological samples is difficult (Wilson et al. 2001) and proved so here with protein losses occurring for both catalase and ECSOD. Nonetheless, the enzymes were eventually used at concentrations that contained less than 0.125EU/mL. The impact of residual endotoxin contamination in recombinant proteins in primary human monocytes and THP-1 cells has been examined elsewhere with LPS at 0.2-2ng/mL increasing IL-1β and TNFα secretion (2-20EU/mL) (Schwarz et al. 2014). In agreement with this, endotoxin caused cytokine secretion when as low as 0.5EU/mL were added in primary human monocytes here. The final results from the endotoxin-free antioxidants used here were validated by the fact that endotoxin at 0.125EU/mL (the cut-off for the LAL assay) or lower had no effect on immune responses in THP-1 or primary human monocytes, and therefore the effect of the endotoxin-free enzymes were considered to be a true effects.

Finally, the toxicity of Gpx3, even at very low concentrations, was unexpected. Gpx3 stimulations were completed prior to endotoxin removal and it is therefore impossible to rule out that this toxicity was not an effect of endotoxin or other contaminant. It is also possible that other (non-LPS contaminants) might be present which could influence immune responses in these cells. Of course, this could also explain the increase in cytokine secretion by ECSOD even after endotoxin-removal. This should be addressed prior to future use of recombinant proteins. Analysis of the proteins using mass spectrometry would allow detection of the presence and mass of any impurity, potentially allowing identification of its origin (Rhodes and Laue 2009). Inactive mutants of the recombinant enzymes should also be used as negative controls in future experiments to help distinguish whether results are caused by a contaminant or the enzymatic activity of the antioxidant.

In conclusion, the inhibition of ROS levels in primary human monocytes using both small molecule antioxidants and antioxidant enzymes, did not have any effect on IL-1β secretion in response to MSU crystals. Together with the findings presented in chapter 3, these results indicate that ROS are not required for the activation of the inflammasome in these cells. The only suggestion that ROS were involved NLRP3 signalling was the trend for reduced Pam3-induced cytokine secretion by NAC. This effect, however, was not replicated by catalase or ECSOD. Further investigation into the effect of NAC, and other antioxidants, on the gene expression of IL1B and NLRP3 is required to confirm if ROS is a required signal for NLRP3 priming in primary human monocytes.
Chapter 5 - Effect of hyperuricemia on activation of NLRP3
5.1 Introduction

Uric acid is the major antioxidant in the plasma, providing around 60% of the free-radical scavenging capacity (Ames et al. 1981). It is this protective function that is thought to have led to the evolutionary loss of the uricase gene in humans, resulting in much higher circulating concentrations of uric acid in humans compared to other mammals (Wu et al. 1992). Paradoxically, elevated plasma uric acid is also associated with the development of a range of metabolic diseases and can affect immune cell function during inflammation and disease (Kutzing and Firestein 2008). In chapter 4, ECSOD, another extracellular antioxidant enzyme, caused a marked increase in cytokine secretion from primary human monocytes, suggesting that perhaps extracellular antioxidants may influence the function of the inflammasome. Elevated serum uric acid is also the greatest risk factor for the development of gout, a disease driven by MSU-induced IL-1β secretion. Investigation of the effect of soluble uric acid on the activation of the inflammasome in primary human monocytes might therefore provide insight into whether changes to the redox environment might influence IL-1β mediated inflammatory responses.

In humans, uric acid is the end product of purine metabolism and is formed by enzymatic degradation of hypoxanthine and xanthine, a reaction catalysed by xanthine oxidoreductase (Maiuolo et al. 2016). In most mammals uric acid is then further oxidised by uricase into allantoin, which is subsequently excreted via the kidneys and urine (Alvarez-Lario and Macarron-Vicente 2010). At some point in their evolutionary history, humans and higher primates acquired a series of mutations in the uricase gene, resulting in complete loss of function of the enzyme (Wu et al. 1992). This loss of uricase, combined with the efficient retention of around 90% of uric acid in the blood via glomerular reabsorption, means that the average human serum uric acid level is 10-fold greater than for most other mammals (Kono et al. 2010). The exact reason for the evolutionary loss of uricase is unknown, yet several hypotheses advocate that its loss was of evolutionary advantage. These hypothesised advantages include its protective function as an antioxidant (Ames et al. 1981; Alvarez-Lario and Macarron-Vicente 2010), its role in maintaining blood pressure during periods of low salt consumption (Watanabe et al. 2002), and its role in neuroprotection (Scott and Hooper 2001) due to data from meta-analyses showing inverse correlations between serum uric acid and neurological diseases such as Parkinson’s disease (Shen and Ji 2013) and Alzheimer’s (Du et al. 2016). Interestingly, uric acid can also function as a pro-oxidant, forming free radicals in a variety of systems via its interaction with peroxynitrite (Vasquez-Vivar et al. 1996; Sautin and Johnson 2008). Perhaps then the evolutionary loss
of uricase occurred to allow increased serum uric acid concentrations to support redox sensitive signalling pathways involved in immune responses (discussed in chapter 1).

Although the loss of uricase is generally described as a beneficial mutation, elevated serum uric acid, known as hyperuricemia, is associated with the pathology of a wide range of diseases (Kratzer et al. 2014), including metabolic syndrome (Chen et al. 2007), hypertension (Feig 2012), chronic kidney disease (Madero et al. 2009) and cardiovascular disease (Alderman 2001). Hyperuricemia is defined as a plasma uric acid concentration in excess of 6.8 mg/dL, at which point uric acid becomes saturated and MSU crystals are likely to precipitate (Richette and Bardin 2010). MSU crystals trigger the development of gout, and hyperuricemia is therefore the single greatest risk factor for gout pathogenesis. A 15 year study of 2046 healthy men examined the association between uric acid concentration and gout, and showed that the annual incidence of gout increased substantially with elevated baseline levels of serum uric acid. A baseline concentration of <7mg/dL had an annual incidence of 0.1%, which increased to 4.9% when the baseline concentration increased to >9mg/dL (Campion et al. 1987; Perez-Ruiz and Lioté 2007). The concentration of serum uric acid has been documented to increase in excess of 6.8mg/dL in patients who are both intercritical and suffering an acute gout attack. A study which combined the results of two trials testing the efficacy of etoricoxib in the treatment of gout documented that of 339 patients suffering from acute gout (samples studied within 48 hours of onset of attack), 55% had a serum uric acid concentration of >8mg/dL with the mean concentration for patients not on treatment being 8.5mg/dL (Schlesinger et al. 2009). More recently, the role of lower concentrations of uric acid in disease has been examined and there is growing evidence that uric acid may play a role in the pathophysiology of cardiovascular and renal diseases even concentrations lower than clinically defined hyperuricemia, suggesting that pathological influence of uric acid might occur independently of crystal formation (Desideri et al. 2014). Thus, despite its beneficial role as an antioxidant uric acid is increasingly being associated with disease, although the exact causative role of uric acid in disease pathogenesis is not fully understood.

Gout is an inflammatory joint disease with a causal link to hyperuricemia due to MSU crystals which form in peripheral joints during periods of elevated plasma uric acid concentrations (Agudelo and Wise 1991). As previously discussed, MSU crystals activate the NLRP3 inflammasome and trigger a severe inflammatory response mediated by IL-1β that presents as a gouty arthritic attack (Martinon et al. 2006). Gout is the most common inflammatory arthritis in the UK with prevalence increasing from 1.4% in 1999 to 2.49% in 2012 (Kuo et al. 2015). Intriguingly, despite the strong associations between hyperuricemia
and gout, only around 10% of hyperuricemic individuals ever develop gout (Campion et al. 1987; Grassi et al. 2013) and the presence of MSU crystals within joints does not necessarily result in an inflammatory attack (Pineda et al. 2011; De Miguel et al. 2012). Thus, there are clearly other undefined factors involved in the pathophysiology of gout. Interestingly, recent studies have demonstrated that soluble uric acid can influence both the priming and activation of the NLRP3 inflammasome, even in the absence of MSU crystal formation. Soluble uric acid increases the production of IL-1β in human PBMCs from patients with gout compared to healthy controls and this secretion correlated with serum uric acid concentration (Crisan et al. 2015). Furthermore, 24-hour *in vitro* preincubation of PBMCs and monocytes from healthy donors with soluble uric acid significantly increased IL-1β secretion in response to TLR ligation by LPS alone or when LPS was added alongside MSU crystals (Crisan et al. 2017). This increased response to LPS and MSU was caused by a shift in IL-1β/IL-1 receptor agonist (IL-1Ra) balance with cells exposed to soluble uric acid producing less IL-1Ra, a shift that was demonstrated to reinforce an enhanced response to subsequent MSU crystal stimulation in PBMCs (Crisan et al. 2015). Interestingly, soluble uric acid also activates the NLRP3 inflammasome in murine BMDMs, increasing IL-1β secretion when added alongside LPS in a ROS-dependent manner (Braga et al. 2017). These recently demonstrated effects of uric acid on secretion of IL-1β could provide insight into possible roles for uric acid in activating the NLRP3 inflammasome. Given the antioxidant effects of uric acid, there is also the possibility that uric acid could influence activation of the inflammasome via its effects on oxidative stress.

Recent studies have therefore shown soluble uric acid to have pro-inflammatory effects in human PBMCs and murine BMDMs; however these findings may not reflect the effects on primary human monocytes which are the major producers of IL-1β during an acute gout attack (Malawista et al. 2011). Thus, the effects of high concentrations of uric acid on activation of NLRP3 in primary human monocytes were tested. Monocytes from healthy donors and gout patients were isolated to provide unique insights into the effects of longer-term hyperuricemia on monocyte immune responses and gene expression of components of the NLRP3 inflammasome.
5.2 Soluble uric acid and MSU crystals induce ROS generation in primary human monocytes

Uric acid was dissolved in cell culture media by incubation for one hour at 37°C with occasional agitation. Following sterile filtering, the solution was diluted to a working concentration of 30mg/dL and added to uric acid assay. A value of 30mg/dL was obtained (Fig. 5.1) confirming full solubility. Crystallisation was never observed in wells during stimulations, with soluble uric acid only precipitating out of solution when left at room temperature for 24-48 hours. MSU crystals were diluted to 10mg/dL (as used in stimulation experiments) in RPMI and incubated overnight at 37°C. Media was spun to pellet crystals and the MSU conditioned media analysed by uric acid assay. A small increase in uric acid concentration in the media was observed (1mg/mL) although this was well below concentrations considered to be hyperuricemic (6.8mg/dL).

Uric acid has been shown to have both pro- and anti-oxidant properties depending on its environment (Sautin and Johnson 2008). To determine its effect on ROS generation in vitro, primary human monocytes were exposed to soluble uric acid (30mg/dL) either alone or alongside the TLR2 ligand Pam3 for 2 hours and ROS generation assessed by ROS-Glo assay. Both soluble uric acid and MSU crystals induced a 2-fold increase in ROS-generation when added alone, but this did not reach statistical significance. Co-incubation of uric acid and MSU with Pam3 induced a significant increase in ROS-generation compared to Pam3 stimulated cells (Fig. 5.2). Interestingly, the addition of Pam3 appeared to increase uric acid induced ROS generation compared to uric acid alone, although this did not reach statistical significance ($p=0.068$).

Soluble uric acid concentrations were selected to allow direct comparison to the concentrations of MSU crystals (10-20mg/dL) used so far throughout the study (chapter 3). The concentrations were subsequently increased to cover a wide range of hyperuricemic conditions. Uric acid was used at a concentration of 30mg/dL for most experiments in this study because of initial experiments which showed a small reduction in cell viability when uric acid was used at 40mg/dL in THP-1 monocytes (discussed below; Fig. 5.4 B). At a concentration of 30mg/dL we could be confident that no cell toxicity would occur, particularly for longer incubations, and being 4-fold greater than clinically defined hyperuricemia would provide the best opportunity to see any effects of uric acid on monocytes in vitro.
Figure 5.1 Solubility of uric acid. Soluble uric acid was resuspended in fully supplemented RPMI at a concentration of 30mg/dL and was confirmed to have fully entered solution by uric acid assay.

Figure 5.2 Both soluble uric acid and MSU crystals induce ROS generation in primary human monocytes. Primary human monocytes were exposed to soluble uric acid and MSU crystals with or without Pam3 for 2 hours and ROS measured by ROS-Glo assay. Data represents means from 6 individual donors ± SEM. Significance was determined by one-way ANOVA with Tukey’s post-hoc test (*P<0.05, ****P<0.0001).
5.3 Soluble uric acid does not activate NF-κB

ROS have been demonstrated to play an essential role in the priming of the NLRP3 inflammasome (Bauernfeind et al. 2011), and priming requires the activation of transcription factor NF-κB (Bauernfeind et al. 2009). To assess the effect of soluble uric acid on NLRP3 priming, NF-κB activation was measured in vitro using the monocytic THP1-Blue cell line which has a stably integrated NF-κB inducible SEAP reporter, allowing measurement of NF-κB activation (see section 2.16).

In previous experiments (chapter 4), Pam3 was shown to robustly activate NF-κB after 6 hours. To examine whether NF-κB activation increased with time, THP1-Blue monocytes were stimulated for up to 18 hours with Pam3. Pam3 induced a time-dependant increase in NF-κB activation over an 18 hour period (Fig. 5.3). An 18-hour stimulation was selected to provide the best opportunity to see any effect of soluble uric acid on NF-κB activation.

Pam3 induced a significant increase in NF-κB inducible SEAP secretion compared to unstimulated cells (Fig. 5.4). Hyperuricemia is defined as a serum uric acid concentration of 6.8mg/dL or greater (Richette and Bardin 2010). Here, THP-1 Blue monocytes were exposed to concentrations ranging from 10mg/dL through to 40mg/dL (1.5 - 5.8 times higher than defined hyperuricemia). Soluble uric acid did not activate NF-κB signalling when added alone or when added alongside Pam3 (Fig. 5.4 A). MSU crystals were added at 10-20mg/dL as a comparison to soluble uric acid having confirmed optimal concentrations previously (Fig. 3.2 A). MSU crystals had no effect on NF-κB activation when added alone, but increased Pam3-induced NF-κB activation at 20mg/dL.

A small increase in toxicity was caused by uric acid at 40mg/dL, causing a 10-15% reduction in viability (Fig. 5.4 B). Soluble uric acid at 10-30mg/dL and MSU crystals at 10-20mg/dL had no effect on viability. As discussed, uric acid was used at a concentration of 30mg/dL for most future experiments.
Figure 5.3 TLR2 ligation activates NF-κB in THP1-Blue cells. THP1-Blue cells were stimulated with Pam3 for 2-18 hours. NF-κB activation was measured by QUANTI-Blue analysis every hour up to 6 hours and then finally at 18 hours. Data represent means from three technical replicates ± SD.
Figure 5.4 Soluble uric acid does not activate NF-κB in THP-1 monocytes. (A) THP1-blue monocytes were stimulated for 18 hours with soluble uric acid or MSU crystals at a range of concentrations with or without Pam3. NF-κB activation was measured by QUANTI-blue analysis. (B) Cell viability was measured by cell-titre blue (CTB) assay. Data represents means of 3 independent experiments ± SEM. Significance was determined by one-way ANOVA with Tukey’s post-hoc test (**P<0.01, ****P<0.0001).
5.4 Soluble uric acid does not alter expression of NLRP3 inflammasome components in THP-1 monocytes

The effect of soluble uric acid on gene expression of the different inflammasome components was also assessed. Cells were exposed to stimulants for 3 hours, as determined by previous optimisation experiments (Fig. 3.9). Studies by Crisan et al. (2015; 2017) and Braga et al. (2017) demonstrated the largest effect of soluble uric acid at 50mg/dL, 7.35-fold higher than clinically defined hyperuricemia. Here, cells were stimulated with 30mg/dL due to the small increase in cell death observed in response to treatment with soluble uric acid at 40mg/dL in previous experiments (Fig. 5.4). This concentration was also similar to that used for MSU crystals, and only being 4-fold higher than clinically defined hyperuricemia, can be considered less pharmacological than other studies.

Overall, uric acid had no effect on the expression of the inflammasome components CASP1, IL1B, NLRP3 or PYCARD, when added alone, or together with Pam3 (Fig. 5.5), confirming that it does not activate NF-κB in human monocytes.
Figure 5.5 Soluble uric acid does not affect transcription of inflammasome components in THP-1 monocytes. THP-1 monocytes were stimulated for 3 hours with soluble uric acid (30mg/dL) ± Pam3 (100ng/ml). cDNA was prepared and gene expression of (A) CASP1, (B) IL1B, (C) NLRP3 and (D) PYCARD was quantified by qPCR. Data represents means from 3 biological replicates ± SEM. Significance was determined by Kruskal-Wallis with Dunn's post-hoc test. Data for control and Pam3 are the same as data presented in figure 3.9 in which cells were exposed to MSU crystals and uric acid at the same time.
5.5 Crystallisation of uric acid is required for activation of the NLRP3 inflammasome

To examine what effect soluble uric acid may have on the activation of the inflammasome, THP-1 monocytes were stimulated for 18 hours with soluble uric acid or MSU crystals added alone or alongside Pam3 and IL-1β secretion quantified as an indirect measure of NLRP3 activation. Soluble uric acid had no effect on IL-1β secretion when added alone or when added alongside Pam3, demonstrating its inability to activate the inflammasome (Fig. 5.6 A). As routinely demonstrated, MSU crystals caused a robust increase in IL-1β secretion when added alongside Pam3. Viability was assessed by CTB and confirmed minimal cell death caused by MSU crystals or soluble uric acid (Fig. 5.6 B).

Primary human monocytes were also exposed to increasing concentrations of soluble uric acid and MSU in the presence or absence of Pam3 (Fig. 5.7 A). Neither uric acid, nor MSU crystals induced a significant increase in IL-1β secretion when added alone. As seen previously, Pam3 induced an increase in IL-1β secretion. This response was almost 3-fold greater in primary human monocytes compared to THP-1 monocytes. Similar to THP-1 responses, soluble uric acid had no effect on Pam3-induced IL-1β secretion, whereas MSU crystals induced a 6-fold increase in IL-1β secretion compared to Pam3 alone. Interestingly, the addition of Pam3 caused an increase in CTG luminescence compared to unstimulated cells (Fig. 5.7 B). This was unaffected by the addition of soluble uric acid or MSU crystals alongside Pam3, however MSU crystals alone also increased CTG luminescence compared to unstimulated controls. CTG is a metabolically driven viability assay, and this could suggest an increase in metabolism in response to these stimuli.
Figure 5.6 Crystallisation of uric acid is required for activation of the NLRP3 inflammasome in THP-1 monocytes. THP-1 monocytes were stimulated for 18 hours with soluble uric acid or MSU crystals with or without Pam3. (A) IL-1β secretion was determined by ELISA. (B) Cell viability was assessed by CTB assay. Data represents means from 4 independent experiments ± SEM. Significance was determined by one-way ANOVA with Tukey’s post-hoc test (**P<0.001, ****P<0.0001).
Figure 5.7 Crystallisation of uric acid is required for activation of the NLRP3 inflammasome in primary human monocytes. Primary human monocytes were stimulated for 18 hours with soluble uric acid or MSU crystals with or without Pam3. (A) IL-1β secretion was determined by ELISA. (B) Cell viability was assessed by CTG assay. Data represents means from 5 individual donors ± SEM. Significance was examined by Kruskal-Wallis with Dunn’s post-hoc test.
5.6 Preincubation of monocytes in soluble uric acid does not alter priming or activation of the NLRP3 inflammasome

In hyperuricemic individuals, circulating monocytes are exposed to elevated concentrations of soluble uric acid for prolonged periods. To examine whether longer-term exposure of monocytes to soluble uric acid may alter NF-κB activation and/or IL-1β secretion, cells were exposed to soluble uric acid (30mg/dL) for up to 48 hours prior to stimulation.

The majority of cytokine secretion data in this study was collected following an 18 hour stimulation (Mullen et al. 2015). To assess the feasibility of completing longer cultures in primary human monocytes, cells from two separate donors were seeded in fully supplemented RPMI or uric acid (30mg/dL) and viability was measured between 18-48 hours. A 25% reduction in viability was seen between 18 and 24 hours (Fig. 5.8). By 36 hours cell viability was reduced by 50% for both donors. Uric acid had no effect on viability of the cells, with the reductions in viability occurring at the same rate as unstimulated controls. As such, preincubation of 24 hours or more were completed in THP-1 monocytes instead of primary human monocytes. Where possible primary human monocytes were cultured for a maximum of 18 hours, but on some occasions were stimulated for up to 22-24 hours.

ROS generation was measured following preincubation of primary human monocytes in soluble uric acid for 16 hours prior to a 2 hour stimulation with MSU crystals or soluble uric acid ± Pam3. ROS-generation induced by soluble uric acid and MSU crystals was less robust in these donors compared to previous data (Fig. 5.2), yet a 1.5-fold increase was still observed (Fig. 5.9 A). Interestingly, monocytes preincubated in soluble uric acid appeared to have increased basal levels of ROS compared to monocytes preincubated in culture media. ROS levels were almost 1.5-fold greater in monocytes pre-exposed to soluble uric acid when unstimulated or when exposed to Pam3 alone, although this was not statistically significant. The 16 hour exposure to soluble uric acid prior to stimulation had no effect on ROS-generation induced by MSU or soluble uric acid stimulation (Fig. 5.9 A).

Gene expression of IL1B, CASP1 and NLRP3 were also assessed following preincubation of primary human monocytes in soluble uric acid for 16 hours prior to a 6 hour stimulation with Pam3. Expression of IL1B, CASP1 and NLRP3 was identical between the groups (Fig. 5.9 B-D). In validation of the lack of effect of soluble uric acid on inflammasome priming, NF-κB activation in THP1-Blue monocytes was also unaffected by pre-exposure to soluble
uric acid for 24 or 48 hours (Fig. 5.10). As seen previously, Pam3-induced a robust increase in $IL1B$ gene expression and NF-κB activation, confirming the priming of the inflammasome.

Finally, the effect of long-term soluble uric acid exposure on IL-1β secretion was investigated. Primary human monocytes were preincubated for 10 hours prior to a 12 hour stimulation with MSU and uric acid ± Pam3. IL-1β secretion was induced by Pam3 alone and further increased by addition of MSU crystals. Preincubation in soluble uric acid had no effect on IL-1β secretion from cells pre-exposed to soluble uric acid (Fig. 5.11 A). The same findings were observed in THP-1 monocytes exposed to soluble uric acid for 24 hours (Fig. 5.11 B) and 48 hours (Fig. 5.11 C) prior to an 18 hour stimulation.
Figure 5.8 Viability of primary human monocyte decreases over time *in vitro*. Primary human monocytes from two separate donors were plated in fully supplemented RPMI ± uric acid. Viability was assessed after 18, 24, 36 and 48 hours using a CTG assay. Data represents means from three technical replicates ± SD.
Figure 5.9 Prolonged exposure to soluble uric acid does not affect ROS generation or gene expression. (A) Primary human monocytes were plated in culture media (black bars) or soluble uric acid (30mg/dL – white bars) for 16 hours prior to 2 hour stimulation with soluble uric acid or MSU crystals ± Pam3. (B-D) Primary human monocytes were exposed to soluble uric acid for 16 hours prior to 6 hour stimulation with Pam3 (100ng/mL). cDNA was prepared and gene expression of (B) IL1B, (C) CASP1 and (D) NLRP3 was determined by qPCR. Data represent means from 4 individual donors ± SEM. Significance was determined by multiple t-tests comparing RPMI vs. uric acid preincubation for each stimulation using Holm-Sidak’s multiple comparison correction.
Figure 5.10 Prolonged exposure to soluble uric acid does not alter NF-κB activation in THP-1 monocytes. THP1-Blue monocytes were preincubated in cell culture media (black bars) or 30mg/dL soluble uric acid (white bars) for (A) 24 or (B) 48 hours. Cells were then stimulated for 18 hours with soluble uric acid or MSU crystals ± Pam3 (100ng/ml). NF-κB activation was determined by QUANTI-Blue analysis. Data represent means from 3-4 biological repeats ± SEM. Significance was determined by multiple t-tests comparing RPMI vs. uric acid preincubation for each stimulation using Holm-Sidak’s multiple comparison correction test.
Figure 5.11 Prolonged exposure to soluble uric acid does not affect IL-1β secretion in human monocytes. (A) Primary human monocytes were preincubated for 10 hours in RPMI (black bars) or soluble uric acid (30mg/dL – white bars) before stimulation with MSU or soluble uric acid ± Pam3 for 12 hours. (B-C) THP-1 monocytes were incubated for (B) 24 hours or (C) 48 hours prior to 18 hour stimulation with MSU or soluble uric acid ± Pam3. IL-1β secretion was determined by ELISA. Data represent means from 4 separate donors (A) or 3-4 individual experiments (B-C) ± SEM. Significance was determined by multiple t-tests comparing RPMI vs. uric acid preincubation for each stimulation using Holm-Sidak’s multiple comparison correction test.
5.7 Activation of the NLRP3 inflammasome in monocytes isolated from gout patients

To investigate whether serum uric acid may influence inflammasome responses in monocytes from gout patients, whole blood was collected from healthy controls \((n=16)\) or gout patients \((n=16)\). Details for patients included in this study can be found in appendix 1. Monocytes were isolated by positive selection using CD14+ magnetic beads. Monocyte purity was initially assessed by flow cytometry. As previously described (section 3.4) a large initial gate was applied to select lymphocytes and monocyte populations according to their scatter profile (Fig. 5.12 A). A second gate was then applied to ensure that only singlet cells were included in the analysis (Fig. 5.12 B). Successful isolation of a highly pure population of human monocytes consisting of 84% CD14+CD16- monocytes and 14.4% CD14+CD16+ monocytes was demonstrated (Fig. 5.12 C-D).

Serum was also isolated from each donor and serum uric acid concentration measured. Serum uric acid concentrations were significantly higher in gout patients compared to healthy controls, although only 25% of the gout population were clinically hyperuricemic \((\geq 6.8\text{mg/dL})\) at the time of sampling (Fig. 5.13 A). No difference in IL-1\(\beta\) secretion was observed between monocytes from healthy controls or gout patients in response to Pam3 (Fig. 5.13 B), nor when MSU and Pam3 were added together (Fig. 5.13 C). However, a trend towards increased responsiveness to MSU crystals was seen in monocytes from gout patients when IL-1\(\beta\) data was normalised to Pam3-induced secretion (Fig. 5.13 D).

To determine whether this trend could be explained by increased expression of genes coding for components of the NLRP3 inflammasome in gout patients, constitutive expression levels of caspase-1 \((\text{CASP1})\), pro-IL-1\(\beta\) \((\text{IL1B})\), NLRP3 \((\text{NLRP3})\) and ASC \((\text{PYCARD})\) in monocytes from gout patients and healthy controls was measured. Gene expression was determined by absolute qPCR and copy numbers were normalised to expression of \(\text{B2M}\) and \(\text{RPL32}\). There were no differences in the expression of any of the genes tested between healthy controls and gout patients (Fig. 5.14).

To further evaluate what influence serum uric acid may have on monocyte inflammasome responses, serum uric acid was correlated with IL-1\(\beta\) secretion and copy numbers for each inflammasome component. Uric acid concentration in sera from healthy controls and gout patients did not correlate with IL-1\(\beta\) secretion or expression of the NLRP3 inflammasome components (Fig. 5.15).
Figure 5.12 Purity of monocytes isolated via CD14+ affinity purification. Monocytes from a single healthy donor were stained with CD14+ and CD16+ antibodies. (A) Cells were separated by size according to their forward and side scatter profiles. A broad gate was applied to select viable mononuclear cells and lymphocytes. (B) A second gate was then applied to account for single cell populations whilst discounting doublets. (C) Q1 represents CD14+CD16- cells, Q2 represents CD14+CD16+ cells, Q3 represents CD14-CD16- cells and Q4 represents CD14-CD16+ cells. (D) Final population statistics were calculated by Diva software.
Figure 5.13 Serum uric acid concentration and monocyte sensitivity in healthy controls and gout patients. (A) Uric acid concentration in sera from healthy controls \( (n=16) \) and gout patients \( (n=15) \) was measured by uric acid assay. Monocytes from healthy donors \( (n=16) \) and gout patients \( (n=16) \) were stimulated for 18 hours with (B) Pam3 or (C) Pam3 and MSU together. IL-1β secretion was determined by ELISA. (D) IL-1β secretion in response to MSU was normalised to secretion induced by Pam3 alone. Individual points represent means from 3-6 technical replicates for each donor. Error bars represent mean ± SEM. Significance was determined using a two-tailed unpaired Mann-Whitney U-test \( (*P<0.05) \).
Figure 5.14 Constitutive expression of NLRP3 inflammasome components in monocytes from healthy controls and gout patients. Expression of (A) CASP1, (B) IL1B, (C) NLRP3 and (D) PYCARD was determined by qPCR. Absolute copy numbers were calculated using standard curves of known copy numbers for each gene of interest. Data was normalised to the expression of reference genes B2M and RPL32 for each cDNA. Individual points represent means from 3-6 technical replicates for each donor. Error bars represent means ± SEM (Healthy n=16, Gout n=16). Significance was determined using a two-tailed unpaired Mann-Whitney T-test.
Figure 5.15 Serum uric acid concentration does not correlate with IL-1β secretion and gene expression of inflammasome components. Uric acid concentration in sera from healthy controls and gout patients were plotted against matched (A) Pam3-induced and (B) MSU+Pam3-induced IL-1β secretion. Uric acid concentration in sera from healthy controls and gout patients were plotted against matched (C) CASP1, (D) IL1B, (E) NLRP3 and (F) PYCARD gene expression data. Individual points represent means from 3-6 replicate measurements for each donor. Linear regression was plotted for each sample group. Correlation coefficient (r) and significance was calculated using Spearman non-parametric analysis.
5.8 Discussion

Uric acid is the major antioxidant in the plasma, accounting for more than half of the antioxidant capacity and, like ECSOD, is a scavenger of superoxide (Ames et al. 1981). Interestingly, recent studies have demonstrated a role for uric acid in both the priming and activation of the NLRP3 inflammasome in murine BMDMs and primary human PBMCs (Crisan et al. 2015; Braga et al. 2017; Crisan et al. 2017). Here, the effect of uric acid on signalling events involved in NLRP3 activation in human monocytes was assessed.

Despite uric acid being the major antioxidant in human plasma, there is a well-established paradoxical role for uric acid having been shown to exert both pro-oxidant and anti-oxidant effects depending on its setting (Sautin and Johnson 2008). Under these conditions, soluble uric acid was a pro-oxidant, significantly increasing ROS generation in primary human monocytes. Regardless of these effects on ROS, soluble uric acid had no effect on either the priming or activation of the NLRP3 inflammasome in either THP-1 monocytes or primary human monocytes. In contrast, treatment of cells with similar concentrations of MSU crystals alongside Pam3 induced a robust increase in NLRP3-dependant IL-1β secretion (also demonstrated in chapters 3 and 4). The lack of effect of uric acid on IL-1β secretion despite this increase in ROS generation is consistent with results presented previously (chapters 3 and 4) demonstrating that ROS are not required for activation of the inflammasome.

Activation of the transcription factor NF-κB is an integral event in NLRP3 priming as it is required for transcription of NLRP3 and pro-IL-1β (Bauernfeind et al. 2009). Uric acid had no effect on NF-κB activation in THP-1 monocytes. Previous work in PBMCs and monocytes showed that uric acid potentiates proinflammatory cytokine production by increasing the expression of IL-1β mRNA and via reduction of IL-1Ra and activation of the AKT-PRAS40 pathway but without causing phosphorylation of the p65 subunit of NF-κB (Crisan et al. 2017). Here, soluble uric acid had no effect on NF-κB activation, nor did it affect expression of any of the NLRP3 inflammasome components in THP-1 cells when added alone or alongside Pam3. Taken together with data detailed elsewhere (Crisan et al. 2017) it is clear that soluble uric acid does not alter NF-κB activation in human monocytes.

A previous study demonstrated that 24-hour exposure to soluble uric acid at 50mg/dL potentiated the immune response of human PBMCs which secreted greater levels of IL-1β following subsequent stimulation with Pam3±MSU than PBMCs not pre-exposed to uric acid (Crisan et al. 2015). This was not replicated here, with monocytes exposed to uric acid for
up to 48 hours prior to stimulation secreting near identical quantities of IL-1β compared to monocytes exposed to RPMI alone. This is also in contrast to findings from a subsequent study which showed increased IL-1β secretion in human monocytes stimulated with LPS and MSU following an initial 24 hour preincubation in uric acid (Crisan et al. 2017). It is important to note the difference in uric acid concentration used here compared to these other studies. Cell toxicity was seen when using uric acid at 40mg/dL and as such a maximum of 30mg/dL was used for long-term preincubation and stimulations. Crisan et al. (2015) only reported significant increases in Pam3+MSU-induced IL-1β secretion when PBMCs were initially preincubated in 50mg/dL uric acid, an effect which was lost when cells were preincubated in 25mg/dL uric acid, suggesting that this could be a concentration issue. However, one could argue that 50mg/dL is an extreme concentration which is unlikely to occur in vivo with individuals suffering acute gout normally exhibiting a serum uric acid concentration of ~8-9mg/dL (Schlesinger et al. 2009). A later study did, however, demonstrate increases in LPS+MSU-induced IL-1β secretion following 24 hour preincubation of human monocytes in 25mg/dL uric acid (Crisan et al. 2017), something which could not recapitulated here because of reductions in primary human monocyte viability after 18 hours in vivo. To overcome this, THP-1 monocytes were used for longer preincubations, and this could be a reason for not seeing an effect of prolonged uric acid exposure on IL-1β secretion. The mechanism by which preincubation with uric acid increased IL-1β secretion upon subsequent stimulation with Pam3 was via downregulation of the IL-1 receptor antagonist (IL-1Ra) which allows binding of the IL-1β produced to the IL-1 receptor (IL-1R) (Crisan et al. 2015). Importantly, THP-1 cells cannot respond to IL-1β in this manner because they do not express IL-1R (Spriggs et al. 1990; Sims et al. 1993). Thus, any downregulation of IL-1Ra in response to uric acid would have had little effect on THP-1 monocytes. It is of course important to note the fact that concentrations of serum uric acid used in these and other studies are well in excess of clinically defined hyperuricemia, up to 7.35-fold greater in some instances. There are no reports of an individual with a plasma uric acid concentration at the concentrations used in this thesis or in other studies.

Another possible explanation for the differences in the results presented here compared to published data is the use of a pure monocyte population. PBMCs are a mixed population of monocytes and lymphocytes, containing on average approximately 8-10% monocytes and were used in other studies showing uric acid to influence NLRP3 signalling (Crisan et al. 2015). It is well known that different cell types interact with each other in vivo via the secretion of various molecules, such as chemokines which mediate the migration of leukocytes towards sites of inflammation (Cline and Swett 1968; Hanifin and Cline 1970).
Interestingly, uric acid has been shown to activate T cells (Webb et al. 2009) and activated T-cells can generate microparticles that are able to induce IL-1β secretion from human monocytes (Scanu et al. 2008). Perhaps then, the interactions between activated T cells and monocytes in the PBMC stimulations could influence IL-1β secretion, an effect that would not be seen in the pure monocyte cultures used here.

In addition to its lack of effect on the priming of the inflammasome, soluble uric acid did not activate the NLRP3 inflammasome inducing no IL-1β secretion when added alone or alongside Pam3. Soluble uric acid has been shown to activate the inflammasome in murine BMDMs, causing a robust increase in IL-1β secretion when added alongside LPS (Braga et al. 2017). Of course, there are known differences in the processing and secretion of IL-1β between monocytes and macrophages which may provide an explanation for these contrasting results. For example, macrophages display lower constitutive pro-IL-1β mRNA and secrete less IL-1β in response to LPS or other PAMPs compared to monocytes (Herzyk et al. 1992; Madej et al. 2017). Monocytes also secrete IL-1β in response to TLR ligation by a priming signal alone whereas macrophages require a distinct second activator (Netea 2009). This suggests that NLRP3 signalling could be cell-type specific and the differences in results seen here compared to others might be related to this. Another potential explanation for the contrasting results is the use of murine cells in other studies. Interestingly, and in confirmation of results here, the increased IL-1β secretion in response to soluble uric acid could not be replicated in human monocytes (Crisan et al. 2015) or human monocyte-derived macrophages (Braga et al. 2017), suggesting that these differences could in fact be species-specific. As discussed in section 1.4.4, there are known differences in immune responses between humans and mice. Mice also have the capacity to synthesise uricase meaning that serum uric acid concentrations are around 10-fold lower in mice than humans (Kono et al. 2010). Therefore, normal uric acid concentrations in humans are likely to be pathological in mice which may explain why innate immune responses are activated by uric acid in mouse cells but not in human cells.

Gout is an inflammatory arthritis driven by MSU crystals which form during periods of hyperuricemia (Agudelo and Wise 1991; Martinon et al. 2006). Monocytes isolated from gout patients were used to gain insight into the effects of longer term elevation of uric acid on monocyte responses. Monocytes isolated from gout patients secreted similar levels of IL-1β to healthy controls but did show a tendency to produce more IL-1β in response to MSU crystals. Uric acid concentrations in sera of gout patients were higher than in healthy controls which could indicate that prolonged exposure to uric acid predisposes monocytes to produce more IL-1β upon stimulation. Indeed, this is a theory supported by previous
studies in PBMCs, which showed elevated IL-1β secretion in PBMCs from gout patients compared to healthy controls and that increased IL-1β secretion correlated with serum uric acid concentration (Mylona et al. 2012; Crisan et al. 2015). However, despite the trend for increased responsiveness to MSU crystals in monocytes from gout patients observed here serum uric acid concentrations did not correlate with IL-1β secretion, nor did it influence the expression of inflammasome components. This was consistent with our THP-1 and primary human monocyte gene expression data where preincubation with uric acid did not influence inflammasome gene expression or cytokine secretion. However, these data are different to those described by Crisan et al. (2015) who demonstrated increased relative IL1B expression in monocytes compared to healthy controls although this data was generated from a limited number of samples: 3 healthy controls and 8 patients with a history of gout. As only 25% of the gout patients tested here were clinically defined as hyperuricemic, it would be desirable to increase patient numbers with the aim of achieving a larger percentage of hyperuricemic individuals to allow a more comprehensive comparison between immune responses of normouricemic and hyperuricemic gout patients.

An important question which requires further investigation is whether uric acid is able to enter monocytes in these experiments. Uric acid has antioxidant effects in the extracellular environment, but has pro-oxidant effects intracellularly (Kang and Ha 2014). The increases in ROS in response to soluble uric acid seen here suggests that uric acid was able to enter the cell because of its intracellular pro-oxidant properties, although we cannot discount that uric acid induced ROS outside of the cells in these in vitro experiments. This effect on ROS was lost following longer-term exposures of uric acid which could be indicative of antioxidant compensation, with cells increasing antioxidant activity to account for any ROS increased caused by uric acid. It currently remains unclear whether uric acid can enter monocytes. Uric acid has been shown to enter adipocytes (Sautin et al. 2007) and vascular smooth muscle cells (Corry et al. 2008) via specific uric acid transporters and intake of uric acid into these cells causes increases in ROS. Leukocytes do express a Glut9a uric acid transporter which is known to regulate plasma uric acid concentrations (Augustin et al. 2004; So and Thorens 2010), however there are no evidence that Glut9a actually transports uric acid into monocytes. The fact that we see increased ROS in monocytes upon incubation with uric acid could point towards intracellular effects of uric acid in these cells. However, this could also be mediated by uric acid induced changes in redox state of membrane receptors and proteins that can in turn exert intracellular effects.

The data presented here demonstrate that crystallisation of uric acid is fundamental to activating the NLRP3 inflammasome in primary human monocytes. No influence of soluble
uric acid on NLRP3-priming or activation was observed throughout this study and we therefore conclude that soluble uric acid does not play a role in IL-1β secretion from human monocytes. The lack of correlation between serum uric acid concentration and production of IL-1β in monocytes from gout patients supports this conclusion. It remains particularly interesting that only around 10% of hyperuricemic individuals develop gout and that the presence of MSU crystals within joints does not always induce a gout attack (Campion et al. 1987; Pineda et al. 2011). Uric acid did increase ROS production here and one possibility is that hyperuricemia results in higher levels of oxidative stress that, in healthy individuals, is dealt with via efficient antioxidant mechanisms. This idea is consistent with the loss of uric acid- and MSU-induced ROS in the longer term ROS measurements. It would be interesting to examine whether gout patients have less efficient antioxidant mechanisms than healthy individuals or individuals with asymptomatic hyperuricemia to determine whether reduced antioxidant capacity may contribute to oxidative stress and predispose certain individuals to inflammation.
Chapter 6 - Antioxidant capacity and IL-1β secretion in patients with gout, CKD and RA
6.1 Introduction

Oxidative stress is an imbalance between the levels of ROS and the antioxidant defences that regulate these levels (Betteridge 2000). It is clear from the data presented thus far that if there is any relationship between oxidative stress and NLRP3 activation it is likely to be more subtle than simply increased ROS generation, involving oxidative stress in its entirety. Uric acid has both pro- and anti-oxidant properties depending on its setting (Sautin and Johnson 2008), and in primary human monocytes uric acid induced a robust increase in ROS generation but had no effect on NLRP3 priming or activation (chapter 5). Uric acid concentration in the serum can have both positive and negative effects on health and can influence the antioxidant capacity of the blood (Ekpenyong and Akpan 2014; Fabbrini et al. 2014). Despite hyperuricemia being the single greatest risk factor for the development of gout, it remains that relatively few hyperuricemic individuals ever suffer from gouty arthritic attacks, with some individuals avoiding inflammation even when MSU crystals are present (De Miguel et al. 2012; Grassi et al. 2013). Therefore, there must be other unknown factors that govern inflammasome activation and IL-1β secretion in these individuals.

Hyperuricemia could perhaps cause alterations in redox regulation which may in turn predispose certain individuals to enhanced NLRP3 activation and IL-1β secretion. Or perhaps some individuals have altered intracellular or extracellular antioxidant capacity which makes them more susceptible to IL-1β secretion and inflammation caused by oxidative stress. Alterations to antioxidant capacity have been demonstrated in a range of inflammatory diseases. Rheumatoid arthritis (RA), for example, is a systemic inflammatory disease in which both NLRP3 activation and oxidative stress is implicated (Choulaki et al. 2015), and blood from RA patients has been shown to have reduced antioxidant capacity compared to healthy controls (Sarban et al. 2005; Quinonez-Flores et al. 2016). Oxidative stress is also implicated in the pathophysiology of chronic kidney disease (CKD) and as kidney function worsens so do imbalances in redox status (Krata et al. 2018). End stage renal disease patients present with both compromised antioxidant capacity and a consequent overabundance of ROS (Poulianiti et al. 2016). The reduced renal function in these patients results in reduced uric acid excretion and CKD patients often present with hyperuricemia (Eleftheriadis et al. 2017). Interestingly however, monocytes from CKD patients have previously been shown to be hyporesponsive to MSU crystal stimulation, secreting lower levels of IL-1β compared to healthy controls (Schreiner et al. 2000). Studies investigating redox regulation in gout patients are lacking, but one study has demonstrated increased oxidative stress in gout patients, with increased serum malondialdehyde, a biomarker for lipid peroxidation, as well as reduced erythrocyte SOD and catalase.
antioxidant capacity (Acharya et al. 2014). Perhaps then, susceptibility to gout attacks is a consequence of a reduced ability to maintain redox homeostasis, because of reduced extracellular or intracellular antioxidant capacity resulting in elevated levels of ROS.

The aim of this chapter was to investigate the role of oxidative stress on IL-1β production in primary human monocytes with a particular focus on intracellular and extracellular antioxidant defences rather than on the more commonly studied ROS. Blood samples from healthy controls as well as gout, CKD and RA patients were collected. In chapter 5, monocytes from gout patients showed a tendency for heightened responses to stimulation with MSU than healthy controls, but this was unaffected by serum uric acid concentration. Here, intracellular and extracellular antioxidant capacity was determined to analyse their effects on monocyte responses. Blood samples from CKD patients was also collected. CKD patients were included to provide a patient cohort in which there are known differences in antioxidant capacity and an association between oxidative stress and disease progression (Poulianiti et al. 2016; Krata et al. 2018). Finally, blood samples from RA patients were collected because of the known altered extracellular antioxidant capacity in these patients and the recent data suggesting that NLRP3 activation is involved in disease progression (Choulaki et al. 2015).
6.2 Serum antioxidant capacity in gout, CKD and RA patients compared to healthy donors

Whole blood was collected from healthy controls, gout, CKD and RA patients. Patient characteristics are detailed in appendix 2. For each donor, serum was isolated and uric acid concentration, as well as the activities of two extracellular antioxidant enzymes, Gpx3 and ECSOD, were measured.

Gout patients had significantly greater serum uric acid concentrations than healthy controls. CKD patients had lower serum uric acid levels than healthy individuals (Fig. 6.1 A) and sera from both gout and CKD populations had significantly reduced GPx activity (Fig. 6.1 B). However, CKD patients also had significantly elevated ECSOD activity (Fig. 6.1 C), which was not seen in gout or RA. Total antioxidant capacity (TAC) was used as a measure of non-enzymatic antioxidants. All four groups showed comparable levels of serum total antioxidant capacity demonstrating no differences in non-enzymatic antioxidant capacity (Fig. 6.1 D). Sera from RA patients did not show any significant differences in serum uric acid, antioxidant enzyme activity, or TAC.

Uric acid is the major antioxidant in the blood and it was therefore hypothesised that perhaps hyperuricemia may result in alterations to the overall redox environment, which in turn might impact on the responsiveness of monocytes to NLRP3 activation. To examine the effect hyperuricemia may have on other serum antioxidants, serum uric acid was correlated with the matched values for Gpx3 and ECSOD activities, as well as to the TAC measurement for each donor (Table 6.1). Serum uric acid did not correlate with antioxidant enzyme activity for any of the patient cohorts. As expected, soluble uric acid concentration correlated significantly with TAC for each of the patient groups as the TAC assay also measures uric acid.
Figure 6.1 Serum antioxidant capacity in gout, CKD and RA patients. Serum was isolated from blood of healthy controls (n=52), gout (n=49), CKD (n=42) or RA (n=36) patients. (A) Uric acid concentration was determined using an amplex-red based uric acid assay. (B) GPx and (C) SOD activities were measured using kinetic assays. (D) Total antioxidant capacity (TAC) was assessed using an antioxidant assay kit and quantified using a Trolox standard curve. Serum enzyme activities and TAC are expressed per mg of protein determined by BCA assay. Individual points represent means from 2-3 replicate measurements for each donor. Error bars represent means ± SEM. Significance was determined by Kruskal-Wallis with Dunn’s post-hoc test (*P<0.05, **P<0.01, ****P<0.0001).
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Table 6.1 Correlation of serum uric acid concentration with other serum antioxidants.

Correlation coefficients (r) and significance were calculated using two-way spearman correlation tests. Significant correlations are highlighted in red. A negative r value signifies an inverse correlation between the paired data.
6.3 IL-1β secretion from monocytes isolated from gout, CKD and RA patients

PBMCs from gout patients secrete significantly greater IL-1β in response to Pam3 and MSU stimulation compared to healthy controls (Crisan et al. 2015). Another study has demonstrated reduced sensitivity to MSU crystals in monocytes from patients with chronic renal failure (Schreiner et al. 2000). To examine whether cytokine secretion differed in this cohort, monocytes from healthy, gout, CKD or RA donor peripheral blood were isolated using CD14+ magnetic bead positive selection and stimulated in vitro with Pam3 ± MSU or CPPD crystals. IL-1β secretion was measured by ELISA as an indirect measure of NLRP3 activation and immune cell sensitivity. Purity of monocyte isolations had previously been assessed by flow cytometry (chapter 5).

Under resting conditions, monocytes from CKD and RA patients appeared to be less active, secreting significantly less IL-1β than monocytes from healthy donors (Fig. 6.2 A). This was replicated when cells were exposed to MSU or CPPD crystals in the absence of Pam3 (Fig. 6.2 C, D), though it is important to note that the levels of IL-1β secreted were very low. The addition of Pam3 caused an increase in IL-1β secretion for all disease groups compared to unstimulated controls (Fig. 6.2 B). Interestingly, monocytes from gout patients were more sensitive to Pam3 stimulation, secreting significantly greater concentrations of IL-1β than monocytes from healthy donors (Fig. 6.2 B). As seen throughout this work (chapters 3-5), the addition of MSU or CPPD crystals alongside Pam3 induced a marked increase in IL-1β secretion compared to Pam3 alone. Monocytes from gout patients did not secrete significantly more IL-1β than healthy donor monocytes in response to MSU crystals, although there was a trend for increased secretion from these donors ($p=0.106$) (Fig. 6.2 D). A significant increase in IL-1β secretion from gout patient monocytes was seen in response to CPPD crystals added alongside Pam3 (Fig. 6.2 F). Monocytes from both CKD and RA patients secreted comparable levels of IL-1β in response to MSU crystals than healthy monocytes (Fig. 6.2 D, F). The increased secretion in response to Pam3 and CPPD+Pam3 from monocytes isolated from individuals with a history of gout suggests that these cells were more sensitive to NLRP3 activation than monocytes from healthy individuals. Viability was routinely assessed using an MTT assay for each donor to ensure the stimulation conditions did not cause cell death. MTT data for all donors was pooled and shown in figure 6.3, demonstrating that the stimulants used did not cause cell death.

Serum uric acid concentration was correlated with matched IL-1β secretion data for each donor. Differences in serum uric acid concentration were seen for gout and CKD patients compared to healthy. No differences in serum uric acid were seen for RA samples and they
were therefore not included in this analysis. In agreement with previous findings (chapter 5), serum uric acid did not significantly correlate with IL-1β secretion in response to Pam3 ± MSU crystals (Fig. 6.4), although there was a trend for increased IL-1β secretion in Pam3-stimulated monocytes from gout patients with elevated serum uric acid concentration (Fig. 6.4 C). There were no correlations observed between CPPD+Pam3 induced IL-1β secretion and serum uric acid concentration for any of the disease groups (Table 6.2).

Finally, serum ECSOD and Gpx3 activity, as well as TAC, was correlated with IL-1β secretion to determine whether extracellular antioxidant capacity might influence a monocytes immune response when exposed to Pam3 with or without MSU and CPPD crystals. There appeared no clear correlation with the levels of these antioxidants and the secretion of IL-1β in response to these stimuli. However, there was a trend for increased Gpx3 activity to negatively correlate with IL-1β secretion from monocytes from gout patients but not from healthy controls or CKD patients (Table 6.3).
Figure 6.2 Secretion of IL-1β from monocytes isolated from gout, CKD and RA patients. Monocytes were isolated from whole blood of healthy (n=40), gout (n=40), CKD (n=37) and RA (n=33) donors. Monocytes were stimulated for 18 hours with (A) RPMI, (B) Pam3 (100ng/mL), (C) MSU crystals (10mg/dL), (D) MSU+Pam3, (E) CPPD crystals (2.5mg/dL) or (F) CPPD+Pam3. IL-1β secretion was determined by ELISA. Individual points represent means from 3-6 replicate measurements for each donor and error bars represent means ± SEM. Significance was determined by Kruskal-Wallis with Dunn’s post-hoc test (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).
Figure 6.3 Viability of monocytes following stimulation. Overall MTT viability data from the entire cohort was pooled. Data represent means ± SEM.
Figure 6.4 Serum uric acid does not correlate with IL-1β secretion in primary human monocytes. Pam3 ± MSU crystal induced IL-1β secretion from monocytes from healthy controls (A & B), gout patients (C & D) and CKD patients (E & F) was correlated with serum uric acid concentration for each donor. The correlation coefficient (r) and significance was determined using a two-tailed Spearman correlation.
Table 6.2 Correlation of serum uric acid concentration with IL-1β secretion. The correlation coefficient (r) and significance were calculated using a two-way spearman correlation test. A negative r value signifies an inverse correlation between the paired data.

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**Table 6.3 Correlation of extracellular antioxidants with IL-1β secretion.** The correlation coefficient (r) and significance were calculated using a two-way spearman correlation test. Significant correlations are highlighted in red. A negative r value signifies an inverse correlation between the paired data.
6.4 Intracellular antioxidant expression in monocytes from gout, CKD and RA patients

Significant differences in extracellular antioxidant capacity was seen in sera from healthy controls, gout and CKD patients (Fig. 6.1). It was previously hypothesised that elevated concentrations of serum uric acid, as would occur during hyperuricemia, might influence the intracellular redox environment in patient monocytes, which may in turn affect the immune response of the cells.

The expression of two key intracellular antioxidants, SOD2 (gene for SOD2) and TXNRD1 (gene for thioredoxin reductase 1) were measured by qPCR in the monocytes from healthy donors as well as gout and CKD patients. No differences in serum antioxidant capacity was seen between RA patients and healthy controls and RA samples were therefore not included in this analysis. There were no significant differences in SOD2 expression between the donor groups (Fig. 6.5 A), although there was a trend for increased expression in monocytes from CKD patients compared to healthy controls ($p=0.052$). Interestingly, monocytes from gout patients did express significantly greater TXNRD1 than healthy controls (Fig. 6.5 B). Intracellular total antioxidant capacity was also measured in a subset of donors and no changes in intracellular TAC were seen between patients and healthy controls (Fig. 6.5 C). Unfortunately, only a subset of each disease group were available for the measurement of intracellular TAC. This was because for the majority of donors all monocytes were used for the stimulation or gene expression experiments. The donors used for intracellular TAC analysis here all had high monocyte yields which enabled some to be used in further experiments.

To examine whether hyperuricemia could influence intracellular antioxidant capacity, serum uric acid concentration for each donor was correlated with expression data for SOD2 and TXNRD1. There was no correlation between serum uric acid and antioxidant gene expression (Table 6.4) demonstrating that hyperuricemia does not influence intracellular antioxidant capacity in respect to SOD2 and TXNRD1.

Additionally, the expression data for SOD2 and TXNRD1 were correlated with monocyte IL-1β secretion in response to Pam3 ± MSU or CPPD crystals. There were no significant correlations between the expression of these genes and IL-1β secretion in monocytes from healthy donors and gout patients (Table 6.5). However, both TXNRD1 and SOD2 expression showed a significant negative correlation with IL-1β secretion in Pam3 and crystal stimulated monocytes from CKD patients.
Figure 6.5 Monocytes from gout patients express higher levels of TXNRD1 but total antioxidant capacity is unchanged. cDNA was prepared from monocytes isolated from healthy \((n=37)\), gout \((n=43)\) or CKD \((n=36)\) donors. Expression of (A) \(SOD2\) and (B) \(TXNRD1\) was assessed by absolute qPCR. Copy numbers were normalised to reference genes \(B2M\) and \(RPL32\). (C) Monocytes from healthy \((n=9)\), gout \((n=13)\) and CKD \((n=5)\) donors were lysed and intracellular TAC and protein content measured. Individual points represent means from 2-3 replicate measurements for each donor. Error bars represent means ± SEM. Significance was assessed by Kruskal-Wallis with Dunn’s post-hoc analysis (*\(P<0.05\)).
Table 6.4 Correlation of serum uric acid concentration with \textit{SOD2} and \textit{TXNRD1} expression. The correlation coefficient (r) and significance were calculated using a two-way spearman correlation test. A negative r value signifies an inverse correlation between the paired data.

<table>
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Table 6.5 Correlation of \textit{SOD2} and \textit{TXNRD1} expression with IL-1β secretion. The correlation coefficient (r) and significance were calculated using a two-way spearman correlation test. Significant correlations are highlighted in red. A negative r value signifies an inverse correlation between the paired data.

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6.5 Expression of SOD2 and TXNRD1 in response to Pam3 and uric acid

To examine whether soluble uric acid might alter SOD2 and TXNRD1 expression in primary human monocytes *in vitro*, cells were exposed to of uric acid (30mg/dL) for 16 hours and then stimulated with Pam3. This was completed in addition to the patient data, to examine whether greater concentrations of serum uric acid might influence the redox environment within a cell at a transcriptional level.

Optimal timing for SOD2 and TXNRD1 gene expression was assessed via a time course experiment. Primary human monocytes from two donors were stimulated for 3-6 hours with Pam3 and gene expression of SOD2 and TXNRD1 was assessed by qPCR. Gene expression was calculated using the Livak delta delta CT (Livak and Schmittgen 2001) method for this experiment because a standard curve was not created for each GOI. Being an optimisation experiment, the relative expression of the GOIs was still considered useful for determining at what time point the largest differences in gene expression occurred in response to the stimuli. Pam3 induced a sharp time-dependent increase in SOD2 expression, with the largest increase occurring after 6 hours (Fig. 6.6 A). TXNRD1 expression increased from 0-3 hours regardless of stimulant (Fig. 6.6 B). For one donor, Pam3 caused an increase in TXNRD1 expression at 6 hours, but this difference was much less apparent for the second donor. Nonetheless, the biggest difference between Pam3-induced TXNRD1 was seen at 6 hours and this time point was used for future experiments. Although an increase in TXNRD1 expression was observed in the absence of Pam3, this would be controlled in future experiments by only comparing cells exposed to Pam3 with or without uric acid preincubation.

Primary human monocytes were subsequently preincubated in RPMI or soluble uric acid for 16 hours prior to a 6 hour stimulation with or without Pam3. Uric acid concentration was maintained during the 6 hour stimulation. Pam3 induced a marked 28-fold increase in SOD2 expression (Fig. 6.7 A), but had little effect on TXNRD1 gene expression (Fig. 6.7 B). In confirmation of the patient expression data, uric acid did not influence the expression of either SOD2 or TXNRD1 demonstrating that it does not increase intracellular antioxidant capacity. It is interesting to note the basal copy numbers of each antioxidant gene, with SOD2 present in 35-fold greater copy numbers than TXNRD1. TAC was also measured following the same stimulation conditions. Neither uric acid exposure nor Pam3 stimulation had any influence on the intracellular TAC of primary human monocytes (fig 6.7 C).
Figure 6.6 SOD2 and TXNRD1 gene expression in primary human monocytes in response to Pam3. Primary human monocytes from 2 donors were stimulated for 3 and 6 hours with Pam3 (100ng/mL) and then cDNA prepared. Gene expression of (A) SOD2 and (B) TXNRD1 was assessed by qPCR. Changes in gene expression were calculated using the Livak (delta delta CT) method and were normalised to reference genes B2M and RPL32. Data points represent means from three technical replicates ± SD.
Figure 6.7 TLR2 activation increases expression of SOD2 but not TXNRD1 in primary human monocytes. Primary human monocytes were exposed to cell culture media or uric acid (30mg/dL) for 16 hours prior to stimulation for 6 hours with or without Pam3 (100ng/mL). Gene expression of (A) SOD2 and (B) TXNRD1 were quantified by qPCR. Absolute copy numbers were calculated using a standard curve for each GOI and normalised to B2M and RPL32 expression. (C) Primary human monocytes were stimulated as above and then lysed and TAC measured in cell lysates. TAC was expressed per mg of protein as determined by BCA assay. Data represent means from 3 (A-B) or 6 (C) separate donors ± SEM. Statistical analysis was completed comparing RPMI with uric acid preincubations using multiple t-tests with Holm-Sidak's multiple comparison correction test.
6.6 Gene expression of NLRP3 inflammasome components in monocytes from gout, CKD and RA patients

Gene expression of inflammasome components in unstimulated monocytes was assessed to determine if there were any differences in the constitutive expression of inflammasome components in these patient samples. There were no significant differences between the expression of CASP1, IL1B, NLRP3 or PYCARD in monocytes from gout patients compared to healthy donors (Fig. 6.8). However, it is interesting to note that of the 10 samples with the highest expression of CASP1, 7 were from patients with gout. Overall, CASP1 expression was reduced in monocytes from CKD and RA patients (Fig. 6.8 A). PYCARD expression was also reduced in CKD patients (Fig. 6.8 D).

The expression of these genes were plotted against matched serum uric acid concentrations to determine whether serum uric acid concentration might alter the expression of inflammasome components in monocytes. Overall, serum uric acid did not correlate with the expression of any of the inflammasome components measured. The only significant correlation observed was in RA patients where serum uric acid concentration was inversely correlated with CASP1 expression (Table 6.6).

Gene expression of the inflammasome components was correlated with IL-1β secretion from matched donors. Overall there was no clear pattern suggesting that the expression of any of the inflammasome components would influence the secretion of IL-1β from stimulated donors (Table 6.7 and 6.8). Nonetheless, there were a few significant correlations that should be noted. CASP1 expression significantly correlated with IL-1β secretion in monocytes from gout patients stimulated with Pam3 and CPPD+Pam3 but did not influence IL-1β secretion from monocytes of healthy, RA or CKD donors. Furthermore, an increased NLRP3 expression correlated with increased CPPD+Pam3-induced IL-1β secretion in monocytes from CKD patients but not any other group. Finally, PYCARD expression correlated with increased Pam3-induced IL-1β secretion in healthy monocytes but interestingly was inversely correlated with CPPD+Pam3-induced IL-1β secretion from RA monocytes. Overall it appeared that the expression of these inflammasome components had little influence on the sensitivity of monocytes to activation by Pam3 or crystal stimuli.
Figure 6.8 Constitutive expression of NLRP3 inflammasome components in monocytes from gout, CKD and RA patients. cDNA was prepared from human monocytes from healthy (n=37), gout (n=43), CKD (n=36) and RA (n=36) donors. Expression of CASP1, IL1B, NLRP3 and PYCARD were measured by absolute qPCR. Individual points represent means from 2-3 replicate measurements for each donor and error bars represent means ± SEM. Significance was determined by Kruskal-Wallis analysis with Dunn’s post-hoc test (*P<0.05, **P<0.01).
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Table 6.6 Correlation of serum uric acid concentration expression of inflammasome components. The correlation coefficient ($r$) and significance were calculated using a two-way Spearman correlation test. Significant correlations are highlighted in red. A negative $r$ value signifies an inverse correlation between the paired data.
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**Table 6.7 Correlation of CASP1 and IL1B expression with IL-1β secretion.** The correlation coefficient (r) and significance were calculated using a two-way spearman correlation test. Significant correlations are highlighted in red. A negative r value signifies an inverse correlation between the paired data.
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Table 6.8 Correlation of NLRP3 and PYCARD expression with IL-1β secretion. The correlation coefficient (r) and significance were calculated using a two-way spearman correlation test. Significant correlations are highlighted in red. A negative r value signifies an inverse correlation between the paired data.
6.7 Discussion

Measurement of serum antioxidants revealed several differences between gout and CKD patients compared to healthy individuals. Gout patients had significantly increased serum uric acid and interestingly monocytes from gout patients also secreted significantly greater IL-1β when stimulated with Pam3 ± CPPD crystals than monocytes from healthy individuals. This is in agreement with previous studies that found increased Pam3-induced IL-1β secretion in PBMCs from gout patients compared to healthy donors (Mylona et al. 2012; Crisan et al. 2015). In contrast to the results presented here, these studies also showed increased production of IL-1β when MSU crystals were added alongside Pam3 (Mylona et al. 2012; Crisan et al. 2015), a response that was seen in monocytes here. These findings are also in contrast to recent studies suggesting that uric acid can both prime and activate the inflammasome (Braga et al. 2017; Crisan et al. 2017), although this was not assessed directly in this study. The lack of correlation between serum uric acid concentration and monocyte IL-1β secretion are in confirmation of the findings presented in chapter 5 in a smaller patient cohort. This was the case for all disease cohorts involved, although there was a small but insignificant trend for increased Pam3-induced IL-1β secretion in monocytes from gout patients. This lack of correlation is further validated by the IL-1β secretion data from the CKD patients, which demonstrated reduced levels of serum uric acid compared to healthy controls but comparable levels of IL-1β from monocytes stimulated with Pam3 ± crystals compared to healthy controls. Overall, the findings of this chapter are consistent with those of chapter 5 demonstrating that serum uric acid does not affect IL-1β secretion in human monocytes.

Serum Gpx3 activity was reduced in both gout and CKD patients compared to controls, and CKD patients also exhibited increased levels of ECSOD activity. CKD patients have previously been documented to have reduced Gpx3 activity (Roxborough et al. 1999; Wong et al. 2016). However, the activities of these antioxidants did not correlate with Pam3 or crystal induced IL-1β secretion in the monocytes isolated from healthy donors or patients with gout or CKD. However, one interesting trend was that Gpx3 activity negatively correlated with MSU+Pam3 induced IL-1β secretion in gout monocytes suggesting that Gpx3 might regulate inflammasome activation in monocytes. There is a lack of published studies investigating both Gpx3 and primary human monocyte inflammatory responses, but serum Gpx3 has been shown to play an anti-inflammatory role in a colitis-associated carcinoma mouse model (Barrett et al. 2013). However, if inflammasome activation is regulated by Gpx3 activity, one would expect this to be the case in the other patient cohorts, particularly in the CKD cohort who, like the gout patients, had reduced Gpx3 activity. Gpx3
activity did not correlate with IL-1β secretion in healthy, CKD or RA donors. Thus, it can be concluded that antioxidant capacity of the serum did not affect IL-1β secretion in monocytes isolated from healthy donors or patients with gout or CKD, signifying that the extracellular redox environment does not influence inflammasome activation.

The different levels of each serum antioxidant between healthy, gout and CKD donors could be indicative of a compensatory measure to maintain the overall antioxidant capacity. Antioxidant systems cooperate to maintain redox homeostasis, such that modifying the level of one antioxidant can cause compensatory changes in the levels of others (Poljsak et al. 2013). This idea of compensation has been used to explain why dietary antioxidant supplementation has limited benefit with the dietary supplementation of a single antioxidant causing a compensatory change in the levels of other antioxidants which maintains the level of antioxidant capacity already present (Poljsak et al. 2013). Potential redox compensation can be seen in the serum of CKD patients in this study which contained significantly lower levels of uric acid but elevated levels of ECSOD. Both uric acid and ECSOD are scavengers of superoxide, and thus the elevated ECSOD may well have compensated for the reduced levels of uric acid, explaining why no changes in total antioxidant capacity were observed.

Intracellular antioxidant expression also differed in monocytes from gout patients compared to healthy controls, but again this did not influence monocyte immune responses. Monocytes from gout patients had higher expression of TXNRD1 compared to healthy controls. Thioredoxin reductase 1 is a cytoplasmic enzyme which plays an important role in the thioredoxin system (Turanov et al. 2010) which is regarded as one of the main regulators of intracellular redox state (Witte et al. 2005). Increased gene expression of TXNRD1 could be indicative of an increased oxidative state in the cytoplasm of monocytes from gout patients. Indeed, there is evidence for an increased thioredoxin system in cardiovascular disease, a disease increasingly linked to overproduction of ROS and also associated with hyperuricemia (Waring et al. 2000; Whayne et al. 2015; Cervantes Gracia et al. 2017). Interestingly, the suppression of thioredoxin reductase has been shown to inhibit inflammasome-dependent IL-1β secretion in LPS-stimulated murine macrophages by suppressing TLR2 mediated transcription of pro-IL-1β and NLRP3 (Isakov et al. 2014). The authors suggested that the thioredoxin system is required for the activity of NF-kB thus positively regulating the NLRP3 inflammasome (Isakov et al. 2014). It is therefore interesting that monocytes from gout patients expressed significantly greater levels of TXNRD1 and were also more sensitive to activation by Pam3 compared to healthy controls. This suggests that perhaps the activity of thioredoxin reductase within a cell might influence inflammatory responses following TLR ligation. However, TXNRD1 expression did not
correlate with Pam3- or crystal-induced IL-1β secretion in monocytes from healthy donors or any of the disease cohorts, which would suggest that thioredoxin reductase activity is not associated with inflammatory responses in these cells.

The constitutive expression of inflammasome components, CASP1, IL1B, NLRP3 and PYCARD also had no apparent influence on the secretion of IL-1β from monocytes in this study, suggesting that none of the disease cohorts were predisposed to an increased inflammatory response. Interestingly, the expression of NLRP3 and IL1B, both transcriptionally regulated via the priming pathway (Bauernfeind et al. 2009), did not influence IL-1β secretion in human monocytes suggesting that monocytes from gout patients are not ‘primed’ prior to exposure to MSU crystals in vivo. Interestingly though a significant correlation between CASP1 expression and IL-1β secretion was observed in monocytes from gout patients following stimulation with Pam3 ± CPPD crystals. Gout patients also demonstrated significantly increased IL-1β secretion under these stimulatory conditions compared to healthy controls. The expression of CASP1 did not differ in gout patients compared to healthy controls, demonstrating that this association with increased IL-1β secretion is not a result of overexpression of the gene. However, it was of interest that of the patients with the top 10 CASP1 expression levels, 7 had gout. It is possible that the correlation is related to the efficiency of pro-caspase-1 cleavage in gout patients. CASP1 is not transcriptionally regulated by the priming pathway (Bauernfeind et al. 2009; Ghonime et al. 2014) (chapters 3 and 5), and perhaps the availability of non-transcriptionally regulated inflammasome components may influence NLRP3 activation. It is also interesting that monocytes from both CKD and RA patients exhibited significantly reduced CASP1 expression and also reduced basal IL-1β secretion compared to healthy controls. Caspase-1 is regulated post-translationally by cleavage following incorporation of pro-caspase-1 into the inflammasome complex (Lu and Wu 2015). Human monocytes are also known to have constitutively active caspase-1 (Kahlenberg and Dubyak 2004; Netea 2009), yet the reason for this remains unknown. Further research is required to investigate whether the extent of constitutive activation of caspase-1 differs in different disease contexts thus predisposing some individuals to more robust inflammatory responses upon monocyte recognition of PAMPs or DAMPs. Of course, it is important to consider that gene expression does not necessarily result in increased protein levels, with numerous regulatory processes occurring between mRNA expression and protein translation (Vogel and Marcotte 2012). Therefore, it would also be interesting to examine whether the availability of pro-caspase-1 protein differs between healthy individuals and gout patients, and whether the cleavage of pro-caspase-1 occurs more rapidly in monocytes from gout patients.
Interestingly, Pam3 induced an increase in SOD2 gene expression, but not TXNRD1. In agreement with these findings an increase in SOD activity but unaffected thioredoxin reductase activity has also been demonstrated in Pam3-stimulated THP-1 cells (Karwaciak et al. 2017). SOD2 and TXNRD1 are mitochondrial and cytoplasmic antioxidant enzymes, respectively, and the increase in SOD2 could be indicative of mitochondrial ROS production upon TLR2 ligation in these cells. Indeed, mitochondrial ROS has been widely linked to the NLRP3 inflammasome, with studies showing increases in mitochondrial ROS (Heid et al. 2013), the release of oxidised mitochondrial DNA (Shimada et al. 2012; Carlos et al. 2017), and the mobilisation of inflammasome components towards the mitochondria (Subramanian et al. 2013) in response to NLRP3 activating stimuli. However, a measurable increase in ROS has not been observed in response to Pam3 so far in this thesis (chapters 3-5). The increased transcription of SOD2 downstream of NF-κB activation is well established (Jones et al. 1997; Kairisalo et al. 2007). SOD2 has previously been shown to have a dual role in microglia, acting as an antioxidant as well as a negative regulator of NF-κB activation (Ishihara et al. 2015), and interestingly SOD2 has been shown to suppress the expression of NLRP3 inflammasome components in a fibroblast cell line (Yoon et al. 2018). As such, this increase in SOD2 is likely part of a negative regulation loop in which NF-κB is self-limiting. These results also confirm that uric acid does not activate NF-κB in human monocytes (chapter 5) as it had no effect on the expression of SOD2.

The results of this chapter show that IL-1β production is independent of intracellular or extracellular changes in antioxidant capacity. There were several alterations in extracellular antioxidants between healthy controls, gout and CKD patients, but none of these differences consistently correlated with IL-1β secretion from monocytes. A compelling finding of these results is the lack of any strong correlation between serum uric acid, IL-1β secretion and inflammasome gene expression, despite recent reports suggesting that hyperuricemia potentiates NLRP3 priming and activation. Gout patients did secrete greater levels of IL-1β than healthy controls following exposure to Pam3 and CPPD crystals added alongside Pam3, suggesting that there are additional unknown factors that regulate the production of IL-1β in individuals with gout but these factors certainly appear to be independent of intracellular or extracellular antioxidant status.
Chapter 7 - Discussion
7.1 Summary of results

This study sought to investigate the role of ROS and oxidative stress in activating the NLRP3 inflammasome in human monocytes. Methods for activating the inflammasome using crystalline stimuli were optimised and ROS levels measured in human monocytes during exposure to these activating stimuli. MSU crystals consistently caused robust increases in both ROS and IL-1β secretion when added in combination with the TLR2 ligand, Pam3, initially suggesting that increases in ROS could be involved in activating the inflammasome. However, the importance of ROS in this system was questioned by the fact that CPPD crystals, which like MSU induced secretion of IL-1β, did so in the absence of measurable increases in ROS production. The role of MSU-induced ROS in activating the inflammasome was subsequently investigated using the small molecule antioxidant NAC and the recombinant antioxidant enzymes ECSOD and catalase. All three of these antioxidants inhibited MSU-induced ROS increases, with NAC almost completely scavenging ROS in this system. However, secretion of IL-1β from primary human monocytes exposed to MSU crystals alongside Pam3 was unaffected by these decreases in ROS. These data clearly demonstrated that ROS are not necessary for activation of the inflammasome in response to MSU or CPPD crystals in primary human monocytes.

The hypothesis that more subtle changes in cellular redox environment would influence activation of the inflammasome was then explored. Uric acid is the major antioxidant in the blood, but at high concentrations is the major risk factor for developing gout. Uric acid can precipitate to form MSU crystals, inducing NLRP3-dependent IL-1β secretion within peripheral joints and can lead to gout attacks. Soluble uric acid had no effect on priming or activation of the NLRP3 inflammasome in primary human monocytes, despite causing increases in ROS in these cells, nor did the concentration of serum uric acid correlate with secretion of IL-1β from primary human monocytes isolated from healthy donors or gout patients. These observations demonstrated that crystallisation of soluble uric acid is required for activation of the inflammasome and that changes in the concentration of this extracellular antioxidant do not alter NLRP3 expression or activation in human monocytes. This was further explored using blood samples from healthy individuals as well as patients with a history of gout, CKD or RA. Various differences in antioxidant capacity were observed in both serum and monocytes from these donors; however none of these alterations correlated with IL-1β secretion in response to Pam3, MSU or CPPD crystals. Interestingly, monocytes from gout patients did secrete more IL-1β in response to Pam3 or CPPD+Pam3 than healthy controls, suggesting that an, as yet unknown, factor may increase the inflammatory responses of these monocytes. The expression of inflammasome
components in patient monocytes were measured to examine whether they were
differentially expressed in disease, and to determine if differences in constitutive expression
of these components could influence the monocyte inflammatory response. Differences in
expression of CASP1 and PYCARD were observed between CKD and healthy monocytes,
but overall the expression of these components did not correlate with monocyte IL-1β
secretion following exposure to Pam3, MSU or CPPD, nor were the expression of these
components influenced by differences in intracellular or extracellular antioxidant capacity.

7.2 Conclusions

The overall conclusion of this study is that the NLRP3 activation is not redox regulated in
primary human monocytes. This is an important finding, particularly in the light of previous
work showing that ROS are involved in this pathway in THP-1 cells, mouse BMDMs, human
PBMCs and human monocyte-derived macrophages (Dostert et al. 2008; Zhou et al. 2010b;
Heid et al. 2013; Simard et al. 2013). Therefore, it appears that the extent to which the
NLRP3 inflammasome is redox regulated is dependent on cell-type which may in turn be
influenced by the cell’s environment. Indeed, there are known differences in the mechanism
of inflammasome activation in human monocytes and macrophages (Netea 2009; Gaidt et
al. 2016). Unlike macrophages, monocytes secrete IL-1β in response to TLR-ligation alone
due to autocrine activation by ATP and the presence of constitutively active caspase-1
(Netea 2009). These differences likely reflect evolutionary adaptations to their environment
with the two-step mechanism for macrophages in tissue prevent unnecessary inflammation
whereas the one-step mechanism in monocytes allowing a rapid response to infection.
Monocytes are also more efficient at maintaining redox homeostasis, quickly increasing
antioxidant capacity in response to LPS (Carta et al. 2011). Perhaps differences in redox
homeostasis between cell types might therefore influence the extent to which the
inflammasome is redox regulated.

Furthermore, the importance of species differences became evident when comparing the
effects of uric acid on NLRP3 activation in human and mouse cells, where uric acid had no
effect on IL-1β secretion from human cells (this study), but induced cytokine release from
mouse macrophages (Braga et al. 2017). Although a role for ROS in the activation of the
inflammasome in primary human monocytes has been documented (Hewinson et al. 2008;
Shin et al. 2012; Carta et al. 2015) these studies were done using DPI to inhibit ROS which,
as discussed in chapter 4, has various off-target effects. In contrast, DPI has also been
shown to have no effect on IL-1β secretion in primary human monocytes, instead
demonstrating NLRP3 activation to be dependent on mitochondrial ROS in these cells (Chen et al. 2017).

The precise role of ROS in activating NLRP3 could thus be influenced by the origin or type of ROS in the cell. Intriguing results, suggesting that ROS generated by NADPH oxidase actually inhibit the secretion of IL-1β, were obtained in human monocytes isolated from patients with chronic granulomatous disease (CGD) which lack NADPH-oxidase generated ROS. These cells secreted greater levels of IL-1β in response to MSU than healthy donors (van de Veerdonk et al. 2010). This inhibitory role for ROS has been confirmed in SOD1 deficient cells which have elevated ROS and secrete reduced levels of IL-1β (Meissner et al. 2008; Tassi et al. 2009). In contrast, there are many reports of the role of mitochondrial ROS in activating NLRP3 (Zhou et al. 2010b; Heid et al. 2013; Chen et al. 2017). Thus, it is possible that the intracellular source of ROS is important and may result in an overall effect on IL-1β secretion depending on which system (mitochondria versus NADPH oxidase) is producing most ROS under particular physiological conditions. This could explain the contrasting results in the literature whereby small changes in cell culture conditions or in cell isolation methods could lead to alterations in cell metabolism and function leading to changes in the relative quantities of ROS produced by the two systems. It is possible that the increases in ROS had no effect on IL-1β production in the experiments described here due to the balance of ROS generated by mitochondria versus NADPH oxidase.

The results presented here provide strong evidence that ROS and oxidative stress are dispensable for activation of NLRP3 in human monocytes and these results do not rely solely on the use of chemical inhibitors of ROS. Rather, several approaches were employed to test whether ROS could influence NLRP3 activation including: testing the association between increases in cellular ROS and production of IL-1β; testing the effects of removal of ROS on production of IL-1β; and investigating the more subtle effects of disturbances in antioxidant capacity and potentially altered redox regulation on expression and activity of NLRP3. The results obtained from all of these approaches converged on the same conclusion: that NLRP3 activation is not regulated by ROS or redox regulation in primary human monocytes. There are a few other studies in primary human monocytes that concluded that ROS are not required for activation of NLRP3 in these cells reporting similar findings to those presented here (van de Veerdonk et al. 2010; Meunier et al. 2012; An et al. 2014). Two of these studies used particulate stimuli, either double-walled carbon nanotubes (Meunier et al. 2012) or MSU crystals (An et al. 2014), demonstrating that NLRP3 activation by crystals or particles in primary human monocytes is independent of ROS, instead requiring potassium and calcium flux, as well as cathepsin B release.
7.3 Limitations and future experiments

A particular limitation of this study is the use of a single readout, namely an IL-1β ELISA, as evidence for NLRP3 activation. Despite IL-1β secretion being the endpoint of NLRP3 activation, the cytokine can be released in its non-active pro-form during cell stimulation and this pro-IL-1β can be measured by ELISA under these conditions. MCC950, a small molecular inhibitor of NLRP3 (Coll et al. 2015), was used to confirm the activation of the NLRP3 inflammasome and did indeed cause a marked reduction in IL-1β secretion. However, it would be important to further validate NLRP3 activation in future experiments by examining the cleavage of pro-IL-1β and pro-caspase-1 by western blot - a common experiment completed in studies involving the NLRP3 inflammasome (Martinon et al. 2006; Hornung et al. 2008; Netea et al. 2009). Validating the specific activation of the NLRP3 inflammasome would strengthen the findings of the study and would confirm the finding that NLRP3 activation occurs in a ROS-independent manner in primary human monocytes.

As mentioned in chapter 3, an interesting discrepancy in this study compared to others is the lack of cell death caused by MSU and CPPD crystals despite causing the activation of the NLRP3 inflammasome. NLRP3-dependent caspase-1 activation is known to precede pyroptotic cell death and this cell lysis is the mechanism by which active IL-1β is released from immune cells (Shi et al. 2015; Sborgi et al. 2016). Throughout this study, no significant cell death has been observed in response to either crystal, despite a large increase in IL-1β. As discussed in chapter 3, this is likely a result of the use of metabolic viability assays in this study which do not provide a measure of pyroptosis. In future experiments it would be of particular interest to examine the release of lactate dehydrogenase, a common marker for assessing pyroptosis, as well as using a gasdermin-D inhibitor such as necrosulfonamide (Rathkey et al. 2018) to examine whether pyroptosis is indeed the mechanism by which IL-1β is released from primary human monocytes. In addition, the measurement of gasdermin-D activation and pyroptosis would also validate the activation of the NLRP3 inflammasome under these experimental conditions, as gasdermin-D activation is mediated by active caspase-1. A recent study has shown that gasdermin-D induced release of IL-1β can occur in murine BMDMs and BMDCs in the absence of cell lysis, suggesting that stimuli at certain concentrations can induce low level IL-1β release without inducing the death of the cell (Heilig et al. 2018). This would be worth examining under the experimental conditions and concentrations used in this study to determine whether MSU and CPPD crystals are able to induce gasdermin-D pores without affecting monocyte viability.
Finally, the sole use of crystalline stimuli for investigating the activation of the inflammasome in this study means that the conclusion that ROS are not involved in the activation of NLRP3 can only be applied to crystalline stimuli. It would be interesting to use non-crystalline positive controls such as ATP and nigericin to examine the importance of ROS in inflammasome assembly in response to non-crystalline stimuli in human monocytes. This would provide insight into whether the findings of this study are specific to phagocytosis and lysosomal destabilisation occurring upstream of NLRP3 inflammasome activation, or whether the findings can be applied to the overall mechanism of inflammasome activation in primary human monocytes. ATP and nigericin are both commonly used in the investigation of the NLRP3 inflammasome (Ferrari et al. 2006; Katnelson et al. 2015) and would therefore also provide useful comparison to other studies within the field.

7.4 Concluding remarks

Monocytes play important roles in development of inflammatory responses, particularly in conditions associated with metabolic disturbances such as gout and cardiovascular disease. The results presented here provide important insights into the mechanisms involved in activation of NLRP3 in monocytes and contribute to our understanding of how these cells can mediate inflammation in metabolic disorders. However, it is important to note the use of pure populations of monocytes in this study, which did not seek to elucidate the effects of redox regulation of monocyte interactions with other cell types. There are known interactions between monocytes and lymphocytes (Cline and Swett 1968) which might explain why some studies using human PBMCs have documented ROS-dependent activation of the inflammasome (Van Bruggen et al. 2010) compared to the ROS-independent activation in primary human monocytes documented here. It could be possible that other cell types are activated by ROS and in turn alter the function of monocytes via secretion of other factors.

The association between inflammation and oxidative stress has long been recognised, and the hypothesis that oxidative stress can drive disease is particularly interesting because of the potential use of antioxidants for treating a wide range of inflammatory conditions. However, it remains difficult to convincingly demonstrate a causal role for ROS in generating inflammation (Ghezzi et al. 2017). The NLRP3 inflammasome was a potential mechanism by which oxidative stress could drive sterile inflammation and inflammatory disease. However, this has not proved to be the case. Based on these findings, targeting oxidative stress for the treatment of diseases where activation of NLRP3 is known to be a major contributor to the pathophysiology should not be considered a viable therapeutic strategy.
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Appendices

Appendix 1. Patient characteristics for chapter 5. DMARDs – disease modifying antirheumatic drugs

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**Treatments:**

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**Drug prescriptions:**

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<td>Steroid (%)</td>
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<td>6</td>
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Appendix 2. Patient characteristics for chapter 6. DMARDs – disease modifying antirheumatic drugs

<table>
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<tr>
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<th>Gout (n)</th>
<th>CKD (n)</th>
<th>RA (n)</th>
</tr>
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<tr>
<td>Total collected</td>
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<td>52</td>
<td>41</td>
<td>36</td>
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<tr>
<td>M/F (%M)</td>
<td>15/38 (28.3)</td>
<td>41/11 (78.8)</td>
<td>28/12 (68.3)</td>
<td>12/23 (33.3)</td>
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<tr>
<td>Mean age at collection (range)</td>
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<td>68 (39-85)</td>
<td>62 (21-93)</td>
<td>64 (37-88)</td>
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<tr>
<td>Mean years since diagnosis (range)</td>
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<td>12 (0-51)</td>
<td>6 (0-49)</td>
<td>14 (0-51)</td>
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<tr>
<td>Mean CRP (mg/L) ± SD</td>
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<td>6.0 ± 5.9</td>
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<td>Mean GFR (mL/min) ± SD</td>
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<td>N/A</td>
<td>8.2 ± 2.52</td>
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Treatment information:

| Patients with drug prescriptions (%)  | 0           | 73        | 0       | 78     |
| Patients on DMARDs (%)               | 0           | 2         | 0       | 66     |
| Patients with multiple drug combinations (%) | 0         | 8         | 0       | 28     |
| Receiving haemodialysis (%)          | 0           | 0         | 100     | 0      |

Drug prescriptions:

<table>
<thead>
<tr>
<th>Drug</th>
<th>Healthy (%)</th>
<th>Gout (%)</th>
<th>CKD (%)</th>
<th>RA (%)</th>
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<tr>
<td>Adalimumab</td>
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<td>Allopurinol</td>
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<td>Benzbromarone</td>
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<td>Colchicine</td>
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<td>Etanercept</td>
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</table>
Publications arising from thesis

Alberts, B. M., C. Bruce, K. Basnayake, P. Ghezzi, K. A. Davies & L. M. Mullen (2019) Secretion of IL-1β from monocytes in gout is redox independent. Front Immunology, 10, 70. doi: 10.3389/fimmu.2019.00070