Regulation of oxidoreductase enzymes during inflammation

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Abstract

Autoimmune diseases such as rheumatoid arthritis (RA) occur when immune responses erroneously target host cells and proteins. The triggers for this dysregulated inflammation are unknown, but Toll-like receptors (TLRs) and oxidative stress are contributing factors \(1, 2\). Peroxiredoxin (PRDX) and thioredoxin (TXN) are intracellular protein thiol-disulphide oxidoreductase enzymes which link the TLRs to oxidative stress. These enzymes are induced during inflammation and oxidative stress, and are released from cells following inflammatory stimuli. In the extracellular environment they can act as danger signals, triggering TLR activation and perpetuating inflammation. However, knowledge of oxidoreductase regulation and release is limited. The hypotheses of this study were that responses of oxidoreductases vary depending on the TLR stimulated, and that serum oxidoreductases could be used as biomarkers for RA.

To investigate TLR-induced oxidoreductase responses, concentrations of TLR agonists required to induce release were examined and tested for effects on tumour necrosis factor (TNF) production and cell viability in RAW 264.7 cells. Cells were stimulated with optimised concentrations in serum free OptiMEM (24h) for cell lysate and supernatant gel electrophoresis and Western blot experiments, and cell culture media (6-18h) for real time Taqman qPCR. PRDX1 and TXN1 release was found to occur primarily following TLR3 and TLR4 stimulation. Each oxidoreductase had a different pattern of gene expression to the TLRs: \(PRDX1\) only increased with TLR4 stimulation, whilst \(TXNRD1\) increased with TLR4, TLR7 and TLR8, and heme oxygenase \((HO1)\) increased with these TLRs plus TLR1/2 and TLR3.

To investigate the role of reactive oxygen and nitrogen species (ROS and RNS respectively) on oxidoreductase release, RAW 264.7 cells were stimulated with PMA or interferon \((IFN)\gamma + lipopolysaccharide (LPS)\). Griess assay was used to assess nitrite production. \(IFN\gamma + LPS\) increased TXN1 release but abrogated PRDX1 release. PRDX1 protein underwent NO-mediated proteasomal degradation following \(IFN\gamma + LPS\) stimulation. Gene expression of all oxidoreductases investigated increased with \(IFN\gamma + LPS\) stimulation compared to LPS alone, and inhibition of protein synthesis prevented \(PRDX1\) gene expression, suggesting a secondary signal protein is required.
To investigate serum PRDX2, serum was isolated from whole blood from RA patients and healthy donors. ELISA was used to quantify both PRDX2 and the inflammatory marker C-reactive protein (CRP). No difference was found between RA and healthy serums, and PRDX2 did not correlate with CRP.

The findings from this project show that oxidoreductase regulation and responses are fine-tuned depending on the TLR stimuli, and that serum PRDX2 is not a suitable biomarker for RA.
Courage doesn’t always roar. Sometimes courage is the little voice at the end of the day that says “I’ll try again tomorrow”
- Mary Anne Radmacher
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Declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. This 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

Sonia Louise Ingram
Chapter 1
1. Introduction

1.1 Inflammation and immunity

1.1.1 The Immune System

Despite being constantly surrounded and inhabited by microbes, we rarely become sick. The immune system has a variety of roles required for keeping the host organism healthy. These roles include recognition of and protection from invading organisms, self-regulation to prevent over-activation and self-harm, wound healing and repair, and memory of previous infections (3).

All cells of the immune system are derived from haematopoietic stem cells (HSCs) which reside primarily in the bone marrow. As stem cells, when HSCs divide the daughter cells can either self-renew, that is, to create a clone of the HSC, or differentiate into progenitor cells of one of two lineages: lymphoid or myeloid. Based on these two lineages, the immune system is divided into two distinct but overlapping factions, innate and adaptive (Figure 1.1).
**Figure 1.1: Cells of the immune system.** Adapted from Hayden and Ghosh 2011 (4). Haematopoietic stem cells are the precursors to almost* all cells of the immune system. Cells are divided into two classes: lymphoid and myeloid. Progenitor cells are the precursors to final differentiated immune cells.

* Several tissue-resident macrophage subsets self-renew from a common precursor established during embryonic development (5, 6). Original in colour.
**1.1.1.1 The innate immune system**

The innate immune system is an ancient system, with features conserved throughout plant and animal life (3, 7). The innate system is the first line of defence to infection, with response times of <96h (3). It can be summarised by four main roles:

1. Provision of physical and molecular barriers
2. Recognition and removal of pathogens
3. Production of inflammatory signalling molecules
4. Activation of adaptive immune system

Physical barriers such as epithelial membranes of the skin, gastrointestinal tract, respiratory tract, and genitalia prevent microbial entry into the body. Skin has an acidic pH which dissuades colonisation by some bacteria, and skin peeling or drying out can help to dislodge bacteria (3). Furthermore, a chemical barrier such as the production of mucus, tears, and saliva can assist with removing pathogens or can inhibit their activity.

When pathogens break through physical barriers three stages of innate immune response occur:

1. Immediate immunity (<4h) – preformed soluble molecules (the complement system) target pathogens for lysis or phagocytosis, antimicrobial enzymes (lysozymes) digest bacterial cell walls, antimicrobial peptides (defensins) directly lyse bacterial membranes
2. Early induced responses (4-96h) – pattern recognition receptors (PRRs) allow effector cells (neutrophils, macrophages) to recognise pathogens, phagocytose them or produce microbicidal agents (reactive oxygen/nitrogen species) to directly kill them. More effector cells are recruited through production of proinflammatory cytokines and chemokines
3. Adaptive immune responses (>96h) – professional antigen presenting cells (APCs) (dendritic cells, macrophages) activate adaptive cells using the Major Histocompatibility Complex (MHC), enabling a targeted response

Cells of the innate immune system are primarily of the myeloid lineage, with the exception of natural killer (NK) cells which are of the lymphoid lineage. Sentinel cells such as tissue-resident macrophages and dendritic cells are often the first immune cells to encounter pathogens. These cells phagocytose pathogens and on activation
release proinflammatory signals. Neutrophils are the most numerous and fast responding phagocytes, arriving within minutes, and are a hallmark of acute inflammation (3). Activated neutrophils release further inflammatory signals, amplifying the response and attracting more immune cells to the site of infection.

APCs phagocytose pathogens and then retreat into the lymphatic system where they activate cells of the adaptive immune system by presenting pathogenic fragments to them. This is the beginning of the adaptive system-mediated targeted immune response [see 1.1.2].

Pathogen Recognition Receptors (PRRs) are responsible for recognising highly conserved molecular patterns displayed by microbes. These include certain peptidoglycans, polysaccharides, DNA or RNA. Binding of these molecular patterns activates sentinel cells, enabling phagocytosis of the pathogen, and triggering the initial inflammatory response. Recognition of foreign or dangerous molecules is the key step in enabling inflammatory signalling and therefore activation of the adaptive system [see 1.5].

1.1.1.2 The adaptive immune system

This project focusses on innate immune responses, but the innate and adaptive immune systems work in concert to clear pathogens and prevent reinfection.

The adaptive immune system derives from the lymphoid cell lineage and arose around 500 million years ago in jawed fish (8) and is conserved throughout all jawed vertebrates (9). The adaptive system is responsible for generating a highly specific, targeted immune response with the aim of eliminating the pathogen or at least limiting growth. Initial activation of this system is reliant on antigen presentation and activation via the innate immune system.

The adaptive immune system is comprised of highly specialised systemic cells, T and B lymphocytes, derived from the lymphoid lineage of immune cells (Figure 1.1). T cells mature in the thymus and B cells in the bone marrow. Each T cell displays a unique T cell receptor, a vast array of which can be generated from a set number of genes using gene rearrangement (3). This allows for huge variation in the T cell population and increases the probability that a variety of antigens will be recognised. Upon receptor binding, which is mediated through APCs and the MHC, the bound T cell becomes activated, undergoes monoclonal expansion and activates other immune cells, enabling widespread defence against the pathogen. B cells are then activated to produce antibodies, using somatic hypermutation and recombination to
create a vast array of antibodies until a strong match to the antigen is formed which is then produced in large quantities. T and B lymphocytes are able to create memory T and B cells following pathogen clearance. This enables immunological memory which can be activated upon reinfection allowing a faster, amplified response upon subsequent interactions with previously encountered pathogens.
1.1.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an autoimmune disease of the joints which affects 1-2% of the global adult population (10). RA is one of the most prevalent chronic inflammatory diseases (11) and affects 2-3 times more women than men (12, 13). It has a strong genetic component, with a heritability risk of ~50% for seropositive and 20% for seronegative patients (14). Over 100 genetic loci have been associated with RA risk, which is further increased with environmental factors such as smoking (11, 15-17). RA joints follow a symmetrical pattern of inflammation, for example both hands will be affected rather than one or other. The exact cause of RA is unknown, but it is thought that environmental stimuli trigger inflammation which then becomes dysregulated in genetically susceptible individuals (18, 19).

RA is predominantly a disease of the synovium (the thin membrane that encapsulates joints). For unknown reasons the synovium becomes inflamed and hypertrophied leading to pannus formation around the joint. Increased proinflammatory mediator production and macrophage infiltration occurs, followed by lymphocyte invasion. Germinal centres (sites of B and T cell proliferation) develop within the joint (20). Angiogenesis becomes dysregulated and there is an increase in straight, branching vasculature (21). As the disease progresses, a more heightened state of inflammation occurs resulting in cartilage degradation and in some cases bone degradation and remodelling (Figure 1.2, (3). In fact, in some individuals bone loss can occur very early in disease prior to onset of clinical symptoms (22, 23).

Symptoms of RA include chronic pain, stiffness, swelling and loss of function in the joint. However, rather than being isolated within the joint RA is actually a disease of systemic inflammation, therefore many co-manifestations can occur affecting organs, skin or the circulatory system (17). RA was initially thought to be a disease of the T and B cells. However, further research has shown that it is more complex than first thought, involving both innate and adaptive immune systems. Aetiology is difficult to study in patients as symptoms only present at a relatively late stage of the inflammatory process (24).

Treatment strategies for RA aim to reach a target of sustained remission or low disease activity within six months. If this is not achieved, more targeted treatment strategies should be employed (25). Disease Modifying Anti-Rheumatic Drugs (DMARDs) are the therapeutics used in RA. There are four classes of DMARD:
1. Conventional synthetic DMARDs (csDMARDs) e.g. methotrexate, leflunomide, sulfasalazine, hydroxychloroquine
2. Targeted synthetic DMARDs (tsDMARDs) e.g. tofacitinib, baricitinib
3. Biological originator DMARDs (boDMARDs) e.g. TNF-inhibitors: adalimumab, certolizumab pegol, etanercept, golimumab, infliximab
4. Biosimilar DMARDs (bsDMARDs) e.g. Abatacept, rituximab, or tocilizumab, as well as other IL-6 pathway inhibitors, sarilumab and/or sirukumab, once approved (25)

Treatment begins with the application of csDMARDs, usually methotrexate (which is the anchor drug (26)) plus short-term glucocorticoids. If the treatment target is not attained within 6 months, prognostic markers (including autoantibodies, high disease activity, and failure of csDMARDs) are used to determine the next treatment strategy: if no unfavourable markers are present a tsDMARD can be added to the therapy combination, but if unfavourable prognostic markers are observed, patients should be moved onto bDMARDs (encompasses boDMARD and bsDMARD) in combination with csDMARDs. There are currently no guidelines as to which bDMARD should be used first, but if patients do not respond to this therapy they can be moved on to any other bDMARD (25). As new treatment options are becoming available the outlook for refractory RA is improving, but an improved understanding of the aetiology of disease is essential for identifying novel drug targets, monitoring disease severity, and achieving successful disease remission in all patients.
Figure 1.2: Diagram of a healthy vs rheumatoid arthritis joint. The rheumatoid arthritis joint is characterised by inflammation of the synovial membrane which leads to joint remodelling through cellular infiltration, bone and cartilage degradation and pannus formation. Original in colour.
1.1.3 Biomarkers of disease

In 2001, the Biomarkers Definitions Working Group defined biomarkers as “biological characteristics that can be objectively measured and evaluated as an indication of normal biological or pathogenic processes, or pharmacologic responses to therapeutic interventions” (27, 28). These can range from gene expression patterns through to protein or molecular concentrations in biological fluids, and even changes in electrical activity in the brain. Biomarkers are divided into two main classes: descriptive, which give an indirect indication of disease activity, and mechanistic, which are directly involved in disease pathogenesis and so give a better guide for clinical decision making (29). These can then be further divided into four groups according to their application: diagnostic, staging of disease, prognostic, and biomarkers to monitor clinical responses to therapeutic interventions (30).

The personalised medicine approach acknowledges that the same disease can have different causes, and therefore therapeutic efficacies, in different patients. Each patient and their treatments should therefore be considered on an individual basis (31). Careful biomarker monitoring is important throughout each stage of disease, enabling early diagnosis and providing prognostic information which is important in guiding clinical decisions.

Various biomarkers are used for diagnosis of RA, even before clinical symptoms develop. As a systemic disease, there are increased circulating proinflammatory cytokines (tumour necrosis factor (TNF), interleukin (IL)-6, IL-12 and IL-17), and approximately two-thirds of patients are seropositive for autoantibodies. Rheumatoid factor (RF) which is an autoantibody directed against the Fc portion of IgG antibodies is present, often at high titre, in 70-80% of RA patients. The long term risk of developing RA is almost 26 times greater with high RF titres (>100 IU/ml) (32), whilst the risk of developing extra-articular manifestations is increased 4-fold when the IgA isotype of RF is present (33).

Most patients positive for RF are also positive for anti-citrullinated peptide antibodies (ACPA) (17, 34-39). ACPA can be detected in patients years before clinical disease onset, and this early biomarker is associated with a poorer long term prognosis than ACPA negative patients, poorer responses to anti-TNF therapy, and increased risk of early bone loss (20, 23, 40). More recently, anti-carbamylated protein (anti-CarP),
and anti-human α-enolase (ENO1) antibodies have been proposed as biomarkers for early RA (41, 42).

Biomarkers used to monitor RA disease activity include erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) in addition to monitoring clinical parameters using the 28-point disease activity score (DAS28), simple disease activity index (SDAI), and clinical disease activity index (CDAI). Current research is focused on establishing biomarkers to predict treatment responses. This is an essential step for personalised medicine, and will allow the most promising treatment strategy for each patient to be administered directly, without compromising patient health by using less targeted therapeutics before the correct therapy is found (27).
1.1.4 Inflammation

Inflammation is classically described as a local protective response resulting in swelling, heat, redness, pain and loss of function (43). These symptoms all result from blood vessel dilatation at the site of injury/infection, with the purpose of immune cell and effector molecule delivery, blood clot formation (generation of a physical barrier), and tissue repair (3). Inflammation is invoked following microbial infection or tissue injury and is a fine balance between effective elimination of the pathogen without causing significant damage to the host.

As summarised above, the process of inflammation is a complex interplay of multiple different factors. An essential component of inflammation is the communication between cells to generate a coordinated response. There are various mechanisms of intercellular communication:

- Direct cell-cell signalling
- Cytokines and chemokines
- Extracellular vesicles

1.1.4.1 Cell-cell communication

Direct cell-cell signalling is a mechanism of immediate signal transfer between cells using various cell membrane receptors. An important example of this communication occurs between APCs and T cells, whereby the APCs utilise MHC to present pathogenic molecular patterns to T cells through binding with the T cell receptor. This receptor complex acts as the first signal for T cell activation. For full T cell activation, a secondary signal is required which occurs via CD28 on the T cell binding with B7.1 on the APC [see 1.4].

1.1.4.2 Cytokines and chemokines

Cytokines and chemokines are essential molecules for intercellular communication. These small proteins (~25kDa) are produced by many cells of the body and elicit their effects by binding with specific receptors. The behaviour of cytokines can be autocrine, where the effect occurs on the producer cell, paracrine, where the effect occurs on adjacent cells, or where cytokines are very stable, endocrine, where the effect occurs on distant cells (3).

Immune cells can produce cytokines with pro- or anti-inflammatory effects, as well as chemokines which signal for chemotaxis (cell movement). There are many different cytokines, sometimes with multiple receptors which elicit different effects.
The naming system of 'interleukin' followed by a number was coined in an attempt to standardise the naming of cytokines secreted by and acting on leukocytes, but as different structures and functions emerged cytokines have broadly been divided into families, though the interleukin designation is still used. These families include colony stimulating factors which stimulate growth, interferons, interleukins and the TNF family. Members of all of these families have diverse roles in both the innate and adaptive systems.

The APCs secrete a structurally diverse group of cytokines in response to pathogen detection. This group includes the proinflammatory cytokines TNF, IL-1β, IL-6, IL-12 and interferon (IFN)-γ (44). TNF and IFNγ are the cytokines considered in this project.

1.1.4.2.1 TNF

The first description of a tumour necrotising factor occurred in 1975 by Carswell who found that TNF was produced in response to endotoxin and could cause necrosis of sarcoma Meth A and other transplanted tumours (45). Ten years later, Beutler et al showed that anti-TNF could protect mice from the lethal effect of LPS, thus giving the first evidence of TNF’s inflammatory importance (46). Since then, a further 18 members of the TNF superfamily, plus 29 receptors, have been identified based on sequence similarities to TNF (47).

TNF is a key fast-response proinflammatory cytokine. Activation of the TNF gene occurs in response to a variety of cellular activating factors including calcium signalling, osmotic stress, oxidative stress and pathogen recognition (48). TNF is an immediate early gene which is transcribed minutes after its activation peaking at 2-4h, protein synthesis then peaks at 4-6h after activation (49). Transcription of TNF is an example of direct transcription which does not rely on de novo protein synthesis and is regulated by enhanceosomes, which are higher-order protein complexes comprised of transcription factors and coactivators (50). Enhanceosomes assemble at the promoter or enhancer region of a gene and mediate its expression (51). Regulation of TNF is complex as the formation of the enhanceosome is cell-type and stimulus-specific, allowing for transcription by different transcription factors following specific stimuli (48, 52, 53). Regulation of TNF protein expression is further complicated by various regulatory steps at the post-transcriptional, translational and post-translational stages (54, 55).
TNF protein is initially expressed as a trimer on the cell membrane (56, 57). Upon cleavage of the 26kDa membrane-bound form by TNF-converting enzyme, the soluble 17kDa form can be released (58). Soluble TNF is primarily produced by activated macrophages, but can also be produced by lymphocytes, mast cells, dendritic cells and fibroblasts (59-61). Its signalling can elicit numerous responses, from inflammation, proliferation and cell survival through to differentiation and apoptosis (62). The proinflammatory effect of soluble TNF is due to the activation of nuclear factor (NF)-κB following TNF/TNF receptor (TNF-RI)-1 binding. This results in upregulation of NF-κB induced proinflammatory cytokines including IL-6, IL-8, IL-18, chemokines, inducible nitric oxide synthase (iNOS), and of TNF itself (48, 63, 64).

The outcome of TNF signalling, be it apoptosis, cell survival, proliferation or inflammation, is not well understood. It is likely that cells receive all of these signals concomitantly and their fate is dictated by the balance of these signals as well as the presence of other cytokines and cellular stimuli (47).

There are two receptors for TNF: TNF-RI and TNF-RII. TNF-RI is a ubiquitous receptor and its activation results in a signalling cascade culminating in activation of caspase-3 (apoptosis), activator protein (AP)-1 (differentiation, proliferation and apoptosis), and NF-κB (inflammation) (47). TNF-RII is primarily expressed by lymphocytes and is involved with their activation and proliferation, but there is extensive crosstalk between TNF-RI and TNF-RII enabling synergy or inhibition of each other’s functions (65). TNF-RII has also been suggested to have a higher affinity for immature, membrane-bound TNF (66). This direct cell-cell signalling between membrane TNF and TNF-RII has anti-inflammatory effects as it causes induction of IL-10, an inhibitor of proinflammatory cytokine production and macrophage functions, and expansion of the anti-inflammatory regulatory T cell subset (67-69).

Dysregulated TNF production can be detrimental, leading to a state of chronic inflammation. The role of TNF in autoimmune diseases is demonstrated by the efficacy of TNF targeting drugs such as anti-TNF monoclonal antibody therapeutics and recombinant soluble TNF receptors in diseases including RA. Anti-TNF biologic drugs feature in the top 10 global block buster drugs, and the three most popular formulations (Humira, Remicade & Enbrel) reached sales of more than US$ 30 Billion in 2015 for rheumatic disease (70). However, anti-TNF therapies are only
effective in two-thirds of RA patients and this response is lost in 23-46% of those patients over time (71-75).

In this project, TNF gene expression was used as a control for direct gene transcription and TNF protein secretion was used as a control for cellular activation following stimulation.

1.1.4.2.2 IFNγ
Following the discovery of interferons (IFNs) by Isaacs and Lindenmann in 1957, in 1965 Wheelock reported IFN-like antiviral activity in supernatants of human leukocytes stimulated with the plant lectin phytohemagglutinin (76). As with the previously discovered IFNs, this inhibitor was macromolecular yet soluble but was less resistant to heat and acid than the other IFNs. It was not until 1972 that Falcoff was able to deduce that in fact there were two types of IFN: Type I induced by viruses and Type II by anti-lymphocyte antibodies (77). This was later supported in a landmark study by Youngner and Salvin who showed that Type II IFN was also induced by Bacille Calmette Guerin (BCG) (78).

There are seven IFN species in humans (IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-ν, IFN-γ), all of which are Type I with the exception of IFNγ which is the only Type II IFN (79). IFNγ is a 17kDa cytokine which binds its receptors as a dimer or tetramer (80, 81). The primary producers of IFNγ are NK cells, although cluster of differentiation (CD)-4 and CD8 T lymphocytes can also be primed to produce IFNγ (82). Transcription factors for IFNγ include cAMP response element binding protein (CREB), activating transcription factor (ATF), and activator protein 1 (AP-1) (83). NK cells constitutively produce IFNγ messenger (m)-RNA, allowing for immediate protein production following stimulation of NK cell receptors in the absence of inhibitory receptor signalling, or following stimulation with cytokines such as IL-12 (84).

The IFNγ receptor (IFNGR) complex is formed from two heterodimers of IFNGR chains: IFNGR1 and IFNGR2. These heterodimers are assembled prior to IFNγ dimer or tetramer binding, which occurs with two heterodimers concomitantly. This results in a complex formed of two/three IFNγ, two IFNGR1 chains and two IFNGR2 chains (85-87). Following receptor binding, the JAK/STAT intracellular signalling pathway is triggered, resulting in immunostimulatory and immunomodulatory effects such as macrophage activation, skewing T lymphocytes towards Th1 phenotype, increased phagocytosis and antigen presentation, and priming production of
proinflammatory cytokines and antimicrobial molecules including reactive oxygen species (ROS) and reactive nitrogen species (RNS) (88, 89).

Production of IFNγ is an essential component of viral, bacterial and tumour immune responses. However, aberrant production of IFNγ is implicated in a variety of autoimmune and chronic inflammatory diseases. Elevated IFNγ has been documented in RA synovial joints, and T lymphocytes (particularly CD8+ T cells) from RA patients display increased IFNγ production following activation (90-92). Paradoxically IFNγ deficiency can exacerbate RA disease. This is suggested to be due to activated T cells differentiating towards the highly proinflammatory T helper (Th)-17 subset in the absence of IFNγ to skew them towards Th1 phenotype (35, 93-96).

1.1.4.3 Extracellular vesicles

In 1983, within a week of one another, Harding and Stahl, and Pan and Johnstone published findings that transferrin receptors associated with small vesicles were expelled from reticulocytes into the extracellular space (97, 98). These vesicles, are the most recently discovered mechanism of intercellular signalling and research into them has exploded in the last 30 years.

Extracellular vesicles (EVs) are small phospholipid bilayer bound vesicles which are released from most cell types. EVs can be found in all biological fluids including saliva, blood serum and urine, and are divided into two classes: exosomes and microvesicles (MV)s (Figure 1.3). These groups are divided based on size and mechanism of formation: MVs are 100-1000nm in size and are formed through blebbing directly from the cell membrane, whilst exosomes are 40-100nm and are formed by invagination of endosomes to form multivesicular bodies (MVBs) which can fuse with the cell membrane to release their contents (99-101). This project focusses on exosomes rather than MVs.
Figure 1.3: Mechanisms of extracellular vesicle formation. Extracellular vesicles are divided into microvesicles and exosomes, based on the size and mechanism of formation. Microvesicles are larger than exosomes (100-1000nm) and are formed through blebbing of cell membranes. Exosomes are small (40-100nm) and are formed through invagination of endosome membranes to form multivesicular bodies (MVB). Exosomes are then released through MVB fusion with the cell membrane. Original in colour.
Exosomes are able to fulfil their role as intercellular signalling packages by displaying adhesion molecules, tetraspanin proteins and MHC molecules on their membrane, and by containing mRNAs, microRNAs and metabolic enzymes (102-106). Exosomes have important roles in immunity and can exert their functions through a number of means including cytokine secretion (107), direct membrane protein contact, such as MHC binding (108, 109), membrane fusion and deposition of exosome contents (102), and endocytosis (110). They are also trafficked to and retained in the draining lymph nodes where they are taken up by macrophages and B cells to disseminate complex messages (111).

Due to their role in immunity and inflammation, exosomes have been investigated in various autoimmune diseases, including RA. Synovial exosome numbers are increased in RA patients and correlate with disease severity (112, 113), and certain exosomal markers such as amyloid A and lymphatic vessel endothelial hyaluronic acid receptor-1 have also been found to correlate with RA disease activity (114). Synovial exosomes in RA can play pathogenic roles by causing proinflammatory activation of synovial fibroblasts (113, 115, 116). However it seems that the origin of exosomes and the activation state of their producing cell dictates their function; platelet-derived exosomes appear to be proinflammatory, whereas mesenchymal stem cell-derived exosome are anti-inflammatory (117). Furthermore, exosomes derived from IL-10 treated dendritic cells (DC), or genetically modified DC expressing Fas Ligand, are anti-inflammatory and immunomodulatory (118, 119).

1.1.5 Macrophages

Monocytes and macrophages are instrumental cells in recognising pathogen and generating an immune response. Monocytes are peripheral macrophage precursor cells and are systemic until they migrate into tissues to differentiate into macrophages, which are relatively long-lived cells. There are also macrophage subsets in several tissues, such as liver Kupffer cells, epidermal Langerhans cells, and microglia which self-renew from a common precursor established during embryonic development, independent of monocytes and HSCs (5, 6).

As almost all tissues contain resident macrophages, these cells are often the first immune cells to encounter invading pathogens (3). Recognising and attenuating pathogen invasion, whilst alerting other immune cells to attack are the principle roles of macrophages. In order to carry out these roles, macrophages use cell surface and intracellular receptors to identify pathogens, phagocytosis or essential secretory
molecules to kill or impede them, MHC to present pathogenic fragments to other cells, and proinflammatory cytokines to raise the alarm (3, 120).

Macrophages have traditionally been classed into two main phenotypes:

- **M1 macrophages** are proinflammatory and produce TNF, IL-1β, IL-6, IL-12, IL-23, ROS, RNS, upregulate MHC class II expression and skew T cells towards proinflammatory Th1 and Th17 phenotypes

- **M2 macrophages** are geared towards resolution of inflammation, pathogen clearance and wound healing (121). They produce anti-inflammatory cytokines IL-10, transforming growth factor (TGF)-β, upregulate scavenger and phagocytosis receptors, and produce chemokines to attract regulatory T cells and Th2 cells (122)

Polarisation towards the M1 phenotype follows stimulation by inflammatory cytokines such as IFNγ and TNF, pathogen-associated molecular patterns (PAMPs), and damage associated molecular patterns (DAMPs). The M2 phenotype is achieved following stimulation with effector cytokines associated with parasitic infections (IL-4), anti-inflammatory cytokines (IL-10), and apoptotic cells (123, 124). Recently, however, it has come to light that the M1/M2 paradigm oversimplifies the highly complex process of macrophage polarisation; rather than two distinct phenotypes, macrophages are a heterogeneous and plastic population which can reshape themselves in response to environmental changes (122, 125, 126).

Pattern recognition receptors (PRRs) are germline-encoded receptors which recognise and bind conserved molecular patterns, PAMPs and DAMPs. The concept of these receptors which function to link the innate and adaptive immune systems was first suggested by Janeway in 1989 (127). There are four types of PRRs, grouped according to location and function: free receptors in the serum (such as the mannose binding lectin of the complement system), membrane-bound phagocytic receptors (C-type lectin receptors), membrane-bound signalling receptors (Toll-like receptors), and cytoplasmic receptors (nucleotide oligomerisation receptors and RIG-1 like receptors) (128). The latter three groups are expressed by macrophages, enabling them to efficiently recognise and respond to pathogens.

Phagocytosis is an important mechanism of pathogen ingestion by specialised immune cells. There are three kinds of phagocytic cell in the immune system:
granulocytes (a term encompassing eosinophils, neutrophils, and basophils),
dendritic cells, and monocytes and macrophages. Phagocytosis is mediated by
phagocytic receptors such as the C-type lectin-like family, scavenger receptors, and
complement receptors. When bound to antigen, receptor clustering occurs which
causes the cell membrane to enclose around the antigen, eventually resulting in
internalisation of the antigen within a membrane-enclosed vesicle called a
phagosome. Acid proteases such as L-cathepsin help to degrade phagosomal
pathogens into peptides. Phagosomes can then fuse with MHC class II-containing
vesicles for antigenic presentation (outlined below), or with lysosomes to form
phagolysosomes which degrade all pathogenic material.

A variety of toxic molecules are produced which are required for pathogen killing
and degradation, including ROS. Superoxide (O$_{2}^-$), a species of ROS, is produced
by the enzyme NADPH oxidase (NOX), which assembles in the phagolysosome
membrane releasing O$_{2}^-$ into the phagolysosome for pathogen killing (129). NOX is
also located on macrophage cell membranes where it can rapidly produce large
volumes of O$_{2}^-$ for direct microbial killing in a process known as respiratory burst
[see 1.2.1].

The final role of macrophages during inflammation is as APCs. This is a role shared
with dendritic cells and also B cells. Dendritic cells are traditionally thought of as the
professional APCs, able to activate and differentiate naïve T cells, whilst
macrophages are primarily scavenger cells which can present antigen to previously
primed or memory T cells. However, more recent studies show that macrophages
can indeed activate naïve T cells, and depending on the macrophage activation
state, polarise them towards particular phenotypes (130). It has also been
suggested that the current nomenclature for defining macrophages, monocytes and
dendritic cells based on surface markers is insufficient, causing confusion and a
method based on cell ontogeny should be employed instead (131).

The process of antigen presentation occurs following macrophage stimulation by
pathogen binding, and cytokine stimulation (such as IFNγ) which causes
upregulation of MHC class II molecules. These are then packaged into the Golgi
and transported to the cell membrane in membrane-enclosed vesicles budded off
from the endoplasmic reticulum. On the way to the cell surface, these vesicles fuse
with phagosomes containing pathogenic peptides. These bind with MHC molecules
and are presented on the surface of macrophages to T cells.
1.1.6 Toll-like receptors

Discovery of the Toll-like receptors (TLRs) stemmed from research into the IL-1β receptor (IL-1R1). This receptor was cloned in 1988 with the aim of deciphering its intracellular signalling pathways, but the cytosolic motifs were not recognised, and appeared to be unique to IL-1R1 (132). Unexpectedly, in 1991 Gay and Keith found that the IL-1R1 cytosolic domain was homologous with the cytosolic domain of a protein called Toll in the fruit fly Drosophila melanogaster, at the time known only for its function in embryo polarisation (133, 134). In 1992 Heguy et al found that IL-1R1 and Toll share certain amino acids essential for NF-κB activation, and this section of the cytosolic domain was named the Toll Interleukin Receptor (TIR) domain (135). Then, in 1996, the lab of Jules Hoffman published findings that Toll mutations in D. melanogaster prevented effective defence against bacteria or fungi, therefore showing that the product of the Toll gene was involved in sensing and mounting responses against pathogenic microorganisms (136).

In 1997 Medzhitov, Preston-Hurlburt, and Janeway found the first human Toll homologue. In their landmark paper, they demonstrated that a constitutively active form in a human monocytic cell line (THP-1) induced NF-κB activation resulting in inflammatory cytokine production and expression of B7.1, the costimulatory molecule required for T cell activation. Finally, evidence of the link between the innate and adaptive immune systems had emerged (137). The following year, after a decade of searching, Bruce Beutler’s lab were able to identify TLR4 as the lipopolysaccharide (LPS) receptor by showing that TLR4 mutation renders mice resistant to endotoxic shock (138). In 2011, Hoffman and Beutler were jointly awarded The Nobel Prize in Physiology or Medicine from these discoveries.

The TLRs are an ancient and highly conserved family of PRRs, found in four animal phyla: Nematoda, Arthropoda, Echinodermata, and Chordata (139). There are 10 TLRs in humans (13 in mice), each recognising and responding to distinct PAMPs, such as LPS which is integral to the outer membrane of gram-negative bacteria. TLRs also have the capacity to bind and recognise host-derived DAMPs, such as host DNA/RNA, heat shock protein (Hsp)-60 and High mobility group box 1 (HMGB1) providing a link between TLRs and autoimmune disease [see 1.1.5.5] (140-144).
1.1.6.1 TLR expression and ligand binding

TLRs are single transmembrane proteins expressed on cell surface or endosomal membranes of innate immune cells such as macrophages and dendritic cells. The cell membrane TLRs: TLR1/2, TLR2/6, TLR4, and TLR5 bind bacterial PAMPs; whilst the endosomal TLRs: TLR3, TLR7, TLR8 and TLR9 bind viral PAMPs and nucleic acids (Figure 1.4, Table 1.1, (145). TLR2 and TLR4 are unique in that they can signal from both the cell membrane and endosome (146-149). The structure of TLRs consists of an extracellular domain which forms a 'C' shape due to a high density of leucine-rich repeat (LRR) motifs. As mentioned, they contain an intracellular TIR domain within the cytoplasmic tail, and this interacts with other TIR domain-containing proteins during intracellular signalling (150).

The extracellular LRR domain is the antigen binding site, both on the concave and convex sides. Each TLR binds distinct PAMPs: TLR2 heterodimerises with TLR1 and binds bacterial triacylated lipopeptides, or with TLR6 to bind bacterial diacylated lipopeptides; TLR3 binds viral double-stranded RNA; TLR4 binds LPS from gram-negative bacterial cell walls; TLR7 and TLR8 bind viral single-stranded RNA; and TLR9 binds hypomethylated CpG motifs, which are increased in bacterial and viral DNA compared with human DNA. The ligand of TLR10 is currently unknown.
Figure 1.4: Cellular location of the Toll-like receptors. The TLRs are distributed on the cell or endosomal membranes. Each TLR binds a specific molecular pattern: cell membrane TLRs bind bacterial patterns whereas endosomal TLRs bind nucleic acids. Following binding, TLRs homo- or hetero-dimerise which brings their TIR domains into contact. Five adaptor proteins enable intracellular signalling: MAL, MyD88, TRIF, TRAM and TICAM-1. TLR2 and TLR4 are able to signal from both the cell membrane and the endosome. Activation of TLR4 requires coactivator proteins MD2, CD14 and LBP. Original in colour.

Abbreviations: Myeloid differentiation primary-response protein 88 (MyD88), TIR domain-containing adaptor protein inducing IFNβ (TRIF), MyD88-adaptor-like protein (MAL), TRIF-related adaptor molecule (TRAM), TIR domain-containing adaptor molecule (TICAM)-1, LPS binding protein (LBP)
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<th>TLR</th>
<th>PAMP ligands</th>
<th>DAMP ligands</th>
</tr>
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<tbody>
<tr>
<td>TLR1/2</td>
<td>Lipomannans (mycobacteria)</td>
<td>Heat shock proteins</td>
</tr>
<tr>
<td>heterodimer</td>
<td>Lipoproteins</td>
<td>HMGB1</td>
</tr>
<tr>
<td>TLR2/6</td>
<td>Lipoteichoic acids (gram-positive bacteria)</td>
<td>Serum amyloid A</td>
</tr>
<tr>
<td>heterodimer</td>
<td>Cell wall β-glucans (bacteria and fungi)</td>
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</tr>
<tr>
<td></td>
<td>Zymosan (fungi)</td>
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<td>TLR3</td>
<td>Double-stranded (ds)RNA (viruses)</td>
<td>Self dsRNA</td>
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<tr>
<td>TLR4</td>
<td>LPS (gram-negative bacteria)</td>
<td>Heat shock proteins</td>
</tr>
<tr>
<td></td>
<td>Lipoteichoic acids (gram-positive bacteria)</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td></td>
<td>Envelope proteins (RSV, MMTV)</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HMGB1</td>
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<tr>
<td></td>
<td></td>
<td>Oxidised LDL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Serum amyloid A</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin (bacteria)</td>
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</tr>
<tr>
<td>TLR7</td>
<td>Single-stranded (ss)RNA (viruses)</td>
<td>Self ssRNA</td>
</tr>
<tr>
<td>TLR8</td>
<td>ssRNA (viruses)</td>
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</tr>
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<td>Self DNA</td>
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<tr>
<td>TLR10</td>
<td>Unknown</td>
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</tr>
</tbody>
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**Table 1.1: Toll-like receptors and their ligands.** Adapted from Murphy 2014 (3, 151). Respiratory syncytial virus (RSV), Mouse mammary tumour virus (MMTV), High mobility group box 1 (HMGB1), Low-density lipoprotein (LDL)
Following ligand binding, TLRs are activated only upon dimerisation or oligomerisation. The LRR domains encase the ligand and the cytoplasmic TIR domains can then interact, leading to downstream signalling (3, 150, 152). TLR4 is unique in that its activation requires formation of a complex comprising of coactivator proteins MD2, CD14 and LPS binding protein (LBP) (153-155).

Following dimerisation and TIR domain binding between the TLR monomers, intracellular TIR domain-containing adapter proteins propagate the signal. There are two main signalling pathways: myeloid differentiation primary-response protein 88 (MyD88) and TIR domain-containing adaptor protein inducing IFNβ (TRIF). All TLRs use the MyD88 pathway with the exception of TLR3 which only uses TRIF and TLR4 which can use both pathways. Adaptor molecules are used by many TLRs: TLR2 and TLR4 use MyD88-adaptor-like protein (MAL) to bind with MyD88, and TLR4 uses TRIF-related adaptor molecule (TRAM) to bind TRIF, whilst TLR3 uses TIR domain-containing adaptor molecule (TICAM)-1 to bind TRIF (156). The final adapter protein, sterile α- and armadillo-motif containing protein (SARM) interacts with and inhibits TRIF function (157). Traditionally, TLR3 and TLR4 were thought to be the only TLRs to use the TRIF/TRAM signalling pathway (158). However, more recently TLR1/2 was also found to use this pathway, and can activate TRIF in a MyD88 dependent mechanism (145, 159, 160).

Following activation of the TRIF/TRAM or MyD88/Mal pathways, complex intracellular signalling cascades occur, culminating in the activation of one of four transcription factors:

- **NF-κB**: A rapid-acting, phosphorylation-dependent transcription factor with over 150 target genes, predominantly involved in immune responses. Target genes include TNF, IFNγ and MHC molecules, and multiple coactivator proteins are required for transcription (161, 162)

- **AP-1**: AP-1 are a family of phosphorylation-dependent transcription factors, expression of which is co-regulated by members of the NF-κB family (163). Target genes include TNF, IL-6, TGFβ, and IL-10 (164)

- **CREB**: CREB is a phosphorylation-dependent transcription factor with both pro- and anti-inflammatory roles. CREB activity requires binding with the co-activators CREB binding protein (CBP) or p300, to transcribe its target genes. It competes for these co-activators with NF-κB, thus CREB activity and the amount of available CBP can limit NF-κB functions (165, 166). CREB target
genes include anti-inflammatory cytokine IL-10, as well as proinflammatory TNF (166)

- Interferon-regulatory factors (IRFs): The IRFs are a family of phosphorylation-dependent transcription factors (IRF1-9) which transcribe IFNα/β and are essential for anti-viral responses

1.1.6.2 TLRs and PKC
Protein kinase C (PKC) are a family of 9 phospholipid-dependent serine/threonine kinases which are an integral component of many intracellular signalling cascades. The isoforms are each transcribed from separate genes and can be divided into 3 subfamilies based on their activation requirements (167):

- Conventional isoforms require calcium, diacylglycerol, and phosphatidylserine, (PKC-α, -βI, -βII, and –γ)
- Novel isoforms require diacylglycerol and phosphatidylserine but are calcium-independent (PKC-δ, -ε, -η, and –θ)
- Atypical isoforms require only phosphatidylserine (PKC-ζ and λ/ι)

Interest in the connection between PKC and the TLRs began before TLR4 had even been discovered when several groups showed that PKC was involved in LPS-mediated production of proinflammatory cytokines such as TNF and IL-1β (168-170). Since then much research into the PKC isoforms and their links with TLR signalling has been undertaken [Table 1.2].
<table>
<thead>
<tr>
<th>PKC isoform</th>
<th>TLR</th>
<th>Model</th>
<th>Method of inhibition</th>
<th>Ref</th>
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<td>PBMC-derived monocytes</td>
<td>Calphostin C</td>
<td>(171)</td>
</tr>
<tr>
<td></td>
<td>TLR4</td>
<td>PBMC-derived monocytes</td>
<td>Rottlerin</td>
<td>(172)</td>
</tr>
<tr>
<td></td>
<td>TLR2, TLR4</td>
<td>Murine bone marrow-derived neutrophils</td>
<td>Gö6976, GF109203X</td>
<td>(173)</td>
</tr>
<tr>
<td></td>
<td>TLR3</td>
<td>PBMC-derived dendritic cells</td>
<td>Gö6976, siRNA</td>
<td>(174)</td>
</tr>
<tr>
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<td>TLR2</td>
<td>Murine bone marrow-derived dendritic cells</td>
<td>PKCa−/− mice</td>
<td>(175)</td>
</tr>
<tr>
<td>β</td>
<td>TLR4</td>
<td>PBMC-derived monocytes</td>
<td>Calphostin C</td>
<td>(171)</td>
</tr>
<tr>
<td></td>
<td>TLR4</td>
<td>Murine peritoneal macrophages</td>
<td>LY379196</td>
<td>(176)</td>
</tr>
<tr>
<td></td>
<td>TLR2, TLR4</td>
<td>Murine bone marrow-derived neutrophils</td>
<td>Gö6976, GF109203X</td>
<td>(173)</td>
</tr>
<tr>
<td>δ</td>
<td>TLR4</td>
<td>PBMC-derived monocytes</td>
<td>Calphostin C</td>
<td>(171)</td>
</tr>
<tr>
<td></td>
<td>TLR4</td>
<td>PBMC-derived monocytes</td>
<td>Rottlerin</td>
<td>(172)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RAW264.7 cells</td>
<td>shRNA</td>
<td>(177)</td>
</tr>
<tr>
<td>ε</td>
<td>TLR4</td>
<td>PBMC-derived monocytes</td>
<td>Calphostin C</td>
<td>(171)</td>
</tr>
<tr>
<td></td>
<td>TLR4</td>
<td>Murine embryonic fibroblasts</td>
<td>PKCe−/− mice</td>
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<td>Murine peritoneal macrophages</td>
<td>PKCe−/− mice</td>
<td>(179)</td>
</tr>
<tr>
<td></td>
<td>TLR4</td>
<td>Murine bone marrow-derived neutrophils</td>
<td>GF109203X, siRNA</td>
<td>(180)</td>
</tr>
<tr>
<td></td>
<td>TLR4</td>
<td>PBMC-derived dendritic cells</td>
<td>Inhibitory peptides</td>
<td>(181)</td>
</tr>
<tr>
<td>ζ</td>
<td>TLR2</td>
<td>PBMC-derived monocytes</td>
<td>Inhibitory peptides</td>
<td>(182)</td>
</tr>
<tr>
<td></td>
<td>TLR4</td>
<td>RAW264.7 cells</td>
<td>siRNA</td>
<td>(183)</td>
</tr>
</tbody>
</table>

Table 1.2: Protein kinase C isoforms involved in Toll-like receptor signalling.
Peripheral blood mononuclear cell (PBMC)
1.1.6.3 TLRs and IFNγ

As outlined in 1.1.4.2.2, IFNγ is an essential proinflammatory cytokine for both innate and adaptive immune responses. When cells are activated with TLR agonists in the presence of IFNγ, the effects of both stimuli synergise causing macrophage differentiation towards a proinflammatory phenotype, upregulation of proinflammatory cytokines and inflammatory lipid mediators (prostaglandins), T cell skewing towards Th1 and Th17 phenotypes, and upregulation of iNOS and nitric oxide production (184-187). Furthermore, IFNγ suppresses macrophage production of inflammation-resolving cytokines such as IL-10 (188), and prevents the establishment of LPS tolerance (189, 190), further accentuating the proinflammatory phenotype. LPS tolerance is a transient state that occurs following chronic low-level LPS exposure where TLR4 responses become reduced (191). LPS tolerance can be reversed by PKC activation (192, 193).

1.1.6.4 TLRs and exosomes

Stimulation of TLR3 or TLR4 induces exosome release (194-197). The resulting exosomes communicate complex signals to distal cells. Exosome contents, and therefore the resulting signals vary depending on the initial TLR agonist and can induce stronger epigenetic responses in distal cells than free TLR ligands (196). In a feedback loop, exosomes themselves stimulate TLR4, inducing proinflammatory effects (198, 199). The exact mechanisms of this TLR4 activation are unknown but are suggested to be due to microRNA (miRNA) transfer.

MiRNAs are short RNA sequences of around 22 nucleotides in length, which bend back on themselves to form hairpins. These bind with the 3’ untranslated region of mRNA sequences, preventing translation (200-203). MiRNAs are ‘fine-tuners’ of the immune response, and have been found to regulate TLR functions through three mechanisms:

1. Targeting and inhibiting components of TLR signalling pathways
2. Regulation of miRNA expression through TLR activation, forming a negative feedback loop with (1)
3. Direct binding and activation of RNA sensing TLRs

Three miRNAs in particular are important in TLR signalling: miR-146a, miR-155, and miR-21 (200). Inflammatory responses to TLR4 stimulation with LPS are promoted by miR-155 and inhibited by miR-146a and miR-21, all of which are exosomal miRNAs (200, 204-210).
1.1.6.5 TLRs and RA

In recent years the TLRs have become potential drug targets for RA. This is due to their hierarchical role in inflammation (as their activation is required for other proinflammatory processes to occur), and their activation by both PAMPs and DAMPs which opens various possibilities of how inflammation is induced and perpetuated. DAMPs are endogenous molecules released from stressed or necrotic cells. These molecules have been found to bind and activate the TLRs either directly or indirectly through adaptor proteins [Table 1.1]. Synovial fluid from RA patient joints contains many potential DAMPs as a result of the highly proinflammatory and stressful environment (1). DAMPs such as Hsp96 and Hsp22 are increased in RA synovial fluid, and activate TLR2 and TLR4 respectively (211, 212). Particular subsets of RA patients have also been found to have TLR4 driven disease, potentially through TLR4 activation by citrullinated fibrinogen (213, 214). In recent years an anti-TLR4 biologic, NI-0101, was developed by NovImmune and Genentech and is currently in Phase II clinical trials for use in RA (215, 216).

Expression of many TLRs increases in synovial tissues, even in the early stages of RA: TLR2, TLR3, TLR4, and TLR7 expression increase in RA synovial fibroblasts (217-219), whilst sites of bone and cartilage erosion display increased TLR2 (220). Macrophage and endothelial cell TLR5 and TLR7 expression also correlates with disease activity score (DAS28) and joint TNF (221-224). Ligation of TLR5 was found to promote angiogenesis, and myeloid cell infiltration and differentiation into mature osteoclasts in RA joints; whilst TLR7 ligation by the miRNA miR-let-7b skewed naive myeloid cells towards the M1 macrophage phenotype (225). Furthermore, single-stranded RNA present in RA synovial fluid can act as an endogenous ligand for TLR7, causing increased TNF production (224). Synovial fibroblasts and macrophages from RA patients also exhibit increased sensitivity to TLR activation, resulting in increased production of proinflammatory cytokines, chemokines and other chemical mediators (219, 226, 227).

1.1.7 NF-κB

In 1986, Ranjan Sen and David Baltimore identified a novel DNA binding factor. They decided to call this nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) as it was a nuclear factor that selectively bound the immunoglobulin κ enhancer and was found in extracts of B-cell tumours but not in other cell lines (228). They then went on to show that NF-κB expression was induced by LPS treatment. Furthermore, this expression did not require de novo protein synthesis, suggesting
pre-formed but inhibited NF-κB complexes in the cell (229). Two years later, it became evident that NF-κB expression was not restricted to B cells (230), and we now know that it is expressed in almost all cell types and is highly conserved (231, 232).

NF-κB is a family of five related proteins, RelA, c-Rel, RelB, p50, and p52, which work together as homodimers or heterodimers to yield up to 15 different NF-κB complexes (232, 233). These proteins all have high sequence homology in their Rel homology region (RHR), which is important for dimerisation, DNA binding, and inhibitor binding (233). The multiple NF-κB complexes allow increased specificity and control of target gene transcription as different dimer combinations control distinct types of genes. Many other regulatory mechanisms regulate NF-κB activity and transcription of its target genes, including competition with other transcription factors and regulation of NF-κB itself (234).

Under resting conditions, NF-κB dimers are held inactive in the cytoplasm by the inhibitory proteins IκB (inhibitors of κB). These proteins contain five to seven tandem ankyrin repeats which bind sections of the RHR to prevent NF-κB DNA binding and nuclear localisation (235). This mechanism of inhibition allows for immediate NF-κB response following stimulation, which can occur through a variety of mechanisms including PRR receptor binding, cytokine receptor binding, and oxidative stress. Two signalling pathways are used for NF-κB activation, the canonical and alternative:

- **The canonical pathway** is the predominant pathway and allows rapid activation of NF-κB. This pathway utilises the IκB kinase (IKK) complex which is comprised of two catalytic subunits, IKKa, IKKβ, and a regulatory subunit, NF-κB essential modifier (NEMO). Following activation, IKK phosphorylates IκB, which is then ubiquitinated by a cullin-RING ubiquitin ligase family SCFβTRCP complex, which specifically recognises and degrades phosphorylated IκB, allowing NF-κB to move into the nucleus (Figure 1.5) (236)

- **The alternative pathway** mainly occurs in B cells in response to a subset of the TNF receptor family, and has a slower onset and longer duration than the canonical pathway (237, 238). Following stimulation, NF-κB-inducing kinase activates IKKα which goes on to phosphorylate p100 (the p52 precursor), leading to degradation of its IκB-like domain and production of p52 which is
then able to traffic into the nucleus with its binding partner RelB (237, 239, 240)

NF-κB is also a redox-sensitive transcription factor. Direct oxidation of Cys-62 in the p50 subunit of its active site can inhibit NF-κB DNA binding (241-244). This cysteine is also susceptible to S-glutathionylation and S-nitrosylation (245-247). Oxidised NF-κB can be returned to its reduced, active form by the redox enzyme thioredoxin (TXN) (248).

NF-κB is a central player in immunity: it is activated by both innate and adaptive immune systems and transcribes genes essential for coordinated immune responses including cytokines, chemokines, receptors and proteins involved in antigen presentation (249). It also has key roles in autophagy, senescence, cell differentiation, survival, and proliferation. This is an essential transcription factor and mutations in regulatory proteins or NF-κB itself can cause immune disease or even embryonic fatality (234). NF-κB can transcribe hundreds of target genes through recognition of the κB motif in their promoter or enhancer regions (228). The κB motif is a short DNA sequence with some variability: 5'-GGGRNWYYCC-3' (N, any base; R, purine; W, adenine or thymine; Y, pyrimidine) (234, 250). NF-κB transcription targets include a vast array of genes essential for immunity including proinflammatory cytokines (such as TNF and pre-IL-1), chemokines and their modulators, antigen presentation proteins, receptors and transcription factors (249).

Cessation of NF-κB signalling can occur through many negative feedback loops. The simplest is that IκB genes contain κB motifs, enabling upregulation of IκB following NF-κB activation (251). The upregulated IκB can then displace NF-κB from its target genes in the nucleus and shuttle it back into the cytoplasm (231, 252). Furthermore, promoter-bound NF-κB can be directly degraded by the proteasome when signalling is required to stop (253). Post-translational modifications and regulation of individual NF-κB subunits are also employed to allow for signal-specific responses (252, 254).
Figure 1.5: The NF-κB canonical pathway. Adapted from Rahman 2011 (255). The IkB kinase (IKK) complex is comprised of two catalytic subunits, IKKα, IKKβ, and a regulatory subunit, NF-κB essential modifier (NEMO). Following activation, IKK phosphorylates IkB, which is degraded by a cullin-RING ubiquitin ligase (CRL) family SCFβTRCP complex. NF-κB can then translocate into the nucleus where it can recognise and bind κB sequences in its target gene promoter regions. Original in colour.
Its central role in inflammation has led NF-κB and its signalling pathways to become therapeutic targets for immune disease and cancer, with over 700 candidate therapeutics in development in 2006 (256-258). The multiple myeloma drug raloxifene exerts some of its functions through NF-κB inhibition as well as via estrogen receptor modulation (259). However, the sheer complexity of NF-κB signalling ensures that finding successful drug candidates will not be an easy task: unexpected and counterintuitive side effects have been found with NF-κB inhibitor candidates, including neuropathy, nephrotoxicity, and even relapse of more aggressive cancers (260). To be successful, NF-κB inhibition would need to be highly targeted to individual subunits within specific signalling pathways. At this stage a deeper understanding of the NF-κB subsets, their roles and regulation is required for this to be possible (234).

1.1.8 Nrf2

In 1994 Moi et al used expression cloning with an oligonucleotide containing the binding motif of the transcription factor NF-E2 DNA to find closely related proteins. They found that two of the isolated clones had a remarkable similarity to the NF-E2 transcription factor: NF-E2 related factor 1 (Nrf1) which had already been recently discovered, and a novel transcription factor which they named Nrf2 (261, 262). In 1996 Venugopal and Jaiswal found that Nrf1 and Nrf2 bound and induced expression of genes containing the Antioxidant Response Element (ARE), a cis-acting element in the promoter region of redox-sensitive genes (263). Later studies confirmed that Nrf2 is the main transcription factor for ARE-containing genes, which include many antioxidant, detoxification and stress-response enzymes such as NAD(P)H:quinone oxidoreductase (NQO)-1, glutathione S-transferase (GST), heme-oxygenase (HMOX)-1, thioredoxin reductase (TXNRD)-1, peroxiredoxin (PRDX)-1 and Nrf2 itself (263-268). These enzymes reduce electrophiles and free radicals into less toxic molecules (269). Due to its roles in regulating these gene families, Nrf2 has earnt the title of master regulator of the antioxidant response [Table 1.3].
<table>
<thead>
<tr>
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<th>Symbol</th>
<th>Name</th>
</tr>
</thead>
<tbody>
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<td>GCLC</td>
<td>glutamate-cysteine ligase, catalytic subunit</td>
</tr>
<tr>
<td></td>
<td>GCLM</td>
<td>glutamate-cysteine ligase, modifier subunit</td>
</tr>
<tr>
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<tr>
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<td></td>
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<td>SLC7A11</td>
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<td></td>
<td>TKT</td>
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<tr>
<td></td>
<td>UGDH</td>
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</table>

Table 1.3: Nrf2 target genes with antioxidant functions. Adapted from Hayes and Dinkova-Kostova 2014 (270). GSH = glutathione, TXN = thioredoxin, NADPH = Nicotinamide adenine dinucleotide phosphate
Nrf2 is important for regulating and orchestrating cellular redox homeostasis, with important cytoprotective roles in response to oxidative and electrophilic stress (271). Nrf2 is a ubiquitous transcription factor encoded by the NFE2L2 gene and is highly conserved, with orthologues described in zebrafish and nematodes (261, 272, 273). Nrf2 is a member of the Cap 'N' Collar subfamily of basic leucine zipper transcription factors, alongside Nrf1, Nrf3, and the transcriptional repressors Bach1 and Bach2 (274). It is a 605 amino acid long protein, containing seven highly conserved Nrf2-ECH homology (Neh) domains (271). In the nucleus, Nrf2 forms a heterodimer with members of the small Maf (musculoaponeurotic fibrosarcoma oncogene homolog) protein family through interactions of the Neh1 domain, facilitating binding to its target gene ARE sequences (275). To effectively transcribe its target genes, Nrf2 recruits transcriptional machinery, including co-activators such as receptor-associated coactivator (RAC3) (276).

Under resting conditions, Nrf2 activity is inhibited by the Kelch ECH-associating protein (KEAP)-1 homodimer, which sequesters Nrf2 in the cytoplasm via interactions with the actin cytoskeleton (277). KEAP1 is also an adapter protein for cullin (Cul)-3 which maintains low levels of cellular Nrf2 protein by ubiquitinating it at two degradation sites in the Neh2 domain, thus targeting it for proteasomal degradation (278-282). Nrf2 also contains two degradation sites in the Neh6 domain, which are not recognised by Cul3 and are redox-independent, but can be phosphorylated by glycogen synthase kinase (GSK)-3β, allowing subsequent ubiquitination by the E3 ubiquitin ligase β-TrCP and proteasomal degradation (283-285). Nrf2 cellular expression is therefore tightly controlled through a number of mechanisms, further demonstrating its cellular importance.

Under conditions of cellular stress, KEAP1 releases Nrf2 which can then localise to the nucleus and transcribe its target genes (Figure 1.6). KEAP1 is a highly redox-sensitive protein due to the 27 cysteine residues within its structure (286). These cysteines appear to have specific functions, with certain chemicals or electrophiles targeting and modifying specific cysteine residues to elicit different effects (287-292). The thiol reactivity of different electrophiles also corresponds with their ability to activate Nrf2 (288). KEAP1 thiols can be modified through changes of their redox state or alkylation, causing changes in the tertiary protein structure and enabling Nrf2 release (288, 293). Furthermore, KEAP1 cysteines can also be modified through S-nitrosylation and glutathionylation (292, 294, 295). Nrf2 can also dissociate itself from KEAP1 via PKC-mediated phosphorylation of its Serine40
residue, it can be activated by sulforaphane (derived from cruciferous vegetables) without the need for dissociation from KEAP1, and can be mediated by miRNAs, demonstrating further complexity of control (296-299).
Figure 1.6: Mechanisms of Nrf2 activation. Adapted from Bellezza 2010 (300).
Under resting conditions Nrf2 is held in the cytoplasm by KEAP1 which interacts with the actin cytoskeleton. Nrf2 is then ubiquitinated by Cul3 and degraded by the proteasome. Following oxidative stress or external stimuli Nrf2 is released from KEAP1, either through oxidation of KEAP1, or through direct phosphorylation of Nrf2 by PKC. Nrf2 can then localise to the nucleus and bind to the ARE sequence of its target genes. Original in colour. Abbreviations: Kelch ECH-associating protein (KEAP)-1, cullin-3 (Cul3), NF-E2-related factor 2 (Nrf2), protein kinase C (PKC), Antioxidant Response Element (ARE), peroxiredoxin (PRDX, thioredoxin reductase (TXNRD), sulfiredoxin (SRX)
Knockout of Nrf2 in mice is not embryonic fatal, as with Nrf1 knockout, and haematopoiesis, growth, and development are unaffected (301). However, Nrf2−/− mice are more susceptible to cellular stressors such as cigarette smoke, allergen, and antigen challenge (302-304). Knockout experiments have also revealed that Nrf2 is a major regulator of cellular lipid disposition in the liver (305). Knockout of KEAP1 is fatal postnatally due to overactivation of Nrf2 causing hyperkeratosis in the oesophagus and forestomach (306).

As such a central player in immune and antioxidant responses, Nrf2 is implicated in a wide variety of diseases, including cardiovascular disease, autoimmunity, cancer and neurological disease, leading it to be identified as a potential drug target (298, 307-312). Many Nrf2 activators have been identified, but due to lack of specificity, few make it to clinical trial. The most successful to date has been dimethyl fumarate which is approved for the treatment of multiple sclerosis (313). Another synthetic Nrf2 activator, [2-cyano-3, 12-dioxo-oleana-1,9(11)-dien-28-oic acid, methyl ester] (CDDO-Me), developed by Reata Pharmaceuticals, and sulforaphane, a compound extracted from broccoli, are both currently in clinical trials for a wide range of conditions (314). More Nrf2 activators and inhibitors are expected in the coming years (315).

1.1.9 Nrf2 and NF-κB cross-signalling
Macrophages use both Nrf2 and NF-κB transcription factors to orchestrate inflammatory responses. NF-κB is the principle proinflammatory transcription factor, whilst Nrf2 is an anti-inflammatory transcription factor due to its antioxidant roles. Following inflammatory or danger signals, NF-κB and Nrf2 are activated to upregulate genes for the required response. These two transcription factors have been found to co-regulate one another through a number of different mechanisms:

- NF-κB can directly upregulate gene expression of Nrf2 as the NFE2L2 gene contains an NF-κB binding site (316)
- The Nrf2 inhibitor KEAP1 can bind and enable ubiquitination of the NF-κB activator protein IKKβ, thus inhibiting activation of NF-κB (317, 318)
- The Rho GTPase RAC1 can activate NF-κB, a process which is downregulated by Nrf2 (319)
- NF-κB induces expression of inflammatory mediators such as cyclooxygenase (COX)-2 which produce electrophilic oxo-derivative molecules, which regulate Nrf2 activity (320)
NF-κB can repress Nrf2 activity by competing for the coactivator protein CBP (321)

Taken together, these findings show that cross-regulation of NF-κB and Nrf2 is highly complex, and there are likely many as yet unknown mechanisms involved. A fine balance is required to ensure that inflammatory responses are appropriate to the situation and that the combination of signals encountered are integrated to deliver a context-specific response (322). It is likely that each of the mechanisms above are activated dependent on circumstances, and are probably separated by kinetics: NF-κB activation is immediate whereas Nrf2 is delayed (319, 323, 324).
1.2 Reduction-Oxidation (Redox) Biology

In the 18th century, Georg Ernst Stahl found that during combustion, substances lose mass. He attributed this to the loss of phlogiston, a theoretical incombustible substance, to the air. It was not until 1777 when Antoine-Laurent Lavoisier, who discovered oxygen, was able to disprove Stahl’s theory of phlogiston and show that the loss of mass was due to loss of oxygen as a gas. The term ‘reduction’ was therefore used to describe the loss of oxygen, and ‘oxidation’ to describe compounds arising from the gain of oxygen, as with metal oxides (325). Following the discovery of the electron in 1896, by J. J. Thomson, the terms oxidation and reduction were expanded to encompass the atomic loss or gain of electrons.

Redox reactions occur where reduction and oxidation occur simultaneously and cannot exist independently, such as acid-base reactions. These types of reactions are essential in molecular biology for example during cellular respiration or redox signalling as they can cause changes in structure, function, and activity of different proteins and molecules.

1.2.1 Reactive oxygen species

Oxygen (O$_2$) is a peculiar gas as it contains two unpaired electrons on separate atomic orbitals, but with the same spin direction. This property restricts oxygen reactions as it can only bind with atoms that have electron spin directions which complement its own. To circumvent this spin restriction electrons are added to oxygen one at a time, which results in the production of oxygen intermediates during its reduction into water (326). These are collectively known as ROS and comprise superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (•OH) and hydroxide ion (OH$^-$). The reactivity of ROS and their propensity to gain electrons (i.e. become reduced) means that they are strong oxidants and play important roles in redox biology.

Oxidative stress is a condition in biological systems first described in 1985 by the grandfather of the topic, Helmut Sies, as “a disturbance in the pro-oxidant-antioxidant balance in favour of the former” (327). Following the discovery of redox signalling, the term oxidative stress is generally replaced by ‘redox homeostasis’ (328-330). However, it is still used to describe conditions of abnormally high ROS, where biological damage can occur such as aberrant oxidation of lipids, protein or DNA.
In addition to ROS, two other elements are also important in redox biology through forming reactive species; RNS and reactive sulphur species (RSS) [Table 1.4].
### Table 1.4: Reactive species. Adapted from Sies et al 2017 (330)
Exogenous or endogenous sources can contribute to ROS production. Cellular exposure to environmental factors such as UV light, tobacco smoke or pesticides can all result in ROS generation (331). Inside the cell, several different systems are in place to generate ROS. The primary ROS species is $\text{O}_2^{-\cdot}$, which is the first to be produced through the reduction of $\text{O}_2$. Superoxide can then be further reduced into other ROS species through the actions of biomolecules and reactive metals. Different cellular compartments have different preferences for ROS generation, and also for optimal ROS concentrations. There are many intracellular ROS sources, which can either produce ROS intentionally or as a by-product of their primary function:

- **Mitochondrial electron transport chain** – During respiration in all cells the mitochondrial electron transport chain reduces $\text{O}_2$ into $\text{H}_2\text{O}$ to enable adenosine triphosphate (ATP) synthesis. Oxygen intermediates are formed during this process, and in certain mitochondrial compartments (primarily complex I and complex III), $\text{O}_2^{-\cdot}$ is generated as a by-product (332, 333).

- **NOX** – As mentioned in section 1.1.4, phagocytic cells generate $\text{O}_2^{-\cdot}$ for pathogen killing in the phagolysosome and during respiratory burst. Electrons are transferred from NADPH to $\text{O}_2$ to generate $\text{O}_2^{-\cdot}$ by the NOX2 enzyme complex which contains a flavin and haem catalytic subunit [Formula 1].

\[
\text{Formula 1: } 2\text{O}_2 + \text{NADPH} \rightarrow 2\text{O}_2^{-\cdot} + \text{NADP} + \text{H}^+
\]

NOX2 is comprised of two subunits, gp91phox (β subunit) and p22phox (α subunit) which are integral membrane proteins. Upon PRR stimulation of the cell a complex of three cytosolic proteins, p40phox, p47phox, and p67phox, is phosphorylated and traffics to NOX2 in the membrane (334). Two cytosolic guanine nucleotide-binding proteins, Rac2 and Rap1A, also traffic to the membrane, and are required for activation of the NOX2 complex (335). Rapid $\text{O}_2^{-\cdot}$ production then occurs as respiratory burst, and upon phagocytosis the NOX2 containing cell membrane is internalised to become the interior membrane of the phagolysosome, allowing pathogen killing (336). The importance of this method of $\text{O}_2^{-\cdot}$ production for pathogen killing is demonstrated by the genetic disease chronic granulomatous disease (CGD). Patients with CGD are more susceptible to infections due to defective NOX2 or associated subunits and therefore impaired $\text{O}_2^{-\cdot}$ production (337-339).
Although NOX2 is unique to phagocytes, other cells express different NOX isoforms to purposely produce O$_2^-$ for redox signalling (340-342).

- **Xanthine oxidase** – functions in the degradation of purines: converting hypoxanthine to xanthine, and then xanthine to uric acid. A by-product of this reaction is superoxide (O$_2^-$).
- **Nitric oxide synthase** – produce O$_2^-$ as an essential step in nitric oxide synthesis (343)
- **Cyclooxygenases** – produce O$_2^-$ as a by-product of prostanoid formation (344)
- **Lipoxygenases** – produce ROS during catalysis of polyunsaturated fatty acids into fatty acid hydroperoxide (345)

Superoxide dismutase (SOD) is the enzyme responsible for converting O$_2^-$ into H$_2$O$_2$ [Formula 2]. As H$_2$O$_2$ is more stable than other ROS it can travel further from its generation site, and is one of the most important transcription-independent signalling molecules, alongside Ca$^{2+}$ and ATP (346, 347). H$_2$O$_2$ is able to diffuse across cell membranes, allowing immediate communication of both intra- and extracellular redox signals.

Formula 2:  
\[ O_2^-( + e^-) \rightarrow O_2^{2-}( + e^-) \rightarrow H_2O_2( + e^-) \rightarrow HO'( + e^-) \rightarrow H_2O \]

Multiple enzyme families including peroxiredoxins (PRDX), catalase and glutathione peroxidases (GPX) are also involved with peroxide scavenging, including H$_2$O$_2$ reduction [Formula 2].

1.2.2 Reactive nitrogen species

Nitric oxide (NO), a small, reactive molecule which is important in signalling and has many physiological roles ranging from regulating vascular tone to immune responses (348-350). It is produced during the NADPH-dependent oxidation of L-arginine into L-citrulline by Nitric Oxide Synthase (NOS) enzymes. There are three NOS isotypes: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). Both nNOS and eNOS are constitutively expressed in their respective tissues and their production of NO is regulated by levels of intracellular calcium (351, 352). These isotypes produce low concentrations of NO which is considered anti-inflammatory, causing reduced expression of adhesion molecules (353), and inhibition of NF-κB binding to target genes (354). The third isoform, iNOS, can be expressed in any cell
type and is upregulated via NF-κB in response to proinflammatory stimuli, such as LPS or proinflammatory cytokines like IFNγ (355). iNOS produces NO in much larger quantities and for a longer duration than the other two isoforms (356). This high concentration NO production can have proinflammatory and microbicidal effects, but can also activate the antioxidant transcription factor Nrf2 (357, 358).

NO is an unstable molecule which is rapidly oxidised into more stable nitrites (NO$_2^-$) and nitrates (NO$_3^-$) which act as a storage pool for NO (359-361). Conversely, during inflammation O$_2^•$ is produced, which can rapidly react with NO to form the peroxynitrite radical (ONOO$^•$), a powerful oxidising and nitrating agent capable of modifying many proteins and molecules, and essential for the microbicidal activity of NO (362-364). Transition metals and O$_2$ can also react with NO to produce further RNS (365).

1.2.3 Reactive sulphur species

The most recently discovered yet arguably most important reactive species are the RSS (366). As this is such a new field, the nomenclature of RSS has not been definitively decided. Currently, the term RSS is used to describe both organic and inorganic reactive sulphur-containing molecules, outlined in Table 1.4 (366, 367). Important questions also currently remain unanswered: are RSS species created as a result of ROS/RNS, and if not which RSS species are capable of initiating the redox signal and which are simply signal conveyers (366, 367). Inorganic RSS species are similar to ROS species likely due to the similar atomic structure of sulphur and oxygen with six valent electrons. Sequential oxidation of hydrogen sulphide (H$_2$S) mirrors the products formed through sequential oxidation of H$_2$O [Formula 3] (368, 369):

\[
\text{Formula 3: } \text{S}_2( + e^-) \rightarrow \text{S}_2^•( + e^-) \rightarrow \text{H}_2\text{S}_2( + e^-) \rightarrow \text{HS}^•( + e^-) \rightarrow \text{H}_2\text{S}
\]

Sulphur is more stable than oxygen when bound with itself, and so can form polysulfides (H$_2$S$_n$) (370). Furthermore, unlike H$_2$O, H$_2$S can act as a reactive species. The realisation that H$_2$S, a gas known for its putrid smell and toxicity, was physiologically important was a surprise for the scientific community (366). Although it had been known since the 1940s that H$_2$S was produced in mammalian cells it was dismissed as a toxic by-product and its role in redox signalling did not come to light until the 1990s (371). Enzymes of the transsulfuration pathway generate cysteine from dietary methionine and serine, and in alternative reactions can also generate mammalian H$_2$S (372-375). The main pathway for H$_2$S consumption is via
the mitochondrial electron transport chain (376), though it can also react with reduced oxygen or nitrogen species, transition metals and with oxidized thiol products (371, 377). Functions of \( \text{H}_2\text{S} \) and its derivatives include control of cellular redox homeostasis, signalling, metabolism, and mitochondrial function (378).

1.2.4 Cysteine and the free thiol

Previously considered as passive recipients of redox modifications, proteins with sulphur-containing functional groups such as cysteines are now considered reactive stressors in their own right (organic RSS) (366, 379-381). This change in thinking was triggered by the discovery that the functional cysteine of PRDX, an enzyme which reduces peroxides, could be over-oxidised to a non-functional sulfinic acid (382), and then returned to a functional reduced state by another enzyme, sulfiredoxin (SRX) (383). These novel findings demonstrated that redox was being used to regulate PRDX enzyme activity and in doing so could regulate the amount of peroxide in the intracellular environment (384, 385). Thereafter, research into thiol redox biology exploded, finding that cysteine containing redox enzymes are likely to be the central regulators in controlling cellular responses to oxidative stress via redox signalling (366, 386-389).

Cysteine is one of the least abundant amino acids, yet certain cysteine motifs are highly expressed in almost all life forms, indicating its importance throughout evolution. Cysteine and methionine are the only two sulphur containing amino acids, but methionine is much less reactive than cysteine as its sulphur is arranged in a thioether form (R-S-R) as opposed to the free thiol of cysteine (R-SH) (390). The free thiol enables cysteine to undergo a wide variety of redox modifications due to its nucleophilicity and redox sensitivity (390, 391). These properties make cysteine a useful amino acid for regulation of protein functions including enzyme catalysis, transcriptional regulation, and protein folding and structure (392).

The reactivity of cysteine free thiol allows it to be ionised (through deprotonation), yielding a highly reactive thiolate (R-S⁻); it can also be oxidised to yield a disulphide bond (R-S-S-R), or into higher oxidation states such as sulfenic acid (R-SOH), sulfinic acid (R-SO₂H), or sulphonic acid (R-SO₃H) (390). Thiols can also be S-glutathionylated (R-S-S-G) following attack by another biological thiol, glutathione (GSH), or S-nitrosated (R-SNO) following attack by NO⁻ or ONOO⁻ (Figure 1.7) (389, 393). What makes thiol modifications an effective signalling mechanism is that for the most part they are reversible, either by molecular attack or enzymatic action.
Figure 1.7: Redox modifications of the cysteine free thiol.

The free thiol residue of the cysteine amino acid is highly nucleophilic and redox sensitive. This enables numerous redox modifications including deprotonation, oxidation, glutathionylation and nitrosation.
1.2.5 The reactive species interactome

A growing understanding of the reactive species, their role in thiol modifications, and the wider impact of those roles led to the coining of the term ‘reactive species interactome’ (RSI) by Miriam Cortese-Krott and Peter Nagy in 2017 (378). The authors propose that the RSI can sense multiple cellular stressors and adjust metabolic needs accordingly through activation of signalling pathways, thus ensuring the organism can respond to environmental change and stay fit for purpose.

The complex chemistry of reactive species and their ability to modify thiols allows cellular responses to be fine-tuned. For example, in addition to the numerous species of ROS, RNS, and RSS, these molecules can interact with each other to form further species: a prime example being NO' reacting with O2- to form ONOO-. RNS and RSS can also react to form products such as HSNO/SNO- and SSNO- (377, 394, 395), and RSS can be oxidised by ROS. The product of these interactions can have greatly differing properties to the parent molecules: highly reducing (RSSH, O2-) or highly oxidizing (HO•, NO2); highly electrophilic (RSOH, H2O2, HNO) or highly nucleophilic (RSSH), and good hydrogen atom donors (HNO, RSSH) or potent hydrogen atom abstractors (HO-, NO2) (378). To ensure that the correct reactive species is generated, spatiotemporal and kinetic controls are employed. For example, to create the very potent ONOO-, O2- and NO', both highly reactive molecules with short half-lives must be generated in the same location and at the same time and rate (396). Regulation of reaction kinetics and substrate levels is the role of oxidoreductase enzymes.

Functions of the RSI are varied and depend on the reactive species and the functional cysteine-containing target protein. Functions of protein kinases and phosphatases, ion channels, transporters, and enzymes can be immediately regulated for short periods of time through modification of cysteine thiols (378). Furthermore, the RSI is able to activate/inactivate redox-sensitive transcription factors such as Nrf2/KEAP1 and NF-κB, demonstrating a significant role in inflammation; particularly as the effects of these transcription factors include ROS/RNS generation and antioxidant expression (241, 248, 286, 397).
1.3 Oxidoreductase enzymes

Major regulators of the RSI are a group of redox enzymes known as the oxidoreductases. Through controlling reaction substrates and kinetics (such as the availability of O$_2^-$ or H$_2$O$_2$), and by directly regulating the redox state of cysteines (by the TXN system), oxidoreductases are able to influence the overall outcome of the RSI (378, 398). This project examines, in particular, a group of oxidoreductases called protein thiol-disulfide oxidoreductases (PDOR). These enzymes catalyse protein thiol-disulphide exchange reactions and therefore regulate protein redox state. Three families of PDOR are the main focus of this project: PRDX, TXN and TXN reductase (TXNRD1). The key conserved structure of these enzymes is the thioredoxin fold (399, 400). The thioredoxin fold consists of a central four-stranded $\beta$-sheet surrounded by three $\alpha$-helices (Figure 1.8). A cis-proline residue is located before $\beta$3, and the active site motif, Cys-X-X-Cys, is located on the loop connecting $\beta$1 and $\alpha$1 ($X$ denotes any amino acid) (401-403). Thioredoxin fold-like proteins use their active site cysteine residues to carry out thiol-disulphide exchange reactions. The two intervening amino acids of the active site dictate the redox potential and therefore have a strong influence on the catalytic site function (404).
Figure 1.8: One molecule of the human protein thioredoxin, a representative of the thioredoxin protein fold class. Image source: Wikipedia, image licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license. The thioredoxin fold consists of a central four-stranded β-sheet surrounded by three α-helices. Original in colour.
1.3.1 Thioredoxin

In 1964, Laurent et al were the first to isolate and characterise TXN and TXNRD from *Escherichia coli* (405). TXN was found to transfer electrons to ribonucleotide reductase (RNR), an essential enzyme which forms the deoxyribonucleotide precursors of DNA. Oxidised TXN could then be returned to its reduced form by TXNRD using NADPH as an electron donor. The human form of TXN was identified in 1974 by Blomback et al (406). The structure of TXN was elucidated in *E.Coli* in 1975 by Holmgren (401), and in humans in 1991 by Forman-Kay et al (407).

TXN occur in two forms: TXN1 (cytosolic) and TXN2 (mitochondrial) (408). Both enzymes are small, 12kDa oxidoreductases which are very highly conserved, from archaea and bacteria to humans (399). The TXN fold of TXN1 contains an additional α strand and β sheet compared to the basic structure, and the active site residues are Cys32-Gly-Pro-Cys35. Within this active site, the N-terminal cysteine thiol (Cys32) has an unusually low pK$_a$ (~6.3 in hTXN (409)) enabling easy deprotonation under physiological conditions and therefore allowing TXN1 to primarily function as a protein disulphide reductase [Formula 4] (410).

Formula 4:  \[
\text{Protein-S-S + TXN-(SH)$_2$} \rightarrow \text{Protein-SH + TXN-S-S}
\]

Deprotonation results in Cys32 becoming a thiolate (\(-\text{R-S}^-\)), enabling nucleophilic attack of disulphide bonds in target proteins. A mixed disulphide bond between Cys32 and the target cysteine is generated following nucleophilic attack, which is then resolved by proton donation by Cys35 (the second cysteine of the TXN active site), resulting in formation of a disulphide bond between Cys32 and Cys35, and two free thiols in place of the disulphide bond on the target protein (Figure 1.9, (410)). The TXN active site disulphide bond is returned to its reduced (free thiol) state by a specific enzyme, TXNRD [Formula 5]. In the absence of TXNRD or under oxidising conditions, TXN can switch function from a reductant to a powerful oxidant, triggering protein thiol oxidation (disulphide formation) rather than reduction (411).

Formula 5:  \[
\text{TXN-S-S + NADPH + H}^+ \rightarrow \text{TXN-(SH)$_2$ + NADP}^+ \]

TXN1 is ubiquitous and despite not having a secretion signal peptide it can be secreted from cells via a leaderless pathway (412). It is able to interact with a wide variety of both intra- and extra-cellular substrate proteins and as a result of this promiscuity is involved in a large variety of essential functions. Homozygous knockout of either TXN1 or TXN2 is embryonic lethal, demonstrating its importance.
TXN functions as an electron donor for reductive enzymes such as RNR (essential for DNA synthesis), and PRDX (402, 414). TXN is also a disulphide reductant for proteins including insulin, oxytocin, and fibrinogen (402), and has other key roles in inflammation such as skewing macrophages to the M2 phenotype (415) and mediating redox control of NF-κB (248, 397, 416).

TXN1 itself is redox-controlled: to carry out its primary reductant functions the cysteines of its active site must be reduced (-SH). Furthermore, in contrast to TXN2, TXN1 contains three additional non-active site cysteines, two of which (Cys-62 and Cys-69) can form an intramolecular disulphide bond which can disrupt functioning of the active site. This secondary redox regulation mechanism is catalysed via PRDX1 in the presence of high H$_2$O$_2$ concentrations (417). This highly oxidised form cannot function as a disulphide reductase or be returned to function by TXNRD; instead, it is reliant on reduction by another oxidoreductase, glutaredoxin (GRX) (Figure 1.10, (418). Similar to PRDX, TXN function is inhibited under oxidising conditions to allow propagation of oxidative signals.
Figure 1.9: Thioredoxin mechanism of protein reduction.

1. The N-terminal cysteine thiol (Cys32) is easily deprotonated under physiological conditions. 2. Deprotonation results in Cys32 becoming a thiolate, enabling nucleophilic attack of disulphide bonds in target proteins. 3. A mixed disulphide bond between Cys32 and the target cysteine is generated following nucleophilic attack, which is then resolved by proton donation by Cys35 (the second cysteine of the TXN active site). 4. An intramolecular disulphide bond forms, and the target protein disulphide bond is reduced. Original in colour.
1.3.2 Thioredoxin reductase

Thioredoxin reductases (TXNRD) are a family of three oxidoreductase enzymes: TXNRD1 (cytosolic), TXNRD2 (mitochondrial) and TXNRD3, which is specific to the microsomal fraction of testis tissue (419). The primary function of TXNRD is to reduce oxidised TXN through transfer of NADPH-derived electrons (Formula 5, Figure 1.10). Knockout of TXNRD1 and TXNRD2 are embryonic lethal demonstrating their importance (420-422). TXNRD is also capable of reducing non-disulfide substrates, such as lipid hydroperoxides (423), and H₂O₂ (424). Like TXN1, TXNRD1 is secreted from the cell, and this occurs via the Golgi pathway (425).

Active TXNRD consists of a homodimer arranged in a head to tail formation. Each 55kDa monomer contains a FAD-binding domain, an NADPH binding domain, an interface domain, and a discrete active centre (426, 427). The discrete active centre is comprised of a disulphide bond and is located proximal to the FAD and NADPH binding domains. The interface domain is conserved in all mammals and is comprised of a 16-residue C-terminal tail containing a selenocysteine (Sec) as the penultimate residue of the sequence -Gly-Cys-Sec-Gly-COOH (428-430). This Sec residue is held in a selenenylsulfide bond (R-Se-S-R) with its neighboring cysteine. Sec is essential for the correct functioning of TXNRD, enabling a lower pKₐ and stronger nucleophilic properties. Replacing Sec with Cys inhibits TXNRD activity (428, 431).

Two NADPH molecules are required for TXNRD function; these bind the NADPH binding domain enabling electron travel via the FAD domain to first reduce the discrete active centre disulphide and second reduce the selenenylsulfide bond. The selenocysteine residue is then free for nucleophilic attack and the interface domain swings out like an arm to transfer electrons from the discrete active centre to TXN, and so reducing its disulphide bond (426). The active centre cysteines can then reduce the selenocysteine and return to a disulphide formation (Figure 1.11, 428).
1. Under normal conditions TXNRD uses NADPH-derived electrons to reduce oxidised TXN, allowing TXN to then reduce oxidised PRDX which in turn reduces peroxides. This process occurs in a continuous cycle. 2. During mild oxidative stress, the non-active site cysteines of TXN become oxidised, preventing its reduction by TXNRD. Instead GRX reduces the non-active site cysteines, allowing TXN to then be fully reduced by TXNRD. GRX itself is reduced by glutathione via glutathione reductase. 3. Under conditions of high oxidative stress, PRDX becomes over-oxidised to sulfinic acid which can be reduced in a multistage reaction by SRX. 4. Under very high oxidative stress PRDX is oxidised to sulfonic acid, which is irreversible. Original in colour.

Abbreviations: Thioredoxin reductase (TXNRD), thioredoxin (TXN), peroxiredoxin (PRDX), glutaredoxin (GRX), sulfiredoxin (SRX), ‘SH’ represents cysteine free thiol
Figure 1.11: Thioredoxin reductase mechanism of electron exchange. Adapted from Zhong et al 2000 (428). (1) Active TXNRD consists of a homodimer arranged in a head to tail formation. Each monomer contains a FAD-binding domain, an NADPH binding domain, a discrete active centre and an interface domain. The discrete active centre contains a disulphide bond, whilst the interface domain contains a selenenylsulfide bond. (2) NADPH binding enables electron travel via the FAD domain to first reduce the discrete active centre disulphide (3) and then the selenenylsulfide bond. (4) The selenocysteine residue is then free for nucleophilic attack and transfers electrons from the discrete active centre to reduce TXN. (5) The active centre cysteines can then reduce the selenocysteine and return to a disulphide formation. Original in colour.
1.3.3 Peroxiredoxin

In 1985, Kim et al. found that purified yeast glutamine synthetase degraded and was inactivated over time when stored in a reducing buffer (containing β-mercaptoethanol or dithiothreitol (DTT)). However, glutamine synthetase prepared from crude yeast extracts did not degrade under these conditions (432). From this observation, they were able to identify a protective enzyme which prevented glutamine synthetase degradation, and named this enzyme Thiol Specific Antioxidant (TSA), as it was thought that its antioxidant function was limited to cysteine RSS (433, 434). This enzyme was induced by oxidative stress and was detectable in rat tissues (435). In 1994, cloning and sequencing of TSA isolated from yeast, rat, and human tissues showed 65% amino acid homology between human and yeast, demonstrating high evolutionary conservation (436, 437). Identification of protein sequences similar to TSA were identified in a variety of organisms in the early 1990s, all of which contained an absolutely conserved active cysteine residue (438-442), and around the same time TSA was found to reduce peroxides and use TXN and NADPH as electron donors in this reaction (443). The TSA protein family was later named the peroxiredoxins (PRDX) (444, 445).

PRDX are very highly conserved: expressed ubiquitously from bacteria to mammals, and they are one of the most highly expressed cellular proteins comprising 0.1-0.8% of the total soluble protein in most mammalian cells (446). Their primary role is peroxide reduction (including \( \text{H}_2\text{O}_2 \), and \( \text{ONOO}^- \)), carried out using TXN as an electron donor [Formula 6]. Through this mechanism, PRDX are involved with many redox signalling systems, from embryonic development through to immune responses (447, 448). The TXN fold of PRDXs contain several additions to the basic form: an N-terminal extension, an insertion between the second β-strand and α-helix and sometimes a C-terminal extension (449).

\[
\text{Formula 6: } \text{H}_2\text{O}_2 + \text{TXN}-(\text{SH})_2 \rightarrow 2\text{H}_2\text{O} + \text{TXN-S-S} \]

There are six PRDX isoforms in mammalian cells, PRDX1-6, which are divided into three subfamilies based on reactive cysteines: 2-Cys (PRDX1-4), atypical 2-Cys (PRDX5), and 1-Cys (PRDX6) (400, 436, 444). PRX-1, 2, 4 and 6 are primarily located in the cytosol, PRDX3 is exclusively located in mitochondria, and PRDX5 can be found in mitochondria, peroxisomes and the cytosol (400, 450). Each 2-Cys PRDX resides as a homodimer, with each subunit containing two cysteines, the
peroxidatic (CP) and the resolving (CR), which form an intersubunit disulphide bond during peroxide reduction [see below]. The atypical 2-Cys PRDX5 exists as a monomer and forms an intramolecular disulphide during catalysis, and the 1-Cys PRDX6 forms a disulphide with other proteins or small thiols (451-453).

This project focusses on the 2-Cys PRDXs, PRDX1 and PRDX2. These are both small (~23kDa) enzymes with high rate constants for H$_2$O$_2$ reduction (1-4x10$^7$ M$^{-1}$s$^{-1}$), which is very fast compared with free thiols (1x10$^1$ M$^{-1}$s$^{-1}$); this together with their abundance makes them favourable targets for oxidation by H$_2$O$_2$ (454-456). They are TXN-dependent peroxidases, using TXN1 to donate NADPH-derived electrons to reduce their active site cysteines (Figure 1.10, (457, 458). As with TXN1, PRDX1 and PRDX2 can be secreted from cells via a leaderless pathway (197, 412). Although PRDX1 and PRDX2 are 91% homologous and contain 78% identical amino acid sequences, the locations of their active cysteines differ slightly; the CP and CR are Cys52 and Cys173 respectively in PRDX1, and Cys51 and Cys172 in PRDX2. Furthermore, PRDX1 contains an additional Cys83 which is not present in PRDX2 (459).

The peroxidatic cysteine (CP) has a very low pK$_a$, making it particularly sensitive to and reactive with peroxides even at low levels (460). Each 2-Cys PRDX monomer contains both CP and CR cysteines. The active enzyme features two monomers arranged in a head-to-toe formation, with the CP of one monomer pairing with the CR at the C-terminus end of its opposite monomer (461). The reaction commences when the CP initiates nucleophilic attack of peroxide molecules such as H$_2$O$_2$: this causes oxidation of the cysteine resulting in formation of a cysteine sulfenic acid intermediate (Cys-S$_p$OH) and reduction of H$_2$O$_2$ into H$_2$O. The sulfenic acid then attacks the free thiol of the CR, to form a disulphide bond which is ultimately reduced by TXN (Figure 1.12, (400). As mentioned in section 1.2.4, in high H$_2$O$_2$ environments, PRDX can become over-oxidised wherein the CP is oxidised to sulfinic acid (Cys-S$_p$O$_2$H) which can be reduced by SRX, or sulfonic acid (Cys-S$_p$O$_3$H), which is irreversible (382).
Figure 1.12: Role of the peroxidatic and resolving cysteines during peroxiredoxin redox reactions

Each 2-Cys peroxiredoxin (PRDX) monomer contains both peroxidatic (CP) and resolving (CR) cysteines. The reaction commences when the CP initiates nucleophilic attack of peroxide molecules such as H₂O₂: this causes oxidation of the cysteine resulting in formation of a cysteine sulfenic acid intermediate (Cys-SpOH) and reduction of H₂O₂ into H₂O. The sulfenic acid then attacks the free thiol of the CR, to form a disulphide bond which is ultimately reduced by thioredoxin (TXN).
PRDX and TXN are both key components of redox signalling and H₂O₂ sensing, and a number of mechanisms are likely to be at play:

1. The floodgate hypothesis - H₂O₂ hyper oxidises PRDX, inactivating its peroxidase activity and allowing direct H₂O₂ mediated redox signalling to occur at the target protein. Furthermore, TXN is freed from its competitive inhibition by PRDX, allowing reduction of other oxidised proteins (462)
2. H₂O₂ oxidises PRDX, which can then transfer the oxidation signal to target proteins
3. H₂O₂ oxidises PRDX, which is then reduced by TXN. The oxidised TXN then transfers the oxidation signal to target proteins (463)

An additional function of PRDX is as a molecular chaperone, which is independent of peroxidase activity (464). Hyperoxidised PRDX assembles into decamers which form double or multiple layers (464-466). The exact functions of this formation have not yet been fully established, though it appears to be protective under oxidative stress (465, 467). Furthermore, in the extracellular environment decameric PRDX1 can activate TLR4 (468). The additional Cys83 in PRDX1 appears to regulate decamer formation (459). This cysteine is located at the putative dimer-dimer interface and its substitution with serine prevents decamer formation. This suggests that PRDX1 has more molecular chaperone functions than PRDX2.

Post-translational modifications of PRDX are also used to regulate their function (469). As with all cysteine thiol proteins, PRDX are susceptible to ROS, RNS, and RSS, but can also be phosphorylated and acetylated to further fine-tune their functions (470).

1.3.4 Redox and inflammation

There is an intimate link between the RSI, oxidoreductases, and inflammation. As mentioned in section 1.2, reactive species are produced following inflammatory stimuli such as TLR activation and can be used for direct pathogen killing, as well as in redox signalling for inflammation (336). The RSI has an important influence on two key transcription factors involved in inflammation and its resolution, NF-κB, and Nrf2.

NF-κB is able to regulate ROS through upregulation of antioxidant enzymes such as MgSOD, and regulation of Nrf2 [see 1.1.8] (319, 471, 472). Conversely, ROS
and also RNS can activate Nrf2 via oxidation/S-nitrosylation of KEAP1, allowing increased oxidoreductase expression and subsequent ROS reduction (286, 358). ROS is also able to regulate NF-κB in a phase-dependent manner: in the early phase H2O2 can activate NF-κB mainly via the classical IKK-dependent pathway (though the exact mechanisms are cell-specific) (473, 474), and during the late phase, ROS can inhibit NF-κB (475). The mechanisms of this inhibition are unclear but could be due to oxidation of IKK (476), or H2O2-induced degradation of the NF-κB regulator Akt/protein kinase B (PKB) (477). Furthermore, redox signalling via TXN1 has been reported to have regulatory effects on NF-κB-DNA binding (478).

The RSI can also influence earlier stages of inflammation; there is evidence that multiple checkpoints throughout the TLR signalling pathways are redox-regulated; for example, the functions of IL-1R-associated kinase-1 (IRAK-1) and IRAK-4, involved in early TLR4 signalling, are oxidant dependent and can be inhibited by antioxidants (479). Furthermore, the inflammatory MAPK pathway can be regulated through PRDX/TXN redox signalling (480).

With so many roles in inflammation, it is no surprise that the RSI is an active component of RA pathogenesis (2). ROS are required for redox signalling, potentiate the inflammatory response, and can directly degrade cartilage in RA (481, 482). Leukocyte mitochondria from RA patients have been found to produce up to 5-fold more ROS than healthy control leukocytes (483), and lipid peroxidation, protein oxidation and oxidative damage causing somatic mutations have been reported in blood, lymphocytes and synovial tissues of RA patients (484-486). To counter increased oxidative stress, increased antioxidant defence mechanisms are required such as glutathione and oxidoreductase enzymes, which are impaired in RA (487-490).

1.3.5 Oxidoreductases and inflammation

The oxidoreductases PRDX1, PRDX2, and TXN1 are all intracellular enzymes with important roles in redox signalling and therefore inflammation. However, these enzymes can be oxidised and glutathionylated to enable release from cells via a non-classical, exosomal pathway (197, 491). Release occurs following stress or inflammatory stimuli (e.g. TLR4 stimulation) or as a result of cell necrosis. Inclusion of oxidised proteins such as the oxidoreductases into exosomes is a method of intercellular communication of stress responses (492). When freely circulating in the extracellular environment PRDX has cytokine-like properties, whilst TXN1
stimulates lymphocyte proliferation and has chemokine-like properties; they can thus be named redox-active cytokines, or redoxkines (491, 493-496). Their proinflammatory functions could be due to redox signalling (via oxidation-activated receptors) or to direct PRR binding. No specific redoxkine receptor has been identified, but PRDX1 can directly activate TLR4 causing proinflammatory cytokine production (468).

Elevated circulating TXN1 levels have been reported in multiple diseases including diabetes, severe burns, coronary disease and hepatocellular carcinoma (497-500). Extracellular TXN1 is likely to have many functions: it can prevent initiation of the complement cascade and, as mentioned, can activate lymphocyte proliferation and function as a chemokine (496, 501). TXN1 can also be cleaved after the 80-84 N-terminal residues to give Trx80, a truncated and solely extracellular form of TXN1 (502). Trx80 is secreted from monocytes and T lymphocytes under proinflammatory conditions, existing in the extracellular milieu as a dimer (502). TXN1 and Trx80 seem to perform opposing functions with regard to inflammation (503, 504); whilst the roles of TXN1 appear to primarily promote homeostasis and immune regulation, Trx80 acts as a proinflammatory cytokine causing activation of the innate immune system, cytokine release and complement activation, without any redox activity (502, 505, 506).

Extracellular PRDX1 and -2 are increased in patients with HIV, lung and pancreatic cancers, acute stroke, acute lung injury and also with systemic inflammatory response syndrome (507-512). Elevated PRDX2 levels have been reported in lymphocytes, synovial tissues, synovial fluid and plasma from patients with RA (513, 514).

Autoantibodies to some redoxkines have occurred in systemic autoimmune diseases. Development of PRDX1 and -2 autoantibodies have been reported, with 33% of patients with systemic autoimmune diseases developing autoantibodies to PRDX1 (19% of RA and 57% of lupus patients), and up to 60% of patients with systemic vasculitis developing autoantibodies to PRDX2 (515, 516). Autoantibodies to TXN1 have not been documented.

Elevated levels of circulating PRDX1 and -2 and TXN1, together with their important roles in inflammation and redox signalling have highlighted them as potential disease biomarkers. A better understanding of their inflammatory functions and
roles in diseases such as RA could enable better understanding of disease aetiology and development of more targeted therapeutics.
Aims

Inflammation is essential for eliminating invading pathogens, repairing wounds and for overall well-being. However, in autoimmune disease inflammation itself is the problem. RA is one example of autoimmune disease where inflammation has become dysregulated and contributes to disease pathogenesis. The initial triggers of inflammation in conditions such as RA are unknown, but many contributing factors exist. The TLRs and oxidative stress are two such factors, which are linked by thiol-oxidoreductase enzymes PRDX and TXN. These intracellular enzymes are induced during inflammation and oxidative stress, and are released from cells following inflammatory stimuli. When in the extracellular environment they can act as danger signals, triggering activation of the TLRs and perpetuating inflammation. However, knowledge of oxidoreductase regulation and release is limited. The hypothesis of this study was that responses of oxidoreductases to TLRs vary according to the TLR stimulated.

This project will pursue four lines of investigation to address this hypothesis:

1. Extracellular release of PRDX and TXN is known to occur in response to TLR4 stimulation. Chapter 3 aims to expand on this knowledge by investigating their release in response to other members of the TLR family.

2. Induction of PRDX and TXN reductase (TXNRD) gene expression is known to occur in response to TLR4 stimulation. Chapter 4 aims to investigate the regulation of oxidoreductase gene expression in response to other members of the TLR family.

3. Nrf2, the transcription factor responsible for oxidoreductase transcription is redox sensitive. Chapter 5 aims to investigate if reactive oxygen or reactive nitrogen species (ROS and RNS respectively) are involved in oxidoreductase release or gene induction.

4. PRDX2 is released from cells under inflammatory conditions and has been shown to increase in lymphocytes and plasma of people with rheumatoid arthritis (RA) (513). Chapter 6 aims to investigate if PRDX2 is detectable in RA serum, and if so, if this correlates with inflammatory markers.
Chapter 2
2. Methods and Materials

2.1 Materials

2.1.1 Instruments

<table>
<thead>
<tr>
<th>Product name</th>
<th>Manufacturer</th>
<th>Manufacturer Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accublock™ Digital Dry Bath</td>
<td>Labnet</td>
<td>Edison, New Jersey, USA</td>
</tr>
<tr>
<td>CKX41 Inverted microscope</td>
<td>Olympus</td>
<td>Shinjuku, Tokyo, Japan</td>
</tr>
<tr>
<td>C-Mag MS7 Stirrer</td>
<td>IKA®</td>
<td>Staufen, Germany</td>
</tr>
<tr>
<td>Hera Cell 150 Incubator</td>
<td>Thermo Fisher Scientific</td>
<td>Waltham, Massachusetts, USA</td>
</tr>
<tr>
<td>Heraeus™ Fresco™ 17 Microcentrifuge</td>
<td>Thermo Fisher Scientific</td>
<td>Waltham, Massachusetts, USA</td>
</tr>
<tr>
<td>Hypercassette</td>
<td>GE Healthcare Life Sciences</td>
<td>Little Chalfont, UK</td>
</tr>
<tr>
<td>Mini Trans-Blot® Cell</td>
<td>Biorad</td>
<td>Hercules, California, USA</td>
</tr>
<tr>
<td>Mini-PROTEAN® Tetra Vertical Electrophoresis Cell</td>
<td>Biorad</td>
<td>Hercules, California, USA</td>
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<tr>
<td>Mx3000P qPCR System</td>
<td>Stratagene California</td>
<td>La Jolla, California, USA</td>
</tr>
<tr>
<td>Neubauer Improved Haemocytometer</td>
<td>Marienfeld Superior</td>
<td>Lauda-Königshofen, Germany</td>
</tr>
<tr>
<td>PF-402 Top pan balance</td>
<td>Thermo Fisher Scientific</td>
<td>Waltham, Massachusetts, USA</td>
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<tr>
<td>PIPETMAN® Pipettes</td>
<td>Gilson Incorporated</td>
<td>Middleton, Wisconsin, USA</td>
</tr>
<tr>
<td>SevenCompact pH Meter</td>
<td>Mettler-Toledo</td>
<td>Greifensee, Switzerland</td>
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<tr>
<td>SRX-101A Developer</td>
<td>Konica Minolta</td>
<td>Marunouchi, Tokyo, Japan</td>
</tr>
<tr>
<td>Synergy HT Plate Reader</td>
<td>Biotek</td>
<td>Winooski, VT, USA</td>
</tr>
<tr>
<td>Veriti 96-Well Thermal Cycler</td>
<td>Applied Biosystems</td>
<td>Foster City, California, USA</td>
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*Table 2.1: Instrument list.*
<table>
<thead>
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<th>Product</th>
<th>Manufacturer</th>
<th>Manufacturer Address</th>
</tr>
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<tr>
<td>2-propanol</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
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<tr>
<td>30% Acrylamide/Bis Solution 29:1</td>
<td>Bio-Rad Laboratories</td>
<td>Hercules, California, USA</td>
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<tr>
<td>5x RT buffer</td>
<td>Invitrogen</td>
<td>Carlsbad, California, USA</td>
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<tr>
<td>Absolute ethanol</td>
<td>Thermo Fisher Scientific</td>
<td>Waltham, Massachusetts, USA</td>
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<tr>
<td>Ammonium persulfate (APS)</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
</tr>
<tr>
<td>Brilliant III Ultra-Fast qPCR Master Mix</td>
<td>Agilent Technologies</td>
<td>Santa Clara, California, USA</td>
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<tr>
<td>Bromophenol Blue</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
</tr>
<tr>
<td>Dimethyl Sulfoxide</td>
<td>Thermo Fisher Scientific</td>
<td>Waltham, Massachusetts, USA</td>
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<tr>
<td>Dithiothreitol (DTT)</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
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<tr>
<td>dNTP</td>
<td>GE Healthcare Life Sciences</td>
<td>Little Chalfont, UK</td>
</tr>
<tr>
<td>Dulbecco’s Phosphate Buffered Saline (sterile)</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
</tr>
<tr>
<td>ECL Blotting Detection Reagents</td>
<td>GE Healthcare Life Sciences</td>
<td>Little Chalfont, UK</td>
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<td>Ethanol</td>
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<td>St. Louis, Missouri, USA</td>
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<tr>
<td>Glycerol</td>
<td>Melford Laboratories</td>
<td>Ipswich, Suffolk, UK</td>
</tr>
<tr>
<td>Glycogen</td>
<td>Invitrogen</td>
<td>Carlsbad, California, USA</td>
</tr>
<tr>
<td>Griess reagent system (G2930)</td>
<td>Promega Corporation</td>
<td>Madison, Wisconsin, USA</td>
</tr>
<tr>
<td>Heat Inactivated Foetal Calf Serum</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
</tr>
<tr>
<td>HyClone® Trypan blue solution 0.4%</td>
<td>Thermo Fisher Scientific</td>
<td>Waltham, Massachusetts, USA</td>
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<tr>
<td>Hyperfilm ECL</td>
<td>GE Healthcare Life Sciences</td>
<td>Little Chalfont, UK</td>
</tr>
<tr>
<td>IGEPAL® CA-630</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
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<tr>
<td>Methanol</td>
<td>Thermo Fisher Scientific</td>
<td>Waltham, Massachusetts, USA</td>
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<tr>
<td>MMLV-RT</td>
<td>Invitrogen</td>
<td>Carlsbad, California, USA</td>
</tr>
<tr>
<td>N-Ethylmaleimide (NEM)</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
</tr>
<tr>
<td>Nitrocellulose membrane (Protran™ Premium 0.45μm)</td>
<td>GE Healthcare Life Sciences</td>
<td>Little Chalfont, UK</td>
</tr>
<tr>
<td>Chemical or Reagent</td>
<td>Supplier</td>
<td>Location</td>
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<tr>
<td>-------------------------------------------</td>
<td>---------------------------------</td>
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<tr>
<td>OptiMEM I medium</td>
<td>Thermo Fisher Scientific</td>
<td>Waltham, Massachusetts, USA</td>
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<tr>
<td>PageRuler™ Plus Prestained Protein Ladder</td>
<td>Thermo Fisher Scientific</td>
<td>Waltham, Massachusetts, USA</td>
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<tr>
<td>Penicillin-Streptomycin</td>
<td>Thermo Fisher Scientific</td>
<td>Waltham, Massachusetts, USA</td>
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<tr>
<td>QIAzol Lysis Reagent</td>
<td>Qiagen</td>
<td>Hilden, Germany</td>
</tr>
<tr>
<td>Random Primers</td>
<td>Promega Corporation</td>
<td>Madison, Wisconsin, USA</td>
</tr>
<tr>
<td>Restore™ Western Blot Stripping buffer</td>
<td>Thermo Fisher Scientific</td>
<td>Waltham, Massachusetts, USA</td>
</tr>
<tr>
<td>RNAse out</td>
<td>Invitrogen</td>
<td>Carlsbad, California, USA</td>
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<td>Roswell Park Memorial Institute (RPMI) 1640 medium</td>
<td>Sigma-Aldrich Corporation</td>
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<td>Sodium chloride</td>
<td>Thermo Fisher Scientific</td>
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<td>Sodium dodecyl sulphate (SDS)</td>
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<td>St. Louis, Missouri, USA</td>
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<td>Sodium hydroxide</td>
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<td>Tetrathylmethylenediamine (Temed)</td>
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<td>Thiazolyl Blue Tetrazolium Bromide (MTT)</td>
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<td>St. Louis, Missouri, USA</td>
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<td>Triton X-100</td>
<td>Thermo Fisher Scientific</td>
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<td>Trizma hydrochloride</td>
<td>Sigma-Aldrich Corporation</td>
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<td>Trizma® base</td>
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<td>Tween 20</td>
<td>Thermo Fisher Scientific</td>
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<tr>
<td>Whatman™ 3MM Chromatography Paper 15cm x 100m</td>
<td>Thermo Fisher Scientific</td>
<td>Waltham, Massachusetts, USA</td>
</tr>
<tr>
<td>β-Mercaptoethanol</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
</tr>
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</table>

**Table 2.2: Chemicals and reagent list.**
2.1.3 Plastics

<table>
<thead>
<tr>
<th>Product</th>
<th>Manufacturer</th>
<th>Manufacturer Address</th>
</tr>
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<tbody>
<tr>
<td>BD Vacutainer® Serum Tubes</td>
<td>Becton, Dickinson and Company</td>
<td>Franklin Lakes, New Jersey, USA</td>
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<tr>
<td>Corning® 75cm² U-Shaped Canted Neck Cell Culture Flask</td>
<td>Corning Inc.</td>
<td>Corning, New York, USA</td>
</tr>
<tr>
<td>Culture Pipette, sterile (5, 10 and 25ml)</td>
<td>Elkay Laboratory Products (UK) Ltd</td>
<td>Basingstoke</td>
</tr>
<tr>
<td>DNA LoBind Tubes (1.5 or 2ml)</td>
<td>Eppendorf Ltd</td>
<td>Stevenage, UK</td>
</tr>
<tr>
<td>Eppendorf PhysioCare Concept® Tubes®</td>
<td>Eppendorf Ltd</td>
<td>Stevenage, UK</td>
</tr>
<tr>
<td>Nunclon™ Delta cell culture plates (96 and 24 well)</td>
<td>Thermo Fisher Scientific</td>
<td>Waltham, Massachusetts, USA</td>
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Table 2.3: Plastic ware list.

2.1.4 Solutions, Buffers and Media

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<th>Reagent</th>
<th>Composition</th>
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<tr>
<td>Cell culture media</td>
<td>RPMI 1640 media containing 2mM L-glutamine, supplemented with 1% (v/v) penicillin-streptomycin and 10% (v/v) heat inactivated Foetal Calf Serum</td>
</tr>
<tr>
<td>ELISA reagent diluent</td>
<td>1x PBS with 1% BSA</td>
</tr>
<tr>
<td>ELISA wash buffer</td>
<td>1x PBS with 0.01% (v/v) Tween-20</td>
</tr>
<tr>
<td>Laemmli sample buffer (6X)</td>
<td>375 mM Tris-HCl, 2% SDS, 40% glycerol, 0.004% Bromophenol blue pH 6.8</td>
</tr>
<tr>
<td>MTT stop solution</td>
<td>10% SDS in 0.01M HCl</td>
</tr>
<tr>
<td>PBS-T+IGEPAL</td>
<td>PBS-T with 0.05% IGEPAL</td>
</tr>
<tr>
<td>PBS-Tween (PBS-T)</td>
<td>10% PBS 10X, 0.075% Tween-20</td>
</tr>
<tr>
<td>Phosphate buffered saline (PBS) (10X)</td>
<td>1.37M NaCl, 27M KCl, 80M Na₂HPO₄, and 20M KH₂PO₄ in distilled water</td>
</tr>
<tr>
<td>SDS-Page resolving gel buffer (4X)</td>
<td>375mM Trizma® (Tris)-hydrochloride (HCl) pH8.8, 7.5-15% Acrylamide/Bis solution, 0.1% (v/v) APS, 0.1% (v/v) SDS, 0.1% Temed</td>
</tr>
<tr>
<td>SDS-Page running buffer (10X)</td>
<td>250mM Tris base, 1.92M glycine, 1% SDS</td>
</tr>
<tr>
<td>SDS-Page stacking gel buffer (4X)</td>
<td>500Mm Tris-HCl pH6.8, 16.5% Acrylamide/Bis solution, 0.15% (v/v) APS, 0.1% (v/v) SDS, 0.1% Temed</td>
</tr>
<tr>
<td>Western blot transfer buffer (10X)</td>
<td>250mM Tris base, 1.92M glycine</td>
</tr>
<tr>
<td>Western blot transfer buffer (1X)</td>
<td>20% methanol, 10% 10X Transfer buffer 70% distilled water</td>
</tr>
</tbody>
</table>

Table 2.4: Solution and buffer composition list.
2.2 Software and Analysis

2.2.1 ImageJ 1.48v

ImageJ is a public domain image processing software programme, developed by the National Institute of Health, USA. ImageJ can be used to analyse scanned images of Western Blots for band density. The generated arbitrary densitometry units can then be used to statistically compare bands within blots.

2.2.2 Microsoft Excel 2010

Microsoft Excel is spreadsheet software featuring calculation, graphing tools, pivot tables and a macro programming language. Excel was used in this project for basic analyses, data storage and compilation.

2.2.3 GraphPad Prism 7.03

GraphPad Prism is a commercial graphing and statistics software developed by GraphPad Software, Inc. GraphPad was specifically designed for experimental biologists, and was used in this project for the majority of statistical analyses.
2.3 Cell culture and stimulation

2.3.1 RAW 264.7 cells

RAW 264.7 cells are a murine cell-line, created in 1978 through intraperitoneal injection of a male BALB/c mouse with Abelson Leukaemia Virus (A-MuLV) and isolated from a resulting tumour ascites. These cells are macrophage-like adherent, immortalised cells capable of pinocytosis, phagocytoses and secretion of proinflammatory cytokines. These cells were used in this project as they express all TLRs with the exception of TLR5, and produce and release oxidoreductase enzymes (197, 491, 517, 518). RAW 264.7 cells were kindly provided by Dr. Jon Mabley (University of Brighton, UK).

2.3.2 RAW 264.7 cell culture

RAW 264.7 cells were stored in liquid nitrogen (LN\textsubscript{2}) until required. Cells were stored in 1ml cryotubes of 10 million cells in 90% FCS and 10% DMSO. To thaw, cryotubes were removed from LN\textsubscript{2} and immediately held in a 37°C water bath until just thawed. Cells were then transferred into 10ml pre-warmed cell culture media and centrifuged 10mins at 297 x G at room temperature. Supernatants were removed and cell pellets set up in 15ml fresh cell culture media in T75 flasks. Cells were cultured for at least 4 days prior to use.

Cells were cultured in 75cm\textsuperscript{2} flasks in 15ml cell culture media. Cells were routinely passaged via cell scraping when 80% confluence was reached or used directly for experiments.

2.3.3 Cell stimulations

For cell stimulations, adherent RAW 264.7 cells were scraped from flask, then seeded at 1.25x10\textsuperscript{5} per well (24-well plate) or 2.5x10\textsuperscript{4} per well (96-well plate) in 1ml or 200μl cell culture media respectively. Cells were incubated overnight to allow adhesion. TLR stimulation was carried out for 6-24 hours at 37°C and 5% CO\textsubscript{2} using TLR agonists (Table 5), or alternative cell stimuli (Table 6) in cell culture media or OptiMEM serum-free media.

OptiMEM serum free media was used in experiments investigating oxidoreductase release from cells where Western blot was used for analysis. The presence of supplemental serum interferes with protein migration during SDS-PAGE and increases non-specific antibody binding. Short-term cell stimulations using OptiMEM negates the need for supplemental serum.
<table>
<thead>
<tr>
<th>Product</th>
<th>TLR</th>
<th>Stock conc.</th>
<th>Working conc.</th>
<th>Manufacturer</th>
<th>Manufacturer address</th>
</tr>
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<tbody>
<tr>
<td>Pam3CSK4</td>
<td>TLR1/2</td>
<td>1mg/ml</td>
<td>1μg/ml</td>
<td>Axxora</td>
<td>New York 11735, USA</td>
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<tr>
<td>Poly IC</td>
<td>TLR3</td>
<td>1mg/ml</td>
<td>12.5μg/ml</td>
<td>Invivogen</td>
<td>San Diego, CA, USA</td>
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<tr>
<td>Lipopolysaccharide</td>
<td>TLR4</td>
<td>200μg/ml</td>
<td>10ng/ml</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
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<tr>
<td>FSL-1 (Pam2CGDP KHPKSF)</td>
<td>TLR2/6</td>
<td>200μg/ml</td>
<td>0.2μg/ml</td>
<td>Invivogen</td>
<td>San Diego, CA, USA</td>
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<tr>
<td>R848</td>
<td>TLR7/8</td>
<td>1mg/ml</td>
<td>1μg/ml</td>
<td>Enzo Life Sciences</td>
<td>Lausen, Switzerland</td>
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<tr>
<td>CLO75</td>
<td>TLR7/8</td>
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<td>10μg/ml</td>
<td>Source Bioscience</td>
<td>Nottingham, UK</td>
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<tr>
<td>ODN 2216</td>
<td>TLR9</td>
<td>500μM</td>
<td>5μM</td>
<td>Invivogen</td>
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Table 2.5: TLR agonist list.
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</thead>
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<tr>
<td>Neoseptin-3</td>
<td>15mg/ml (31.6mM)</td>
<td>25μM (519)</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
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<tr>
<td>Phorbol 12-myristate 13-acetate (PMA)</td>
<td>1mg/ml</td>
<td>500nM (520)</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
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<tr>
<td>N-acetylcysteine (NAC)</td>
<td>82mg/ml (0.5M)</td>
<td>10mM (521)</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
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<tr>
<td>Staurosporine</td>
<td>1mM</td>
<td>100nM (522)</td>
<td>Calbiochem® (Merck), Darmstadt, Germany</td>
<td></td>
</tr>
<tr>
<td>Recombinant rat IFN-γ</td>
<td>2x10⁶U/ml</td>
<td>100U/ml (523)</td>
<td>Research and Diagnostic Systems, Inc.</td>
<td>Minneapolis, Minnesota, USA</td>
</tr>
<tr>
<td>Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME)</td>
<td>5mg/ml (30mM)</td>
<td>0.3mM (524)</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
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<tr>
<td>N⁵-Methyl-L-Arginine Acetate (L-NMMA)</td>
<td>5mg/ml (30mM)</td>
<td>0.3 and 1mM (524)</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
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<tr>
<td>MG132</td>
<td>10mg/ml (21mM)</td>
<td>0.2μM (525)</td>
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<td>St. Louis, Missouri, USA</td>
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<tr>
<td>DETA/NO</td>
<td>61.28mM</td>
<td>0.1 and 0.2mM (526)</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
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<tr>
<td>Cycloheximide</td>
<td>5mg/ml (17.8mM)</td>
<td>5μM (527)</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
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</tbody>
</table>

Table 2.6: Cell stimuli.
2.3.4 Cell viability assay

To assess toxicity of cell stimuli, viability assays were conducted using MTT assay. This method allows viable cell quantification based on reduction of MTT dye to insoluble crystals (formazan), by nicotinamide adenine dinucleotide (NADH), a coenzyme found in all living cells (Figure 2.1). RAW 264.7 cells were seeded at a density of $1.25 \times 10^5$/ml in a 96 well plate in cell culture media for 24/48h according to experimental protocol. MTT reagent (0.5mg/ml) was added directly to the cells (100µl/well) and incubated for 4h at 37°C. MTT stop solution was then added to wells (100µl/well) and incubated for 16h at 37°C. Absorbance was measured at 590nm.
Figure 2.1: MTT reduction into Formazan. Modified from Stockert et al 2012 (528). The MTT assay is a colorimetric reaction used to assess cell viability. MTT substrate is reduced into insoluble formazan dye using protons derived from NADPH flux. Formazan dye can be dissolved MTT stop solution and measured for light absorbance between 500 and 600nm.
2.4 Ethical approvals, patients and recruitment

2.4.1 Healthy control samples
Recruitment of participants for this study was approved by Brighton and Sussex Medical School Research Governance and Ethics Committee (RGEC), under project “Pharmacomodulation of cytokines” (R&D Ref No: 09/043/GHE). Information sheets explaining the reason for the study and the role of blood donations were provided to all participating donors. Informed consent and blood samples were collected by phlebotomy trained members of staff at Brighton and Sussex Medical School. Donor data remained anonymised throughout the study. Healthy donors were excluded if they had taken medication other than the contraceptive pill during the week prior to the blood donation.

2.4.2 Rheumatoid arthritis samples

2.4.2.1 Recruitment of study participants
Recruitment of participants for this study was approved by Brighton and Sussex University Hospitals (BSUH), South East Coast Brighton and Sussex Research Ethics Committee and the National Research Ethics Service (NRES), under projects “Role of Oxidative Stress in Inflammation” (REC reference: 15/NS/0083) and “Autoimmune Inflammatory Mechanisms (AIM)” (REC reference: 14/NW/1114). Patient information sheets explaining the reason for the study and the role of patient samples were provided to all participating patients. Informed consent and blood samples were collected by the clinical care team or a trained member of NHS staff. Patient data remained anonymised and was stored securely according to Caldicott principles.

2.4.2.2 Patient characteristics
Inclusion criteria:

- Patients referred to the rheumatology clinic for assessment and treatment of rheumatoid arthritis
- Participants able to give informed consent
- Participants 18 years of age or over

Exclusion criteria:

- Patients unable to provide informed consent
- Pregnant women
- Patient withdrawal from study
- Participants known to have an acute or chronic infection, positive for human immunodeficiency virus, anti-hepatitis C virus antibodies, hepatitis B surface antigen or syphilis
- Patients less than 18 years of ages

2.4.2 Serum Collection
Blood samples were collected by trained staff in silica spray-coated 5ml serum collection tubes. Tubes were left to coagulate for 1h at room temperature, and then centrifuged at 1300 x G for 10 minutes at room temperature to ensure elimination of all blood cells. Serum was extracted and stored at -80°C.
2.5 SDS-PAGE and Western Blot

SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were used throughout this project to investigate released and intracellular oxidoreductase proteins. Unlike techniques such as ELISA [see 2.8], Western blot is only semi-quantitative, but it allows differentiation of protein oligomers. Characterisation of oxidoreductase oligomers provides information on their oxidation state, and therefore insights into their functions [see 1.3].

Western blot is an analytical technique for protein separation and detection which was developed in 1979 by Harry Towbin. Protein samples are denatured and then separated based on size and charge by SDS-PAGE (Figure 2.2, (529)). Electrophoretic transfer enables protein transfer from the polyacrylamide gel onto nitrocellulose membrane which has a high affinity for proteins. Membranes must be blocked to prevent non-specific antibody binding before incubation with a primary antibody, raised against the protein of interest. A secondary antibody is then used to detect the primary antibody and is visualised depending on the entity it is conjugated to: this can be a fluorophore but in this case secondary antibodies were conjugated with horseradish peroxidase (HRP). HRP then catalyses a multistep redox reaction resulting in reduction of H$_2$O$_2$ and oxidation of luminol into its light emitting form which is detectable (428nm) using photographic film (Figure 2.3). Chemical substrates such as modified phenols can chemically enhance light emission from this reaction by up to 13 fold, this is known as enhanced chemiluminescence (ECL).

Due to the separation of proteins of different molecular weights, Western blot allows differentiation of protein oligomers when gels are run under non-reducing conditions. For example, depending on its oxidation state PRDX can exist as a monomer (22kDa), a dimer (44kDa) or a decamer (220kDa). Each of these formations will run at different rates through the gel and can therefore be separated and individually characterised. This is in contrast to other techniques such as ELISA where the total protein, regardless of oligomerisation, will be quantified.
Figure 2.2: Western blot protocol.

(1) Acrylamide gels are cast with the resolving gel at the bottom and stacking gel on top (2) Samples are loaded in Laemmli buffer using gel loading pipette tips (3) Electrophoresis is performed enabling protein migration down gel (4) Electrophoretic transfer of proteins from gel onto nitrocellulose membrane (5) Membrane is blocked in 5% bovine serum albumin to prevent non-specific binding (6) Primary antibody binds protein of interest (7) HRP-conjugated secondary antibody binds primary antibody (8) Secondary antibody is detected with ECL (9) ECL is visualised with photographic film or detection devices such as Li-Cor. Original in colour. HRP = horseradish peroxidase, ECL = enhanced chemiluminescence
Figure 2.3: Western blot antibody detection and chemiluminescence reaction

In a multistep reaction, HRP oxidises ECL luminol into 3-aminophthalate which can emit light at 428nm. The ECL component enhances light emission by up to 13 fold. Original in colour. ECL = enhanced chemiluminescence  HRP = horseradish peroxidase
2.5.1 Polyacrylamide gel preparation

The polyacrylamide gel for Western blot is comprised of two sections: the running gel and the stacking gel (Figure 2.2). The stacking gel comprises the upper 20% of the final gel and is the gel into which wells are formed for protein loading. Stacking gel has a higher acidity than running gel (pH 6.8 vs pH 8.8), and a lower acrylamide content (Tables 2.7 and 2.8). These properties enable proteins within the stacking gel to form thin, well defined bands when a current is applied. The proteins then separate out according to size and charge on reaching the running gel (530).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>7.5% gel</th>
<th>10% gel</th>
<th>12% gel</th>
<th>15% gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide/bis solution</td>
<td>2.5ml</td>
<td>3.3ml</td>
<td>4ml</td>
<td>5ml</td>
</tr>
<tr>
<td>Running buffer (4x)</td>
<td>2.5ml</td>
<td>2.5ml</td>
<td>2.5ml</td>
<td>2.5ml</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
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<tr>
<td>APS (10%)</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
<td>100μl</td>
</tr>
<tr>
<td>Temed</td>
<td>10μl</td>
<td>10μl</td>
<td>10μl</td>
<td>10μl</td>
</tr>
<tr>
<td>H₂O</td>
<td>4.9ml</td>
<td>4.067ml</td>
<td>3.4ml</td>
<td>2.4ml</td>
</tr>
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</table>

**Table 2.7: Running gel composition.**

<table>
<thead>
<tr>
<th>Reagent</th>
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<tr>
<td>Acrylamide/bis solution</td>
<td>835μl</td>
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<tr>
<td>Running buffer (4x)</td>
<td>1.25ml</td>
</tr>
<tr>
<td>SDS (10%)</td>
<td>50μl</td>
</tr>
<tr>
<td>APS (10%)</td>
<td>75μl</td>
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<tr>
<td>Temed</td>
<td>5μl</td>
</tr>
<tr>
<td>H₂O</td>
<td>2.86ml</td>
</tr>
</tbody>
</table>

**Table 2.8: Stacking gel composition.**
2.5.2 Western blot sample preparation

2.5.2.1 Cell lysates
Stimulated cells were gently washed in PBS which was removed completely before 150μl 1.5% Laemmli sample buffer containing 50mM N-Ethylmaleimide (NEM) (to prevent modification of protein free thiols) was added to each well. Adherent cells were resuspended in Laemmli buffer, transferred into 1ml eppindorf tubes and boiled for 5 minutes at 100°C. For reducing conditions, DTT (0.1M) was added to Laemmli buffer. Each well was loaded with 5-10μl of lysate sample.

N-Ethylmaleimide
NEM is an organic derivative of maleic acid. It is an alkene and adds nucleophiles such as free thiols, to form a very strong C-S bond which is practically irreversible. NEM is therefore an irreversible inhibitor of thiol peptidase enzymes such as those of the thioredoxin fold family, preventing thiol-disulphide exchange reactions and any changes in thiol oxidation state.

2.5.2.2 Cell supernatants
Supernatants were centrifuged 5000 x G for 5 minutes at 4°C to remove any remaining cells or cellular debris. For reducing conditions DTT (0.1M) was added to 1x Laemmli buffer. Each well was loaded with 23μl of supernatant sample.

2.5.2.3 Serum
Serum samples were first diluted 1:50 in ultrapure water, then thoroughly mixed with 1x Laemmli buffer and 10% β-mercaptoethanol. Serum samples were not boiled prior to gel loading. Each well was loaded with 10μl of serum sample.
2.5.3 SDS-PAGE and Western blot

Gels of varying acrylamide percentages were used according to the molecular mass of the protein of interest (e.g. 15% for TXN1 and 12% for PRDX1). Gels were made in house according to Tables 2.7 and 2.8.

1. **Sample preparation and gel electrophoresis**: Samples (prepared as per 2.5.2) were boiled for 5 minutes with 1x Laemmli sample buffer immediately prior to SDS-PAGE and run in running buffer for 1.5-2h at 120 volts

2. **Protein transfer**: Proteins were transferred to nitrocellulose membrane via wet transfer at 4°C (325mA, 60mins)

3. **Blocking and primary antibody**: Membranes were blocked in 5% (wt/vol) BSA/PBS for 1h at room temperature, then incubated with primary antibody in 5%BSA/PBS overnight at 4°C (Table 9)

4. **Secondary antibody**: Membranes were washed 3x 5mins in PBS-T+IGEPAL, then incubated 1h at room temperature with HRP conjugated secondary antibody in 5%BSA/PBS

5. **Development**: Membranes were washed 3x 5mins in PBS-T+IGEPAL, and 1x 5mins in PBS. Membranes were incubated 5mins in ECL substrate (1ml per blot) and exposed to Hyperfilm ECL in a hypercassette and dark room

To ensure even protein loading between wells, cell lysate blots were stripped in 10ml Western blot stripping buffer, rinsed in PBS then blocked and reprobed for GAPDH. There is no defined loading control protein that can be used for cell supernatants. The most reliable loading control alternative was Ponceau red staining which non-specifically stains all protein bands. This could not be used as in cases such as LPS stimulation where it is known that almost 50% more proteins are secreted than with other TLR stimulations, normalisation to Ponceau red staining would skew results (196).
### 2.5.4 Antibodies

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Species</th>
<th>Clonality</th>
<th>Dilution*</th>
<th>Manufacturer</th>
<th>Manufacturer address</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human/mouse PRDX-1</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1:1000</td>
<td>Dr Eva-Maria Hanschmann</td>
<td>University of Greifswald, Germany</td>
</tr>
<tr>
<td>Human/mouse PRDX-2</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1:1000</td>
<td>Dr Eva-Maria Hanschmann</td>
<td>University of Greifswald, Germany</td>
</tr>
<tr>
<td>Mouse TXN-1</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1:1000</td>
<td>IMCO Corporation Ltd AB</td>
<td>Stockholm, Sweden</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Rabbit</td>
<td>Monoclonal</td>
<td>1:1000</td>
<td>Cell Signaling Technology</td>
<td>Danvers, Massachusetts, USA</td>
</tr>
<tr>
<td>Mouse Hsp70</td>
<td>Rabbit</td>
<td>Polyclonal</td>
<td>1:1000</td>
<td>Abcam plc</td>
<td>Cambridge, UK</td>
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</table>

<table>
<thead>
<tr>
<th>HRP-conjugated</th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit IgG</td>
<td>Goat</td>
<td>Polyclonal</td>
<td>1:25000</td>
<td>Sigma-Aldrich Corporation</td>
<td>St. Louis, Missouri, USA</td>
</tr>
</tbody>
</table>

*All dilutions were performed in 5% BSA/PBS*

**Table 2.9: Western blot antibody list.**
2.6 Polymerase Chain Reaction (PCR)

Oxidoreductase gene expression in response to TLR agonists and other cell stimuli was quantified using reverse transcription (RT) followed by real time Taqman qPCR. This technique is fast, highly sensitive and specific.

The polymerase chain reaction (PCR) was first developed in 1983 by Kary Mullis who later won the Nobel Prize for his work, together with Michael Smith (531, 532). PCR is a technique used to amplify a segment of DNA across several orders of magnitude. Real time or quantitative PCR (qPCR) monitors amplification over time, allowing for quantification of the amount of starting DNA.

2.6.2 Reverse transcription

Reverse transcription is used to reverse transcribe RNA strands into complimentary (c)DNA transcripts by reverse transcriptase enzymes. These cDNA transcripts can then be investigated by PCR, in this project real time Taqman qPCR. As mRNA is only generated when genes are being actively transcribed, this allows investigation of active gene expression. Random primers were used in this project to transcribe total RNA.

2.6.1 Polymerase chain reaction

The PCR reaction is based on repeated cycles of heating and cooling a DNA mixture containing the DNA of interest, oligonucleotide primers, a mix of deoxynucleotides (dNTP), and a DNA polymerase enzyme. The high temperatures required for denaturing DNA during PCR also denatures many DNA polymerase enzymes, meaning that fresh enzymes would need to be added at the elongation step of each cycle. The thermostable DNA polymerase, *Taq* polymerase, alleviates this step. *Taq* polymerase is named after the thermophilic bacterium it was originally isolated from, *Thermus aquaticus*, and allows PCR reactions to take place in a closed tube for their duration.

The PCR reaction occurs in 3 stages which are repeated for 30-40 cycles:

1. Denaturation: The PCR mixture is heated to 94-96°C causing dsDNA to denature into two ssDNA
2. Annealing: The mixture is cooled to 50-65°C allowing oligonucleotide primers to anneal to the 3’ end of the gene of interest
3. Elongation: The temperature is adjusted to that optimal for the DNA polymerase used; for Taq polymerase this is 65-75°C. The DNA polymerase uses available dNTPs to extend the primer strand in the 5’ to 3’ direction.

2.6.3 Real time quantitative polymerase chain reaction

Real time quantitative PCR uses fluorescent dyes or fluorescently labelled DNA probes which allow the amplification of DNA at each cycle to be monitored. The amount of DNA in the starting mixture can then be calculated from the amplification curve in relation to a housekeeping gene.

2.6.3.1 Non-specific detection: DNA-binding dyes

DNA amplification can be measured using intercalating DNA-binding dyes such as SYBR green. These dyes bind with dsDNA and only fluoresce when bound. Fluorescence therefore increases with each PCR amplification cycle and can be quantified. This method is non-specific as all dsDNA is bound by the dye, including nonspecific PCR products such as primer dimers.

2.6.3.2 Specific detection: Taqman qPCR

The Taqman method of qPCR employs a sequence-specific, fluorescently labelled oligonucleotide probe in addition to the sequence-specific PCR primers. The probe is labelled with a fluorescent reporter at the 5’ end and a quencher at the 3’ end. When in close proximity, the reporter is prevented from emitting photons by the quencher and so cannot fluoresce by fluorescence resonance energy transfer (FRET). After DNA is denatured and if the target sequence is present, the Taqman probe anneals to single DNA strands downstream of the PCR primer. Taq DNA polymerase then lengthens the primer strand in a 5’ to 3’ direction. When Taq polymerase encounters the bound probe it hydrolyses the probe, cleaving the fluorescent reporter, allowing it to move away from the quencher and fluoresce freely (Figure 2.4). The Taq polymerase then removes the rest of the probe and continues to the end of the strand. Each PCR cycle results in more reporter cleavage, resulting in accumulation of fluorescent reporters which is proportional to the amount of amplicon produced.
2.6.4 qPCR protocol

1. **Cell stimulation**: RAW 264.7 cells were seeded at 1.25x10^5 cells/well of 24 well plates and incubated overnight at 37°C. Cells were then stimulated for 6h or 18h.

2. **Cell lysis**: Supernatants were removed and cells were gently washed in PBS before lysis in Qiazol.

3. **RNA extraction**: RNA was extracted with chloroform and precipitated with isopropanol, then washed with ethanol. RNA quantification was performed by Nanodrop.

4. **Reverse transcription**: Reverse transcription was performed on 250ng RNA using a two-step method: RNA was first heated to 65°C with random primers and dNTPs to facilitate primer binding, then a mixture consisting of the enzymes MMLV-RT (a reverse transcriptase) and RNAse OUT (a ribonuclease inhibitor), as well as DTT (a reducing agent to loosen RNA secondary structures), was added and run on the reverse transcription programme:
   - 25°C for 10min
   - 37°C for 60min
   - 70°C for 15min
   - Samples were held at 4°C until use.

5. **qPCR**: Real time Taqman qPCR was performed on cDNA generated in step 4 using Brilliant III Ultra-Fast qPCR Master Mix, PCR primers and probe as outlined in Table 2.10. The qPCR programme held samples for 3 minutes at 95°C then ran 40 cycles of:
   - 15 seconds at 95°C
   - 20 seconds at 65°C
<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer/probe mix</th>
<th>Manufacturer</th>
<th>Manufacturer Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRDX1</td>
<td>Mm01621996_s1</td>
<td>Life Technologies (Applied Biosystems division)</td>
<td>Carlsbad, California, USA</td>
</tr>
<tr>
<td>TXNRD1</td>
<td>Mm00443675_m1</td>
<td>Life Technologies (Applied Biosystems division)</td>
<td>Carlsbad, California, USA</td>
</tr>
<tr>
<td>HO1</td>
<td>Mm00516005_m1</td>
<td>Life Technologies (Applied Biosystems division)</td>
<td>Carlsbad, California, USA</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Mm00446968_m1</td>
<td>Life Technologies (Applied Biosystems division)</td>
<td>Carlsbad, California, USA</td>
</tr>
<tr>
<td>TNF</td>
<td>Mm00443258_m1</td>
<td>Life Technologies (Applied Biosystems division)</td>
<td>Carlsbad, California, USA</td>
</tr>
</tbody>
</table>

Table 2.10: qPCR primer/probe mixes.
Figure 2.4: Reverse transcription and real time Taqman quantitative polymerase chain reaction

(A) Messenger RNA is isolated, dNTPs are available for reverse transcription (B) mRNA is reverse transcribed to create complimentary (c)DNA strands (C) Taqman qPCR primers bind gene of interest cDNA at 3’ end (D) Taqman probe anneals to single cDNA strands downstream of the PCR primer (E) Taq DNA polymerase lengthens the primer strand in a 5’ to 3’ direction. When Taq polymerase encounters the bound probe it hydrolyses the probe, cleaving the fluorescent reporter, allowing it to move away from the quencher and fluoresce freely (F) Taq polymerase continues to the end of the strand, removing the rest of the probe. Each PCR cycle results in more reporter cleavage, resulting in accumulation of fluorescent reporters which is proportional to the amount of amplicon produced. Original in colour.
2.6.5 qPCR Data analysis

Data obtained from qPCR can be analysed through two principle methods: absolute quantification and relative quantification. Absolute quantification uses a standard curve run alongside PCR samples to determine the input copy number. Reference gene copy numbers can be quantified for each sample to allow normalisation before the gene of interest copy number is calculated using the standard curve. Relative quantification also normalises sample gene of interest amplification to reference genes, but unlike absolute quantification the PCR signal of a treated sample is calculated in relation to an untreated, control sample rather than a standard curve.

In this project, relative quantification was used and so the Livak (2-ΔΔCt) method of analysis was used (Table 2.11). The raw data obtained from real time qPCR is an amplification graph for each sample (Figure 2.5). A cycle threshold (Ct) value is defined as the number of cycles required for the fluorescent signal to cross the threshold (exceed background fluorescence levels), 0.1 was used for all analyses in this project. By subtracting the reference gene Ct value from the gene of interest Ct value, the ΔCt is generated. Sample ΔCts are then subtracted from a single control sample ΔCt to determine the difference between them, known as ΔΔCt. To determine fold change, 2-ΔΔCt is calculated.

During this project data analyses were performed investigating the effect of cell stimulations on gene expression compared to control (untreated) cells. To enable multiple experimental repeats to be combined into a single analysis, ΔΔCt was calculated using a single control sample from one experiment to normalise each sample of all combined experiments. This enables multiple experiments to be analysed together, and maintains the variability of control sample populations so that statistical analysis can be performed.

<table>
<thead>
<tr>
<th></th>
<th>Gene of interest Ct</th>
<th>Housekeeping gene Ct</th>
<th>ΔCt vs. reference</th>
<th>ΔΔCt vs control</th>
<th>2-ΔΔCt FC vs control</th>
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<tr>
<td>Control</td>
<td>20.35</td>
<td>19.07</td>
<td>1.28</td>
<td>0.00</td>
<td>1.00</td>
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<tr>
<td>Treated</td>
<td>16.28</td>
<td>18.92</td>
<td>-2.64</td>
<td>-3.92</td>
<td>15.14</td>
</tr>
</tbody>
</table>

Table 2.11: Calculation of fold change (FC) by real time qPCR.
Figure 2.5: Amplification graphs from qPCR experiment

The data generated from qPCR can be visualised in amplification graphs. Fluorescence is plotted for each sample following each PCR cycle. The threshold cycle value (Ct value) is defined as the number of cycles required for the fluorescent signal to cross the threshold (exceed background) and here is shown by the horizontal blue line at 0.1. Ct values are then used to calculate gene expression. Original in colour.
2.7 Griess reaction assay

Griess assay was used in this project to quantify nitrite concentration in supernatants from stimulated cells.

The Griess reaction is an analytical method to detect nitrite ions in solution. The reaction forming the basis of the Griess reaction was originally described in 1858 by Johann Peter Griess in his quest to find novel dyes (533).

Nitric oxide is readily produced in cells, primarily via NOS enzymes which catalyse the oxidation of L-arginine to L-citrulline, producing NO as a byproduct. Oxidation of NO results in the generation of the more stable nitrite (NO$_2^-$) and nitrate (NO$_3^-$) species, which form a reservoir of bioactive NO which can be called upon as required (361). The Griess reaction allows quantification of nitrite using a two-step diazotization reaction; that is, the conversion of primary aromatic amines into diazonium salts. This occurs first by nitrite reacting with sulphanilamide to form a diazonium ion. This ion then couples with N-(1-naphthyl) ethylenediamine (NED) to form the pink coloured diazonium salt which can be read for absorbance at 540nm (Figure 2.6). A nitrite standard curve is used to calculate total nitrite in each sample.

2.7.1 Griess protocol

A Griess reagent system kit (see Table 2.2) was used for all assays. Supernatants from cell stimulations were added to a 96 well plate alongside a nitrite standard curve. Sulphanilamide (1% sulfanilamide in 5% phosphoric acid) (100μl) was added to samples for 10 minutes, then NED (0.1% NED in water) (100μl) was added for a further 10 minutes and kept out of direct light. Plates were read for absorbance at 540nm.
Figure 2.6: Mechanism of Griess reaction. Adapted from Giustarini et al 2008 (534). The Griess reaction allows quantification of nitrite using a two-step diazotization reaction: primary aromatic amines are converted into diazonium salts. Nitrite reacts with sulphanilamide to form a diazonium ion which then couples with N-(1-naphthyl) ethylenediamine (NED) to form the pink coloured diazonium salt which can be read for absorbance at 540nm.
2.8 Enzyme-linked immunosorbent assay

In this project sandwich enzyme-linked immunosorbent assay (ELISA) was used for quantification of cell supernatant TNF, and human serum PRDX2 and C-reactive protein (CRP) (Table 2.12).

ELISA is used to quantify proteins or antigens (analytes) in liquid samples. It was developed independently in two laboratories in 1971; those of Peter Perlmann and Eva Engvall in Sweden, and Anton Schuurs and Bauke van Weemen in the Netherlands (535, 536).

This technique uses a solid phase (a polystyrene ELISA plate) onto which proteins or antibodies are adsorbed, these are then bound by antibodies which enable a detection step. The most commonly used detection step uses biotinylated detection antibodies which are then bound with streptavidin conjugated with HRP. HRP then catalyses the oxidation of TMB into a blue TMB product. The reaction is stopped using sulfuric acid, which turns the reaction yellow (Figure 2.7).

Protein quantification by ELISA is fast, highly sensitive and specific compared with other techniques such as radiolabelling. It is therefore very widely used. However ELISA quantifies the total concentration of a particular protein and is unable to differentiate between protein subtypes such as proteins with different posttranslational modifications or oligomers.

<table>
<thead>
<tr>
<th>ELISA kit</th>
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<td>Mouse TNF-alpha DuoSet ELISA</td>
<td>DY410</td>
<td>31.3pg/ml</td>
<td>Research and Diagnostic Systems, Inc.</td>
<td>Minneapolis, Minnesota, USA</td>
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<tr>
<td>Human Peroxiredoxin 2 DuoSet ELISA</td>
<td>DY3489</td>
<td>0.78ng/ml</td>
<td>Research and Diagnostic Systems, Inc.</td>
<td>Minneapolis, Minnesota, USA</td>
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<tr>
<td>C Reactive Protein ELISA kit</td>
<td>EIA-1952</td>
<td>&lt; 1 µg/mL</td>
<td>DRG Diagnostics</td>
<td>Marburg, Germany</td>
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</tbody>
</table>

Table 2.12: ELISA kits.
Figure 2.7: TMB reaction mechanism

TMB substrate is oxidised by horseradish peroxidase (HRP) and converted into a blue TMB product. The reaction is stopped using sulfuric acid, which turns the reaction yellow and can be read for light absorbance at 450nm. Original in colour.
There are four types of ELISA (Figure 2.8):

2.8.1 Direct ELISA
The direct ELISA requires adsorption of the analyte of interest directly onto the ELISA plate via charge interactions. The plate is then blocked to prevent non-specific binding and an enzyme-linked antibody is applied, which binds with the analyte. A solution of enzyme substrate is applied, enabling catalysis which results in a colour change. Absorbance can then be measured and a standard curve is used to determine analyte concentration.

2.8.2 Indirect ELISA
The indirect ELISA is essentially the same as the direct ELISA, except that the primary antibody is not enzyme-linked. Instead a secondary enzyme-linked antibody is applied to bind to the Fc region of the primary antibody. This can amplify the signal as more than one secondary antibody can bind each primary antibody, and also is cost effective as the enzyme-linked antibody can be used in multiple ELISAs.

2.8.3 Sandwich ELISA
The sandwich ELISA is used when the analyte of interest cannot adsorb to the ELISA plate itself, instead a capture antibody is required. A known amount of the capture antibody adsorbs to the plate and the antigen binds with this. The primary antibody is then applied to bind with the analyte, followed by either an enzyme-linked secondary antibody targeted to the Fc region of the primary antibody, or a biotinylated secondary antibody which is then bound by HRP-linked streptavidin. The TMB substrate is then applied and absorbance measured.

2.8.4 Competitive ELISA
The competitive ELISA is a slightly more complex variation of the ELISA technique, and is used when the analyte is too small for two antibodies to bind it, or if only one antibody can be raised against it. In this technique, the antibody is first incubated in solution with the analyte. The antibody/analyte solution is then applied to an ELISA plate pre-coated with a known amount of analyte. Any unbound antibodies will bind with the plate-bound analyte, whilst antibody/analyte complexes will be removed in wash steps. A secondary, enzyme-linked antibody and substrate are then applied as with other ELISA variations. The resulting signal output inversely correlates with the amount of antigen in the sample (537).
There are four variations of the ELISA technique. Direct, indirect and competitive ELISAs all use analyte which is directly adhered to the plate. Direct ELISA primary antibody can be detected directly; indirect ELISA requires a secondary antibody to enable detection, competitive ELISA removes antigen-bound antibody before being applied to the plate and so output inversely correlates with the amount of antigen in the sample. Sandwich ELISA, which was used in this project, is similar to the indirect ELISA but uses a plate-bound capture antibody to bind analytes. Original in colour.
Chapter 3
3. Peroxiredoxin and Thioredoxin
Release Following Toll-Like
Receptor Stimulation

3.1 Introduction
Circulating levels of the oxidoreductase enzymes PRDX and TXN are elevated in a variety of diseases [see 1.3.5]. In the extracellular environment these enzymes have proinflammatory properties and act as redoxkines (491, 493, 494, 538). Therefore, understanding the triggers for release of these enzymes is of key importance for understanding their roles in inflammation.

The first paper to suggest that inflammatory stimuli induced active PRDX release from live cells rather than release as a product of cell necrosis was in 2014 when Liu et al stimulated the immortalised human bronchial epithelial cell line BEAS-2B with LPS for 12h and assessed PRDX1 release using Western blot and ELISA (538). At the same time, Salzano et al published a paper which aimed to identify glutathionylated proteins that were secreted from the murine macrophage-like cell line RAW 264.7 following 24h LPS stimulation. Using mass spectrometry they identified PRDX2 as a released protein. Further, Western blot and ELISA were used to show that TXN1 was co-released with PRDX2, which was released in an oxidised, glutathionylated form. It was also suggested that PRDX2 release occurred through a non-classical secretory pathway similar to HMGB1 (491). The following year, Mullen et al showed that PRDX1 and PRDX2 were both released as oxidised dimers, and that the active site cysteine residues (Cys51 and Cys172 in PRDX2, and Cys52 and Cys173 in PRDX1) were essential for release. Ultracentrifugation was used to isolate exosomes from cell supernatants and Western blot showed that PRDX1 and PRDX2 were associated with this fraction and increased in response to LPS and TNF stimulation (197). PRDX1 has also been identified as one of the 100 most common proteins identified in exosomes (539).

To date, investigation into PRDX1/2 and TXN1 release has focussed on stimulation of TLR4 with LPS. However, studies comparing protein changes with different TLR
stimulations are limited: in 2014, Sjoelund et al compared phosphorylation signalling cascades activated following up to 30min TLR2, TLR4 and TLR7 stimulation of immortalized macrophages from C57BL/6 mice (540). Using this model they were able to show that the phosphoproteomic response differed for each TLR: only TLR2 and TLR4 induced phosphorylation of proteins involved with phagocytosis, and the kinetics of phosphorylation involved with endocytosis differed between TLR2 and TLR4. Building on these findings in 2016, Tarasova et al characterised whole proteome changes following stimulation of each TLR in the human monocytic cell line THP-1, and found that monocyte activation and differentiation are induced to different levels with stimulation of different TLRs (541). Taken together, these findings demonstrate that although all TLRs culminate in the activation of a limited number of transcription factors such as NF-κB, each TLR fulfils a unique role using distinct signalling pathways. Therefore it seems likely that stimulation of different TLRs will have differing effects on PRDX1/2 and TXN1 release.

In contrast to the TLR-induced intracellular protein pool (the proteome), the secreted protein pool (the secretome, encompassing proteins secreted through both classical and non-classical pathways, exosomes, and through membrane shedding) has been less well characterised and is an emerging area of research. Exosome release following TLR stimulation has been investigated by a number of groups. In 2016, Szul et al and Zhou et al showed that stimulation of TLR4 and TLR3 respectively induce exosome release (194, 195). Szul et al also showed that RNA interference of TLR4 in murine airway epithelial cells abrogated this release. In 2017, Srinivasan et al used a human ovarian adenocarcinoma cell line (HEY) to show that exosomes derived following TLR3 or TLR4 stimulation could be isolated and applied to distal cells. Microarray analysis was then used to assess the resulting distal cell activation, which was found to relate to the TLR stimulation of the cell of origin (196). Exosomes isolated from TLR3-stimulated cells induced activation of NF-κB and chemokine signalling pathways in distal cells, and skewed macrophages to an M1 phenotype, whilst exosomes isolated from TLR4-stimulated cells induced LPS tolerance in distal cells.

PRDX1 and TXN1 are known to be released from the RAW 264.7 macrophage-like cell line following TLR4 stimulation (197, 491), and exosome release has been documented following TLR3 and TLR4 stimulation (194, 195). The questions we hoped to address in this chapter were if release of PRDX1 and TXN1 is a property unique to TLR4, or if it is a property shared by some or all of the TLRs. Also, if
release of PRDX1 and TXN1 correlates with that of the exosomal marker Hsp70 (542-544). To address these questions a RAW 264.7 cell model was used. PRDX1 release was first assessed using increasing concentrations of TLR agonists. The resulting concentrations were then tested for effects on cellular activation and viability by assessing TNF production (ELISA), and cell metabolism (MTT assay) respectively. SDS-PAGE and Western blot of cell supernatants were used to assess PRDX1, TXN1, and Hsp70 release.

3.2 Aims

- Establish working concentrations of TLR agonists to ensure cellular activation without loss of cell viability
- Assess PRDX1 and TXN1 release from RAW 264.7 cells following 24h stimulation with each TLR agonist
- Investigate if TLR-induced PRDX1 and TXN1 release correlates with TLR-induced release of the exosome marker Hsp70
3.3 Results

3.3.1 Optimisation of TLR agonist concentrations

Cellular release of PRDX1 and TXN1 is induced following TLR4 stimulation with LPS (491). This chapter aims to build on this knowledge by investigating PRDX1 and TXN1 release in response to stimulation of the other TLR family members.

To investigate the concentration of each TLR agonist required to induce PRDX1 release, RAW 264.7 cells were stimulated for 24h with increasing concentrations of each TLR agonist. Cell supernatant Western blots for PRDX1 and TXN1 showed that release was highest in response to stimulation of the cell membrane TLRs (TLR1/2, TLR2/6 and TLR4), and to TLR3 (Figure 3.1 and 3.2). Even at high concentrations (10µg/ml), CL075 (TLR7/8) failed to elicit any PRDX1 release. Stimulation of the remaining endosomal TLRs (R848, TLR8 and ODN2216, TLR9) induced slight PRDX1 release. Furthermore, PRDX1 release following all TLR stimulations was as oxidised dimers, this is the state previously observed with TLR4 stimulation (197). The lowest concentrations to elicit PRDX1 release were used for all subsequent experiments.

To ensure that the observed PRDX1 release was not due to cell necrosis, TLR agonist concentrations that elicited sufficient release were assessed for their effect on cell viability (Figure 3.3). These results show that viability was affected very little following stimulation in cell culture media (Figure 3.3A), but there was increased variability in viability when serum-free OptiMEM was used: Pam3 and ODN2216 caused a statistically significant decrease (P<0.05 vs untreated cells) of 19.3% and 19.5% respectively (Figure 3.3B).

To ensure that the tested TLR agonist concentrations were sufficient to activate proinflammatory responses, TNF secretion was measured by ELISA (Figure 3.4). In both cell culture media and serum-free OptiMEM it was established that TNF was secreted in response to each TLR agonist.
Figure 3.1: Optimisation of TLR agonist concentrations for PRDX1 release.

Western blot was used to analyse PRDX1 protein release from RAW 264.7 cells stimulated for 24h with TLR agonists at increasing concentrations in serum-free OptiMEM. Supernatants (23μl) from three separate samples were loaded onto gels for each condition. Duplicate blots are shown from two independent experiments.
Figure 3.2: Optimisation of TLR agonist concentrations for TXN1 release.

Western blot was used to analyse TXN1 protein release from RAW 264.7 cells stimulated for 24h with TLR agonists at increasing concentrations in serum-free OptiMEM. Supernatants (23μl) from three separate samples were loaded onto gels for each condition. Duplicate blots are shown from two independent experiments.
Figure 3.3: TLR agonists have limited effect on cell viability.

MTT assay was used to determine effect of TLR agonists on RAW 264.7 cell viability. MTT assay was performed following 24h TLR stimulations in (A) cell culture media \((n=5)\), or (B) serum-free OptiMEM \((n=6-11)\). TLR agonist concentrations: Pam3= 1μg/ml, FSL-1= 0.2μg/ml, LPS= 10ng/ml, Poly IC= 12.5μg/ml, CL075= 10μg/ml, R848= 1μg/ml, ODN2216= 5μM. Bars represent mean±SD.

*P<0.05 vs untreated cells by one-way ANOVA and Dunnett’s multiple comparisons test.
Figure 3.4: TNF is secreted by RAW 264.7 cells in response to all TLR stimulations.

TNF secretion in response to TLR agonists was assessed to confirm RAW 264.7 cell activation. Cells were stimulated with TLR agonists for 24h in (A) cell culture media (n=3), and (B) serum-free OptiMEM (n=6). Supernatant TNF was quantified by ELISA. Dotted line depicts assay sensitivity limit. TLR agonist concentrations: Pam3= 1μg/ml, FSL-1= 0.2μg/ml, LPS= 10ng/ml, Poly IC= 12.5μg/ml, CL075= 10μg/ml, R848= 1μg/ml, ODN2216= 5μM. Bars represent mean±SD. **P<0.01, ***P<0.001 and ****P<0.0001 vs untreated cells by one-way ANOVA and Dunnett’s multiple comparisons test.
3.3.2 PRDX and TXN release in response to different TLR agonists

Following optimisation of TLR agonist concentrations, the effect of each TLR agonist on PRDX1 and TXN1 release could be established. Cells were stimulated with TLR agonists for 24h in serum-free OptiMEM. SDS-PAGE and Western blot were used to assess PRDX1 and TXN1 protein release. All Western blots were analysed by densitometry. These results are shown for each agonist on paired graphs, where each line is representative of a single Western blot (Figures 3.5, 3.6 and 3.7). Densitometry values are expressed as percentage of control.

These results show that PRDX1 and TXN1 release occurred primarily in response to Poly IC (TLR3) and LPS (TLR4). Low PRDX1 and TXN1 release was observed with the other TLRs, particularly Pam3 (TLR1/2) and FSL-1 (TLR2/6), but Poly IC and LPS were the only TLR agonists to reliably induce greater release and achieve statistical significance.
Figure 3.5: Densitometry analysis of supernatant Western blots.

(A) Western blot densitometric analysis was performed using ImageJ software. (B) Average densitometry of the control samples was then calculated, which was then used to calculate the percentage of control for each sample on that blot. (C) Results are shown for each agonist on paired graphs, where each line is representative of a single Western blot.
Figure 3.6: TLR3 and TLR4 stimulation induce PRDX1 release.

PRDX1 protein release from RAW 264.7 cells stimulated for 24h with TLR agonists in serum-free OptiMEM. Western blot was performed on cell supernatants and densitometric analysis was performed on each blot. Each point represents one condition within a single Western blot (n=3), mean±SD. Each line therefore represents the change in release from untreated cells on an individual Western blot. Densitometry values are expressed as percentage of control. TLR agonist concentrations: Pam3= 1μg/ml, FSL-1= 0.2μg/ml, LPS= 10ng/ml, Poly IC= 12.5μg/ml, CL075= 10μg/ml, R848= 1μg/ml, ODN2216= 5μM.

*P<0.05 vs untreated cells by Wilcoxon matched-pairs signed rank test.
Figure 3.7: TLR3 and TLR4 stimulation induce TXN1 release.

TXN1 protein release from RAW 264.7 cells stimulated for 24h with TLR agonists in serum-free OptiMEM. Western blot was performed on cell supernatants and densitometric analysis was performed on each blot. Each point represents one condition within a single Western blot (n=3). Each point represents one condition within a single Western blot (n=3), mean±SD. Each line therefore represents the change in release from untreated cells on an individual Western blot. Densitometry values are expressed as percentage of control. TLR agonist concentrations: Pam3=1μg/ml, FSL-1= 0.2μg/ml, LPS= 10ng/ml, Poly IC= 12.5μg/ml, CL075= 10μg/ml, R848= 1μg/ml, ODN2216= 5μM.

*P<0.05 vs untreated cells by Wilcoxon matched-pairs signed rank test.
3.3.3 PRDX and TXN release corresponds with Hsp70 release

Our lab previously found that PRDX1 and TXN1 are released via exosome (197, 491). These experiments aim to investigate if release of the exosomal marker Hsp70 correlates to PRDX1 and TXN1 release. A disparity between Hsp70 release and PRDX1/TXN1 release could suggest that these proteins are specifically incorporated into exosomes following certain TLR stimulations. Conversely, consistent release of all three proteins together suggests that PRDX1 and TXN1 are constituent exosome components.

Western blot for PRDX1, TXN1 or Hsp70 showed that Hsp70 was released following TLR stimulation in the same pattern as PRDX1 and TXN1: primarily in response to Poly IC and LPS (TLR3 and TLR4 respectively) (Figure 3.8), suggesting that PRDX1 and TXN1 are constituent exosome components and that exosome release predominantly occurs in response to these TLRs.
Figure 3.8: Exosome marker Hsp70 release corresponds to PRDX1 and TXN1 release.

Western blot analysis of released PRDX1 (A), TXN1 (B), and Hsp70 (C) protein from RAW 264.7 cells stimulated with LPS (TLR4), FSL-1 (TLR2/6) or Poly IC (TLR3) for 24h in serum-free OptiMEM. Representative blots of 4 experimental repeats. TLR agonist concentrations: FSL-1= 0.2μg/ml, LPS= 10ng/ml, Poly IC= 12.5μg/ml.
3.4 Results summary

The aims of this chapter were to investigate the release of PRDX1 and TXN1 from RAW 264.7 cells in response to stimulation of each TLR. TLR agonist concentrations were first titrated to investigate which concentrations were required to induce release. The lowest concentrations to induce release were then tested to ensure sufficient cell activation (TNF secretion) was achieved without causing toxicity. Optimised TLR agonist concentrations were tested on cells to assess PRDX1 and TXN1 release, and whether or not this coincided with Hsp70 release (an exosome marker). These findings show that PRDX1, TXN1 and Hsp70 release occurred primarily in response to TLR3 and TLR4 stimulation (Poly IC and LPS respectively), though low levels of release occurred following stimulation of the other TLRs. PRDX1 was released as oxidised dimers following stimulation of all TLRs.

3.5 Discussion

TLR3 and TLR4 are the only TLRs documented to induce exosome release (194-196). The results presented in this chapter show that of the TLRs, TLR3 and TLR4 stimulation caused the greatest PRDX1 and TXN1 release (Figures 3.6 and 3.7). These are the only TLRs to use the TRIF intracellular signalling pathway. Interestingly, the TRIF signalling pathway culminates in the activation of IRF-3 and induction of Type I IFNs, which are traditionally known as anti-viral cytokines, and exosomes have been found to confer anti-viral activity between cells (545-547). PRDX1 and PRDX2 have also been reported to have antiviral activity following HIV infection (548).

A recent study by Koppenol-Raab et al used mass spectrometry based proteomics to assess the secretome and proteome of RAW 264.7 cells following stimulation of TLR2, TLR4 and TLR7 for up to 24h (549). The authors found that 298 secretome proteins were common to all three TLR stimulations, but an additional 19 secretome proteins were distinct for TLR2, 324 for TLR4 and 106 for TLR7, demonstrating that each TLR uniquely affects secretion. Furthermore, this study identified PRDX1 as one of the 298 secretome proteins common to all three TLR stimulations. Traditional mass spectrometry directly identifies molecules, but does not give an indication of their quantity. The results presented in this thesis build on these results by providing semi-quantitative data showing that low amounts of PRDX1 are indeed released in response to TLR2 and TLR7, but much greater release is observed with TLR4, and also with TLR3 stimulation which was not investigated by Koppenol-Raab.
Furthermore, these results show that all PRDX1 release was in the oxidised, dimeric formation, which does not alter between TLRs.

This work was carried out using RAW 264.7 cells, which express all TLRs with the exception of TLR5 and are a good *in vitro* model of innate inflammatory responses (518). It would be interesting for future work to build on these findings by investigating PRDX1 and TXN1 release from human primary monocytes and macrophages in response to TLR stimulation.

SDS-PAGE and Western blot were used to investigate oxidoreductase release in this chapter to allow differentiation of oxidative states. However, there are a number of limitations with this technique. Firstly, to perform Western blot analysis of cell supernatants and avoid extensive non-specific antibody binding, cells had to be cultured in serum-free OptiMEM. This media was not optimal for maintaining cell viability, particularly with Pam3 and ODN2216 stimulations (Figure 3.3). Oxidoreductase release may therefore have increased in these populations due to apoptosis or necrosis. However, the release observed with Pam3 and ODN2216 was low compared to LPS- and Poly IC-induced release, with which cell viability was not an issue.

A further limitation of Western blot is that it is a semi-quantitative technique. In this project Western blot was utilised to observe the oxidative state of oxidoreductases, but it would be interesting to use a more quantitative technique such as ELISA. This is a sensitive and specific technique which would allow direct oxidoreductase quantification. One consideration for using ELISA for PRDX1 and TXN1 is their localisation within exosomes, which can limit antibody binding in an ELISA [see Chapter 6].

Hsp70 is a commonly used exosome marker (542-544). However recent research has shown that it is expressed by other extracellular vesicles such as microvesicles (550). Likewise, PRDX1 and PRDX2 have been identified in both exosomes and microvesicles (197, 551). Therefore further research is required to confirm if TLR-induced oxidoreductase release is via exosomes, microvesicles, or both. This could be achieved using ultracentrifugation to separate the two extracellular vesicle populations based on size. Furthermore, it would be interesting to confirm if these proteins are specifically incorporated into exosomes or their inclusion is simply due to the high abundance of these proteins in cells (463).
The envelopment of PRDX and TXN in extracellular vesicles, and the oligomeric formation of PRDX as dimers rather than decamers, suggests that this TLR-induced release is not intended to take advantage of their role as proinflammatory DAMPs, but is a more controlled method of intercellular communication (468). Recent research has shown that TLR3 and TLR4 mediated exosome release can communicate anti-microbial activity amongst cells (195, 196, 552), and prime distal cells for stressors such as oxidative stress (553). Although the majority of exosomal communication has been attributed to RNA transfer (196, 553), proteins such as the anti-viral stimulator of IFN genes (STING) also have roles in intercellular communication (554). The inclusion of PRDX1 and TXN1 may prime distal cells for impending oxidative stress, the result of microbial defence. Alternatively, their inclusion could be a mechanism of redox signalling whereby the oxidised proteins are delivered into recipient cells to spark a thiol-disulphide exchange reaction cascade to communicate proinflammatory signals.

To gain a more complete view of the role of oxidoreductases in intercellular communication, it would be interesting to investigate release of other oxidoreductase enzymes, particularly TXNRD1 which contains a secretion signal peptide and has been reported in microvesicles (551), and SRX which has also been associated with pathogenesis of oxidative-stress mediated diseases (555).

The results presented in this chapter show that PRDX1, TXN1, and Hsp70 are released following TLR stimulation, with the greatest release occurring in response to TLR3 and TLR4 stimulation. Further research is required to define which extracellular vesicles these proteins are released through, and the exact function of this release.
Chapter 4
4. Nrf2 target gene expression following Toll-like receptor stimulation

4.1 Introduction

Many proteins of the thioredoxin fold family, including PRDX1, TXN1, and TXNRD1 are transcribed by Nrf2, which is often described as a master regulator of redox state [see Table 1.3] (270, 556). Control of cellular redox state enables Nrf2 to regulate inflammatory responses through redox-sensitive transcription factors, such as NF-κB, and via redox signalling (304, 319). Increased understanding of oxidoreductase transcription will enable a deeper understanding of their regulation and contribution to inflammatory responses.

Nrf2 can be activated through a variety of mechanisms [see 1.1.7], including via TLR stimulation. In 2011, Vijayan et al used Nrf2 immunofluorescent staining in a RAW 264.7 cell model to show that TLR4 stimulation with LPS activates Nrf2 nuclear translocation, which can be abrogated with use of a TLR4 pathway inhibitor, LFM-A13 (557). The authors also used a luciferase-based ARE reporter system and qPCR to show that RAW 264.7 cell stimulation of TLR2/6, TLR4, TLR7 and TLR9, but not TLR3, upregulated expression of the archetypal Nrf2 gene HO1, which was abrogated by LFM-A13 treatment. Another paper by Yin et al showed that stimulation of TLR2, TLR3, and TLR4 caused increased HO1 protein expression in RAW 264.7 cells, but HO1 gene expression was not directly investigated (558).

As mentioned in Chapter 3, although there are a limited number of adapter proteins involved in TLR signalling, each TLR has a distinct role. Demonstrating this, in 2009 Amit et al performed genome-wide expression profiling of murine bone marrow dendritic cells (BMDCs) stimulated with TLR2, TLR3, TLR4, TLR7 and TLR9 agonists (559). They showed that the TLRs can be divided into two groups based on which of the 1800 genes affected by TLR stimulation were induced: inflammatory-like (enriched for NF-κB and inflammatory responsive genes), and anti-viral-like (enriched for IRFs, viral- and interferon-responsive genes). TLR4 responses were found to be a combination of the two. Although some TLRs showed a large degree of overlap (82% of TLR7 and TLR9 responses overlapped with TLR4
responses), stimulation of each TLR gave a distinct expression signature and a small number of genes were specific to a single stimulus. In 2011, Chevrier et al showed that in murine BMDCs, 280 genes encoding signalling proteins were differentially expressed with TLR2, TLR3 or TLR4 stimulations, showing that TLR signalling pathways diverge to affect a wide variety of target genes (560).

The results from Chapter 3 show that release of PRDX1 and TXN1 proteins occurred primarily in response to TLR3 and TLR4 stimulation. Previous research in our lab and others showed that TLR4 stimulation of RAW 264.7 cells induced PRDX1, TXNRD1, and HO1 gene expression but failed to elicit significant induction of TXN1 (Figure 4.1, (549, 561). Building on these results, this chapter aims to investigate gene expression of PRDX1 and TXNRD1 in response to each member of the TLR family. Expression of HO1 will also be assessed as a benchmark of Nrf2 activation. This research will enable a better understanding of the role of PRDX1 and TXNRD1 in the context of innate inflammation and their relationship with each TLR.

4.2 Aims

- Assess PRDX1, TXNRD1 and HO1 gene expression in RAW 264.7 cells following stimulation of each TLR
- Investigate the mechanisms of Nrf2 activation by assessing the effect of ROS and protein kinase inhibition on PRDX1, TXNRD1 and HO1 gene expression
Figure 4.1: Protein thiol-disulphide oxidoreductase gene expression is induced by TLR4 stimulation.

Microarray analysis of oxidoreductase gene expression following 6h LPS (10ng/ml) stimulation in RAW 264.7 cells. Data are expressed as fold change vs untreated cells (n=3). Bars represent mean±SD.

Data were obtained from reanalysis of microarray data from Diotallevi et al (561)**

** Raw data in standard format from the microarray experiment have been deposited in the Gene Expression Omnibus (GEO) Database of NCBI (http://www.ncbi.nlm.nih.gov/geo) and are accessible through GEO series accession number GSE79397.
4.3 Results

4.3.1 Oxidoreductase gene expression following TLR stimulation

Investigation of oxidoreductase gene expression in response to TLR stimulation showed *PRDX1* increased on average 2.5 fold compared with untreated cells in response to LPS alone. No other TLR agonist induced *PRDX1* gene expression (Figure 4.2A). In contrast, both *TXNRD1* and *HO1* were increased in response to CL075 (TLR7) and R848 (TLR8) in addition to LPS (TLR4), and *HO1* expression also increased with Pam3 (TLR1/2) and Poly IC (TLR3), showing that each of these genes are controlled separately (Figures 4.2B and 4.2C).

To confirm that TLR4 signalling rather than an off-target effect of LPS stimulation induced this expression, a synthetic TLR4 agonist, neoseptin, was used to stimulate RAW 264.7 cells for 6h (Figure 4.3). Neoseptin induced expression of each of the investigated oxidoreductase genes to an equal or greater extent than LPS, confirming that a TLR4 pathway is responsible for this induction.
Figure 4.2: Oxidoreductase expression induced by agonists of different Toll-like receptors.

Real time Taqman qPCR analysis of PRDX1 (A), TXNRD1 (B) and HO1 (C) gene expression in RAW 264.7 cells stimulated for 6h with TLR agonists. Data were normalised for HPRT1 and expressed as fold change vs one control sample (n≥4). Bars represent mean±SD. TLR agonist concentrations: Pam3= 1μg/ml, FSL-1= 0.2μg/ml, LPS= 10ng/ml, Poly IC= 12.5μg/ml, CL075= 10μg/ml, R848= 1μg/ml, ODN2216= 5μM.

****P<0.0001, ***P<0.001, **P<0.01, *P<0.05 vs control by one-way ANOVA followed by Dunnett’s multiple comparisons test.
Figure 4.3: PRDX1, TXNRD1 and HO1 gene expression is increased with the TLR4 agonist neoseptin.

Real time Taqman qPCR analysis of PRDX1 (A), TXNRD1 (B) and HO1 (C) gene expression in RAW 264.7 cells stimulated for 6h with LPS (10ng/ml) or neoseptin (25μM). Gene expression values were normalised for HPRT1 and expressed as fold change vs one control sample (n=12). Bars represent mean±SD.

****P<0.0001, ***P<0.001 vs control by one-way ANOVA followed by Tukey’s multiple comparisons test.
4.3.2 Involvement of ROS with oxidoreductase gene expression
TLR4 stimulation induces production of ROS, which could activate Nrf2 as it is a redox-sensitive transcription factor (288, 479, 562, 563). Therefore, to investigate the effect of ROS on oxidoreductase gene expression, RAW 264.7 cells were incubated with the antioxidant N-acetyl-cysteine (NAC) for 1h prior to stimulation with LPS or PMA for 6h. PMA is a strong inducer of ROS (564) and so we hypothesized that if ROS are involved, PMA should activate Nrf2 and therefore increase oxidoreductase gene expression, whilst the antioxidant NAC should abrogate this response. PMA treatment was found to induce expression of all oxidoreductases, to a similar extent as LPS (Figure 4.4). PRDX1 and TXNRD1 gene expression were unaffected by NAC treatment suggesting that these genes are not ROS regulated. However, HO1 gene expression decreased with NAC treatment of LPS- and PMA- stimulated cells, suggesting that ROS are involved with regulation of HO1 gene expression (Figure 4.4C).
Figure 4.4: NAC reduces LPS- and PMA-induced HO1 gene expression.
Real time Taqman qPCR analysis of PRDX1 (A), TXNRD1 (B) and HO1 (C) gene expression in RAW 264.7 cells stimulated for 6h with LPS (10ng/ml) or PMA (500ng/ml), with or without NAC (10mM). Data were normalised for HPRT1 and expressed as fold change vs one control sample (n=12). Bars represent mean±SD. **P<0.01, *P<0.05 vs control by two-way ANOVA and Tukey’s test for multiple comparisons.
4.3.3 Involvement of protein kinases in the induction of oxidoreductase gene expression

As NAC treatment failed to inhibit PMA-induced *PRDX1* and *TXNRD1* expression, it appeared that another target of PMA must be involved. PMA is structurally similar to diacylglycerol, the natural ligand for PKC, and is a potent PKC activator. There are many isoforms of PKC, each with a specific role and many of which have been linked to the TLRs, particularly TLR4 (see Table 1.2, (565). LPS stimulation activates most PKC isoforms, whilst PKC activation can reverse LPS tolerance (173, 178, 193). Furthermore, PKC can directly phosphorylate and activate Nrf2 (297).

Staurosporine is a pan-specific protein kinase inhibitor, including PKC. RAW 264.7 cells were incubated with staurosporine for 30 mins prior to the addition of LPS or PMA for 6h. Staurosporine only decreased LPS-induced *HO1* expression, indicating that protein kinase-mediated phosphorylation is required for LPS-induced *HO1* expression, but not for *PRDX1* or *TXNRD1* (Figure 4.5). Staurosporine also decreased PMA-induced expression of all oxidoreductases, suggesting that protein phosphorylation is required for PMA-induced gene expression.
Figure 4.5: *PRDX1*, *TXNRD1* and *HO1* gene expression is increased with PMA stimulation, and reduced with protein kinase inhibitor staurosporine.

Real time Taqman qPCR analysis of *PRDX1* (A), *TXNRD1* (B) and *HO1* (C) gene expression in RAW 264.7 cells stimulated for 6h with LPS (10ng/ml), PMA (500ng/ml) with or without staurosporine (100nM). Gene expression values were normalised for *HPRT1* and expressed as fold change vs one control sample (n=12). Bars represent mean±SD.

***P<0.001, *P<0.05 vs control by two-way ANOVA and Tukey’s test for multiple comparisons.
4.4 Results summary

The primary aims of this chapter were to investigate PRDX1, TXNRD1, and HO1 gene expression in response to different TLR agonists, and to assess any differences in induction of each of these genes. These results show that PRDX1, TXNRD1, and HO1 are all induced by LPS and by the synthetic TLR4 agonist neoseptin (Figures 4.2 and 4.3). However, there are differences in the extent of gene induction; HO1 is typically induced about 4 fold following LPS stimulation, and PRDX1 and TXNRD1: 1.5-2.5 fold. Analysis of gene expression following stimulation with each of the TLRs showed that PRDX1 was only induced in response to TLR4, whereas TXNRD1 was induced in response to TLR4, TLR7/8 and TLR8, and HO1 to TLR1/2, TLR3, TLR4, TLR7/8, and TLR8 (Figure 4.2).

Incubation with the antioxidant NAC did not affect LPS- or PMA-induced PRDX1 or TXNRD1 gene expression suggesting that ROS are not involved with these pathways, but it did reduce LPS- and PMA-induced HO1 expression (Figure 4.4). Incubation with the pan-specific kinase inhibitor staurosporine decreased all PMA-induced gene expression, and also LPS-induced HO1 expression indicating the requirement for protein phosphorylation, likely via PKC, in these pathways (Figure 4.5).
Figure 4.6: PRDX1, TXNRD1 and HO1 gene expression are induced through different mechanisms.

(A) Induction of PRDX1 gene expression occurs in response to TLR4 stimulation with LPS or neoseptin, or via direct Nrf2 activation with PKC (297). (B) TXNRD1 gene expression is induced with TLR4, TLR7, and TLR8 stimulation, as well as PKC-mediated activation of Nrf2. (C) HO1 gene expression is induced by TLR1/2, TLR3, TLR4, TLR7, and TLR8. TLR1/2 and TLR4 are both able to translocate and signal from the endosome. PKC can directly activate Nrf2, and both PKC and ROS appear to be involved with TLR4-mediated gene induction. Original in colour.
4.5 Discussion

The results from this chapter demonstrate that although PRDX1, TXNRD1, and HO1 are all Nrf2 transcribed genes containing the ARE region, their expression is differentially regulated. The gene expression profiles of each of the investigated oxidoreductases are sufficiently different to suggest that separate regulatory mechanisms are in place for each gene. This could be at the level of histone modification, transcriptional repressors, or other inhibitors of the polymerase complex formation, which are common gene regulation mechanisms (566).

Although HO1 is the archetypal Nrf2 target gene, the results from this chapter show that each of the target genes investigated behave quite differently to TLR stimulation, with five TLRs causing HO1 expression but only TLR4 inducing expression of PRDX1. These results further confirm that various regulatory mechanisms are likely to be in place for these genes.

Previous studies of the effect of TLR ligands on HO1 gene expression are conflicting. A 2011 paper by Vijayan et al. showed RAW 264.7 cell HO1 expression was induced in response to TLR2/6, TLR4, TLR7, and TLR9, but not to TLR3 stimulation (557). Then in 2015 Yin and Cao showed that TLR2, TLR3 and TLR4 stimulation induced HO1 protein expression in RAW 264.7 cells after 18h (567). The results from this chapter show that HO1 was induced in response to TLR1/2, TLR3, TLR4, TLR7/8, and TLR8, which are all endosomal TLRs or TLRs capable of signalling from this compartment (Figure 4.2C, (146-149). These results are in contrast to the paper by Vijayan et al., but agreement with Yin and Cao. With the exception of LPS and Poly IC, the TLR agonists used differed in each study: the TLR2 agonist used by Yin and Cau was peptidoglycan; Vijayan et al. used lipoteichoic acid (TLR2/6); whereas Pam3 (TLR1/2) or FSL-1 (TLR2/6) were used in this project.

PRDX1 gene expression was uniquely induced by TLR4 stimulation and no other TLR. The synthetic TLR4 agonist neoseptin was used to confirm that TLR4 stimulation, rather than an off-target effect, was responsible for gene expression. Experiments using the antioxidant NAC showed that NAC had no effect on LPS-induced PRDX1 nor TXNRD1 expression, but did reduce HO1 expression suggesting that this is a more redox-sensitive gene (Figure 4.4). Further work investigating the redox-sensitivity of these genes would be interesting. It is known that PRDX1, TXNRD1 and HO1 are induced by oxidative stress, but the results
presented here suggest that some genes may be more susceptible to changes in redox state than others (568).

In this chapter, PMA was used in its capacity as an inducer of ROS to investigate its effect on gene expression. It was found that PMA could induce expression of all oxidoreductase genes investigated, and that, with the exception of HO1, this was independent of its role of ROS inducer as NAC did not reduce its effect (Figure 4.4). Instead, it was found that protein kinases activated by PMA were involved in the gene induction, as induction was inhibited by staurosporine (Figure 4.5). Staurosporine also inhibited LPS-induced expression of HO1 (Figure 4.5C). Many PKC isoforms are involved with the TLR4 signalling pathway (Table 1.2), and therefore may be involved with signalling for HO1 gene expression but not for PRDX1 or TXNRD1 as LPS-dependent induction was unaffected by staurosporine for these genes. It would be interesting for future work to investigate the involvement of different PKC isoforms in this pathway.

The significant reduction in PMA-induced expression of all oxidoreductase genes when staurosporine was used indicates that the pathway used by PMA for induction of PRDX1 and TXNRD1 is much more reliant on protein kinase activation than the pathway used by TLR4. For example, PMA-induced gene expression may be a result of direct Nrf2 activation: one PKC isoform, PKCe has already been identified to directly phosphorylate Nrf2 (297, 569).

In conclusion, the results from this chapter have demonstrated that, although each of these genes is transcribed by Nrf2, different regulatory mechanisms are in place to fine-tune their expression (Figure 4.6). The intracellular signalling pathways used by the TLRs are highly complex. This work gives a small insight into two of the mechanisms used in the TLR-Nrf2 pathway to regulate oxidoreductase expression, namely redox signalling and protein kinases.
Chapter 5
5. The Effect of Nitric Oxide on Peroxiredoxin Production

5.1 Introduction

Chapters 3 and 4 explored the regulation of PRDX1 protein release and gene expression in TLR stimulated cells, finding that TLR4 is implicit to PRDX1 regulation. The work in this chapter, therefore, began in an attempt to increase the LPS-induced PRDX1 release seen in Chapter 3, by priming cells with IFNγ. LPS and IFNγ act in synergy to augment TLR4-mediated proinflammatory responses, and pre-stimulation with IFNγ prevents LPS tolerance (190, 570-572). This led to the hypothesis that IFNγ+LPS stimulation should increase PRDX1 release. A key effect of IFNγ and LPS co-stimulation is the increased activation of iNOS and subsequent NO production, which has been reported to increase extracellular vesicle release (572-574). RA synovial joints, and T lymphocytes from RA patients display increased IFNγ production following activation (90-92). The TLRs are also activated by DAMPs during RA (Table 1.1). Therefore this model is relevant to RA pathology.

The roles of NO and PRDX1 are interlinked: the NO derivative ONOO⁻ is reduced by PRDX, whilst NO-mediated S-nitrosylation of KEAP1 allows release and activation of the PRDX1 transcription factor Nrf2 (358, 448, 575-577). Another target of Nrf2 is the oxidoreductase SRX, which reverses the over-oxidation of PRDX (578). NO is also implicated in direct prevention of PRDX over-oxidation, through S-nitrosylation of PRDX which inhibits PRDX activity and oligomerisation (469, 576, 579).

Nitrosative stress has been implicated in proteasomal degradation of PRDX. In 2010, Nasu et al used HEK 293T cells, a tandem affinity purification procedure, mass spectrometry and immunoprecipitation to show that PRDX1 is poly-ubiquitinated for proteasomal degradation by the ubiquitin protein ligase E6-associated protein (E6AP) (580). Song et al then showed that redox control mediated PRDX2 degradation, with E6AP targeting PRDX2 active site cysteines over-oxidised to sulfonic acid (SO₃H) (581). In 2014 Tao et al showed that the ONOO⁻ donor 3-morpholinosydnonimine (SIN-1) could induce E6AP activation and PRDX1 degradation in the human brain microvascular endothelial cell (HBMEC) line, therefore linking the regulation of PRDX1 with nitrosative stress (582).
Following on from the results of Chapters 3 and 4, this chapter aims to investigate if ROS or RNS are involved with PRDX1 and TXN1 release from cells, and if RNS are involved with PRDX1, TXNRD1 and HO1 gene expression.

5.2 Aims

- Investigate PRDX1 and TXN1 release from RAW 264.7 cells following ROS and RNS induction with PMA and IFNγ+LPS respectively
- Assess effects of RNS donors, inhibitors and proteasome inhibitors on PRDX1 protein
- Investigate PRDX1, TXNRD1 and HO1 gene expression following ROS and RNS induction with PMA and IFNγ+LPS respectively
5.3 Results

5.3.1 PRDX1 protein loss with IFNγ+LPS stimulation

Cell stimulation with LPS, PMA, or IFNγ+LPS show that contrary to our hypothesis, PRDX1 release was not increased with IFNγ+LPS but was lost (Figure 5.1A). In contrast, TXN1 release did increase (Figures 5.2A). Inspection of cell lysate Western blots showed that PRDX1 was not retained inside the cell, but had considerably reduced (Figures 5.1B, 5.1C).

To further investigate the decreased PRDX1, a time course was performed over 24h (Figure 5.3). These results show that PRDX1 begins decreasing as early as 6h, but is clearly reduced by 18h post-stimulation (Figure 5.3A).

A key mechanism for the synergy between IFNγ and LPS is due to upregulation of iNOS, therefore the nitrite content of the time course supernatants was investigated by Griess reaction (Figure 5.3B). The timing of nitrite concentration was found to coincide with PRDX1 decrease, peaking at 18h, suggesting that this could be NO-mediated. When cells were stimulated in a larger media volume (1ml), the kinetics of reaction altered resulting in the PRDX1 decrease occurring at 3h and 6h, but by 18h protein had been restored to the same level, if not slightly increased, as control (Figure 5.3C). This difference in kinetics when a larger volume is used may be due to increased NO diffusion from the cell and a resulting decrease in intracellular NO concentration.
Figure 5.1: Intracellular and released PRDX1 following IFNγ, LPS, and PMA stimulation.

Western blot analysis of cell supernatant (A) and lysate (B and C) PRDX1 protein from RAW 264.7 cells stimulated for 24h with IFNγ (100U/ml), LPS (10ng/ml), PMA (500ng/ml) or Poly IC (12.5μg/ml) in serum-free OptiMEM. Reducing gels (C) were run with the addition of DTT (0.1M).
Figure 5.2: Intracellular and released TXN1 following IFNγ, LPS, and PMA stimulation.

Western blot analysis of TXN1 protein from RAW 264.7 cell supernatant (A) and lysates (B). Cells were stimulated for 24h with LPS (10ng/ml), PMA (500ng/ml), or IFNγ (100U/ml) in serum-free OptiMEM. Representative blots of three independent experiments.
Figure 5.3: Time course of IFNγ+LPS-induced PRDX1 protein decrease and NO production.

(A) Western blot analysis of intracellular PRDX1 protein from RAW 264.7 cells stimulated with IFNγ (100U/ml) and LPS (10ng/ml) for 24h in cell culture media in 300μl volumes. Two representative blots from three independent experiments. (B) Griess assay of RAW 264.7 cell supernatant (n=6). Bars represent mean±SD. **P<0.01, ***P<0.001 by Kruskal–Wallis one-way analysis of variance. (C) Western blot analysis of intracellular PRDX1 protein from RAW 264.7 cells stimulated with IFNγ (100U/ml) and LPS (10ng/ml) for 24h in cell culture media in 1ml volume.
5.3.2 NO mediates PRDX1 protein decrease

To confirm that the increased nitrite production observed at 18h (Figure 5.3) was due to the combined effect of IFNγ and LPS, Griess reaction was performed on supernatants from cells stimulated with IFNγ, LPS, and IFNγ + LPS (Figure 5.4A). These results show that the combination of IFNγ and LPS is required to significantly increase nitrite.

Inhibitors of iNOS were then investigated in our model, and assessed for their effect on nitrite production. RAW264.7 cells were incubated with l-NAME or l-NMMA (0.3mM) in cell culture media for 1h prior to the 2h stimulation with IFNγ, and subsequent 24h stimulation with LPS. Griess reaction was performed on cell supernatants (Figure 5.4B). These results show that in this model, l-NMMA was the only iNOS inhibitor to significantly reduce nitrite production, by 28.4% (P<0.001) compared to 9.2% with l-NAME (P<0.01). At the higher concentration of 1mM, l-NMMA reduced nitrite production, by 35% (Figure 5.5).
Figure 5.4: IFNγ+LPS induced nitrite production and inhibition.

(A) Nitrite levels of supernatants from RAW 264.7 cells stimulated for 2h with IFNγ (100U/ml), then LPS (10ng/ml) for 24h in cell culture media. (B) l-NAME or l-NMMA (both 0.3mM) were applied 1h prior to IFNγ+LPS to inhibit iNOS. Bars represent mean±SD. **P<0.01, ***P<0.001, ****P<0.0001 by one-way ANOVA and Dunnett's (A) or Tukey's (B) multiple comparisons test (n=9).
Figure 5.5: IFNγ+LPS induced nitrite production is inhibited with l-NMMA.

Nitrite levels of supernatants from RAW 264.7 cells stimulated for 2h with IFNγ (100U/ml), then LPS (10ng/ml) for 24h in cell culture media. l-NMMA (1mM) was applied 1h prior to IFNγ+LPS to inhibit iNOS. Bars represent mean±SD.

****P<0.0001 by one-way ANOVA and Tukey’s multiple comparisons test.
5.3.3 PRDX1 degradation can be rescued with iNOS or proteasome inhibition

The effect of L-NMMA on IFNγ+LPS induced PRDX1 decrease was then investigated. RAW 264.7 cells were incubated with L-NMMA (1mM) for 1h prior to IFNγ+LPS stimulation. These results show that iNOS inhibition rescues IFNγ+LPS induced PRDX1 decrease (Figure 5.6A). The PRDX1 levels do not return to those of untreated cells, but this is likely due to the reduction in nitrite achieved with L-NMMA being 35% (Figure 5.5).

The possible degradation of PRDX1 was further investigated using the proteasome inhibitor MG132. RAW 264.7 cells were incubated with MG132 (0.2µM) in cell culture media for 1h prior to IFNγ+LPS stimulation. Inhibition of the proteasome with MG132 prevented degradation of PRDX1 induced by IFNγ+LPS (Figure 5.6B).
Figure 5.6: iNOS and proteasome inhibition rescue IFNγ+LPS-induced PRDX1 protein degradation.

Western blot analysis of intracellular PRDX1 protein from RAW 264.7 cells incubated for 1h with MG132 (0.2μM) or L-NMMA (1mM), then 2h with IFNγ (100U/ml) and 24h with LPS (10ng/ml) in cell culture media. Two representative blots of three independent experiments.
5.3.4 Exogenous NO donor can induce PRDX1 degradation

To further confirm that the PRDX1 degradation observed was due to NO production, the effect of the NO donor DETA/NO was investigated. Of the available NO donors, DETA/NO was selected due to its long half-life and steady NO release (583). DETA/NO treatment did not appear to induce degradation and instead converted PRDX1 from the dimeric to the monomeric form, likely due to S-nitrosylation (Figures 5.7A-C). Griess reaction results show that the concentration of DETA/NO used generated levels of nitrite in the supernatant comparable to those generated by IFNγ+LPS (Figure 5.7D). Although the supernatant nitrite concentrations were comparable, as DETA/NO is an exogenous NO donor it is likely that the intracellular NO concentrations differed between IFNγ+LPS and DETA/NO-stimulated cells. Therefore, a higher concentration of 0.2mM DETA/NO was investigated for its effect on PRDX1 (Figure 5.8). At this higher concentration, DETA/NO caused significant PRDX1 loss (Figure 5.8A). However, the supernatant nitrite levels were significantly increased from IFNγ+LPS treatment (Figure 5.8B).
Figure 5.7: Addition of an NO donor alters PRDX1 oxidation state.

(A) Western blot analysis of intracellular PRDX1 protein of RAW 264.7 cells stimulated for 24h with LPS (10ng/ml) and IFNγ (100U/ml), or DETA/NO (0.1mM) in cell culture media. Representative blots of 3 independent experiments. Densitometric analysis was performed on the Western blot upper band (PRDX1 dimer) (B) and lower band (PRDX1 monomer) (C) and normalised for equal loading with GAPDH densitometry. (D) Griess reaction was performed on RAW 264.7 cell supernatants (n=9). Bars represent mean±SD. *P<0.05, ***P<0.001, ****P<0.0001 vs untreated cells by two-way ANOVA (B-C), or one-way ANOVA (D) and Dunnett's multiple comparisons test.
Figure 5.8: Addition of an NO donor causes PRDX1 loss.
Western blot analysis of intracellular PRDX1 protein (A) and Griess assay of cell supernatants (B) from RAW 264.7 cells stimulated for 24h with LPS (10ng/ml) and IFN\(\gamma\) (100U/ml), or DETA/NO (0.2mM) in cell culture media (\(n=3\)). Bars represent mean±SD.
#P<0.0001 vs untreated cells, ****P<0.0001 by one-way ANOVA and Dunnett's multiple comparisons test.
5.3.5 NO mediates \textit{PRDX1} gene expression

The finding that iNOS mediates PRDX1 protein degradation led to investigation of the effect of IFN\textgamma+LPS on \textit{PRDX1} gene expression. These results show that the combination of IFN\textgamma+LPS increased \textit{PRDX1} gene expression to almost double that of LPS alone, which was unexpected as the same conditions caused large reduction of PRDX1 protein (Figure 5.9). IFN\textgamma alone did not induce \textit{PRDX1} gene expression. With \textit{TXNRD1} and \textit{HO1}, IFN\textgamma+LPS induced gene expression to the same or a lesser extent than LPS alone.

The kinetics of gene expression were investigated following TLR4 stimulation to find if each of these three oxidoreductase genes behave in the same manner. Therefore gene expression was analysed over a time course of 24h following LPS or IFN\textgamma+LPS stimulation (Figure 5.10). \textit{PRDX1} was induced slowly with both sets of stimuli, with expression peaking at 24h (Figure 5.10A), whereas \textit{TXNRD1} was induced much faster, peaking at 3h (Figure 5.10B). LPS-induced \textit{HO1} peaked at 6h, whereas IFN\textgamma+LPS stimulation peaked at 18h (Figure 5.10C).

Expression of \textit{PRDX1} and \textit{HO1} were significantly increased after 18h with IFN\textgamma+LPS stimulation compared to LPS alone, increasing 10 fold and 20 fold respectively (Figures 5.10A and 5.10C).
Figure 5.9: PRDX1, TXNRD1 and HO1 gene expression is increased by IFNγ+LPS.

Real time Taqman qPCR analysis of PRDX1 (A), TXNRD1 (B), and HO1 (C) gene expression in RAW 264.7 cells stimulated for 6h with LPS (10ng/ml), and/or IFNγ (100U/ml). Data were normalised for HPRT1 and expressed as fold change vs one control sample (n=12). Bars represent mean±SD. #P<0.05 vs untreated cells, ****P<0.0001 by one-way ANOVA followed by Dunnett’s multiple comparisons test.
Figure 5.10: Time course of LPS and IFNγ+LPS-induced PRDX1, TXNRD1 and HO1 gene expression.

RAW 264.7 cells were stimulated with IFNγ (100U/ml) for 2h then LPS (10ng/ml) for 24h. PRDX1 (A), TXNRD1 (B) and HO1 (C) gene expression were assessed by real time Taqman qPCR over time. Data were normalised for HPRT1 and expressed as fold change vs one control sample (n=8). Bars represent mean±SD.

****P<0.0001 vs LPS stimulated cells by two-way ANOVA followed by Dunnett’s multiple comparisons test.
5.3.6 *PRDX1* and *HO1* gene expression is likely iNOS mediated

To confirm if the observed gene expression was due to iNOS generated NO, L-NMMA was incubated with cells for 1h prior to 18h IFNγ+LPS stimulation. *PRDX1* and *HO1* gene expression were investigated (Figure 5.11). IFNγ+LPS-induced gene expression was significantly reduced as a result of iNOS inhibition.

To further investigate if the generation of iNOS was responsible for the increased *PRDX1* gene expression with IFNγ+LPS treatment, the effect of cycloheximide (CHX), a protein synthesis inhibitor, was investigated (Figure 5.12). By inhibiting protein synthesis, we could confirm if the gene expression observed was due to the production of a secondary message (iNOS). RAW 264.7 cells were incubated with CHX (5µM) for 30mins prior to stimulation with IFNγ+LPS in cell culture media. Real time Taqman qPCR was used to investigate *PRDX1* gene expression, and *TNF* gene expression was used as a control as this is a direct induction pathway that is unaffected by CHX (584). *PRDX1* gene expression was abolished with CHX treatment, at both 6h and 18h. *TNF* gene expression was unaffected by CHX treatment at 6h but was reduced at 18h to the level observed at 6h. This is likely due to CHX preventing the synthesis of cytokines and other proteins which further amplify *TNF* expression at longer time points, e.g. the positive feedback loop of TNF itself (585). These results suggest that even at the shorter time point of 6h, *PRDX1* gene expression requires the generation of a secondary signal; likely iNOS.
Figure 5.11: Effect of an iNOS inhibitor on IFN\(\gamma\)+LPS-induced PRDX1 and HO1 gene expression.

Real time Taqman qPCR analysis of PRDX1 (A) and HO1 (B) gene expression in RAW 264.7 cells stimulated for 18h with LPS (10ng/ml) and IFN\(\gamma\) (100U/ml), with or without the addition of iNOS inhibitor L-NMMA (1mM). Data were normalised to HPRT1 and expressed as fold change vs one control sample (\(n=12\)). Bars represent mean±SD. ****P<0.0001 by two-way ANOVA followed by Dunnett’s multiple comparisons test.
Figure 5.12: Effect of a protein synthesis inhibitor on IFNγ+LPS-induced PRDX1 and TNF gene expression.

Real time Taqman qPCR analysis of PRDX1 (A) and TNF (B) gene expression in RAW 264.7 cells stimulated for 6h or 18h with LPS (10ng/ml) and IFNγ (100U/ml), with or without the addition of cycloheximide (5μM). Data were normalised to HPRT1 and expressed as fold change vs one control sample (n=8). ****P<0.0001 by three-way ANOVA and Tukey's multiple comparisons test.
5.4 Results summary

The aims of this chapter were to investigate the effects of ROS and RNS on PRDX1 and TXN1 protein release from RAW 264.7 cells, and of RNS on PRDX1, TXNRD1 and HO1 gene expression.

The results from this chapter show that IFNγ+LPS-induced NO has many effects on oxidoreductase enzymes. Stimulation with IFNγ+LPS increased release of TXN1 as we had hypothesized (Figure 5.2). However, PMA treatment increased intracellular PRDX1 and TXN1 protein but did not stimulate release, and IFNγ+LPS caused loss of total PRDX1 protein (Figure 5.1). A time course of IFNγ+LPS-induced PRDX1 loss showed that its timing coincided with increased supernatant nitrite (Figure 5.3).

Use of iNOS inhibitors showed that L-NMMA decreased nitrite production more effectively than L-NAME, and that L-NMMA could prevent IFNγ+LPS-induced PRDX1 loss (Figures 5.4, 5.5, and 5.6A). The proteasome inhibitor MG132 could also prevent IFNγ+LPS-induced PRDX1 loss, confirming that PRDX1 was undergoing proteasomal degradation (Figure 5.6B). Use of the NO donor DETA/NO could induce PRDX1 loss (Figure 5.8).

Gene expression of PRDX1, TXNRD1 and HO1 was induced following IFNγ+LPS treatment, and with PRDX1 and HO1 this was significantly increased from LPS alone after 18h stimulation (Figure 5.10). Incubation with L-NMMA significantly reduced IFNγ+LPS-induced PRDX1 and HO1 gene expression (Figure 5.11). Incubation with cycloheximide reduced IFNγ+LPS-induced PRDX1 gene expression further still (Figure 5.12). These results support previous findings that NO is an important signalling molecule for cellular redox control.
5.5 Discussion

The finding that IFNγ+LPS stimulation caused PRDX1 protein degradation supports previous studies by Nasu et al (2010) who showed that PRDX1 is polyubiquitinated by the ubiquitin protein ligase, E6AP, and Tao et al (2014) who showed that E6AP is activated by nitrosative stress (580, 582). This chapter builds on these studies by clearly demonstrating PRDX1 loss with IFNγ+LPS, and that this loss can be rescued through inhibition of iNOS or proteasome (Figure 5.6), or induced through application of an NO donor (Figure 5.8). Furthermore, this work shows loss of total PRDX1, demonstrated by decreased protein observed under reducing conditions (where all oligomers will be reduced to the monomer at 22kDa), whereas Tao et al show loss of a high molecular weight oligomer PRDX1 at >118kDa, and do not show this under reducing conditions. However, a study by Diet et al (2007) found that IFNγ+LPS stimulation of murine bone marrow-derived macrophages actually caused an increase in PRDX1 protein after 20h stimulation (576). Furthermore, they observed increased PRDX1 protein following DETA/NO stimulation as well. These results are in direct contrast to those observed here. One explanation for this could be a difference in the kinetics of degradation between models, or a difference in response to NO donors. The difference in kinetics is a plausible explanation because as we observed here, an increased media volume can alter the kinetics of the degradation (Figure 5.3). This is potentially due to the larger volume enabling increased NO diffusion out of cells and so intracellular NO concentrations decrease faster, though it would be interesting for future work to confirm this by measuring nitrite production in cell lysates. Furthermore, as there is a concomitant and significant increase of PRDX1 gene expression that occurs by 18h in our model, it suggests that at a later time point or when redox equilibrium is restored, the mRNA is translated to give increased amounts of PRDX1 protein.

In this study, we observed that the iNOS inhibitor L-NMMA reduced nitrite by 28.4% compared with a 9.2% reduction with L-NAME. Both L-NMMA and L-NAME are chemically modified from L-arginine: the terminal guanidine nitrogen is substituted with NO₂ and CH₃ in L-NMMA and NO₂ in L-NAME which also contains an alkyl esterification at the C terminus (586). These compounds can, therefore, block the conversion of L-arginine to L-citrulline. To exert its inhibitory function L-NAME must first be hydrolysed to its free acid form, L-NOARG which in vivo is performed by esterase enzymes (587). It is likely that L-NAME was less effective than L-NMMA in
our model as it was not being sufficiently hydrolysed to its active form during cell metabolism.

Previous studies have shown that PRDX1 can be directly S-nitrosylated: Diet et al found that S-nitrosylation was protective to PRDX1, preventing over-oxidation by H₂O₂ and formation of sulfinic acid at the peroxidatic cysteine (576); Engelman et al then found that S-nitrosylation inhibits the peroxidase activity of PRDX1, inhibits formation of the PRDX1 decamer, and inhibits regeneration of S-nitrosylated PRDX1 back to its reduced form through inhibition of the TXN/TXNRD1 system (579); and Riquier et al suggested that the inactivation of PRDX1 by S-nitrosylation may also be due to S-homocysteinylation (469). These studies, together with our findings demonstrate that under nitrosative stress PRDX1 is inactivated, either through S-nitrosylation, inhibition of regeneration back to the reduced form, or ubiquitination and degradation. Control mechanisms of which outcome is employed are currently unclear: perhaps the degree or duration of nitrosative stress dictates whether PRDX1 should be inactivated or degraded. This theory is possible as the study by Engelman et al used S-nitrosocysteine as the NO donor, whereas DETA/NO was used here. These NO donors have drastically different NO release profiles: S-nitrosocysteine causes rapid, unsustained NO release (lasting <4h), whereas DETA/NO release is gradual and longer-term (lasting <24h) (583).

In this chapter we found that: IFNγ+LPS stimulation induced expression of the Nrf2 transcribed genes PRDX1, TXNRD1, and HO1; that this expression was reduced with iNOS inhibition for PRDX1 and HO1; and that PRDX1 gene expression was further reduced with inhibition of protein synthesis. Together these findings suggest that Nrf2 activation increases with NO stimulation, and also that PRDX1, in particular, is reliant on the induction of iNOS, or another secondary signal, for its expression. Our results support the findings of previous studies which demonstrate a requirement for S-nitrosylation of KEAP1 by iNOS-derived NO for Nrf2 activation (358, 576, 577), and more specifically that PRDX1 expression increases with IFNγ+LPS (576), and HO1 induction is reliant on NO (588).

Activation of iNOS is augmented by the synergy of IFNγ+LPS stimulation but can be induced to a lesser degree by LPS or IFNγ alone (589, 590), therefore expression of Nrf2 target genes is still increased, though not to the same extent following one or other stimuli. Furthermore, Nrf2 is not only activated by S-nitrosylation of KEAP1, but also by oxidation, glutathionylation and electrophilic attack (286, 591, 592). Nrf2
can also be activated independently of KEAP1: by increased gene expression through autoregulation (593), regulation via other transcription factors such as NF-κB (319, 594) or by microRNAs (269, 595); by direct phosphorylation of Nrf2 by a variety of kinases (297, 596, 597); or through binding with direct binding partner proteins (598, 599). The interactome and regulome of Nrf2 is vast so it is likely that multiple methods of regulation occur, though the fine-tuning of this regulation is not well understood (600).

The results from this chapter show that there is a disparity between NO-mediated regulation of PRDX1 gene and protein expression; NO was found to increase PRDX1 gene expression, but cause degradation and loss of the protein. Protein concentration changes are a result of mRNA concentration and stability, translation efficiency and protein degradation. Low protein-per-mRNA ratios are not unusual and on average multicellular organisms show the lowest correlations between mRNA and protein concentrations (as low as 40%) though this varies depending on the gene of interest and on cellular conditions (601). In this case, we do not know if PRDX1 mRNA is not translated into protein, if all translated protein is immediately degraded, or if both mechanisms are at play. This would be an interesting area of investigation for future work. In this project, we did not investigate the response of HO1 protein to IFNγ+LPS stimulation, but it would be interesting to see if HO1 behaves in the same way as PRDX1, as a recent study by Jez et al showed that HO1 can be ubiquitinated and degraded following valproic acid treatment, though the exact mechanisms of action of this are unclear (602).

These findings show that RNS are important redox signalling molecules involved in the regulation of both PRDX1 gene expression and protein degradation.
Chapter 6
6. Peroxiredoxin-2 in Serum of Rheumatoid Arthritis Patients and Healthy Controls

6.1 Introduction

The aetiology and pathophysiology of RA are incompletely understood, but multiple studies have implicated oxidative stress as a contributing factor (2). Free radical species can contribute to inflammation indirectly via redox signalling and activation of redox sensitive transcription factors such as NF-κB, or directly through oxidation of lipids, proteins and DNA. Cartilage degradation occurs following ROS-mediated oxidation of proteoglycan and inhibition of its synthesis (482). ROS can also oxidise lipids and peptides, disrupting cell membranes and potentially creating neoepitopes against which immune responses can be raised (603). Therefore, antioxidant defence mechanisms are equally important and implicated in RA.

Impaired antioxidant capacity has been well characterised in RA. In the last year two studies separately reported decreased total antioxidant capacity in RA patient serum: Zhou et al investigated oxidant and antioxidant status in 112 RA patients and found that total oxidant status and oxidative stress increased, while total antioxidant capacity decreased in RA patient blood serum compared to control. Furthermore, oxidative status strongly correlated with RA disease activity (604). The second study by Rodriguez-Carrio et al similarly looked at blood serum from 113 RA patients and showed that increased TNF and monocyte chemotactic protein inversely correlated with total antioxidant capacity (605). In 2018 Kardes et al investigated non-enzymatic superoxide radical scavenger activity in blood plasma of 45 RA patients and found an inverse correlation with clinical disease parameters (606).

Investigation of the enzymatic antioxidant systems in RA have mainly centred on SOD, catalase, glutathione peroxidase, and glutathione reductase, reporting decreased activity in these enzymes, with the exception of SOD where the results are conflicting and either increased or decreased compared to healthy controls (486, 487, 607-609).
Studies of the role of the PDOR enzyme family in RA are limited, but multiple researchers have shown the main non-enzymatic thiol antioxidant, glutathione, is decreased in RA (487, 608, 610). Furthermore a number of studies have shown that TXN protein and activity is increased in RA synovial fluid, plasma and serum (489, 490, 611, 612). However, little research has investigated the peroxiredoxins in RA.

Our lab previously investigated PRDX1 in plasma from people with RA and no differences were found compared to healthy control plasma (unpublished data). In 2006, Kim et al showed that PRDX2 was elevated in synovial fluid from patients with RA compared to synovial fluid from patients with osteoarthritis using two-dimensional gel electrophoresis and mass spectrometry (514). Then in 2012, Szabo-Taylor et al used gel electrophoresis to show that PRDX2 was increased over two-fold in lymphocytes from RA patients vs healthy lymphocytes, and flow cytometry to show exofacial PRDX2 expression was significantly increased in B lymphocytes and Th17 lymphocytes from RA patients (513). The authors also detected elevated PRDX2 in a small number of RA patient plasma and synovial fluid samples using Western blot. Taken together, these studies suggest that PRDX2 could be a potential predictive biomarker for RA as it is increased in synovial fluid and detectable in plasma. The work in this chapter aims to investigate if PRDX2 is increased in blood serum of patients with RA compared with healthy control blood donors. We aim to quantify PRDX2 and correlate the results with the inflammatory marker, CRP.

6.2 Aims

- Establish reliable method of quantifying PRDX2 in serum
- Measure PRDX2 in RA patient and HC donor serum
- Correlate serum PRDX2 with CRP
6.3 Results

6.3.1 Serum PRDX2 detection unreliable using Western blot

Serum PRDX2 from RA patients and healthy donors was assessed using SDS-PAGE and Western blot. PRDX2 was clearly detectable and increased compared with HC in RA samples under both reducing and non-reducing conditions (22kDa and 44kDa respectively) (Figure 6.1A and 6.1B). There was however a very strong high molecular weight band visible (at 95Kda and 250kDa in reducing and non-reducing conditions respectively). This band could be the PRDX2 decamer, which is estimated to run at around 220kDa, but if so this complex should have broken down into its constituent monomers (22kDa) under reducing conditions, which was not observed. Therefore it is unclear what exactly this band represents. It could be non-specific staining from the PRDX2 or secondary antibodies, or it could be that serum is so concentrated with protein that the samples are prevented from running into the gel adequately.

Attempts to repeat these results were carried out extensively, but proved unsuccessful. Therefore conclusions cannot be drawn from the results of this technique. On closer inspection it was found that PRDX2 levels observed with Western blot varied tremendously between each experimental repeat. On one occasion a donor would show high levels of PRDX2, and another time the same donor would show no PRDX2. As suggested above, this variability could be due to the viscous nature of blood serum preventing the samples from running into the gel effectively, rendering this technique ineffective for this experiment.
Figure 6.1: PRDX2 protein detection in human serum.
Western blot analysis of human blood serum PRDX2 from patients with rheumatoid arthritis (RA), or healthy control donors. Serum samples were diluted 1:50 in ultrapure water, then gel electrophoresis was performed under non-reducing (A), or reducing conditions (10% β-mercaptoethanol) (B).
6.3.2 Serum PRDX2 quantification using ELISA

Due to the unreliability of results obtained using Western blot, a commercial ELISA kit for PRDX2 was used to quantify serum PRDX2. Although this method could not differentiate between PRDX2 redox states, it could allow direct calculation of PRDX2 serum concentrations. Results from the ELISA showed that there was no significant difference between HC and RA samples, and that there was a wide range of PRDX2 concentrations in both groups (Figure 6.2). As with Western blot, it was found that the reproducibility of these results could vary hugely if different aliquots of the same serum sample were used. It was hypothesised that the variability observed within samples could be due to PRDX2 protein being enclosed within exosomes: antibody binding is prevented when PRDX2 is inside exosomes, but when released from exosomes free PRDX2 is able to bind. Freeze-thawing exosomes can disrupt their membrane integrity, particularly at temperatures around -25°C where exosomes are least stable (613, 614). Although all serum samples were stored at -80°C where exosome integrity is stable, different rates of thawing could lead to uneven disruption of exosomes between identical samples therefore showing PRDX2 variation between different aliquots of the same serum. Similarly, during the Western blot experiments, in order to prevent protein aggregation which would impair gel electrophoresis, serum samples were not boiled prior to gel loading which may have also impeded PRDX2 release from exosomes and have led to the unreliable results discussed in 6.3.1.

A technique for disrupting the exosomal membrane to allow ELISA binding was outlined by Sasaki et al (2016), whereby the sample is incubated for 10mins with sodium hydroxide (NaOH, 0.4N) and a detergent (Triton X-100, 0.5%), then neutralised with hydrochloric acid (HCl, 0.4N) prior to use in ELISA (613, 615). This exosome disruption technique has previously been used only in urine (613, 615) and so its use in serum is novel.

Results using this method showed greatly increased PRDX2 concentrations in treated samples compared to untreated (Figure 6.3), and increased sample reliability suggesting that exosomal containment was indeed impairing the ELISA. The exosome disruption technique was then implemented for the full ELISA (Figure 6.4). These results showed an even greater range of PRDX2 concentration in both HC and RA samples, but there was no difference between groups.
Figure 6.2: Serum PRDX2, measured by ELISA.

Blood serum PRDX2 protein in patients with rheumatoid arthritis (RA), or healthy control was quantified by ELISA (n=16 healthy control and 22 RA patient). Bars represent mean ± SEM and Welch's unequal variances t-test was used for analysis.
Figure 6.3: Serum PRDX2 increases following NaOH+Triton X-100 treatment. The effect of exosome membrane disruption on serum PRDX2 was quantified by ELISA. Each serum sample was divided in two, with one half treated for 20 minutes with NaOH (0.4N) and 0.5% Triton X-100, then neutralised with HCl (0.4N) to disrupt exosome membranes, and the other half left untreated ($n=6$). *$P<0.05$ by paired t-test.
Figure 6.4: PRDX2 in rheumatoid arthritis and healthy control blood serum.

Rheumatoid arthritis (RA) and healthy control (HC) serum was treated for 20 minutes with NaOH (0.4N) and 0.5% Triton X-100, then neutralised with HCl (0.4N). Serum PRDX2 was then quantified by ELISA. Bars represent mean ± SEM and Welch's unequal variances t-test was used for analysis.
6.3.3 Comparison of PRDX2 with C-reactive protein

To investigate the implication of PRDX2 in RA serum, PRDX2 concentrations were compared with CRP, an inflammatory marker. Serum CRP concentrations were quantified using a commercially available ELISA kit, and plotted against PRDX2 concentrations for each sample. PRDX2 data were normally distributed, but CRP data were not and so were transformed (log_{10}) to allow Gaussian distribution and analysed for correlation using the Pearson correlation coefficient, which confirmed no correlation between PRDX2 and CRP (r= -0.03) (Figure 6.5).
Figure 6.5: Serum PRDX2 protein does not correlate with CRP.
Rheumatoid arthritis C-reactive protein (CRP) levels were quantified by ELISA. CRP
data were transformed ($\log_{10}$) and plotted against peroxiredoxin (PRDX)-2 values
for each serum sample ($n=36$).
6.4 Results summary

The aims of this chapter were to establish a reliable assay to measure blood serum PRDX2, to quantify PRDX2 in RA patient and HC serum, and to correlate this with the inflammatory marker CRP. These results show that gel electrophoresis allowed detection of PRDX2, but was highly unreliable. Serum PRDX2 was quantifiable using ELISA but this method was unreliable without prior disruption of exosomes to allow PRDX2 release. Exosome disruption enabled detection of increased PRDX2, but there was no significant difference between RA patient and HC serum PRDX2. Furthermore, there was no correlation between PRDX2 and CRP.

6.5 Discussion

The primary aims of this chapter were to investigate PRDX2 in the serum of RA patients compared with HC donors, and to investigate these results in the context of inflammation by comparing with the inflammatory marker CRP. PRDX2 is an inflammatory mediator, which is involved with cytoprotection through regulation of ROS, and with induction of inflammatory cytokines, such as VEGF, TNF-α, IL-6, and IL-1β (491, 616). Its increased expression and extracellular release following inflammatory stimuli led to the hypothesis that in a chronic inflammatory disease such as RA, there would be a detectable increase in PRDX2 in patient blood serum (491, 617, 618). However these findings show that there was no significant increase in serum PRDX2, and that the release which did occur did not correlate with inflammation (CRP). These results are in contrast to those obtained by Szabo-Taylor et al, who found elevated PRDX2 in RA patient plasma, though this study used gel electrophoresis to calculate PRDX2 concentrations (513). Western blot is a semi-quantitative method, and as discussed in 6.3.1 it is unreliable for serum detection of PRDX2. Furthermore, the sample size used in this chapter was larger than that used by Szabo-Taylor, reducing the risk of Type I error (false positive results) (n=36 vs n=9 respectively).

The results from Kim et al and Szabo-Taylor showed increased PRDX2 in RA synovial fluid and peripheral blood lymphocytes (513, 514). Together with the findings presented here that PRDX2 is not elevated in RA serum, these results suggest that PRDX2 may be upregulated but not released in peripheral blood in RA. Plasma PRDX2 has been shown to increase in early stage liver fibrosis (619), and autoantibodies to PRDX2 have been documented in systemic vasculitis, a chronic inflammatory condition of the blood vessels, suggesting PRDX2 cellular release can
occur in these conditions (515). Perhaps in RA PRDX2 release is localised to the site of inflammation, and occurs following specific stimulation such as TLR stimulation by DAMPS. If this is the case intracellular PRDX2 may increase (as was observed by Szabo-Taylor et al), but any release would be localised to the joint and so would not be detectable in blood serum, as we have observed here. It may therefore be interesting for future work to quantify PRDX2 in RA synovial fluid and joint tissues.

A more recent publication by Szabo-Taylor et al investigated PRDX1 and PRDX2 in plasma using flow cytometry and found extracellular vesicles containing PRDX1, but not PRDX2, were increased in RA compared to healthy controls (620). This is in contrast to previous findings in our lab carried out using ELISA, but highlights that the method of detection is extremely important. It would be interesting to investigate PRDX1 in RA further, using the exosome disruption technique described here.

This research used CRP to measure PRDX2 against as it is one of the most commonly used markers of inflammation. However, there is not always good correlation between CRP and clinical parameters of RA disease (621). The multi-biomarker disease activity (MBDA) score, which assesses levels of 12 serum biomarkers (vascular cell adhesion molecule-1, epidermal growth factor, vascular endothelial growth factor-A, IL-6, TNF-RI, YKL-40, matrix metalloproteinase (MMP)-1, MMP-3, leptin, resistin, Serum amyloid A, CRP), is now being recognised as a more effective and reliable indicator of disease activity (622). A recent study by Curtis et al found that the MBDA score was associated with RA disease activity, obesity, and age, and was negligibly affected by common comorbidities. Furthermore, almost one-third of patients with normal CRP had high MBDA scores (623). Therefore, the MBDA would be a better parameter to compare oxidoreductase expression with in future work.

Descriptive biomarkers such as CRP, erythrocyte sedimentation rate, and the MBDA score provide information about the state of inflammation during RA. Therefore, the efficacy of therapeutic interventions can be monitored using these and other clinical parameters which comprise the DAS-28 score. However, it can take time for the efficacy of a treatment to be reflected by clinical parameters and so there has been great interest in identifying pharmacodynamic biomarkers which respond early to treatment, allowing effective and efficient decision making with regards to treatment strategy, as well as faster progress of new drugs through
clinical trials when used in place of clinical response as an end point (624). These biomarkers can be treatment specific, for example analysis of peripheral blood B cells and plasmablasts can be used to assess response to rituximab therapy, which targets CD20 expressing B cells for NK cell mediated killing (625-627).

As the key diagnostic biomarkers of RA, many studies have investigated anti-CCP and RF for their potential as indicators of response to anti-TNF therapy. However a 2014 meta-analysis found their status not to be associated with therapeutic responses (628). Other auto-antibodies directed towards citrullinated peptides may be better candidate pharmacodynamics biomarkers, such as anti citrullinated vimentin, early disappearance of which is associated with better response to treatment (629).

Due to the varied mechanisms of action and effects of different RA therapeutics, stratification of patients based on treatment is an important consideration for research into potential biomarkers. It is difficult to predict the effect of different therapeutics on the oxidoreductase enzymes. Inhibition of TNF would reduce NF-κB activation, which could either increase Nrf2 activation (as NF-κB can repress Nrf2 activation by competing for the coactivator protein CBP), or decrease Nrf2 activity (the NFE2L2 gene contains an NF-κB binding site). The effect of methotrexate on Nrf2 could be an altogether different picture due to its varied mechanisms of action. Investigation into Nrf2 activation in stratified patient subsets could therefore be an interesting area for future research.

As a heterogeneous disease with multiple patient subsets starting to be identified, there is a need for more RA biomarkers to stratify patients and enable a personalised medicine approach. The findings presented in this chapter show that serum PRDX2 would not be a suitable biomarker for RA. Further investigation is required to establish if it is elevated locally within the joint.
Chapter 7
7. Discussion and conclusions

Understanding the mechanisms underlying inflammation is essential for identifying therapeutic targets and treating inflammatory diseases, such as RA. The PDOR enzymes have been known for a long time. As some of the most highly expressed and highly conserved proteins their importance is undeniable, but still their exact roles during inflammation remain unknown. These enzymes provide a key link between the pathogen sensing TLRs and oxidative stress, two contributory factors for RA.

The oxidoreductases PRDX and TXN are intracellular enzymes which are released from cells in exosomes through a non-classical secretory pathway following TLR4 stimulation (197, 491, 618). When freely circulating in the extracellular environment they gain cytokine- and chemokine-like properties (491, 493-496). These properties could be a result of redox signalling or receptor binding though no specific receptor has been identified. However, PRDX1 can directly activate TLR4 causing proinflammatory cytokine production (468).

The work presented in this thesis aimed to characterise the release and gene expression of oxidoreductase enzymes following stimulation of each TLR family member. The effects of ROS and RNS on oxidoreductase regulation were also investigated. Finally, this project aimed to investigate the potential for use of PRDX2 as a biomarker for RA.

7.1 TXN1 and PRDX1 release is TLR-specific

The hypothesis for Chapter 3 was that oxidoreductase release varies according to the specific TLR stimulated. Analysis of PRDX1 and TXN1 release after TLR stimulation showed that release primarily occurred in response to TLR3 and TLR4. These two TLRs have been previously identified to induce exosome release (194, 195). Furthermore, PRDX1 and TXN1 release correlated with the exosome marker protein Hsp70, supporting previous findings that PRDX and TXN are released through exosomes following TLR stimulation (197, 491).

Increased TLR expression has been documented in synovial fibroblasts from people with RA (217-219), and there are increased DAMPs in synovial fluid available to activate TLRs (Table 1.1, (1, 211, 212). Synovial exosomes have been shown to increase and correlate with disease severity in RA (110-113). The findings
presented in this thesis show that TLR3 and TLR4 are key inducers of PRDX1 and TXN1 (therefore exosome) release. Consequently, one can surmise that DAMP-mediated stimulation of TLR3 and TLR4 could be a contributing factor to RA, particularly in patients with elevated synovial exosomes. These findings highlight an additional benefit of TLR-targeted therapeutics such as the anti-TLR4 antibody NI-0101 which is being developed by NovImmune and Genentech to inhibit TLR4 (215, 216), and suggest that blockade of TLR3 signalling may also have potential as a therapeutic target.

### 7.2 PRDX1, TXNRD1 and HO1 genes are independently regulated

The hypothesis for **Chapter 4** was that PRDX1, TXNRD1 and HO1 gene expression varies according to the specific TLR stimulated. PRDX1 gene expression only increased in response to TLR4, whereas TXNRD1 and HO1 increased in response to stimulation of three or more TLRs. These results show that although PRDX1, TXNRD1 and HO1 are all Nrf2-transcribed genes, they are each independently regulated. Furthermore, HO1 is known as the archetypal Nrf2 gene, but these results suggest that it has a more complex signalling pathway than the other two Nrf2 genes as its induction by TLR4 was affected by both ROS- and protein kinase inhibition whereas PRDX1 and TXNRD1 were not. It would be interesting for future work to define the protein kinase (likely a PKC isoform) and the redox sensitive elements involved with LPS-induced HO1 expression, but which are not required for PRDX1 and TXNRD1 induction.

Three Nrf2 activators are already in clinical trials for various disease states: dimethyl fumarate (approved and marketed for multiple sclerosis), CDDO-Me and sulforaphane (313, 314), with many more in development (315). Activation of Nrf2 should result in increased antioxidant enzyme production and therefore decreased oxidative stress. In addition, activation of Nrf2 should result in decreased NF-κB activation due to Nrf2 and NF-κB cross-signalling (317-319). Both of these mechanisms should be beneficial for autoimmune disease. However, the Nrf2 targets PRDX1 and TXN1 are upregulated in multiple diseases (489, 490, 507-511, 611, 612) and display proinflammatory functions in the extracellular environment (491, 493-496). Therefore nonspecific Nrf2 activation could inadvertently worsen inflammation through upregulation of these oxidoreductases.
The results presented here show that specific regulatory mechanisms are in place for each Nrf2 target gene. Therefore, nonspecific Nrf2 activation could disrupt the oxidoreductase balance, upregulating one enzyme e.g. HO1 over others. This could disrupt the RSI which would have many cellular consequences. As the regulation, interplay and functional roles of Nrf2 target genes in inflammation are still incompletely understood it is difficult to predict what the outcome of Nrf2 activation might be in different diseases and whether it would be detrimental or beneficial. A deeper understanding of oxidoreductases and their transcriptional regulation would greatly assist the development of Nrf2 therapeutics.

7.3 RNS induce PRDX1 gene expression which is dependent on a secondary signal

The synergy between IFNγ and LPS, IFNγ-mediated inhibition of LPS tolerance (190, 570, 571), and the activation of Nrf2 following S-nitrosylation of its inhibitor KEAP1 (294, 295) led to the hypothesis for Chapter 5 that oxidoreductase gene expression would increase with IFNγ+LPS stimulation. The results presented in this thesis show PRDX1, TXNRD1 and HO1 gene expression increase with IFNγ+LPS stimulation. Furthermore, these results show that a secondary signal, likely iNOS, is an essential requirement for PRDX1 and HO1 gene expression. Further work is required to definitively prove that iNOS is the essential secondary signal.

Transcription of iNOS is NF-κB-regulated (630). If Nrf2 activation is iNOS-mediated this could be an additional mechanism of cross-signalling between NF-κB and Nrf2. One can postulate that inhibition of NF-κB and therefore of iNOS could contribute to the unexpected and often counterintuitive side effects of NF-κB-targeted therapeutics by interfering with oxidoreductase expression and therefore with redox state and signalling (234, 260)

7.4 RNS induce PRDX1 proteasomal degradation

The second hypothesis for Chapter 5 was that oxidoreductase release would increase with induction of ROS and RNS by PMA and IFNγ+LPS respectively. The findings presented here show that TXN1 release increased following RNS induction, whereas PRDX1 was degraded by the proteasome. PMA was not found to induce release of PRDX1 or TXN1.

Increased IFNγ has been documented in synovial joints and T cells of people with RA (90-92). This increase may exacerbate disease by preventing both production
of inflammation-resolving cytokines such as IL-10, and the onset of LPS (TLR4) tolerance (188-190).

The different regulation of PRDX1 and TXN1 proteins suggests that they have different roles in this scenario. Almost complete degradation of PRDX1 frees TXN1 from competitive inhibition, enabling TXN1 to reduce other proteins (462). This may be an important step for intercellular signalling, enabling communication of different messages to those when PRDX1 is incorporated alongside TXN1 in exosomes. Furthermore, degradation of PRDX1 prevents its molecular chaperone activities, which are not fully understood, though this could be an important mechanism (464).

7.5 PRDX2 is not elevated in RA serum and does not correlate with CRP

The hypothesis for Chapter 6 was that PRDX2 would be elevated in serum from RA patients compared to healthy donors, and that this would correlate with the inflammatory marker CRP. This is the first study to quantitatively examine PRDX2 in serum. The results presented in Chapter 6 show that serum PRDX2 concentrations spanned a large range, did not show differences between the healthy and RA groups, and did not correlate with serum CRP.

In light of the results from Chapter 5 and the increased IFNγ known to occur during RA (90-92), it is possible that the PRDX1 and PRDX2 are degraded and therefore undetectable during disease (90-92). If this is the case TXN1 would be preferentially released and therefore increased in RA, which has been shown in multiple studies (489, 490, 611, 612). Therefore, perhaps TXN1 is the key redox signalling enzyme in RA.

The results presented in this thesis suggest that two mechanisms of redox control of TXN1 may exist. The floodgate hypothesis states that PRDX is inactivated by over-oxidation, therefore freeing TXN from competitive inhibition and allowing reduction of other proteins, as well as direct H2O2 mediated redox signalling to occur at the target protein (462). The findings of this thesis show that a second mechanism may also occur where PRDX is degraded and therefore frees TXN of competitive inhibition.

A hypothesis for the key difference between these two mechanisms is that under conditions of oxidative stress when PRDX becomes over-oxidised, it is likely that TXN will also be oxidised. Therefore, perhaps it is under these conditions that TXN
switches from a reductant to a powerful oxidant (411). Conversely, when PRDX is degraded by the proteasome under normal redox conditions TXN can remain a reductant and so is free to reduce proteins other than PRDX. A study by Lemarechal et al showed that serum from RA patients exhibited increased TXN activity and decreased TXNRD activity compared to healthy controls (611). This disparity in enzyme activity could be due to TXN acting as an oxidant in RA and therefore not requiring TXNRD-mediated reduction for its activity.

As both TXN and IFNγ have been reported to increase in RA, it would be interesting to investigate TXN redox state in this disease. This would enable better understanding of the role of TXN in redox signalling and inflammation during RA.

7.6 Concluding remarks

Redox regulation and signalling are important mechanisms of inflammation. The oxidoreductase enzymes are key mediators of these processes, but their regulation and specific roles in inflammation are not well understood.

The research presented in this thesis is the first to show that oxidoreductases are regulated in many ways following TLR stimulation. Cellular release occurred following TLR3 and TLR4 stimulation and this correlated with the exosome marker Hsp70. Gene expression was independently regulated for each oxidoreductase. These results show that although all Nrf2 transcribed genes, oxidoreductase responses to TLR stimulation are finely-tuned, varying according to the oxidoreductase and the specific cell stimulation.

This project is the first to consolidate previous research showing that RNS simultaneously induce both PRDX1 gene expression and protein degradation, and to show that a secondary signal, likely iNOS, is required for PRDX1 gene expression. Furthermore, this thesis puts forward the hypothesis that PRDX1 degradation is a mechanism to liberate TXN from competitive inhibition under redox equilibrium.

A further novel finding from this project is that serum PRDX2 from RA patients is not increased compared to that of healthy donors, and does not correlate with the inflammatory marker CRP. These findings suggest that serum PRDX2 would not be an effective biomarker for RA.
Taken together, the results from this project show that oxidoreductases are uniquely regulated during the innate immune response. Further research is required to understand their roles and the impact they may have on diseases such as RA, which may then enable identification of novel therapeutic targets.
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