**Neutrophils and redox stress in the pathogenesis of autoimmune disease**

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**Abstract**

Polymorphonuclear leukocytes, or neutrophils, are specialist phagocytic cells of the innate immune system. Their primary role is host defence against micro-organisms, which they kill via phagocytosis, followed by release of reactive oxygen species (ROS) and proteolytic enzymes within the phagosome. ROS are generated via the action of the NADPH oxidase (also known as NOX2), in a process termed the ‘Respiratory Burst’. This process consumes large amounts of oxygen, which is converted into the highly-reactive superoxide radical O2- and H2O2. Subsequent activation of myeloperoxidase (MPO) generates secondary oxidants and chloroamines that are highly microbiocidal in nature, which together with proteases such as elastase and gelatinase provide a toxic intra-phagosomal environment able to kill a broad range of micro-organisms. However, under certain circumstances such as during an auto-immune response, neutrophils can be triggered to release ROS and proteases extracellularly causing damage to host tissues, modification of host proteins, lipids and DNA and dysregulation of oxidative homeostasis. This review describes the range of ROS species produced by human neutrophils with a focus on the implications of neutrophil redox products in autoimmune inflammation.

**Keywords**

Neutrophil, ROS, Redox, Auto-immune, Rheumatoid arthritis, Systemic Lupus Erythematosus, Vasculitis, Behçets disease, Psoriasis, Multiple Sclerosis

**Abbreviations**

|  |  |
| --- | --- |
| ANCA | Anti-neutrophil cytoplasmic antibody |
| APS | Anti-phospholipid syndrome |
| ERK | Extracellular signal–regulated kinase |
| FcγR | Fcγ Receptor |
| FMLP | f-Met-Leu-Phe |
| GM-CSF | Granulocyte/macrophage-colony stimulating factor |
| IgG | Immunoglobulin G |
| MAPK | Mitogen-activated protein kinase |
| MPO | Myeloperoxidase |
| mtROS | Mitochondrial ROS |
| NADP | Nicotinamide adenine dinucleotide phosphate |
| NADPH | Nicotinamide adenine dinucleotide phosphate, reduced form |
| NET | Neutrophil extracellular trap |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NOX | NADPH oxidase |
| Nrf2 | Nuclear factor erythroid 2-related factor |
| Phox | Phagocyte oxidase |
| PMA | Phorbol 12-myristate 13-acetate |
| PR3 | Proteinase 3 |
| RA | Rheumatoid arthritis |
| SLE | Systemic lupus erythematosus |
| TNF | Tumour necrosis factor |
| VEGF | Vascular endothelial growth factor |

**Introduction**

Neutrophils are specialist cells of the innate immune system that play a major role in host defence through phagocytosis and generation of reactive oxygen species (ROS). Production of ROS within the phagosome occurs via the action of NADPH oxidase (NOX2) and myeloperoxidase (MPO), and together with release of proteases from intracellular vesicles into the phagosome, provides a defensive arsenal against a broad spectrum of microscopic pathogens. During infection, ROS and proteases may be released extracellularly causing local tissue damage at the site of infection[1]. This damage is normally resolved by resident macrophages, which phagocytose apoptotic neutrophils and damaged tissue as part of the normal process of inflammation resolution[2]. Neutrophils also contribute to inflammation and tissue damage in inflammatory disease, whereby they become inappropriately activated by cytokines, chemokines and auto-antibodies[1]. Auto-immune neutrophils function in a multitude of ways to direct the inflammatory response, including release of proteases which damage host tissue and activate soluble proteins[3], secretion of cytokines and chemokines which direct both the innate and adaptive immune responses[4], shedding of receptors such as the interleukin-6 receptor to initiate trans-signalling[5,6], release of neutrophil extracellular traps (NETs) providing a source of auto-antigens[7], and production of ROS[8,9]. This review will provide an overview of the production of ROS by neutrophils via NOX2 and MPO, the importance of ROS for normal neutrophil functions during infection, and then present the evidence for neutrophil-derived ROS in the pathogenesis of auto-immune disease. Specifically, we will review the contribution of neutrophil-derived ROS in the pathogenesis of rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), anti-phospholipid syndrome (APS), psoriasis, vasculitis and multiple sclerosis.

**Redox reactions in neutrophils**

*NADPH Oxidase (NOX) derived oxidants*

Neutrophils are innate immune cells which primarily function in host defence through phagocytosis of micro-organisms and release of ROS via the ‘Respiratory Burst’. The production of ROS within the neutrophil phagosome, in concert with the secretion of granule proteases, provides a broad-spectrum cytotoxic environment sufficient to kill a wide range of bacterial and fungal pathogens. The primary source of immune-active ROS in neutrophils is the NADPH oxidase (NOX2). NOX2 is a multi-component enzyme, which is assembled at the phagosomal and plasma membranes of neutrophils upon receipt of a “priming” signal, e.g. activation of a cytokine receptor[10,11]. Priming facilitates assembly of NOX2 via mobilisation and phosphorylation of granular and cytosolic components to the phagosomal membrane in readiness for killing of phagocytosed pathogens[11,12]. There are at least six components of the NOX2: p22phox and gp91phox (which together comprise cytochrome b558), p40phox, p47phox, p67phox and Rac (Rac-1 or Rac-2). Around 90% of cytochrome b558 is located on the membranes of specific-granules in resting neutrophils[12]. Priming also induces mobilisation of granules to the plasma membrane, which can result in the localisation of up to 80% of cellular cytochrome b558 on the plasma membrane of an active neutrophil depending upon the stimulus[13,14]. Assembly of NOX2 at the plasma membrane leads to the release of oxygen radicals into the extracellular environment, and is a major cause of redox stress in auto-immune disease.

The cytosolic *phox* components of the oxidase are inactive in the cytosol of resting neutrophils. However, upon priming by agents such as lipopolysaccharide, TNF and GM-CSF, the *phox* proteins are rapidly phosphorylated by protein tyrosine kinases (including p38 MAPK, ERK, and protein kinase C[10,15,16]), and mobilised to the plasma membrane where they complex with cytochrome b558 (Figure 1)[17]. Phosphorylated p40phox and p47phox act as adaptor proteins, enabling binding of p67phox to cytochrome b558[18]. Finally, following translocation to the phagosomal membrane, the GTPase Rac is released from its complex with Rho-GDI, whereupon it binds with p67phox inducing a conformational change that allows interaction with the assembled NADPH oxidase complex[19-21]. Simultaneous binding of all cytosolic components to cytochrome b558 is essential for the formation of an efficient catalytic enzyme complex[22]. A number of proteins, such as phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and S100A8/A9, have been identified as enhancing NOX2 activity at the phagosomal membrane either by controlling the rate of mobilisation of *phox* components or enhancing electron transferase activation[23].

Activation of the respiratory burst normally requires a second signal following priming of the cell and assembly of NOX2. This is usually the activation of Fcγ and/or complement receptors following phagocytosis of opsonised pathogens, or activation of the f-Met-Leu-Phe (fMLP) receptor. Upon activation, assembled NOX2 catalyses the reduction of O2 to the superoxide radical O2- in the reaction NADPH + 2O2 → 2O2- + H+ + NADP+ (Figure 1). The superoxide radical is unstable, and rapidly dismutates either spontaneously or enzymatically (via superoxide dismutase) into hydrogen peroxide (H2O2), or in the presence of iron or copper salts, it may form the hydroxyl free radical (HO●). Whilst O2- is relatively inactive, in the presence of nitric oxide it can form peroxynitrite (ONOO-), a highly reactive oxygen species[24]. The hydroxyl radical is also extremely reactive and microbiocidal, causing DNA mutation/modification, enzyme inactivation and lipid peroxidation[25,26]. Hydrogen peroxide is membrane-permeable, microbiocidal at high concentrations, and the major substrate of myeloperoxidase, a neutrophil granule enzyme implicated in the production of highly-reactive, secondary oxidants.

Despite the generation of acidic compounds during the respiratory burst, the pH within the phagosome remains within the range of pH 7.5-8 and may, on occasions, rise as high as pH 9[27]. Protons are consumed during the formation of H2O2 and HO●, and K+ ions are actively pumped into the phagosome by calcium-activated K+ channels to compensate for the influx of electrons during NOX2 activity. This results in a transient increase in local pH, and a hypertonic K+-rich environment, which provides an optimal environment for the activity of granule proteases, such as elastase and cathepsin G[27,28].

*Myeloperoxidase derived oxidants*

The major source of neutrophil secondary oxidants is via the action of myeloperoxidase (MPO), a haem peroxidase stored within azurophilic neutrophil granules[29]. MPO catalyses the oxididation of halides (Cl-, Br-) and thiocyanate (SCN-) by H2O2 into hypochlorous (HOCl), hypobromous (HOBr), hypothiocyanous (HOSCN) acids (Figure 1), all of which have potent microbiocidal properties including chemical modification of proteins, lipids and DNA. For example, HOCl and HOBr react with methionine and cysteine residues to produce methionine sulfoxide and disulfides, promoting irreversible cross-linking in and between protein molecules, and also between DNA and protein[30]. HOCl oxidizes DNA bases to form 5-hydroxyluracil, 5-hydroxycytosine, and thymine glycol[31], and uniquely generates chlorinated bases 8-chloroguanosine, 8-chloroadenosine, 5-chlorocytosine, and 5-chlorouracil[32]. HOCl also produces a range of microbiocidal mono- and di-chloroamines, molecules which are long-lived and able to penetrate hydrophobic microbial membranes to modify intra-cellular targets[33,34]. HOCl and its chloroamine derivatives are many times more microbiocidal than H2O2, at ~1000-fold lower concentrations. The production of HOCl, by MPO, is a critical step in the production of neutrophil extracellular traps (NETs, described below)[35], and is implicated in DNA damage and modification of histones associated with inflammatory disease[36].

*Mitochondrial oxidants*

Whilst the main source of ROS in neutrophils is via NOX2 reactions, ROS produced by mitochondria (mtROS) may play a significant role in tissue damage, activation of the inflammasome and NET production. Small amounts of mitochondrial superoxide reacts with superoxide dismutase in the mitochondrial matrix to form H2O2. This can then cross the mitochondrial outer membrane to activate cytoplasmic targets such as transcription factors and the inflammasome. H2O2 nanodomains exist at the interface between the mitochondrial membrane and endoplasmic reticulum, which regulate Ca2+ signalling and mitochondrial activity[37]. Whilst mitochondrial catalase acts as a scavenging enzyme to convert H2O2 to H2O, minimising the damaging effect of mtROS, changes in the expression levels of superoxide dismutase and catalase may lead to a dysregulation of this anti-oxidant system and increased leakage of mtROS to the cytoplasm[24,38]. Xanthine oxidase reactions may also produce ROS, particularly under conditions of hypoxia which are often associated with inflammatory disease[24].

**Anti-oxidant systems in health**

In health, several anti-oxidant systems operate to limit the damage caused by ROS. Superoxide dismutase converts oxygen radicals to H2O2, and is present within both the mitochondria and secretory vesicles in neutrophils, as well as within blood sera. During neutrophil activation and degranulation, secretory vesicles mobilise to the plasma membrane and release superoxide dismutase extracellularly to limit damage to surrounding tissues by superoxide radicals[39]. Whilst H2O2 is a key substrate for MPO, it may also be metabolised by catalase and other peroxidases, including glutathione peroxidase, all of which transform H2O2 into H2O. However, superoxide dismutase, catalase and glutathione peroxidase may be inactivated if HOCl is present as a result of MPO reactions[40]. Expression of anti-oxidant enzymes including superoxide dismutase and glutathione peroxidase is under the control of antioxidant response elements[41].

Antioxidant response elements are short sequences of DNA within a gene promoter, which are primarily under control of the transcription factor nuclear factor erythroid 2-related factor (Nrf2). Under conditions of increased ROS, Kelch-like ECH-associated protein 1 (Keap1) is oxidised and dissociates from Nrf2, allowing translocation to the nucleus and activation of antioxidant response elements transcription targets[42]. Redox factor-1 can be oxidised by ROS and translocate to the nucleus, where it interacts with thioredoxin and activates key transcription factors, including activator protein 1 (AP-1) and NF-κB[43]. The phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway can also be activated by ROS, through oxidation and inactivation of phosphatase and tensin homolog (PTEN), a phosphatase which dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to phosphatidylinositol 4,5-bisphosphate (PIP2), normally inhibiting the signal transduction from PI3K and subsequent activation of pleckstrin homology domain containing proteins, such as 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and protein kinase B (AKT)[44].

Dietary anti-oxidants such as glutathione, vitamins A, C and E, and co-enzyme Q10 also play a key role in limiting damage from redox reactions via the reduction of H2O2, and may be present in sera at μM concentrations[45,46]. In disease, in addition to over production of oxygen radicals by activated neutrophils, many elements of the anti-oxidant system may be present or produced at lower than normal concentrations, leading to dysregulation of redox homeostasis and resulting in redox stress.

**Redox control of normal neutrophil functions**

*Apoptosis*

Apoptosis is the default setting for inactive circulating neutrophils, which survive in the blood for less than 24 hours[47]. This constitutive rate of apoptosis in inactive neutrophils is regulated by the intrinsic pathway of apoptosis and the B-cell lymphoma 2 (Bcl-2) family of proteins, of which neutrophils express only a few restricted members, the anti-apoptotic myeloid cell leukaemia 1 (Mcl-1) and Bcl-2-related protein A1 (A1/Bfl-1), and the pro-apoptotic Bcl-2 associated X protein (Bax), Bcl-2 homologous antagonist killer (Bak) and BH3 interacting-domain death agonist (Bid). Mcl-1 and Bfl-1 maintain mitochondrial integrity but are short-lived proteins that are readily degraded by the proteosome allowing for the oligomerisation of Bax and Bak. These Bax:Bak complexes disrupt the mitochondrial membrane, releasing cytochrome c which complexes with apoptotic protease activating factor-1 (APAF1) to form the apoptosome; this cleaves and activates pro-caspase-9, which in turn cleaves and activates the executioner caspase, pro-caspase-3[47,48] initiating apoptosis. Constitutive neutrophil apoptosis is delayed following exposure to agonists including GM-CSF[49,50], TNFα[51], leukotriene B4[52] and lipopolysaccharide[53]. These agonists delay apoptosis through the increased production or stabilisation of Mcl-1 and Bfl-1[50,51,54]. Leukotriene B4-delayed apoptosis has been shown to be NOX2-generated ROS dependent[55].

Following phagocytosis neutrophils initiate a specialised programme of cell death termed phagocytosis-induced cell death. During phagocytosis-induced cell death, neutrophils up-regulate expression of phosphatidylserine on their extracellular membrane as an “eat me” signal to initiate removal by nearby macrophages[56]. By engaging in this process of efferocytosis, neutrophils prevent the release of their toxic cytoplasmic contents, which would otherwise cause significant damage to surrounding tissue. Whilst phagocytosis increases the rate of phagocytosis-induced cell death, cytokines at inflammatory sites may still exert an anti-apoptotic effect on neutrophils[57-59]. This mixture of pro- and anti-apoptotic signals ensures maximal phagocytosis of pathogens prior to phagocytosis-induced cell death. There is mounting evidence that phagocytosis-induced cell death is regulated by NOX2-derived ROS, and that intracellular concentrations of ROS may influence regulators of apoptosis. GM-CSF both increases ROS production and delays apoptosis in phagocytosing neutrophils[60]. During phagocytosis of *S. aureus*, NOX2-derived ROS prevents activation of caspase-3, delaying apoptosis[61]. Alternatively, TNF-α accelerates phagocytosis-induced cell death via a NOX2-dependent process, where NOX2 generated ROS increases caspase-8 activation in phagocytic neutrophils[60]. Activation of NOX2 and generation of H2O2 is critical for the exposure of phosphatidylserine as a pro-phagocytic “eat me” signal to macrophages[56,61]. The NOX2 inhibitor diphenyleneiodonium decreases expression of phosphatidylserine on the surface of neutrophils, and prevents phagocytosis by macrophages[56].

*NETosis*

Neutrophil extracellular traps (NETs) are mesh like DNA structures decorated with histones, MPO and other antimicrobial proteins expelled from neutrophils in response to infectious or inflammatory stimuli[62]. They are an alternative defence mechanism by which neutrophils trap and possibly kill microbes[62,63]. Some NET proteins are post-translationally modified, including histones which have been found to be methylated, acetylated[64] and citrullinated[65-68], leading to speculation NETs may be a source of auto-antigens in auto-immune disease[8]. The most potent inducer of NETosis *in vitro* is phorbol myristate acetate (PMA), which activates protein kinase C and NOX2 in both human[69,70] and murine neutrophils[71]. PMA-induced NETosis relies on NOX2 activation as evidenced by the fact that neutrophils from patients with chronic granulomatous disease patients, who have genetic defects preventing assembly of a functional NADPH oxidase, produce neither ROS nor NETs; diphenyleneiodonium also inhibits PMA-induced NETosis[69,72,73]. Calcium ionophores such as ionomycin and A23187 induce the release NETs, and in particular, citrullinated histones on NETs[65-68]. Several physiological agonists have also been reported to induce NET release, including fMLP, IL-8, lipopolysaccharide, nitric oxide, TNFα and others[74].

ROS production is required for several steps of NET formation, causing increases in membrane permeability and release of neutrophil elastase from granules, which in turn degrades the linker histone H1 and the core histones, driving chromatin decondensation[75]. ROS promote morphological changes that occur during NETosis[70] and inactivate caspases to block apoptosis and trigger autophagy[75]. Autophagy, the conserved process of lysosome-mediated intracellular degradation enabling routine turnover of proteins and organelles, has been shown to be intimately associated with ROS. In particular the level of intracellular ROS determine whether autophagy reactions lead to NETosis[70]. Production of secondary oxidants such as HOCl is a critical step in PMA-induced NETosis[75-78]. Absence of extracellular Cl-, a substrate for MPO, decreases NET production *in vitro*, and whilst exogenous addition of H2O2 and HOCl stimulates NETosis, only exogenous HOCl can rescue NETosis in the setting of MPO inhibition[35].

NETosis induced by calcium ionophores such as A23187 appears to occur in a different manner from that of PMA-induced NETosis, and appears to be independent of NOX2 activity and thus is often referred to as NOX-independent NET formation[79,80]. This NOX-independent NETosis is dependent on calcium and activates peptidyl arginase deiminases leading to cellular hypercitrullination[65,79]. It also relies upon production of mtROS via the calcium-activated small conductance potassium channel member SK3[79].

*Signalling*

At low concentrations, ROS act as signalling molecules in both intra- and extra-cellular signal transduction, influencing a variety of cellular processes, such as proliferation, metabolism, differentiation and survival[81-85]. The main mechanism for this is through oxidation of sulphur-containing cysteine and methionine residues resulting in altered protein structure, function and DNA binding capacity. Cysteine thiols (-SH) are oxidised to sulfenic acid (-SOH), which can then form di-sulphide bonds or be further oxidised to sulfinic (-SO2H) and sulfonic acid
(-SO3H), with increasing irreversibility, whereas methionine sulphides (-SR) are oxidised to sulfoxide (-SOR), and further to sulfonyl (-SO2R). These reactions are largely reversible, with a primary act of ROS signalling being their own regulation, or oxidative homeostasis: as ROS levels increase, mechanisms to reduce ROS and return to an equilibrated state are activated. Dedicated reductase enzymes reduce the oxidised residues back to their normal state, whilst a plethora of antioxidant mechanisms act to scavenge loose oxidants and prevent damage.

Mitogen-activated protein kinases (MAPK) respond to ROS in a cell type and stimulus specific manner[85]. In neutrophils, endogenous production of H2O2 is regulated by p38-MAPK and c-Jun n-terminal kinase (JNK) and, and can induce phosphorylation of ERK1/2[86]. The apoptosis signal-related kinase 1 (ASK1) is activated by ROS, causing dissociation of thioredoxin and subsequent oxidation of Cys-32 and Cys-35 followed by formation of a di-sulphide bond[87]. Whilst ASK1 can signal in apoptosis, via p38-MAPK and JNK, it has also been shown to regulate cytokine production[88] and cell differentiation pathways[89]. Several ROS-activated protein kinase families, including protein kinases -A, -C and -G, have been implicated in MAPK signalling[90]. Inhibition of MAPK phosphatases by ROS can also modulate MAPK activity. Oxidation of many protein tyrosine phosphatases blocks their ability to inhibit MAPK activation[91-93]. Increased intracellular ROS has also been shown to activate the inflammasome, which recruits caspases -1 and -12 and cleaves the inflammatory cytokines pro-IL-1 and pro-IL-18 to their active forms, prior to secretion[94].

Specifically in neutrophils, ROS are implicated in migration to and from wounds and may be necessary for resolution of neutrophil-mediated inflammation. The Src family kinase Lyn acts as a sensor for H2O2 gradients, generated from epithelial wounds by dual oxidase, attracting neutrophils to the site of injury[95]. Adhesion molecule expression can also be sensitive to the ROS environment. Adhesion of neutrophils to endothelial cells, and subsequent extravasation into tissues, involves ROS-induced phosphorylation of focal adhesion kinase pp125FAK, paxillin and p130cas[96].

**Neutrophil oxidants in autoimmune disease**

*Rheumatoid arthritis*

Rheumatoid arthritis (RA) is an inflammatory disease of the joints which causes irreversible damage to synovial tissues, including degradation of cartilage and underlying bone. The disease is characterised by the growth of a hyperplastic inflammatory tissue (pannus) within the joint, which is enriched with synovial fibroblasts and leukocytes that drive the inflammatory process. Neutrophils have long been implicated in the pathogenesis of RA due to their ability to release inflammatory cytokines, ROS and matrix proteases via the secretome. In RA, immune complexes such as rheumatoid factor and anti-citrullinated protein antibodies activate neutrophils via Fcγ receptors[97]. This triggers neutrophils to degranulate either into the synovial fluid or directly onto the articular surface forming a microenvironment of concentrated ROS, proteases and cytotoxic factors[8]. These processes result in a heightened presence of ROS in the joint environment, causing damage to the articular cartilage and underlying bone[8]. Patients with RA have evidence of increased ROS production in their circulating blood and synovium, with several studies demonstrating a significant correlation between ROS levels and disease activity[98-100]. Production of O2- and H2O2 by both blood and synovial fluid neutrophils is significantly higher in RA patients compared to healthy control blood neutrophils[100]. The low-density granulocyte population, elevated in RA, is thought to comprise immature neutrophils which demonstrate altered transcriptome and ROS generating properties[101]. ROS production in response to fMLP is lower in RA low-density granulocytes primed by TNF-α compared to paired blood neutrophils, likely due to their lower expression of TNF-receptors[101]. The expression of TNF-α, a key modulator of RA activity, can be enhanced in monocytes under increased oxidative stress[102,103], and TNF-α itself induces ROS production[104].

Markers of ROS-mediated damage, including lipid peroxidation, protein oxidation and DNA damage are increased in RA blood, whereas antioxidant defence mechanisms, both enzymatic such as catalase and superoxide dismutase, and non-enzymatic such as vitamin C and reduced glutathione, are retarded[105]. Antioxidants have a demonstrable effect on lowering ROS production in *ex vivo* neutrophils and on *in vitro* matrix degradation[106,107], and oxidative stress within RA synovial tissues decreases during therapy, such as with TNF-α inhibitors[108]. The clinical evidence for therapeutic use of antioxidants is however, unconvincing[109-111].

Dysfunctional immunoglobulin G (IgG), can be found in the sera of patients with RA. ROS-mediated modification of human IgG generates novel antigens and increases immunogenicity and production of rheumatoid factor immune complexes[112]. Both soluble and synovial tissue embedded complexes of IgG are the main activator of ROS release by RA neutrophils, acting via FcγR2a and FcγR3b[113]. Complexes of soluble IgG stimulate the extracellular release of O2- and H2O2 species *in vitro* from neutrophils primed with cytokines such as GM-CSF and TNF[113]. This extracellular release of ROS is due almost entirely to activation of neutrophils via FcγR3b[114]. Interestingly, removal or blockade of FcγR3b does not impact significantly on bacterial phagocytosis and killing, identifying this receptor as a potential, ROS-specific therapeutic target in RA[114].

Whilst ROS production in the joint can act directly to modify, denature and breakdown cell and matrix components, increasing levels in the joint environment can also disrupt oxidative homeostasis and induce ROS-mediated signalling pathways in both neutrophils and surrounding cells. Regulation of matrix metalloproteinases[115,116], collagen degradation[107], fibronectin fragment formation[117] and chondrocyte hypertrophy and apoptosis[118] can all be linked to ROS activity. Cyclooxygenase (COX)-2, an enzyme involved in pro-inflammatory prostaglandin synthesis and a common target of non-steriodal anti-inflammatory drugs, is highly expressed in RA and can be induced in synovial fibroblasts by ROS-induced MAPK and NF-κB activation via phosphorylation of transforming growth factor beta-activated kinase 1 (TAK1)[119]. T cell proliferation, differentiation and apoptosis may be regulated by local ROS production[120,121], and oxidative stress inhibits expression of IL-17 and interferon-γ *in vitro* and *ex vivo* in CD4+ T cells, suggesting a role for ROS in directing specific adaptive immune responses.

*Systemic lupus erythematosus*

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease with chronic or episodic inflammation in several organ systems, including the skin, joints, central nervous system and kidneys. Patients are at increased risk of both cardiovascular events and subclinical atherosclerosis. SLE may be characterised by multiple autoantibodies, circulating immune complexes at high levels, and low complement levels. Many clinical manifestations of SLE, including organ damage, can be explained by the release of enzymes from neutrophil granules and increased production of ROS in response immune complexes deposited in tissues[122-124].

Several studies have shown that SLE neutrophils produce oxygen intermediates (O2-, H2O2, HO●) more rapidly and in higher concentrations than healthy controls[125-127], and basal H2O2 production by SLE neutrophils is the highest in neutrophils from patients with lupus nephritis[127]. Levels of intracellular anti-oxidants, including glutathione and γ-glutamyl-transpeptidase, are decreased in SLE neutrophils[128]. SLE disease course varies between patients, with most patients experiencing both recurrent ‘active’ SLE flares and periods of ‘inactive’ disease. Neutrophils from SLE patients in active flare have been shown, paradoxically, to produce lower levels of ROS *in vitro* than neutrophils from SLE patients with inactive disease. However, ‘active SLE’ neutrophils exhibit higher levels of intracellular oxidative damage, including decreased levels of malondialdehyde, a marker of lipid peroxidation, and high levels of protein oxidation, evidenced by increased carbonyl groups and decreased thiol (SH) levels, suggesting exposure to oxidative stress *in vivo* prior to isolation from the blood[126]. Lower ROS production by ‘active SLE’ neutrophils in response to IgG immune complexes *in vitro* may be explained by lower expression of FcγRII (CD32) and complement receptor 1 (CR1)[129]. Neutrophils from SLE patients with both circulating immune complexes and cytotoxic antibodies produce the highest O2- response to FcγR/complement receptor stimulation[130]. SLE serum induces O2- generation in healthy neutrophils, with O2- production correlating positively with the presence of immune complexes and negatively with complement levels [131].

SLE is an incredibly heterogeneous disease, both in terms of organ involvement and antibody profiles. It is estimated that 11% of SLE patients have neutrophil cytosolic factor 1 (Ncf1) polymorphisms[132]. The missense single nucleotide polymorphism, rs201802880, NCF1-339 T allele, decreases extracellular ROS release by SLE neutrophils but increases expression of type 1 interferon-regulated genes, and in addition is associated with a younger age of diagnosis. This suggests that ROS may be less important drivers of disease in individuals carrying this Ncf1 variant[132]. These findings echo those from an Ncf1 (m1J) mutated mouse model, which showed that the unexplained connection between ROS deficiency and increased susceptibility to autoimmunity could be explained by the discovery that activation of interferon signalling is a major pathway downstream of a deficient NOX2 complex in both mice and humans[133].

Neutrophils from SLE patients exhibit markers of oxidative stress, including increased DNA damage[134]. The DNA in NETs from SLE neutrophils has a higher content of 8-hydroxyguanosine, an oxidized self-DNA which may function as a damage-associated molecular pattern, promoting and exacerbating the inflammatory response, for example via the production of interferons[135,136]. SLE low-density granulocytes express increased levels of pro-inflammatory cytokines and type I-interferon compared to neutrophils[137,138] and in addition spontaneously undergo enhanced NETosis *ex vivo*[7,139]. It has been suggested that SLE low-density granulocyte undergo NETosis in response to the production of mtROS, with SLE low-density granulocyte NETs containing mitochondrial DNA[136]. This phenomenon is also observed in chronic granulomatous disease low-density granulocytes, which lack functional NOX2 but can produce mtROS[136].

*Anti-phospholipid syndrome*

One third of SLE patients have anti-phospholipid syndrome (APS), the major clinical manifestation of which is thrombosis and/or pregnancy loss. The presence of one of these clinical features, plus anti-phospholipid antibodies such as anti-cardiolipin, anti-β2-glycoprotein-I or lupus antibody in the blood, are used for diagnosis of APS. APS may also occur in the absence of any other related disease, where it is termed primary APS[140]. H2O2 production by neutrophils is increased in APS, and glutathione levels are significantly decreased[141]. Levels of the antioxidant transcription factor Nrf2 are decreased in APS along with significant losses in mitochondrial membrane potential[141]. Ubiquinol, also known as reduced co-enzyme Q10, is vital component of the mitochondrial complexes I, II and III, and is currently undergoing a clinical trial in APS[142] after patients treated with co-enzyme Q10 for 1 month showed improved functions in many cell types. In particular, co-enzyme Q10 decreases NET production by APS neutrophils and down-regulates levels of intracellular elastase and MPO[143]. Catalase and glutathione peroxidase activities are decreased in monocytes and neutrophils of APS patients, perhaps because of the overproduction of H2O2[144].

Anti-phospholipid antibodies can activate neutrophil degranulation, ROS release and IL-18 production[145,146]. Anti-β2-glycoprotein-I antibodies stimulate both APS and healthy neutrophils to produce H2O2, leading to NET release via activation of toll-like receptor-4 and NOX2[147]. Additionally, oxidative stress has been shown to be a risk factor for modifications to β2-glycoprotein-I that promote anti-phospholipid antibody:β2-glycoprotein-I interactions[148]. Anti-cardiolipin antibodies play a role in oxidative status, by inducing ●NO and O2- production, resulting in enhanced levels of plasma peroxynitrite[149]. In humans, anti-phospholipid antibodies alone induce only minor activation of neutrophils, including oxidative burst at high antibody concentrations. However, in the presence of lipopolysaccharide, the activation threshold is markedly lower indicating a synergistic activation pathway of anti-phospholipid antibodies and toll-like receptor-4 in neutrophils. The toll-like receptor-2 ligand Pam3Cys is also synergistic with anti-phospholipid antibodies. This is in keeping with the fact that infections may be an additional factor potentiating the impact of anti-phospholipid antibodies in APS[146].

Neutrophil-derived ROS may be a cause of miscarriage in APS. In this process, anti-phospholipid antibodies bind to trophoblasts, where they activate the complement cascade leading to the generation of C5a, attracting and activating neutrophils. C5a engages with its receptor C5aR, causing neutrophils to express tissue factor and protease activated receptor 2 (PAR2). Tissue factor forms a ternary complex with factor VIIa and factor Xa that activates PAR2, ROS production, trophoblast injury and ultimately fetal death[150-152]. Pravastatin, shown to prevent miscarriage in anti-phospholipid antibody-treated mice[153], also improves pregnancy outcomes in humans with APS[154]. Pravastatin downregulates tissue factor and PAR2 expression on neutrophils, inhibiting activation[151]; this might explain why trophoblasts are not damaged and thus why pregnancies continue[151].

*Vasculitis*

Vasculitis comprises a spectrum of disorders in which blood vessels are destroyed by inflammation. In anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, neutrophils are both the direct target of auto-antibodies and the main protagonist of vascular damage through the production of ROS. ANCA-associated vasculitis is classified as granulomatosis with polyangiitis (formerly Wegener’s granulomatosis), microscopic polyangiitis or eosinophilic granulomatosis (formerly Churg-Strauss syndrome). Auto-antibodies are produced against neutrophil granule proteins MPO or proteinase-3 (PR3), with anti-MPO ANCAs being most commonly associated with microscopic polyangiitis and anti-PR3 ANCAs being present in >90% of patients with granulomatosis with polyangiitis[9]. Anti-MPO ANCAs stimulate the production of ROS by unprimed, healthy control neutrophils[155], and anti-PR3 ANCAs stimulate the production of ROS by vasculitis neutrophils, with levels of ROS produced correlating with the NET production[156]. Activation of ROS by ANCAs is enhanced by the presence of high mobility group box-1 (HMGB1), an inflammatory protein which is elevated in the serum of vasculitis patients and which is associated with increased levels of neutrophil migration and vascular damage[157]. Increased production of MPO-containing NETs by vasculitis neutrophils and low-density granulocytes may exacerbate damage to vascular tissue[158,159]. NET remnants (nucleosome:MPO complexes) are found in the serum of vasculitis patients; these are elevated during active disease but do not correlate to ANCA titres[159]. Blockade of phosphatidylinositol-4,5-bisphosphate 3-kinase-γ (PI3Kγ) in a mouse model of microscopic polyangiitis abrogated MPO-ANCA-induced NET production and ameliorated disease[160]. Pharmacological inhibition of PI3Kγ decreased NET production by healthy human neutrophils[160]. Oxidised lipoproteins, associated with an elevated risk of atherosclerosis in vasculitis patients, are elevated in vasculitis serum and may form as a result of increased production of MPO-derived oxidants such as HOCl[161]. As well as inducing damage to the endothelial layer itself, neutrophil-derived ROS enhance activation and ligand-binding activity of αMβ2 integrin and increase interactions of neutrophils with both platelets and endothelial cells under flow conditions[162].

Behçets disease is a form of vasculitis with multi-system involvement, including the eyes, mouth, genitals and brain. Although ANCAs are not present in patients with Behçets disease, activated neutrophils are found at sites of inflammation. Serum from Behçets patients stimulates production of both O2- and H2O2 species, and more specifically, serum from patients with active disease stimulates the production of higher levels of H2O2 than are produced in response to PMA *in vitro*[163]. A recent study showed that, not only is spontaneous NETosis increased in Behçets neutrophils, but that the factor in patient plasma responsible for increased NET and ROS production is sCD40L[163] which is present at significantly higher concentrations in Behçets patients compared to healthy controls and patients with SLE.Neutrophils from Behçets patients have also been shown to release high levels of ROS when stimulated with fMLP *in vitro*[164,165], and levels of lipid peroxidation are higher in both Behçets serum, erythrocytes and neutrophils compared to healthy controls[166,167]. As well as increased ROS production by neutrophils, vascular damage in Behçets disease may be mediated by dysregulation of the anti-oxidant system, including increased levels of superoxide dismutase and decreased levels of glutathione peroxidase[167]. A separate study reported lower superoxide dismutase levels in Behçets disease, which was associated with lower serum and erythrocyte glutathione peroxidase, lower erythrocyte catalase, higher adenosine deaminase (a marker of T-cell and neutrophil activation) and increased lipid peroxidation[168]. Increased ROS production by Behçets neutrophils may be a direct cause of fibrinogen oxidation, which leads to slower fibrin polymerisation and resistance to plasmin-induced lysis[169], a possible cause of coronary symptoms associated with the disease. High levels of plasma MPO activity have also been reported in Behçets patients, along with increased levels of plasma nitrate/nitrite, which are substrates for MPO and cause the formation of the reactive nitrogen dioxide (●NO2) oxidising agent[165]. Consumption of nitrate/nitrite by MPO, leading to depletion of these substrates for nitric oxide synthase reactions, decreases the production of nitric oxide (●NO), an essential regulator of smooth muscle contraction and vasodilation[170]. In addition, increased expression of cyclooxygenase-2 in Behçets neutrophils may contribute to increased vascular damage via the synthesis of pro-inflammatory prostaglandins[165].

Neutrophil-derived ROS are also implicated in development of fibrosis and vascular damage in systemic sclerosis. Auto-antibodies directed against angiotensin II type 1 receptor and endothelin-1 type A receptor purified from systemic sclerosis sera can activate ROS production by healthy control neutrophils[171], and neutrophils from systemic sclerosis patients produce a heightened ROS response to fMLP following priming[172].

*Psoriasis*

Psoriasis is an auto-inflammatory disease of the skin characterised by the hyperproliferation of keratinocytes with increased cell turnover leading to the formation of plaques on the skin surface. Hallmark histological feature of psoriasis, such as the pustules of Kogoj and microabscesses of Munro, are formed by the accumulation of neutrophils within the dermis and epidermis respectively. Despite observed neutrophilia in plaques, psoriasis is thought to be a largely T cell driven disease, characterised by the high expression of type 1 cytokines in both plaques and serum[173]. However, the pathophysiology of psoriasis involves cells of both the innate and adaptive immune systems. A simplified pathway of psoriasis plaque formation has been proposed[174] in which dendritic cells express TNF-α and IL-23, activating Th1 cells, Th17 cells and resident macrophages. Activated Th17 cells express both IL-22, which promotes keratinocyte proliferation, and IL-17 which acts in concert with TNF-α to induce IL-8 production by keratinocytes. Activation of keratinocytes also causes increased expression of vascular endothelial growth factor (VEGF) leading to vascularisation of the plaques. Neutrophils are recruited to the site by IL-8 and once there readily degranulate, releasing proteolytic enzymes and ROS causing local tissue damage and potentiating inflammation[174]. Whist cytokine-driven pathways are hugely important in the pathogenesis of psoriasis, there is considerable evidence for the additive role of a pro-oxidant:antioxidant imbalance[175].

As one of the most abundant cell types at active sites of psoriasis, the contribution of neutrophils to disease pathology is thought to be significant. Neutrophils isolated from patients with psoriasis degranulate both primary and secondary granules more readily than neutrophils from healthy controls. Furthermore, increased MPO and catalase activity is reported in psoriatic neutrophils[176]. It has also been reported that psoriatic neutrophils exhibit enhanced NOX2 activity and that they can produce more ROS than healthy neutrophils, even in the absence of autologous serum[177]. Whilst this indicates that psoriatic neutrophils have a constitutively enhanced capacity to produce ROS, it does not mean that psoriatic sera does not contribute to increased neutrophil activation, as healthy neutrophils demonstrate increased O2- generation when incubated in serum from psoriasis patients[178]. As well as increased ROS generation, neutrophils from patients with psoriasis have been reported to have lower activity of key antioxidant enzymes, superoxide dismutase and glutathione peroxidase,[179,180], and decreased activity of glutathione peroxidase also correlates with increase disease severity.

NETs have been observed in psoriatic lesions and neutrophils isolated from the blood of psoriasis patients more readily undergo the ROS-driven process of NETosis than neutrophils from healthy controls. Interestingly, in these individuals spontaneous NETosis correlates with disease severity. As has been shown in the case of neutrophil ROS production, this phenomenon is not a primary psoriatic neutrophil defect, as psoriatic serum also induces NETosis in healthy neutrophils[181]. Interestingly, this NET formation is likely to be ROS dependent as it is blocked by dimethyl fumurate in a ROS-dependent manner[182].

The effect of the local tissue environment on neutrophil ROS generation in psoriasis has recently been further delineated. It has been demonstrated that neutrophils in psoriatic lesions have an increased capacity for activation, mediated by both keratinocytes and T cells. Psoriatic keratinocytes produce significantly more TNF-α, monocyte chemoattractant protein 1 (MCP-1), IL-8 and GM-CSF than keratinocytes from healthy individuals[183]. These cytokines are all well known priming agents of neutrophils. Importantly, O2- production is 1.5 fold greater in healthy neutrophils incubated with psoriatic keratinocytes compared with healthy keratinocytes[184].

There have been two waves of interest in neutrophil involvement in psoriasis pathogenesis. The first wave, between the late 1970s and early 1990s focussed on neutrophil adhesion, phagocytosis, degranulation and ROS generation. With the discovery of T cell involvement, particularly Th17 cells, interest in psoriatic neutrophils waned[185]. However, in recent years, a number of papers have reported that neutrophils express IL-17, the signature cytokine of Th17 cells and a major driver of psoriatic disease[186,187]. Despite the controversy around these reports[188,189], it has led to a renewed interest in neutrophil involvement in psoriasis, and though welcome, it may be poorly directed. It would be wholly more interesting and logical to concentrate attention on the involvement of neutrophil-derived ROS, and on VEGF expression by keratinocytes. Both healthy and psoriatic keratinocytes produce high concentrations of ROS; however, unlike cytokines, VEGF is not up-regulated in keratinocytes during co-incubation with IL-2-treated T cells and so increased VEGF expression must be mediated by some other cell type[183]. VEGF is known to be increased in response to H2O2[190] and it is therefore not inconceivable to suggest that psoriatic neutrophils, which have low glutathione peroxidase activity[180], low superoxide dismutase activity[179], greater NOX2 activity and oxidative bursts[177], and which degranulate more readily[176] might be the driver of keratinocyte VEGF production through aberrant H2O2 release.

*Multiple Sclerosis*

Multiple sclerosis is an inflammatory demyelinating disorder of the central nervous system, characterised by the formation of sclerotic lesions in the white matter of the brain, loss of oligodendrocytes leading to demyelination of neurons, and systemic inflammation. Whilst multiple sclerosis is traditionally believed to be a T-cell-mediated auto-immune disease[191], there is an increasing amount of evidence for the involvement of neutrophils in disease pathology. Neutrophils isolated from the peripheral blood of multiple sclerosis patients have an activated phenotype, expressing increased levels of several cell surface markers of activation and lower levels of apoptosis[192]. Production of ROS by multiple sclerosis neutrophils in response to fMLP is significantly increased compared to healthy controls, and serum concentrations of neutrophil elastase are elevated[192]. NETs containing complexes of DNA and MPO are not only present in multiple sclerosis sera[192], but are also significantly elevated in male patients, who generally have a worse prognosis than females[193]. Levels of lipid peroxidation are higher in multiple sclerosis neutrophils and sera, and this is associated with lower levels of cellular glutathione peroxidase and serum vitamins A and E[166].

However the precise role of neutrophils in the pathogenesis of multiple sclerosis is still under question; whilst peripheral blood neutrophils are activated in multiple sclerosis they are not found within the central nervous system, and neutrophil products (such as NETs) are not found within cerebral spinal fluid. A potential role for neutrophil proteases such as matrix metalloproteinases in opening the blood:brain barrier has been proposed[194]. In mouse models of the disease, around 55% of MPO-positive leukocytes found in the brain are neutrophils[195], with MPO-positive neutrophil numbers remaining elevated in the brain during disease remission but increasing further during relapse. However, in humans the main source of MPO and ROS within brain lesions is believed to be macrophages[196]. A direct role for neutrophil-induced demyelination of the corpus callosum has been demonstrated in a cuprizone-induced murine model of multiple sclerosis[91].

**Closing remarks**

This review of recent literature clearly demonstrates that neutrophils are primed to release higher levels of ROS in a number of auto-immune disease settings, and that damage mediated to host tissue by neutrophil-derived oxidants (both primary and secondary) contributes to disease pathology (summarised in Figure 2). However, there is insufficient detail in the literature about the involvement, relevance, and concentrations of specific ROS species *in vivo* in auto-immune diseases. Many studies measure the ability of *ex vivo* patient neutrophils to produce ROS in response to non-physiological stimuli (e.g. PMA) or pathological agonists such as autologous serum or auto-antibodies, with little regard for the species of ROS being produced. As this review and the work of others clearly shows, the production of different ROS species is highly relevant both in terms of the cellular microenvironment, disease pathology and the manifestation of oxidative damage at the cellular and tissue level. As technologies and techniques for the study of redox reactions become more sophisticated, it is important to revisit some of the experiments reviewed here, to understand the role of specific neutrophil-derived oxidants in finer detail.

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**Figures**

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**Figure 1. Production of reactive oxygen species (ROS) within the neutrophil phagosome.** Priming of a neutrophil, e.g. by a cytokine, triggers mobilisation of cytoplasmic *phox* and Rac proteins and membrane-expressed cytochrome b558 (Cyt b) to assemble the NADPH oxidase (NOX2) at the phagosomal membrane. Reduction of oxygen in the presence of NADPH by NOX2 produces O2- within the phagosome. This in turn dismutates enzymatically (via superoxide dismutase) or spontaneously into H2O2. Priming also stimulates fusion of neutrophil granules with the phagosome leading to release of myeloperoxidase (MPO) which produces secondary HOCl from reactions with H2O2.



**Figure 2. Neutrophil-derived oxidants in the pathogenesis of auto-immune disease.** Production of ROS including O2-, H2O2 and HOCl, both intracelullarly and extracellulary, regulates a myriad of neutrophil functions, as well as inducing oxidative damage at the molecular, cellular and tissue level which contributes to disease pathogenesis and damage to host tissue. ARE, antioxidant response element; NET, neutrophil extracelllular trap; NOS, nitric oxide synthase; TF, transcription factor.