**Human Neutrophils in Auto-immunity**

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**Abstract**

Human neutrophils have great capacity to cause tissue damage in inflammatory diseases via their inappropriate activation to release reactive oxygen species (ROS), proteases and other tissue-damaging molecules. Furthermore, activated neutrophils can release a wide variety of cytokines and chemokines that can regulate almost every element of the immune system. In addition to these important immuno-regulatory processes, activated neutrophils can also release, expose or generate neoepitopes that have the potential to break immune tolerance and result in the generation of autoantibodies, that characterise a number of human auto-immune diseases. For example, in vasculitis, anti-neutrophil cytoplasmic antibodies (ANCA) that are directed against proteinase 3 or myeloperoxidase are neutrophil-derived autoantigens and activated neutrophils are the main effectors cells of vascular damage. In other auto-immune diseases, these neutrophil-derived neoepitopes may arise from a number of processes that include release of granule enzymes and ROS, changes in the properties of components of their plasma membrane as a result of activation or apoptosis, and via the release of Neutrophil Extracellular Traps (NETs). NETs are extracellular structures that contain chromatin that is decorated with granule enzymes (including citrullinated proteins) that can act as neo-epitopes to generate auto-immunity. This review therefore describes the processes that can result in neutrophil-mediated auto-immunity, and the role of neutrophils in the molecular pathologies of auto-immune diseases such as vasculitis, rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). We discuss the potential role of NETs in these processes and some of the debate in the literature regarding the role of this phenomenon in microbial killing, cell death and auto-immunity.

**Highlights**

* Neutrophils modulate the immune response and, if inappropriately activated, are a source of auto-antigens
* ANCA-associated vasculitis is characterized by specific auto-immunity against the neutrophil components PR3 and MPO
* PR3 is a danger signal that can shape both the innate and the adaptive immune response
* NETs are released from neutrophils under inflammatory conditions and might be a source of auto-antigens

**Abbreviations**

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| AAV | ANCA-associated vasculitis |
| ACPA | anti-citrullinated protein antibodies |
| ANCA | anti-neutrophil cytoplasmic antibodies |
| APRIL | a proliferation-inducing ligand |
| BAFF/BLyS | B-cell activating factor/ B Lymphocyte Stimulator |
| LDG  EGPA | Low density granulocyte  eosinophilic granulomatosis with polyangiitis |
| G-CSF | Granulocyte-colony stimulating factor |
| GM-CSF | Granulocyte/macrophage-colony stimulating factor |
| GPA | granulomatosis with polyangiitis |
| ICAM | Intercellular Adhesion Molecule |
| IFN | Interferon |
| IL | Interleukin |
| JSLE | Juvenile systemic lupus erythematosus |
| LDG | Low density granulocyte |
| LPS | lipopolysaccharide |
| MMP | Matrix metalloproteinase |
| MPA | microscopic polyangiitis |
| MPO | Myeloperoxidase |
| NADPH oxidase | nicotinamide adenine dinucleotide phosphate-oxidase |
| NET | Neutrophil extracellular trap |
| PAD | protein-arginine deiminase |
| pDCs | Plasmacytoid dendritic cell |
| PMA | phorbol 12-myristate 13-acetate |
| PR3 | Proteinase 3 |
| RA | Rheumatoid arthritis |
| RAGE | receptor for advanced glycosylation endproducts |
| RANKL | Receptor activator of nuclear factor kappa-B ligand |
| RNP  ROS | Ribonucleoprotein  Reactive oxygen species |
| SLE | Systemic lupus erythematosus |
| STAT | Signal Transducer and Activator of Transcription |
| TLR | Toll-like receptor |
| TNF | Tumour necrosis factor |

**1. Introduction**

**1.1 Neutrophils and host defence**

Neutrophils play key roles in the control of bacterial and fungal infections, via their ability to migrate from the circulation to sites of infection, and when at these sites, to recognize and destroy the invading pathogens[1]. Neutrophils are therefore highly-specialised killing cells, containing a wide variety of degradative enzymes (e.g. proteases, hydrolases, nucleases) in their granules, plus the ability to generate reactive species (ROS) via an activated NADPH oxidase in combination with myeloperoxidase[2-4]. These cytotoxic components can, by acting together, rapidly and effectively kill a wide range of microbial targets[4]. These properties of neutrophils make them uniquely adapted for this killing role, and indeed they have the highest cytotoxic potential of all immune cells.

In order to perform this role in host defence, inactive neutrophils in the circulation must respond to regulatory or chemotactic signals (e.g. cytokines, chemokines and host- or pathogen-derived factors) and move from the circulation to the site of infection. This process involves “priming” of their functions which occurs via activation of kinase cascades, changes in the surface properties of the cells via movement of cytoplasmic granules to the cell surface and activation of *de novo* gene expression[5-7]. All of these mechanisms contribute to the processes that result in a “primed” neutrophil with a greater cytotoxic capacity, an extended lifespan and enhanced functions that allow these cells to mount an effective challenge during the acute inflammatory response[6]. During these processes, neutrophils themselves can secrete cytokines, chemokines and other regulatory molecules that can promote inflammation (including recruitment and activation of other neutrophils) and also regulate other elements of the immune system[8, 9]. Once neutrophil function is complete (e.g. the infection is cleared) they undergo cell death by apoptosis and inflammation normally resolves[6].

**1.2. Neutrophils, inflammation and inflammatory damage**

Whilst this key role of neutrophils in host defence has been appreciated for many years, the ability of these cells to contribute to the tissue damage associated with inflammation and inflammatory diseases is also recognised. Observations from human studies and animal models implicate neutrophils and their products of activation (e.g. proteases and ROS) in the tissue- and organ-damage associated with human diseases, that include rheumatoid arthritis, vasculitis, chronic obstructive pulmonary disease and inflammatory bowel disease[10-13]. In such diseases, neutrophils can infiltrate tissues and become inappropriately activated, e.g. as a result of infection or via immune complexes[14] to secrete molecules that are normally retained in phagocytic vesicles following phagocytosis of pathogens[15]. These secreted molecules can attack host tissues if they overwhelm endogenous tissue levels of anti-proteinases or anti-oxidants[16].

In addition to their direct role in initiating tissue damage in inflammatory diseases, neutrophil-derived cytokines, chemokines and other regulatory molecules (e.g. eicosanoids) can also orchestrate the functions of other immune cells in these inflammatory conditions[8-10]. Far from being passive cells that can only respond to inflammatory signals by generating a cytotoxic response, it is now recognised that neutrophils are key players in the regulation of almost every element of the immune response: from control of haematopoiesis to modulation of T and B cell function[8, 17]. In inflammatory diseases such as rheumatoid arthritis, a variety of cytokines and chemokines are implicated in disease pathology[18, 19], and this phenomenon has formed the basis for the development of a range of anti-cytokine biologic drugs (typified by TNF inhibitors, TNFi) which can result in dramatic improvements in disease activity[20]. Neutrophil-derived cytokines may, at least in part, contribute to the dysregulated cytokine/chemokine signalling networks that characterize these diseases[8, 19].

More recently, it has become recognised that neutrophils may also be the source of auto-antigens, via a number of mechanisms (Figure 1). These include: neutrophil degranulation (which releases granule enzymes into the extracellular environment and also changes the properties of their plasma membrane); apoptosis, the process of regulated cell death, which also results in changes to the properties of the plasma membrane of apoptotic neutrophils; neutrophil extracellular trap (NET) formation. This review will focus therefore on the processes by which neutrophils can expose or generate auto-antigens that result in the generation of autoantibodies that characterize a number of human diseases, and hence how neutrophils may contribute to immune dysregulation favouring auto-immunity. For a comprehensive review of the use of animal models to determine the role of neutrophils in autoimmune diseases, the reader is referred to [21] and the article by Lowell and co-workers in this issue of Seminars in Immunology.

**1.3 Mechanisms of exposure of neutrophil-derived auto-antigens**

**1.3.1. Neutrophil activation and degranulation**

During phagocytosis, internalised microbes or immune complexes are localized within phagolysosomes, the membranes of which contain an activated NADPH oxidase (that generates ROS) while the matrix of these phagocytic vesicles becomes enriched with activated granule enzymes (such as myeloperoxidase, defensins and proteases) following fusion of granules with the phagocytic vesicle[4]. Generally during this process of phagocytosis, very few, if any of these cytotoxic molecules are released extracellularly from the phagocytosing neutrophil. However, there are a number of circumstances in which neutrophil contents, especially granule enzymes and ROS, can be released extracellularly, and this processes can result in oxidative-modification of serum proteins to enhance their antigenicity, thereby converting them to auto-antigens (Figure 1)[22]. Circumstances under which this secretion can occur include when the phagocytic target is too large to be ingested (e.g. a large fungal or protozoal target) or during “frustrated phagocytosis”, for example, when cartilage or another surface becomes deposited with immune complexes and hence recognised by neutrophil immunoglobulin receptors[10, 23]. During this latter process the concentrations of released neutrophil-derived products into this confined zone, can be so high as to easily saturate endogenous levels of anti-proteinases and anti-oxidants[16].

Alternatively, when neutrophils have been “primed”, for example by cytokines, to alter their functional responsiveness to ligands, soluble agonists (e.g. soluble immune complexes or bacterial-derived peptides (of the fMet-Leu-Phe family) can induce a rapid (within minutes) and extensive release of ROS and granule enzymes into the external environment (Figure 1)[5, 24]. Activation in this way can also result in changes to the plasma membrane of the activated neutrophils and the cell surface expression of granule proteins, such as myeloperoxidase and proteinase 3[25]. This process has been implicated in the pathogenesis of vasculitis (Figure 2)[11]. Additionally, released neutrophil granule enzymes and ROS may modify the structures of host proteins and other targets to again alter their properties to expose neo-epitopes that may lead to loss of immune tolerance.

**1.3.2. Apoptosis**

Neutrophils have a very short half-life and exhibit high rates of constitutive apoptosis[26, 27]. During culture *in vitro*, their half-life is estimated to be approximately 12h[28-30], but *in vivo* this may be longer, although the precise survival time of these cells is not easy to measure[31]. As a consequence of this short half-life in the circulation, the bone marrow releases vast numbers of neutrophils on a daily basis, estimated to be 5-10x1010, and this number can be greatly increased during infections[32]. During inflammatory challenge *in vivo*, and during incubation with a variety of pro-inflammatory agents *in vitro*, neutrophil lifespan can be extended[33, 34] and consequently this delayed apoptosis can then enable neutrophils to survive for long enough to successfully carry out their functions during inflammation or infection.

Many of the mechanisms that control neutrophil apoptosis have now been delineated and death receptor signalling and the Bcl-2/caspase protein families play key regulatory roles[35, 36]. Human neutrophils are unusual in that while they express a large number of pro-apoptotic members of the Bcl-2 family, the only anti-apoptotic protein of this family that has been equivocally identified is Mcl-1. Human neutrophils express high-levels of mRNA for BCL2A1 (Bfl-1), but identification of this protein in these cells is still hampered by availability of an antibody that unambiguously identifies this protein in these cells[33]. Mcl-1 is an unusual family member in that it has a very short half-life of normally 2-3 h (dependent on the cell type), but this half-life can be extended or shortened by post-translational modifications that regulate its rate of turnover[34, 37]. This short half-life of the protein makes it ideally suited to control cell death in these normally short-lived cells, and changes in its stability by post-translational modifications have the consequence that neutrophil lifespan can be rapidly modulated without processes that require *de novo* biosynthesis[37]. Likewise, cytosolic proliferating cell nuclear antigen (PCNA) is degraded by the proteasome upon apoptosis. PCNA is a protein originally thought to be only involved in DNA synthesis but has since been shown to control neutrophil survival by sequestering pro-caspases[38].

When neutrophils undergo apoptosis, many changes occur on their plasma membrane[39, 40]. The expression levels of many ligand-binding receptors decreases (via shedding or internalization) and this down-regulates the ability of the dying cells to respond to extracellular ligands. In addition phosphatidylserine, which is normally localized on the inner leaflet of the plasma membrane, appears on the cell surface of apoptotic neutrophils[40]. This process provides a convenient assay to detect apoptotic neutrophils, as phosphatidylserine, in the presence of Ca2+, binds annexin V, which may be fluorescently-tagged. These changes in the surface properties of apoptotic neutrophils can be recognised by macrophages or other phagocytic cells, which then phagocytose apoptotic neutrophils by processes that do not trigger the release of pro-inflammatory cytokines[40]. This clearance mechanism therefore provides for a “safe” removal of apoptotic neutrophils that does not result in spillage of neutrophil degradative enzymes (that would occur if the cells underwent cell death by necrosis) without triggering inflammation.

However, the plasma membrane changes that occur during neutrophil apoptosis can also result in the appearance of auto-antigens, such as DNA and proteins on their cell surface[41]. Therefore, if there are increases in the rate of neutrophil apoptosis or else a defect in their rate of clearance, apoptotic cells may accumulate in the circulation or in tissues. This failure to clear apoptotic cells could then lead to the exposure of auto-antigens leading to activation of dendritic cells, especially plasmacytoid dendritic cells (pDCs) that are crucial for the immune tolerance associated with the clearance of apoptotic cells[42] as illustrated in auto-immune vasculitis (Figure 2)[43]. In addition, exposure of modified proteins or other molecules on the cell surface can promote the generation of autoantibodies which can play a role in the pathology of JSLE and SLE[41] particularly in the generation of anti-dsDNA antibodies (Figure 3).

**1.3.3 Formation of NETs**

The discovery that, in response to pathogens and other agents, neutrophils can extrude chromatin (DNA and associated histones) that is decorated with granule proteins to form extracellular Neutrophil Extracellular Traps or NETs[44], has prompted extensive interest in this phenomenon. NETs are commonly induced *in vitro* by incubation of neutrophils for a few hours with phorbol 12-myristate 13-acetate (PMA), which induces a whole range of non-physiological functions in neutrophils via supra-activation of protein kinase C. NETs comprise fibres of DNA containing 30-50 nm clusters of histones and antimicrobial proteins[45]. The following processes have been described during the sequence of events leading to NET formation. First, the generation of ROS by the NADPH oxidase appears to be important in initiation of NET formation because neutrophils from patients with Chronic Granulomatous Disease, which have a defective oxidase, cannot form NETs[46]. There is, however, evidence to suggest that some stimuli can induce NETs independently of NADPH oxidase activity and so the properties of the NETs that are formed might depend on the type of stimulus used[47]. This is followed by activation of PAD4 (protein-arginine deiminase 4) whose substrates include histones H2A, H3 and H4, and this enzyme can induce arginine to citrulline conversions on key residues of these proteins[48]. This conversion of uncharged citrulline from charged arginine results in histone decondensation within the nucleus. Pharmacological inhibition of PAD4 activity has been shown to be sufficient to disrupt mouse and human NET formation[49]. Granule enzymes, such as elastase may then migrate into the nucleus and further assist in chromatin unfolding[50]. These changes in chromatin structure can alter the shape of the normally multi-lobed neutrophil nucleus and then the nuclear membrane disrupts to release the chromatin into the cytoplasm, where it can further bind to released granule proteins. The decorated chromatin may then be extruded via disruptions in the plasma membrane.

The effects of PMA on human neutrophils are complex and result from supra-activation of protein kinase C. For example, PMA-induced cell death involves morphological changes that are quite different from those of typical apoptosis or necrosis and instead result in a uniform decrease in nuclear contents of chromatin preceding lysis of the nuclear envelope[51]. PMA-induced neutrophil death is also dependent on NADPH oxidase activation. Since the first description of NETs[44], many groups have investigated this phenomenon of DNA release associated with granule proteins, to evaluate their "pro-inflammatory" capacities in various inflammatory or infectious conditions. In some cases, NET formation was found to correlate with ROS production, suggesting that it could be another consequence of NADPH oxidase activation[52]. Many groups therefore use PMA-induced NET generation as an index to characterize the state of activation of neutrophils in various conditions, but there are some caveats to this approach. First, the relevance of using PMA to stimulate NET formation is of little physiological or pathological relevance and second, the protocols used to characterize NETs are variable, thus rendering it difficult to compare data obtained between different laboratories[53].

Many pathogens can also induce NET formation *in vitro*[54] and sometimes this NET formation does not lead to cell death[55]. Evidence for genuine NETosis *in vivo* (as a regulated death process, rather than extrusion of DNA and non-specific binding of cationic granule enzymes that may also be released as neutrophils lyse) is difficult to obtain. To date, the molecular mechanisms underlying plasma membrane rupture and cell death by NETosis are unknown and might have similarities with pathogen-induced neutrophil lysis. Whether NETosis involves the receptor-interacting protein kinase 3 (RIPK3), involved in necroptosis, is not yet clear since conflicting results have been reported[56, 57]. Several reports show that NETs contribute to defence against infection, although supporting evidence to justify such a conclusion has been indirect. Whether NETs can directly contribute to bacterial killing has been discussed[58] but their ability to immobilize bacteria and other pathogens, could well limit the dissemination of infections *in vivo*[54]. However, a recent report investigating the functions of neutrophils from patients with Papillon Lefèvre Syndrome has provided some new insights into the role of NETs in bactericidal activity[59]. Neutrophils from these patients lack active serine proteinases and are unable to generate PMA-induced NETs, but they show a normal capacity to kill bacteria, which is consistent with the notion that NET-associated proteinases are not required for full bactericidal potential in human neutrophils[60].

The concept of NETosis as a novel form of cell death arose from the observation that DNA extrusion from neutrophils or eosinophils can be triggered by physiological stimuli or pathogens without triggering other forms of cell death[61]. Importantly, in this vital and active process, the DNA was of mitochondrial origin[62]. It is likely, therefore, that nuclear-derived and mitochondrial-derived NETs might be activated by different mechanisms and elicit different host functions[63]. This notion of vital NET formation (which has been referred to as "vital NETosis"[64]) has been convincingly shown *in vivo* in different animal models. Vital NET formation exhibits features that differ from those of the originally-described NETosis process, which is now referred to by some as "suicidal NETosis"[64]. Vital NET formation requires the presence of activated platelets, occurs within minutes and involves budding of DNA-containing vesicles from neutrophils without perforating the plasma membrane[64]. The role of this process *in vivo*, and the patho-physiological conditions that may regulate this NET formation in disease, are topics of extensive and hot debate in the neutrophil scientific community[45, 64, 65]. Nevertheless, the description of NETs has demonstrated that neutrophils can release extracellular DNA upon activation and/or death at a site of infection and inflammation[66]. Importantly, this DNA may be associated with numerous neutrophil-derived cationic proteins, including antimicrobial proteins and histones, thus adding potential novel functions of these structures, including the exposure of neo-epitopes that can break immune tolerance. NETs are decorated with citrullinated histones and granule enzymes (MPO, elastase, lactoferrin, MMP-9, LL37), that themselves may also have altered immunogenicity via modifications, such as by oxidation. While NET-associated proteins may have antimicrobial roles (as discussed above), they may also serve as auto-antigens in diseases such as SLE, JSLE and RA and hence contribute to auto-immunity. Indeed, these diseases are characterised by high titres of auto-antibodies, such as anti-cyclic citrullinated peptide (Anti-CCP) antibodies in RA and anti-dsDNA antibodies in lupus[67-69].

**2. Definition of ANCA-associated auto-immune vasculitis**

Neutrophils play a pivotal role in the pathophysiology of anti-neutrophil cytoplasmic antibody (ANCA)-positive vasculitis because (a) they can be the source of auto-antigens, (b) are activated by the ANCA and (c) are effector cells of damage to the endothelium (Figure 2)[11].This deleterious role of activated neutrophils in vasculitis lesions is suggested by their presence in the arterial, arteriolar and capillary perivascular infiltrates (including glomerular and pulmonary vessels), and development of ANCA directed against two neutrophil enzymes, proteinase 3 (PR3) and myeloperoxidase (MPO). Vasculitides associated with ANCA are classified into three distinct clinical entities: (i) granulomatosis with polyangiitis (GPA, formerly known as Wegener’s granulomatosis); (ii) microscopic polyangiitis (MPA) and (iii) eosinophilic granulomatosis with polyangiitis (EGPA, formerly known as Churg-Strauss syndrome). These diseases are characterized by necrotizing inflammation of small vessels[70]. Vascular lesions may be at the origin of the clinical symptoms, and will vary depending on the location, size of the affected vessels, and pathogenic mechanism(s) involved[71]. However, it is striking to note that of the multiple proteins contained in neutrophil azurophil granules, MPO and PR3, while being biochemically very different, are the preferred targets for ANCA-associated vasculitides, but the clinical symptoms are different depending on which of these two proteins is involved. In systemic GPA, characterized by impaired renal and pulmonary functions, >90% patients have anti-PR3 ANCA during flares, and <10% have anti-MPO ANCA. In contrast, anti-MPO ANCA are observed in 60-70% of patients with MPA and in 30-38% of patients with EGPA. It is extremely rare for these two types of antibodies to be detected in a same patient. Genome-wide association studies (GWAS)[72], have highlighted the different pathophysiological mechanisms underpinning this antigenic specificity in ANCA-associated vasculitis[73]. How specific anti-MPO or anti-PR3 antibodies arise in these different forms of the disease is still unknown. The theory of complementary of PR3 proposes another hypothesis, in which a peptide encoded by the reverse DNA strand to PR3 encodes a complementary PR3 which has homology with certain *Staphylococcus aureus* proteins. The immune response to *S. aureus* therefore generates antibodies that cross-react with complementary PR3 but also to anti-PR3 via the anti-idiotypic antibody network[74]. However, anti-complementary PR3 is not detected in all GPA patients[75]. It should be noted that other target antigens, such as lysosomal-associated membrane protein 2 (LAMP2), have been described in both GPA and MPA and might play an additional role in vascular inflammation[76]. Importantly, when present, these anti-LAMP2 antibodies coexist with anti-MPO or with anti-PR3, the latter being mutually exclusive[77]. An important point to note is that ANCA epitopes can exist in different conformational states, which vary during the course of the disease. This must be considered when characterising new target antigens.

**2.1 Biochemical and functional characteristics of the prototypic target auto-antigens: proteinase 3 (PR3) and myeloperoxidase (MPO)**

Although these two proteins are localized in neutrophil azurophil granules and are both involved in neutrophil microbiocidal mechanisms, their structures and functions are very different. They have common pro-inflammatory properties and can also modulate the inflammatory process via their synergistic activities[78]. Myeloperoxidase (MPO) is highly abundant in neutrophils (up to 5% of the dry weight) and is exclusively found in azurophilic granules. It is a key component of the phagocyte oxygen-dependent intracellular microbiocidal system[2], and is composed of two subunits linked by a disulfide bridge with each subunit containing a heavy chain of 57.5 kDa, a 14-kDa light chain and a haem group. It has the unique property, not shared by other peroxidases, to generate chlorinated oxidants including hypochlorous acid (HOCl-) and chloramines[79]. Hypochlorous acid exerts toxic effects, not only on microorganisms (bacteria, fungi and parasites) but also on host cells. This is because hypochlorous acid can oxidize a variety of molecules ranging from intracellular enzymes involved in essential processes, such as mitochondrial respiratory chain components, nucleotides and lipids, and can hence alter membrane components essential for cell metabolism. However, it has become clear that MPO is not solely a bactericidal protein, but also a key player in the balance between innate and adaptive immunity through its pro- and anti-inflammatory functions. MPO is present in atherosclerotic plaques where it can oxidize low density lipoproteins and extracellular matrix proteins within the blood vessel walls, implicating MPO in the physiopathology of atherosclerosis[80] that is now considered as an "auto-immune" disease.

Proteinase 3 (PR3), also called myeloblastin[81], is expressed by neutrophils and monocytes. In contrast to MPO whose biological activities are unique, PR3 belongs to the neutrophil serine protease family (the serprocidins) and is classically localized in azurophilic granules along with its homologs: elastase, cathepsin G and azurocidin[82]. After phagocytosis of pathogens, PR3 is secreted into the phagolysosome to carry out its microbiocidal function. Moreover, these serine proteinases have pro-inflammatory activity[83] as shown in many animal models, such as mice genetically-deficient in elastase or cathepsin G or double deficient in elastase and PR3. In addition, mice deficient in dipeptidylpeptidase cannot cleave the pro-sequence of these serine proteases and so are protected against rheumatoid arthritis[83]. Although PR3 shares more than 60% sequence homology with neutrophil elastase, it has some unique structural and functional properties[84]. One specific feature of PR3 is its bimodal membrane expression on the resting neutrophil surface, such that some neutrophils lack membrane PR3 (mPR3-) whereas others express PR3 (mPR3+). Interestingly, patients with ANCA-associated vasculitis have an increased proportion of mPR3+cells[25]. CD177 (also called human neutrophil antigen B1, NB1), a glycosylphosphatidylinositol (GPI)-linked membrane receptor, is co-expressed on the same neutrophil subset that expresses membrane PR3[85, 86]. It has been suggested that NB1 could bind PR3, thereby acting as a receptor for PR3. PR3 associates with membranes in a hydrophobic patch that regulates its interaction with lipids and its membrane anchorage[87]. PR3 can be externalized during apoptosis in association with specific partner proteins, including the phospholipidscramblase1[88] and calreticulin, a chaperone protein involved in the recognition of apoptotic cells by macrophages[89].

**2.2 Dysregulated neutrophil functions in vasculitis: role of ANCA**

Neutrophils have a major role in the pathophysiology of ANCA-associated vasculitis, being involved both in the mechanisms of endothelial injury and in the immune deregulation associated with these diseases. Indeed, neutrophils can secrete a variety of cytokines, chemokines and lipid mediators that many immune cells (monocytes, dendritic cells, T lymphocytes and B lymphocytes) respond to. For example, neutrophils are a source of B-cell Activating Factor (BAFF/BLyS) and A Proliferating-Inducing Ligand (APRIL), which are both members of the TNF superfamily and involved in the fundamental processes of B lymphocyte homeostasis[90, 91]. In particular, serum BAFF levels are high in GPA relative to levels in healthy controls[92]. The classical pathological process of ANCA-positive vasculitis involves the accumulation of neutrophils at the inflammatory site that can be initiated by the priming of neutrophils (see above). Priming enhances the membrane expression of PR3 and MPO, which can then bind ANCA to trigger neutrophil activation. TNF-primed neutrophils incubated *in vitro* with IgG purified from sera containing anti-PR3 or anti-MPO ANCA are capable of producing superoxide anion and release granular proteins[93]. However, this activation *in vitro* by anti-MPO or anti-PR3 ANCA is observed only when neutrophils are pre-treated with cytochalasin B[93], a pharmacological agent that destabilizes the actin cytoskeleton in cells. In this model, it has been shown that ANCA-induced neutrophil activation requires firstly antigen binding to Fcγ receptors (FcγRIIa or FcγRIIIb) and involvement of β2-Integrins. Recent reviews describe activation of kinase networks in which PI3-kinase plays a key role in ANCA-induced neutrophil activation[94]. Animal models of anti-MPO ANCA-induced vasculitis[95] have provided evidence that the alternative pathway of the complement system [96] and serine proteases[97] play pathological roles. It has been suggested that the formation of NETs, composed of DNA expelled by dying neutrophils, and cationic proteins derived from granules, including PR3 and MPO, may be involved in the pathophysiology of ANCA-positive vasculitis[98]. However, this phenomenon does not fully explain the selectivity of ANCA towards PR3 and MPO that is observed in GPA and MPA, respectively. Intravascular "vital NET" formation has been observed *in vivo* and might be an important aspect in thrombosis-induced vasculitis[99], but no data are available yet in the anti-MPO-induced vasculitis model. Nonetheless, in a murine model of atherosclerosis, it has been shown that protease-mediated cytokine processing by PR3 was a key determinant in atherosclerosis and that NET release did not appear to play a key role[100]. Microarray analysis of whole blood from patients with ANCA-associated vasculitis (AAV)[101] identified a gene expression signature that overlapped with a low density granulocyte (LDG) signature previously identified in SLE[102] which will be discussed later in this review. Elevated transcripts for PR3 identified in AAV PBMCs were associated with high levels of disease activity and a lack of response to therapy[101]. Subsequently, neutrophils and LDGs isolated from patients with AAV were observed to undergo increased spontaneous NETosis in culture, with AAV NETs staining positive for PR3 and MPO[101].

**2.3 Defects in the resolution of inflammation**

Inflammation in ANCA-induced vasculitis is sustained and fails to resolve, leading to vessel necrosis, granuloma formation and ultimately promotion of auto-immunity. Indeed, a delay in the phagocytosis of apoptotic cells may favour auto-immunity, as is the case in systemic lupus erythematosus (SLE)[103] (see below). Dendritic cells play a key role in antigen presentation and soluble PR3 may activate these cells via the cleavage of the protease-activated receptor 2 (PAR2)[104]. In another study, PMA-induced NET formation has been shown to mediate transfer of MPO and PR3 to myeloid dendritic cells[105]. As mentioned above, membrane expression of PR3 interferes with the phagocytosis of apoptotic neutrophils by macrophages[88]. Moreover, PR3 expressed on the membrane of apoptotic cells triggers a pro-inflammatory response in macrophages via their secretion of inflammatory cytokines, (including interleukin-1β), chemokines and the expression of nitric oxide synthase 2[43]. This PR3 effect is dependent on its membrane anchorage and its enzymatic activity, and strongly suggests another novel “auto-inflammatory” component in this disease. Notably, the microenvironment produced by the macrophages after the phagocytosis of apoptotic cells expressing PR3 could regulate pDCs to polarize T Helper lymphocytes toward the Th9/Th2 phenotype[43]. This function can completely abrogate the generation of regulatory T cells, thus favouring auto-immunity[42]. Most importantly, a similar T cell polarization was found in patients with GPA[43]. Finally, macrophages, pDCs and T cells are all found in close proximity in the granulomatous lesions in lungs from these patients[43, 106]. In GPA, the auto-antigen therefore appears to play a double role, acting as an auto-antigen and a danger signal disturbing the resolution of inflammation and promoting auto-immunity.

**2.4 Defects in neutrophil functions in vasculitis**

During a disease flare, neutrophils of patients with a GPA strongly express the genes for PR3 and MPO[107], genes that are normally expressed only during the promyelocytic phase of granulocytic differentiation, according to the theory of "Targeting by timing"[108]. Gene expression profile studies performed on whole blood leukocytes from AAV patients have shown a signature consisting of more than 200 genes expressed in neutrophils, whereas a lymphocyte signature was observed in SLE patients[109]. Some vasculitis patients present without anti-PR3 or anti-MPO antibodies, but activated neutrophils are still implicated in endothelial damage[110]. Neutrophils from patients with AAV show an enhanced rate of apoptosis *in vitro* (compared to healthy controls) when activated by ANCA[111]. In contrast, spontaneous apoptosis of neutrophils was delayed in patients with AAV[112]. Whether these disturbances in the balance of survival/apoptosis impact upon the clearance of apoptotic neutrophils that is normally required for the resolution of inflammation[113] is not known, and requires further investigations.

**3. Neutrophils in SLE**

SLE is an auto-immune disease characterized by autoantibody production against nuclear antigens, immune complex deposition in tissues, infiltration of tissues (such as kidneys) with T and B cells, neutrophils and macrophages, and subsequent inflammatory tissue injury[114]. Clinical symptoms range from mild skin rashes to life-threatening multi-organ manifestations. In addition to the dysregulation of B and T cells, recent studies have established the active role of cells of the innate immune system including macrophages, DCs and neutrophils in driving the auto-immune response and tissue damage in SLE. Leukopenia is a common finding in patients with SLE, primarily due to lymphopenia, whilst neutropenia is reported in 20-40% of SLE patients[115, 116]. However, SLE patients who receive immunosuppressive drugs might also be at risk of developing neutropenia.

**3.1. A dysregulated neutrophil population in SLE**

Neutrophils isolated from the blood of SLE patients display a number of abnormal features in their phenotype and function, such as increased aggregation[117-120], impaired phagocytic capabilities[121, 122], inability to be cleared by the C1q/calreticulin/CD91-mediated apoptotic pathway[123], abnormal oxidative activity, increased apoptosis that may lead to neutropenia, and enriched numbers of LDGs in the peripheral blood (Figure 3)[117-120]. LDGs display an activated phenotype and express surface markers of mature neutrophils, but their nuclear morphology resembles that of an immature cell[117, 120, 124]. Isolated LDGs have elevated levels of somatic alterations that are consistent with genetic damage or genomic instability[125]. An increased circulating LDG population, which correlates with dsDNA antibody concentration and scores of disease activity, has been also observed in juvenile SLE (JSLE) patients, suggesting that this subset of neutrophils may be a useful biomarker[126]. Neutrophils from SLE patients express a decreased expression of C5aR and CD62L, but normal CD11b expression[127, 128], indicating their phenotype is altered rather than activated.

**3.2. Dysregulated neutrophil functions and pro-inflammatory role of neutrophil proteases in SLE**

Levels of defensins released by neutrophils and/or LDGs are increased in SLE sera[129, 130]. Neutrophil-specific proteins are also found in the urine of SLE patients and can be used as a surrogate marker of disease activity[131]. The cathelicidin, LL-37, released from the C-terminal domain of the hCAP18/LL-37 precursor protein by proteolytic cleavage by PR3, can also trigger inflammatory cytokine production. Lupus LDGs significantly overexpress mRNA for various immuno-stimulatory bactericidal proteins and alarmins (e.g. CTSG, proteinase 3, and neutrophil elastase), relative to normal density neutrophils in SLE patients and healthy controls[102, 132-134].

The increased numbers of apoptotic neutrophils in lupus, with enhanced surface expression of auto-antigens, and their impaired removal by phagocytes, could enhance the processes that lead to the development of auto-immunity (Figure 3)[135]. In addition, low levels of ROS production by granulocytes has been associated with disease severity in auto-immune conditions including SLE[128] as well as in Behçet’s disease, Guillain-Barre syndrome and multiple sclerosis[136-138] and might be an important common denominator in the pathogenesis of auto-immunity. This lower ROS production was associated with a decreased number of newly-released CD10-/CD16low neutrophils from the bone marrow[128]. This suggests that decreased ROS production may indicate altered neutrophil behaviour rather than generally impaired functions. Neutrophil ROS can regulate humoral autoimmunity through inhibition of IL-15 and thus IFN-γ production by NK cells[139]. This regulatory role for neutrophils in SLE has been validated *in vivo*, where neutrophil depletion resulted in spontaneous activation of NK cells and autoimmune B cells [139].

**3.3. The effect of the SLE inflammatory environment on neutrophil function**

The cytokine milieu, especially IL-6, IL-10, IL-17, IL-18, IL-21, TNF-α and interferon (IFN)-α is integrally involved in the pathogenesis of SLE[140]. Although SLE is suggested to be a Th2-driven disease, there is emerging evidence to propose a critical pathogenic role of IL-17[141, 142]. IL-17A amplifies the immune response by inducing the local production of chemokines and cytokines, and plays an indirect role in recruiting neutrophils[143, 144]. The pro-inflammatory activity of IL-17A has been associated with the pathogenesis of SLE. For example, levels of IL-17A were higher in patients with new-onset SLE, JSLE, and in pregnant women with SLE[145-149]. Neutrophils enhance immunoglobulin production by B cells through a mechanism that involves BAFF, APRIL and IL-21. Interestingly, BAFF production is increased in SLE patients[150] while microarray analysis has demonstrated the role of IFN-α in the pathogenesis of this disease[151]. Chronic activation of pDCs by circulating immune complexes, causes them to secrete type-I IFNs, which is established as an early trigger of auto-immunity in patients with SLE. Genome-wide association studies provide strong genetic evidence that type-I IFNs (with IFN-α as the dominant mediator) are important for SLE risk[152] and an over-representation of IFN-inducible transcripts is detected in neutrophils from SLE patients[153]. The accumulated data indicate that levels of IFN-α in the circulation are significantly elevated in lupus patients compared with control subjects, and high levels of IFN-α are associated with more severe measures of disease activity[154]. Analysis of transcripts from cell subsets of SLE and AAV patients revealed that whereas the granulopoiesis signature was common to both diseases, the type-I IFN and plasmablast signatures were restricted to SLE[132]. LDGs from SLE patients have been reported to express transcripts for IFN-α in response to PMA and G-CSF[117], although this needs to be confirmed by other groups. In the pathogenesis of non-autoantibody-mediated haematological manifestations of SLE, bone marrow biopsies have revealed that the anaemia is due to erythroid dyspoiesis with morphological evidence of death of erythroid precursors. Intense phagocytosis of nuclear material by mature neutrophils, was linked with TNFα production (and not IFN-α)[155]. Animal models suggest that TNFα production selectively damages erythroid precursors through a TLR7-driven neutrophil activation, leading to the anaemia often seen in SLE and RA[155-157]

Ribonucleoprotein (RNP)-containing immune complexes stimulate immune cells, including myeloid cells, and this requires activation of both FcγR and TLRs[158, 159]. Genome-wide association studies, experimental mouse models and analysis of clinical samples have provided evidence for the involvement of TLRs, including TLR2, TLR4, TLR5 and TLR7/8/9, in SLE pathogenesis[61, 160]. TLR7 is required in the recognition of RNP-associated auto-antigens, while TLR9 is involved in the detection of DNA or DNA-associated auto-antigens. Environmental factors also play a role in the onset of SLE and recognition of pathogens through TLRs, is critically involved in autoantibody production and glomerulonephritis in lupus-prone animal models[161-163]. Whereas the pathological role of TLR7 in human SLE and lupus nephritis in mouse models is relatively well-established[164-166], the role of TLR9 is paradoxical since mice lacking TLR9 have exacerbated disease, despite lacking anti-nucleosome antibodies[167-172]. MyD88 is a common adaptor protein required for most TLR signalling and in experimental murine models, recruitment of granulocytes requires a MyD88-dependent pathway[164, 173, 174]. Human neutrophil subsets express all members of the TLR family with the exception of TLR3, enabling them to initiate immune responses upon recognition of exogenous or endogenous ligands[61, 175]. In addition, high–mobility group box 1 (HMGB1), which is released during SLE pathogenesis, and binds both DNA and pathogenic anti-DNA autoantibodies through its receptor RAGE (receptor for advanced glycosylation end-products), may trigger recruitment of neutrophils and may also be involved in anti-DNA autoantibody-induced kidney damage in lupus nephritis.

**3.4. Role of NETs in SLE**

The propensity of SLE neutrophils and LDGs to form NETs containing nuclear auto-antigens has been extensively studied. Lupus neutrophils may be activated by autoantibodies and nucleosomes, and display a tendency to form aggregates. It has been suggested that neutrophil death is linked with pDC activation and type-I IFN production in SLE[176] and that SLE neutrophils die upon exposure to SLE-derived anti-RNP antibodies, thereby releasing NETs. SLE NETs contain DNA as well as large amounts of LL37 (the C-terminal peptide derived from the human cathelicidin hCAP-18) and HMGB1, neutrophil proteins that facilitate the uptake and recognition of mammalian DNA by pDCs. Type-I IFN and immune complexes trigger further activation of neutrophils, releasing more NETs[68], establishing a vicious cycle at the core of SLE pathogenesis (reviewed in [102]). It may be envisaged that NETs associate with modified granule proteins, some of which result in the formation of damage-associated molecular pattern molecules (DAMPs) perhaps recognized by the same innate receptors as pathogen-associated molecular pattern molecules (PAMPs). In support of this idea, NETs and LL37 can activate the NLRP3 inflammasome in macrophages to induce the synthesis of IL-1β and IL-18, both of which can result in imbalanced immune homeostasis[177].

Since neutrophil-derived antimicrobial peptides and DNA form complexes that can lead to TLR9-mediated inflammatory responses by pDCs[68], it may be speculated that NET formation is a pathological mechanism leading to development of SLE. However, auto-immune-prone mice that lack functional TLR9 invariably develop more severe clinical disease and have shortened lifespans[167-169, 171, 178] suggesting a protective role of TLR9 activation. Several immune mechanisms may explain this paradoxical response: (i) TLR9 signalling regulates anti-DNA B cells and helps purge the repertoire of peripheral auto-reactive cells[179]; (ii) distinct roles for TLR7 and TLR9 in the differentiation of auto-reactive B cells that explain the capacity of TLR9 to limit, as well as TLR7 to promote, the clinical features of SLE[180]; (iii) RNP-associated auto-antigens may be more pathogenic because they trigger different activation pathways or the Abs directed against the RNP-associated auto-antigens have unique properties of [181]. Taking into account the reported tolerogenic role of TLR9 in SLE (from the murine studies, see above), NET formation may, in some circumstances, drive a protective rather than a pathological response (Figure 3).

The potential contribution of NET formation to SLE pathogenesis[182] is intriguing, but contradictory results have been obtained in experimental murine models. Since NET formation requires the NADPH oxidase[46], SLE pathogenesis may be expected to rely on the presence and activity of this enzyme. Deficiency of the NADPH oxidase component Nox2, therefore would be expected to inhibit disease pathogenesis in lupus-prone mice, but in fact it was found to exacerbate disease[183], arguing against the role of NETs in SLE pathogenesis. It was also reported that neutrophils from lupus-prone MRL/Faslpr mice showed similar levels of spontaneous NET formation compared to wild-type mice[183]. Also, the use of inhibitors of peptidylarginine deiminases (PAD), which block NET formation, modulated the changes in vascular phenotype normally seen in the experimental NZM mouse model of lupus[184]. Another unresolved question is why different auto-immune disease are associated with the presence of different autoantibodies? For example, if NETs provide a source of auto-antigens in inflammatory disorders such as AAV[98] and SLE[185], it is unclear why the auto-antibodies to PR3 or MPO (present in AAV) are not expressed in SLE patients and inversely, why anti-ribonucleoprotein and anti-DNA antibodies are absent in AAV patients. Another unresolved issue is related to the fact that NETs are proposed to be formed during bacterial infections, but if so, it is unclear how auto-immunity is avoided under these conditions of NET formation.

**4. Neutrophils in the pathogenesis of rheumatoid arthritis**

Rheumatoid arthritis (RA) is a systemic auto-immune disease which causes damage to synovial joints and long term disability[186]. Patients with RA often suffer from additional inflammatory comorbidities, such as cardiovascular disease, inflammatory eye disease and stroke[187, 188]. The disease is typified by dysregulation in both innate and adaptive immune function, including increased production of inflammatory cytokines (including TNFα, IL-6, GM-CSF, IL-1β, IL-17) and loss of tolerance to self-antigens, such as citrullinated peptides[10, 18, 20, 186]. A key feature of RA is swollen joints, containing excess synovial fluid and a hyperplastic synovial lining which has undergone angiogenesis, leading to the growth of an invasive, inflammatory tissue or pannus across the surface of synovial joints[10, 186]. This inflammatory pannus comprises activated synovial fibroblasts, macrophages, lymphocytes and neutrophils. Synovial neutrophils secrete inflammatory molecules (cytokines, prostaglandins) and collagen-degrading enzymes, whilst at the pannus-cartilage interface, inappropriately activated osteoclasts are activated to resorb bone, leading to irreversible joint destruction[10].

**4.1 Pro-inflammatory role of neutrophil proteases and ROS in RA**

Neutrophils play a key role in the pathogenesis of RA through the release of cytotoxic ROS, collagen-degrading proteases and inflammatory cytokines and chemokines[10]. Immune complexes within synovial fluid induce degranulation and ROS release[189] into the synovial fluid and via “frustrated phagocytosis”, a process whereby activated neutrophils adhere to immune complexes embedded in synovial tissue, causing degranulation directly onto the surface of the joint (Figure 1)[10, 23]. Neutrophil MMP-8 and -9, elastase, gelatinase, cathepsin G, lipocalin and proteinase 3 are all found at elevated levels in RA synovial fluid[10, 190-194]. These neutrophil-derived proteases can cleave collagen within the cartilage matrix, digest hyaluronic acid, process pro-cytokines (such as IL-33) into mature forms, and cleave cytokine receptors, such as the IL-6R to enable trans-signalling in neighbouring cells[83, 195-199]. Released neutrophil granule enzymes may also mediate immune responses. For example, Cathepsin G is a chemoattractant for monocytes[200], lactoferrin is a survival factor and inducer of adhesion for neutrophils[191, 201], and lipocalin is implicated in the activation of MMP-9[194]. RA synovial neutrophils display an activated phenotype that is similar in many ways to that of tissue macrophages, secreting a large repertoire of inflammatory cytokines and chemokines (including IL-8, IL-1β, TNF, RANKL, BAFF/BLyS, oncostatin M, CCL2, CCL20, CXCL10)[6, 10, 202-206] and expressing MHC class II[207]. Secretion of cytokines, such as GM-CSF by synovial fibroblasts[208], in concert with the hypoxic environment of the synovial joint[209], delays synovial neutrophil apoptosis[210] via increased levels of Mcl-1[209].

**4.2 Activated neutrophil phenotype in RA**

As discussed above, in healthy individuals blood neutrophils are relatively inactive, requiring a “priming” signal to initiate mobilisation of adhesion molecules (ICAMs, FcγRs) facilitating migration from peripheral blood into inflamed tissues[5, 6]. In the absence of priming and activation, healthy neutrophils undergo controlled apoptosis within several hours of release from the bone marrow. In RA, peripheral blood neutrophils have an activated phenotype, with dysregulation of apoptosis via up-regulation of Mcl-1[204, 211], activation of transcription factors such as NF-κB[204], and FoxO3a[212], increased chemotactic ability via up-regulation of C-C chemokine receptor 2 (CCR2)[213], increased phagocytic capacity[214], and up-regulation of FcγRs which trigger production of ROS by immune-complexes, including rheumatoid factor[215-217]. RA peripheral blood and synovial fluid neutrophils produce significantly higher amounts of ROS compared to healthy control neutrophils, without the need for *in vitro* priming[218], and in addition have increased p47phox-Ser345, ERK1/2 and p38 MAPK phosphorylation suggesting they have already been exposed to priming agents such as TNFα or GM-CSF *in vivo*[219]. Levels of MPO are elevated in RA sera[220], and whilst only around 50% of serum MPO is biologically active[221] it may still be present at sufficient levels to contribute to oxidative stress, a process which results in DNA damage, oxidation of lipids, and molecular changes in immunoglobulins implicated in the development of rheumatoid factor[22, 222].

The transcriptome of RA blood neutrophils differs significantly from that of healthy individuals[223], and includes activation of STAT proteins and expression of a type-I IFN-induced gene expression signature[224]. Importantly, gene expression signatures in RA neutrophils can be used to stratify patients into responders and non-responders to TNF-inhibitor therapy based on the presence of specific gene biomarkers[224] (and Wright unpublished). Neutrophil phenotype in RA is closely associated with clinical response to therapies, such as corticosteroids, methotrexate and TNF-inhibitors. Changes in blood neutrophil function are observed during therapy, including abrogation of delayed apoptosis[211], decreased production of TNFα[204] and S100A12[225, 226], decreased membrane expression of proteinase 3 [227], and a decreased rate of chemotaxis in patients who respond to therapy[228].

**4.3 NETs and NET-derived auto-antigens in RA**

Emerging evidence implicates neutrophils and NET production as a source of tissue damage and auto-antibody production in RA[10]. A feature of severe, erosive RA is the presence of anti-citrullinated protein antibodies (ACPA). Citrullinated peptides are preferentially recognised by the HLA-DRB1\*04:01/04 allele, which enables presentation of citrullinated peptides to auto-reactive T cells, and which has a strong association with the development of RA[229]. Spontaneous NET production by RA neutrophils in culture is enhanced compared to healthy controls[69, 230], with RA neutrophils having significantly more nuclear PAD4 and citrullinated histone H3 (Figure 1)[230]. RA sera and synovial fluid can induce NET production[69, 230] by RA and healthy control neutrophils, and ACPA from RA sera cross-reacts with citrullinated histone H4 derived from NETs[231]. Depletion of ACPA from RA sera abrogates the production of NETs[230]. Enhanced production of NETs in response to PMA, TNFα, IL-17 and LPS has been reported in RA blood neutrophils[69], and analysis of the NET proteome of healthy control neutrophils in response to very high concentrations of TNFα (100ng/mL) or RA IgG (100mg/mL) identified 25 NET-bound proteins, including citrullinated vimentin and α-enolase[69]. However, these experiments used high, non-physiological concentrations of TNFα and LPS, and IL-17, and it is difficult to rationalise the effects of the latter agonist as freshly-isolated human neutrophils lack a functional IL-17 receptor[144, 206].

RA synovial fluid cells (normally >80% neutrophils) show marked hypercitrullination of intracellular proteins across a large range of molecular masses[232]. Whilst histone citrullination is a key step in neutrophil activation and NET release, hypercitrullination is not induced by any form of cell death but instead may be mediated by perforin and the membrane attack complex (MAC)[232]. A recent study of individual CD19+ B cells from RA synovial tissue identified significant production of antibodies to citrullinated histones H2A/H2B, citrullinated vimentin and citrullinated fibronectin[233]. Anti-citrullinated H2A/H2B-reactive RA recombinant monoclonal antibodies selectively recognised NETs produced by RA blood and synovial neutrophils[233]. Immuno-histochemical staining of synovial tissue identified cathelicidin LL37 (a NET auto-antigen in SLE) in association with neutrophils in RA but not healthy controls[234].

**4.4 Neutrophils in the initiation of RA**

Whilst the cause of RA is not completely understood, a number of genetic risk factors have been identified, including specific HLA haplotypes as described earlier, and loci within genes PTPN22, TNFAIP3, C5-TRAF1, CTLA4 and PADI4[186, 235]. A recent study implicated the C1858T (R620W) SNP in PTPN22 with enhanced PAD4 activity and spontaneous NET production[236] in healthy individuals carrying the T allele of the SNP (C1858T). This genetic variant is also associated with enhanced neutrophil migration, enhanced calcium release and enhanced ROS production in neutrophils from healthy controls and patients with RA[237]. The pivotal role of neutrophils in both joint damage and disease progression in animal models of RA is demonstrated by the K/BxN mouse model. In this model, the F1 offspring of KRN mice (transgenic for a T-cell receptor for bovine RNase42-56 on I-Ak and glucose-6-isomerase282-294 on I-Ag7) and non-obese diabetic mice spontaneously develop inflammatory arthritis at around 5 weeks of age that closely resembles human RA[238]. Serum from K/BxN mice induces development of the disease when injected into normal mice, but most strikingly has no effect in neutrophil-depleted mice, which are completely resistant to the effects of K/BxN serum[238]. K/BxN serum transfer also has no effect in mice with neutrophils lacking a functional Syk kinase, Syk being critical in the neutrophil response to immune complexes via FcγRs.[239]. Whilst NETs and citrullinated histone H4 are detected in synovial tissues of K/BxN serum transfer animals, PAD4 knock out does not prevent initiation of inflammatory arthritis or a decrease in measures of disease severity in this model[240]. However, in the glucose-6-phosphate isomerase-induced arthritis mouse model, PAD4 knock out is associated with lower levels of disease activity including mean arthritis score, cell infiltration, cartilage destruction, bone erosion and serum IL-6 concentration[241].

**4.5 Neutrophils in the resolution of inflammation in RA**

Neutrophils may play an additional, important role in the resolution of inflammation in RA. Neutrophil-derived microvesicles, present in high concentrations in RA synovial fluid, express the anti-inflammatory pro-resolving protein annexin A1, which in animal models has been shown to enter damaged cartilage and stimulate chondrocyte activation and cartilage protection[242]. Neutrophil-derived lactoferrin also modulates chondrocyte activation, increasing production of bone morphogenic protein (BMP)-7, a protein that plays an important role in synthesis of collagen types II and IV[243].

**4.6 Neutrophils in juvenile arthritis**

In addition to their key role in the pathophysiology of adult inflammatory arthritis, neutrophils are implicated in the pathogenesis of juvenile idiopathic arthritis (JIA), of which there are at least six forms including systemic and polyarticular JIA[244, 245]. JIA constitutes the most common, chronic paediatric auto-immune disease and is defined as joint inflammation in children (under the age of 16) persisting for at least 6 weeks with no other identified cause. Neutrophils are found in high abundance in JIA synovial fluid, while in systemic JIA, elevated serum levels of neutrophil-derived calgranulins S100A8, -A9 and -A12 are both a diagnostic biomarker and indicator of disease activity[244, 246, 247]. Gene expression studies in JIA report an abnormal neutrophil transcriptome, possibly activated by IL-8 and IFN-γ, which persists even after successful drug therapy[247, 248], and there is evidence that JIA neutrophils have undergone ROS production and degranulation *in vivo*[247], which as in RA, is implicated in joint damage and oxidative stress.

**5. Summary and Conclusions**

From the evidence presented in this review, it is clear that neutrophils can contribute to the processes that lead to auto-immunity and hence the pathologies of a number of auto-immune diseases. In some of these diseases, the role of neutrophils is very clear, for example in the case of ANCA-associated vasculitis, in which the auto-antigens are specifically directed against the neutrophil proteins, PR3 and MPO. On the one hand, the anti-MPO ANCA via its ability to activate neutrophils, has been directly implicated *in vivo* in the vascular damage in vasculitis: on the other hand, the target antigen PR3 expressed at the membrane of neutrophils can act as a danger signal and subvert the immune silencing that is associated with the clearance of apoptotic cells.

There has been a considerable amount of interest in the literature surrounding the ability of neutrophils to release NETs and the role of these structures in microbial killing, neutrophil death and the exposure of auto-antigens that could contribute to the generation of anti-dsDNA and anti-citrullinated peptide antibodies in SLE and RA, respectively. However, the evidence to support the role of NETs in the former two processes is less convincing than that supporting their ability to expose neo-epitopes, particularly *in vivo* in disease. However, the speculated role of these DNA:granule protein complexes in the pathogenesis of SLE (through induction of inflammatory immune responses via TLR9) is questionable, as increasing evidence demonstrates a paradoxical protective role of TLR9 in SLE. One may thus question the immune-modulatory role of NETs and/or neutrophils in the induction of tolerogenic B cells and protective immune responses in SLE. With regards to ANCA-associated vasculitis, since there is no immune complex deposition and no anti-DNA antibodies, the involvement of NETs in immune dysregulation is less clear. Nonetheless, the potential importance of intravessel "vital NET" formation may, in turn, play a pivotal role in disseminating unresolved inflammation associated with thrombosis in ANCA-associated vasculitis. Further studies, especially using the anti-MPO vasculitis murine model, will be required to resolve this issue.

Although the NET-based hypothesis of auto-immunity is attractive, it is unlikely to be responsible for all systemic inflammatory diseases. Major unanswered questions exist around why different auto-immune diseases are associated with different auto-antibody profiles, if NETs are responsible for initiating auto-immunity. However, a careful analysis of the underestimated role of neutrophils in the pathophysiology of each disease should provide us with reliable information to develop novel therapeutic strategies to target neutrophils.

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**Figure Legends**

**Figure 1. Generation of auto-antigens by neutrophils during inflammation.** Neutrophils are activated during inflammation, for example by immune complexes (soluble or tissue-embedded) which bind to Fcγ receptors, or by recognition of opsonized bacteria via complement- and Fcγ receptors. This activation induces either the secretion of ROS and proteases followed by controlled apoptosis of the neutrophil, or the release of NETs and death by NETosis. Whilst these responses are critical to host defence, they can also induce modification of host proteins (indicated by an asterisk) leading to the generation of neoepitopes and the exposure of both nuclear and cytoplasmic proteins, and DNA, to the immune system. These modified host-proteins are recognised by pDCs as “foreign”, thereby initiating an auto-immune response.

**Figure 2. Role of the auto-antigen proteinase 3 in ANCA-associated vasculitis.** Proteinase 3 (PR3) is stored in the azurophil granules of circulating neutrophils. During vascular inflammation, neutrophils are activated by ANCA and undergo apoptosis. During this process, they can express PR3 at the plasma membrane, which can activate macrophages through the MYD88/interleukin 1 pathway inducing the production of inflammatory cytokines and chemokines. PR3 acts as a danger signal for macrophages resulting in a microenvironment favouring activation of pDCs, which are key cells in the immune silencing associated with the phagocytosis of apoptotic cells. Phagocytosis of apoptotic cells expressing PR3 results in an inhibition of the generation of regulatory T cells and a polarization of CD4 positive T helper cells into a Th9 profile. In addition, anti-PR3 ANCA further enhances the generation of Th17 cells thus potentiating inflammation. Generation of G-CSF potentiates PR3 synthesis in myeloid precursors leading to increased PR3 expression in mature neutrophils, and thus in turn potentiating inflammation. Indicates an increase in concentration or amount.

**Figure 3. Altered properties of neutrophils in SLE.** Neutrophils from SLE patients have an altered differentiation and activation program: 1) SLE patients have decreased levels of a subset of newly released CD10-/CD16low neutrophils from the bone marrow which produce low levels of ROS. This results in negative modulation of IL-15 levels, thereby inhibiting IFN-γ production by natural killer (NK) cells, which leads to an increased humoral response that is associated with organ damage in SLE patients; 2) LDGs represent a subset of neutrophils with increased ability to release proteases, defensins, cathelicidins as well as pro-inflammatory cytokines such as IFN-α; 3) The pro-inflammatory cytokines released by neutrophils, such as IFN-α and TNF-α modify the local micro-environment and interact with antigen-presenting B- and T-cells, while BAFF and APRIL will enhance tolerogenic B cell activation; 4) NETs release DNA which forms complexes with anti-microbial proteins and activate pDCs to produce IFN-α. From *in vivo* models, TLR9 recognition of DNA:granule protein complexes favours a regulatory immune response that could (?) lead to the generation of tolerogenic B cells; 5) Macrophages from SLE patients show a defect of apoptotic cell clearance, which leads to increased debris and aberrant expression of autoantigens. Amongst those, RNP and RNP-protein complexes activate antigen-presenting cells via TLR7, leading to high levels of IFN-α production by pDCs and production of pathogenic antibodies by auto-reactive B cells. indicates an increase, while indicates a decrease.

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