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**Review**

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Review

# Neutrophils in autoimmunity: when the hero becomes the villain

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## Summary

Neutrophils were long considered to be a short-lived homogenous cell population, limited to their role as first responders in anti-bacterial and -fungal immunity. While it is true that neutrophils are first to infiltrate the site of infection to eliminate pathogens, growing evidence suggests their functions could extend beyond those of basic innate immune cells. Along with their well-established role in pathogen elimination, utilizing effector functions such as phagocytosis, degranulation, and the deployment of neutrophil extracellular traps (NETs), neutrophils have recently been shown to possess antigen-presenting capabilities. Moreover, the identification of different subtypes of neutrophils points to a multifactorial heterogeneous cell population with great plasticity in which some subsets have enhanced pro-inflammatory characteristics, while others seem to behave as immunosuppressors. Interestingly, the aberrant presence of activated neutrophils with a pro-inflammatory profile in several systemic and organ-specific autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), systemic sclerosis (SSc), multiple sclerosis (MS), and type 1 diabetes (T1D) could potentially be exploited in novel therapeutic strategies. The full extent of the involvement of neutrophils, and more specifically that of their various subtypes, in the pathophysiology of autoimmune diseases is yet to be elucidated.

**Keywords:** neutrophils, autoimmune diseases, therapeutics, LDG, LDN

**Abbreviations:** APC: antigen presenting cell; CMP: common myeloid progenitor; CRAMP: cathelicidin-related antimicrobial peptide; G-CSF: granulocyte colony stimulating factor; GMP: granulocyte-monocyte progenitor; ICAM-1: intercellular adhesion molecule-1; IFN-γ: interferon gamma; IL-1β: interleukin 1 beta; LDG: low density granulocytes; LDN: low density neutrophils; LFA: lymphocyte-function associated antigen; mAb: monoclonal antibody; MHC: major histocompatibility complex; MMP9: matrix metallopeptidase 9; MPO: myeloperoxidase; MS: multiple sclerosis; NADPH: nicotinamide adenine dinucleotide phosphate; NDN: normal density neutrophils; NE: neutrophil elastase; NET: neutrophil extracellular trap; NOD: nonobese diabetic; PAD4: peptidyl arginine deiminase 4; PR3: proteinase 3; RA: rheumatoid arthritis; ROS: reactive oxygen species; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; T1D: type 1 diabetes; TLR: toll-like receptor; TNF-α: tumour necrosis factor alpha.

## Introduction

Neutrophils are the heroes of the immune system. First to infiltrate sites of inflammation, they not only deploy numerous strategies to eliminate invading pathogens, but also send out signals to alert other immune cells of the invasion. Their strategies for pathogen elimination include, but are not limited to degranulation, the stimulated release of anti-microbial granule proteins; phagocytosis, the engulfment and subsequent elimination of the pathogen; and reactive oxygen species (ROS) production [1]. Another somewhat unique strategy of neutrophils is the release of DNA entangled with anti-microbial granule proteins in a mesh-like structure called NETs, a mechanism that targets pathogens too large for phagocytosis [2]. Moreover, neutrophils secrete a multitude of cytokines (i.e. interleukin [IL]-1β, tumour necrosis factor [TNF]-α, interferon [IFN]-γ) and chemokines (i.e. chemokine [C-C motif] ligand [CCL] 2, chemokine [C-X-C motif] ligand [CXCL] 1) to recruit and activate other immune cell types [3]. Interestingly, neutrophils are also capable of processing extracellular proteins and presenting antigenic epitopes to T cells, proving that they are much more than the short-lived innate immune cells they were thought to be [4].

Contrary to past perceptions, neutrophils appear to be a heterogeneous cell population with subtypes, such as low-density granulocytes (LDGs)/low-density neutrophils (LDNs), first identified in patients with SLE, RA, and acute rheumatic fever [5, 6]. Recent studies into the pathology of certain autoimmune diseases have revealed that LDGs/LDNs are enriched in these patients and present with enhanced pro-inflammatory characteristics, which can have deleterious effects [7]. In fact, uncontrolled NET formation and ROS production have been demonstrated to have an exacerbating effect on the pathology of SLE, RA, SSc, T1D, and MS [8–14]. In light of these discoveries, a more comprehensive investigation into the role of neutrophils in autoimmunity is needed as the full extent of their capabilities demonstrates that when uncontrolled, they can be extremely dangerous.

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Here, we give a brief discussion on the development and functions of neutrophils but mainly focus on the emerging evidence from recent publications that propose novel concepts and mechanistic understandings of key neutrophil characteristics that make them detrimental in the pathophysiology of autoimmune diseases. Moreover, we briefly review the known neutrophil-targeted therapeutic strategies in these diseases.

## Neutrophils: from bone to tissue

### An origin story

Neutrophil development begins in the bone marrow and continues in extramedullary tissues like the spleen with the help of granulocyte colony stimulating factor (G-CSF) [15, 16]. As cells of the myeloid lineage, neutrophils originate from common myeloid progenitor (CMP) cells that differentiate to establish the granulocyte-monocyte progenitor (GMP) cell pool, which can in turn differentiate into either cells of the granulocyte lineage or cells of the monocyte/macrophage lineage. GMP cells fated to become neutrophils transition through a series of developmental stages, broadly defined by two phases: a proliferative phase comprising of promyelocytes and myelocytes, followed by a non-proliferative phase that transitions from metamyelocytes to band cells and finally into mature segmented neutrophils [17] (Fig. 1). Traditionally, the various stages of granulopoiesis were defined on the basis of cellular size, nuclear condensation, and granule content, which may not accurately reflect their functional properties and identify truly distinct stages of neutrophil development. More recently, advances in single cell transcriptomics and mass cytometry have identified three distinct developmental stages of post-mitotic human bone marrow neutrophils: precursor, immature, and mature neutrophils, based on varying surface expression levels of CD101, CD49d, CD10, CD15, CD16, and CD11b [18]. While neutrophils lose the expression of CD49d when transitioning from precursor to immature cells, they gain CD101 and CD16 expression. Both immature and mature neutrophil subsets express CD62L, which is downregulated following activation or during physiological aging in the absence of inflammation, whereas mature neutrophils exclusively express CD10 and present a segmented nucleus (extensively reviewed by Ng et al. [19]).

Whilst it is clear that neutrophil development largely occurs in the bone marrow, there is uncertainty whether the final differentiation into a mature subset is finalized in the bone marrow or following release into the peripheral circulation. Immature neutrophils are retained in the bone marrow by cell surface expression of chemokine receptor CXCR4, which maintains them in close contact with CXCL12-expressing stromal cells [20]. CXCR1 and CXCR2 are upregulated on the surface of cells undergoing differentiation into a mature neutrophil, facilitating emigration from the bone marrow in response to chemokines such as CXCL8/IL-8 [21]. However, 'emergency granulopoiesis', distinct from steady-state granulopoiesis, is characterised by accelerated proliferation and differentiation of neutrophil progenitors and reduced

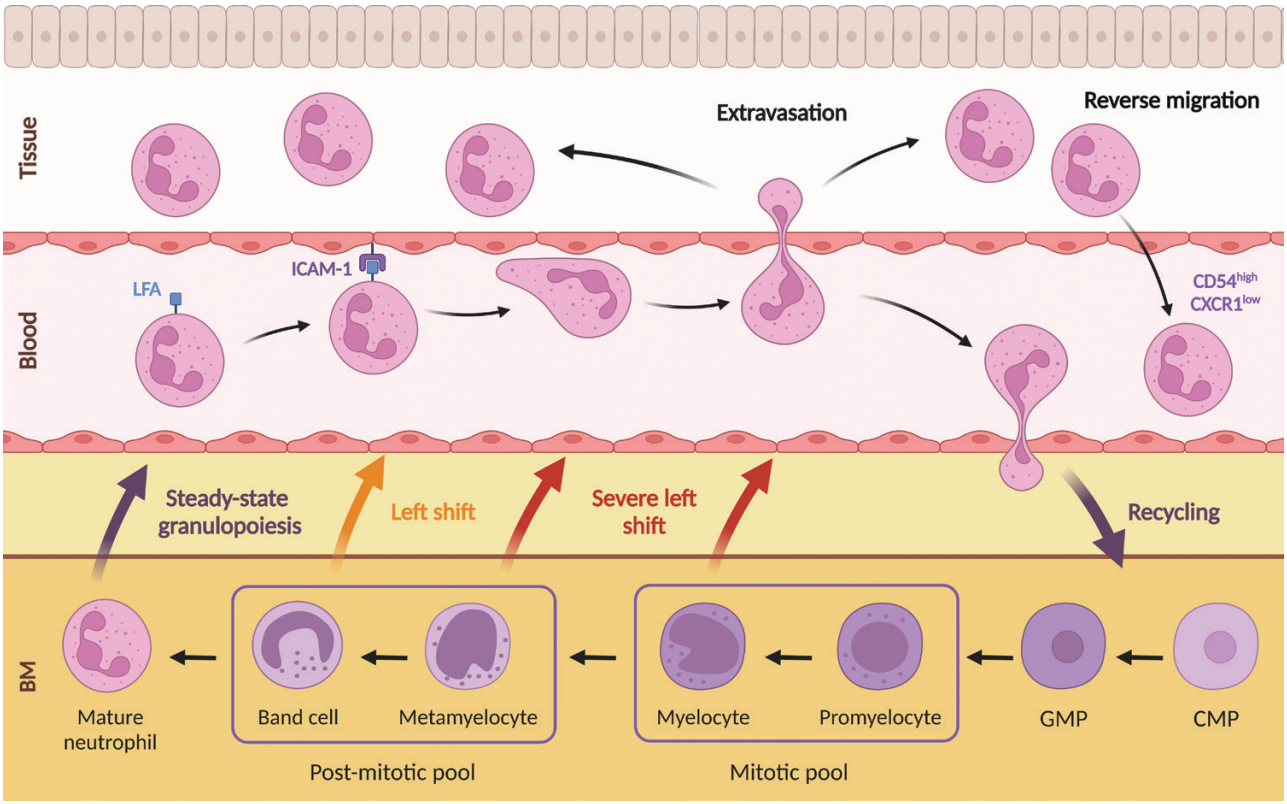



**Figure 1:** Neutrophil mobility between bone marrow, blood and tissue. Stages of neutrophil development in the bone marrow, from common myeloid progenitor (CMP) to mature neutrophil, are illustrated. Steady-state and emergency granulopoiesis ('left shift' and 'severe left shift'), as well as neutrophil recycling in the bone marrow are indicated in thick arrows. Neutrophil mobility in the periphery and extravasation into tissue are indicated in thin arrows. Neutrophils that have undergone reverse migration are phenotypically distinct ($CD54^{high}$ and $CXCR1^{low}$). Abbreviations: BM: bone marrow; CMP: common myeloid progenitor; GMP: granulocyte-monocyte progenitor; ICAM-1: intercellular adhesion molecule-1; LFA: lymphocyte-function associated antigen. Created with *BioRender.com*.

lymphopoiesis and monocytopoiesis [22]. This process has been shown to occur not only under circumstances of severe infection but also during chronic inflammation, such as in autoimmune diseases [23]. Along with the rapid mobilization of mature neutrophils, emergency granulopoiesis can also be a source of immature neutrophils in the periphery, caused by the processes referred to as 'left shift' and 'severe left shift' depending on the precursor that is mobilized [24] (described in Fig. 1). These processes of immature neutrophil mobilization into the periphery suggest that tissue-localized inflammation can influence the properties of circulating neutrophils and thus explain the existence of neutrophil subtypes presenting with altered functional and phenotypic characteristics in some autoimmune diseases.

### Tissue infiltration: not a one-way street?

Upon completing their step-wise development in the bone marrow, neutrophils are released into the circulation at a rate of approximately $10^{11}$ cells per day. Following emigration from the bone marrow, they are recruited to sites of inflammation and infiltrate the tissue in a series of ordered steps mediated by adhesion receptors. These receptors, such as intercellular adhesion molecule-1 (ICAM-1) and lymphocyte-function associated antigen (LFA), are induced on the surface of recruited neutrophils and activated endothelial cells, facilitating their entry into peripheral sites [1] (mechanistic details have been extensively reviewed by Kolaczkowska et al. [25]). Neutrophils have a unique ability to perform this extravasation even in conditions of high sheer stress imposed by blood flow using unique mechanisms of cell flattening and membrane tethering [26]. Once guided by chemokines and pro-inflammatory cytokines, the tissue-infiltrated neutrophils then deploy a multitude of effector functions in their arsenal to eliminate invading pathogens. Single cell RNA sequencing and mass cytometry experiments have recently revealed that neutrophils are capable of tissue-driven adaptations where they gain distinct functional and phenotypical characteristics [27]. Unlike other myeloid cells, neutrophils are believed to be relatively short-lived suggesting that these tissue-specific properties are acquired at a remarkably rapid rate. The exact mechanisms behind functional and phenotypic plasticity in tissue-infiltrating neutrophils remain to be clarified.

Furthermore, recent evidence suggests that neutrophils are capable of migrating back into the vasculature under either physiological or pathological conditions [28]. Mathias *et al.* first made the observation in transgenic zebrafish expressing the green fluorescent protein in neutrophils and proposed that this reverse migration from a wound site may be an additional mechanism for curtailing inflammation alongside their clearance through efferocytosis by macrophages at the site of inflammation [29, 30]. Reverse migrating neutrophils were shown to be phenotypically ($CD54^{high}$, $CXCR1^{low}$) and functionally (enhanced ROS production) distinct, suggesting a potential role in disseminating localized inflammation to secondary organs (Fig. 1). Interestingly, neutrophils presenting with a phenotype indicative of reverse migration were augmented in the circulation of people with RA and other chronic inflammatory diseases, like severe atherosclerotic disease of the aorta [31]. This suggests that reverse migration can either be an efficient method to resolve inflammation or have pathophysiological implications, depending on the context. Finally, 'aging' neutrophils down-regulate CXCR2 expression and re-express CXCR4 on the cell surface to migrate back to the bone marrow for clearance [32, 33]. Casanova-Acebes *et al.* demonstrated that aged neutrophils re-entering the bone marrow using the CXCR4-CXCL12 homing axis are cleared by stromal macrophages, which regulates the release of hematopoietic progenitor cells into the circulation in a circadian rhythm-dependent manner [32].

### Neutrophil subtypes

More recently, it has been proposed that neutrophils are not simply a homogenous cell population, but rather a complicated cell type with phenotypically and functionally distinct subtypes. These neutrophil subtypes include low-density granulocytes (LDG), or more specifically low-density neutrophils (LDN) as they will be referred to hereafter, which were first identified in patients with SLE, RA, and acute rheumatic fever in 1986, where a 'contamination' with 'lower buoyant density' neutrophils at the interface of Ficoll-Hypaque gradients was observed [6]. The LDN cell population was initially thought to consist exclusively of immature neutrophils due to their less segmented nuclei compared to mature neutrophils [7]. Gene expression analysis revealed high levels of primary granule protein-encoding mRNAs, typically associated with the promyelocytic stage of neutrophil development, in support of the immature phenotype hypothesis [34]. However, more recent studies have shown that a significant proportion of the LDNs express markers of mature neutrophils such as CD10 and CD15 [35]. Taken together, the current consensus is that LDNs are a subset of neutrophils consisting of both mature and immature populations, each with distinct morphological and functional anomalies. While the origin of the immature LDNs can be explained by the aforementioned processes of 'left shift' and 'severe left shift', mobilizing neutrophil precursors into the periphery in response to inflammatory cues, it is not clear how the mature LDNs acquire their distinct characteristics. One hypothesis states that these characteristics are acquired in the tissue and mature LDNs are neutrophils that have undergone reverse migration, resulting in the emergence of mature LDNs in circulation that are phenotypically and functionally distinct from normal density neutrophils (NDNs) [5]. This supports the idea that the properties of circulating neutrophils can be influenced by microenvironmental cues under various inflammatory conditions. Of note, LDNs have been identified in healthy donors, where they present with comparable rates of NETosis, similar proportions of granule proteins localized in NETs, and a similar extent of ROS production compared to NDNs following activation [36]. Furthermore, a subtype of neutrophils, termed polymorphonuclear-myeloid-derived suppressor cells (PMN-MDSCs), presenting with distinct immunosuppressive characteristics, has been described in cancer patients [37]. This further highlights the emerging concept of neutrophil plasticity in various inflammatory contexts. Additional studies are needed to elucidate the role of these subtypes and their functions, under both physiological and pathological conditions. Due to their relevance in autoimmunity, we will focus on the LDN subtype in this review.

## Neutrophils: weapons in their arsenal

Neutrophils are capable of a myriad of effector functions, equipped to deal with any threat the host may encounter. Coupled with their sheer abundance in the circulation and the ability to mobilize rapidly to sites of inflammation, the

full extent of their functional capabilities makes neutrophils extremely effective first-responders to infection.

## Neutrophil anti-microbial functions

Neutrophils are armed with granules: membrane-bound vesicles packed with proteins that play important roles in all the known antimicrobial functions of neutrophils. Four different types of granules have been characterized: azurophilic (primary), specific (secondary), gelatinase (tertiary), and secretory vesicles, differing in their content, structure, and function. The different granule subtypes are formed during specific stages of neutrophil development and are characterized by their contents. Azurophilic granules contain antimicrobial proteins (i.e. myeloperoxidase [MPO]), proteases (i.e. neutrophil elastase [NE], proteinase 3 [PR3]) and membrane-permeabilizing molecules (i.e. lysozyme, defensin). Specific and gelatinase granules consist of a mixture of anti-microbial proteins and various proteins that aid in vascular extravasation and response to cytokines/chemokines (i.e. gelatinase, lactoferrin). Secretory vesicles are the source of a variety of receptors and are triggered to fuse with the plasma membrane following cellular activation. Granule protein content, as well as the mechanisms of granule release and regulation, have been extensively reviewed by Yin and Heit [38].

In addition to their direct release during degranulation, neutrophil granules are essential for phagocytosis and NET formation. Phagocytosis is mediated by opsonic receptors that recognize antibodies, complement proteins, mannose binding lectins, and other host-derived proteins that target and bind specific structures on the surface of pathogens [39]. Following uptake, a fusion of the phagosome with preformed granules dooms the internalized microbe for destruction via activation of various cytolytic enzymes or through the generation of ROS when coupled with nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. With the help of MPO, activation of NADPH oxidase promotes the generation of superoxide anion ($O_2^-$) followed by the production of other ROS, resulting in a significant increase in oxygen consumption, known as the respiratory burst [40].

NET formation, the extrusion of DNA and chromatin entangled with anti-microbial granule proteins, is another efficient method for pathogen destruction. With the help of NADPH oxidase, MPO-derived ROS activate NE, which subsequently cleaves histones and actin, leading to chromatin decondensation, and NET release [2]. NET formation can also occur independently of NADPH oxidase and MPO, through the activity of peptidyl arginine deiminase (PAD) 4 that can induce chromatin decondensation by converting arginine to citrulline on histone residues causing the loss of a positive charge [41]. The intricate cell signalling and cytoskeletal mechanisms involved in NET formation have been extensively reviewed by Thiam *et al*. [42]. Pyroptosis is another highly inflammatory mechanism of neutrophil degradation, characterized by pro-inflammatory caspase-1 and inflammasome activation. While pyroptosis is distinguished from NETosis by the retention of DNA within the lysing cell, some studies suggest that pyroptosis can also lead to a non-canonical form of NETosis through the activation of caspase-11 [43]. In both pyroptosis and NETosis, the membrane rupture is dependent on the cleaving and subsequent activation of the pore-forming protein gasdermin-D, which can also result in the release of inflammatory cytokines, such as IL-1β [44]. The various forms of neutrophil death, as well as the underlying mechanism are extensively reviewed by Pérez-Figueroa *et al*. [45].

These anti-microbial functions, coupled with their abundance in the circulation and rapid recruitment to sites of inflammation, make the humble neutrophil an indispensable player in the early stages of anti-microbial immunity.

## Neutrophil interactions with other cell types

Perhaps the largest impact of neutrophils at the site of inflammation lies beyond their anti-microbial effector functions but in their immune-modulating capabilities. Indeed, as first-responders to infection, the true potential of neutrophils lies in their ability to influence various aspects of the ensuing immune response through the release of cytokines, chemokines, NETs, or even via direct cell-to-cell contact with other types of immune cells, eliciting either pro- or anti-inflammatory responses [46].

Neutrophils secrete a variety of cytokines and chemokines that recruit cells of both the innate and adaptive immune systems to the site of inflammation. Chemokine CCL2 and the pro-inflammatory cytokines TNF-α and IL-1β recruit macrophages and dendritic cells (DCs), whereas the chemokines CXCL1, CXCL7, CCL19, and CCL20 recruit T cells [3]. Neutrophils can also interact with platelets, which bind various leukocytes but preferentially interact with neutrophils, through surface expression of P-selectin. Platelets are capable of activating neutrophil functions through direct contact or secreted microparticles, and neutrophils subsequently participate in the phagocytic removal of platelets [47–49]. Neutrophils also help to bridge the gap between innate and adaptive immunity, indirectly boosting antigen-specific T-cell responses with cytokines or through direct interaction with DCs. Antigens captured by neutrophils through phagocytosis can be passed to DCs that then present them to T cells. In addition, neutrophils can induce either a Th1 or Th2 polarization in activated CD4 T cells, through the production of IL-12 or IL-4, respectively [50]. Some neutrophil-derived factors can also influence B cells, such as B cell-activating factor of the tumour necrosis family (BAFF), and a proliferation-inducing ligand (APRIL), which drive B cell expansion and plasma cell differentiation [51]. Moreover, in response to signals from sinusoidal endothelial cells in the marginal zones of the spleen, neutrophils produce NET-like structures and cytokines that promote immunoglobulin G (IgG) class switching, somatic hypermutation, and antibody production in activated B cells [51]. Nucleic acids present in NETs can stimulate pattern recognition receptors and drive cytokine production from a number of cells types. Monocytes can recognize DNA complexed with citrullinated histone H3 (citH3) in NETs via toll-like receptor (TLR) 4, whereas both DNA and RNA in NETs, when complexed with the antimicrobial self-peptide LL37, can activate TLR8 signalling [52, 53]. MicroRNAs (miRNA) are also detectable within NETs and can elicit particular effects on cells, such as miRNA-142-3p which enhances TNF-α production in macrophages [54].

Furthermore, under certain conditions neutrophils can display features of antigen presenting cells (APCs) and have even been shown to have direct contact with T cells [55]. To activate naïve T cells, an APC must be able to internalize exogenous antigens, process them into smaller peptide subunits, load them into major histocompatibility molecules, and present them on the cell surface. Ligation of the B7 molecules (i.e. B7-1/CD80 and B7-2/CD86) with CD28 on the T cell

provides co-stimulatory signals, which is required for the activation of naïve T cells. As professional phagocytes, neutrophils are more than capable of internalizing antigens via phagocytosis, pinocytosis, or receptor-mediated endocytosis. Indirect evidence for antigen processing by neutrophils exists based on the expression of HLA-DM, a chaperone protein that is required for the proper loading of antigenic peptides onto MHC-II molecules, the absence of which results in defective peptide loading. Expression of HLA-DM was confirmed in cytokine-stimulated HLA-DR positive neutrophils but was not detected in HLA-DR negative neutrophils. Moreover, neutrophils pulsed with Bet v 1, the major allergen in birch pollen, were able to activate a panel of Bet v 1-specific T-cell clones, showing that they were capable of processing and presenting antigenic peptides to T cells [56]. Neutrophils are capable of expressing both classes of major histocompatibility complex (MHC) proteins, as well as an array of costimulatory molecules. In their quiescent state, neutrophils neither express MHC-II nor the co-stimulatory molecules CD80 and CD86. GM-CSF and IFN-γ induce neutrophils to express MHC-II on their surface, as well as high concentrations of IL-3 and TNF-α [57, 58]. Conflicting evidence exists for the ability of neutrophils to express the ligands for CD28, the absence of which promotes T-cell anergy. While some studies could show that neutrophils stimulated with cytokines up-regulated expression of CD80 and CD86, others failed to show expression in response to GM-CSF, IL-3, or IFN-γ [57, 58]. Neutrophils are also capable of migrating to lymph nodes by expressing the lymph node-homing receptor CCR7. Moreover, neutrophils isolated from the arm-draining lymph nodes following vaccination were capable of presenting the vaccine antigen to antigen-specific memory CD4 T cells *ex vivo* [57]. However, the kinetics of naïve vs. memory T-cell activation in response to cognate antigen differ significantly, and evidence for neutrophils being able to activate naïve CD4 T cells is still lacking. Whether neutrophils are able to traffic antigens to lymph nodes and specifically activate naïve CD4 T cells, the physiological relevance of neutrophils as APCs in the activation of adaptive immune responses, and how this compares to other professional APCs, remains unclear. In addition, considerable differences between neutrophils of mice and men limit the translatability of these results to the human condition. Nevertheless, the scope of these immune-modulating capabilities demonstrates the important role of neutrophils in perpetuating or curtailing inflammation. These features have significant implications, not only for the successful clearance of invading pathogens but also for initiating and exacerbating autoimmune disease.

## Neutrophils in autoimmunity: when the hero becomes the villain

Despite remarkable differences in the underlying pathological mechanisms and presentation of clinical symptoms, diseases like SLE, RA, SSc, MS, and T1D have a common element at the core of their pathologies: an immune-mediated attack. At odds with their supportive roles in anti-microbial immunity and tissue regeneration/repair, neutrophils have been shown to play a deleterious role in the pathology of several autoimmune diseases. This is not surprising considering their wide range of functions, their biotoxicity, and their sheer abundance in the circulation. Rapidly deployed to the site of inflammation and ready to unleash their anti-microbial and immunomodulatory functions, neutrophils are capable of inflicting a lot of collateral damage to the surrounding tissue when activated in the context of autoimmunity.

Neutrophil-derived factors, such as cytokines, chemokines, ROS, NETs, as well as other antimicrobial peptides, all contribute significantly to the autoimmune process (summarized in Fig. 2). In a murine model of MS, central nervous system (CNS)-infiltrating neutrophils were shown to secrete TNF-α, IL-6, IFN-γ, and IL-12, assisting in the maturation of DCs that subsequently activate myelin-specific T cells [59]. In non-obese diabetic (NOD) mice, an animal model of T1D, neutrophils were shown to activate plasmacytoid DCs (pDCs) through the secretion of cathelicidin-related antimicrobial peptide (CRAMP), which subsequently drives the T cell-mediated autoimmune response against pancreatic beta cells [60]. Neutrophil-derived ROS are increased in the circulation and synovial tissue of SLE and RA patients, respectively, which in abundance can cause extensive tissue damage and even modify certain molecules rendering them immunogenic, unable to perform their original function, or less susceptible to degradation [8, 9]. For instance, elevated levels of oxidized IgG and self-DNA in RA and SLE patients respectively, were linked to enhanced immune activation in these diseases [61, 62]. Despite conflicting evidence on the levels of ROS production in T1D neutrophils, these toxic chemicals can initiate the destruction of the insulin-producing beta cells [10]. While neutrophils produce ROS and "fibrogenic" cytokines such as transforming growth factor (TGF)β, vascular endothelial growth factor (VEGF), and IL-6, that can cause endothelial damage and subsequent fibrosis in SSc, their involvement in the pathophysiology of the disease remains to be clarified [3, 63].

Amongst their plethora of capabilities, NETosis is by far the most prominent neutrophil function associated with autoimmune pathologies, shown to contribute significantly to the immuno-pathological processes of SLE, T1D, and RA [11, 12]. NET components such as PR3, MPO, and NE, activated and released during NETosis, are cytotoxic and have been shown to cause direct damage to the endothelium [2]. In T1D, pancreas-infiltrating neutrophils prone to undergoing NETosis were identified in newly diagnosed patients and people at high risk of developing the disease, correlating with elevated NET-associated NE and PR3 in the circulation [64–66]. While less is known about the role of neutrophils and NETosis in MS, studies have reported elevated MPO and DNA–MPO complexes in the serum of MS patients [67, 68]. NETing neutrophils isolated from SLE patients induced type I IFN production in DCs, further perpetuating the autoimmune response [69]. Neutrophils with a propensity for NET formation were also present in the blood and synovial fluid of people with RA, as well as in the circulation of SSc patients [13, 70]. Interestingly, excessive NETosis was linked to a dysregulated platelet-neutrophil interaction resulting in improper clearance of platelets and subsequent accumulation of platelet-derived microparticles in SSc patients [14]. While beneficial in physiological conditions, the interaction of neutrophils with platelets can have detrimental consequences in an inflammatory context [47, 49]. Platelet-neutrophil aggregates were found in the blood of T1D patients, as well as in the synovial fluid of RA patients, where the aberrant NET formation is a key part of the pathophysiology [71, 72]. Moreover, platelet-induced NETosis was shown to occur through the induction of autophagy in neutrophils by the

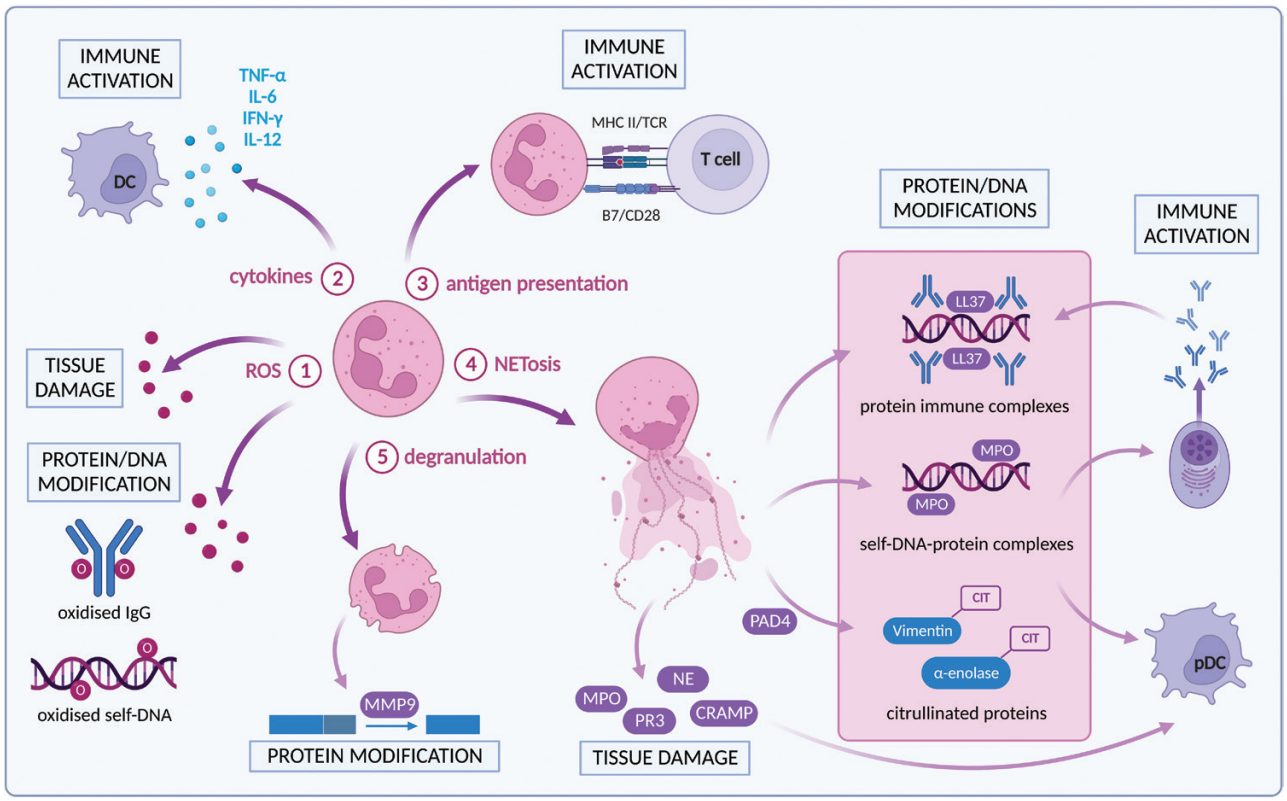



**Figure 2:** Neutrophil functions in autoimmunity. Neutrophil functions (numbered 1-5) shown to be implicated in autoimmune diseases, as well as their effects and consequences are indicated. (1) ROS produced by neutrophils can cause tissue damage and oxidise (indicated by an 'O') proteins and self-DNA. (2) Cytokines (i.e., TNF-α, IL-6, IFN-γ, IL-12) can aid in the maturation of DCs and cause immune activation. (3) Neutrophils express MHC-II and B7 molecules and can present antigens to T cells, activating the adaptive immune system. (4) NETosis can cause immune activation through protein and self-DNA modifications directly or through PAD4 activation (CIT: citrullination). NET-associated granule proteins (i.e. MPO, PR3, NE, CRAMP) can also cause tissue damage and/or activate pDCs. (5) Granule proteins such as MMP9 released during degranulation can modify proteins. Abbreviations: CRAMP: cathelicidin-related antimicrobial peptide; MHC: major histocompatibility complex; MMP9: matrix metallopeptidase 9; MPO: myeloperoxidase; NE: neutrophil elastase; NET: neutrophil extracellular trap; PAD4: peptidyl arginine deiminase 4; PR3: proteinase 3; ROS: reactive oxygen species. Created with *BioRender.com*.

expression of high mobility group Box 1 (HMGB1) on activated platelets [73]. Autophagy, an important mechanism for cell maintenance, is not only required for NETosis but also essential to many other neutrophil functions, including degranulation, phagosomal maturation, and ROS generation [74–77]. Characterized by the formation of autophagosomes that contain cytosolic components destined for degradation, autophagy is not only vital for the survival and functional integrity of neutrophils, but has also been shown to contribute to various autoimmune pathologies when dysregulated [78]. In fact, autophagy was upregulated in neutrophils from the synovial fluid and blood of patients with RA and SLE, respectively [79, 80]. Increased levels of autophagy in SLE neutrophils were associated with enhanced release of NETs containing tissue factor and IL-17A, which contributes to the development of fibrosis in SLE patients [80]. Thus, platelet- and autophagy-induced NET formation are possible culprits in the pathophysiology of autoimmune diseases.

Perhaps the most distinctive role of NETs and NET components in autoimmunity is their capacity to generate autoantigens. NET components such as MPO and PR3 can be recognized as autoantigens and generate autoantibodies in certain autoimmune diseases such as anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis [81]. Furthermore, self-DNA-peptide complexes, produced as a result of enhanced NETosis, were shown to trigger pDC activation and autoantibody production in SLE patients [51]. Neutrophils and NETs can also trigger or exacerbate autoimmunity through the formation of neoantigens via the post-translational modification of self-proteins. In RA, PAD4 released during NETosis was shown to citrullinate structural proteins such as vimentin and α-enolase, inducing the formation of anti-citrullinated protein autoantibodies (ACPA) [70]. Neutrophil granule proteins are also capable of generating neoantigens. Proteolytic cleavage of myelin base protein and collagen, in MS and RA respectively, by neutrophil granule proteins (i.e. matrix metallopeptidase 9 [MMP9], gelatinase B) was shown to create remnant epitopes that drive autoimmunity [82, 83]. Whilst autoantibodies against beta-cell neoantigens have been implicated in T1D, the role of neutrophils and NET-derived autoantigens in the autoimmune process remains to be clarified [84]. Aberrant NETosis, modifications of self-proteins, and the formation of autoantibodies against these modified proteins create a vicious cycle that perpetuates the autoimmune response.

In addition to their relevance in the pathogenesis, dysregulated NETosis is also associated with complications of autoimmune diseases. In a murine model of SLE, excessive NET formation was linked to diffuse alveolar haemorrhage, a pulmonary complication that often leads to respiratory

failure in SLE patients [85]. In T1D, aberrant NET formation was shown to be closely associated with diabetes-induced microvascular complications. NET components such as self-DNA, NE, and PR3 were not only elevated in the blood of T1D subjects but also identified in the diabetic foot ulcers of these patients [86]. NETs and NET components were also shown to be implicated in diabetic retinopathy and delayed wound healing, thus contributing to the chronic inflammatory response in T1D [87, 88].

Increasing evidence partially attributes the aforementioned altered functions, such as ROS and NET formation to the LDN subtype (Fig. 3). Enhanced spontaneous NETosis by LDNs contributes to tissue damage and autoantigen externalization in SLE [89]. In RA LDNs, ROS production and apoptosis are decreased, whereas granule protein transcript levels are increased [70, 90]. LDNs were also identified as a heterogeneous neutrophil subset, primed for immune activation, in MS and ANCA-associated vasculitis patients [91, 92]. More work is needed to decipher the importance of neutrophil subtypes, such as LDNs, in the autoimmune processes of SLE, RA, and MS, as well as their involvement in other autoimmune diseases, such as T1D and SSc.

Of note, it is important to consider the stages of disease development at which neutrophils and their various subtypes are implicated. However, whether they contribute to the initiation of disease or are simply responsible for maintaining inflammation, neutrophils are progressively recognized to have a prominent role in several autoimmune diseases. In addition to their already described roles in autoimmunity, we suspect additional functions of neutrophils are yet to come to light. For example, although neutrophils have been shown to migrate to lymph nodes, express HLA-DR, efficiently process antigens and present them to T cells (reviewed by Polak *et al.* [4]), the implication of their antigen presenting capabilities in the initiation and/or exacerbation of autoimmune disease remains to be elucidated. A deeper insight into the roles of neutrophils in autoimmunity, including their phenotypic and functional heterogeneity, may help stratify patients accordingly and lead to the development of novel neutrophil-targeted therapies, and more personalized treatment options.

## Neutrophil-targeted therapeutics

Long term use of glucocorticoids, typically used to treat symptoms of autoimmune inflammation, is often associated with severe side effects that can potentially increase the burden of the disease [93, 94]. Thus, more targeted therapeutic strategies are needed to ameliorate the quality of life of these patients. Given that neutrophils are increasingly recognized to play a vital role in the initiation and progression of autoimmunity, this has led to the development of many neutrophil-targeted therapies for the treatment and potential prevention of various autoimmune diseases such as SLE, RA, SSc, MS, and T1D. Depending on the stage of disease progression, there are numerous neutrophil-targeted therapies that can be employed to reduce their deleterious effects on autoimmunity, such as altering their abundance in the circulation, inhibiting migration into inflammatory sites, and dampening effector functions.

Reducing neutrophil numbers in the circulation and their infiltration into inflamed tissues is an effective way of reducing neutrophil-driven autoimmune inflammation at the earlier

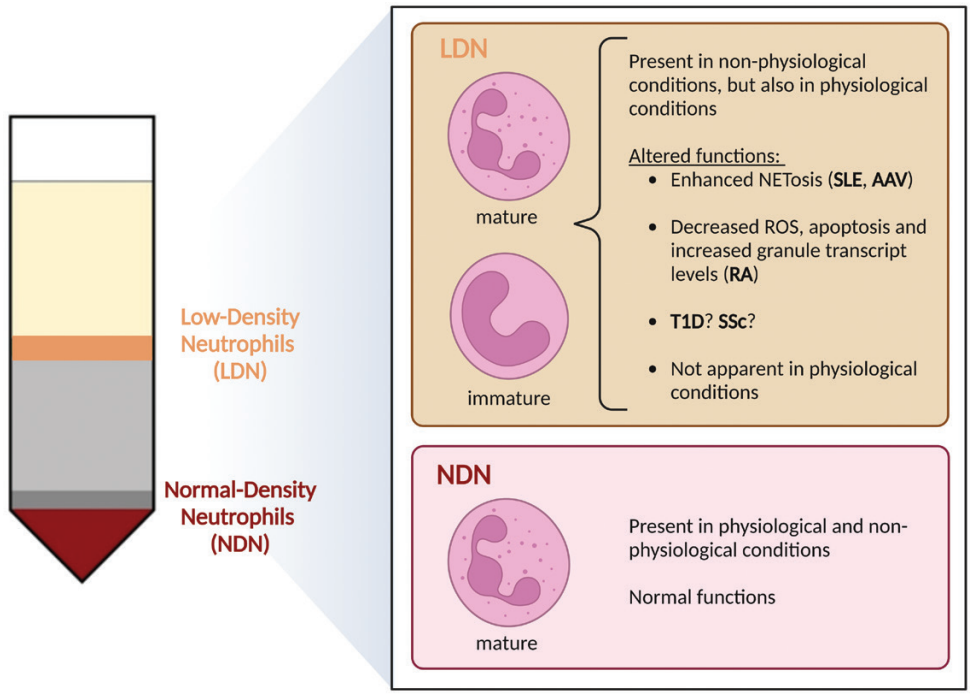



**Figure 3:** Normal- and low-density neutrophils in health and disease. After gradient separation, neutrophils found in the pellet and in the PBMC layer are termed normal-density neutrophils (NDN) and low-density neutrophils (LDN), respectively. While the NDN subtype is composed of mainly mature cells, studies have shown that the LDN subtype can contain both mature and immature neutrophils. LDNs have been shown to have altered functions in various autoimmune diseases such as SLE, RA and AAV, while their role and functions in T1D and SSc remain to be elucidated. Both subtypes were found in physiological and non-physiological conditions. However, the role of LDNs in physiological conditions remains to be clarified. Created with *BioRender.com*.

stages of disease development. In a pre-clinical study, blocking the G-CSF receptor with a monoclonal antibody (mAb) showed that it can regulate G-CSF-mediated neutrophilia, without affecting basic neutrophil functions [95]. Testing anti-G-CSF mAbs in an animal model of RA inhibited neutrophil infiltration into the joints and effectively halted the progression of the disease without causing neutropenia or affecting basic neutrophil antimicrobial effector functions [96]. Alternatively, neutrophil migration into inflammatory sites can be disrupted by targeting CXCR1 and CXCR2, receptors that direct the chemotactic migration of neutrophils. Blocking these chemokine receptors inhibited autoimmune insulitis and even reversed T1D in NOD mice [97]. The authors postulated that the CXCR1/2 inhibitors also act on other myeloid cells implicated in T1D since neutrophil depletion alone was not as effective in preventing T1D. The exact mechanisms behind the inhibition of autoimmune insulitis remain to be clarified. Despite these positive pre-clinical results, a recent multicentre double-blind study showed no significant effect of ladarixin, a CXCR1/2 receptor-blocking agent, on preserving residual beta-cell function in newly diagnosed T1D patients [98]. Neutrophil chemokines that bind these receptors, can also be targeted with inhibitors. However, clinical trials of CXCL8/IL-8 neutralizing antibodies in psoriasis and RA have shown no significant effect on disease pathology [99].

An alternative approach is to target signal transduction pathways activated by these cytokines and chemokines, thus interfering with the recruitment, activation, and subsequent effector functions of neutrophils. The Janus kinase (JAK)/ signal transducers and activators of the transcription (STAT) pathway are essential for neutrophil activation and migration in response to signals provided by cytokines [100]. Tofacitinib, an inhibitor of JAK3, which controls CXCL8/IL-8-mediated neutrophil chemotaxis, reduces symptoms and improves physical function in people with RA and has been approved for use in these patients [101]. Its efficacy in reducing both cutaneous and pulmonary fibrosis in SSc patients in a recent study points to a potential use in treating the disease [102]. A recent phase 1 clinical trial in people with mild-to-moderate SLE showed that tofacitinib treatment decreased peripheral type I IFN gene signature, as well as reducing the levels of circulating NET components and LDNs. The authors also demonstrated that the drug is safe and well-tolerated, without significant adverse effects such as an increase in bacterial infections [103]. The authors argued that the increase in LDNs in the placebo group only partially explained the significant reduction of circulating LDNs, without affecting the total neutrophil counts. Further studies are necessary to determine the mechanisms of this reduction, which can potentially lead to LDN-specific therapies in autoimmune diseases. Other JAK molecules, such as JAK1 and JAK2, which mediate the response to cytokines such as IL-2, IL-6, and IFNs [104], have shown efficacy in reducing immune cell infiltration in various autoimmune diseases. Inhibitors of JAK1 and JAK2 block neutrophil, DC, and B cell infiltration into the CNS and reduce clinical symptoms in animal models of MS [105]. A selective JAK1 inhibitor reduced CD8 T cell proliferation, as well as MHC-II upregulation on beta cells in a preclinical model of T1D [106]. However, there is no evidence of the direct effect of the inhibitor on neutrophil migration or function. Another signalling molecule important in neutrophil function is Bruton's tyrosine kinase (BTK). Evobrutinib, a BTK inhibitor, has been shown to be safe and effective in improving symptoms in patients with relapsing MS [107]. Other BTK inhibitors are being investigated for the treatment of RA and SLE (extensively reviewed by Neys *et al*. [108]). An important consideration for these strategies, however, is that targeting signal transduction pathways is not neutrophil specific and thus also has effects on other types of immune cells, such as lymphocytes, and other myeloid cells. Considering the deleterious role of these immune cells in the pathophysiology of autoimmune disease, the overall effect of targeting signal transduction molecules may be beneficial. However, further investigation is necessary to determine if and to what extent anti-microbial immunity may be compromised in patients receiving these treatments.

Finally, specifically targeting neutrophil effector functions is a viable option for the treatment and potential prevention of various autoimmune diseases, such as targeting neutrophil-derived cytokines that play an important role in autoimmunity. Infliximab and tocilizumab, mAbs against TNF-α and IL-6, respectively, are approved treatments for people with RA and have been shown to reduce CXCL-8/IL-8-mediated neutrophil infiltration in the joints, along with other immune cells [109, 110]. Tocilizumab is also an approved treatment for a subtype of SSc patients where it improved clinical symptoms of interstitial lung disease [111]. NET formation is a process that is highly dependent on enzymes such as MPO, NE, and PAD4, which promote chromatin decondensation, making them suitable candidates for NET-targeted therapeutics [112, 113]. The pan-PAD inhibitor BB-Cl-amidine reduced protein citrullination and Th1/Th17 responses, subsequently reducing inflammation and joint destruction in an animal model of RA [114]. This was also the case in a pre-clinical model of T1D, with the addition of a significant reduction in NET formation and prevention of disease onset [115]. While pan-PAD inhibitors can have off-target cytotoxic effects, isoform-specific inhibitors such as PAD4 inhibitors were shown to be nontoxic, as well as efficient in reducing inflammation in pre-clinical models of RA [116, 117]. PAD inhibitors that are currently available or undergoing testing are extensively reviewed by Bruggeman *et al*. [118]. Moreover, NET components, that are deleterious in the case of dysregulated NETosis, can also be targeted with inhibitors. Selective inhibitors of MPO and NE, reduced disease activity in immune complex vasculitis and bronchiectasis, respectively [119, 120]. Another potential NET-targeting strategy involves the disruption of NET structures with recombinant (r) DNase I which has shown efficacy in improving lung function in cystic fibrosis [121]. The use of rDNAse I was shown to be safe and tolerable in SLE, however further studies are necessary to determine its effectiveness in reducing clinical manifestations of the disease, as well as their use in other autoimmune pathologies [122]. Low molecular weight heparin (LMWH) has also been shown to prevent NET formation by interfering with autophagy, degranulation, and platelet-neutrophil interaction [123, 124]. While shown to be potent in reducing NETs and inflammation in COVID-19 patients, the efficacy of LMWH in autoimmune diseases remains to be determined [125]. Other indirect NET inhibitors are discussed by Chamardani *et al*. [126].

In summary, when treating autoimmune disease, neutrophils can be targeted in a number of different ways and at various stages of disease development. Although some of these neutrophil-targeted therapies are currently being tested or are approved for use in one type of autoimmune disease (summarized in Fig. 4), the deleterious pro-inflammatory role that

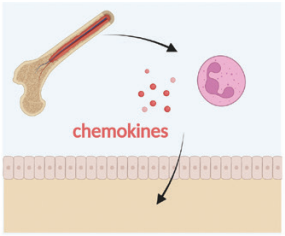
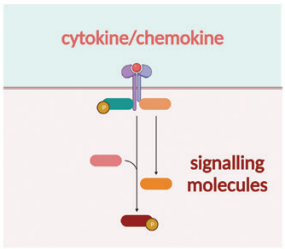
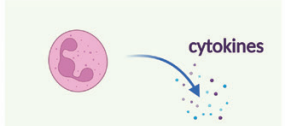
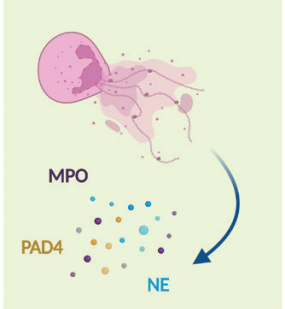
| Therapeutic target | | | Efficacy | References |
|---|---|---|---|---|
| Neutrophil mobilization |   | G-CSF receptor | Reduced neutrophil trafficking and local inflammation in pre-clinical model of RA | Campbell et al., 2016 |
| | | CXCR1/2 | No effect in multicenter double-blind study in T1D | Piemonti et al., 2022 |
| | | CXCL8/ IL-8 | No effect in RA and psoriasis | Bachelerie et al. 2014 |
| Signal transduction |   | JAK 3 | Reduced symptoms and improved physical condition in RA and SSc Decreased type 1 IFN and LDGs in SLE | Fleischamann et al., 2012 Hasni et al., 2021 |
| | | JAK 1/ 2 | Blocked neutrophil infiltration in CNS and improved clinical symptoms in pre-clinical model of MS | Liu et al., 2014 |
| | | BTK | Reduced symptoms in patients with refractory MS | Montalban et al., 2019 |
| Cytokines |   | TNF-α | Reduced neutrophil and other immune cell infiltration in RA Approved in RA and SSc | Taylor et al., 2000 |
| | | IL-6 | | Wright et al., 2014 |
| NETosis |   | PAD enzymes | Reduced inflammation and NET formation in pre-clinical models of RA and T1D | Kawalkowska et al., 2016 Sodré et al., 2021 |
| | | PAD4 | Reduced inflammation without off-target effects in pre-clinical model of RA | Willis et al., 2017 Martin Monreal et al., 2021 |
| | | NET DNA | Reduced NETs in cystic fibrosis Safe and tolerable in SLE | Khan et al., 2019 Davis et al., 1999 |
| | | MPO, NE | Reduced disease activity in immune complex vasculitis and bronchiectasis | Zheng et al., 2015 Stockley et al., 2013 |

**Figure 4:** Non-exhaustive list of neutrophil-targeted therapeutics. Therapeutics targeting various aspects of neutrophil biology are described, as well as their efficacy in autoimmune diseases. Among drugs targeting neutrophil mobilization are those that block G-CSF, CXCR1 and CXCR2 receptors, as well as their ligands like CXCL8/IL-8. Inhibitors of JAK3, JAK 1/2, BTK are drugs that target signal transduction in response to cytokines/chemokines. Cytokines such as TNF-α and IL-6 can also be blocked with monoclonal antibodies. To target NETosis, NET components such as DNA, PAD enzymes, as well as granule proteins (i.e. MPO, NE) can be inhibited. Abbreviations: BTK: Bruton's tyrosine kinase; G-CSF: granulocyte colony stimulating factor; JAK: Janus kinase; MPO: myeloperoxidase; NE: neutrophil elastase; NET: neutrophil extracellular trap; PAD: peptidyl arginine deiminase. Created with *BioRender.com*.

neutrophils play in various autoimmune pathologies supports the potential use of these therapies in other types of autoimmune disease. The caveat is however that the important contribution of neutrophils to host defence must be properly considered when designing and testing neutrophil-targeted therapeutics. The particularly deleterious role of neutrophil subtypes like LDNs in the pathophysiology of autoimmune diseases makes them suitable candidates for more targeted therapies. However, the remarkable subtleties in the phenotypical and functional differences between LDNs and NDNs present a difficulty in developing a therapeutic strategy specific to this subset of neutrophils. A deeper understanding of neutrophil plasticity and the resulting subsets is needed to safely target LDNs in autoimmune diseases.

## Concluding remarks

Neutrophils are a unique subset of cells, owing to their vast capabilities in not only anti-microbial immunity but also their effects on other cells. They are highly efficient in eliminating pathogens but can cause serious problems for the host if misguided. Although they have been shown to be heavily implicated in the pathology of numerous autoimmune diseases, more work is needed to elucidate the exact role and mechanism of action of neutrophils in these diseases. Their relatively short lifespan, their susceptibility to spontaneous activation due to enhanced sensitivity to external factors and their low RNA content present a substantial technical difficulty. The recent revelations on neutrophil heterogeneity due to tissue-specific and micro-environmental cues further highlight the importance of careful interpretation of experimental results. Furthermore, in light of recent discoveries of novel neutrophil subtypes presenting with heightened pro-inflammatory characteristics in diseases like SLE, RA, and MS, questions arise on their exact role in autoimmunity. For instance, are these subtypes intrinsically different in patients compared to healthy controls or are they strictly the product of a tissue-specific inflammatory environment? Which immune

modulators influence the activation and survival of these neutrophil subtypes? How do the pro-inflammatory neutrophils affect the progression and perpetuation of other autoimmune diseases? Further studies are needed to understand the role of neutrophils and their various subtypes, which could lead to novel therapeutic strategies in both the treatment and prevention of autoimmune diseases.

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## Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

S.B. performed the literature review, wrote the manuscript, and prepared the figures. D.E., C.M., and C.G. critically reviewed and edited the manuscript. All authors edited and approved the final version of the manuscript.

## Ethics Approval

Not applicable.

## Data Availability

Not applicable.

## Permission to Reproduce

Not applicable.

## Clinical Trial Registration

Not applicable.

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