Polymorphonuclear Neutrophils in Rheumatoid Arthritis and Systemic Lupus Erythematosus: More Complicated Than Anticipated

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Abstract: Polymorphonuclear neutrophils (PMN) are the most abundant leucocytes in the circulation in humans. They represent a heterogeneous population exerting diverse functions through several activities. Usually described as typical pro-inflammatory cells, immunomodulatory properties of PMNs have been reported. Among others, once activated and depending on the stimulus, PMNs expel neutrophil extracellular traps (NET) in the extracellular space. NETs are complexes made of DNA and granule proteins representing an innate immune mechanism fighting infections. Nevertheless, an excess of NET formation might be involved in the development of inflammatory or autoimmune responses. Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are two chronic, inflammatory, autoimmune diseases of unknown etiology and affecting mostly women. Several abnormal or non-classical functions of PMNs or PMN sub-populations have been described in SLE and RA. Particularly, NETs have been suggested to trigger pro-inflammatory responses by exposing pro-inflammatory mediators. Likewise, NETs may be the targets of autoantibodies or even might trigger the development of autoantibodies by exposing autoantigens. In the present review, we will summarize heterogeneous properties of human PMNs and we will discuss recent evidence linking PMNs and NETs to the pathogenesis of both SLE and RA.

Keywords: neutrophils; neutrophil extracellular traps; rheumatoid arthritis; systemic lupus erythematosus; inflammation; autoimmunity; immunomodulation; extracellular chromatin

1. Introduction

Polymorphonuclear neutrophils (PMN) represent more than 50% of blood leukocytes in humans. Circulating PMNs were thought classically to have a relatively short half-life. However, more recently, their in vivo lifespan has been estimated to 5.4 days in human blood [1] and to 120 h for tissue PMNs in vivo in zebrafish [2]. Moreover, PMN survival is increased under inflammatory conditions.

PMNs are among the first cells recruited to sites of inflammation. They are classically described as pro-inflammatory cells. Their classical functions comprise: (1) phagocytosis (via direct recognition of pathogens through receptors or via receptors for opsonins); (2) degranulation (different types of granules being present in PMNs with primary granules (containing, for example, proteinase 3, myeloperoxidase (MPO), cathepsin G, neutrophil elastase or defensins (belonging to antimicrobial peptides)), secondary granules (containing, for example, lactoferrin, cathelicidin (LL-37 in humans, an antimicrobial peptide)), tertiary granules (containing, for example, gelatinases, such as matrix metalloproteinase (MMP) 2 and MMP9) and secretory vesicles (containing, for example, receptor for the C1q complement protein)); (3) production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) which display bactericidal activities or are involved in cell-cell communication; (4) secretion of cytokines (e.g., tumor necrosis factor (TNF)) and chemokines
(e.g., interleukin (IL)-8). More recently, activated PMNs have been shown to produce and release neutrophil extracellular traps (NET), complexes of DNA and proteins from granules [3]. Nevertheless, immunomodulatory and regulatory or even immunosuppressive functions have also been attributed to PMNs.

The cell surface molecule CD66b is exclusively expressed by granulocytes in humans [4], especially PMNs and eosinophils. However, as in blood 95% of them are PMNs, CD66b is classically used as a PMN marker. Eosinophils can be further excluded by CD16 staining to discriminate neutrophils (CD16+) from eosinophils (CD16-). It should be noted that in mice Ly-6G should be used as a PMN marker, and not Gr-1, which is also detected, for example, on monocytes [5]. Nevertheless, PMNs are not a homogenous population. Naïve circulating PMNs are CD54low CXCR1high whereas naïve PMNs activated in vitro and tissue-resident PMNs are CD54low CXCR1low. Interestingly, a subset of human PMNs able of retrograde migration from tissues to peripheral blood (reverse transmigration) is characterized by the CD54high CXCR1low phenotype [6]. During systemic inflammation or after severe injury, three circulating PMN populations with different morphologies and phenotypes have been described: band cells (CD16dim/CD62Lbright), PMNs with hypersegmented nucleus (CD16bright/CD62Ldim) and PMNs with a conventional segmented nucleus (CD16bright/CD62Lbright), similar to PMNs found under normal conditions [7]. Likewise, a PMN subset defined as CD49d+VEGFR1highCXCR4high has been observed during hypoxia [8]. PMNs from the peripheral blood can also be classified according to their density after isolation by density-gradient centrifugation. High-density neutrophils correspond to conventional PMNs (also named normal-density neutrophils (NDN)), whereas low-density neutrophils (LDN) are detected at higher concentrations in patients with pathologic conditions (essentially autoimmune and autoinflammatory diseases, cancer, infections) or some non-pathologic situations (e.g., pregnancy). LDNs were first described as cells contaminating peripheral blood mononuclear cells (PBMC) prepared by Ficoll-Hypaque density centrifugation from patients with systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA) [9] and were later purified, characterized and defined as low-density granulocytes (LDG) in SLE patients [10]. PMN heterogeneity is also observed in tissues. Several phenotypes and functions of tissue PMNs have been reported, especially in tumors. PMNs able to infiltrate tumor tissue are termed tumor-associated neutrophils (TAN). Their heterogeneity has been observed during tumor progression as their phenotype and functions vary greatly, as reviewed in [11], with the description of anti- and pro-tumor TANs, initially described as N1 and N2 neutrophils [12]. Origin of TAN subsets may result from the activation status of PMNs or their differentiation from progenitors depending on the environment. However, the latter TAN subsets will not be further developed in the present review.

Upon activation, PMNs may produce NETs. Firstly described as a defense mechanism against bacteria [3], NETs can be induced by a variety of pathogens and during sterile inflammation as well, in response to several damage-associated molecular patterns (DAMP). According to the stimulus triggering NET formation, NET composition differs and NET formation is associated or not with PMN death. Likewise, NET formation has been associated with a range of activities, from antibacterial to immunomodulatory or even pro-inflammatory activities. Therefore, by exposing pro-inflammatory mediators, NETs may behave as a DAMP or be a source of DAMPs. Moreover, NETs may be a source of autoantigens by exposing or releasing in the extracellular space self-proteins (either in excess, or normally not exposed, or modified (e.g., citrullinated or after proteolytic cleavage)).

As a consequence of the different properties mentioned above, PMNs participate not only in the host defense against pathogens, but also in sterile inflammation, tumor progression or control, wound healing and tissue repair. Regarding sterile inflammation, PMNs have been suggested to play a key role in several pro-inflammatory autoimmune diseases, particularly in RA and SLE.

SLE is a systemic chronic inflammatory disease affecting 0.05% of the population, especially women (9♀/1♂) between 15 to 50 years old. It is characterized mainly by skin,
kidney, joint and central nervous system manifestations. SLE is triggered by a combination of genetic and environmental factors. It is defined as an autoimmune disease and is associated with the production of more than 100 different autoantibodies [13]. Among the targets recognized, nucleosome (the basic DNA packing unit in chromatin) is a major lupus autoantigen. It is found at higher concentrations in the circulations of SLE patients [14–16] and both autoreactive nucleosome-specific B [17] and T-helper [18] cells have been reported. Actually, SLE patients develop autoantibodies recognizing the whole nucleosomal complex (nucleosome-restricted antibodies) or its constituents, namely either histones or DNA. Of note, anti-double-stranded (ds) DNA autoantibodies represent a lupus marker.

RA is also a chronic, autoimmune, inflammatory disease. It affects 0.5% of the population with a 3♀/1♂ ratio. RA leads to joint destruction and is associated with systemic manifestations. Particularly, RA is a major risk factor for cardiovascular diseases. As many autoimmune diseases, RA is triggered by a combination of genetic (essentially genes coding for HLA-DR molecules) and environmental (e.g., smoking) factors. RA is associated with the production of the disease-specific anti-citrullinated protein antibodies (ACPA) [19,20], which are present in about 70% of patients and are useful for both RA diagnosis and eventually prognosis [21]. ACPA-positive RA patients develop a more severe and erosive disease with extra-articular manifestations. ACPAs recognize different target proteins and some ACPAs are pathogenic (e.g., by inducing osteoclastogenesis [22]).

The aim of the present review is to summarize evidence linking human PMNs to the pathogenesis of SLE and RA, with a special focus on NETs. We will describe how human PMNs are activated, the consequences on immune cell activation, their potential involvement in inducing autoantibody production and the downstream pathogenic mechanisms. To better understand the potential pathogenicity of PMNs, we will first briefly introduce the complexity of human PMNs and NET formation.

2. Interaction of PMNs with Other Immune Cells and Non-Classical Functions of PMNs

Insight into PMNs has evolved during recent years and non-classical functions of PMNs have been identified. First of all, PMN plasticity has been demonstrated. Indeed, PMNs may acquire phenotypic and functional properties of antigen-presenting cells, like cell surface expression of MHC II and costimulatory molecules as well as antigen presentation [23,24]. The latter results suggest that PMNs are also directly involved in adaptive immune responses. Even cross-presentation was reported [25,26]. PMN plasticity in terms of phenotype and function may depend on the microenvironment.

PMNs or PMN subsets with unexpected activities have been reported. Steady-state PMNs from healthy individuals express a functional cell surface Toll-like receptor (TLR) 9 [27] and part of them are able to secrete interferon (IFN)-α upon activation [28]. Even a sub-population expressing a functional T-cell receptor leading to IL-8 secretion upon specific engagement has been reported [29].

PMNs communicate with diverse immune cells and link innate and adaptive immune systems. Thus, PMNs interact and activate dendritic cells (DC) [30]. Likewise, PMNs and the pro-inflammatory Th17 lymphocytes chemoattract each other [31]. In addition, PMNs help B lymphocytes to produce immunoglobulins (Ig) [32]. PMNs also interact and/or affect macrophages/monocytes, for example, by promoting the secretion of mature IL-1β by lipopolysaccharide (LPS)-primed macrophages [33] or by favoring adhesion of classical monocytes through the release of cathelicidins [34]. Particularly, PMNs display immunomodulatory activities. We have reported that PMNs cooperate with regulatory T lymphocytes (Treg) to sustain their activity [35]. Likewise, PMNs may produce the regulatory cytokine IL-10 under particular conditions [36]. Activated PMNs also secrete soluble CD66b (soluble CEACAM8) [37], which has a dual activity as it is known to costimulate B lymphocytes or to inhibit TLR2 response. Interestingly, even immunosuppressive functions have been reported for PMNs. In healthy individuals, CD11c<sup>bright</sup>/CD62L<sup>dim</sup>/CD11b<sup>bright</sup>/CD16<sup>bright</sup> PMNs inhibiting T-cell proliferation have been observed following endotoxin challenge [7]. These PMNs exhibit a decreased adhe-
sion to activated endothelium [38]. Likewise, immunosuppressive CD10+ PMNs inhibiting IFN-γ production by T lymphocytes have been discovered in healthy individuals treated with granulocyte colony-stimulating factor for stem cell mobilization [39]. This subset belongs to LDNs. Actually, immunosuppressive NDNs have also been reported, but only in pathological situations such as cancer patients and HIV-1 infection. PMNs have also been shown to decrease IFN-γ secretion by invariant natural killer T cells, a mechanism requiring cell–cell contact and live PMNs [40]. Likewise, PMNs impair pro-inflammatory cytokine secretion by PBMCs in response to C. Albicans or a TLR4 agonist (LPS); however, here probably via PMN-derived proteases [41].

Similarly, PMNs may also be immunomodulatory via the production of NETs. We have shown that NETs inhibit the secretion of the pro-inflammatory cytokine IL-6 by LPS-stimulated macrophages [42]. Similarly, NETs inhibit myeloid DC activation in response to LPS [43]. NETs have also been shown to control inflammation by degrading cytokines, in situations where PMN concentrations are huge, as in gout [44]. In this context, NETs aggregate and act via serine proteases. One may thus ask whether the mechanism described by Gresnigt et al. does not partly rely on NETs or NET-derived proteases. On the contrary, NETs can prime T lymphocytes [45], suggesting that an excess of NETs might become pathogenic. Similarly, NETs induce polyclonal activation of memory B lymphocytes from healthy individuals [46]. One can hypothesize that PMNs might also communicate with other cell types through NET formation.

Nevertheless, excessive PMN activation and NET release can be deleterious, and are for example associated with SARS-CoV-2 pathogenesis as recently reviewed [47].

3. Neutrophil Extracellular Trap Formation, Function and Clearance

NETs were first described in 2004 [3]. In response to phorbol myristate acetate (PMA, a protein kinase C agonist), IL-8 or LPS, PMNs were originally shown to release in the extracellular environment structures composed of nuclear DNA filaments decorated with histones (namely chromatin filaments) and associated with granule proteins, but devoid of membrane. Importantly, these fibers are capable of trapping and killing gram-positive and gram-negative bacteria, highlighting a new innate immune mechanism to neutralize and remove pathogenic bacteria by neutrophils. Since this original description, the definition of NETs has evolved, both in terms of composition and function [48]. Thus, in addition to bacteria, NETs are involved in host defense against parasitic [49] and fungal [50,51] infections. NETs also display antiviral properties [52]. It has been proposed that NETs are one of the means used by PMNs to degrade microbes that have a too large diameter to be phagocytosed [53].

NETs bind to pathogens by electrostatic interactions between histones or DNA constituents of NETs and the membrane of microbes, thus preventing their dissemination. NETs inactivate virulent factors of the captured pathogens and eliminate them. To do this, numerous proteins have been shown associated with NETs. Twenty-four proteins have been identified by Urban et al. in PMA-induced NETs, including some cytoplasmic proteins [54]. Since then, proteomic analyses revealed that the composition of NETs prepared from PMNs purified from healthy individuals is more complex and may vary according to the stimulus [55,56]. Interestingly, the antimicrobial effect mediated by NETs has been shown to pass especially through histones [3], LL-37 [57] and calprotectin (S100A8/A9) [54]. Some of these proteins kill microbes by forming membrane pores. Likewise, an antimicrobial activity has been reported for DNA [58]. Conversely, as a defense mechanism, bacteria have developed evading strategies against NETs, for example, via the secretion of nucleases degrading NETs [59] or virulence factors inhibiting the activity of antimicrobial peptides [57]. Although, reciprocally, LL-37 confers resistance of NETs against bacterial nucleases [60]. However, NETs also act as a physical barrier to limit bacterial biofilm dissemination [61].

The original description by Brinkmann et al. was associated with the death of PMNs and was named NETosis [3], a process different from apoptosis or necrosis [62]. NETosis,
sometimes named suicidal NETosis, occurs in 2–4 h and is the canonical mechanism. Since then, different mechanisms and pathways of NET formation have been described [63,64], leading to PMN death or not, depending on the stimulus. In the latter case, this process is named vital (or live) NETosis. Therefore, it is recommended to use “NETosis” only when NET extrusion is accompanied with PMN death, or at least “suicidal” or “vital” should be mentioned. Otherwise, the correct term is “NET formation” [48]. Accordingly, NET formation is triggered by several stimuli: parasites [65], bacteria like Staphylococcus aureus and their products such as LPS [62], Candida albicans [66,67] or HIV-1 [52] but also by immobilized immune complexes (IC) [68], activated platelets [69] or cytokines like IL-8 [3].

Vital NETosis is accomplished by rapidly (less than one hour) ejecting mitochondrial DNA, instead of nuclear DNA, bound to granule proteins [70]. Mitochondrial NETs are generally produced after priming PMNs with granulocyte-macrophage colony-stimulating factor, followed by stimulation with LPS or complement factor 5a (C5a) and preserve membrane integrity. These NETs were subsequently shown to kill bacteria [71,72].

Another non-suicidal pathway of NET formation has been described, leading to NETs made of nuclear DNA. Activation of PMNs by Staphylococcus aureus in vivo and in vitro results in nuclear condensation which is followed by the separation of inner and outer membranes of the nucleus. Subsequently, transport vesicles containing nuclear DNA are formed and burgeon through the plasma membrane into the extracellular space, without breach of the plasma membrane in rapid kinetics (5–60 min). Once in the external environment the vesicles rupture and NETs are released [73,74]. This mechanism is also bactericidal, although those NETs display a limited proteolytic activity.

Recently, NET formation was compared in response to several stimuli [64]. All the five stimuli tested induced NETs composed essentially of chromosomal DNA, displaying bactericidal activity and leading to PMN death (NETosis). Nevertheless, different pathways were triggered during NET formation. However, stimulation by Staphylococcus aureus or granulocyte-macrophage colony-stimulating factor followed by stimulation with LPS or C5a was not tested in that study. Therefore, one cannot firmly conclude that all stimuli inducing NET formation lead to NETs made of nuclear DNA and in a suicidal way.

Thus, several mechanisms of NET formation exist, triggered by different pathways and leading or not to PMN death. Depending on the stimulus, formed NETs differ in their composition (nuclear versus mitochondrial DNA, containing histones or not, eventually enriched in some post-translational modifications, . . .). A minimal common definition for NETs may be complexes made of DNA and proteins from granules (e.g., neutrophil elastase, MPO), eventually with other associated proteins.

Once released, NETs are cleared by different mechanisms. NETs are normally degraded by deoxyribonuclease 1 (DNase1) present in sera from healthy individuals [75] and can be degraded in vitro by DNase1-like 3 secreted by myeloid DCs [76]. Moreover, NETs are engulfed by macrophages, a process facilitated when NETs are opsonized by C1q [77] or LL-37 [76], and are then degraded intracellularly.

4. Involvement of PMNs in RA and SLE

Although PMNs are beneficial in a physiological situation, they may behave differently in a pathological context through increased release of ROSs and increased secretion of pro-inflammatory cytokines. For instance, activated PMNs release granules containing proteases able to process pro-inflammatory cytokines into mature active forms [78]. Particularly, NETs are protective in response to several types of infections, but NET formation is also triggered during sterile inflammation and may cause tissue damages or immune cell activation. Therefore, uncontrolled activation of PMNs or deficient resolution of inflammation, e.g., due to prolonged PMN survival, may be deleterious. Similarly, an excess of NET formation or impairment of the NET clearance mechanisms described above may lead to enhanced release of DAMPs and autoantigens, potentially modified by post-translational modifications or PMN proteases, generating neo-epitopes.
Former studies have shown that PMNs are activated in SLE [79] and RA [80,81]. As mentioned above, PMN activation may lead to NET formation. Moreover, PMNs are present in affected tissues both in SLE [82] and RA [83,84] patients. Because anti-dsDNA autoantibodies are a SLE marker and because circulating chromatin is detected at higher concentrations in SLE patients [15] and correlates with disease activity [16], NETs have been considered as a putative lupus autoantigen. Similarly, because ACPAs are a RA marker and because NET formation is associated with protein citrullination in some cases, it has been hypothesized that NETs might induce or be recognized by ACPAs. In both cases, NETs may form ICs with those autoantibodies and trigger downstream pathogenic mechanisms. More recently, anti-NET antibodies have been reported in RA and SLE [85].

For all these reasons, PMNs and NETs have been intensively studied in the last years in both diseases. We will here discuss the potential pathogenic roles of PMNs and particularly of NETs in both RA and SLE. Key observations supporting these hypotheses are presented in Table 1 (PMNs) and Table 2 (NETs). Potential pathogenic mechanisms involving PMNs and NETs are depicted in Figure 1A (SLE) and Figure 1B (RA).

4.1. Involvement in SLE Pathogenesis

Some alterations were reported for PMNs in SLE. PMNs express CD11b (a subunit of Mac-1) and single-nucleotide polymorphisms in the ITGAM locus (coding for CD11b) are associated with SLE. PMNs from individuals with nonsynonymous variant alleles of ITGAM show a significant impaired Mac-1-mediated and IFN-γ-mediated phagocytosis as well as impaired adhesion [86]. Similarly, activated PMNs from SLE patients shed FcγRIIA, potentially leading to an impaired clearance of ICs [87]. Moreover, an increase in PD-L1-expressing PMNs has been reported in SLE, especially in patients with high disease activity [88]. Likewise, the frequency of IL-17+ PMNs is increased in SLE patients in comparison to healthy individuals [89]. In addition, lupus PMNs extrude oxidized mitochondrial DNA-protein complexes and these complexes are interferogenic, as they stimulate plasmacytoid DC (pDC) to secrete IFN-α [90], a key lupus cytokine. Indeed, IFN-α promotes e.g., the differentiation of SLE monocytes into DCs. A subset of LDNs, named LDGs, has been observed in SLE [10]. In contrast to immunosuppressive LDNs described earlier, LDGs are pro-inflammatory cells. They display an activated phenotype and secrete higher levels of pro-inflammatory cytokines, have a normal bactericidal activity and capacity to synthesize H2O2, but show an impaired phagocytic activity. Importantly, LDGs express increased levels of IFN-α mRNA. Particularly, LDGs are non-suppressive in SLE and activate T lymphocytes to produce pro-inflammatory cytokines [91].

In SLE, extracellular chromatin (especially nucleosomes) is found in the circulation and deposits in kidneys [92]. We have shown that nucleosomes are not only a major lupus autoantigen but also behave like a DAMP. Particularly, extracellular nucleosomes trigger PMN activation, leading to the secretion of the pro-inflammatory cytokine IL-8 [93]. The latter is also a chemokine and is particularly efficient in recruiting PMNs, leading to an amplification loop. Interestingly, recognition of nucleosomes by PMNs occurs independently of TLR9 [94], a classical DNA receptor. Importantly, in response to nucleosomes PMNs secrete IFN-α [28], a cytokine involved in SLE pathogenesis, and produce NETs [28], fueling the microenvironment in autoantigens and pro-inflammatory mediators. PMNs primed to produce IFN-α were actually observed in the bone marrow of SLE patients [95]. These PMNs also produce B-cell factors like BAFF and APRIL, whereas alterations in B-cell development were observed in the bone marrow. Cell-free chromatin activates also natural killer cells, at least partly by up-regulating plasma membrane MICA expression on monocytes or PMNs [96]. In addition to free nucleosomes, lupus ICs containing DNA and anti-DNA antibodies also stimulate PMNs to secrete IL-8 [97].
concentrations in SLE patients [15] and correlates with disease activity [16], NETs have
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formation is also triggered during sterile inflammation and may cause tissue damages or

immune cell activation. Therefore, uncontrolled activation of PMNs or deficient resolu-

Figure 1. Potential pathogenic mechanisms involving PMNs and NETs in SLE (A) and RA (B). PMNs are activated either spontaneously or in response to disease-associated stimuli, potentially leading to NET formation. The consequences of PMN activation (e.g., antigen presentation, cytokine secretion) and NET formation (e.g., release of immunomodulatory molecules and autoantigens, potentially modified, such as by released PADs) on different cell types and activation of the complement system are depicted. NETs (modified or not) may act directly or via NET-containing immune complexes. Amplification loops are shown. The figure focuses on PMNs and NETs; other pathogenic mechanisms are involved. The downstream consequences of cytokines, immune complexes or activated target cells are not presented. ACPA, anti-citrullinated protein antibody; Ag, antigen; autoAb, autoantibody; autoAg, autoantigen; B, B lymphocyte; carb, carbamylated; cit, citrullinated; ds, double-stranded; EC, endothelial cell; FLS, fibroblast-like synoviocyte; IC, immune complex; IFN, interferon; IgG, immunoglobulin G; IL, interleukin; LDG, low-density granulocyte; mDC, myeloid dendritic cell; mitoDNA, mitochondrial DNA; MΦ, macrophage; OC, osteoclast; PAD, peptidyl-arginine deiminase; pDC, plasmacytoid dendritic cell; RNP, ribonucleoprotein; Th, T-helper lymphocyte; TNF, tumor necrosis factor.
Many studies focused on the role of NETs in SLE. LDGs have a higher capacity to form NETs and NETs are observed in skin and kidney lesions of patients [82]. Importantly, anti-dsDNA autoantibodies purified from SLE patients trigger NET formation in PMNs from healthy individuals [98]. Moreover, several NET clearance mechanisms are impaired in SLE. Serum DNase1 activity is decreased in SLE patients [99] and loss-of-function variants of DNase1-like 3 have been reported [100]. Likewise, in a subset of SLE patients, NET degradation by serum DNase1 is impaired, due to either the presence of DNase1 inhibitors or anti-DNase1 antibodies, which is associated with kidney manifestations [75]. Moreover, serum C1q concentrations are decreased in SLE patients [101] and therefore the NET clearance mechanism described for macrophages [77] is probably impaired. This may result in activation of the complement cascade upon deposition of residual C1q on NETs [102].

Several studies analyzed the capacity of NETs to activate immune cells, supporting their role as a source of DAMPs, inducing important SLE cytokines. Lupus LDGs spontaneously produce NETs which stimulate IL-1β and IL-18 secretion by LPS-primed macrophages from healthy individuals [103]. Once secreted, IL-18 induces NET formation. Both secretion of mature (cleaved) IL-1β and activation of caspase-1 were noted, indicating involvement of inflammasome. Similarly, NETs from healthy donors and SLE patients increase calcium flux in macrophages from healthy donors and SLE patients [104]. Moreover, NETs from lupus LDGs are enriched in MMPs and damage endothelial cells in vitro [105]. NETting PMNs are able to stimulate IFN-α secretion by pDCs [106], whereas PMNs stimulated by ribonucleoprotein-containing ICs release oxidized mitochondrial DNA inducing IFN-α secretion in PBMCs [107]. Similarly, PMNs stimulated with anti-ribonucleoprotein autoantibodies produce NETs able to stimulate IFN-α secretion by pDCs [108]. Those NETs contain LL-37, a DNA-binding protein facilitating uptake by pDCs and recognition by TLR9. The NET activity in SLE may depend on a specific NET composition. A particular protein composition of NETs has been associated with lupus nephritis [109], whereas in patients with active SLE, NETs are enriched in IL-17A [110]. Likewise, IL-33 is complexed with NETs and amounts of such complexes are correlated with disease activity. These NETs induce IFN-α production by pDCs in an IL-33 receptor-dependent manner [111].

In agreement with the hypothesis that NETs may be a source of autoantigens, patient antibodies binding NETs have been reported [75,85,102]. Those antibodies recognize the NET structure, without additional information on their fine specificity, and can, therefore, be named anti-NET autoantibodies. Compared to NETs from healthy individuals, SLE NETs contain increased amounts of acetylated and methylated histones [112] and some of these post-translational modifications are targeted by lupus IgG autoantibodies (e.g., acetylation of histone H4 at lysine 16) [113]. Recently, human monoclonal anti-DNA antibodies generated from SLE B cells have been demonstrated to recognize NETs [114]. Moreover, autoantibodies against NET proteins are present in SLE patients, as anti-neutrophil elastase, anti-LL-37, anti-MPO, anti-cathepsin G or anti-lactoferrin antibodies (as reviewed in [115,116]) and, inversely, some antibody reactivities associated with SLE target NET proteins or NET-binding proteins (e.g., anti-C1q antibodies). These data are reminiscent of anti-neutrophil cytoplasmic antibodies (ANCA) associated with the pathogenesis of autoimmune small vessel vasculitis (SVV). Most SVV patients have detectable ANCAs specific for proteinase 3 or MPO. Interestingly, NETs have been shown to be a source of proteinase 3 and MPO, whereas ANCAs induce NET formation in normal PMNs [117]. Former studies have shown that mice immunized with human PMNs develop anti-MPO and anti-lactoferrin antibodies [118]. Although the role of ANCAs is less evident in SLE, lupus patients have circulating ANCAs, including anti-LL-37 antibodies [106]. Interestingly, memory B lymphocytes purified from SLE patients produce ANCAs, including anti-LL37 antibodies, when stimulated with NETs [46].

As a consequence of all those NET-recognizing autoantibodies, NETs may also be pathogenic in ICs. For instance, induction of IFN-α secretion by pDCs was observed with NETs in the presence of anti-LL-37, anti-DNA or anti-human neutrophil peptide antibodies
and was stronger than with NETs alone [106]. Interestingly, IFN-α secretion was abolished in the presence of a TLR9 antagonist, suggesting the recognition of the DNA moiety of NETs. Therefore, in addition to classical downstream pathogenic mechanisms induced by ICs in SLE, NET-containing ICs trigger mechanisms mediated by their particular composition.

4.2. Involvement in RA Pathogenesis

In RA, PMNs are present in the synovial fluid of patients [83]. In addition, PMNs are found in large numbers at the pannus-cartilage interface in early RA [84] and heavily infiltrate the synovial tissue in the first weeks of RA onset [119].

As described in SLE, RA PMNs also display particular characteristics and alterations. Thus, RA synovial fluid PMNs express class II MHC molecules and induce T-cell proliferation in a class II MHC-dependent manner [120]. Moreover, synovial fluid PMNs trans-differentiate into DC-like cells [121], evidencing plasticity. Actually, RA synovial fluid is anti-apoptotic on PMNs cultured under hypoxia [122], which mimics conditions existing in vivo within joints. RA synovial fluid PMNs express a high amount of BAFF at the mRNA level [123], suggesting a role of PMNs in supporting B-cell activation. In fact, RA synovial fluid PMNs express a lower amount of plasma membrane BAFF than blood PMNs, whereas TNF induces BAFF release, suggesting that joint infiltrating PMNs shed BAFF upon local stimulation by synovial fluid TNF [124]. PMNs also produce TNF, a key cytokine in RA, as well as IL-8 and IL-6, cytokines also associated with RA and particularly RA synovial fluid PMNs spontaneously express those three cytokines at the mRNA level [123]. RA synovial fluid PMNs display a gene signature of oxidative stress [125]. Interestingly, RA synovial fluid contains high amounts of mitochondrial DNA and the latter induces RANKL expression in normal PMNs. Regarding circulating cells, RA blood PMNs express RANKL [126], a key molecule in osteoclast differentiation, linking PMNs to bone erosions in patients. Membrane TNF expression is increased on RA PMNs before TNF inhibitor therapy in comparison to PMNs from healthy individuals, but returns to baseline expression after successful therapy with TNF inhibitor [80]. In addition, apoptosis is delayed in blood PMNs from RA patients before TNF inhibitor therapy [80]. We have recently reported that PMNs cooperate with regulatory T lymphocytes to sustain the activity of the latter in healthy individuals, whereas this mechanism is deficient in RA patients [35]. By comparing paired blood and synovial fluid PMNs from RA patients with active disease, synovial fluid PMNs had increased expression of chemokines [127]. Particularly, hypoxia-inducible factor-1α signaling is up-regulated in synovial fluid PMNs. Importantly, a combination of PMN biomarkers may predict response to TNF inhibitor therapy in RA [128]. Finally, LDGs have also been observed in RA patients. By comparing RA PMNs and RA LDGs, both populations differ by their transcriptome and RA LDGs may represent an immature population with lower constitutive rates of apoptosis [129]. Although the pro-inflammatory activity of LDGs might be less evident in RA than in SLE, only RA LDGs were able to secrete IL-1β after stimulation [129].

**Table 1. Involvement of PMNs in SLE and RA.**

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<td>PMNs express/produce key disease cytokines</td>
<td>IFN-α [28]</td>
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¹ IFN, interferon; LDGs, low-density granulocytes; PMNs, polymorphonuclear neutrophils; TNF, tumor necrosis factor.

Numerous studies suggest that NETs are involved in RA pathogenesis. RA PMNs have a higher capacity to produce NETs in vitro, either spontaneously [130,131] or after stimulation [42,130]. Particularly, pro-inflammatory cytokines induce NET formation,
especially TNF and IL-17A [130]. Moreover, NETs are present not only in the synovial fluid of RA patients [132], but also in rheumatoid nodules [130]. NET formation is associated with the release of active peptidyl-arginine deiminase (PAD) 2 and 4, enzymes involved in protein citrullination, either as free extracellular PAD2/PAD4 or bound to NETs [132]. Importantly, active PADs are present in RA synovial fluids [132]. The latter results link NET formation to local citrullination in vivo and the potential induction of ACPA production in RA patients. Interestingly, we and others have shown that ACPA-rich IgGs purified from RA patients bind to NETs [42,130] or even induce NET formation [130]. NET binding and NET induction were confirmed with purified ACPAs [133]. Similarly, sera from RA patients recognize activated PMNs and NETs (and especially citrullinated histone H4) [134] and monoclonal antibodies generated from RA synovial B lymphocytes have a strong reactivity against citrullinated histones and PMA-induced NETs [135]. It should be noted that citrullination can occur during NET formation (at different levels and in response to some stimuli), and thus NETs may be a source of RA autoantigens and be either the targets of ACPAs or even the true autoantigen triggering ACPA production. Likewise, RA patients develop also antibodies against carbamylated proteins (anti-CarP) which are predictive for a more severe disease [136]. Yet, NETs externalize carbamylated proteins and carbamylated NETs from RA patients are particularly efficient in activating fibroblast-like synoviocytes (FLS) and macrophages [137]. Especially, NETs up-regulate RANKL in RA FLSs, linking NETs to osteoclastogenesis. In addition, some RA patients were shown to develop ANCAs [138]. ANCAs may recognize different NET proteins and NETs may become pathogenic in the form of ICs. For instance, anti-lactoferrin antibodies are found in the serum of RA patients and lactoferrin-containing ICs stimulate TNF secretion by macrophages [139]. Moreover, classical effector functions of ICs might be triggered, as mentioned for SLE patients. In turn, PMNs are activated by ICs [140], especially by ICs from the synovial fluid of RA patients [141], leading to an amplification loop.

Potential roles of NETs in RA physiopathology have been investigated. NETs activate RA FLSs, which are critical cells involved in joint damage, leading to pro-inflammatory cytokine secretion by FLSs [130]. NETs are also internalized by FLSs, triggering class II MHC molecules up-regulation, and this is associated with presentation of citrullinated antigens to T-cells; RA FLSs loaded with NETs activate citrullinated-vimentin-specific CD4+ T lymphocytes from RA patients [133]. Importantly, NETs are directly involved in articular cartilage damage by degrading aggrecan in a neutrophil elastase-dependent manner [142]. We have shown that NETs are pro-inflammatory on resting macrophages and PMNs. NETs activate both cell types to secrete pro-inflammatory cytokines, but not IL-10. Moreover, RA NETs were more active than NETs from healthy individuals in terms of their capacity to induce IL-8 secretion, while inducing minimal IL-10 secretion [42]. Similarly, activation of myeloid DCs from healthy individuals by RA NETs is stronger than with NETs from healthy individuals [131].

Interestingly, although the source of chromatin is unknown, extracellular cell-free chromatin is also observed in the synovial fluid in RA [83] and deposits on the cartilage surface, and in addition chromatin-containing ICs deposit in the joint tissue of RA patients [143]. Thus, some of the DAMP activities of chromatin on PMNs described above in SLE may also occur in RA, like PMN activation [93], as observed with RA PMNs [28], leading to the secretion of the key RA cytokine TNF in addition to IL-8 and IL-6 [94], as well as induction of NET formation [28]. Moreover, we have reported that chromatin-stimulated PMNs secrete a soluble form of CEACAM8 (also named CD66b), a granulocyte-specific protein, and that concentrations of soluble CEACAM8 are elevated in the synovial fluid of RA patients [37]. Interestingly, RA LDGs express higher levels of CEACAM8 mRNA than RA blood PMNs [129]. As in SLE, the origin of extracellular chromatin in RA is unknown and part of it may come from NETs. Therefore, cell-free chromatin may behave as a DAMP in a disease non-specific manner. Reciprocally, if part of this chromatin has a different composition in RA in comparison to SLE, or if the response of RA PMNs to chromatin differs from SLE PMNs, chromatin may act in a more SLE-specific way.
Table 2. Involvement of NETs in SLE and RA.

<table>
<thead>
<tr>
<th></th>
<th>SLE</th>
<th>RA</th>
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<tbody>
<tr>
<td>Higher NET formation</td>
<td>[82]</td>
<td>[42,130,131]</td>
</tr>
<tr>
<td>Impaired NET clearance</td>
<td>[75,102]</td>
<td>[144]</td>
</tr>
<tr>
<td>Anti-NET antibodies</td>
<td>[75,85,102]</td>
<td>[85]</td>
</tr>
<tr>
<td>NETs detected in affected tissues</td>
<td>Skin, kidney [82]</td>
<td>Rheumatoid nodules [130]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Synovial fluid [132]</td>
</tr>
<tr>
<td>Disease-specific antibodies bind NETs</td>
<td>Anti-dsDNA [114]</td>
<td>ACPA-rich IgGs [42,130]</td>
</tr>
<tr>
<td>Disease-specific antibodies induce NETs</td>
<td>Anti-dsDNA [98]</td>
<td>Purified ACPAs [133]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACPA-rich IgGs [130]</td>
</tr>
<tr>
<td>NETs directly activate immune cells or key cells involved in disease pathogenicity</td>
<td>Immune cells [103,104,106–108]</td>
<td>Immune cells [42,131,137]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Endothelial cells [105]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FLSs [130,137]</td>
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<tr>
<td></td>
<td></td>
<td>[106]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>[139]</td>
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</tbody>
</table>

1 ACPAs, anti-citrullinated protein antibodies; ds, double-stranded; FLSs, fibroblast-like synoviocytes; NETs, neutrophil extracellular traps.

Defects in NET/chromatin clearance mechanisms might participate in RA pathogenesis, although this area of investigation is less developed than in SLE. Actually, sera from RA patients display a lower capacity to degrade NETs in vitro than sera from healthy individuals [144]. Likewise, the capacity of RA sera to degrade DNA in vitro is impaired in comparison to healthy individuals, especially in RA patients with high disease activity [145]. According to the literature, serum DNase1 may at least partly contribute to NET degradation. Although DNase1-like 3 was shown to be unaffected in RA patients [146], the missense variant rs35677470 at the DNase1-like 3 locus is potentially associated with the development of RA [147]. Other clearance mechanisms might be involved and defective in RA.

According to the above data, accumulation of NETs and/or cell-free chromatin may be deleterious in RA as a source of DAMPs and autoantigens. As mentioned, NET formation is increased in RA PMNs. More recently, several studies have reported that cell-free chromatin or NET-derived products are found at higher concentrations in the circulation of RA patients. Indeed, ELISAs have been developed to measure extracellular MPO-DNA complexes or neutrophil elastase-DNA complexes as well as cell-free nucleosomes. Although they might be indicative in some approaches, those ELISAs are only surrogate markers of NET formation.

5. Conclusions

Many data support a key role for PMNs in RA and SLE pathogenesis. Many of them suggest that PMNs are involved through the formation of NETs while others highlight PMN dysfunctions. Several pathogenic mechanisms are potentially common to RA and SLE, but they are probably finely regulated in response to stimuli which are more disease specific. The next step will be to determine the nature of the disease-specific stimuli triggering PMN activation and NET formation in vivo. Another key step will consist in determining the precise PMN response in each disease, the mechanisms and pathways governing NET activities, and to which extent NET composition varies in different pathologies.

Indeed, NET composition likely affects their activities. Using PMNs prepared from healthy individuals and activated in vitro, it has been shown that the composition of NETs depends on the stimulus [55,56]. Most importantly, NET composition also varies depending on the disease [55]. One study reported a NET composition specific to PMNs isolated from SLE patients with nephritis [109]. The origin of NETs and/or chromatin (PMN sub-populations, tissue or circulating PMNs) might also contribute to different activities.

Other cell types (eosinophils [148], basophils [149], mast cells [150], monocytes [151] and lymphocytes [152]) have been reported to release extracellular traps or DNA and, therefore, their contribution to the mechanisms described above should be analyzed in RA and SLE.

In conclusion, PMNs (and NETs) can be both pro- and anti-inflammatory and this probably partly depends on the stimuli triggering PMN activation/NET formation and...
the microenvironment. Uncontrolled PMN activation, excessive NET formation, and/or impaired NET clearance may become pathogenic. As a consequence, NETs may behave both as an adjuvant and as a source of DAMPs and autoantigens, influencing both innate and adaptive immunity. The complex NET composition and the repertoire of antibodies recognizing NETs suggest a broad family of anti-NET antibodies may exist, as described for the anti-nucleosome antibody family in SLE patients.

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References


58. Derré-Bobiliot, A.; Cortes-Perez, N.G.; Yamamoto, Y.; Kharrat, P.; Couvé, E.; Da Cunha, V.; Decker, P.; Boissier, M.C.; Escartin, F.; Cesselin, B.; et al. Nucleic Acid A (Gs6061), an extracellular nuclease of Streptococcus agalactiae, attacks the neutrophil extracellular traps and is needed for full virulence. *Mol. Microbiol.* 2013, 89, 518–531. [CrossRef]


78. Farrera, C.; Fadeel, B. Macrophage clearance of neutrophil extracellular traps is a silent process. *J. Immunol.* 2013, 191, 2647–2656. [CrossRef]


82. Wright, H.L.; Moots, R.J.; Edwards, S.W. The multifactorial role of neutrophils in rheumatoid arthritis. *Nat. Rev. Rheumatol.* 2014, 10, 593–601. [CrossRef]


111. Georgakis, S.; Gkirtzimanaki, K.; Papadaki, G.; Gakiopoulou, H.; Drakos, E.; Eloranta, M.I.; Makridakis, M.; Kontostathi, G.; Zoidakis, J.; Baira, E.; et al. NETs decorated with bioactive IL-33 infiltrate inflamed tissues and induce IFNalpha production in SLE patients. JCI Insight 2021, 6, e147671. [CrossRef]


120. Cross, A.; Bucknall, R.C.; Cassatella, M.A.; Edwards, S.W.; Moots, R.J. Synovial fluid neutrophils transcribe and express class II major histocompatibility complex molecules in rheumatoid arthritis. Arthritis Rheum. 2003, 48, 2796–2806. [CrossRef]


