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An emerging role of neutrophils and NETosis in chronic inflammation and fibrosis in systemic lupus erythematosus (SLE) and ANCA-associated vasculitides (AAV): Implications for the pathogenesis and treatment

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ABSTRACT

Neutrophils derive from hematopoietic stem cells (HSCs) with systemic inflammation driving their activation and differentiation to myeloid progenitors to ensure enhanced myelopoiesis. Epigenetic reprograming and re-education of these HSCs produces neutrophils primed towards elimination of pathogens and increased inflammatory response. Neutrophils -an important component of acute inflammation- are not present in chronic inflammatory tissues leading to the false assumption that they may not be as important for the latter. Activated neutrophils may release Neutrophil Extracellular Traps (NETs) during a distinct form of cell death, named NETosis; NETs are rich in bioactive molecules that promote thrombosis (including atherothrombosis), inflammation and fibrosis. Thus, although neutrophils may not be present in chronic inflammatory lesions, their remnants may amplify the inflammatory response beyond their short life-span in the tissues. Herein, we review current evidence supporting a role of neutrophils and NETosis in tissue injury and dysfunction in systemic autoimmunity using as disease paradigms Systemic Lupus Erythematosus (SLE) and the ANCA-associated vasculitides (AAV). We also discuss the mechanisms involved and their potential as targets for novel therapy and drug repositioning.

1. Introduction

Neutrophils, the most abundant cells of the innate immune system, derive from the differentiation of hematopoietic stem cells (HSCs) which reside in the bone marrow in a quiescent state, being ready to respond to stress, such as severe infection, systemic inflammation, or iatrogenic myeloablation. Systemic inflammation drives HSC activation and differentiation to myeloid progenitors to enhance myelopoiesis [1].

Neutrophil Extracellular Traps (NETs) are extracellular networks of DNA scaffolds decorated with granular components, histones and

Abbreviations: HSCs, Hematopoietic stem cells; NET, Neutrophil Extracellular Trap; MPO, Myeloperoxidase; NADPH, Nicotinamide adenine dinucleotide phosphate; PAD4, Peptidylarginine deiminase IV; DDIT4/REDD1, Regulated in DNA Damage and Development 1; RIPK3/MLKL, Receptor-interacting protein kinase-3/mixed lineage kinase domain-like; SLE, Systemic Lupus Erythematosus; AAV, ANCA-associated vasculitides; ANCA, Anti-neutrophil cytoplasmic antibodies; TLR, Toll-like receptors; IFN, Interferon; LDGs, Low-density granulocytes; HMGB1, High mobility group box protein 1; pDCs, Plasmacytoid dentritic cells; mBCs, Memory B cells; AIM2, Absent in melanoma 2; STING, Stimulator of IFN genes; DAI, DNA-dependent activator of IFN regulatory factors; MDSCs, Myeloid-derived suppressor cells; ROS, Reactive oxygen species; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; TNFa, Tumor necrosis factor alpha; MMP9, Matrix metalloproteinase 9; APS, Antiphospholipid syndrome; TF, Tissue factor; IL-, Interleukin-; HIF-1α, Hypoxia-inducible factor-1α; ET-1, Endothelin-1; PR3, Proteinase 3; MPA, Microscopic polyangiitis.; GPA, Granulomatosis with polyangiitis; EGPA, Eosinophilic granulomatosis with polyangiitis; mDCs, Myeloid DCS; LAMP-2, Lysosomal membrane protein 2; LC3B, Light chain 3B; C5a, Complement 5a; NOX, Nicotinamide adenine dinucleotide phosphate oxidase; CMP, Common myeloid progenitors; GMP, Granulocyte-macrophage progenitors; MMP9, Matrix metalloproteinase 9

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cytoplasmic proteins [2,3]. Under physiological conditions, neutrophils release NETs as a defense mechanism to entrap and kill bacteria [2,4–7], fungi [8,9] and viruses [10]. Sterile stimuli, such as phorbol 12-myristate 13-acetate (PMA), monosodium urate and calcium pyrophosphate dehydrate crystals, also induce NET formation [11–14].

Initially, neutrophils were thought to release NETs during a distinct form of cell death, named NETosis [2,3,15]. Current evidence suggests that neutrophils may remain viable and functional even after NET extrusion, mainly when the scaffold is composed of mitochondrial DNA [16–18]. Several mechanisms are involved in NET formation. Nuclear and granular membranes disintegrate and enzymes, such as neutrophil elastase and myeloperoxidase (MPO), are released [19,20]. Elastase alters the neutrophil's cytoskeleton and enters the nucleus where it synergizes with MPO to decompensate chromatin [19,21]. *Via* nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (NOX) [3,22] or other mechanisms [23–27], the enzyme peptidylarginine deiminase IV (PAD4) converts arginine residues into citrulline residues, leading to histone deamination, loss of positive charge and chromatin decompensation [11,22,28,29]. Autophagy, a process normally involved in degradation and recycling of cellular components, is also involved in NET formation [30]. Inhibition of the mTOR pathway increases autophagosome formation and accelerates NET formation [31], whereas early-stage or late-stage autophagy inhibitors attenuate NET release [12,32,33]. Recently, we described that the hypoxia-response and stress-induced protein Regulated in DNA Damage and Development 1 (DDIT4/REDD1) is a mediator of NET release by lowering the threshold of autophagy activation [34,35]. Necroptosis, a receptor-interacting protein kinase-3/mixed lineage kinase domain-like (RIPK3/MLKL)-dependent lytic death, was also demonstrated to control NET generation [36–38]. Of note, controversial data exist, reporting the formation of NETs in either the absence of PAD4 [39] or in Atg5-knockout [40] or RIPK3-knockout [41] neutrophils.

Deregulated NET formation is implicated in several diseases [42], such as sepsis [33], autoimmune [43–46] and autoinflammatory [34] diseases, vein and arterial thrombosis [47], acute myocardial infarction [48,49], cancer [50,51] and fibrosis [52]. Depending on the pathophysiologic context of each disease, NETs are decorated with distinct bioactive proteins that may account for their differential contribution to disease pathogenesis and phenotype. For instance, the expression of tissue factor (TF), the main *in vivo* initiator of the coagulation, on NETs contributes to thromboinflammation in sepsis [33], vasculitis [53], myocardial infarction [49] and Systemic Lupus Erythematosus [35]; interleukin (IL)-1β-decorated NETs promote inflammation in familiar Mediterranean fever and Still's disease [54]. In rheumatoid arthritis, ANCA-associated vasculitides (AAV) and Systemic Lupus Erythematosus (SLE), NETs are enriched in immunogenic autoantigens and damage associated molecular patterns [55]. Also, NETs promote fibrosis in congestive heart failure [56] and IL-17-bearing NETs promote fibrosis in interstitial lung disease [57]. Together these suggest that NETs are implicated in the pathogenesis of a variety of autoimmune diseases promoting autoreactivity and tissue injury, including their major comorbidities such as accelerated atherosclerosis, thrombosis and increased risk for infection [58].

Herein, we present current evidence supporting a role of neutrophils and NETosis in tissue injury and dysfunction in systemic autoimmunity, using as disease paradigms SLE and the AAV. We also discuss their mechanisms involved and their potential as targets for novel therapy and drug repositioning.

2. Systemic Lupus Erythematosus (SLE) and neutrophil extracellular traps

SLE is the prototypic systemic autoimmune disease characterized by loss of tolerance to self-antigens (mainly located in the nucleus), abnormal T and B cell responses, and autoantibody production. SLE pathogenesis is complex and involves defective clearance of immune complexes and debris containing nucleic acids, excessive innate immune activation involving Toll-like receptors (TLR) and type I interferons (IFN), and aberrant lymphocyte activation. Despite vigorous research, the etiology of SLE still remains elusive [59,60].

Table 1
Neutrophils and Neutrophil Extracellular Traps (NETs) in Systemic Lupus Erythematosus (SLE).

Neutrophils	Ref.
• Neutropenia	[61]
• Decreased granulocyte–macrophage colony-forming units in the bone marrow	[62,63]
• Increased apoptosis, altered phagocytosis and oxidative metabolism	[64–68]
• Granulopoiesis gene signature in peripheral blood and bone marrow mononuclear cells	[69–71]
• Gradual enrichment of neutrophil transcripts in the blood during progression to active lupus nephritis	[72]
• Robust demethylation of interferon genes in Low-density granulocytes	[73]
Neutrophil extracellular traps (NETs)	
Impaired degradation of NETs	
• due to DNase-1 inhibitors in serum	[43,68,74]
• due to anti-NET antibodies that prevent access of DNase-1 to NETs	
• due to impaired DNase-1 function	
• due to C1q on NETs that directly inhibits DNase-1	
• associated with higher type 1 interferon and anti-NET antibodies	
Mechanisms and mediators of NET release	
• type I IFN	[35,5–77]
• anti-ribonucleoprotein antibodies	
• TLR7/8 activation	
• Chromatin	
• HIF-1α -and endothelin-1-mediated REDD1/ autophagy pathway	
Proteins on SLE NETs	
• LL37	[35,55,74,75,85]
• HMGB1	
• C1q	
• Neutrophil elastase	
• Interleukin-17	
• Matrix metalloproteinase 9	
• Tissue factor	
Target cells	
• Plasmacytoid dendritic cells	[35,55,75,80,81,85]
• Memory B cells	
• Macrophages	
• Endothelial cells	
• Skin fibroblasts	

Neutropenia is a common finding in patients with SLE [61]. Bone marrow from patients with SLE has reduced granulocyte–macrophage colony-forming units [62,63] and neutrophils from patients with SLE exhibit increased apoptosis, altered phagocytosis and disordered oxidative metabolism [64–68]. Gene expression studies revealed a granulopoiesis gene signature in the peripheral blood of patients with SLE [69]. By the use of cDNA microarrays, we also demonstrated that bone marrow mononuclear cells from patients with active SLE are characterized by an apoptotic and a granulopoiesis gene signature [70,71]. More recently, Banchereau R. et al. profiled the blood transcriptome of pediatric SLE patients and observed gradual enrichment of neutrophil transcripts during progression to active lupus nephritis [72]. Epigenome profiling revealed that low-density granulocytes (LDGs) -an inflammatory subset of neutrophils- demonstrate robust demethylation of IFN genes [73].*Together these data point towards the involvement of neutrophils and NET release in the pathogenesis of SLE (*Table 1*)*. Upstream mediators of NET formation in SLE, bioactive proteins on SLE NETs and effector functions of SLE NETs are presented in Fig. 1A.

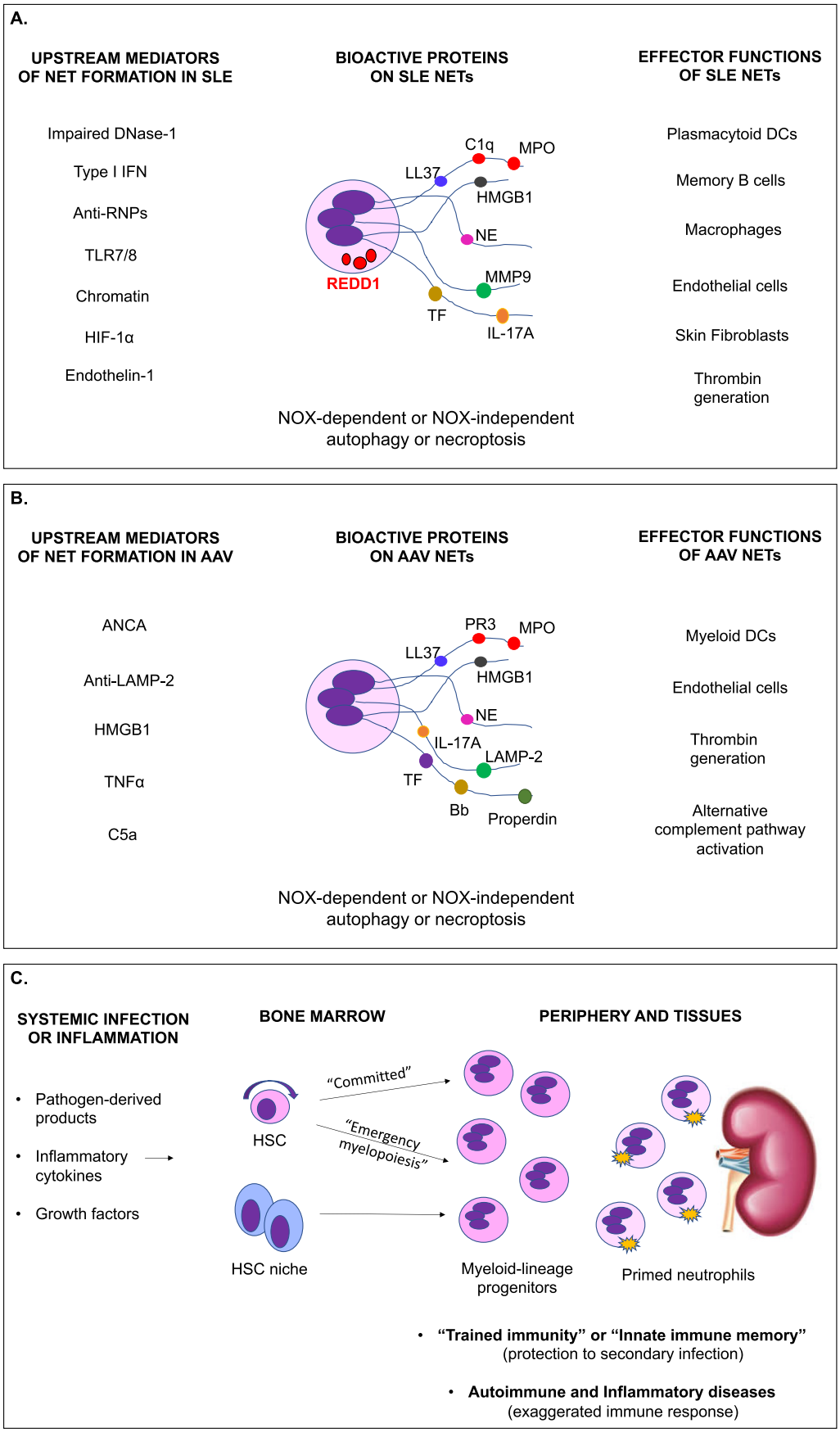

Fig. 1. (A) Upstream mediators of NET formation in SLE, bioactive proteins on SLE NETs and effector functions of SLE NETs. Upstream mediators, such as type I interferon (IFN), anti-ribonucleoprotein antibodies (anti-RNPs), hypoxia-inducible factor-1α (HIF-1α) and endothelin-1 (ET-1), prime neutrophils towards NET release that is mediated through a NADPH-oxidase (NOX)-dependent or NOX-independent autophagy or necroptosis process. NETs in SLE are decorated with bioactive proteins such as LL37, high mobility group box protein 1 (HMGB1), complement 1q (C1q), myeloperoxidase (MPO), neutrophil elastase (NE), matrix metalloproteinase 9 (MMP9), interleukin-17A (IL-17A) and tissue factor (TF). NETs from SLE patients activate plasmacytoid dendritic cells (pDCs), macrophages, memory B cells, endothelial cells and skin fibroblasts. (B) Upstream mediators of NET formation in AAV, bioactive proteins on AAV NETs and effector functions of AAV NETs. Upstream mediators, such as anti-neutrophil cytoplasmic antibodies (ANCA), anti-lysosomal membrane protein 2 antibodies (anti-LAMP-2), high mobility group box protein 1 (HMGB1), complement 5a (C5a) and tumor necrosis factor α (TNFα), prime neutrophils towards NET release that is mediated through a NADPH-oxidase (NOX)-dependent or NOX-independent autophagy or necroptosis process. NETs in AAV are decorated with bioactive proteins such as LL37, HMGB1, MPO, NE, LAMP-2, IL-17A and TF. NETs from AAV patients activate myeloid DCs and endothelial cells, and mediate thrombin generation and alternative complement pathway activation. (C) Hematopoietic stem cells (HSC) and cells from the HSC niche differentiate into myeloid-linage progenitors. In systemic infection or inflammation, pathogen-derived products, inflammatory cytokines and growth factors activate HSC and HSC-supportive cell populations' differentiation towards the myeloid lineage, in a process called "emergency myelopoiesis". Myeloid confer protection to secondary infection, in a phenomenon termed "trained innate immunity" or "innate immune memory" or result in exaggerated immune responses, contributing to autoimmune and inflammatory diseases, such as SLE or AAV. Myeloid progenitors released from the bone marrow may infiltrate tissues in the periphery resulting in the generation *in situ* of primed neutrophils that may lead to exaggerated responses within the affected tissues.

2.1. Impaired degradation of NETs in SLE

Hakkim A. et al. were the first to observe impaired degradation of NETs in serum from patients with SLE. This was caused by the presence of DNase-1 inhibitors and anti-NET antibodies that prevented access of DNase-1 to NETs [43]. Impaired DNase-1 function and impaired NET degradation were related with kidney involvement [43], disease activity and low C3 and C4 in the blood [74]. Leffler J. et al. observed that patients with sera that degraded NETs less efficiently demonstrated higher type I IFN activity and higher levels of autoantibodies against NETs. NETs activated the complement *in vitro* and C1q deposited on NETs directly inhibited DNase-1. Autoantibodies on NETs caused more C1q deposition, leading to a vicious circle of inefficient NET degradation and proinflammatory reactions [74]. It was also demonstrated that the efficiency of NET degradation was impaired in patients with anti-double stranded DNA (anti-dsDNA) antibodies but not anti-extractable nuclear antigen (anti-ENA) antibodies [68]. *Thus impaired degradation of NETs in patients with active SLE leads to increased NET release that is associated with disease activity and kidney involvement.*

2.2. Mechanisms of NET release in SLE

Garcia-Romo G.S. et al. found that type I IFN primed neutrophils *in vivo*; exposure of neutrophils to anti-ribonucleoprotein antibodies resulted to neutrophil death and release of NETs decorated with the antimicrobial proteins LL37 and high mobility group box protein 1 (HMGB1) [75]. TLR7/8 activation led to cleavage of the N-terminal part of FcgRIIA, shifting neutrophils from immune-complex clearance towards NETosis. These neutrophils also mediated the cleavage of FcgRIIA on pDCs and monocytes resulting to generalized inefficient clearance of immune-complexes and increased complement 5a (C5a) generation, suggesting that blocking of TLR7/8 activation could decrease the inflammatory potential of circulating immune-complexes [76]. Chromatin-induced neutrophil activation was associated with upregulation of DNA-sensors, such as AIM2 (absent in melanoma 2), STING (stimulator of IFN genes) and DAI (DNA-dependent activator of IFN regulatory factors), leading to increased NET release [77]. We also demonstrated that the inflammatory microenvironment of lupus in NZB/W-F1 lupus-prone mice resulted to increased elimination of granulocytic myeloid-derived suppressor cells that was attributed to ROS-mediated extracellular trap formation [78].

Autophagy is a key mechanism underlying NET release. To delineate the mechanism regulating NET release in SLE, we analyzed autophagy levels in peripheral blood neutrophils from patients with SLE, and observed increased basal autophagy levels when compared to neutrophils from patients with inactive SLE or healthy individuals. Active SLE neutrophils demonstrated increased NET release that was attenuated by early-stage or late-stage autophagy inhibitors, such as wortmannin or hydroxychloroquine. As evidenced by *in vitro* stimulation of healthy neutrophils with serum from active SLE patients, increased autophagy levels and subsequent NET release were not cell-intrinsic effects but were mediated by the inflammatory microenvironment of SLE [35]. REDD1 regulates NET release through lowering the threshold of autophagy activation [34]; thus we next examined whether REDD1/autophagy pathway is involved in NET release in SLE. We observed that serum from active SLE patients induced REDD1 expression in neutrophils and subsequent autophagy activation and NET release. These were mediated by hypoxia-inducible factor-1α (HIF-1α) [a hypoxia-sensitive transcription factor affecting numerous immune cells and involved in the mammalian target of rapamycin (mTOR) system] and endothelin-1 (a potent vasoconstrictor involved in the mTOR system), as evidenced by their specific inhibition with L-ascorbic acid or bosentan, respectively. Thus, by upregulating the REDD1/autophagy pathway in neutrophils, HIF-1α and endothelin-1 were revealed as upstream regulators of NET release in SLE. *Our findings suggest multiple potential therapeutic targets, such as HIF-1α, endothelin-1 and autophagy inhibitors, to attenuate NET release in SLE* [35].

2.3. Effector functions of NETs in SLE: implications for the endothelial and vascular injury, thromboinflammation and fibrosis

Depending on the pathophysiologic context of each disease, NETs are decorated with *distinct bioactive proteins* that may contribute to the disease pathogenesis and phenotype. LL37 and HMGB1 are antimicrobial proteins that mediate the uptake of mammalian DNA by plasmacytoid dendritic cells (pDCs). In SLE, it was shown that LL37- and HMGB1-decorated NETs were potent pDCs activators and IFN inducers in an FcgRIIa-, NADPH- and TLR7-dependent manner [75]. LL37 was required for pDCs activation and protection of DNA from nuclease degradation. Circulating immune-complexes were composed of neutrophil peptides and LL37 complexed with DNA, suggesting that NETs represent an origin of immune-complexes [79]. *Via* TLR9, NETting neutrophils containing LL37-DNA complexes activated human memory B cells (mBCs) towards the production of NET-specific autoantibodies [80]. In macrophages from SLE patients, LL37 on NETs activated the NLRP3 inflammasome *via* the P2X7 receptor-mediated potassium efflux, resulting to release of active IL-1β and IL-18, leading to further NETosis. *This feedback loop could lead to flare and organ injury* [81].

In addition to the nuclear origin, a mitochondrial origin of DNA on NETs was also demonstrated. Lood C. et al. demonstrated that activation of neutrophils by ribonucleoprotein immune-complexes resulted to translocation of mitochondria to the neutrophil's surface to release ROS-mediated oxidized mitochondrial DNA that increased the inflammatory response in a STING-dependent manner. NETs released from LDGs from SLE patients were enriched in oxidized mitochondrial DNA, leading to increased proinflammatory and IFN responses. Administration of mitochondrial ROS scavengers attenuated lupus-like disease in MRL/*lpr* lupus-prone mice [82]. In renal biopsy specimens from patients with lupus nephritis, mitochondrial DNA was observed on NETs, and mitochondrial-DNA NETs were more efficient than dsDNA-NETs to activate pDCs towards IFNα production [83].

Cardiovascular disease remains one of the leading causes of mortality and morbidity in SLE [84] and data propose the involvement of neutrophils to atherogenesis and plaque rupture during myocardial infarction [49]. LDGs play an important role in endothelial and vascular injury in SLE; Villanueva E. et al. observed that circulating LDGs from SLE patients underwent increased NETosis leading to externalization of dsDNA, LL37, neutrophil elastase and IL-17. NETting LDGs from SLE patients mediated endothelial cell toxicity in a NET-mediated manner and were observed in glomeruli from patients with lupus nephritis and in affected lupus dermis and subcutis [55]. Matrix metalloproteinase 9 (MMP9) on NETs induced endothelial cell apoptosis and impaired aortic endothelium-dependent vasodilation in murine [85]. Similarly, impaired NET-dependent vascular injury and kidney or skin injury were observed in NZM 2328 and MRL/*lpr* murine lupus models [86,87]. *Together these suggest that NETs may be associated with endothelial dysfunction and premature cardiovascular disease in patients with SLE.*

Emerging evidence also implicates NET formation with venous and arterial thrombosis. NETs have been observed in thrombi from patients with myocardial infarction undergoing thrombectomy [48,49], in thrombi from patients with sepsis [88] and thrombotic microangiopathies [89], and in adhering cells of hemodialysis membranes in hemodialysis patients [90]. NETs entrap erythrocytes and platelets, and bind fibrinogen, fibronectin, von Willebrand factor and tissue factor, promoting clot formation and stabilization [91]. In patients with primary antiphospholipid syndrome (APS), neutrophils were predisposed to release NETs spontaneously in a ROS- and TLR4-mediated manner, and circulating NETs correlated with a history of arterial thrombosis [92]. Blood from APS patients was enriched in LDGs and NETs were resistant to degradation [93]. In a mouse model of APS, both neutrophils and NETs were required for immunoglobulins to accelerate the thrombotic phenotype [94]. In mice, thrombi induced by IgG from

patients with APS were enriched for citrullinated histone 3 and treatment with either DNase or a neutrophil depleting antibody reduced thrombosis [95].

Based on these data, we reasoned that the increased thrombogenicity and fibrosis observed in patients with active SLE could be attributed to NET-related proteins. TF expressed on NETs is a mediator of thromboinflammation in several conditions [33]. In addition, IL-17A that is implicated in SLE pathogenesis and severe lupus nephritis [96], promotes NET-dependent lung fibrosis [57]. Thus, we first investigated if TF and IL-17A decorate NETs in human SLE. By immunofluorescence and immunoblotting on neutrophils and NET structures, we found that serum from active SLE patients mediated autophagy induction and subsequent release of NETs bearing bioactive IL-17A and TF, mediating thrombin generation. Next, to study whether SLE NETs mediate tissue injury, we treated human skin fibroblasts with active SLE NETs. We observed that REDD1-mediated NETs bearing bioactive TF and IL-17A could activate and differentiate human skin fibroblasts towards collagen production. TF- and IL-17A-decorated NETs were present in kidney biopsies of patients with proliferative lupus nephritis and skin biopsies from patients with active discoid lupus, suggesting that TF- and IL-17-bearing NETs may promote thromboinflammation and fibrosis in SLE by activating resident cells [35].

2.4. Post-translational modifications in SLE NETs

Several NET components may undergo post-translational modifications. Histone modifications -more specifically acetylated H4-K8, 12, 16, acetylated B2B-K12 and tri-methylated H3−K27− are abundant in NETs from SLE patients, contributing to the immunostimulatory potential of NETs [97]. To investigate whether NETs and their histone post-translational modifications may induce autoantibody production against histones, Liu C.L. et al. developed an *in vitro* method to study histone post-translational modifications of NETs and found that serum from SLE patients with anti-histone antibodies reacted with acetyl-histone H2B proteins. IgM reactivity to multiple H3 and H4 post-translational modification epitopes and widespread IgM reactivity to methyl-H3 post-translational modification epitopes were observed. Reactivity to citrullinated epitopes was observed at low levels for IgM and IgG. Histones within SLE NETs harbored methylation marks such as mono-, di- and tri-methyl H3 at K4, K9, K27, K36 and H4 at K20. Also hypercitrullination and marks associated with transcriptional repression were identified [98].

Microparticles isolated from patients with SLE were positive for annexin V and apoptosis-modified chromatin, and primed neutrophils towards NETosis [99]. In patients with lupus nephritis, cells undergoing apoptosis released microparticles that contain hyperacetylated histones. Microparticles enriched in acetylated chromatin accumulated in the peripheral blood and rapidly released NETs in a ROS-independent manner [100].

Differential ubiquitin concentration in NETs was observed in NETs from healthy individuals, normal-density granulocytes from SLE patients and LDGs from SLE patients. NETs from SLE patients expressed less ubiquitinated proteins than NETs from healthy individuals. NETs from LDGs had the lowest ubiquitin levels, suggesting that ubiquitination is less in cells demonstrating high proinflammatory activity. SLE patients demonstrated increased titers of anti-ubiquitinated-MPO antibodies that correlated with SLEDAI score and decreased complement. Upon stimulation with SLE NETs, macrophages from SLE patients demonstrated increased TNFa and IL-10 production [101].

2.5. Targeting NETs in lupus-prone mice and human lupus

NET release is implicated in the pathogenesis of SLE through altering immune responses and mediating end-organ injury. Thus, modulating the process of NET release could offer therapeutic potential in the disease. Therapeutic targeting of NETs could be done by (a) suppressing NET formation or (b) disrupting the architecture of NETs or (c) targeting bioactive proteins expressed on NETs.

Fuchs T.A. et al. demonstrated that the NADPH oxidase inhibitor diphenylene iodonium (DPI) prevented NET release upon activation of neutrophils with PMA or *S. aureus* [3]. It was also demonstrated that inhibition of NADPH oxidase prevented intracellular chromatin decompensation *in vitro* [30]. Similarly, DPI and the chemical TLR4 inhibitor, TAK-242, abrogated NET formation and ROS production *in vitro* in APS. [92]. Treatment of neutrophils with nocodazole that interferes with tubulin polymerization into microtubules or cytochalasin D, an inhibitor of actin filamentation, reduced the release of chromatin; pretreatment of neutrophils with M1/70, an integrin adhesion receptor antibody, reduced deployment of chromatin into NETs [102].

To test if NET inhibition may ameliorate lupus manifestations, Knight J.S. et al. treated NZM 2328 lupus-prone mice with Cl-amidine, a chemical inhibitor of PAD enzymes. Cl-amidine blocked NET release *in vitro* and *in vivo*, and also altered complement levels and the autoantibody profile of mice. It also decreased MPO, IgG and C3 deposition within the kidneys, improved endothelium-dependent vasodilation and vasculogenesis, and delayed thrombosis development *in vivo* [86]. The same was tested in MRL/*lpr* lupus-prone mice by the use of either Cl-amidine or BB-Cl-amidine that exhibits enhanced cellular potency. Both agents blocked NET formation *in vitro* and *ex vivo* without blocking NOX-dependent ROS generation. *In vivo*, PAD inhibition improved vascular function, decreased IFN signature in the bone marrow and kidneys, improved skin involvement, reduced immune-complex deposition within the kidneys and reduced proteinuria [87].

Disrupting the architecture of NETs could be done by the administration of DNase-1. In NZB/W-F1 lupus-prone mice, the intraperitoneal administration of recombinant murine DNase postponed the development of lupus and extended the period from disease onset to death [103]. In patients with lupus nephritis, the administration of recombinant human DNase-1 was well tolerated without significant adverse effects, however no improvement in serum markers of the disease activity was observed [104].

Recently, we identified upstream regulators mediating NET release and downstream molecules expressed on NETs in SLE, linking immunometabolism, thromboinflammation and fibrosis towards end-organ injury. To this end, we proposed a multi-step model mediating end-organ injury in SLE that could be targeted at multiple stages through repositioning of existing drugs. More specifically, we identified endothelin-1 and HIF-1α in the inflammatory microenvironment of SLE as upstream mediators of REDD1/autophagy-mediated NET release in SLE. In murine lupus nephritis, the administration of the specific endothelin-A receptor antagonist, FR139317, ameliorated lupus nephritis [105]. In human SLE bosentan has been administered in SLE patients exhibiting pulmonary arterial hypertension [106,107]. Treatment of neutrophils with L-ascorbic acid -a HIF-1α inhibitor- or bosentan -an endothelin-1 receptor antagonist- ameliorated NET release, indicating that endothelin-1 and HIF-1α inhibition could be used to suppress the "pre-NETotic" step of NET formation in human SLE. Hydroxychloroquine, a disease modifying anti-rheumatic drug and a late-stage autophagy inhibitor, is used to prevent flares and increase survival in patients with SLE. NET formation could be targeted by autophagy inhibition with hydroxychloroquine [35].

Our data also identified IL-17A and TF as downstream bioactive molecules expressed on SLE NETs, mediating thromboinflammation and fibrosis [35]. Interestingly, induction of lupus by pristane did not occur in IL-17- or IL-17F-deficient mice [108,109] and IL-17RA knockout mice were protected from IFN-I-dependent crescentic glomerulonephritis and renal infiltration with activated macrophages [110]. In an FcgammaR2b-deficient mouse model of lupus, loss of signaling by IL-17 cytokines improved survival and protected mice from glomerulonephritis, by elimination of recruitment of inflammatory cells, mainly neutrophils, in the kidneys [111]. Biologic agents that directly or indirectly block the IL-17 pathway are being studied in

Table 2
Neutrophil Extracellular Traps (NETs) in ANCA-associated vasculitides (AAV).

Mechanisms and mediators of NET release	Ref.
• Impaired degradation of NETs • Anti-neutrophil cytoplasmic antibodies (ANCA) • Antibodies against lysosomal membrane protein 2 (anti-LAMP-2) • HMGB1 • Semaphorin 4D • Tumor Necrosis Factor a (TNFα) • Complement 5a (C5a) • Autophagy • Necroptosis	[38,45,53,115,117–121,124]
Proteins on AAV NETs	
• Proteinase 3 (PR3) • Myeloperoxidase (MPO) • LL37 • High mobility group box protein 1 (HMGB1) • Neutrophil elastase • Interleukin-17 • Tissue factor (TF) • Lysosomal membrane protein 2 (LAMP-2) • Bb protein and properdin	[45,53,116–118,124,125]
Target cells and effector functions	
• Myeloid dendritic cells • Endothelial cells • Alternative complement pathway activation • Thrombin generation	[38,53,122]
Tissues expressing AAV NETs	
• Kidneys (glomeruli, interstitium, interlobular arteries) • Small arterioles • Nerves • Pulmonary capillaries • Nose • Bronchoalveolar lavage	[45,53,126–128]

several autoimmune diseases, however neither the targeting of the IL-17 pathway nor the IL-17 expressed on NETs has been studied as a therapeutic target in SLE. Inhibition of IL-17A or TF expressed on NETs prevented the activation and differentiation of human skin fibroblasts and collagen production, indicating that targeting bioactive proteins on SLE NETs with either anti-IL-17 agents or thrombin inhibitors or PAR blockers could inhibit the activation of resident tissue cells mediating thromboinflammation and fibrosis in SLE.

3. ANCA-associated vasculitides (AAV) and Neutrophil Extracellular Traps

AAV are a group of systemic autoimmune diseases characterized by the presence of antibodies against either proteinase 3 (PR3) or MPO, activating neutrophils at the site of the endothelium, leading to necrotizing small-vessel vasculitis. AAV include microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), eosinophilic granulomatosis with polyangiitis (EGPA) and drug-induced AAV [112,113]. Several pieces of data support the involvement of NETs in AAV (Table 2). Upstream mediators of NET formation in AAV, bioactive proteins on AAV NETs and effector functions of AAV NETs are presented in Fig. 1B.

3.1. Mechanisms of NET release in AAV

Kossenbrock K. et al. were the first to observe in 2009 that PR3-specific ANCA autoantibody-stimulated neutrophils to release NETs decorated with PR3 and MPO [45]. Active AAV patients expressed higher levels of circulating NET remnants when compared to patients in remission [114], and serum from MPO-ANCA MPA patients exhibited impaired degradation of NETs that was partially recovered upon depletion of IgG [115]. NETs expressing PR3 and MPO were also spontaneously released from LDGs of AAV patients [116].

Data on the role of antibodies against lysosomal membrane protein 2 (anti-LAMP-2) in the pathogenesis of AAV are controversial. However, *in vitro*, anti-LAMP-2 antibodies activated neutrophils towards the release of NETs expressing autoantigens and antimicrobial peptides [117], and neutrophils from AAV patients released NETs expressing LAMP-2 [118].

Studies demonstrated that HMGB1 levels reflect disease activity in AAV and that HMGB1 increases the translocation of ANCA for ANCA-mediated respiratory burst and neutrophil degranulation. In neutrophils treated with HMGB1 and ANCA-positive IgG, Ma Y.H. et al. demonstrated that HMGB1 interacted with TLR2, TLR4 and RAGE in a NOX-dependent manner mediating increased NET release [119]. Also, Nishide M. et al. observed that semaphoring 4D -a neural guidance factor in neuronal development- on neutrophils was bound to plexin B2 on endothelial cells, decreasing NET formation. Through semaphoring 4D's intracellular domain, recombinant plexin B2 suppressed Rac1 activation in neutrophils and inhibited ANCA-induced oxidative burst and NET release [120].

In addition to SLE, autophagy mediates NET release in AAV too. Neutrophils treated with ANCA-positive IgG demonstrated autophagy vacuolation, LC3B accumulation and autophagy-mediated NET release [121]. Autophagy inhibition with 3MA and LY294002 reduced anti-LAMP-2-mediated NET release [118]. Schreiber A. et al. showed that NETs in AAV are formed *via* RIPK1/3-MLKL-dependent necroptosis signaling [38].

3.2. Effector functions of NETs in AAV: implications for the autoimmunity, endothelial injury and thromboinflammation

To test whether NETs favor the uptake of neutrophil proteins to antigen-presenting cells in AAV, NETtotic neutrophils were co-cultured with myeloid DCs (mDCs). NETtotic neutrophils stably interacted with mDCs, and NET components -such as DNA, PR3 and MPO- were uploaded onto mDCs. Naïve mice immunized with mDCs uploaded with NET components demonstrated induction of ANCA and anti-dsDNA, that was associated with the development of vasculitis in renal and pulmonary tissue. Notably, DNase treatment prevented uploading onto mDCs and autoimmunity induction [122].

In a rat model of MPO-AAV, immunization with NETs induced by PMA in combination with propylthiouracil, produced MPO-ANCA and induced pulmonary capillaritis [123], demonstrating a feedback loop where ANCAs induce NET release that mediate small-vessel vasculitis. This could be explaining the development of AAV in patients receiving anti-thyroid medications, such as propylthiouracil or methimazole. NETs isolated from neutrophils treated with anti-MPO mediated endothelial cell injury and alternative complement activation. Of note, DNase-1 treatment protected mice from developing necrotizing crescentic glomerulonephritis; the same was observed in RIPK3-deficient mice immunized with anti-MPO [38].

Patients with AAV are displaying a high rate of thromboembolic disease during their active phase. Due to the important role of neutrophils in inflammation-associated thrombosis, we examined if TF was implicated in the thrombotic aspect of AAV. TF-decorated NETs were released from peripheral blood neutrophils and neutrophils from bronchoalveolar lavage from patients with active AAV. Importantly, TF on NETs was bioactive as evidenced by thrombin generation, and TF-decorated NETs

were observed in nasal and renal biopsy specimens. We further demonstrated that TNFa primed neutrophils towards NET release [53]. Others demonstrated that C5a in serum from patients with AAV mediated TF-decorated NET release [124]. TNFa-primed neutrophils stimulated with ANCA-positive IgG released NETs bearing proteins of the alternative complement pathway, such as Bb and properdin, and ANCA-induced NETs activated the alternative complement pathway in a TF- and thrombin-dependent manner [125].

3.3. NET formation in end-organ tissues in AAV and therapeutic targeting

In vivo, NETs were found in kidney biopsy specimens from patients with small-vessel vasculitis next to neutrophil infiltrates in glomeruli, interstitium [45] and along the interlobular arteries [126]. NETs were also identified in vasculitic small arterioles of MPO-ANCA-positive patients with MPA, nerve biopsy samples from patients with ANCA-associated peripheral nerve vasculitis [127], and pulmonary capillaries of patients with MPA [128]. We observed NETs in the bronchoalveolar lavage of patients with active AAV and TF-decorated NETs in nasal and renal biopsy specimens of patients with AAV [53].

As for SLE, targeting of NETs could offer therapeutic potential in AAV. C5a in serum from patients with AAV mediates NET release and treatment of neutrophils with anti-CD88 antibody or NDT9513727, agents that block the C5a receptor, resulted in reduced NET formation [124]. Similarly, C5a receptor blockade with anti-CD88 antibody abrogated the priming of neutrophils from supernatants of ANCA-activated neutrophils [129]. To examine if PAD inhibitors suppress MPO-ANCA production *in vivo*, Cl-amidine was injected in BALB/c mice treated with PMA combined with propylthiouracil. Citrullination in the peritoneum and serum MPO-ANCA titer were significantly reduced [130]. Kimura H. et al. administered an extract from *Candida albicans* to induce ROS generation and NETosis in a NOX-dependent manner. Mice demonstrated MPA-like vasculitis. Genetic ablation of PI3K-gamma in mice reduced NETosis and ANCA production. To assess the efficacy of NET inhibition *in vivo* in MPA, AS252424 -a PI3K-gamma-specific inhibitor- was administered to the mouse model of MPA. ANCA titers and histological severity of renal and pulmonary injury were reduced, indicating that inhibition of *in vivo* NETosis through PI3K-gamma blockage is effective in improving MPA [131]. NET treatment with DNase prevented DC uploading and autoimmunity induction in a vasculitis model [122] and protected mice from developing necrotizing crescentic glomerulonephritis [38], indicating that the integrity of the NET scaffold is necessary for the bioactivity of the NET proteins.

To our knowledge, currently no clinical trials study the therapeutic targeting of NETs in AAV.

4. Systemic inflammation drives hematopoietic stem cell (HSC) activation and differentiation to myeloid progenitors, to enhance myelopoiesis: trained immunity and neutrophils

Neutrophils derive from HSCs which reside in the bone marrow in a quiescent state, being ready to respond to stress, such as severe infection, systemic inflammation, or iatrogenic myeloablation [1]. Thus, during systemic infection or inflammation there is activation of hematopoietic progenitors in the bone marrow, resulting in their proliferation and differentiation towards the myeloid lineage originating not only from committed myeloid progenitors but also from HSCs in a processed called *emergency myelopoiesis.* To this end, pathogen-derived products or inflammatory cytokines and growth factors activate HSC differentiation towards the myeloid lineage. The inflammatory mediators driving demand-adapted myelopoiesis target not only HSCs but also HSC-supportive cell populations such as mesenchymal stem cells collectively known as the HSC niche [132].

Inflammatory cytokines, and myeloid specific growth factors, including IL-1 and granulocyte macrophage growth factor (GM-CSF) [132], drive the reprogramming of HSC towards myeloid lineage increasing the production of platelets, granulocytes and monocytes at the expense of lymphopoiesis. This is mediated by epigenetic modifications and induction of lineage specific transcriptional networks involving transcription factors. The modifications in the epigenome of hematopoietic progenitors result in their enhanced adaptation to inflammatory and hematopoietic stress and the generation of myeloid cells that (a) confer protection to secondary infection, in a phenomenon termed “trained innate immunity” or “innate immune memory” [133,134] or (b) result in exaggerated immune responses and thus contributing to autoimmune and inflammatory diseases, such as arthritis [135,136], SLE [137] or atherothrombosis [138–140]. It is conceivable that in SLE or vasculitis, granulocytic progenitors such as common myeloid progenitors (CMP) or granulocyte-macrophage progenitors (GMP) released from the bone marrow may infiltrate tissues in the periphery resulting in the generation *in situ* of primed neutrophils that may lead to exaggerated responses within the affected tissues (Fig. 1C).

5. Conclusions and perspectives

Initially, neutrophils were thought to release NETs during a distinct form of cell death, named NETosis. Nowadays, evidence suggests that neutrophils may remain viable and functional even after NET extrusion. Several NOX-dependent or NOX-independent mechanisms are involved in NET formation, including REDD1-mediated autophagy induction and necroptosis.

Deregulated NET formation is implicated in several diseases. Depending on the pathophysiologic context of each disease, NETs are decorated with distinct bioactive proteins that may account for their differential contribution to disease pathogenesis and phenotype. Although neutrophils may be not present in sites of chronic inflammation, their remnants may amplify the inflammatory response beyond their short life-span in the tissues. In systemic autoimmunity, evidence clearly demonstrates the role of neutrophils and NETs in tissue injury and dysfunction. Better understanding of the mechanisms mediating NET release and how NETs affect end-organ tissues will reveal possible novel therapeutic targets.

Neutrophils derive from HSCs which reside in the bone marrow in a quiescent state, being ready to respond to stress. During systemic inflammation there is activation of hematopoietic progenitors in the bone marrow, resulting in their proliferation and differentiation towards the myeloid lineage increasing the production of platelets, granulocytes and monocytes at the expense of lymphopoiesis. These HSCs are uniquely primed to respond to acute inflammation but may have a poor response to infectious challenges. *Re*-establishment of the appropriate lymphoid *versus* myeloid balance in systemic autoimmune diseases may improve immune function decreasing the risk of infection and resolution of inflammation.

Take-home messages

- Depending on the pathophysiologic context of each disease, NETs are decorated with distinct bioactive proteins that may account for their differential contribution to disease pathogenesis and phenotype.
- Although neutrophils may be not be present in sites of chronic inflammation, their remnants may amplify the inflammatory response beyond their short life-span in the tissues.
- NET formation in systemic autoimmunity has implications for the activation of the immune response
- NET formation in autoimmunity mediates endothelial injury, thromboinflammation and fibrosis
- Upstream molecules mediating NET release or downstream proteins expressed on NETs could be therapeutically targeted
- During systemic inflammation there is activation of HSCs in the bone marrow, resulting in their proliferation and differentiation

towards the myeloid lineage.

- These HSCs are uniquely primed to respond to acute inflammation but may have a poor response to infectious challenges

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