Neutrophil extracellular traps in vasculitis, friend or foe?

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1 **Neutrophil extracellular traps in vasculitis, friend or foe?**

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Abstract

Purpose of review: Neutrophil extracellular traps (NETs) can be found at the sites of vascular lesions and in the circulation of patients with active small vessel vasculitis. Neutrophils from vasculitis patients release more NETs *in vitro*, and NETs have properties that can harm the vasculature both directly and indirectly. There are several ways to interfere with NET formation, which open for new therapeutic options. However, there are several types of NETs and different mechanisms of NET formation, and these might have different effects on inflammation. Here we review recent findings regarding the pathogenesis and therapeutic potentials of NETs in vasculitis.

Recent findings: Experimental mouse models support a role for NETs in promoting vascular damage, where histones and mitochondrial DNA appear to be driving forces. However, impaired formation of NETs in an SLE-like mouse model leads to more severe disease, suggesting that NETs can be important in limiting inflammation. Studies on drug-induced vasculitis reveal that levamisole can induce NETosis via muscarinic receptors, predisposing for the generation of autoantibodies, including anti-neutrophil cytoplasmic autoantibodies (ANCA). This supports the notion that NETs can bridge the innate and adaptive immune systems.

Summary: NETs can participate in the pathogenesis of vasculitis, but in some models there also seem to be protective effects of NETs. This complexity needs further evaluation with experimental models that are as specific as possible for human primary vasculitis.

Keywords

Vasculitis, Neutrophil extracellular traps (NETs), ANCA, Autoantigens, Inflammation

Introduction

The formation of neutrophil extracellular traps (NETs) was initially described as a mechanism to ensnare and kill invading microorganisms (1), but in recent years NETs have attracted increased attention in a wide variety of medical conditions such as cancer, thromboembolism, arteriosclerosis, and autoimmune diseases (2). Kessenbrock and co-workers suggested a role for NETs in the pathogenesis of vasculitis in 2009 (3), and since then there has been a growing body of literature on the connections between NETs and vasculitis. Here we give an overview of recent advances pertaining to altered NET formation in vasculitis, the relation between NETs and vascular damage, NETs as a source of autoantigens, the utility of biomarkers associated with NETs, and finally some possible therapeutic implications of NET formation in vasculitis.

Definitions and nomenclature

The formation of NETs, called NETosis, was originally proposed to be a form of programmed cell death initiated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation followed by chromatin decondensation, breakdown of the nuclear membrane, and mixing of the chromatin with granule constituents (4). The process is dependent on myeloperoxidase (MPO), neutrophil elastase, and peptidyl arginine deiminase (PAD) 4 (5, 6) and results in the extrusion of a tangle of DNA decorated with citrullinated histones and other proinflammatory molecules (7). However, subsequent research has questioned whether each step in this pathway is necessary for NETosis. It was, for example, recently shown that saliva can induce NETosis independently of NADPH oxidase and

neutrophil elastase (8). Neutrophils are not the only cells that can extrude extracellular traps (ETs), and eosinophils, basophils, mast cells, and monocytes also have such capacity (9, 10), and the term ETosis has been coined as a general term for cells releasing ETs. It has been shown that extrusion of NETs is not necessarily associated with cell death, and today many authors distinguish between NETosis involving cell death (suicidal NETosis) and NETosis where the neutrophils remain viable (vital NETosis). NETs released during vital NETosis can consist of nuclear or mitochondrial (mt) DNA and can be released in an NADPH-oxidase and/or reactive oxygen species (ROS)-independent manner (10-13).

Primary systemic vasculitis encompasses a wide variety of diseases with idiopathic vascular inflammation as their common defining feature. According to the current nomenclature, they are divided into groups based on the size of the vessels that are predominantly affected in the individual diseases (14). The small-vessel vasculitides are further grouped according to immunofluorescence findings of biopsies into immune-complex vasculitides and pauci-immune vasculitides. The latter are also referred to as anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis (AAV) because of their relationship to ANCA, and this group contains the diseases granulomatosis with polyangiitis (GPA, formerly Wegener's granulomatosis), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA, formerly Churg–Strauss syndrome) (14). More common than primary vasculitis is vasculitis as a complicating feature of other autoimmune diseases, infections, malignancies, or adverse drug reactions (15).

Vasculitis is associated with increased NETosis

The pathogenesis varies between different forms of vasculitis, but at least in small-vessel vasculitis neutrophils have a prominent role. Neutrophils produce ROS and release

destructive enzymes, and they attract other players to the scene through the production of cytokines and chemokines. It is often difficult to distinguish the contribution of NETs relative to activation, degranulation, and other forms of neutrophil cell death than NETosis. There are several investigations showing increased NETosis in active vasculitis, and NETs and/or remnants of NETs can be found both in the affected tissues and in the blood circulation of AAV patients (16). Co-expression of granule proteins (such as MPO and neutrophil elastase) and chromatin (primarily citrullinated histone 3) is often considered as evidence of NETosis. However, one needs to be aware when screening for NETs that they can also be mitochondrial-derived and thus would not contain histones.

NETs in AAV were first reported on in kidney biopsies (3), which was later confirmed by others (17-20), and then also in skin specimens (21-23) and in thrombi (20, 24) of these patients. Neuropathy is another common feature of vasculitis, and NETs were recently shown to also be common in nerve biopsies from AAV patients, but not seen in patients with non-vasculitic demyelinating neuropathy (25). More NETs were seen in ANCA-positive MPA patients compared to ANCA-negative MPA patients and in patients with vasculitis secondary to rheumatoid arthritis (25). Increased levels of breakdown fragments of NETs (NET remnants) in the circulation have also been reported in vasculitis (3, 26, 27).

In vitro studies on neutrophils from AAV patients show that they are more prone to undergo spontaneous NETosis (18, 26, 28) and are more responsive to NET-inducing stimuli (29). Similar findings have been reported in other autoimmune diseases where NETosis is implicated in the pathogenesis (30-32). It was recently also revealed that NETosis is negatively regulated by interaction between plexin B2 on endothelial cells and semaphorin

4D on neutrophils, and NETosis in humans cells can be inhibited *in vitro* by recombinant plexin B2 (33). Interestingly, neutrophils from AAV patients exhibit reduced expression of semaphorin 4D compared with healthy controls (33), which could be an explanation for the increased amount of NETs in these patients.

Serum, immune complexes, and autoantibodies from vasculitis patients induce NETosis *in vitro*

In vitro studies have shown that IgG and serum from AAV patients can stimulate neutrophils from healthy controls to undergo NETosis to a greater extent than IgG and serum from healthy controls (Table 1) (3, 33-39). For ANCA IgG, this generally requires the neutrophils to be primed in order to increase the membrane expression of proteinase 3 (PR3) and MPO before they will respond to ANCA exposure by undergoing NETosis. This can, for example, be seen in recent reports that have shown an effect of PR3-ANCA IgG and MPO-ANCA IgG on NETosis after priming with high mobility group box 1 (HMGB1) (35) or tumour necrosis factor (36), respectively. The ability of MPO-ANCA IgG to induce NETosis appears to be related to antibody affinity rather than antibody levels (34), and it has been shown that patients with high-affinity MPO-ANCA IgG exhibit higher occurrence of NETs in renal biopsies than patients with low-affinity MPO-ANCA IgG (40). However, IgG-depleted serum (38) and serum from ANCA-negative patients (39) have a similar ability as whole serum to induce NETosis, and this questions the role of ANCA IgG in these experimental settings, which did not include priming of the neutrophils before stimulation.

IgG-containing immune complexes can also induce NETosis, and the most recent study showed that these complexes activate neutrophils via cross-linking of Fc gamma receptor IIIb (41). Another recent study found that heat-aggregated immune complexes from patients with

systemic lupus erythematosus (SLE) and rheumatoid arthritis (where secondary vasculitis is common) induce NETosis, but that study did not look at receptor specificity (42). IgA immune complexes in plasma and synovial fluid from rheumatoid arthritis patients were shown to induce NETosis via Fc alpha receptor I (43). This most probably has a bearing on IgA vasculitis, a disease characterised by IgA immune complex deposition and small-vessel leucocytoclastic vasculitis (14). A recent study on patients with PR3-ANCA-associated vasculitis showed that serum PR3-ANCA IgA levels were more closely related to disease activity than PR3-ANCA IgG levels (44).

NETs and vascular damage

There are several ways in which NETosis can harm the vasculature, both directly and indirectly. The release of noxious substances such as degrading enzymes can directly induce apoptosis in endothelial cells and degrade the basement membrane (45), and histones can be toxic to endothelial cells (46). A recent study showed that endothelial cells can phagocytise NETs, but that excessive amount of NETs promotes vascular leakage by interfering with endothelial cell-cell interactions (47). The same study also showed that NETs can induce endothelial to mesenchymal transformation (EndMT) and that such cells are increased in the glomeruli both in MLR/lpr mice (a mouse model of SLE) as well as in patients with lupus nephritis (47). EndMT is important during vascular repair, but it is also connected to several disease conditions because it contributes to tissue fibrosis (48), which is a common feature in vasculitis. Indirectly, NETs promote vascular damage by activating the alternative complement pathway (49).

Renal injury is common in small vessel vasculitis, including both glomerulonephritis with crescent formation and tubulointerstitial nephritis. Recent studies have shown that NETs are

present in glomeruli and that they contribute to glomerular injury in mouse models of glomerular vasculitis induced by anti-glomerular basement membrane (GBM) antibodies (50) or GBM antiserum (46), as well as in MLR/lpr mice that spontaneously develop SLE-like disease (47, 51). The role of NETs in tubulointerstitial injury was shown in a study of ischemic acute kidney injury (AKI) in mice, where epithelial tubular cells during hypoxia released histones that activated neutrophils to release NETs (52). These NETs in turn induced epithelial cell necrosis with the release of histones from these cells, thus creating a necroinflammation loop leading to enhanced tubular necrosis. This study mimics a possible scenario during excessive inflammation, with hypoxia and kidney injury, as is seen in vasculitis. Tubulointerstitial injury could also retard glomerular blood flow, thus reducing the shear stress, which has been shown to rapidly clear the glomeruli of NETotic neutrophils (50). Regarding immune complex vasculitis, NETs were shown to contribute to vessel destruction and haemorrhage in mouse skin specimens after injection of bovine serum albumin (BSA) and anti-BSA antibodies (53).

NETs can be protective

A recent study showed that saliva can induce NETs, and that this capacity is diminished in Bechet's disease, a form of primary vasculitis characterised by mouth and genital ulcers (8). The authors argued that the absence of NETs leads to diminished protection against bacteria on the mucus membranes and that this promotes ulcer formation. Other examples where reduced NETosis leads to more severe disease are mouse models of SLE (54) and gout (55). These studies suggest that NETs can act as platforms to degrade proinflammatory mediators that would otherwise drive inflammation. Additionally, NETs can impair GM-CSF/IL-4-induced dendritic cell differentiation from monocytes *in vitro*, and can instead promote an alternatively activated macrophage phenotype (56). This subgroup of macrophages is

important for the resolution of inflammation, which is crucial for preventing chronic inflammation.

Antigen exposure in NETs promotes the production of autoantibodies

NETs contain an array of molecular motifs that serve as targets for autoantibodies in autoimmune diseases, including double-stranded DNA in SLE (32), citrullinated peptides in rheumatoid arthritis (31), and MPO and PR3 in AAV (3). NETs can also contain alarmins, such as LL39 and HMGB1 (57, 58), that provide danger signals and thus reduce immunological tolerance. The strongest evidence that NETs actually serve as a source of autoantigens driving autoantibody production in vasculitis comes from studies on drug-induced vasculitis. MPO-ANCA positivity is relatively common in patients treated with the anti-thyroid drug propylthiouracil (PTU), and some of these patients develop a vasculitis-like syndrome (59). Phorbol 12-myristate 13-acetate (PMA) in combination with the anti-thyroid drug PTU induces NETs that resist DNase I degradation, and such NETs cause the production of ANCAs and AAV-like disease in rats (60). Using a similar approach as above but in a mouse model, PMA and PTU again resulted in the production of MPO-ANCA, but did not induce disease (61), indicating that antibody formation is not sufficient to induce full-blown disease. Levamisole, a veterinary compound often found in adulterated cocaine, is also associated with ANCA formation and vasculitis-like syndromes. Contrary to PTU, levamisole directly induces NETosis in neutrophils *in vitro* via the stimulation of muscarinic receptors (23, 62). Also, cocaine itself is able to induce NETosis (62). Patients with cocaine/levamisole-associated autoimmunity possess IgG class autoantibodies against NET components such as neutrophil elastase (62), PR3, MPO, LL-37, and anti-nuclear antibodies (23). Further, IgG from patients with levamisole-associated autoimmunity enhance NETosis induced by cocaine or levamisole, which could possibly create a vicious circle in these patients (62).

NETs as a biomarker

Monitoring of disease activity is an unmet need in vasculitis, and better monitoring will enable more efficient use of the drugs available today and will reduce the side effects of maintenance therapy. As reviewed elsewhere (16), several studies in recent years have reported on increased levels of NETs and NET-associated proteins in the circulation of AAV patients that often correlate with disease activity. However, there is no assay available today that has proven to be clinically useful. Measurements of NETs suffer from a lack of standardisation, as well as from problems with sensitivity and specificity. Because of this there are currently no general values for these parameters regarding the presence of NETs in various diseases. The fact that ANCAs of different affinities appear to vary in their NET-inducing capacity encourages further studies with this approach to evaluate its usefulness to monitor disease activity (34). The capacity for serum to degrade NETs is another tempting approach, and this capacity is reduced in serum from AAV patients (34). DNase I activity did not vary with disease activity in that study, but NET degradation per se with serum from patients with various disease activity was not evaluated. Regardless of the methodological approach, further evaluation of NETs as a biomarker to monitor disease activity in AAV (alone or in combination with other parameters) requires carefully undertaken longitudinal studies.

Implications for treatment

As summarised in Table 2, several approaches have been used in recent studies to block the effect of NETs on vascular damage in different in vivo experimental models. Anti-histone treatment through anti-histone antibodies, heparin, or activated protein C all proved to be efficient in treating anti-GBM-induced vasculitis (46). This was also the case for the PAD inhibitor Cl-amidine (46), which inhibits the citrullination of histones that is an important

233 step during suicidal NETosis. Inhibiting PAD signalling was also shown to be efficient in a
234 mouse model of post-ischemic AKI (52). Injection of adipose tissue-derived mesenchymal
235 stem cells (MSCs) prior to injection of BSA/anti-BSA antibodies in a model of immune
236 complex vasculitis significantly reduced the vessel damage and the amount of NETs that
237 were formed (53). The inhibitory mechanisms of the MSCs included phagocytosis of
238 neutrophils and upregulation of superoxide dismutase 3, an antioxidant, both of which
239 prevented NET formation (53). There is a plethora of other molecules that are being tested for
240 their ability to block NETosis, and these are primarily being evaluated *in vitro*. A recent
241 example is the blockade of neutrophil elastase, which has been shown to inhibit NET-induced
242 disruption of endothelial cell-cell integrity and EndMT (48). Although inhibition of NADPH
243 oxidase is effective in inhibiting suicidal NETosis *in vitro*, recent studies on experimental
244 mouse models of SLE (54) and gout (55), both of which lack NADPH oxidase, show more
245 severe diseases. In line with these studies, patients with chronic granulomatous disease, with
246 defective NADPH oxidase, have a greater incidence of autoimmune diseases (63). Low-
247 density granulocytes are the neutrophil subpopulation that release the most NETs both in
248 AAV (28) and SLE (32). LDG NETs from SLE patients have been shown to be enriched for
249 mtDNA and formation of these NETs can be inhibited *in vitro* with the mitochondrial ROS
250 inhibitor MitoTEMPO (51). Interestingly, treatment of MLR/lpr mice with MitoTEMPO
251 limits release of mtDNA NETs and reduces disease severity (51). Thus, targeting these NETs
252 might also be an interesting approach in AAV. Regarding PAD4, which was shown to be a
253 good target in various mouse models to limit NET-mediated inflammation, it was shown in
254 the same study as for NADPH oxidase that PAD4^{-/-} mice developed worse disease than
255 control mice (54). It appears that a certain amount of ROS signalling and NETosis is
256 important to limit inflammation, while excessive NETs can instead cause disease. A recent
257 example of compounds tested *in vitro* relevant to this aspect is the tetrahydroisoquinolines

that can inhibit NETosis without interfering with ROS production (64). These were shown to inhibit both spontaneous and PMA-induced NETosis in neutrophils from SLE patients.

DNase I, which efficiently degrades DNA and thus degrades NETs, is already being used clinically and has proven to be safe. However, a phase 1 clinical study of DNase I in SLE patients did not show any effect on double-stranded DNA autoantibody production, inflammatory markers, or disease severity (65). When applied in a mouse model of anti-GBM-induced glomerulonephritis, DNase I rescued mice from haematuria, but not proteinuria (50). It appears that components of NETs such as histones and neutrophil elastase can still harm the vasculature after DNase I treatment (66). It is worth noting that the complement fragment C5a can also induce NETosis (11), and a recent randomised controlled trial in AAV patients showed that the C5a receptor inhibitor avacopan had positive effects on disease activity (67).

Conclusion

Several studies have taken different approaches to studying the role of NETs in vasculitis, including in vitro functional studies, drug-induced autoimmunity, and animal models, and these have proposed NETs to constitute a source of autoantibodies and to promote vascular damage. These events can be avoided or reversed by inhibiting NETosis, blocking the proteins that are present in NETs, or by clearing NETs that have already formed. It would, however, be valuable for our understanding of NETs in vasculitis to learn more about why some animal models of autoimmune disease with impaired NETosis show aggravation of disease.

283 **Key points**

- 284 - There are signs of increased NETosis in tissue and blood samples from vasculitis patients.
- 285 - Neutrophils from vasculitis patients are more prone to undergo NETosis *in vitro*, and serum
- 286 and autoantibodies from these patients induce NETosis in neutrophils from healthy controls.
- 287 - NETosis can induce endothelial damage both directly and indirectly, but NETosis may also
- 288 be protective under some circumstances.
- 289 - There are several pharmacological approaches to alter NETosis, but such treatments, like
- 290 the NETs themselves, might prove to be double-edged swords.

291

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294

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298

299 **Conflicts of interest**

300 None

301

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Table 1. Neutrophil extracellular trap-inducing capacity of IgG and serum from vasculitis patients and HCs

Ab/serum	Patients	Disease activity	Priming/activation	Patients vs. HCs (+/-)	Subgroup analyses	Ref
IgG	AAV	Active and remission	TNF	+	NA	3
IgG	MPA	Active	TNF	+	NETosis correlated with disease activity and antibody affinity	34
IgG	AAV	Active	HMGB1	+	NA	35
IgG	AAV	Active	TNF	+	NA	36
Anti-Lactoferrin	EGPA	Active	PMA	+	NETosis correlated with disease activity	37
Whole serum	AAV	Not available	No	+	MPA serum induced more NETosis than GPA serum	38
Whole serum	EGPA	Remission	No	+	MPO-ANCA ⁺ serum induced more NETosis than PR3-ANCA ⁺ and ANCA ⁻ serum	39
Serum from ANCA-negative patients	EGPA	Remission	No	+ (vs. whole serum)	NA	39
IgG-depleted serum	AAV	Not available	No	+ (vs. whole serum)	No difference in NETosis between IgG-depleted serum and whole serum	38

+, increased NETosis; NA, not available; ANCA, anti-neutrophil cytoplasmic autoantibody; AAV, ANCA-associated vasculitis; MPA, myeloperoxidase; EGPA, eosinophilic granulomatosis with polyangiitis; HMGB1, high mobility group box 1; TNF, tumour necrosis factor; PMA, phorbol 12-myristate 12-acetate; HCs, healthy controls; GPA, granulomatosis with polyangiitis; PR3, proteinase 3

Table 2. Therapeutic approaches that inhibit or reduce the effect of NETs on vascular damage

Therapy	Agent/approach	Experimental setup	Ref
Neutrophil clearance	AT-MSC injection, clearing via phagocytosis	Mouse model, BSA/anti-BSA immune complex-mediated vasculitis	53
C5aR inhibitor	Avacopan	Randomised controlled trial in human AAV, phase 2	67
Histone neutralisation	Anti-histone antibodies or Heparin or Activated protein C	Mouse model, sheep anti-rat GBM serum-induced glomerular vasculitis	46
PAD inhibitor	Cl-amidine	Mouse model, sheep anti-rat GBM serum-induced glomerular vasculitis	46
PAD inhibitor	Cl-amidine	Mouse model, post-ischemic AKI	52
DNA degradation	DNase I	Mouse model, anti-GBM-induced glomerular vasculitis	50
ROS scavenging	AT-MSC injection, ROS scavenging via SOD3	Mouse model, BSA/anti-BSA immune complex-mediated vasculitis	53
mtROS inhibitor	MitoTEMPO	Mouse model, spontaneous development of SLE-like disease	51

mtROS, mitochondrial reactive oxygen species; PAD, peptidyl arginine deiminase;
AT-MSC, adipocyte tissue-derived mesenchymal stem cell; SOD3, superoxide dismutase 3;
SLE, systemic lupus erythematosus; GBM, glomerular basement membrane; AKI; acute kidney injury;
BSA, bovine serum albumin; AAV, ANCA-associated vasculitis