Targeting autoimmunity in renal diseases: focus on neutrophil extracellular traps and autoreactive B-cells

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Clinical implications of excessive neutrophil extracellular trap formation in renal autoimmune diseases

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ABSTRACT

Neutrophil extracellular traps (NETs) are extracellular DNA structures covered with anti-microbial peptides, danger molecules and autoantigens that can be released by neutrophils. NETs are an important first line defense mechanism against bacterial, viral, fungal and parasitic infections, but they can also play a role in autoimmune diseases. NETs are immunogenic and toxic structures that are recognized by autoantibodies of patients with antineutrophil cytoplasmic antibodies-associated vasculitis (AAV), (i.e. against myeloperoxidase or proteinase-3) and systemic lupus erythematosus (SLE), (i.e. against double-stranded DNA, histones, or nucleosomes). There is cumulating preclinical and clinical evidence that both excessive formation and impaired degradation of NETs are involved in the pathophysiology of AAV and SLE. These autoimmune diseases give rise to two clinically and pathologically distinct forms of glomerulonephritis (GN), respectively crescentic pauci-immune GN and immune-complex mediated GN. Therefore, it is relevant to understand the different roles NET formation can play in the pathophysiology of these most prevalent renal autoimmune diseases. This review summarizes the current concepts on the role of NET formation in the pathophysiology of AAV and SLE, and provides a translational perspective on the clinical implications of NETs, such as potential therapeutic approaches that target NET formation in these renal autoimmune diseases.

NEUTROPHIL BIOLOGY

Neutrophils are the most abundant (~57%) subpopulation of the circulating white blood cells and represent the most important effector cells of the innate immune system. They are typically recognized by the lobulated nucleus and have a relatively short lifespan of hours to days[1]. Upon an infection, neutrophils are the first responders of the immune system at the site of inflammation, and they recruit and activate other immune cells. To exert their primary defense function, neutrophils have the ability to attack pathogens by phagocytosis and by the release of different granules (called degranulation) that contain anti-microbial peptides and proteases, such as myeloperoxidase (MPO), neutrophil elastase (NE), LL-37 and matrix metalloproteinases[2]. Recently, it has become clear that neutrophils also have the ability to directly attack and restrain pathogens by the release of neutrophil extracellular traps (NETs)[3,4].

NETosis is a process that results in the release of extracellular DNA by neutrophils, which was originally believed to coincide with cell death[5] and is phonetically classified among other regulated cell death (RCD) pathways, such as apoptosis, pyroptosis, necroptosis, and ferroptosis[6]. After the discovery of NETosis, similar processes have been described in other immune cells, including eosinophils[7], monocytes[8] and B-cells[9], which are collectively referred to as 'ETosis' and which are out of the scope of this review. These pathways may be classified by their caspase-dependency and their immunogenicity. Classic apoptosis is typically seen as a caspase-dependent, non-immunogenic regulated cell death that is associated with the preservation of the plasma membrane integrity throughout the process of cell death[10]. In contrast, caspase-independent necroptosis and ferroptosis, as well as caspase-dependent pyroptosis, are all highly-immunogenic forms of regulated cell deaths associated with the loss of plasma membrane integrity[6]. Necroptosis and pyroptosis have been demonstrated to be relevant to fighting bacterial and viral infections[6], whereas ferroptosis has been implicated in cancer cell death and tissue injury[11]. NETosis is a caspase-independent process, but the studies are seemingly unclear on how to classify the process as immunogenic[12,13], or even anti-inflammatory[14]. This is mainly due to the fact that the pathways leading to NETosis are still evolving[15], and many studies have demonstrated that distinct forms of the release of extracellular DNA by neutrophils exist[16,17]. Besides the classical suicidal NET formation that coincides with neutrophil death, it has also been demonstrated that NET formation can occur independently of cell death, which is referred to as vital NET formation[18,19]. NETs can also have anti-inflammatory effects which has been demonstrated in mice models of gout[14] and lupus-prone mice with defects in reduced NAD phosphate (NADPH) oxidase, which show a more severe phenotype[20].

NETosis by neutrophils is an important mechanism in the innate immune system. However, it was recently described that neutrophils can play a role in the adaptive immune response through interaction with antigen presenting cells[21] and lymphocytes[22], both at sites of inflammation and in draining lymph nodes[22]. In mice, it was shown that a subset of neutrophils has the ability to induce antibody production and class switching of marginal zone B-cells by production of B-cell activating factor, a proliferation inducing ligand, IL-21, CD40L expression, and NET formation[23]. Moreover, fewer and hypomutated marginal zone B-cells were observed in patients with congenital neutropenia, which supported this novel function of neutrophils as modulators of the adaptive immune response[23].

THE IMMUNOGENICITY AND TOXICITY OF NETS

The first and foremost assumption on NETs is that the extruded DNA is immunogenic and leads to overt inflammation that can then potentially lead to autoimmune diseases. However, it has long been known that DNA in itself is not immunogenic[24]. Only DNA in combination with danger signals, (i.e. danger-associated molecular patterns), such as LL37[24,25], a cathelicidin anti-microbial peptide, or High Mobility Group Box Protein-1 (HMGB1)[26], can activate antigen presenting cells, in particular, plasmacytoid dendritic cells (DCs)[24] and B-cells[26,27]. This is mediated through Toll-like receptor-9 (TLR9) signaling that results in the production of interferon-α[21] and (auto-)antibodies[27]. Preclinical mouse models demonstrated that *in vitro* monocyte-derived DCs take up DNA particles from neutrophils undergoing NETosis, apoptosis or necrosis[28]. This internalization is mediated via the receptor for advanced glycation endproducts (RAGE)-TLR9 pathway[29,30]. Transfer of these DNA-loaded monocyte-derived DCs, led to production of antibodies against dsDNA, MPO and proteinase-3 (PR3) in mice[28]. Autoantibody production was most significant when mice were injected with DNA-loaded monocyte-derived DCs that were exposed to NET-ting neutrophils. Other studies also demonstrated that nuclear material from NETs was more immunogenic than apoptotic material[12,15].

Besides the immunogenic effects of NETs, they are also believed to have a direct cytotoxic effect on human epithelial and endothelial cells through the externalisation of histones[31-33] and MPO[33,34]. NET-related histones were demonstrated to cause direct cytotoxicity of glomerular endothelial cells, podocytes, and parietal endothelial cells, which led to crescentic glomerulonephritis (GN) in preclinical models[35]. Crescentic GN is typically seen in antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) patients and less frequent in lupus nephritis (LN). Moreover, extracellular MPO was demonstrated to induce oxidative damage[33], which was associated with glomerular and interstitial injury in AAV patients[34]. In addition, endothelial cells have a limited capacity to internalize NETs[36]. An overflow of NETs induced vascular leakage and endothelial-to-mesenchymal transition. In patients with systemic lupus erythematosus (SLE), glomerular presence of NETs was correlated with the severity of proteinuria and glomerular endothelial to mesenchymal transition[36], which emphasized the relevancy of this process.

Overall, break of self-tolerance towards autoantigens is a hallmark for a wide spectrum of systemic autoimmune diseases, including AAV[37] and SLE[38]. NETs are believed to be an important source of autoantigens in systemic autoimmune diseases[12,21,24,25,30,39-48]. Indeed, 74% of these identified NET-associated proteins are recognized by autoantibodies in systemic autoimmune diseases[48]. This NET autoimmunity was most prominent in SLE patients, and subsequently, in AAV patients. Proteomic studies of NETs derived from neutrophils of patients with AAV or SLE, or alternatively healthy neutrophils stimulated with AAV and SLE sera, are lacking. The currently identified range of peptides and enzymes localized to NETs are studied by proteomics of NETs induced by phorbol-12-myristate-13-acetate (PMA)[49]. Translating data from PMA-induced NETs to clinical disease should generally be done with caution, because the *in vivo* relevance of PMA as a chemical compound remains unclear[16]. Nevertheless, several NET-related proteins found on PMA-induced NETs have also been identified with immunofluorescence microscopy studies on AAV- and SLE-induced NETs. The current data on NET-associated molecules, as identified by proteomics or immunofluorescence microscopy studies, that are known autoantigens in AAV and SLE are listed in Table 1.

Table 1. NET-associated molecules that are known autoantigens in AAV and/or SLE.

NET-molecules	Method of detection	Neutrophil localization	Auto-antigen	Role in autoimmune disease	Ref
Azurocidin	Proteomics of PMA-induced NETs[49]	Azurophilic Granules	AAV	Autoantibodies (atypical) present in AAV	[52]
Cathepsin G	Proteomics of PMA-induced NETs[49]	Azurophilic Granules	AAV SLE	Autoantibodies (atypical) present in AAV Autoantibodies are present in SLE	[54,136] [53,137]
Neutrophil Elastase	Proteomics of PMA-induced NETs[49]	Azurophilic Granules	AAV SLE	Anti-elastase antibodies present in AAV Anti-elastase antibodies present in SLE	[54,136,138]
Lactoferrin	Proteomics of PMA-induced NETs[49]	Secondary Granules	AAV SLE	Atypical ANCA in AAV Autoantibodies are present in SLE	[136,139] [53,137,140]
LAMP-2	IF of PMA-induced NETs derived of AAV neutrophils	Lysosomal membrane of granules	AAV	Anti-LAMP autoantibodies are present in AAV patients Detected in AAV kidney biopsies	[55,141,142]
Lysozym C	Proteomics of PMA-induced NETs[49]	Secondary Granules	AAV	Atypical ANCA in AAV	[54,136]

Table 1. Continued.

NET-molecules	Method of detection	Neutrophil localization	Auto-antigen	Role in autoimmune disease	Ref
MPO	Proteomics of PMA-induced NETs[49] IF of AAV-induced NETs & SLE-induced NETs[93]	Azurophilic Granules	AAV	Typical autoantigen for ANCAs in AAV Detected in AAV kidney biopsies Anti-MPO antibodies are sometimes present in SLE	54 34,55 143
PR3	Proteomics of PMA-induced NETs[49] Present on cell bodies of NET-ting neutrophils[50]	Azurophilic granules	AAV	Typical autoantigen for ANCAs in AAV Detected in AAV kidney biopsies	55
Alpha actinin 1/4	Proteomics of PMA-induced NETs[49]	Cytoskeleton	SLE	Autoantigen in LN, also bound by anti-dsDNA antibodies and associated with disease activity in SLE	144-149
AENO	Proteomics of PMA-induced NETs[49]	Glycolytic enzymes	SLE	Autoantigen eluted from LN biopsies. Autoantibody associated with disease activity in SLE	150-152
Annexin A1	Proteomics of SLE-induced NETs/IF[153]	Cytosol	SLE	Autoantigen eluted from LN biopsies. Autoantibodies present in SLE and associated with disease activity	150,152,153
C1q	IF of PMA-induced NETs incubated with SLE serum[102]	-	SLE	Anti-C1q antibodies are present in SLE and associated with disease activity	102,116,151, 154,155
Catalase	Proteomics of PMA-induced NETs[49]	Peroxisomal	SLE	Autoantibodies present in SLE	156
Citrullinated histones	IF AAV-induced NETs[95] and SLE-induced NETs[91]	Cytoplasmic granules and nucleus	SLE	Anti-CCP antibodies are rarely detected in SLE	56

Table 1. Continued.

NET-molecules	Method of detection	Neutrophil localization	Auto-antigen	Role in autoimmune disease	Ref
dsDNA	By definition present	Nucleus	SLE	Anti-dsDNA antibodies are hallmark of SLE and strongly correlate with disease activity	56
Histones (H2A, H2B, H3, H4)	Proteomics of PMA-induced NETs[49]	Nucleus	SLE	Autoantigen in SLE Causing crescentic GN	35
HMGB1	IF of RNP-ICx-induced NETs[21]	Nucleus	SLE	Anti-HMGB1 antibodies levels correlate with disease activity, with anti-dsDNA Abs and with HMGB1 levels in SLE. HMGB1 binds (SLE)-ICx	21,56,157 26,158
HNP/α defensins	Proteomics of PMA-induced NETs[49]. IF of PMA induced NETs & SLE-induced NETs[24]	Azurophilic Granules	SLE	HNP binds SLE-ICx Anti-HNP autoantibodies are present in SLE	24,159
LL-37	IF of PMA induced & SLE-induced NETs[24]	Nuclear	SLE	Anti-LL37 antibodies are present in SLE LL37 binds SLE-ICx	21,27 160,161
mtDNA	IF of RNP-ICx-induced NETs[42]	Mitochondria	SLE	Anti-mitochondrial antibodies are present in SLE patients	42,56
Properdin	IF of AAV-induced NETs[109] IF of PMA induced-NETs[162]	Secondary granules	SLE	Properdin levels are decreased in SLE sera Case report of anti-properdin antibodies in SLE. Properdin is present in AAV kidney biopsies	163 164 165

AAV, antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis; AENO, alpha enolase; ICx, immune complexes; IF, Immunofluorescence; LAMP-2, lysosomal membrane protein-2; LN, lupus nephritis; MPO, myeloperoxidase; NET, neutrophil extracellular trap; PMA, phorbol myristate acetate; PR3, proteinase-3; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus.

The main antigens for ANCAs, (i.e. MPO and PR3), which both originate from the azurophilic granules of neutrophils, were demonstrated on NETs[39,50]. In addition, co-localization of NETs (determined as extracellular histones) with MPO and PR3 was demonstrated in kidney biopsies of AAV patients[34,39]. Autoantigens for atypical ANCAs, including azurocidin[51,52], cathepsin G[53], elastase[53,54], lactoferrin[53,54], lysosomal membrane protein-2[55] and lysozyme C[54], were demonstrated on NETs (Table 1). These atypical ANCAs are sometimes present in AAV patients[51], but also commonly associated with other systemic inflammatory diseases[51].

SLE patients, and especially those with immune complex (ICx)-mediated LN, can present with a wide range of circulating autoantibodies (>180 specificities) that recognize, among others, dsDNA, histones, nucleosomes, and extractable nuclear antigens[56]. Many of these SLE-specific autoantigens can be found on NETs[49] (Table 1), whereas some extractable nuclear antigens, including Ro, La, Smith and ribonucleoprotein have not yet been identified on NETs[40,49,57]. The combination of autoantibodies that recognize autoantigens on NETs convert these structures into highly immunogenic ICxs that can engage with TLRs and Fc-γ receptor (FcγR)IIA[48].

Altogether, these cumulative data demonstrate that i) NETs are immunogenic; ii) NETs can directly mediate cytotoxicity to the glomerular tuft; iii) NETs contain relevant AAV and SLE-autoantigens and contribute to induction of autoimmunity.

TRIGGERS AND PATHWAYS OF NET FORMATION

Since the discovery of NETosis in 2004[3], the triggers and mechanisms of NET formation *in vitro* have been extensively studied, but unfortunately the exact *in vivo* processes remain to be fully elucidated[5,58,59]. A profound understanding of the triggers and intracellular pathways leading to NETosis in autoimmune diseases is important to understand their role in disease pathophysiology and identify potential, novel therapeutic strategies.

Over the years, a wide range of chemical and physiological triggers have been identified that can trigger NET formation *in vitro*. It is important to realize that although different stimuli can result in NET formation (i.e. the release of neutrophil-derived DNA to the extracellular space), it often involves signaling through distinct pathways[16,18,46,60,61]. The main pathways that have been demonstrated to be involved in different forms of NET formation include activation of protein kinase C (PKC)[62], NADPH-oxidase[58,59], reactive oxygen species (ROS)[63], Raf-mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway[64], MPO/neutrophil elastase (NE) complex[65], autophagy[5,55], microtubule polymerization[66] and protein arginine deiminase (PAD)-4/histone citrullination[67-69]. In addition, during NET formation, the breakdown of the nuclear envelope will need to occur, which resembles the nuclear envelope disintegration during mitosis in dividing cells[70]. In the following, we will focus on preclinical studies of known triggers and pathways of NET formation.

In vitro NET formation has been primarily studied after stimulation with PMA[3], a robust chemical compound that induces massive NET formation through PKC signaling, calcium influx and ROS production[16]. Subsequently, the azurosome is activated, which is a complex of MPO, NE, and cathepsin G, which leads to chromatin decondensation[65,71], rupture of the plasma membrane and release of chromosomal DNA[58,61]. PMA-induced NET formation is strictly dependent on NADPH-mediated ROS production[16,58]. This was primarily demonstrated in neutrophils derived from patients with chronic granulomatous disease that have mutations in their NADPH oxidase complex; therefore their neutrophils are unable to produce ROS, and are incapable of NET formation induced by PMA[16,58]. In line with this, PMA-induced NET formation can effectively be blocked by diphenyleneiodonium[72]. In addition, PMA-induced NET formation does not usually involve PAD enzymes, because PMA activates PKCα, which inhibits PAD enzymes intracellularly[73]. Consequently, citrullination of histones is generally low on PMA-induced NETs[16].

The latter is in contrast to another widely used trigger of NET formation, calcium ionophores (CIs), which trigger DNA release through a calcium-dependent hyperactivation of PAD enzymes[17], and results in hypercitrullination of histones[16,17]. This process is independent of PKC and ROS[68]. Importantly, in some studies, PAD enzyme inhibition led only to a limited inhibition of CI-induced NET formation, which implied that citrullination itself might not be a prerequisite[74]. CI-induced NET formation is intrinsically distinct from PMA-induced NET formation, but in the end, both pathways result in neutrophil-derived extracellular DNA release. Importantly, the citrullination of histones, as indicated by citrullinated histone H3 (CitH3) staining, is much more evident on CI-induced NET formation compared with PMA-induced NET formation[17].

The involvement of PAD enzymes in NET formation originally came from the observation that PAD4 deficient mice could not make NETs (as measured by CitH3-positive NETs), when stimulated with CIs[67,75]. Because murine neutrophils are distinct from human neutrophils[76], results from mouse neutrophil experiments do not always directly translate to humans[77]. For instance, there is a different balance of lymphocytes and neutrophils between humans and mice: human blood contains mainly neutrophils (50-70% neutrophils, 30-50% lymphocytes), whereas mouse blood contains mainly lymphocytes (75-90% lymphocytes versus 10-25% neutrophils)[78]. Also in contrast to human neutrophils, murine neutrophils do not express defensins[79], FcαRI, FcγRIIA and FcγRIIC[80] and various chemokines (e.g., IL-8)[76]. Moreover, histones present in NETs can, but will not always, undergo post-translational modifications, such as citrullination[81]. Thus, studies investigating only the presence of CitH3-positive NETs as a quantitative measure for total NET formation potentially neglect CitH3-negative NETs, which are especially present when PAD enzymes are inhibited[16,17,82]. Thus, citrullination of NET-related histones can occur during NET formation, but is not required for NET formation[16,17,73,83]. As such, PAD inhibition can decrease NET formation dependently of the trigger used to induce NETs and will always result in decreased or absent citrullination of histones[17,75]. Therefore, the interpretation of CitH3 as quantitative NET marker should be used with consideration[16,17].

All of the previously described triggers of NET formation involved lysis of the membrane, which is named lytic NET formation[18]. Lytic NET formation is also referred to as suicidal NET formation, which typically takes a few hours, involves NADPH oxidase and ROS, and result in plasma membrane lysis, and consequently, DNA release, after which the neutrophil dies[46].

In contrast, there are studies that demonstrated a non-lytic form of NET formation, which is also referred to as vital NET formation[18,19,84]. During non-lytic NET formation, the neutrophils stay alive and retain their capability of phagocytosis[19]. In contrast to the classic lytic forms, it does not involve the plasma membrane rupture, and DNA is released through blebbing of vesicles[85]. [9,84]

Non-lytic NET formation can be triggered by lipopolysaccharide (LPS)[86], micro-organisms[19,85,87,88], TLR4-activated platelets[86,89], complement proteins together with TLR2 ligands[19], granulocyte-macrophage, colony-stimulating factor in combination with TLR4 or C5a[84], TLR9 triggering by CpG or non-CpG[9], or SLE-specific ICx[21,42,90-92]. Non-lytic NET formation is triggered within minutes[85], and there is still controversy whether this is dependent on NADPH oxidase[42,84] or independent of NADPH oxidase[9,85,86,90]. However, chronic granulomatous disease patients who lack NADPH oxidase rely on mitochondrial ROS and form mitochondrial DNA enriched NETs. Moreover, non-lytic NETs were demonstrated to be enriched for interferogenic mitochondrial DNA[9,84]. The involvement of PAD enzymes and citrullination has not been studied in-depth for vital NET formation[17], but it was shown that Leishmania parasites induce vital NET formation within 10 minutes independently of both ROS and PAD enzymes[88]. Taken together, these data demonstrate that NET formation is a highly specific regulated process that can be triggered by a wide range of different stimuli, all engaged on a different molecular pathway before finally leading to the extrusion of neutrophil-derived DNA in the extracellular environment. The involvement of the different pathways is intrinsically dependent on the specific trigger of NET formation. Therefore, the elucidation of the disease-specific triggers of NET formation and the pathways that are involved is essential to understand the role of NETs in autoimmune diseases such as AAV and SLE.

NETS IN RENAL AUTOIMMUNE DISEASES

NET formation in autoimmune glomerulonephritis

In healthy humans, the formation of NETs has an antimicrobial function[4] and is counter-balanced by the physiological degradation of NETs by DNAse[93]. As expected, both excessive NET formation and impaired NET degradation has been demonstrated to play an important role in the pathogenesis of renal autoimmune diseases, including AAV and SLE[21,24,27,93,94].

Recently, we demonstrated that an excess in *ex vivo* NET formation was characteristic for both patients with active AAV[95] and patients with severe SLE[91]. We also made a side-by-side comparison of AAV- and SLE-induced NET formation using confocal microscopy and immunohistochemistry, and showed lytic NET formation within hours in AAV versus nonlytic NET formation with clustering of NET-ting neutrophils within minutes in SLE (Figure 1) (Chapter 4). Moreover, it was demonstrated that AAV-induced NET formation involved NADPH oxidase and PAD enzymes[95], whereas SLE-induced NET formation was independent of NADPH oxidase[90,92]. Recently, several studies linked necroptosis, a lytic form of cell death that is mediated by receptor interacting protein kinase (RIPK) and mixed lineage kinase domain (MLKL), to lytic NET formation[96], and specifically, to AAV-induced NET formation[97]. In contrast, SLE-induced NETs have immunogenic properties with the presence of HMGB1[21] and oxidized mitochondrial DNA[42], which has not been seen on AAV-induced NETs (Chapter 4). NETs can also frequently contain post-translational modifications (e.g., acetylation, methylation, citrullination)[41,47,68,81]. In SLE, this leads up to the development of autoantibodies against modified histones[47,81], for instance, acetylated and methylated histones[81,98-100], and modified ubiquinated MPO[101]. Of note, ubiquinated-MPO-enriched NETs are highly capable of activating macrophages[101]. In AAV, NETs were specifically enriched for citrullinated histones (Chapter 4), which have been linked to cause crescentic GN in preclinical models[35]. Another important distinction are the triggers of excessive NET formation which is IgG-dependent in SLE[91] but independent of IgG in AAV[95].

Taken together, cumulative evidence demonstrates that NET formation is not equal in SLE- and ANCA-associated renal autoimmune diseases and can be linked to the distinct forms of GN observed in these patients. Features of AAV- and SLE-induced NET formation closely associated with the respective, typical features of pauci-immune, histone-induced crescentic GN in AAV and ICx-mediated full-house LN in SLE (Figure 2).

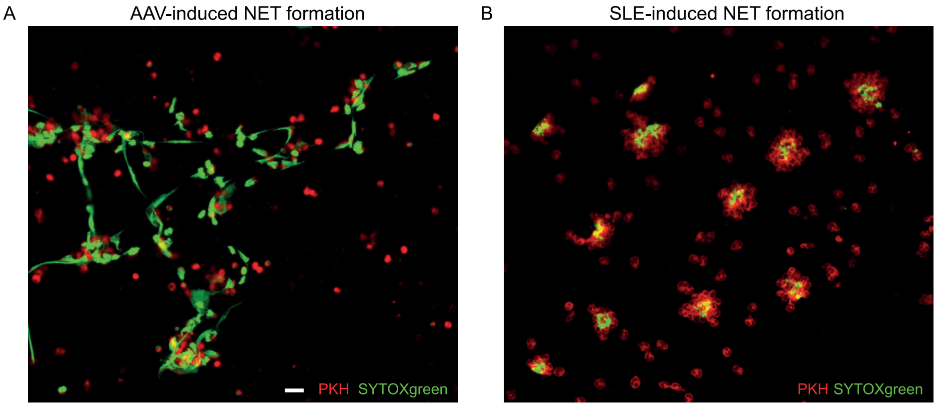

Figure 1. ***Ex vivo* neutrophil extracellular trap formation (NET) in antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) and systemic lupus erythematosus (SLE).** Paul-Karl-Horan (PKH)26-labelled neutrophils (red) derived from a healthy donor were exposed to 10% serum of patients with AAV or SLE for 4 hours to induce neutrophil extracellular trap (NET) formation. Extracellular DNA was stained with SYTOXgreen (green) and NET formation was imaged with immunofluorescence confocal microscopy. Images of AAV- **(A)** and SLE-induced **(B)** NET formation are shown at original magnification x10; scale bar = 20um.

NET degradation in autoimmune glomerulonephritis

As mentioned before, NETs also have a physiological role and become potentially pathogenic when they are not degraded efficiently[93,94]. For SLE patients, impaired degradation of NETs and other apoptotic material was associated with severity of lupus disease, and notably, LN[94,102]. The underlying reason for impaired NET degradation was demonstrated to be dependent upon at least two mechanisms in these SLE patients: (I) the presence of DNase1 inhibitors was shown to reduce the capacity of NET degradation; and (II) the presence of anti-NET antibodies (i.e., a mix of antibodies against nuclear material) formed complexes that prevented the enzymatic degradation of NETs by the DNAse enzyme[24,94]. These phenomena were also shown to be present in MPO^{+} AAV patients who exhibited lower rates of NET degradation[93].

Although impaired DNase1 is proposed as the main regulator NET degradation, it is unclear whether this enzyme can function in the tissues; impaired DNase activity was not associated with disease activity in AAV patients[93,103]. Macrophages have been reported as important effector cells that clear NETs. Defective phagocytosis by macrophages of mice deficient in milk fat globule epidermal growth factor-8 developed GN[104] and also lupus-prone mice with defective macrophages through deficiency of caspase activated DNase resulted in higher anti-DNA antibody levels[104]. Moreover, patients with acute respiratory distress syndrome (ARDS) demonstrated enhanced NET formation and diminished macrophage engulfment[105]. Until now, no study has investigated the clearance of NETs by macrophages in AAV or SLE patients; however, this could be a potential contributing mechanism to the pathophysiology of these autoimmune diseases.

In summary, several studies demonstrated that both excessive NET formation and reduced NET degradation is a common autoimmune phenomenon found in AAV and SLE. However, the triggers and pathways leading to excessive NET formation in these renal autoimmune diseases are intrinsically different.

Excessive NET formation: focus on AAV

The role of NET formation in AAV was initially demonstrated by Kessenbrock *et al*. who observed that isolated ANCA was capable of inducing NET formation, and that NET structures were detected in renal biopsies of AAV patients[39]. Although, these findings were subsequently confirmed[93,106,107] others showed *in vivo* that circulating NET remnants were highly present in AAV patients with active disease but had an inverse correlation to serum ANCA levels[108]. Recently, we demonstrated that NET formation was induced by IgG-depleted AAV sera[95], whereas IgA was not involved. These data suggested that not ANCAs, but other coinciding factors, affected neutrophils to form NETs *in vivo* whereas the exact triggers controlling AAV-induced NET formation still remain unknown.

Importantly, AAV-induced NETs are pro-inflammatory[97], and were demonstrated to mediate vascular injury through inflicting endothelial injury *in vitro*[97,108]. As discussed previously, both processes are linked to glomerular injury and crescentic GN in AAV[34,35]. In addition, AAV-induced NETs are able to activate the alternative pathway of complement[97,109], which is an important contributor to AAV pathogenesis, as demonstrated by the clinical success of C5aReceptor blockade in patients[110].

In summary, NETs have a high clinical relevance in the pathophysiology of AAV because AAV patients display both an excessive formation and impaired degradation of NETs. NETs contain the main autoantigens for AAV and directly cause cytotoxicity, which leads to crescentic lesions in pauci-immune GN in AAV.

Excessive NET formation: focus on SLE

In the earliest publications that claimed NETs were related to SLE disease pathogenesis, investigators found that SLE neutrophils released significantly increased levels of DNA referred to as spontaneous NET formation[24]. It has been known for a long time that neutrophils of SLE patients are different from those of healthy people; the existence of a subgroup of low-density granulocytes was demonstrated in 1986[111]. Later, it was demonstrated that these neutrophils had an increased capability to form NETs[44,112,113].

Morphologically, SLE sera can induce typical clustering of neutrophils[114-116]. This phenomenon of neutrophil clustering preceded the discovery of NETs and has been known since 1990[115,116]. Clustering of neutrophils upon stimulation with SLE sera was correlated with lupus disease activity and was associated with the presence of anti-C1q autoantibodies[116]. During SLE-induced NET formation, we observed a nonlytic form of NET formation that coincided with clustering of neutrophils (Chapter 4). Importantly, SLE-induced NET formation was demonstrated to be NADPH/ROS-independent[90,92] and resulted in release of mitochondrial DNA[84], which are characteristics of nonlytic NET formation. SLE-induced NET formation can be triggered by ICx[21,91] or apoptotic microparticles[92]. Ribonucleoprotein-ICx, which are specifically present in SLE, triggered NET formation in a NADPH-dependent manner. These NETs also contained oxidized mitochondrial DNA[42].

SLE-induced NETs are believed to be highly immunogenic because they contain oxidized mitochondrial DNA[42], HMGB1[21], and LL37[21]. In addition, SLE-induced NETs can form ICx[24,102] and activate the complement system *in vitro*[102]. HMGB1-nucleosome complexes were previously shown to induce an anti-dsDNA response in a TLR2 dependent manner in a non-autoimmune mice model, which supported an important role for the combination of NETs with danger-associated molecular patterns in SLE[117,118]. Recently, it was also demonstrated that LL37-DNA complexes originating from NETs are able to directly trigger autoantibody production by SLE memory B-cells through endosomal uptake of LL37 and subsequent TLR9 receptor signaling[27]. This study identified an important link between NETs and autoreactive B-cells in SLE. Importantly, the excess of circulating NETs in SLE patients was associated with severe organ inflammation, and specifically, LN[94]. Moreover, impaired degradation of NETs was also shown to be associated with SLE flares, disease activity, high autoantibody levels, and complement consumption[102].

In summary, these data demonstrated the clinical relevance of NET formation in SLE, especially in ICx-mediated LN. NETs are involved in the pathophysiology of SLE: NETs induce autoantibodies that lead to ICx formation with NETs, which subsequently trigger more NET formation, causing a perpetuating, vicious cycle in SLE patients.

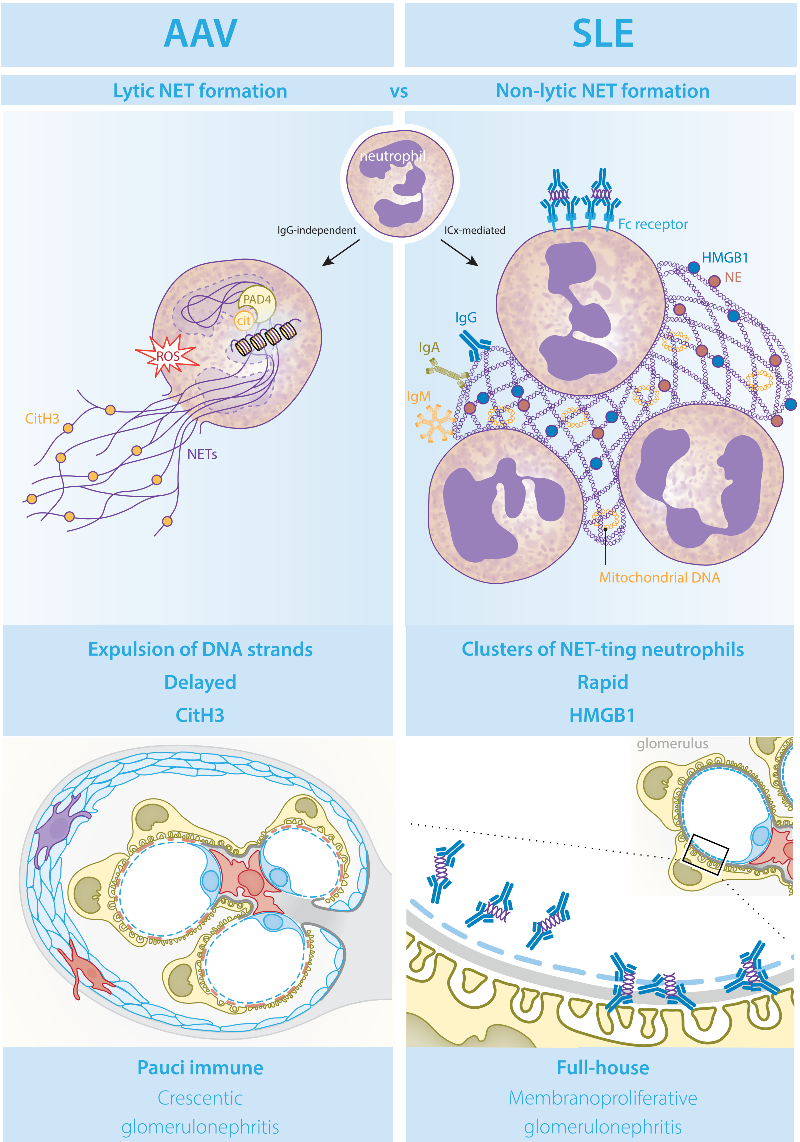

Figure 2. Overview of neutrophil extracellular trap (NET) formation in antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) versus systemic lupus erythematosus (SLE). In AAV, lytic NET formation is induced involving reduced NAD phosphate oxidase and protein arginine deiminase (PAD) enzymes, which results in a lytic expulsion of NETs harboring citrullinated histones within hours. In SLE, non-lytic extrusion of NETs concomitant with clustering of neutrophils is induced within minutes. SLE-induced NETs have immunogenic properties including enrichment for High Mobility Group Box Protein 1 (HMGB1), oxidized mitochondrial-derived DNA and immune complex formation (ICx) which was not the case for AAV-induced NETs. ROS, reactive oxygen species.

Unknowns about NET formation in renal autoimmune diseases

It has become apparent that lytic NET formation is associated with chromosomal DNA subject to post-translational modifications while nonlytic NET formation is enriched for mitochondrial DNA. Despite extensive preclinical studies on the mechanisms underpinning lytic and nonlytic NET formation, the clinical and translational studies in SLE and AAV are much more challenging and less unambiguous. As previously described, controversy remains as to whether NETs can be triggered *in vivo* by ANCA[39] or not[95], whether *in vivo* NADPH oxidase/ROS is involved in SLE[42] or not[90,92], and the extent of mitochondrial DNA versus chromosomal DNA present in SLE-induced NETs[27,42]. Therefore, to better understand NET formation in autoimmune diseases, it is realistic to postulate that there is not only a sole mechanism of NET formation ongoing *in vivo* but rather that different forms of NET formation occur in parallel. Attention will need to be given to the chosen stimulus to induce NETs (e.g. whole serum versus purified autoantibodies) and the use of healthy or AAV- or SLE-derived neutrophils for future studies.

THERAPEUTICS TARGETING NET FORMATION

There are several hypotheses on developing therapeutic targets that could interfere with NET formation. Because different forms of NET formation occur in AAV and SLE patients, it can be anticipated that the effects of potential therapeutic approaches will also be different. Obviously, depletion of neutrophils is not attractive because of the high risk of infection in patients with neutropenia. However, engagement of signal inhibitory receptor on leucocyte-1, which is a specific protein expressed on phagocytes that negatively regulates neutrophil function[119], directly targets neutrophils without depleting them or diminishing their pro-inflammatory capabilities while reducing SLE-induced NET formation *in vitro*[119]. A summary of reported, potential approaches that reduced NET formation *in vitro* are summarized in Table 2 and include targeting ROS with diphenyleneiodonium[62], targeting mitochondrial ROS with MitoTEMPO[42], a mitochondrially targeted antioxidant, or N-acetylcysteinine (NAC)[120]; inhibiting PAD enzymes by chlooramidine[121]; or enhancing breakdown of NETs with DNase1[122]. Thus far, none of these approaches have been successfully applied as a therapeutic approach. Recently, a novel antibody specifically targeting histones 2A and 4, named therapeutic anti-citrullinated protein antibodies (tACPA), demonstrated *in vitro* inhibition of CI-induced NET formation[123]. Also, Tofacitinib, a Janus kinases (JAK)/signal transducer and activator of transcription proteins (STAT) inhibitor reduced both spontaneous and LPS-induced NET formation in a mouse model of lupus[124] and is currently under clinical investigation in SLE patients (NCT02535689). Metformin was evaluated as a proof-of-concept treatment in a large cohort of SLE patients and demonstrated a reduction of *in vitro* PMA-induced NETs through an unknown mechanism and a decrease in flares of SLE patients[125]. In addition, vitamin D decreased PMA-induced NET formation of SLE neutrophils *in vitro*[126]. So far, there are no data on the effect of mycophenolate mofetil, azathioprine, rituximab and cyclophosphamide on NET formation in AAV or SLE. However, corticosteroids, the cornerstone of induction treatment for both AAV and SLE patients, were demonstrated to impair ROS production by granulocytes and inhibit NET formation *in vitro* for both mouse and human neutrophils[127].

Table 2. Potential NET-targeted therapies in glomerular diseases.

Treatment	Target
ANCA-associated vasculitis	
DPI	NADPH
Chlooramidin	PAD enzymes
Corticosteroids	ROS, CLEC7A
C5a receptor antagonist	C5a receptor antagonist
NEC-1, NSA	Necroptosis pathways
Vitamin D	Unknown
Eculizumab	C5a mAb
Systemic lupus erythematosus	
NAC	ROS scavenger
MitoTEMPO	Mitochondrial ROS scavenger
DNase 1	DNA
tACPA	Histones 2A, 4
SIRL-1	SIRL-1
Tofacitinib	Inhibition of JAK STAT
Metformin	Unknown mechanism
Corticosteroids	ROS, CLEC7A
Vitamin D	Unknown
Eculizumab	C5a

Effect on NET formation	Clinical effect
Abrogation of PMA-induced NET formation[62]	Not tested
Decreased NET formation in mouse model[121]	Protection against renal, skin and vascular manifestations in mice models
Decreased *in vitro* (mouse & human) and *in vivo* (mouse) NET formation[166]	Effective and widely used FDA-approved therapy
Decreased NET formation and neutrophil activation[110,167]. Did not affect AAV serum induced NET formation[95]	Effective and safe in phase III study[110]
Decreased AAV-induced NET formation[97]	Not tested
Reduced PMA-induced NET formation *in vitro*[126]	Improved endothelial function in SLE patients
Did not affect AAV serum induced NET formation[95]	Case reports: effective and safe[149]
Decreased NET release[120,168]	Reduced disease activity in patients
Decreased NET formation and decreased oxidation of nucleic acids in NETs leading to decreased immunogenicity and IFN responses[42]	Reduced disease activity in mice
Enzymatic degradation of NETs[122,169]	Reduction of autoantibodies, proteinuria, delayed mortality in mouse model. Safe in phase I study, no change in disease activity
Inhibition of calcium ionophore induced NET formation[123]	Not tested
Inhibition of SLE-induced NET formation[119]	Not tested
Reduced spontaneous and LPS-induced NETs in mouse model of lupus[124]	Not tested
Reduced PMA-induced NET formation, decreased CPG-stimulated pDC IFN production[125]	Decreased clinical flares, prednisone exposure
Decreased *in vitro* (mouse & human) and *in vivo* (mouse) NET formation[166]	Effective and widely used FDA approved therapy
Reduced NET formation *in vitro*[126]	Improved endothelial function in SLE patients
Reduced NET formation and neutrophil activation[170,171]	Improved survival mouse model, safe and decreased haemolytic activity in SLE patients[172]

Table 2. Continued.

Treatment	Target
RTX+BLM	Plasma cells → ICx formation
PIC1	Complement protein 1
HCQ	TLR9
Anifrolumab	IFN inhibitors
Calcineurin inhibitors	T cell activation

AAV, antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis; BLM, belimumab; DPI, diphenyleneiodonium; FDA, Food and Drug administration; ICx, immune-complex formation; HCQ, hydroxychloroquine; IFN, interferon; JAK, Janus kinases; NAC, N-acetyl cysteine; NEC-1, necrostatin-1; NSA, necrosulfanomide; pDC, plasmacytoid dendritic cell; PIC1,

Another potential successful approach can be to target the known triggers of NET formation (Table 2). In AAV, because the exact triggers are still unknown, C5a in combination with granulocyte-macrophage colony stimulating factor was reported to induce NET formation[84], and C5a receptor inhibition with avacopan was demonstrated to be clinically effective in AAV patients[110]. Although C5a, one of the components of the terminal complement system, has an important role in AAV[128,129], *in vitro* C5a receptor blockade or the C5 antibody eculizumab were not able to inhibit AAV-induced NET formation[95]. Another therapeutic approach in AAV was provided by recent studies that indicated that AAV-induced NET formation might involve the RIPK/MLKL-mediated necroptosis pathway. *In vitro* inhibition of the RIPK-complex by necrostatin-1 and inhibition of MLKL by necrosulfanomide both reduced AAV-induced NET formation[97]. Therefore, future studies that investigate the potential of therapeutic RIPK/MLKL pathway inhibitors (ClinicalTrials.gov NCT02903966) are of high interest.

In SLE, ICx are mainly responsible for excessive NET formation; therefore, the effective eradication of ICx could decrease NET formation[91]. Eradication of autoantibodies as defined by seroconversion (to negative) upon immunosuppressive treatment is not

Effect on NET formation	Clinical effect
Decreased NET formation[91]	Reduction of anti-dsDNA, anti-histones, anti-nucleosomes, anti-C1q, decreased disease activity[91]
Inhibition of ICx-induced NET formation Inhibit NET formation by human neutrophils stimulated by PMA, MPO, or immune complex activated human sera[130]	Not tested
Decreased LPS-induced NET formation[131] Decreased IgG production of NET-stimulated SLE B-cells[27]	Effective and widely used FDA approved therapy
Anifrolumab decreased neutrophil NET complexes[132,173]	Reduced disease activity
Modulation of calcium pools Reduced NET formation[133]	Improvement of renal disease[174] Voclosporin: NCT03021499

Peptide Inhibitor of Complement C1; PMA, phorbol myristate acetate; ROS, reactive oxygen species; RTX, rituximab; SIRL-1, Signal inhibitory receptor on leukocytes-1; SLE, systemic lupus erythematosus; STAT, signal transducer and activator of transcription proteins; TLR9, Toll-like receptor-9;

a major endpoint in clinical studies, although significant reductions in autoantibody levels can be observed. Recently, we showed that combined B-cell targeted treatment with rituximab combined with belimumab in patients with severe SLE resulted in a significant decrease of anti-dsDNA antibodies, which also associated significantly with decreased excessive NET formation *in vitro*[91]. Peptide inhibitor of complement factor C1 (compound name: PA-dPEG24) inhibited the activation of the classical complement pathway by ICx *in vitro* and also decreased NET formation when induced by PMA, MPO or heat-aggregated ICx[130]. Hydroxychloroquine, an effective and widely-used therapy in SLE patients inhibits the DNA-sensing TLR9 pathway and was demonstrated to inhibit IgG secretion by B-cells stimulated with NET-derived LL37-DNA complexes[27]. In addition, LPS-induced NET formation was inhibited when human healthy and lupus neutrophils were pre-treated with hydroxychloroquine[131]. Anifrolumab, an interferon-a receptor antagonist was investigated in a randomised clinical trial in SLE patients; it demonstrated an inhibitory effect on NET formation, which was not observed in the placebo arm, and showed promising clinical efficacy[132]. In addition, NET formation assessed by measuring 3 types of DNA complexed to MPO-, NE- or CitH3, as related to NETs, was significantly higher in SLE patients with a simultaneously high interferon signature status. The latter two studies indicated an association of NET formation with the interferon signaling pathway. Finally, there was some evidence that the calcineurin inhibitors, such as cyclosporine A, have an inhibitory effect on NET formation[133]. Calcineurin inhibitors showed promising clinical efficacy for LN patients[134].

In summary, there are several reports on therapeutics that are able to target NET formation in AAV and in SLE. These involve both newly developed but also currently used standard of care therapeutics. The distinct disease-specific forms of NET formation should be taken into account when evaluating targeted therapies at NET formation. Diminishing NET formation in AAV and SLE patients has been suggested to have a beneficial clinical effect based on reported preclinical and a few small clinical studies. Because NETs have a pivotal role in the pathophysiology of renal autoimmune diseases, targeting NETs might be clinically relevant for AAV and SLE patients.

NETS AS A BIOMARKER OF DISEASE ACTIVITY

As mentioned previously, NET formation has been demonstrated to be involved in the pathophysiology of renal autoimmune diseases. Therefore, NET formation could be a potential biomarker for disease activity. Both AAV and SLE are characterized by relapses and remissions of disease. We and others demonstrated that excessive NET formation is predominantly seen in AAV patients with active disease and is low in AAV patients who were in remission or during an infection[95]. In addition, one study demonstrated a longitudinal association of excessive NET formation with active disease within individual AAV patients[108]. In contrast, NETs, as measured by cell free DNA or MPO-DNA complexes, were not associated with disease activity in AAV patients by a third group[103]. We recently also demonstrated that in SLE patients treated with rituximab (RTX) + belimumab (BLM), excessive NET formation correlated with disease activity[91]. Moreover, it was demonstrated that NET formation of SLE neutrophils were significantly correlated with the titer of anti-LL37 autoantibodies in serum of SLE patients[27]. Although the early identification and even prediction of disease flares would be advantageous to manage both AAV and SLE patients, the potential of NET formation as a possible biomarker has not been extensively studied yet. It is important to note that there is no gold standard to measure NET formation. The current methods used to evaluate NET formation in patients range from enzyme-linked immunosorbent assays, immunohistochemistry, immunofluorescence, and flow cytometric assays[135]. All of these methods have their own specificity, objectivity and ways to quantify NETs. Additional complexity is introduced by the different triggers used to induce and measure NETs[16,17]. Nevertheless, it is compelling to postulate that NET formation could be a measure of the autoantigenic load in patients and could plausibly be related to disease activity, remission, or predict relapses. As such, it would be of interest to investigate and quantify autoantigen formation in analogue to autoantibody formation throughout the course of follow-up of AAV and SLE patients.

CONCLUSIONS

The accumulating evidence on the pathogenic role of excessive NET formation in AAV and SLE and its relation to their respective forms of GN confirm the clinical relevance of NET formation in renal autoimmune diseases. NETs are a source of autoantigens in both AAV and SLE, are involved in shaping the humoral autoimmune response and cause direct glomerular inflammation and damage. Excessive NET formation and impaired degradation of NETs are jointly autoimmune phenomena that can lead to disease-relevant autoantibody production. Knowledge on the intrinsically distinct triggers and pathways of NET formation that are involved in AAV and SLE is growing and will undoubtedly foster further investigations into the potential of therapeutically targeting NET formation and the use of NETs as biomarkers.

Future studies on AAV- and SLE-induced NET formation should focus on the identification of specific NET-associated molecules, preferably assessed with proteomic-based approaches (Table 3). In addition, future studies should focus on evaluating if quantifying NET formation could serve as a biomarker for disease activity and/or prognosis in AAV and SLE patients. Finally, therapeutic targets should be identified that could potentially regress excessive NET formation or increase NET degradation in these renal autoimmune diseases. Together, addressing these research questions will increase our understanding of the *in vivo* NET formation processes in AAV and SLE.

Table 3. Research questions for future translational research.

	Research questions
1.	Which NET-associated proteins are specifically present on AAV- and SLE-induced NETs as identified through proteomics?
2.	Could the quantification of NET formation serve as a biomarker for disease activity and prognosis in relation to conventional and novel therapies in AAV and SLE patients?
3.	Which targets can be identified that are capable of regressing excessive NET formation or increase NET degradation that could translate to a therapeutic approach in AAV and SLE?

AAV, antineutrophil cytoplasmic antibodies (ANCA)– associated vasculitis; NET, neutrophil extracellular trap; SLE, systemic lupus erythematosus.

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