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The Netherlands

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

Dam, L.S. van

### Citation

Dam, L. S. van. (2022, February 22). *Targeting autoimmunity in renal diseases: focus on neutrophil extracellular traps and autoreactive B-cells*. Retrieved from <https://hdl.handle.net/1887/3275833>

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**Note:** To cite this publication please use the final published version (if applicable).

# 2

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

Laura S. van Dam, Ton J. Rabelink,  
Cees van Kooten, Y.K. Onno Teng

*Kidney International Reports* 2019

## 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<sup>1</sup>. 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<sup>2</sup>. 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)<sup>3,4</sup>.

NETosis is a process that results in the release of extracellular DNA by neutrophils, which was originally believed to coincide with cell death<sup>5</sup> and is phonetically classified among other regulated cell death (RCD) pathways, such as apoptosis, pyroptosis, necroptosis, and ferroptosis<sup>6</sup>. After the discovery of NETosis, similar processes have been described in other immune cells, including eosinophils<sup>7</sup>, monocytes<sup>8</sup> and B-cells<sup>9</sup>, 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<sup>10</sup>. 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<sup>6</sup>. Necroptosis and pyroptosis have been demonstrated to be relevant to fighting bacterial and viral infections<sup>6</sup>, whereas ferroptosis has been implicated in cancer cell death and tissue injury<sup>11</sup>. NETosis is a caspase-independent process, but the studies are seemingly unclear on how to classify the process as immunogenic<sup>12,13</sup>, or even anti-inflammatory<sup>14</sup>. This is mainly due to the fact that the pathways leading to NETosis are still evolving<sup>15</sup>, and many studies have demonstrated that distinct forms of the release of extracellular DNA by neutrophils exist<sup>16,17</sup>. 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<sup>18,19</sup>. NETs can also have anti-inflammatory effects which has been demonstrated in mice models of gout<sup>14</sup> and lupus-prone mice with defects in reduced NAD phosphate (NADPH) oxidase, which show a more severe phenotype<sup>20</sup>.



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<sup>21</sup> and lymphocytes<sup>22</sup>, both at sites of inflammation and in draining lymph nodes<sup>22</sup>. 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<sup>23</sup>. 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<sup>23</sup>.

## 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<sup>24</sup>. Only DNA in combination with danger signals, (i.e. danger-associated molecular patterns), such as LL37<sup>24,25</sup>, a cathelicidin anti-microbial peptide, or High Mobility Group Box Protein-1 (HMGB1)<sup>26</sup>, can activate antigen presenting cells, in particular, plasmacytoid dendritic cells (DCs)<sup>24</sup> and B-cells<sup>26,27</sup>. This is mediated through Toll-like receptor-9 (TLR9) signaling that results in the production of interferon- $\alpha$ <sup>21</sup> and (auto-)antibodies<sup>27</sup>. Preclinical mouse models demonstrated that *in vitro* monocyte-derived DCs take up DNA particles from neutrophils undergoing NETosis, apoptosis or necrosis<sup>28</sup>. This internalization is mediated via the receptor for advanced glycation endproducts (RAGE)-TLR9 pathway<sup>29,30</sup>. Transfer of these DNA-loaded monocyte-derived DCs, led to production of antibodies against dsDNA, MPO and proteinase-3 (PR3) in mice<sup>28</sup>. 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<sup>12,15</sup>.

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<sup>31-33</sup> and MPO<sup>33,34</sup>. 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<sup>35</sup>. 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<sup>33</sup>, which was associated with glomerular and interstitial injury in AAV patients<sup>34</sup>. In addition, endothelial cells have a limited capacity to internalize NETs<sup>36</sup>. 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<sup>36</sup>, 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<sup>37</sup> and SLE<sup>38</sup>. NETs are believed to be an important source of autoantigens in systemic autoimmune diseases<sup>12,21,24,25,30,39-48</sup>. Indeed, 74% of these identified NET-associated proteins are recognized by autoantibodies in systemic autoimmune diseases<sup>48</sup>. 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)<sup>49</sup>. 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<sup>16</sup>. 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 <sup>49</sup>      | Azurophilic Granules           | AAV          | Autoantibodies (atypical) present in AAV                                                | 52                    |
| Cathepsin G         | Proteomics of PMA-induced NETs <sup>49</sup>      | Azurophilic Granules           | AAV<br>SLE   | Autoantibodies (atypical) present in AAV<br>Autoantibodies are present in SLE           | 54,136<br>53,137      |
| Neutrophil Elastase | Proteomics of PMA-induced NETs <sup>49</sup>      | Azurophilic Granules           | AAV<br>SLE   | Anti-elastase antibodies present in AAV<br>Anti-elastase antibodies present in SLE      | 54,136,138            |
| Lactoferrin         | Proteomics of PMA-induced NETs <sup>49</sup>      | Secondary Granules             | AAV<br>SLE   | Atypical ANCA in AAV<br>Autoantibodies are present in SLE                               | 136,139<br>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<br>Detected in AAV kidney biopsies | 55,141,142            |
| Lysozym C           | Proteomics of PMA-induced NETs <sup>49</sup>      | 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 <sup>49</sup><br>IF of AAV-induced NETs & SLE-induced NETs <sup>93</sup>      | Azurophilic Granules             | AAV          | Typical autoantigen for ANCA in AAV<br>Detected in AAV kidney biopsies<br>Anti-MPO antibodies are sometimes present in SLE | 54<br>34,55<br>143      |
| PR3                    | Proteomics of PMA-induced NETs <sup>49</sup><br>Present on cell bodies of NET-ting neutrophils <sup>50</sup> | Azurophilic granules             | AAV          | Typical autoantigen for ANCA in AAV<br>Detected in AAV kidney biopsies                                                     | 55                      |
| Alpha actinin 1/4      | Proteomics of PMA-induced NETs <sup>49</sup>                                                                 | 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 <sup>49</sup>                                                                 | 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 <sup>153</sup>                                                             | 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 <sup>102</sup>                                               | -                                | SLE          | Anti-C1q antibodies are present in SLE and associated with disease activity                                                | 102,116,151,<br>154,155 |
| Catalase               | Proteomics of PMA-induced NETs <sup>49</sup>                                                                 | Peroxisomal                      | SLE          | Autoantibodies present in SLE                                                                                              | 156                     |
| Citrullinated histones | IF AAV-induced NETs <sup>95</sup> and SLE-induced NETs <sup>91</sup>                                         | 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                                                     | <sup>56</sup>                                      |
| Histones (H2A, H2B, H3, H4) | Proteomics of PMA-induced NETs <sup>49</sup>                                                           | Nucleus                 | SLE          | Autoantigen in SLE<br>Causing crescentic GN                                                                                                | <sup>35</sup>                                      |
| HMGB1                       | IF of RNP-ICx-induced NETs <sup>21</sup>                                                               | Nucleus                 | SLE          | Anti-HMGB1 antibodies levels correlate with disease activity, with anti-dsDNA Abs and with HMGB1 levels in SLE. HMGB1 binds (SLE)-ICx      | <sup>21,56,157</sup><br><sup>26,158</sup>          |
| HNP/ $\alpha$ defensins     | Proteomics of PMA-induced NETs <sup>49</sup> . IF of PMA induced NETs & SLE-induced NETs <sup>24</sup> | Azurophilic Granules    | SLE          | HNP binds SLE-ICx<br>Anti-HNP autoantibodies are present in SLE                                                                            | <sup>24,159</sup>                                  |
| LL-37                       | IF of PMA induced & SLE-induced NETs <sup>24</sup>                                                     | Nuclear                 | SLE          | Anti-LL37 antibodies are present in SLE<br>LL37 binds SLE-ICx                                                                              | <sup>21,27</sup><br><sup>160,161</sup>             |
| mtDNA                       | IF of RNP-ICx-induced NETs <sup>42</sup>                                                               | Mitochondria            | SLE          | Anti-mitochondrial antibodies are present in SLE patients                                                                                  | <sup>42,56</sup>                                   |
| Properdin                   | IF of AAV-induced NETs <sup>109</sup><br>IF of PMA induced-NETs <sup>162</sup>                         | Secondary granules      | SLE          | Properdin levels are decreased in SLE sera<br>Case report of anti-properdin antibodies in SLE. Properdin is present in AAV kidney biopsies | <sup>163</sup><br><sup>164</sup><br><sup>165</sup> |

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<sup>39,50</sup>. In addition, co-localization of NETs (determined as extracellular histones) with MPO and PR3 was demonstrated in kidney biopsies of AAV patients<sup>34,39</sup>. Autoantigens for atypical ANCAs, including azurocidin<sup>51,52</sup>, cathepsin G<sup>53</sup>, elastase<sup>53,54</sup>, lactoferrin<sup>53,54</sup>, lysosomal membrane protein-2<sup>55</sup> and lysozyme C<sup>54</sup>, were demonstrated on NETs (Table 1). These atypical ANCAs are sometimes present in AAV patients<sup>51</sup>, but also commonly associated with other systemic inflammatory diseases<sup>51</sup>.

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<sup>56</sup>. Many of these SLE-specific autoantigens can be found on NETs<sup>49</sup> (Table 1), whereas some extractable nuclear antigens, including Ro, La, Smith and ribonucleoprotein have not yet been identified on NETs<sup>40,49,57</sup>. The combination of autoantibodies that recognize autoantigens on NETs convert these structures into highly immunogenic ICxs that can engage with TLRs and Fc- $\gamma$  receptor (Fc $\gamma$ R)IIA<sup>48</sup>.

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<sup>3</sup>, 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<sup>5,58,59</sup>. 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<sup>16,18,46,60,61</sup>. The main pathways that have been demonstrated to be involved in different forms of NET formation include activation of protein kinase C (PKC)<sup>62</sup>, NADPH-oxidase<sup>58,59</sup>, reactive oxygen species (ROS)<sup>63</sup>, Raf-mitogen-activated protein kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway<sup>64</sup>, MPO/neutrophil elastase (NE) complex<sup>65</sup>, autophagy<sup>5,55</sup>, microtubule polymerization<sup>66</sup> and protein arginine deiminase (PAD)-4/histone citrullination<sup>67-69</sup>. 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<sup>70</sup>. 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<sup>3</sup>, a robust chemical compound that induces massive NET formation through PKC signaling, calcium influx and ROS production<sup>16</sup>. Subsequently, the azurosome is activated, which is a complex of MPO, NE, and cathepsin G, which leads to chromatin decondensation<sup>65,71</sup>, rupture of the plasma membrane and release of chromosomal DNA<sup>58,61</sup>. PMA-induced NET formation is strictly dependent on NADPH-mediated ROS production<sup>16,58</sup>. 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<sup>16,58</sup>. In line with this, PMA-induced NET formation can effectively be blocked by diphenyleneiodonium<sup>72</sup>. In addition, PMA-induced NET formation does not usually involve PAD enzymes, because PMA activates PKC $\alpha$ , which inhibits PAD enzymes intracellularly<sup>73</sup>. Consequently, citrullination of histones is generally low on PMA-induced NETs<sup>16</sup>.

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<sup>17</sup>, and results in hypercitrullination of histones<sup>16,17</sup>. This process is independent of PKC and ROS<sup>68</sup>. 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<sup>74</sup>. 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<sup>17</sup>.

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<sup>67,75</sup>. Because murine neutrophils are distinct from human neutrophils<sup>76</sup>, results from mouse neutrophil experiments do not always directly translate to humans<sup>77</sup>. 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)<sup>78</sup>. Also in contrast to human neutrophils, murine neutrophils do not express defensins<sup>79</sup>, FcαRI, FcγRIIA and FcγRIIC<sup>80</sup> and various chemokines (e.g., IL-8)<sup>76</sup>. Moreover, histones present in NETs can, but will not always, undergo post-translational modifications, such as citrullination<sup>81</sup>. 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<sup>16,17,82</sup>. Thus, citrullination of NET-related histones can occur during NET formation, but is not required for NET formation<sup>16,17,73,83</sup>. 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<sup>17,75</sup>. Therefore, the interpretation of CitH3 as quantitative NET marker should be used with consideration<sup>16,17</sup>.

All of the previously described triggers of NET formation involved lysis of the membrane, which is named lytic NET formation<sup>18</sup>. 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<sup>46</sup>.



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

Non-lytic NET formation can be triggered by lipopolysaccharide (LPS)<sup>86</sup>, micro-organisms<sup>19,85,87,88</sup>, TLR4-activated platelets<sup>86,89</sup>, complement proteins together with TLR2 ligands<sup>19</sup>, granulocyte-macrophage colony-stimulating factor in combination with TLR4 or C5a<sup>84</sup>, TLR9 triggering by CpG or non-CpG<sup>9</sup>, or SLE-specific ICx<sup>21,42,90-92</sup>. Non-lytic NET formation is triggered within minutes<sup>85</sup>, and there is still controversy whether this is dependent on NADPH oxidase<sup>42,84</sup> or independent of NADPH oxidase<sup>9,85,86,90</sup>. 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<sup>9,84</sup>. The involvement of PAD enzymes and citrullination has not been studied in-depth for vital NET formation<sup>17</sup>, but it was shown that *Leishmania* parasites induce vital NET formation within 10 minutes independently of both ROS and PAD enzymes<sup>88</sup>. 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

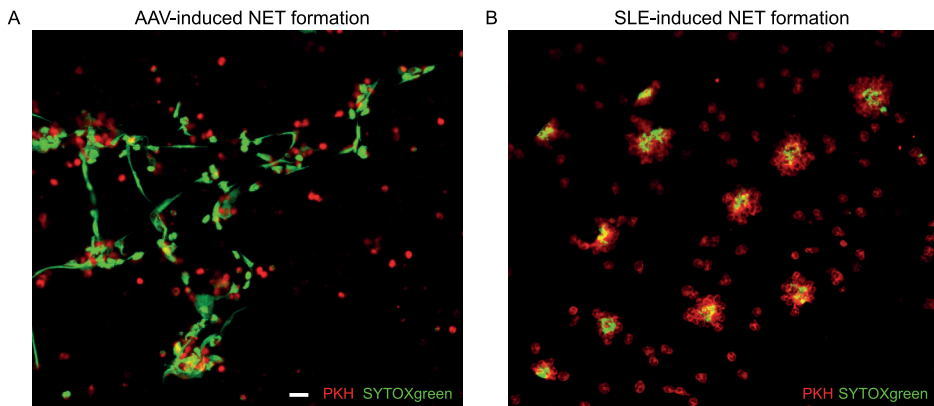
### NET formation in autoimmune glomerulonephritis

In healthy humans, the formation of NETs has an antimicrobial function<sup>4</sup> and is counter-balanced by the physiological degradation of NETs by DNase<sup>93</sup>. 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<sup>21,24,27,93,94</sup>.

Recently, we demonstrated that an excess in *ex vivo* NET formation was characteristic for both patients with active AAV<sup>95</sup> and patients with severe SLE<sup>91</sup>. 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<sup>95</sup>, whereas SLE-induced NET formation was independent of NADPH oxidase<sup>90,92</sup>. 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<sup>96</sup>, and specifically, to AAV-induced NET formation<sup>97</sup>. In contrast, SLE-induced NETs have immunogenic properties with the presence of HMGB1<sup>21</sup> and oxidized mitochondrial DNA<sup>42</sup>, which has not been seen on AAV-induced NETs (Chapter 4). NETs can also frequently contain post-translational modifications (e.g., acetylation, methylation, citrullination)<sup>41,47,68,81</sup>. In SLE, this leads up to the development of autoantibodies against modified histones<sup>47,81</sup>, for instance, acetylated and methylated histones<sup>81,98-100</sup>, and modified ubiquitinated MPO<sup>101</sup>. Of note, ubiquitinated-MPO-enriched NETs are highly capable of activating macrophages<sup>101</sup>. In AAV, NETs were specifically enriched for citrullinated histones (Chapter 4), which have been linked to cause crescentic GN in preclinical models<sup>35</sup>. Another important distinction are the triggers of excessive NET formation which is IgG-dependent in SLE<sup>91</sup> but independent of IgG in AAV<sup>95</sup>.

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<sup>93,94</sup>. For SLE patients, impaired degradation of NETs and other apoptotic material was associated with severity of lupus disease, and notably, LN<sup>94,102</sup>. 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<sup>24,94</sup>. These phenomena were also shown to be present in MPO<sup>+</sup> AAV patients who exhibited lower rates of NET degradation<sup>93</sup>.

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<sup>93,103</sup>. 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<sup>104</sup> and also lupus-prone mice with defective macrophages through deficiency of caspase activated DNase resulted in higher anti-DNA antibody levels<sup>104</sup>. Moreover, patients with acute respiratory distress syndrome (ARDS) demonstrated enhanced NET formation and diminished

macrophage engulfment<sup>105</sup>. 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<sup>39</sup>. Although, these findings were subsequently confirmed<sup>93,106,107</sup> 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<sup>108</sup>. Recently, we demonstrated that NET formation was induced by IgG-depleted AAV sera<sup>95</sup>, 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<sup>97</sup>, and were demonstrated to mediate vascular injury through inflicting endothelial injury *in vitro*<sup>97,108</sup>. As discussed previously, both processes are linked to glomerular injury and crescentic GN in AAV<sup>34,35</sup>. In addition, AAV-induced NETs are able to activate the alternative pathway of complement<sup>97,109</sup>, which is an important contributor to AAV pathogenesis, as demonstrated by the clinical success of C5aReceptor blockade in patients<sup>110</sup>.

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<sup>24</sup>. 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<sup>111</sup>. Later, it was demonstrated that these neutrophils had an increased capability to form NETs<sup>44,112,113</sup>.

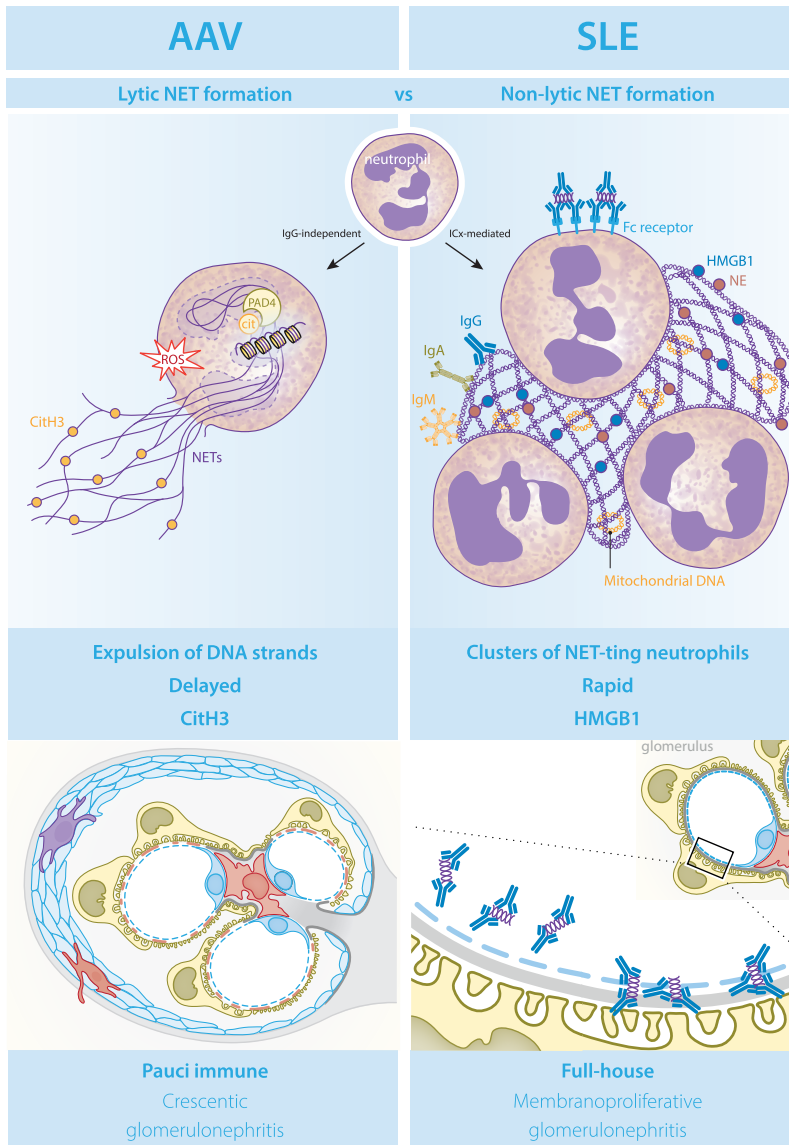




Morphologically, SLE sera can induce typical clustering of neutrophils<sup>114-116</sup>. This phenomenon of neutrophil clustering preceded the discovery of NETs and has been known since 1990<sup>115,116</sup>. Clustering of neutrophils upon stimulation with SLE sera was correlated with lupus disease activity and was associated with the presence of anti-C1q autoantibodies<sup>116</sup>. 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<sup>90,92</sup> and resulted in release of mitochondrial DNA<sup>84</sup>, which are characteristics of nonlytic NET formation. SLE-induced NET formation can be triggered by ICx<sup>21,91</sup> or apoptotic microparticles<sup>92</sup>. Ribonucleoprotein-ICx, which are specifically present in SLE, triggered NET formation in a NADPH-dependent manner. These NETs also contained oxidized mitochondrial DNA<sup>42</sup>.

SLE-induced NETs are believed to be highly immunogenic because they contain oxidized mitochondrial DNA<sup>42</sup>, HMGB1<sup>21</sup>, and LL37<sup>21</sup>. In addition, SLE-induced NETs can form ICx<sup>24,102</sup> and activate the complement system *in vitro*<sup>102</sup>. 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<sup>117,118</sup>. 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<sup>27</sup>. 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<sup>94</sup>. Moreover, impaired degradation of NETs was also shown to be associated with SLE flares, disease activity, high autoantibody levels, and complement consumption<sup>102</sup>.

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<sup>39</sup> or not<sup>95</sup>, whether *in vivo* NADPH oxidase/ROS is involved in SLE<sup>42</sup> or not<sup>90,92</sup>, and the extent of mitochondrial DNA versus chromosomal DNA present in SLE-induced NETs<sup>27,42</sup>. 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<sup>119</sup>, directly targets neutrophils without depleting them or diminishing their pro-inflammatory capabilities while reducing SLE-induced NET formation *in vitro*<sup>119</sup>. A summary of reported, potential approaches that reduced NET formation *in vitro* are summarized in Table 2 and include targeting ROS with diphenyleneiodonium<sup>62</sup>, targeting mitochondrial ROS with MitoTEMPO<sup>42</sup>, a mitochondrially targeted antioxidant, or N-acetylcysteine (NAC)<sup>120</sup>; inhibiting PAD enzymes by chloroamine<sup>121</sup>; or enhancing breakdown of NETs with DNase1<sup>122</sup>. 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<sup>123</sup>. 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<sup>124</sup> 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<sup>125</sup>. In addition, vitamin D decreased PMA-induced NET formation of SLE neutrophils *in vitro*<sup>126</sup>. 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<sup>127</sup>.



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

| Treatment                           | Target                      |
|-------------------------------------|-----------------------------|
| <b>ANCA-associated vasculitis</b>   |                             |
| 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                     |
| <b>Systemic lupus erythematosus</b> |                             |
| 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 <sup>62</sup>                                                                                        | Not tested                                                                                                                       |
| Decreased NET formation in mouse model <sup>121</sup>                                                                                        | Protection against renal, skin and vascular manifestations in mice models                                                        |
| Decreased <i>in vitro</i> (mouse & human) and <i>in vivo</i> (mouse) NET formation <sup>166</sup>                                            | Effective and widely used FDA-approved therapy                                                                                   |
| Decreased NET formation and neutrophil activation <sup>110,167</sup> . Did not affect AAV serum induced NET formation <sup>95</sup>          | Effective and safe in phase III study <sup>110</sup>                                                                             |
| Decreased AAV-induced NET formation <sup>97</sup>                                                                                            | Not tested                                                                                                                       |
| Reduced PMA-induced NET formation <i>in vitro</i> <sup>126</sup>                                                                             | Improved endothelial function in SLE patients                                                                                    |
| Did not affect AAV serum induced NET formation <sup>95</sup>                                                                                 | Case reports: effective and safe <sup>149</sup>                                                                                  |
| Decreased NET release <sup>120,168</sup>                                                                                                     | Reduced disease activity in patients                                                                                             |
| Decreased NET formation and decreased oxidation of nucleic acids in NETs leading to decreased immunogenicity and IFN responses <sup>42</sup> | Reduced disease activity in mice                                                                                                 |
| Enzymatic degradation of NETs <sup>122,169</sup>                                                                                             | 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 <sup>123</sup>                                                                         | Not tested                                                                                                                       |
| Inhibition of SLE-induced NET formation <sup>119</sup>                                                                                       | Not tested                                                                                                                       |
| Reduced spontaneous and LPS-induced NETs in mouse model of lupus <sup>124</sup>                                                              | Not tested                                                                                                                       |
| Reduced PMA-induced NET formation, decreased CPG-stimulated pDC IFN production <sup>125</sup>                                                | Decreased clinical flares, prednisone exposure                                                                                   |
| Decreased <i>in vitro</i> (mouse & human) and <i>in vivo</i> (mouse) NET formation <sup>166</sup>                                            | Effective and widely used FDA approved therapy                                                                                   |
| Reduced NET formation <i>in vitro</i> <sup>126</sup>                                                                                         | Improved endothelial function in SLE patients                                                                                    |
| Reduced NET formation and neutrophil activation <sup>170,171</sup>                                                                           | Improved survival mouse model, safe and decreased haemolytic activity in SLE patients <sup>172</sup>                             |

Table 2. Continued.

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

AAV, antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis; BLM, belimumab; DPI, diphenyleiiodonium; 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, necrosulfanamide; 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<sup>84</sup>, and C5a receptor inhibition with avacopan was demonstrated to be clinically effective in AAV patients<sup>110</sup>. Although C5a, one of the components of the terminal complement system, has an important role in AAV<sup>128,129</sup>, *in vitro* C5a receptor blockade or the C5 antibody eculizumab were not able to inhibit AAV-induced NET formation<sup>95</sup>. 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 necrosulfanamide both reduced AAV-induced NET formation<sup>97</sup>. 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<sup>91</sup>. Eradication of autoantibodies as defined by seroconversion (to negative) upon immunosuppressive treatment is not

| Effect on NET formation                                                                                                                                                                                                                                                                               | Clinical effect                                                                                              |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------|
| Decreased NET formation <sup>91</sup>                                                                                                                                                                                                                                                                 | Reduction of anti-dsDNA, anti-histones, anti-nucleosomes, anti-C1q, decreased disease activity <sup>91</sup> |
| Inhibition of ICx-induced NET formation<br>Inhibit NET formation by human neutrophils stimulated by PMA, MPO, or immune complex activated human sera <sup>130</sup>                                                                                                                                   | Not tested                                                                                                   |
| Decreased LPS-induced NET formation <sup>131</sup><br>Decreased IgG production of NET-stimulated SLE B-cells <sup>27</sup>                                                                                                                                                                            | Effective and widely used FDA approved therapy                                                               |
| Anifrolumab decreased neutrophil NET complexes <sup>132,173</sup>                                                                                                                                                                                                                                     | Reduced disease activity                                                                                     |
| Modulation of calcium pools<br>Reduced NET formation <sup>133</sup>                                                                                                                                                                                                                                   | Improvement of renal disease <sup>174</sup><br>Voclosporin: NCT03021499                                      |
| Peptide Inhibitor of Complement C1; PMA, phorbol myristate acetate; ROS, reactive oxygen species; RTX, rituximab; S1RL-1, Signal inhibitory receptor on leukocytes-1; SLE, systemic lupus erythematosus; STAT, signal transducer and activator of transcription proteins; TLRg, Toll-like receptor-g; |                                                                                                              |

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*<sup>91</sup>. 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<sup>130</sup>. Hydroxychloroquine, an effective and widely-used therapy in SLE patients inhibits the DNA-sensing TLRg pathway and was demonstrated to inhibit IgG secretion by B-cells stimulated with NET-derived LL37-DNA complexes<sup>27</sup>. In addition, LPS-induced NET formation was inhibited when human healthy and lupus neutrophils were pre-treated with hydroxychloroquine<sup>131</sup>. Anifrolumab, an interferon- $\alpha$  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<sup>132</sup>. 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<sup>133</sup>. Calcineurin inhibitors showed promising clinical efficacy for LN patients<sup>134</sup>.

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<sup>95</sup>. In addition, one study demonstrated a longitudinal association of excessive NET formation with active disease within individual AAV patients<sup>108</sup>. 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<sup>103</sup>. We recently also demonstrated that in SLE patients treated with rituximab (RTX) + belimumab (BLM), excessive NET formation correlated with disease activity<sup>91</sup>. 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<sup>27</sup>. 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<sup>135</sup>. 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<sup>16,17</sup>. 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.

## REFERENCES

1. Pillay J, den Braber I, Vrisekoop N, *et al.* In vivo labeling with  $2\text{H}_2\text{O}$  reveals a human neutrophil lifespan of 5.4 days. *Blood*. 2010;116(4):625-627.
2. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect*. 2003;5(14):1317-1327.
3. Brinkmann V, Reichard U, Goosmann C, *et al.* Neutrophil extracellular traps kill bacteria. *Science*. 2004;303(5663):1532-1535.
4. Brinkmann V, Zychlinsky A. Beneficial suicide: why neutrophils die to make NETs. *Nat Rev Microbiol*. 2007;5(8):577-582.
5. Remijnsen Q, Kuijpers TW, Wirawan E, Lippens S, Vandenabeele P, Vanden Berghe T. Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. *Cell Death Differ*. 2011;18(4):581-588.
6. Linkermann A, Stockwell BR, Krautwald S, Anders HJ. Regulated cell death and inflammation: an auto-amplification loop causes organ failure. *Nat Rev Immunol*. 2014;14(11):759-767.
7. Yousefi S, Gold JA, Andina N, *et al.* Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. *Nat Med*. 2008;14(9):949-953.
8. Nakazawa D, Shida H, Kusunoki Y, *et al.* The responses of macrophages in interaction with neutrophils that undergo NETosis. *Journal of autoimmunity*. 2016;67:19-28.
9. Ingelsson B, Soderberg D, Strid T, *et al.* Lymphocytes eject interferogenic mitochondrial DNA webs in response to CpG and non-CpG oligodeoxynucleotides of class C. *Proc Natl Acad Sci U S A*. 2018;115(3):E478-E487.
10. Zhang Y, Chen X, Gueydan C, Han J. Plasma membrane changes during programmed cell deaths. *Cell Res*. 2018;28(1):9-21.
11. Xie Y, Hou W, Song X, *et al.* Ferroptosis: process and function. *Cell Death Differ*. 2016;23(3):369-379.
12. Gupta S, Kaplan MJ. The role of neutrophils and NETosis in autoimmune and renal diseases. *Nat Rev Nephrol*. 2016;12(7):402-413.
13. Lee KH, Kronbichler A, Park DD, *et al.* Neutrophil extracellular traps (NETs) in autoimmune diseases: A comprehensive review. *Autoimmun Rev*. 2017.
14. Schauer C, Janko C, Munoz LE, *et al.* Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. *Nat Med*. 2014;20(5):511-517.
15. Lightfoot YL, Kaplan MJ. Disentangling the role of neutrophil extracellular traps in rheumatic diseases. *Curr Opin Rheumatol*. 2017;29(1):65-70.
16. Kenny EF, Herzig A, Kruger R, *et al.* Diverse stimuli engage different neutrophil extracellular trap pathways. *eLife*. 2017;6.
17. Konig MF, Andrade F. A Critical Reappraisal of Neutrophil Extracellular Traps and NETosis Mimics Based on Differential Requirements for Protein Citrullination. *Frontiers in Immunology*. 2016;7:461.
18. Yipp BG, Kubes P. NETosis: how vital is it? *Blood*. 2013;122(16):2784-2794.
19. Yipp BG, Petri B, Salina D, *et al.* Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med*. 2012;18(9):1386-1393.
20. Campbell AM, Kashgarian M, Shlomchik MJ. NADPH oxidase inhibits the pathogenesis of systemic lupus erythematosus. *Sci Transl Med*. 2012;4(157):157ra141.

21. Garcia-Romo GS, Caielli S, Vega B, *et al.* Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Science Translational Medicine*. 2011;3(73):73ra20.
22. Leliefeld PH, Koenderman L, Pillay J. How Neutrophils Shape Adaptive Immune Responses. *Front Immunol*. 2015;6:471.
23. Puga I, Cols M, Barra CM, *et al.* B cell-helper neutrophils stimulate the diversification and production of immunoglobulin in the marginal zone of the spleen. *Nat Immunol*. 2011;13(2):170-180.
24. Lande R, Ganguly D, Facchinetti V, *et al.* Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Science Translational Medicine*. 2011;3(73):73ra19.
25. Lande R, Gregorio J, Facchinetti V, *et al.* Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature*. 2007;449(7162):564-569.
26. Tian J, Avalos AM, Mao SY, *et al.* Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat Immunol*. 2007;8(5):487-496.
27. Gestermann N, Di Domizio J, Lande R, *et al.* Netting Neutrophils Activate Autoreactive B Cells in Lupus. *J Immunol*. 2018;200(10):3364-3371.
28. Sangaletti S, Tripodo C, Chiodoni C, *et al.* Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity. *Blood*. 2012;120(15):3007-3018.
29. Bertheloot D, Naumovski AL, Langhoff P, *et al.* RAGE Enhances TLR Responses through Binding and Internalization of RNA. *J Immunol*. 2016;197(10):4118-4126.
30. Carmona-Rivera C, Carlucci PM, Moore E, *et al.* Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. *Sci Immunol*. 2017;2(10).
31. Brinkmann V, Zychlinsky A. Neutrophil extracellular traps: is immunity the second function of chromatin? *J Cell Biol*. 2012;198(5):773-783.
32. Chen R, Kang R, Fan XG, Tang D. Release and activity of histone in diseases. *Cell Death Dis*. 2014;5:e1370.
33. Saffarzadeh M, Juenemann C, Queisser MA, *et al.* Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. *PLoS One*. 2012;7(2):e32366.
34. O'Sullivan KM, Lo CY, Summers SA, *et al.* Renal participation of myeloperoxidase in antineutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis. *Kidney Int*. 2015;88(5):1030-1046.
35. Kumar SV, Kulkarni OP, Mulay SR, *et al.* Neutrophil Extracellular Trap-Related Extracellular Histones Cause Vascular Necrosis in Severe GN. *J Am Soc Nephrol*. 2015;26(10):2399-2413.
36. Pieterse E, Rother N, Garsen M, *et al.* Neutrophil Extracellular Traps Drive Endothelial-to-Mesenchymal Transition. *Arterioscler Thromb Vasc Biol*. 2017;37(7):1371-1379.
37. Lamprecht P, Kerstein A, Klapa S, *et al.* Pathogenetic and Clinical Aspects of Anti-Neutrophil Cytoplasmic Autoantibody-Associated Vasculitides. *Front Immunol*. 2018;9:680.
38. Zharkova O, Celhar T, Cravens PD, Satterthwaite AB, Fairhurst AM, Davis LS. Pathways leading to an immunological disease: systemic lupus erythematosus. *Rheumatology (Oxford)*. 2017;56(suppl\_1):i55-i66.
39. Kessenbrock K, Krumbholz M, Schonermarck U, *et al.* Netting neutrophils in autoimmune small-vessel vasculitis. *Nature Medicine*. 2009;15(6):623-625.
40. Villanueva E, Yalavarthi S, Berthier CC, *et al.* Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *J Immunol*. 2011;187(1):538-552.

41. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, *et al.* NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med.* 2013;5(178):178ra140.
42. Lood C, Blanco LP, Purmalek MM, *et al.* Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. *Nat Med.* 2016;22(2):146-153.
43. Carmona-Rivera C, Purmalek MM, Moore E, *et al.* A role for muscarinic receptors in neutrophil extracellular trap formation and levamisole-induced autoimmunity. *JCI Insight.* 2017;2(3):e89780.
44. Grayson PC, Carmona-Rivera C, Xu L, *et al.* Neutrophil-Related Gene Expression and Low-Density Granulocytes Associated With Disease Activity and Response to Treatment in Antineutrophil Cytoplasmic Antibody-Associated Vasculitis. *Arthritis Rheumatol.* 2015;67(7):1922-1932.
45. Grayson PC, Kaplan MJ. At the Bench: Neutrophil extracellular traps (NETs) highlight novel aspects of innate immune system involvement in autoimmune diseases. *J Leukoc Biol.* 2016;99(2):253-264.
46. Jorch SK, Kubes P. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med.* 2017;23(3):279-287.
47. Knight JS, Carmona-Rivera C, Kaplan MJ. Proteins derived from neutrophil extracellular traps may serve as self-antigens and mediate organ damage in autoimmune diseases. *Front Immunol.* 2012;3:380.
48. Darrah E, Andrade F. NETs: the missing link between cell death and systemic autoimmune diseases? *Front Immunol.* 2012;3:428.
49. Urban CF, Ermert D, Schmid M, *et al.* Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. *PLoS pathogens.* 2009;5(10):e1000639.
50. Panda R, Krieger T, Hopf L, *et al.* Neutrophil Extracellular Traps Contain Selected Antigens of Anti-Neutrophil Cytoplasmic Antibodies. *Front Immunol.* 2017;8:439.
51. Schultz H, Csernok E, Herlyn K, *et al.* ANCA against bactericidal/permeability-increasing protein, azurocidin, calprotectin and defensins in rheumatic and infectious diseases: prevalence and clinical associations. *Clinical and experimental rheumatology.* 2003;21(6 Suppl 32):S117-120.
52. Zhao MH, Lockwood CM. Azurocidin is a novel antigen for anti-neutrophil cytoplasmic autoantibodies (ANCA) in systemic vasculitis. *Clin Exp Immunol.* 1996;103(3):397-402.
53. Zhao MH, Liu N, Zhang YK, Wang HY. Antineutrophil cytoplasmic autoantibodies (ANCA) and their target antigens in Chinese patients with lupus nephritis. *Nephrol Dial Transplant.* 1998;13(11):2821-2824.
54. Schultz DR, Tozman EC. Antineutrophil cytoplasmic antibodies: major autoantigens, pathophysiology, and disease associations. *Semin Arthritis Rheum.* 1995;25(3):143-159.
55. Tang S, Zhang Y, Yin SW, *et al.* Neutrophil extracellular trap formation is associated with autophagy-related signalling in ANCA-associated vasculitis. *Clin Exp Immunol.* 2015;180(3):408-418.
56. Yaniv G, Twig G, Shor DB, *et al.* A volcanic explosion of autoantibodies in systemic lupus erythematosus: a diversity of 180 different antibodies found in SLE patients. *Autoimmun Rev.* 2015;14(1):75-79.
57. Chauhan SK, Rai R, Singh VV, Rai M, Rai G. Differential clearance mechanisms, neutrophil extracellular trap degradation and phagocytosis, are operative in systemic lupus erythematosus patients with distinct autoantibody specificities. *Immunol Lett.* 2015;168(2):254-259.

58. Fuchs TA, Abed U, Goosmann C, *et al.* Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol.* 2007;176(2):231-241.
59. Remijsen Q, Vanden Berghe T, Wirawan E, *et al.* Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Res.* 2011;21(2):290-304.
60. Phillipson M, Kubes P. The neutrophil in vascular inflammation. *Nat Med.* 2011;17(11):1381-1390.
61. Delgado-Rizo V, Martinez-Guzman MA, Iniguez-Gutierrez L, Garcia-Orozco A, Alvarado-Navarro A, Fafutis-Morris M. Neutrophil Extracellular Traps and Its Implications in Inflammation: An Overview. *Front Immunol.* 2017;8:81.
62. Gray RD, Lucas CD, MacKellar A, *et al.* Activation of conventional protein kinase C (PKC) is critical in the generation of human neutrophil extracellular traps. *J Inflamm (Lond).* 2013;10(1):12.
63. Nishinaka Y, Arai T, Adachi S, Takaori-Kondo A, Yamashita K. Singlet oxygen is essential for neutrophil extracellular trap formation. *Biochem Biophys Res Commun.* 2011;413(1):75-79.
64. Hakkim A, Fuchs TA, Martinez NE, *et al.* Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Nat Chem Biol.* 2011;7(2):75-77.
65. Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol.* 2010;191(3):677-691.
66. Neeli I, Dwivedi N, Khan S, Radic M. Regulation of extracellular chromatin release from neutrophils. *Journal of innate immunity.* 2009;1(3):194-201.
67. Martinod K, Demers M, Fuchs TA, *et al.* Neutrophil histone modification by peptidylarginine deiminase 4 is critical for deep vein thrombosis in mice. *Proc Natl Acad Sci U S A.* 2013;110(21):8674-8679.
68. Wang Y, Li M, Stadler S, *et al.* Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J Cell Biol.* 2009;184(2):205-213.
69. Fuhrmann J, Clancy KW, Thompson PR. Chemical biology of protein arginine modifications in epigenetic regulation. *Chem Rev.* 2015;115(11):5413-5461.
70. Amulic B, Knackstedt SL, Abu Abed U, *et al.* Cell-Cycle Proteins Control Production of Neutrophil Extracellular Traps. *Dev Cell.* 2017;43(4):449-462 e445.
71. Metzler KD, Fuchs TA, Nauseef WM, *et al.* Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood.* 2011;117(3):953-959.
72. Ostafin M, Pruchniak MP, Ciepiela O, Reznick AZ, Demkow U. Different procedures of diphenyleneiodonium chloride addition affect neutrophil extracellular trap formation. *Anal Biochem.* 2016;509:60-66.
73. Neeli I, Radic M. Opposition between PKC isoforms regulates histone deimination and neutrophil extracellular chromatin release. *Front Immunol.* 2013;4:38.
74. Lewis HD, Liddle J, Coote JE, *et al.* Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. *Nat Chem Biol.* 2015;11(3):189-191.
75. Kusunoki Y, Nakazawa D, Shida H, *et al.* Peptidylarginine Deiminase Inhibitor Suppresses Neutrophil Extracellular Trap Formation and MPO-ANCA Production. *Front Immunol.* 2016;7:227.
76. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol.* 2004;172(5):2731-2738.
77. Bardoel BW, Kenny EF, Sollberger G, Zychlinsky A. The balancing act of neutrophils. *Cell Host Microbe.* 2014;15(5):526-536.
78. Doeing DC, Borowicz JL, Crockett ET. Gender dimorphism in differential peripheral blood leukocyte counts in mice using cardiac, tail, foot, and saphenous vein puncture methods. *BMC Clin Pathol.* 2003;3(1):3.

79. Risso A. Leukocyte antimicrobial peptides: multifunctional effector molecules of innate immunity. *J Leukoc Biol.* 2000;68(6):785-792.
80. Daeron M. Fc receptor biology. *Annu Rev Immunol.* 1997;15:203-234.
81. Liu CL, Tangsombatvisit S, Rosenberg JM, *et al.* Specific post-translational histone modifications of neutrophil extracellular traps as immunogens and potential targets of lupus autoantibodies. *Arthritis research & therapy.* 2012;14(1):R25.
82. Claushuis TAM, van der Donk LEH, Luitse AL, *et al.* Role of Peptidylarginine Deiminase 4 in Neutrophil Extracellular Trap Formation and Host Defense during Klebsiella pneumoniae-Induced Pneumonia-Derived Sepsis. *J Immunol.* 2018.
83. Neeli I, Radic M. Knotting the NETs: analyzing histone modifications in neutrophil extracellular traps. *Arthritis research & therapy.* 2012;14(2):115.
84. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ.* 2009;16(11):1438-1444.
85. Pilsczek FH, Salina D, Poon KK, *et al.* A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. *The Journal of Immunology.* 2010;185(12):7413-7425.
86. Pieterse E, Rother N, Yanginlar C, Hilbrands LB, van der Vlag J. Neutrophils Discriminate between Lipopolysaccharides of Different Bacterial Sources and Selectively Release Neutrophil Extracellular Traps. *Front Immunol.* 2016;7:484.
87. DeSouza-Vieira T, Guimaraes-Costa A, Rochael NC, *et al.* Neutrophil extracellular traps release induced by Leishmania: role of PI3Kgamma, ERK, PI3Ksigma, PKC, and [Ca2+]. *J Leukoc Biol.* 2016;100(4):801-810.
88. Rochael NC, Guimaraes-Costa AB, Nascimento MT, *et al.* Classical ROS-dependent and early/rapid ROS-independent release of Neutrophil Extracellular Traps triggered by Leishmania parasites. *Sci Rep.* 2015;5:18302.
89. Carestia A, Kaufman T, Schattner M. Platelets: New Bricks in the Building of Neutrophil Extracellular Traps. *Front Immunol.* 2016;7:271.
90. Pieterse E, Rother N, Yanginlar C, *et al.* Cleaved N-terminal histone tails distinguish between NADPH oxidase (NOX)-dependent and NOX-independent pathways of neutrophil extracellular trap formation. *Annals of the rheumatic diseases.* 2018.
91. Kraaij T, Kamerling SWA, de Rooij ENM, *et al.* The NET-effect of combining rituximab with belimumab in severe systemic lupus erythematosus. *Journal of autoimmunity.* 2018.
92. Rother N, Pieterse E, Lubbers J, Hilbrands L, van der Vlag J. Acetylated Histones in Apoptotic Microparticles Drive the Formation of Neutrophil Extracellular Traps in Active Lupus Nephritis. *Front Immunol.* 2017;8:1136.
93. Nakazawa D, Shida H, Tomaru U, *et al.* Enhanced formation and disordered regulation of NETs in myeloperoxidase-ANCA-associated microscopic polyangiitis. *Journals of the American Society of Nephrology.* 2014;25(5):990-997.
94. Hakkim A, Furnrohr BG, Amann K, *et al.* Impairment of neutrophil extracellular trap degradation is associated with lupus nephritis. *Proc Natl Acad Sci U S A.* 2010;107(21):9813-9818.
95. Kraaij T, Kamerling SWA, van Dam LS, *et al.* Excessive neutrophil extracellular trap formation in ANCA-associated vasculitis is independent of ANCA. *Kidney International.* 2018.
96. Desai J, Kumar SV, Mulay SR, *et al.* PMA and crystal-induced neutrophil extracellular trap formation involves RIPK1-RIPK3-MLKL signaling. *European Journal of Immunology.* 2016;46(1):223-229.



97. Schreiber A, Rousselle A, Becker JU, von Massenhausen A, Linkermann A, Kettritz R. Necroptosis controls NET generation and mediates complement activation, endothelial damage, and autoimmune vasculitis. *Proc Natl Acad Sci U S A*. 2017;114(45):E9618-E9625.
98. Dieker J, Berden JH, Bakker M, *et al*. Autoantibodies against Modified Histone Peptides in SLE Patients Are Associated with Disease Activity and Lupus Nephritis. *PLoS One*. 2016;11(10):e0165373.
99. van Bavel CC, Dieker J, Muller S, *et al*. Apoptosis-associated acetylation on histone H2B is an epitope for lupus autoantibodies. *Mol Immunol*. 2009;47(2-3):511-516.
100. van Bavel CC, Dieker JW, Kroeze Y, *et al*. Apoptosis-induced histone H3 methylation is targeted by autoantibodies in systemic lupus erythematosus. *Annals of the rheumatic diseases*. 2011;70(1):201-207.
101. Barrera-Vargas A, Gomez-Martin D, Carmona-Rivera C, *et al*. Differential ubiquitination in NETs regulates macrophage responses in systemic lupus erythematosus. *Annals of the rheumatic diseases*. 2018;77(6):944-950.
102. Leffler J, Martin M, Gullstrand B, *et al*. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. *J Immunol*. 2012;188(7):3522-3531.
103. Wang H, Sha LL, Ma TT, Zhang LX, Chen M, Zhao MH. Circulating Level of Neutrophil Extracellular Traps Is Not a Useful Biomarker for Assessing Disease Activity in Antineutrophil Cytoplasmic Antibody-Associated Vasculitis. *PLoS One*. 2016;11(2):e0148197.
104. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Identification of a factor that links apoptotic cells to phagocytes. *Nature*. 2002;417(6885):182-187.
105. Gregoire M, Uhel F, Lesouhaitier M, *et al*. Impaired efferocytosis and neutrophil extracellular trap clearance by macrophages in ARDS. *Eur Respir J*. 2018;52(2).
106. Ohlsson SM, Ohlsson S, Soderberg D, *et al*. Neutrophils from vasculitis patients exhibit an increased propensity for activation by anti-neutrophil cytoplasmic antibodies. *Clin Exp Immunol*. 2014;176(3):363-372.
107. Yoshida M, Sasaki M, Sugisaki K, Yamaguchi Y, Yamada M. Neutrophil extracellular trap components in fibrinoid necrosis of the kidney with myeloperoxidase-ANCA-associated vasculitis. *Clin Kidney J*. 2013;6(3):308-312.
108. Soderberg D, Kurz T, Motamedi A, Hellmark T, Eriksson P, Segelmark M. Increased levels of neutrophil extracellular trap remnants in the circulation of patients with small vessel vasculitis, but an inverse correlation to anti-neutrophil cytoplasmic antibodies during remission. *Rheumatology (Oxford)*. 2015;54(11):2085-2094.
109. Wang H, Wang C, Zhao MH, Chen M. Neutrophil extracellular traps can activate alternative complement pathways. *Clin Exp Immunol*. 2015;181(3):518-527.
110. Jayne DRW, Bruchfeld AN, Harper L, *et al*. Randomized Trial of C5a Receptor Inhibitor Avacopan in ANCA-Associated Vasculitis. *J Am Soc Nephrol*. 2017;28(9):2756-2767.
111. Hacbarth E, Kajdacsy-Balla A. Low density neutrophils in patients with systemic lupus erythematosus, rheumatoid arthritis, and acute rheumatic fever. *Arthritis and rheumatism*. 1986;29(11):1334-1342.
112. Carmona-Rivera C, Kaplan MJ. Low-density granulocytes: a distinct class of neutrophils in systemic autoimmunity. *Semin Immunopathol*. 2013;35(4):455-463.
113. Kaplan MJ. Neutrophils in the pathogenesis and manifestations of SLE. *Nat Rev Rheumatol*. 2011;7(12):691-699.

114. van der Linden M, van den Hoogen LL, Westerlaken GHA, *et al.* Neutrophil extracellular trap release is associated with antinuclear antibodies in systemic lupus erythematosus and anti-phospholipid syndrome. *Rheumatology (Oxford)*. 2018.
115. Jonsson H, Sturfelt G. A novel assay for neutrophil clustering activity of human sera: relation to disease activity and neutropenia in systemic lupus erythematosus. *Annals of the rheumatic diseases*. 1990;49(1):46-50.
116. Sturfelt G, Jonsson H, Hellmer G, Sjöholm AG. Clustering of neutrophil leucocytes in serum: possible role of C1q-containing immune complexes. *Clin Exp Immunol*. 1993;93(2):237-241.
117. Urbonaviciute V, Furnrohr BG, Meister S, *et al.* Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE. *J Exp Med*. 2008;205(13):3007-3018.
118. Urbonaviciute V, Starke C, Pirschel W, *et al.* Toll-like receptor 2 is required for autoantibody production and development of renal disease in pristane-induced lupus. *Arthritis and rheumatism*. 2013;65(6):1612-1623.
119. Van Avondt K, Fritsch-Stork R, Derksen RH, Meyaard L. Ligation of signal inhibitory receptor on leukocytes-1 suppresses the release of neutrophil extracellular traps in systemic lupus erythematosus. *PLoS One*. 2013;8(10):e78459.
120. Garcia RJ, Francis L, Dawood M, Lai ZW, Faraone SV, Perl A. Attention deficit and hyperactivity disorder scores are elevated and respond to N-acetylcysteine treatment in patients with systemic lupus erythematosus. *Arthritis and rheumatism*. 2013;65(5):1313-1318.
121. Knight JS, Subramanian V, O'Dell AA, *et al.* Peptidylarginine deiminase inhibition disrupts NET formation and protects against kidney, skin and vascular disease in lupus-prone MRL/lpr mice. *Annals of the rheumatic diseases*. 2015;74(12):2199-2206.
122. Davis JC, Jr., Manzi S, Yarboro C, *et al.* Recombinant human Dnase I (rhDNase) in patients with lupus nephritis. *Lupus*. 1999;8(1):68-76.
123. Chirivi RG, van Rosmalen JW, Kambas K, *et al.* Netosis-inhibiting t-aca therapy for use in different net-driven human autoimmune diseases. *Annals of Rheumatic Diseases*. 2018;77:205.
124. Furumoto Y, Smith CK, Blanco L, *et al.* Tofacitinib Ameliorates Murine Lupus and Its Associated Vascular Dysfunction. *Arthritis Rheumatol*. 2017;69(1):148-160.
125. Wang H, Li T, Chen S, Gu Y, Ye S. Neutrophil Extracellular Trap Mitochondrial DNA and Its Autoantibody in Systemic Lupus Erythematosus and a Proof-of-Concept Trial of Metformin. *Arthritis Rheumatol*. 2015;67(12):3190-3200.
126. Handono K, Sidarta YO, Pradana BA, *et al.* Vitamin D prevents endothelial damage induced by increased neutrophil extracellular traps formation in patients with systemic lupus erythematosus. *Acta Med Indones*. 2014;46(3):189-198.
127. Natarajaswamy Kalleda JA, Spoorthi Poreddy, Berkan Arslan, Mike Friedrich, Zeinab Mokhtari, Katja Ottmüller, Ana-Laura Jordán-Garrote, Hermann Einsele, Matthias Brock, Katrin G Heinze, and Andreas Beilhack Corticosteroids Impair Granulocyte Transfusion Therapy By Targeting NET Formation and Neutrophil Antifungal Functions Via ROS/Dectin1 Pathways. *Blood* 2016;128(22):2506.
128. Huugen D, van Esch A, Xiao H, *et al.* Inhibition of complement factor C5 protects against anti-myeloperoxidase antibody-mediated glomerulonephritis in mice. *Kidney Int*. 2007;71(7):646-654.
129. Xiao H, Schreiber A, Heeringa P, Falk RJ, Jennette JC. Alternative complement pathway in the pathogenesis of disease mediated by anti-neutrophil cytoplasmic autoantibodies. *Am J Pathol*. 2007;170(1):52-64.

130. Hair PS, Enos AI, Krishna NK, Cunnion KM. Inhibition of Immune Complex Complement Activation and Neutrophil Extracellular Trap Formation by Peptide Inhibitor of Complement C1. *Front Immunol.* 2018;9:558.
131. Smith CK, Vivekanandan-Giri A, Tang C, *et al.* Neutrophil extracellular trap-derived enzymes oxidize high-density lipoprotein: an additional proatherogenic mechanism in systemic lupus erythematosus. *Arthritis Rheumatol.* 2014;66(9):2532-2544.
132. White W, Seto N, Playford M. Alteration of mediators of vascular inflammation by anifrolumab in the phase IIB study in SLE. *Annals of Rheumatic Diseases.* 2018;77:136-137.
133. Gupta AK, Giaglis S, Hasler P, Hahn S. Efficient neutrophil extracellular trap induction requires mobilization of both intracellular and extracellular calcium pools and is modulated by cyclosporine A. *PLoS One.* 2014;9(5):e97088.
134. Kraaij T, Bredewold OW, Trompet S, *et al.* TAC-TIC use of tacrolimus-based regimens in lupus nephritis. *Lupus Sci Med.* 2016;3(1):e000169.
135. Masuda S, Nakazawa D, Shida H, *et al.* NETosis markers: Quest for specific, objective, and quantitative markers. *Clinica Chimica Acta.* 2016;459:89-93.
136. Silva de Souza AW. Autoantibodies in systemic vasculitis. *Front Immunol.* 2015;6:184.
137. Manolova I, Dancheva M, Halacheva K. Antineutrophil cytoplasmic antibodies in patients with systemic lupus erythematosus: prevalence, antigen specificity, and clinical associations. *Rheumatology International.* 2001;20(5):197-204.
138. Nassberger L, Jonsson H, Sjöholm AG, Sturfelt G, Heubner A. Circulating anti-elastase in systemic lupus erythematosus. *Lancet.* 1989;1(8636):509.
139. Schulte-Pelkum J, Radice A, Norman GL, *et al.* Novel clinical and diagnostic aspects of antineutrophil cytoplasmic antibodies. *J Immunol Res.* 2014;2014:185416.
140. Carmona-Rivera C, Kaplan MJ. Detection of SLE antigens in neutrophil extracellular traps (NETs). *Methods Mol Biol.* 2014;1134:151-161.
141. Kain R. L29. Relevance of anti-LAMP-2 in vasculitis: why the controversy. *Presse medicale.* 2013;42(4 Pt 2):584-588.
142. Kain R, Rees AJ. What is the evidence for antibodies to LAMP-2 in the pathogenesis of ANCA associated small vessel vasculitis? *Curr Opin Rheumatol.* 2013;25(1):26-34.
143. Pradhan VD, Badakere SS, Bichile LS, Almeida AF. Anti-neutrophil cytoplasmic antibodies (ANCA) in systemic lupus erythematosus: prevalence, clinical associations and correlation with other autoantibodies. *J Assoc Physicians India.* 2004;52:533-537.
144. Renaudineau Y, Deocharan B, Jousse S, Renaudineau E, Putterman C, Youinou P. Anti-alpha-actinin antibodies: a new marker of lupus nephritis. *Autoimmun Rev.* 2007;6(7):464-468.
145. Seret G, Canas F, Pougnet-Di Costanzo L, *et al.* Anti-alpha-actinin antibodies are part of the anti-cell membrane antibody spectrum that characterize patients with lupus nephritis. *Journal of autoimmunity.* 2015;61:54-61.
146. Renaudineau Y, Croquefer S, Jousse S, *et al.* Association of alpha-actinin-binding anti-double-stranded DNA antibodies with lupus nephritis. *Arthritis and rheumatism.* 2006;54(8):2523-2532.
147. Mason LJ, Ravirajan CT, Rahman A, Putterman C, Isenberg DA. Is alpha-actinin a target for pathogenic anti-DNA antibodies in lupus nephritis? *Arthritis and rheumatism.* 2004;50(3):866-870.
148. Zhao Z, Weinstein E, Tuzova M, *et al.* Cross-reactivity of human lupus anti-DNA antibodies with alpha-actinin and nephritogenic potential. *Arthritis and rheumatism.* 2005;52(2):522-530.

149. Manenti L, Urban ML, Maritati F, Galetti M, Vaglio A. Complement blockade in ANCA-associated vasculitis: an index case, current concepts and future perspectives. *Intern Emerg Med.* 2017;12(6):727-731.
150. Bruschi M, Galetti M, Sinico RA, *et al.* Glomerular Autoimmune Multicomponents of Human Lupus Nephritis In Vivo (2): Planted Antigens. *J Am Soc Nephrol.* 2015;26(8):1905-1924.
151. Mosca M, Chimenti D, Pratesi F, *et al.* Prevalence and clinico-serological correlations of anti-alpha-enolase, anti-C1q, and anti-dsDNA antibodies in patients with systemic lupus erythematosus. *The Journal of rheumatology.* 2006;33(4):695-697.
152. Bruschi M, Sinico RA, Moroni G, *et al.* Glomerular autoimmune multicomponents of human lupus nephritis in vivo: alpha-enolase and annexin A1. *J Am Soc Nephrol.* 2014;25(11):2483-2498.
153. Bruschi M, Petretto A, Vaglio A, Santucci L, Candiano G, Ghiggeri GM. Annexin A1 and Autoimmunity: From Basic Science to Clinical Applications. *Int J Mol Sci.* 2018;19(5).
154. Orbai AM, Truedsson L, Sturfelt G, *et al.* Anti-C1q antibodies in systemic lupus erythematosus. *Lupus.* 2015;24(1):42-49.
155. Horvath L, Czirjak L, Fekete B, *et al.* Levels of antibodies against C1q and 60 kDa family of heat shock proteins in the sera of patients with various autoimmune diseases. *Immunol Lett.* 2001;75(2):103-109.
156. Mansour RB, Lassoued S, Gargouri B, El Gaid A, Attia H, Fakhfakh F. Increased levels of autoantibodies against catalase and superoxide dismutase associated with oxidative stress in patients with rheumatoid arthritis and systemic lupus erythematosus. *Scand J Rheumatol.* 2008;37(2):103-108.
157. Abdulahad DA, Westra J, Bijzet J, Limburg PC, Kallenberg CG, Bijl M. High mobility group box 1 (HMGB1) and anti-HMGB1 antibodies and their relation to disease characteristics in systemic lupus erythematosus. *Arthritis research & therapy.* 2011;13(3):R71.
158. Avalos AM, Kiefer K, Tian J, *et al.* RAGE-independent autoreactive B cell activation in response to chromatin and HMGB1/DNA immune complexes. *Autoimmunity.* 2010;43(1):103-110.
159. Tamiya H, Tani K, Miyata J, *et al.* Defensins- and cathepsin G-ANCA in systemic lupus erythematosus. *Rheumatology international.* 2006;27(2):147-152.
160. Neumann A, Berends ET, Nerlich A, *et al.* The antimicrobial peptide LL-37 facilitates the formation of neutrophil extracellular traps. *Biochem J.* 2014;464(1):3-11.
161. Neumann A, Vollger L, Berends ET, *et al.* Novel role of the antimicrobial peptide LL-37 in the protection of neutrophil extracellular traps against degradation by bacterial nucleases. *Journal of innate immunity.* 2014;6(6):860-868.
162. Yuen J, Pluthero FG, Douda DN, *et al.* NETosing Neutrophils Activate Complement Both on Their Own NETs and Bacteria via Alternative and Non-alternative Pathways. *Front Immunol.* 2016;7:137.
163. Pauly D, Nagel BM, Reinders J, *et al.* A novel antibody against human properdin inhibits the alternative complement system and specifically detects properdin from blood samples. *PLoS One.* 2014;9(5):e96371.
164. Nozal P, Garrido S, Martinez-Ara J, *et al.* Case report: lupus nephritis with autoantibodies to complement alternative pathway proteins and C3 gene mutation. *BMC Nephrol.* 2015;16:40.
165. Hilhorst M, van Paassen P, van Rie H, *et al.* Complement in ANCA-associated glomerulonephritis. *Nephrol Dial Transplant.* 2017;32(8):1302-1313.
166. Kalleda N, Amich J, Arslan B, *et al.* Dynamic Immune Cell Recruitment After Murine Pulmonary *Aspergillus fumigatus* Infection under Different Immunosuppressive Regimens. *Front Microbiol.* 2016;7:1107.

167. Huang YM, Wang H, Wang C, Chen M, Zhao MH. Promotion of hypercoagulability in antineutrophil cytoplasmic antibody-associated vasculitis by C5a-induced tissue factor-expressing microparticles and neutrophil extracellular traps. *Arthritis Rheumatol*. 2015;67(10):2780-2790.
168. Lai ZW, Hanczko R, Bonilla E, *et al*. N-acetylcysteine reduces disease activity by blocking mammalian target of rapamycin in T cells from systemic lupus erythematosus patients: a randomized, double-blind, placebo-controlled trial. *Arthritis and rheumatism*. 2012;64(9):2937-2946.
169. Macanovic M, Sinicropi D, Shak S, Baughman S, Thiru S, Lachmann PJ. The treatment of systemic lupus erythematosus (SLE) in NZB/W F1 hybrid mice; studies with recombinant murine DNase and with dexamethasone. *Clin Exp Immunol*. 1996;106(2):243-252.
170. Wang Y, Hu Q, Madri JA, Rollins SA, Chodera A, Matis LA. Amelioration of lupus-like autoimmune disease in NZB/WF1 mice after treatment with a blocking monoclonal antibody specific for complement component C5. *Proc Natl Acad Sci U S A*. 1996;93(16):8563-8568.
171. van Bijnen STA, Wouters D, van Mierlo GJ, Muus P, Zeerleder S. Neutrophil activation and nucleosomes as markers of systemic inflammation in paroxysmal nocturnal hemoglobinuria: effects of eculizumab. *J Thromb Haemost*. 2015;13(11):2004-2011.
172. Barilla-Labarca ML, Toder K, Furie R. Targeting the complement system in systemic lupus erythematosus and other diseases. *Clin Immunol*. 2013;148(3):313-321.
173. Furie R, Khamashta M, Merrill JT, *et al*. Anifrolumab, an Anti-Interferon-alpha Receptor Monoclonal Antibody, in Moderate-to-Severe Systemic Lupus Erythematosus. *Arthritis Rheumatol*. 2017;69(2):376-386.
174. Lee YH, Lee HS, Choi SJ, Dai Ji J, Song GG. Efficacy and safety of tacrolimus therapy for lupus nephritis: a systematic review of clinical trials. *Lupus*. 2011;20(6):636-640.



