Neutrophil Extracellular Traps in Autoimmune Diseases

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INTRODUCTION

In 2004, NETosis was first reported as an important step to kill bacteria by neutrophils. During the process of NETosis, neutrophil extracellular traps (NETs) that contain large web-like structures of decondensed chromatin decorated with histones and intracellular components, including neutrophil elastase (NE), myeloperoxidase (MPO), high mobility group protein B1 (HMGB1), and proteinase 3 (PR3), are extruded into the extracellular space.[1] The structures of NETs enable the neutrophil to potently catch and kill pathogens at the site of inflammation. Furthermore, increasing studies have identified the presence of NETs in autoimmune diseases. NETs deliver multiple autoantigens to host immune system that induce autoimmune responses and directly release damage-associated molecular patterns to amplify inflammatory responses. Therefore, NETs are commonly described to play a crucial role in the pathogenesis and development of autoimmune diseases in recent years.

PATHWAYS OF NEUTROPHIL EXTRACELLULAR TRAP FORMATION

To date, three different pathways have been identified to mediate the formation of NETs. The best-known pathway is the conventional suicidal NETosis that generally lasts from 2 to 4 h.[2] Suicidal NETosis is initiated by the recognition of several stimuli (e.g., bacteria, viruses, fungi, or ribonucleoprotein immune complexes) through neutrophil receptors (such as toll-like receptors [TLRs] and IgG-Fc receptors).[3] Then, stored calcium ions of the endoplasmic reticulum are released into the cytoplasm, leading to increased protein kinase C activity.[4] This induces

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to assemble into a functional complex (PHOX) which subsequently stimulates the abundant production of reactive oxygen species (ROS).[5] ROS acts as a second messenger in conventional suicidal NETosis and plays a crucial role in promoting the breakdown of the nuclear membrane.[3] Histone deamination by peptidyl arginine deiminase 4 (PAD4) mostly contributes to chromatin decondensation.[6] In addition, in the cytosol, NE and MPO are released from azurophilic granules and then translocate into the nucleus, further promoting unfolding of chromatin. The decondensed chromatin coated with cytoplasmic and granule components is extruded into the extracellular space. Differently, in vital NETosis, neutrophils release NETs without breakdown of nuclear or plasma membrane, remain viable, and retain several conventional functions. Vital NETosis occurs within 5–60 min of neutrophil stimulation and is independent on ROS production.[1] This type of NET formation is induced by the recognition of stimuli through TLRs, the C3 complement receptor, and the interaction between glycoprotein Ib in platelets with β2 integrin. A third form of NETosis (mitochondrial NETosis) dependent on ROS production has been described, in which mitochondrial DNA instead of nuclear DNA is released. This mitochondrial NETosis is identified in neutrophils within 15 min when stimulated with C5a or lipopolysaccharide.[7]

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Interestingly, in patients with some autoimmune diseases, there is a distinct population of neutrophils termed low-density granulocytes (LDGs)[8] which are present in mononuclear cell fractions after density-gradient centrifugation. Compared with normal density neutrophils, these LDGs are more prone to spontaneously release NETs. Although three pathways and multiple molecules are commonly considered to mediate NETosis, the dominant pathways in conventional neutrophils or LDG remain to be further elucidated.

Neutrophil Extracellular Traps in Antineutrophil Cytoplasmic Antibody-Associated Vasculitis

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is referred as a subgroup of small vessel vasculitis characterized by immune depositions and the presence of ANCAs and includes microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), and eosinophilic GPA (EGPA). In 2009, NET deposition recognized as MPO and PR3 complexes was found in inflamed kidneys of patients with ANCA-associated vasculitis, suggesting a link between NETs and autoimmunity.[9] Subsequently, elevated levels of circulating NET remnants were found in patients with active AVV, and the levels were positively associated with disease activity.[10] In AVV patients, apart from high levels in circulation, NETs have been shown to be present in various local lesions, such as glomerulus and skin lesions, as well as in thrombi. In addition, a recent study has identified the presence of NETs in the peripheral nervous system of MPA patients.[11] Where do these abundant NETs in AVV come from? In particular, LDGs have been identified in AVV patients that may be an important source of NETs. Furthermore, products of NETosis can induce NET formation, perpetuating a vicious cycle of NET production. For example, HMGB1 released by NETosis can promote the ANCA-induced NET formation.[12] To more directly investigate the effect of NETs in AVV, Sangaletti's group directly injected myeloid dendritic cells loaded with NET components into naive mice. These mice produced more ANCAs and developed autoimmune vasculitis.[13] However, NETs degraded with DNAse prevented the autoimmunity response.[13] Collectively, these findings emphasized a vital role of NETs in the development of AAV.

Thrombosis is one of the common complications of ANCA-associated vasculitis. Interestingly, a case study of a MPA patient with deep vein thrombosis has found that abundant NETs were enriched in the thrombus.[14] During acute myocardial infarction, thrombin-activated platelets interact with neutrophils at the site of plaque rupture, leading to local NET formation and activation of tissue factor that initiates coagulation and thrombin formation.[15] Activated tissue factor-associated NETs were also found in AVV patients.[16] Other components of NETs, such as extracellular histones, also enhanced the generation of thrombin in a platelet-dependent manner.[17] In addition, NE coupled with externalized nucleosomes showed increased capacity of coagulation and thrombosis formation.[18] These observations have indicated that NETs contribute to thrombosis formation.

Neutrophil Extracellular Traps in Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by overproduction of autoantibodies and involvement of multiple organs. In SLE patients, high serum levels of autoantibodies, for example, anti-DNA antibodies, anti-ribonucleoprotein, and mostly target NET components. Furthermore, elevated levels of NET deposition were detected in the skin and kidneys of SLE patients and lupus-prone mice.[19] Similar to AVV patients, SLE patients also show a small population of circulating LDG.[20] In SLE, these LDGs display enhanced capacity to produce inflammatory cytokines, particularly type I interferons (IFNs).[21] Moreover, NETs released by LDGs from SLE patients contain elevated autoantigens and immunostimulatory proteins such as LL-37 and interleukin (IL)-17.[21] LDGs play an important role in the pathogenesis of SLE, but their origin needs to be further elucidated. Because LDGs also showed elevated activation markers,[8] one of the possible explanations is that LDGs may represent a particular activation status from normal density neutrophils. For example, some neutrophils in vivo are activated to undergo vital NETosis and then release intracellular components but remain viable and retain several conventional functions. Those neutrophils may become an important source of LDGs. It is also possible that LDGs may belong to a subset of neutrophils with specific disruptions during neutrophil development.

Owing to the presence of DNase1 inhibitors or NET-bound autoantibodies that are able to block the access of DNase1 to NETs, SLE patients exhibit a decreased ability to degrade NETs. Excessive NET formation and impaired clearance of NETs may lead to persistent and prolonged existence of NETs in SLE. Unfortunately, prolonged exposure to NETs may promote autoimmune and inflammatory responses, resulting in tissue damage. It is worth noting that NETs efficiently stimulate plasmacytoid dendritic cells (pDCs) to produce a large amount of type I IFN through the recognition of TLR-9 in SLE.[22] This relation between NETs and type I IFNs provides more evidence to explain how NETs promote immune responses. In addition, NETs-derived proteins in SLE patients facilitate the activation of NLRP3 inflammasome and release of active IL-1β and IL-18, further amplifying inflammatory responses. In turn, the active IL-18 is able to induce NETosis, producing a pro-inflammatory feedback loop.[23] In addition, Riveral et al. have indicated that activation of endothelial matrix metalloproteinase-2 (MMP-2) induced by MMP-9 contained in NETs contributed to endothelium damage.[24] NET components significantly mediated oxidation of high-density lipoprotein, leading to SLE-related atherosclerotic cardiovascular disease.[25]

Posttranslational modifications (PTMs) can modify protein structure and function, and the components of NETs are susceptible to undergo PTMs (i.e., ubiquitination, acetylation, and citrullination). These modified NET components may contribute to the dysregulation of immune responses. A recent study[26] has demonstrated that ubiquitinated NET proteins in SLE patients could induce the release of inflammatory cytokines by macrophages. In addition, anti-ubiquitinated MPO antibodies were observed in SLE subjects and the titer of antibody was positively correlated with disease activity. Moreover, the citrullination is commonly present in rheumatoid arthritis (RA) (this part will be discussed in detail later). Therefore, these PTMs of NET proteins play important roles in the development of inflammatory responses in autoimmune diseases.

Neutrophil Extracellular Traps in Rheumatoid Arthritis

RA is a chronic autoimmune disease characterized by synovial joint inflammation and production of autoantibodies to citrullinated protein antigens (ACPAs). Evidence has suggested that citrullinated antigens are mostly derived from NETs. In RA-free first-degree relatives, elevations of sputum anti-CCP were reported to be correlated with increased levels of NETs in sputum.[27] In addition, increased NETs were found in peripheral blood, synovial fluid, rheumatoid nodules, and skin of RA patients, and the NET levels were positively associated with the levels of ACPA.[28] Further, two studies provided more direct evidence for the association of NETs and ACPAs. As reported, antibodies against citrullinated histone H4 in NETs were detected in more than 60% of sera from RA patients.[29] Furthermore, approximately 40% of RA monoclonal antibodies generated by synovial tissue B cells exhibited a strong reactivity against citrullinated histones H2A/H2B, followed by citrullinated fibrinogen and citrullinated vimentin.[30] During NETosis, PAD, in particular PAD4, mediates the citrullination of exposed proteins. A single nucleotide polymorphism at position 1858 (C1858T) in the DNA encoding a protein tyrosine phosphatase (PTPN22) that results in the conversion of an arginine (R620) to a tryptophan (W620) has been found strong connection with RA. Chang *et al.* have indicated that the modification of C1858T disrupted the interaction between PTPN22 and PAD4, resulting in an expansion of citrullinated antigens and increased formation of NETs.[31]

Increased NETosis in oral cavity of periodontitis patients perhaps participates in the initiation of RA. Importantly, abundant NETs have been detected in gingival crevicular fluid from patients with periodontitis.[32] *Porhyromonas gingivalis* is the most important microbe responsible for periodontitis. PAD generated from *P. gingivalis* was shown to promote citrullination of microbial and host protein.[33] Further, it was shown that *P. gingivalis* could induce NET generation.[34] Interestingly, a case–control study has indicated that periodontal treatment markedly decreased the serum levels of NETs in patients with RA and periodontitis.[35]

Except for the significance of ACPAs in the diagnosis for RA, NET components, such as cell-free DNA, cell-free nucleosome, NE protein, and MPO protein, are also increased in RA patients that could also be potential biomarkers for RA diagnosis. As reported recently, the serum levels of MPO-DNA complexes exhibited diagnostic potential with a sensitivity of 91.9% and a specificity of 56.0%.[10] Moreover, plasma levels of cell-free nucleosomes were identified with a sensitivity (91%) and a higher specificity (92%) for RA diagnosis.[36]

Psoriasis and Neutrophil Extracellular Traps

Psoriasis is a chronic immune-mediated skin disease characterized by erythematous lesions with white or silvery scales which can occur at any site of the skin. Stephen *et al.* found that increased NET formation existed in peripheral blood and lesion skin, and the levels of NETs were correlated with disease severity of psoriasis.[37] It has been reported that NET-associated IL-17 deposited frequently in psoriatic lesion epidermis.[38] It is well known that IL-17 plays a central role in psoriasis pathogenesis. Therefore, NETs may participate in the pathogenesis of psoriasis through IL-17. In addition, LL37, one of the NET components, interacts with self-RNA or self-DNA to form the complexes found in psoriatic skin lesions.[39] The self-DNA-LL37 complex is transported to endosomal TLR9 of pDCs, eventually triggering the secretion of IFN-α. Moreover, the complex comprised of self-RNA and LL37 can induce the activation of classical myeloid DCs to produce pro-inflammatory cytokines, such as TNF-α and IL-6, in TLR7 and TLR8-dependent manners.[39] Aside from LL-37, a mixture of human NE (HNE), DNA, and secretory leukocyte proteinase inhibitor (SLPI) was found to colocalize with pDCs in lesion skin of psoriasis patients. The complex of SLPI-HNE-DNA can activate pDCs through intracellular TLR9, leading to a marked production of type I IFN.[40] Collectively, those products contribute to triggering inflammatory responses in psoriasis.

However, NETs may also exhibit a beneficial effect on preventing psoriasis plaques from infection. In psoriasis patients, NETs were found to induce the production of human β-defensin-2, an important antimicrobial peptide, in epidermal keratinocytes.[37] These findings may provide a novel explanation for the low susceptibility of psoriasis plaques to microbial infections.

Neutrophil Extracellular Traps in Antiphospholipid Syndrome

Antiphospholipid syndrome (APS) is a systemic autoimmune disorder associated with the presence of antiphospholipid antibodies, including lupus anticoagulant, anticardiolipin antibodies, and anti-β2-glycoprotein 1 antibodies.[41] APS mostly presents with venous or arterial thrombosis and/or pregnancy morbidity. APS can develop as a primary disease

or be secondary to other autoimmune diseases such as SLE,[41] but there was no difference in NET formation between primary and secondary APS. Several studies have linked NETs with APS. Indeed, the levels of DNA and NETs were found to be increased in sera from patients with APS.[42] Antiphospholipid antibodies purified from APS patients can induce neutrophils from healthy volunteers to produce NETs. Further, those NET generations can be abrogated by inhibiting the production of reactive oxygen species and TLR 4 signaling.[42] On the other hand, Leffler *et al.* revealed that serum from APS patients exhibited impaired NET degradation, which was strongly correlated with antibodies against NETs and specific clinical manifestations in patients with secondary APS.[43]

An *in vivo* study has showed that exaggerated thrombosis occurred in mice treated with IgG from APS patients.[44] Moreover, citrullinated histone H3, a marker of NETs, was enriched in APS thrombi. In addition, the levels of thrombosis were decreased in APS mice when they were treated with deoxyribonuclease that dissolves NETs or with a neutrophil-depleting antibody.[44] These investigations suggest a direct role of NETs in thrombosis development of APS.

Neutrophil Extracellular Traps in Other Autoimmune Diseases

In fact, some studies have examined the link between NETs and other autoimmune diseases such as multiple sclerosis (MS), dermatomyositis (DM), polymyositis (PM), and IgG4-related autoimmune pancreatitis (AIP). Elevated levels of circulating NETs were detected in a subset of MS patients. Interestingly, gender-specific differences were found in circulating NETs in patients with relapsing–remitting MS, suggesting that NETs may underlie gender-specific differences in MS pathogenesis.[45] Zhang *et al.* have suggested that aberrant NET formation may be correlated with the pathogenesis of DM, PM, and the complication of interstitial lung disease (ILD).[46] NET production was increased or degradation decreased in DM/PM patients, and the lowest NET degradation was observed in DM/PM patients with ILD.[46] Recently, the role of NET formation also has been tested in IgG4-related AIP that is characterized by massive infiltration of IgG4-expressing cells into the pancreas, increased serum IgG4 levels, and storiform fibrosis. More importantly, the NET formation was found to contribute to pDC activation, IFN-α generation, and subsequent IgG4 production.[47] A subset of RA patients develops a progressing disorder, Felty's syndrome (FS), which clinically manifests with the coexistence of RA, chronic neutropenia, and spleen enlargement. Indeed, circulating autoantibodies in FS were found to preferentially bind to deiminated histones H3, H4, and H2A.[48]

Owing to the lower incidence of these autoimmune diseases and the lack of appropriate animal models, the relationships between NET formation and these autoimmune diseases have not been deeply studied. Although we believe that NET formation also plays important roles in the development of these autoimmune diseases, there are some questions. For example, dominant antibodies in different autoimmune diseases may target different components of NETs. It might be because that genetic and environment factors also contribute to the pathogenesis and development of autoimmune diseases.

Neutrophil Extracellular Traps and the Drug-Induced Autoimmune Disease

Other than genetic and environmental factors, some drugs are the important factors to induce autoimmune diseases. It has been reported that exposure to some medications, such as propylthiouracil (PTU), procainamide, hydralazine, minocycline, and clozapine, may lead to autoimmune diseases. Approximately 30% of patients treated with PTU produce MPO-ANCA or even develop MPO-ANCA-associated vasculitis. Moreover, about 20% of patients exposed to procainamide develop lupus-like syndrome.[49] Hydralazine-induced lupus or vasculitis-like features are found in approximately 5% of patients.[49,50] The use of minocycline or clozapine was reported to be correlated with the development of autoimmune diseases.[50] However, the studies for the underlying mechanisms of the drug-induced autoimmune diseases are rare. Nevertheless, some studies lead us to speculate that the drug triggers autoimmunity and mediates autoimmune disease in susceptible hosts by promoting NET formation. Both hydralazine and procainamide were found to markedly induce NET generation dependent on NADPH oxidase and PAD4.[51] In addition, rats treated with PMA and PTU produced abnormal NETs that were barely digested by DNase 1.[52] Importantly, Carmelo Carmona-Rivera *et al.* have found that NETs were present in skin lesions from patients with levamisole-induced vasculitis and levamisole-mediated NETs promoted endothelial dysfunction.[53]

NETosis as Therapeutic Targets

Further investigations on the inhibition of NETosis pathway provide potential therapeutic avenue for autoimmune diseases. Several stimuli bind to neutrophil receptors (like TLRs and complement receptors) to activate neutrophils and trigger NETosis. Disrupting these interactions can inhibit the process of NETosis and prevent disease development. TAK-242, a TLR4 inhibitor, reduced NET formation, suggesting a therapeutic effect on autoimmune diseases.[54] Chloroquine, a conventional drug used in SLE treatment, was found to suppress NET formation.[55] Similarly, C5aR inhibition with anti-C5 mAb suppressed ANCA-induced NET formation, delayed onset of proteinuria, and improved survival in lupus-prone mice.[54] Therefore, inhibiting the recruitment of neutrophils or the release of NETs may be an attractive strategy for SLE therapy. Indeed, Huang *et al.*

have indicated that milk fat globule-EGF factor 8 inhibited neutrophil migration and NETosis through downregulation of CXCR2 expression, resulting in attenuated early inflammatory responses in SLE and reduced tissue damage.[56]

Calcium mobilization is required for NETosis and calcineurin inhibitors (for example, cyclosporine A or tacrolimus) would be potential therapeutic agents by modulation of NETosis.[54] As a matter of fact, cyclosporine A and tacrolimus were very effective medications for SLE patients. ROS is necessary for NET formation and a series of ROS scavengers exhibit therapeutic effects on autoimmune diseases. For example, decreased NET generation was observed after treatment with ROS scavengers such as N-acetyl cysteine (NAC). Moreover, administration of NAC improved disease outcome of SLE patients in two clinical studies.[54] *In vivo*, treatment of Mito TEMPO, a specific scavenger for mitochondrial ROS production, blocked spontaneous NETosis and reduced disease severity in a mouse model of lupus. Recently, the effect of PAD inhibitors has been proved in mouse models to delay disease and prevent disease-related tissue damage in mouse models of SLE and RA.[54] Similarly, the inhibition of Cl-amidine on NET generation was believed to reduce atherosclerosis and arterial thrombosis in mice.[57] In addition, MPO, one of NET components, promotes chromatin decondensation during NETosis. As expected, PF1355, an inhibitor of MPO, could reduce vasculitis in a mouse model.[54] Moreover, tofacitinib, a Janus kinase inhibitor, blocked NETosis, leading to reduction of lupus activity and improvement in SLE-associated vascular damage.[58] Compared with conventional treatment, add-on metformin treatment reduced the risk of SLE clinical flares and decreased prednisone exposure possibly through downregulating the NET-mt DNA-pDC-IFNα pathway.[59]

DNAse is most commonly used to dismantle DNA, the major frame structure of NETs. It was shown that administration of DNase 1 led to the degradation of NETs and improvement of disease activity in mouse models of lung injury and lupus.[54] NE is an important by-product of the process of NETosis. Vitamin D 1,25(OH)$_2$D$_3$ prevented NET-induced endothelial damage of SLE patients through inhibiting the externalization of NE.[60] Particularly, NETs can activate the NLRP3 inflammasome to release IL-18 which in turn induces NET formation. Thus, inhibition of NRLP3 inflammasome or targeting IL-18 by monoclonal antibody can interrupt this feed-forward loop and would be more effective strategies for treating NETosis-related diseases. Similarly, in AVV, ANCA can activate neutrophils, monocytes, and endothelial cells to enhance the secretion of IL-8 which can mediate the fee-back loop of NET formation, suggesting that targeting IL-8 may also have a therapeutic potential.[19] Finally, downstream cascade of NETosis was inhibited by monoclonal antibodies targeting IFN-α (sifalimumab and rontalizumab) or B cells (rituximab).[1]

In addition, some microorganisms that can evade the attack of neutrophils by blocking NET formation such as secreting endonucleases.[61] Focusing on these mechanisms may provide us with novel insights into therapeutic targets of autoimmune diseases by deciphering the multiple molecules at different steps of NETosis.

Some clinical drugs targeting different molecules of NETosis have been widely and successfully used in SLE treatment such as chloroquine, cyclosporine A, and tacrolimus. The understanding on the inhibition of NET formation enables physicians to be more confident and more skillful to use those drugs for the treatment of autoimmune diseases. However, most other agents have just been identified to have therapeutic effects in animal models of autoimmune diseases; their significance in treating autoimmune patients needs to be further investigated.

In summary, NETosis is a specific form of cell death of neutrophils and increasing evidence has indicated that NETosis plays an important role in the pathogenesis and development of autoimmune diseases. In addition, the components of NETs may be highly possible candidates of new biomarkers for disease activity, diagnosis, or of therapeutic targets for autoimmune diseases.

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