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Could Neutrophil Extracellular Traps Elucidate the Mysteries of Pathogenesis?

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Abstract

Recently, NETosis emerged as a specific type of neutrophil death that is involved in innate immunity, and its products ''Neutrophil extracellular traps (NETs)'' are now implicated as new candidates in a diversity of pathologic states. NET formation in contact to different pathogens or a variety of stimuli, is dependent on nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and involves the generation of reactive oxygen species (ROS). They consist of processed chromatin bound to granular and selected cytoplasmic proteins and act mainly via toll-like receptors (TLRs) signaling pathway. Pathogens trapped in NETs are killed through dual oxidative and non-oxidative mechanisms, even those so large that they cannot be phagocytosed. NETs participate in clot formation in blood vessels and might be cytotoxic to tumor cells. Conversely, different mechanisms were found to mediate the pathogenic role of NETs in different pathological states such as: vascular disorders; severe sepsis; autoimmune diseases; pulmonary disorders; pregnancy related disorders; cancer and otitis media. Thus, molecules that affect the balance of NET induction and destruction or attack the integrity of the NET structure like: NADPH inhibitors; deoxyribonuclease (DNase); blocking antibodies against histones or ROS scavengers can be of therapeutic value.

Keywords: Neutrophil extracellular traps; NEtosis; Pathogenesis; Histone-citrullination; Plasmacytoid dendretic cells; Autoimmune; Cancer

Could Neutrophil Extracellular Traps Elucidate the Mysteries of Pathogenesis?

Long time ago, necrosis and apoptosis had been defined as the only known subtypes of cell death incriminated in the pathogenesis of variety of diseases. Recently, NETosis emerged as a specific type of cell death that occurs in neutrophils, and its products "Neutrophil Extracellular Traps (NETs)" are now implicated as new candidates in a diversity of pathologic states.

How Nets Form?

Originally, NETs were described to be produced by neutrophils in contact with pathogens such as bacteria, fungi, viruses and protozoa; a variety of stimuli such as proinflammatory cytokines; activated platelets and endothelial cells (ECs); nitric oxide (NO); monosodium urate crystals and various autoantibodies or even chemical compounds (e.g. phorbol-12-myristate-13-acetate) [1,2]. Diverse neutrophil receptors can signal to trigger NETosis, as binding via toll-like receptors (TLRs) or complement receptors (Fc receptors), in addition to cytokine receptors as interleukin (IL)-8, tumor necrosis factor (TNF)-α and interferon (IFN)-ϒ [1].

Following neutrophil activation, a number of nuclear and cytoplasmic events with dramatic alterations in the morphology of the cells must take place to complete NETosis. These events involve peptidyl arginine deiminase (PAD)-mediated histone H3 hyper-citrullination by converting arginine residues to citrulline ones, followed by chromatin decondensation, dissolution of cellular membranes including the nuclear membrane disintegration, and the final combination of both nuclear and cytoplasmic effector proteins before the last step, which is the extrusion of a protein-loaded NET into the extracellular environment [3-9].

Most studies indicate that NET formation is dependent on functional nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation, with the associated generation of reactive oxygen species (ROS) [4,7,10]. How ROS contribute to NETosis is a controversy. One proposition is that ROS directly induce the morphologic alterations occurring in neutrophils during NETosis. A hypothesized alternative is that ROS act to inactivate caspases, thus hindering apoptosis and instead promoting autophagy, a process leading to dissolution of cellular membranes [7]. Besides, myeloperoxidase (MPO) and neutrophil elastase were described to regulate NET release [4,7]. Nonetheless, NETosis might possibly take place in certain circumstances through ROS-independent mechanisms [11].

What is the Structure of NETs?

NETs are meshworks of chromatin fibers with a diameter of 15-17 nm associated with citrullinated histone H3 (H3Cit) and decorated with a number of antimicrobial factors which collaborate to form an extracellular net that traps and kills microbial pathogens [2,7,12]. The protein components of NETs include: MPO, cathepsin G, elastase, proteinases, defensins or bacterial permeability increasing protein (BPI), lactoferrin, gelatinase, Peptidoglycan Recognition Proteins (PGRPs) and calprotectin, delivered by the neutrophil granules [1,4,13].

It has been also noticed that, other immune cells such as mast cells, eosinophils and macrophages can release Extracellular Traps (ETs). These different ETs have some common features, despite of the type of cells from which they originated, but on the other hand, ETs arising from different cell types exhibit as well unique features, different from those originally depicted for neutrophils [11]. What's more, some

reports demonstrated the ability of eosinophils and neutrophils to form ETs using mitochondrial DNA instead of chromosomal DNA without induction of cell death [14]. Thus, NETosis appears to be an event completely independent of caspases. An extra feature that distinguishes NETosis from apoptosis and necrosis is the fact that both the nuclear and the granular membranes undergo fragmentation [11].

How to Recognize NETs?

Recognition of NETs by various techniques relies on different principles such as DNA detection with membrane-impermeable DNA dyes like SYTOX green or detecting changes in nuclear morphology (loss of lobules and expansion of the nucleus) and composition (migration of MPO to the nucleus). This could be done in tissue sections and in secretions or in a supernatant after releasing the NETs with a mild nuclease treatment [1,4,7]. Immunofluorescence microscopy and analysis of transmission or scanning electron microscopy data may be preferred approaches to distinguish NETosis. Importantly, fibrin may mimic NETs in scanning electron microscopy [15].

The Advantageous Effects of NETs

The advantageous effects of NETs have been described in several studies. NETs were at first identified for their anti-microbial activity and were involved in innate immunity. Their DNA fibers form web-like configurations and harbor several antibacterial proteins that assist in trapping and killing bacteria or other microbial pathogens, therefore, NETs play an important role in host defense [1,3,4,7]. While phagocytosis and degranulation typically take minutes to occur after microbial contact, NETosis is a more protracted event that lasts 2-4 hours after initial stimulation [1]. The pathogens trapped in NETs are killed through dual oxidative and non-oxidative pathways, even those so large pathogens that they cannot be phagocytosed, including gram-positive and gram-negative bacteria, yeasts, viruses and protozoan parasites. Though pathogen entrapment within the DNA fibers hinders the spread of microorganisms over the body and offers a higher concentration of antimicrobial effectors at the spot of infection, yet, some pathogens have developed a machinery to escape NETs. Such machinery, identified in Staphylococcus aureus, or Streptococcus pyogenes is based on the secretion of endonucleases which degrade DNA [1,16]. Because trapping occurs via charge interactions between the pathogen cell surface and NET components, some pathogens may evade trapping by altering their surface charge or making a polysaccharide capsule such as that formed by Streptococcus pneumonia [17].

Moreover, it was also discovered that chromatin and proteases discharged into the circulatory system during NET formation can mediate procoagulant and prothrombotic factors and participate in clot formation in blood vessels [18]. Besides, it was speculated that NET components akin to MPO, proteinases and histones might be cytotoxic to tumor cells and can slow down their growth or directly incarcerate tumor cells and thereby prevent their further dissemination [19].

How Can NETs Induce Harmful Effects?

On 2012, Kaplan and Radic [2] described the double-edged sword effect of NETs. Despite their positive role in host defense, the tissue damaging consequences of NET have been observed under many pathological conditions. NETs were found to arise at the expense of injury to the host when occurring at the incorrect time, in the incorrect place, or with incorrect magnitude, thus can guide unfavorable consequences. Therefore, both the generation and the destruction of NETs has to be tightly regulated to provide punctual defense against invading pathogens plus timely coagulation and to evade undesirable effects that are associated with overshooting release or reduced clearance of NETs. So, the mechanisms underlying the harmful effects of NETs might be explained as follows:

1-The prolonged existence of NETs in tissues is linked to the risk for development of autoreactivity against various components in NETs. Thus, NETs have been observed in a variety of inflammatory, autoimmune and vascular diseases [20,21].

2- The close contact of DNA strands carrying their cytotoxic proteases in NETs with the thin-wall blood vessels leads to endothelial damage or uncontrolled thrombus formation as documented in sepsis and small vessel vasculitis [21,22].

3-Degradation of NETs releases their associated proteins consisting mainly of histones, elastase, and other proteins to the extracellular milieu. These proteins are potential contributors to inflammation and tissue injury in sepsis and other inflammatory reactions [23].

4-NETs-associated proteins including histone and myeloperoxidase have direct cytotoxic effects. So, NETs were seen entangled with thin alveolar-capillary surfaces of the lungs during severe influenza inducing direct cytopathic effect to alveolar epithelial and endothelial cells via toll-like receptor-mediated signaling [23,24].

5-The existing fact that extracellular DNA is required for induction of immune responses has released a new prospect about the role of NETs in the pathogenesis of autoimmune diseases as NETs formed in response to infections expose chromatin and neutrophil proteins at inflammatory sites providing a novel sources of autoantigens [21,25]. In Systemic Lupus Erythematosus (SLE), NETs provide autoantigens and immunostimulatory damage-associated molecules. It is noteworthy that, NET formation involves ROS that modify DNA and proteins, making them more immunogenic. Likewise, "NET immunocomplexes" formed of anti-NET antibodies complexed with persistent NETs can be involved in the exacerbations of SLE and could be pathogenic in the development of glomerulonephritis [22]. This view is supported by the observation that infections by pathogens which frequently involve NETosis are principal candidates for initiating or enhancing autoimmune disease [25].

6-NETosis plays a pathogenic role in autoimmune small vessel vasculitis (SVV) by both presenting autoantigens such as proteinase-3 (PR3) and MPO to the immune system and mediating vascular damage. The formed anti-neutrophil cytoplasmic antibodies (ANCAs) notably, anti-PR3 and MPO ANCAs activate neutrophils to release ROS, destructive granular molecules and proinflammatory cytokines, resulting in necrotic inflammation of small blood vessels as shown in vitro and in animal models. Moreover, the levels of circulating NET components are usually elevated in active SVV patients and NETs are detected in kidney biopsies from SVV patients [26].

7-NETs released by tumor-associated neutrophils possess a possible role in cancer metastasis and immuno-editing [19]. NETs, through the action of their protease components could promote extravasation and metastasis. In addition, NETs power the platelet adherence to the metastatic cells by recruiting platelets, so protect the circulating tumor cells and attenuate the host immune response against them [12].

8-On the contrary, lack of NET formation has severe consequences as exemplified by patients suffering from chronic granulomatous disease. Those patients cannot make NETs due to an inactive NADPH oxidase complex and suffer from severe recurrent infections [4,27].

Pathogenic Role of NETs in Different Human Disorders

NET formation was observed in many infectious and non-infectious diseases, autoimmune and inflammatory disorders such as vascular disorders, sepsis, pulmonary diseases, and pregnancy related disorders and even in cancer.

Vascular Disorders

As mentioned formerly, abundance of NETs due to imbalanced production and destruction can lead to endothelial damage and uncontrolled thrombus formation and in turn endothelial cell activation can elicit NETosis. Furthermore, netting neutrophils may play important roles in the promotion of atherosclerosis, vasculitis of different etiology and other vascular disorders [22,23]. For example, disordered regulation of NETs has been implicated in the production of MPO-ANCAs and subsequent development of microscopic polyangiitis (MPA). A recent study has demonstrated the presence of NETs in glomerular crescents as well as in thrombi of MPA patients [28].

Currently, NETs were identified in luminal location within both murine and human atherosclerotic lesions. The pathophysiological mechanism of NET-driven atherogenesis was found to involve the autoimmune activation of plasmacytoid dendritic cells (pDCs). Early in atherogenesis, complexes of self-DNA (most likely NET-borne DNA, but also self-DNA from dying cells) and neutrophil-derived granule proteins (e.g. cathelicidin) stimulate pDCs in the vessel wall, resulting in a strong type I interferon (IFN) response, which drives atherogenesis. While at later stages of atherosclerosis, a NET-promoted vicious circle involving platelets and neutrophils may lead to atherothrombosis. In addition, heteromers of platelet-derived chemokines activate neutrophils to release NETs. These NETs may, in turn, further propagate platelet activation and induce thrombus formation [29,30].

Sepsis

It has been recognized that neutrophils and platelets contribute in the pathogenesis of severe sepsis. A number of cellular events augment trapping of bacteria in blood vessels: platelet TLR4 detect TLR4 ligands in blood and provoke platelet binding to adherent neutrophils. This directs vigorous neutrophil activation and formation of NETs which trap and kill bacteria in circulation. The complete event occurs primarily in the liver sinusoids and pulmonary capillaries, where this antibacterial mechanism brings damage to the endothelium and provides a scaffold and stimulus for widespread thrombus formation [25,31].

Autoimmune Disorders

NETs were known to provide a unique, stimulatory microenvironment that can break normal immune tolerance, and thereby predispose to autoimmunity due to exaggerated NETosis or diminished NET clearance. In autoimmune disease, NETs function via modulating the link between innate and adaptive immune responses by activating plasmacytoid dendritic cells (pDCs) through toll-like receptor 9 (TLR9), an intracellular receptor that recognizes DNA. The autoantibodies against chromatin as well as the DNA-complexed granular proteins and other proteins released by neutrophils during NETosis impel to many autoimmunity syndromes. Clinical and experimental evidence suggests that NETs participate in the pathogenesis of systemic lupus erythematosus (SLE), glomerulonephritis, small-vessel vasculitis (SVV), gout, Felty's syndrome and psoriasis [19,25,32-34]. In addition, IL-17; a proinflammatory cytokine released from Th17 cells and IL-23; a known activator of Th17 differentiation were found to stimulate mast cells to release ETs decorated with IL-17 in a variety of autoimmune diseases such as psoriasis, rheumatoid arthritis and inflammatory bowel disease [33].

SLE is a systemic autoimmune syndrome typified by autoantibodies against DNA, chromatin, and DNA-associated proteins, including NET components. Neutrophils derived from SLE patients display a number of abnormal features in their phenotype and function, such as increased aggregation; increased apoptosis that may lead to neutropenia; impaired phagocytosis that may impair clearance of apoptotic cell debris; and enriched low-density granulocytes (LDG) that has a much enhanced capacity to form NETs and externalize various immunostimulatory proteins in the peripheral blood. Neutrophils from SLE patients are more susceptible to produce NETs and a subset of SLE patients display diminished NET clearance ability by nucleases [32,35,36]. Two mechanisms were proposed to explain the impaired NET clearance in SLE: the presence of DNase1 inhibitors or anti-NET antibodies that protect NETs from degradation. The high numbers of immature neutrophils present in the blood of SLE patients reflect the rapid neutrophil turnover and are associated with an increased expression of neutrophil-associated genes. Furthermore, SLE-associated NETs are able to activate pDCs to produce type I IFNs, a phenomenon that may be mandatory in disease pathogenesis. Therefore, the persistently exposed NET components may directly damage tissues and may also activate complement, thereby amplifying disease [22,25,32,35,36]. Moreover, IL-17 externalization during NETosis might be important in the pathogenesis and organ damage in SLE with implications for lupus nephritis and accelerated atherosclerosis and vascular dysfunction [37].

In psoriasis, local production of type I IFNs, such as IFN-α, by pDCs is an important upstream event in the activation of autoimmune T-cells [38]. More recently, it has also been suggested that the secretory leukocyte proteinase inhibitor (SLPI) derived from NETs, can bind DNA and serve to convert self DNA into an activator of pDCs in psoriatic lesions [34]. However, in Felty's syndrome characterized by rheumatoid arthritis, splenomegaly and neutropenia, autoantibodies were found to be directed against citrullinated proteins especially PAD4-deiminated histones and to induce NETosis [39].

Although gout is not a typical autoimmune disease, it shares the characteristic of acute, sterile inflammation mediated by the pro-inflammatory cytokine IL-1β. The "gout-associated NETs" contain DNA, MPO, and the alarmin, high mobility group box chromosomal protein 1 (HMGB1), and may propagate the inflammatory response [40]. More recently, basophils and eosinophils were shown to release ETs in gouty crystals [41].

Pulmonary Diseases

Several reports have focused on the pro-inflammatory potential of NETs in a diverse range of pulmonary diseases. Previous findings demonstrated the participation of NETs in lung pathogenesis during lethal influenza pneumonia. Recent studies revealed that NETs induced during influenza infection do not participate in bacterial killing, but may further exacerbate lung pathology during secondary bacterial pneumonia [42].

NETs have been also detected in the lungs and plasma of patients with transfusion-related acute lung injury (TRALI) and acute respiratory distress syndrome (ARDS) suggesting that NETs are responsible for the endothelial damage and capillary leakage in the lung [25]. Moreover, NETs-derived DNA-protein complexes have been found in the airway fluids of cystic fibrosis patients where they can increase the viscosity of the sputum, induce lung destruction and provoke a negative impact on the lung functions [19,43].

NET induction has been observed in mycobacterium tuberculosis (Mtb) infection in response to neutrophil activation through both ROS dependent and phagocytosis dependent ways. Yet, NETs seem to be unable to kill mycobacteria; instead they have been shown to cause tissue-damaging effects assigned to the granular proteins and histones associated with the NETs [44]. Additionally, NETs may play a vital role in the partnership between neutrophils and macrophages during granuloma formation in tuberculosis. Experimentally, significant secretion of the cytokines such as IL-6, TNF-α, IL-1β and IL-10 was noticed from macrophages co-cultured with NETs obtained from Mtb-activated neutrophils. Also, NETs binding heat shock protein 72 (Hsp72) were able to trigger cytokine release from macrophages. Thus the immuno-modulatory role of NETs and proteins derived from them may influence not only chronic inflammation during tuberculosis but also immune regulation and autoimmunity [45].

Pregnancy Related Disorders

In preeclampsia, vast numbers of NETs were detected in the intervillous space of affected placentae occluding the site of oxygen exchange between the mother and the fetus. The presence of placental micro-debris in the maternal circulation lead to the activation of neutrophils as assessed by the elevated expression of CD11b. NETs could be also induced by other placentally derived factors, such as the cytokine IL-8 [46]. As preeclampsia is characterized by hypoxia-reperfusion damage, the presence of large numbers of NETs directly in the intervillous space, the site of oxygen exchange between mother and fetus, may contribute to this. NETs may also contribute to the widespread systemic damage to the maternal endothelium and promote thrombosis exacerbating the occlusion of blood flow through the intervillous space. The likelihood of such an event is high, as excessive fibrin deposition and infarction are frequently observed in preeclamptic placentae [47-49, 50].

Currently it is still not clear whether NETs are involved in other pregnancy-related disorders such as intrauterine growth retardation, recurrent fetal loss or preterm labor, but they may be involved in infertility, in recurrent fetal loss mediated by anti-phospholipid antibodies, or perhaps even in fetal abortion triggered by infections with microorganisms such as Listeria monocytogenes or Brucella abortus [50].

Cancer

Cancer immuno-editing refers to the combination of host-protective and tumor-promoting actions of immunity. A possible involvement of NETs in cancer was considered, however, up to this moment, it is difficult to decide whether NETosis plays a pro- or anti-tumorigenic role. It is speculated that NET components like MPO, proteinases and histones possess anti-tumorigenic effects by means of actual killing of tumor cells, inhibiting their growth, activating the immune system or scaffolding directly tumor cells and thereby preventing their further dissemination. Furthermore, probably through histones NETs can kill activated endothelial cell thus damaging tumor-feeding blood vessels [19,23,47]. Alternatively, NETs which harbor potent proteases could be pro-tumorigenic by degradation of the extracellular matrix and promotion of extravasation and metastasis besides helping metastatic cells to evade the immune response as by forming a barrier between cancer cells and the immune system, thus assisting cancer cells to escape immune recognition [12,19].

Otitis Media

Bacterial biofilm was increasingly recognized to play a role in the recurrence and persistence of infections such as otitis media (OM). Both animal and in vitro studies suggest that DNA is important in biofilm formation, stabilization and persistence of bacteria in OM. Such extensive extracellular DNA stranding was demonstrated in middle ear effusion being predominantly neutrophil derived through NET formation or less frequently derived from the bacteria. Haemophilus influenzae and Streptococcus pneumoniae are the most commonly identified otopathogens in the middle ear effusion of children with recurrent acute otitis media. These species can resist NET killing despite high levels of antimicrobial proteins due to production of DNases and through alteration of the bacterial surface. So, the inability of NETs to eliminate these otopathogens may contribute to establishing stable biofilm communities in the middle ear effusion. The later incident combined with the viscosity of the DNA may impede clearance of the middle ear effusion and the associated bacteria. Mast cells and eosinophils are also able to form extracellular traps in recurrent OM, but their role requires further investigation [51-53].

Therapeutic Implications of NETs

As our awareness of NET functioning expands, the modulation of NETosis may open up new paths for the development novel effective therapeutic agents in different disease states. Accordingly, molecules that affect the balance of NET induction and destruction or attack the integrity of the NET structure can be of therapeutic value.

For example, NET-induced cytotoxicity could be abolished or reduced by treatment with NADPH inhibitors that blocks NETosis, deoxyribonuclease (DNase) that disrupts NET, or blocking antibodies against histones or MPO [18,25]. Notably, administration of histone blocking antibody or DNase I protected mice from TRALI and anti-histone H4 antibody treatment reduced the mortality of mice in a sepsis model [25]. Therefore, inhibition of the effects of histones may prove beneficial in various inflammatory conditions or in endothelial damage [2].

In SVV, treatment with DNase1 not only caused inhibition of NET formation but also prevented vessel inflammation [54]. Also,

application of DNase1 in a murine model of deep vein thrombosis (DVT) protected mice from DVT and revealed that the cleavage of NETs by DNase1 prevents the cascade of events leading to thrombosis [55].

Various ROS scavengers are effective at reducing the release of NETs, and similar avenues may be available to repress NETosis in vivo in chronic inflammatory disorders [4]. MPO inhibitors, such as 4-aminobenzoic acid hydrazide, and various PAD4 inhibitors may have similar effects [7]. Furthermore, the role of colchicine or other drugs that destabilize the cytoskeleton, a structure implicated in NETosis, should be explored [56].

Obviously, a better understanding of how NETs are generated and whether NETs formed upon microbial exposure are distinct from those that form under "sterile" conditions, as exist in autoimmune or vasculopathic disorders, may allow the development of compounds that selectively target the deleterious aspects triggered by these networks [2].

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