Neutrophil extracellular traps — the dark side of neutrophils

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Neutrophil extracellular traps (NETs) were discovered as extracellular strands of decondensed DNA in complex with histones and granule proteins, which were expelled from dying neutrophils to ensnare and kill microbes. NETs are formed during infection in vivo by mechanisms different from those originally described in vitro. Citrullination of histones by peptidyl arginine deiminase 4 (PAD4) is central for NET formation in vivo. NETs may spur formation of autoantibodies and may also serve as scaffolds for thrombosis, thereby providing a link among infection, autoimmunity, and thrombosis. In this review, we present the mechanisms by which NETs are formed and discuss the physiological and pathophysiological consequences of NET formation. We conclude that NETs may be of more importance in autoimmunity and thrombosis than in innate immune defense.

Introduction

Neutrophil extracellular traps (NETs) are extracellular strands of decondensed (unwound) DNA in complex with histones and neutrophil granule proteins. NETs were discovered more than a decade ago (1) in a study demonstrating that they are generated in vitro after stimulation of isolated neutrophils with IL-8, a major neutrophil chemoattractant; LPS, a component of Gram-negative bacteria; or PMA, a potent activator of PKC. Further, these in vitro generated NETs possessed antibacterial activity, which was ascribed to the associated histones (2), proteolytic enzymes from granules that might degrade bacterial virulence factors, and enzymatically active myeloperoxidase (MPO). Notably, the antibacterial activity of NETs is abrogated by DNase (1). Induction of NETs by IL-8 and LPS indicates that NETs are formed during inflammation and infection, and NETs are found in vivo during bacterial infections such as appendicitis (1). This seminal publication demonstrating the existence of NETs showed that neutrophils may undergo an alternative death pathway, termed NETosis, which allows them to serve in innate immune defense even after their death. However, NETosis as a neutrophil death mechanism may largely be an in vitro phenomenon, and NETs seem to be generated in vivo by mechanisms different from those described in vitro. The purpose of this Review is to contrast the fundamental mechanisms for NET formation in vitro and in vivo and to discuss the biological relevance of the latter.

Mechanisms of NET formation

Although neutrophils are transcriptionally active cells, most of their DNA is transcriptionally inactive and condensed into heterochromatin within the nucleus. DNA is wrapped around histones to form nucleosomes and further organized into chromatin. Heterochromatin decondensation is mediated by peptidyl arginine deiminase 4 (PAD4), which catalyzes the conversion of histone arginines to citrullines, reducing the strong positive charge of histones and consequently weakening histone-DNA binding. This weakened interaction subsequently unwraps the nucleosomes, a prerequisite for NET formation (ref. 3 and Figure 1). Spikes in intracellular Ca^{2+} are important for propagating intracellular signal transduction during physiological neutrophil activation (4), and PAD4 is activated by Ca^{2+} (5). PAD4-deficient mice are unable to form NETs in response to physiological activators such as bacteria (6, 7). Thus, deimination of histones may be regarded as a sine qua non for NET formation in vivo.

In addition to PAD4, neutrophil elastase (NE) is considered essential for NETosis, as NE cleaves histones during NET formation (8). Accordingly, secretory leukocyte peptidase inhibitor (SLPI), an endogenous inhibitor of elastase (and of cathepsin G), inhibits formation of NETs (9). The central effect of elastase is corroborated by the inability of elastase-deficient mice to undergo NETosis (7, 10). Neutrophils from patients with Papillon-Lefèvre syndrome are devoid of all neutrophil serine proteases and are incapable of generating NETs by PMA stimulation (other inducing agents were not investigated) (11).

Inducers of NET formation

NET formation is more easily investigated in vitro than in vivo. While in vitro studies are easy to control, they are also artificial and make use of neutrophils isolated from blood, selecting for cells that adhere to slides. In vivo, neutrophils are captured by activated endothelium and are guided to sites of infection to undergo NET formation; this extravasation induces profound changes in neutrophils (12, 13). With this in mind, in vitro studies have identified several distinct activation pathways leading to NETosis that are believed, and in some cases have been shown, to be operative in vivo as well. These pathways include activation by integrins (complement receptor) and Toll-like receptors (14–16). Chemokine receptors are necessary for integrin activation and signaling (10, 17). Integrins generate signals that elicit NETosis in response to bacteria, and NETs are not produced when integrins are deficient, such as in leukocyte adhesion deficiency (18). L-Selectin–mediated signaling was recently demonstrated to elicit NETs in vitro (18). Notably, the initial description of NET formation elicited by IL-8 in vitro has not been uniformly confirmed (19).

Conflict of interest: N. Borregaard owns stocks in Novo Nordisk A/S in excess of $10,000.

Reference information: *J Clin Invest*. 2016;126(5):1612–1620. doi:10.1172/JCI84538.

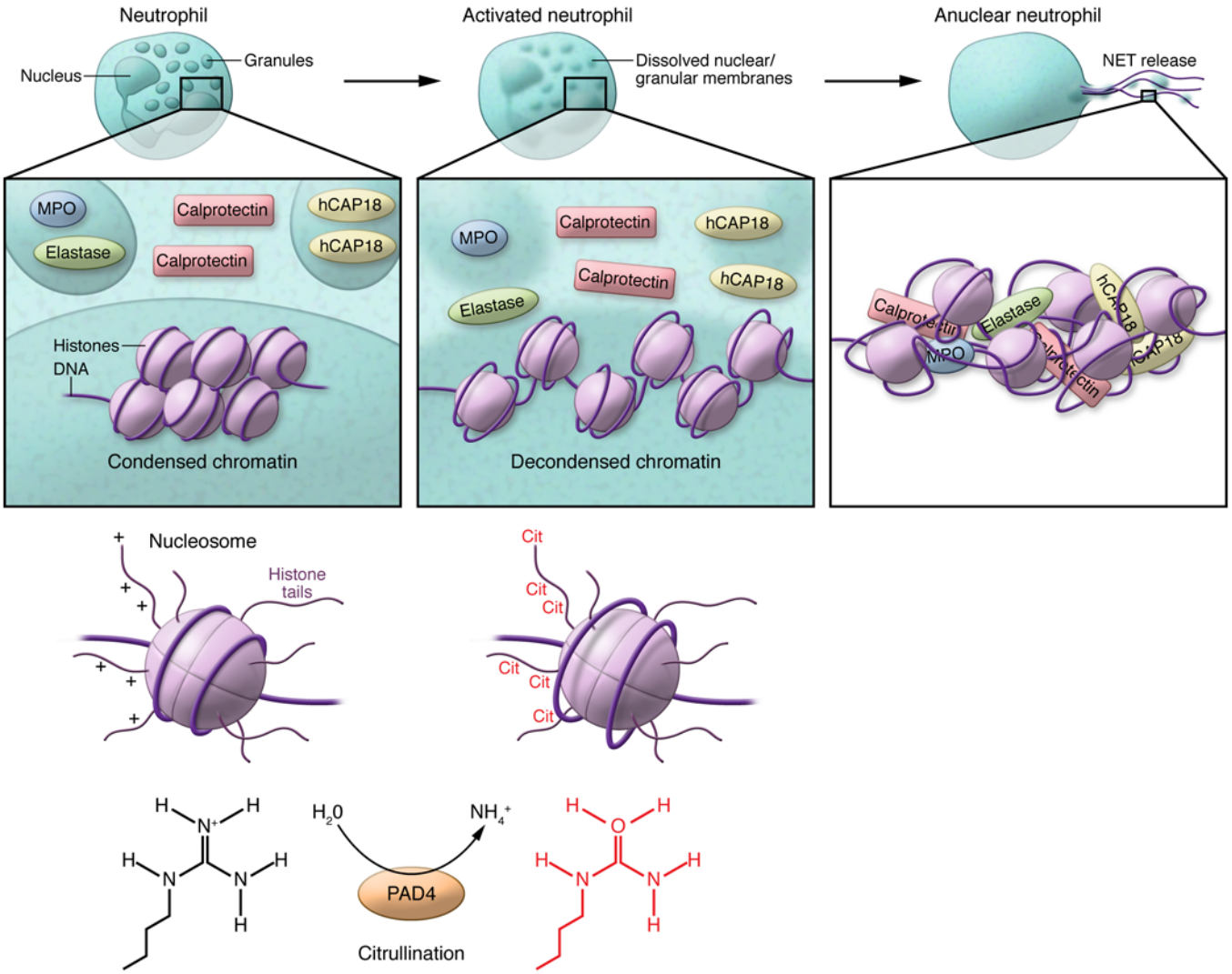

Figure 1. NET formation. In activated neutrophils PAD4 citrullinates certain histone arginines, and the tight electrostatic binding between histones and DNA in nucleosomes is weakened. Nuclear and granule membranes are dissolved. Decondensed DNA with citrullinated histones and granule proteins meet and are expelled from the neutrophil as NETs that may ensnare and possibly kill microbes. The surface membrane reseals and leaves a viable anuclear neutrophil behind. Cit, citrulline.

PMA-induced NETs. Most studies to identify NET formation mechanisms use PMA stimulation of isolated neutrophils. PMA is a potent activator of the neutrophil respiratory burst, which is elicited when activation of neutrophils leads to assembly of multicomponent NADPH oxidase (20). Defects in this process abrogate generation of superoxide and hydrogen peroxide and their derivatives, resulting in the severe immunodeficiency known as chronic granulomatous disease (CGD) (21). In the first description of NETs, generation of hydrogen peroxide was necessary for NETosis, and CGD neutrophils failed to generate NETs in vitro after PMA stimulation and exposure to *Staphylococcus aureus* (22). PAD4 may not be necessary for PMA-induced NETs (23), and PMA does not induce a rise in intracellular Ca^{2+} that could activate PAD4 (24).

MPO, which converts hydrogen peroxide to hypochlorous acid (25), appears to be required for PMA-induced NETosis (26). MPO binds histones and assists elastase during PMA-induced NETosis (8). The enzymatic activity of MPO appears to be essential, as chemical inhibition of MPO halts NETosis. Inhibition of MPO can be rescued by the addition of hypochlorous acid, but not hydrogen peroxide (27). These results suggest that hypochlorite induces chlorination of histones, reducing their positive charge to loosen histone-DNA interactions in a manner similar to histone citrullination. Interestingly, the authors found that MPO does not seem to be essential for NETosis by murine neutrophils under conditions where NETosis by human neutrophils was clearly MPO dependent (27). The mechanistic basis for this difference is unknown, but the nuclear morphology of murine and human neutrophils is very different, with doughnut-shaped circular nuclei prominent in murine neutrophils, and it is possible that this affects the sensitivity to hypochlorite.

While PMA efficiently induces NETs in human neutrophils in vitro, it is an artificial stimulus, bypassing membrane receptors and their signaling pathways. It is therefore unclear whether the components essential for PMA-induced NETosis are also essential

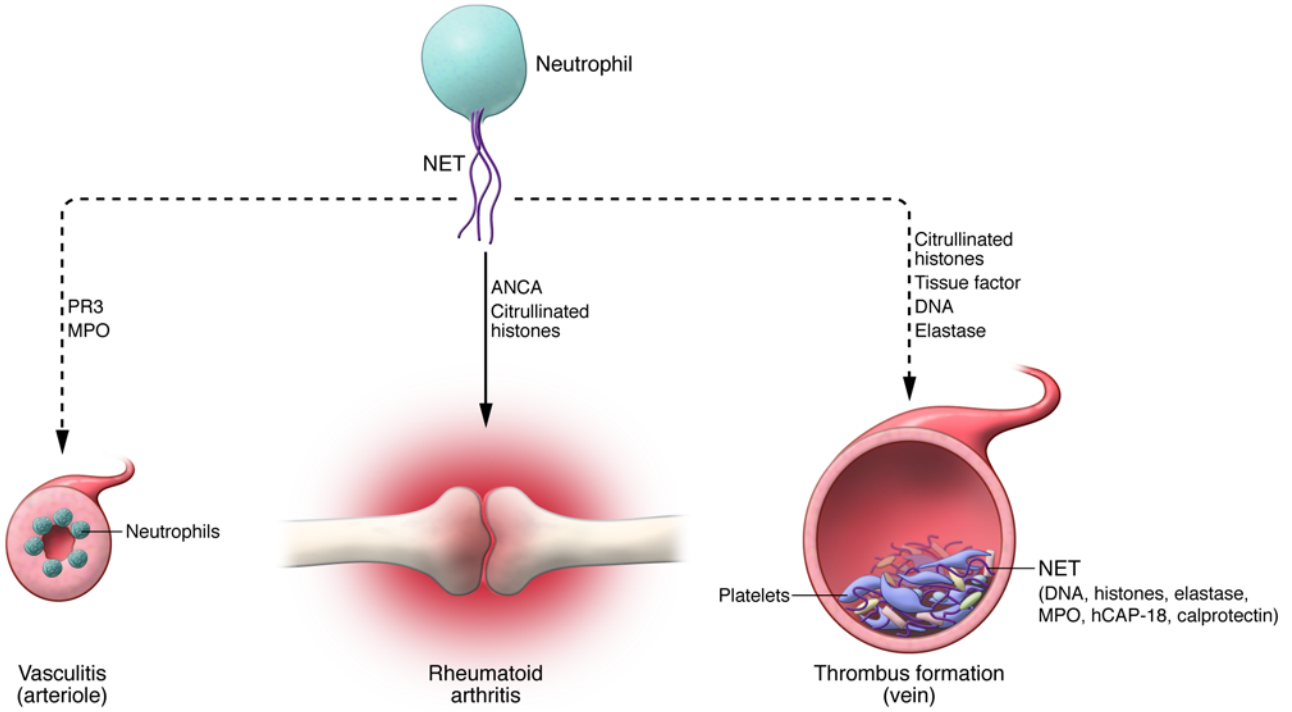

Figure 2. NET-associated histone modifications. NETs contain antigens, some of which are modified histones that act as neoantigens to induce formation of autoantibodies that induce or accelerate vasculitis, rheumatoid arthritis, and thrombus formation.

for physiologically relevant stimuli. The critical role of NADPH oxidase and MPO for NET formation may solely relate to in vitro activation by PMA, since in vivo studies show that generation of NETs is independent of ROS generated by NADPH oxidase (7).

Toxin-mediated NETosis. *S. aureus* secretes toxins that induce nuclear swelling of neutrophils and rapid release of their DNA content, which is still surrounded by membrane. The membrane subsequently disintegrates and releases DNA. This process does not require neutrophil activation (28). Phagocytosed toxin-producing *S. aureus* bacteria were shown to induce neutrophil lysis by activating endogenous mechanisms for necrosis (29). We consider these *S. aureus* toxin–mediated effects cytolysis, a process distinct from NETosis.

NETs formed in vivo. Endotoxin in blood activates platelets via TLR4, and these platelets associate with adherent neutrophils in liver sinusoids and in lungs, inducing NETs that both bind bacteria and mediate significant endothelial cell damage, as shown in mice (30). While LPS stimulation of neutrophils induces NETosis in vitro (1), NETs were only generated in vivo in response to platelet-bound LPS (30). Such NETs were able to ensnare *E. coli* and reduce bacterial dissemination (31).

The liver is exposed to bacteria and bacterial products from the portal blood flow, but the liver was recently shown to be the major organ of clearance of intravenously injected *S. aureus* in the mouse. Sinusoidal Kupffer cells capture Staphylococci from blood, and neutrophils are subsequently recruited and generate NETs with DNA-bound histones and elastase. Remarkably, the NET-bound elastase is enzymatically active despite the presence of plasma containing levels of antiproteases sufficient to completely inhibit NE. The *S. aureus*–induced NETs are anchored to vessel walls by von Willebrand factor (VWF) and cause major but transient liver cell damage. Intravital imaging of the liver showed that NET formation depends on both NE and PAD4 activity but does not depend on NADPH oxidase activity (7).

Microbes often invade through breaches of skin or mucosa and are detected by resident macrophages and mast cells. Signals from these cells activate endothelium, resulting in recruitment of neutrophils. Intravital microscopy revealed that neutrophils expel microvesicles containing decondensed DNA with histones and granule proteins, leaving the neutrophil with an intact surface membrane. Disruption of the microvesicles liberates NETs. This process has been named vital NETosis, as it allows movement of the denucleated neutrophils, which thereby exhibit signs of vitality (Figure 1). The anuclear neutrophils remaining after expulsion of nuclear DNA still contain granules. This is fundamentally different from the NETs generated by PMA (14).

Which cells make NETs?

When stimulated by IL-8, LPS, or opsonized bacteria, all neutrophils respond with a respiratory burst, yet NETs are made by only a fraction (approximately 20%) of neutrophils, depending on the stimulus (32). The reason for this is unknown. It is becoming increasingly clear that neutrophils in peripheral blood are not entirely homogeneous. Neutrophil subsets are known to differ in expression of CD177 (33), taste receptors with chemotactic activity (34), and intragranular proteins (olfactomedin-4 [OLFM4]) (35). Additionally, conditions wherein granulopoiesis is stressed by infections (emergency granulopoiesis) result in an increased number of neutrophils with altered functional phenotype, so-called myeloid-derived suppressor cells (MDSCs), which may be neutro-

phils that have been activated and partially degranulated (36, 37). Whether such phenotypic differences are relevant to the ability of these cells to undergo NETosis is not known, but circumstantial evidence points in this direction, as discussed below.

By definition, NETs can only be made by neutrophils. The terms extracellular traps (ETs) and ETosis have been developed to encompass extracellular decondensed strands of DNA from cells other than neutrophils. The requirements for a respiratory burst, NE, and MPO — as described for the classical NETs induced in PMA-stimulated neutrophils — can obviously only be met by neutrophils. However, eosinophils are also capable of generating a respiratory burst, and eosinophil peroxidase is capable of converting hydrogen peroxide into oxidizing halogen derivatives from iodide and bromide but not chloride (38). These eosinophil ETs (EETs) contain intact eosinophil granules, are able to ensnare bacteria, and can be demonstrated in vivo in eosinophil-rich secretions (39, 40).

Basophils can form ETs (BETs) that kill bacteria. Formation of BETs is not dependent on NADPH oxidase activity (41, 42). In contrast, mast cells were shown to release NADPH oxidase–dependent ETs (MCETs) in vitro when stimulated by *Streptococcus pyogenes* or by PMA (43). Monocyte and macrophage murine cell lines also form ETs when stimulated by PMA (44). Formation of ETs is thus far known to be restricted to cells of myeloid origin. Whether myeloid cells have nuclear characteristics or activities of hydrolytic enzymes in common that are not shared with cells from other lineages is not known.

Nuclear DNA versus mitochondrial DNA in NETs. Mitochondrial DNA (mtDNA) has been described as a major source of neutrophil and eosinophil ETs (45–47). mtDNA is not organized in nucleosomes and is not associated with histones (48), which are essential structures of NETs. While NETs composed of mitochondrial DNA do not seem to be elicited from neutrophils in vivo (14), mtDNA may be important as a stimulus for NET formation. During trauma, including major surgery, mtDNA is released into the circulation and can be sensed by danger-associated molecular pattern (DAMP) receptors such as TLR9 and induce NETs by an NADPH oxidase–independent mechanism (49, 50).

Do NETs kill microbes?

The vast majority of NET-associated proteins are histones (51), and proteins from cytosol and granules account for only 16% of NET-associated proteins, of which elastase constitutes one-third on a molar basis. This analysis is based on PMA-induced NETs and may differ for NETs induced by other methods (18). In general, small cationic proteins kill microbes by binding and inserting themselves into the negative lipid bilayer of microbial surfaces. Neutrophil granules are rich in antibacterial proteins and peptides that associate with NETs due to the negative charge of DNA, but DNA may act as a sink for such proteins, preventing their interaction with bacteria.

DNA itself is highly microbicidal due to the phosphodiester bond of the DNA backbone (Figure 2), which is a powerful chelator of divalent cations. It is therefore likely that citrullination of histones, which loosen their grip on DNA, make it possible for DNA to interact with bacteria and exert the antibacterial effect of NETs (52). Calprotectin, a member of the S100A family of cytosolic proteins, inhibits growth of fungi by chelating divalent metal ions (53). Calprotectin is the most prominent cytosolic protein in neutrophils, accounting for approximately 50% of all cytosolic proteins (54); it was identified in NETs (51) and has been shown to kill *Candida* and *Aspergillus* species (55, 56).

Are NETs important for immune defense?

Following the initial enthusiasm about the discovery of a new antimicrobial activity of neutrophils, the ability of NETs to kill microbes was questioned. Dissolution of NETs by DNase, long after the NET-exposed bacteria had officially been declared dead, allowed revival of the NET-associated microbes (57). Indeed, we found that bacteria bound to PMA-induced NETs are alive, as determined by live/dead staining (18). The report of a patient with chronic granulomatous disease (CGD) and *Aspergillus* infection who was rescued by gene therapy that restored the capacity to mount a respiratory burst and generate NETs has been considered proof of principle of the role of NETs in immune defense (58). The validity of this case as support for the in vivo significance of NETs has been questioned, as the effect might be explained by restoration of the ability to generate L-kynurenine from L-tryptophan by indoleamine 2,3-dioxygenase (IDO), a process that is dependent on superoxide and is essential to dampen the tissue destruction caused by *Aspergillus* infection (59). Furthermore, CGD patients have a selective deficiency in microbial killing (60). Thus, the inability to form NADPH-dependent NETs does not seem to play a major role in the immunodeficiency of CGD. Furthermore, studies on NET formation in vivo do not uniformly support a requirement for ROS in NET formation (7).

A rare autosomal recessive disease known as Papillon-Lefèvre syndrome is characterized by severe juvenile periodontitis and hyperkeratosis palmoplantaris. The condition is caused by inactivating mutations in *CTSC*, which encodes cathepsin C (also known as dipeptidyl peptidase 1) (61). This enzyme is required for removal of two N-terminal amino acids that block the active site of neutrophil serine proteases (62). Failure to remove these two N-terminal amino acids results in the inability to generate active enzyme and complete degradation of the serine proteases before mature neutrophils are formed (11, 63, 64). These patients are therefore completely devoid of all neutrophil serine proteases, including elastase, and hence incapable of generating NETs (11), except by saliva (18). Ironically, saliva is supposed to prevent periodontal disease, which is the hallmark of Papillon-Lefèvre syndrome, but saliva-generated NETs are clearly not sufficient. Do these patients have a severe immunodeficiency? Not as far as larger cohorts of this rare condition have shown (65, 66). Major infections, as seen in CGD, in severe congenital neutropenia, or in leukocyte adhesion deficiencies, are not a problem in Papillon-Lefèvre syndrome. The severe periodontal disease is considered to be due to microbes such as *Actinobacillus actinomycetemcomitans*. These microbes are efficiently killed by the antimicrobial peptide LL-37 (67), which is generated by proteolytic cleavage of cathelicidin (hCAP-18) from neutrophil-specific granules (68). This process is executed by proteinase 3 (PR3) (12), in which Papillon-Lefèvre neutrophils are deficient (11, 69).

As mentioned above, citrullination of histones by PAD4 is pivotal for the generation of NETs. Accordingly, PAD4-deficient mice are incapable of generating NETs; however, these animals do not

appear to have an impaired defense against microbial infections. The cecal ligation puncture (CLP) model to induce peritonitis is a very powerful model of severe infection. PAD4-deficient mice do not demonstrate higher morbidity or mortality than wild-type mice in this model (70). This was investigated both in low-grade CLP with more than 80% survival and in high-grade CLP with less than 20% survival; however, this model may not be appropriate for the study of NET antimicrobial activity. It is possible that the serosal surface of the peritoneum does not allow NETs to adhere and constrain bacteria. While wild-type and *Pad4*$^{-/-}$ mice exhibited significant differences in the levels of circulating DNA and citrullinated histones, the peritoneum was not examined for the presence of NETs. Less dramatic models of infection, such as skin infections or inhalation of pulmonary pathogens, seem more physiological and may provide a better substrate for NETs to capture microorganisms. Subcutaneous infection by *S. pyogenes* is more severe in PAD4-deficient mice, but only when the Streptococcus strain is mutated to delete DNase (6). All in all, current evidence does not point to a significant nonredundant function of NETs in innate immune defense.

NETs in the oral mucosa

Patients with leukocyte adhesion deficiency type 1 (lacking CD18) typically present with severe juvenile periodontitis due to the inability of neutrophils to control the oral mucosa microflora (71). We recently found that saliva contains numerous NETs (18). Sialyl Lewis^x, a carbohydrate structure present on several proteins including mucins in saliva and selectins, stimulates neutrophils present in saliva to rapidly dissolve their nuclear and granule membranes and catapult out decondensed DNA with associated granule proteins, i.e., NETs, through L-selectin–mediated signaling. These NETS are highly antibacterial and resistant to bacterial DNases. Of note, the formation of NETs in saliva is independent of NADPH oxidase activity, of serine proteases including elastase, and of β_2-integrins. The role of PAD4 was not investigated. Saliva from patients with Behçet's syndrome and from patients with attacks of aphthous ulcers fails to induce NETs, indicating a role for NETs in maintaining the integrity of the oral mucosa (18).

Statins and NETs

Treatment with statins was reported to augment PMA-induced NETosis of isolated neutrophils, monocytes, and macrophages (44). Statin treatment of phagocytes was shown to boost their extracellular killing of *S. aureus* in vitro, corresponding to the elevated generation of ETs. The authors further demonstrated augmented generation of extracellular DNA in the peritoneum after thioglycollate challenge and found enhanced ex vivo killing of *S. aureus* by peritoneal exudate cells harvested from simvastatin-treated mice; they demonstrated increased in vivo killing of *S. aureus* in a pneumonia model, but did not analyze the effect on survival of the mice. These results were presented as an explanation for the decreased susceptibility to bacterial infections associated with use of statins (72); however, if statins led to an increased production of NETs, statin users should exhibit enhanced thrombosis and autoimmunity, but the opposite is observed. Statins improve the resolution of experimental thrombi in a mouse model, and notably, fewer NETs were observed in thrombi of statin-treated mice than in controls (73). Use of statins is an independent factor associated with reduced risk of venous thrombosis (74), and statins ameliorate the activity of systemic lupus erythematosus (SLE), systemic sclerosis, and rheumatoid arthritis (RA) and reduce thrombosis in phospholipid syndromes (75). A randomized study to determine the effect of simvastatin in septic pneumonia and in particular the effect on NET formation is underway (76). In this study, elderly patients with septic pneumonia were randomized to simvastatin or placebo treatment. NET production from isolated patient neutrophils was assessed 72–96 hours after ex vivo stimulation with IL-8, *N*-formyl-methionyl-leucyl-phenylanine (fMLP), and LPS. No difference in NET production was observed between the two groups (David R. Thickett, personal communication). These findings indicate that statins do not alter NET production.

The dark side of NETs

NETs and acute respiratory distress. Acute respiratory distress syndrome (ARDS) can be elicited by several mechanisms. Transfusion-related acute lung injury (TRALI) and sepsis are major causes and point to activated neutrophils as being central for the rapid induction of respiratory failure due to interstitial pulmonary inflammation. NET-like structures are detected in the blood of patients with TRALI, NETs are abundant in alveoli of an experimental mouse model of TRALI (77) and in humans, and DNase I treatment inhibits experimental TRALI in mice (78). NETs with citrullinated histones, a marker of PAD4 activity, are present in blood smears of critically ill patients (79). A mouse model for ventilator-induced lung injury depends on platelet-neutrophil interactions in the pulmonary vessels wherein platelet activation provides agonists for neutrophil activation, resulting in intravascular NETs that compromise ventilation and pulmonary microcirculation (10).

NETs and autoimmunity. Autoimmune diseases such as vasculitis and SLE are characterized by the circulation of autoantibodies that recognize intracellular antigens. As mentioned above, NETs are extracellular complexes of components that are normally intracellular, including DNA, histones, and granule proteins, which are frequent targets for autoantibodies.

Antibodies to citrullinated histone H1 present in NETs are observed in SLE and Sjögren's syndrome (80) and in patients with RA (81), and PAD4 released from neutrophils generates additional citrullinated autoantigens in RA (82). Sera from SLE patients contain antibodies reactive to NETs (83). In addition to NETs providing antigens for autoantibody formation, the autoantibodies can induce NETs, and NETs may therefore be central to a vicious cycle that propagates inflammation in these inflammatory disorders (84, 85). Intravascular NETs generated in response to circulating bacteria provide a logical and perhaps pathogenic link between infection and vasculitis. NETs present autoantigens concomitantly with danger signals (extracellular DNA) to an immune system alerted by the infection (86). This potent cocktail of immune stimulators may explain not only the induction of autoantibodies associated with vasculitis but also the flares induced by infection (Figure 2). In antineutrophil cytoplasmic antibody–associated (ANCA-associated) vasculitis, autoantibodies against the NET components MPO and PR3 are dominant and are believed to be pathogenic, while in SLE, circulating immune complexes of anti-DNA antibodies and DNA cause disease by precipitating in the kidneys, skin, and joints (87).

DNase I degrades NETs, and neutralizing antibodies that decrease DNase I activity are associated with SLE (88). It is conceivable that NETs are formed as part of the natural lifespan of neutrophils and that NETs are continuously generated and circulate in plasma at low levels as a major source of circulating DNA (89) (discussed below). Enhanced spontaneous formation of NETs from isolated neutrophils was observed in two mouse models of SLE, the New Zealand mixed model (90) and the MRL/*lpr* model (91). In both cases, PAD4 inhibition significantly reduced NET formation and protected against SLE pathologies, including deposition of immune complexes in kidneys, proteinuria, and areas of skin affected by alopecia (reported only in the MRL/*lpr* model), and enhanced vascular relaxation as a measure of vascular pathology (91). The K/BxN autoantibody-mediated arthritis model depends on antibodies not associated with NETs, and PAD4 is not involved in disease activity, as PAD4-deficient mice develop inflammatory joint disease to the same extent as wild-type mice following transfer of serum from the K/BxN strain (92).

NETs and thrombosis. Platelets play a central role in activating neutrophils to generate NETs in liver and lungs during endotoxemia and sepsis (30), conditions that are associated with neutrophil activation and disseminated intravascular coagulation. However, a key question is whether NETs play a general role in promoting thrombosis independent of infection. NETs with citrullinated histones, which serve as proof of active NET formation, were observed in organizing thrombi, i.e., thrombi that are infiltrated by neutrophils (93), suggesting but not proving a causal link between NETs and thrombosis. The ability of intravenous DNAse I treatment to prevent thrombosis indicates that extracellular DNA as found in NETs is thrombogenic (89, 94), and NETs were found to be a scaffold for thrombus formation in an in vitro model of thrombosis (94). A study demonstrating that PAD4 is essential in a mouse model of venous thrombosis, in which thrombosis is induced by partial ligation of veins, provided proof of a direct role for NETs in thrombosis (95). Notably, mice with myeloid-specific PAD4 deficiency protected against thrombosis to the same extent as mice that are entirely PAD4 deficient, demonstrating that NETs do indeed play a role in venous thrombosis.

An interesting issue to be addressed is whether the partial ligation of veins induces local NET formation, or whether the reduced blood flow allows preexisting NETs to initiate contact with platelets and coagulation factors. In other words, are intravascular NETs formed constitutively, perhaps by neutrophils adherent to the vessel wall as part of the normal life cycle of neutrophils; or do they form only during stasis or endothelial cell damage, such as occurs during atherosclerosis? As recently reviewed, NET DNA can be found circulating under normal conditions, with levels enhanced during infection, inflammation, and cancer, i.e., in conditions where activation of coagulation is a prominent feature, pointing to NETs as contributing factors for thrombosis under both normal and pathological conditions. The issue of neutrophil subpopulations is also relevant when discussing NETs and thrombophilia, since low-density granulocytes (LDGs) increase in number with vasculitis (37), cancer (96), pregnancy (97), and infection (98). All of these conditions are associated with thrombophilia and with increased levels of NETs, possibly due to spontaneous intravascular NET formation from LDGs.

It is not clear which components of NETs are thrombogenic. Tissue factor (99), the contact system (100), DNA, histones, and possibly elastase present on NETs are all recognized activators of coagulation (101). P-selectin, expressed on platelets or in soluble form, induces NETs (102), pointing to an intimate connection between platelet activation and NETs; this again places NETs at center in a vicious cycle that promotes thrombosis.

Endothelial cell–derived VWF binds NETs both in vitro (103) and in vivo (7), and VWF secreted from endothelium binds NETs and recruits neutrophils to generate NETs that were shown to result in significant tissue injury to the myocardium during reperfusion after ischemia (104). It is therefore possible that endothelial cell activation such as occurs during inflammation and infection promotes thrombosis both by secretion of VWF in itself and by promoting NETs with bound VWF, thereby providing a concentration of procoagulant factors, i.e., VWF, P-selectin (which is also secreted from endothelial cells), DNA, histones, elastase, tissue factor, and the contact system.

Antiphospholipid antibodies are thrombogenic. The dominating autoantibodies in antiphospholipid syndromes target β_2-glycoprotein I (105). β_2-glycoprotein I is a circulating phospholipid-binding glycoprotein secreted by the liver, endothelial cells, monocytes, and trophoblasts (which is of relevance for thrombosis of the placenta). It is unclear how antiphospholipid antibodies induce thrombosis, but autoantibodies against β_2-glycoprotein I induce NETs and enhance thrombosis (106). Moreover, sera from patients with antiphospholipid antibodies have reduced capacity to dissolve NETs (107). This strongly indicates that NETs may be central to thrombosis in the antiphospholipid syndromes.

Vaso-occlusive crisis (VOC) is a common and serious complication in sickle cell disease (SCD) that is often precipitated by infections. Elevated levels of circulating NETs are present in blood from SCD patients, and TNF-α treatment of SCD mice results in a profuse and deadly accumulation of NETs in pulmonary vessels. NET accumulation was prevented by pretreatment with DNase I, indicating that NETs are critical for the development of VOC in SCD (108).

If NETs are important for thrombosis in general and for the thrombophilia associated with cancer, a prothrombotic condition that is difficult to control, then the recent development of potent inhibitors of PAD4, which are effective in blocking NET formation (109), may provide new therapy for controlling thrombosis in cancers and other thrombophilias.

NETs and diabetes. High levels of glucose augment NET production from isolated neutrophils in vitro, and high levels of NET components, nucleosomes, and neutrophil elastase were detected in the plasma of type 2 diabetes patients with elevated levels of glycated hemoglobin (HbA1c) (110). Neutrophils from diabetes patients and from diabetic mice generate NETs more readily than normal neutrophils, which corresponds well with the elevated levels of proinflammatory cytokines in diabetes (111). Additionally, wound healing, which is compromised in diabetes, is impaired by NETs, but this impairment was not observed in diabetic PAD4-deficient mice (111).

Conclusion

The initial excitement about the potential role of NETs as a mechanism to constrain and eliminate invading microbes resulted from the description of NETs generated in vitro by PMA may turn out

to be largely of in vitro significance and without physiological impact. Without a doubt, NETs are generated both intravascularly in response to sepsis and in tissues in response to local infection; however, current evidence does not support a major role for NETs in host defense. Killing of microbes is generally executed within the phagocytic vacuole, where microbes are exposed to a very high concentration of antimicrobial peptides and ROS. NETs do not generate ROS, and while NETs supply DNA and histones as antimicrobial agents that are not present in phagocytic vacuoles, their concentration may simply be too low for them to exert sufficient antimicrobial activity. Studies investigating NETs in thrombosis, vasculitis, and autoimmune diseases have highlighted the possible importance of neutrophils and extracellular DNA in these conditions. However, the connection to NETs requires further validation. Current evidence suggests that PAD4 is pivotal for NET formation in vivo. A phenotype in the *Pad4*-KO mice cannot necessarily be taken as definitive proof of NET involvement, as PAD4 may have important functions other than NET formation.

Whether NETs are formed spontaneously as part of the life cycle of neutrophils — and particularly from the LDGs that are present in normal blood and in increased amounts during infections and inflammation (vasculitis) — has not been addressed, but such evidence would explain the apparent causal relationship between infection and thrombosis. While NETs may arise by different mechanisms, deimination of histone arginines is a fulcrum for all in vivo generated NETs. Deimination of histone arginines may create neoantigens that spur autoimmunity. Additionally, this process may serve a central target for therapy in conditions where NETs are pathogenic.

Acknowledgments

N. Borregaard is funded by the Danish Medical Research Council.

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