

REVIEW ARTICLE

Extracellular DNA traps in allergic, infectious, and autoimmune diseases

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Abstract

Extracellular DNA traps are part of the innate immune response and are seen with many infectious, allergic, and autoimmune diseases. They can be generated by several different leukocytes, including neutrophils, eosinophils, and monocytes, as well as mast cells. Here, we review the composition of these extracellular DNA-containing structures as well as potential mechanisms for their production and function. In general, extracellular DNA traps have been described as binding to and killing pathogens, particularly bacteria, fungi, but also parasites. On the other hand, it is possible that DNA traps contribute to immunopathology in chronic inflammatory diseases, such as bronchial asthma. In addition, it has been demonstrated that they can initiate and/or potentiate autoimmune diseases. Extracellular DNA traps represent a frequently observed phenomenon in inflammatory diseases, and they appear to participate in the cross-talk between different immune cells. These new insights into the pathogenesis of inflammatory diseases may open new avenues for targeted therapies.

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The formation of extracellular DNA traps has been reported as an efficient mechanism in host defense used by neutrophils (1), eosinophils (2), mast cells (3), and monocytes (4). These cells of the innate immune system play important roles in fighting against infections, particularly at barrier sites, where the organism comes in contact with the environment. Extracellular DNA traps have been shown to be able to bind and

to kill bacteria (1–4) and fungi (5–7) in the extracellular space, but may also contribute to immunopathology (1, 2) or even cause autoimmunity (8–10). It should be noted that extracellular DNA-containing structures have also been described in plants, protecting the root tips against infections (11). The observations in plants support the concept that DNA, besides its function in maintaining and providing genetic information, can play an additional role in the innate immune system that is conserved from the plants via insects, various vertebrates to man (Fig. 1) (reviewed in 12).

This article summarizes recent findings regarding the molecular composition of extracellular DNA traps, discusses some controversial issues regarding the origin of DNA fibers and the requirements for their release, and reviews the observations on these structures in diseased tissues and organs. We focus mainly on data obtained in human and mouse sys-

Abbreviations

ANCA, antineutrophil cytoplasm antibody; C5a, complement factor 5a; CGD, chronic granulomatous disease; DNase, deoxyribonuclease; EET, eosinophil extracellular trap; LPS, lipopolysaccharide; MCET, mast cell extracellular trap; MET, monocyte extracellular trap; MPO, myeloperoxidase; NET, neutrophil extracellular trap; pDC, plasmacytoid dendritic cell; PR3, proteinase 3; TF, tissue factor; TLR, Toll-like receptor.

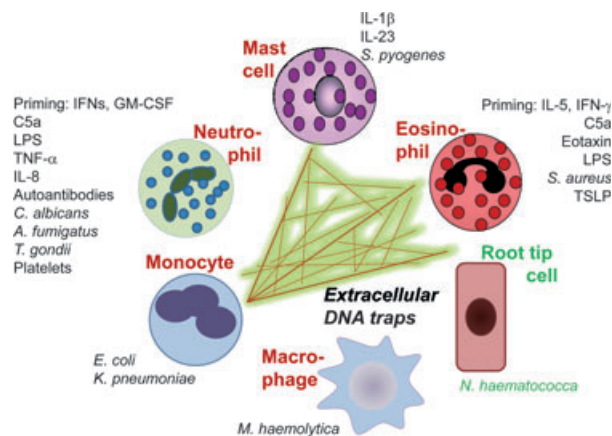


Figure 1 Cells and the triggers (animal: red, plant: green) needed to produce extracellular DNA traps.

tems. We will first discuss the different cell types and the molecular mechanisms leading to extracellular DNA trap formation. In the second part of this work, we will summarize our current knowledge regarding the presence and function of these newly discovered extracellular structures under pathological, particularly inflammatory, conditions.

Neutrophil extracellular traps

Activated neutrophils can form protrusions and prominent extracellular fibrous structures, called neutrophil extracellular traps (NETs) (1). NETs are composed of DNA and contain proteins from azurophilic (primary), specific (secondary), and tertiary granules (1, 13). Interestingly, NETs also contain histones (1), which exhibit strong antibacterial effects (14). Myeloperoxidase (MPO) has been reported to exhibit a dual function in NET formation: It is required for NET formation and mediates a direct antibacterial activity within the NETs (15, 16).

Several observations suggest that NETs are a result of an active process performed by viable neutrophils: First, motile neutrophils generate NETs (17). Second, neutrophil survival factors, such as GM-CSF, interferons, IL-8, C5a, and LPS, stimulate NET formation (1, 17, 18). Third, NET formation is rather rapid and occurs much earlier than any regulated cell death process (NETs can be produced within 5–10 min after activation) (1, 17–19). Fourth, the ejection of DNA appears to require energy (17). Fifth, cytoplasmic markers other than granule proteins are not found within NETs (1, 17). Indeed, NETs were formed by viable neutrophils both under *in vitro* and *in vivo* conditions (17, 19, 20). However, the current opinion held by most investigators is that the DNA release process requires the cell death of neutrophils (21). This type of cell death (as required for NET formation) has been called NETosis as it is believed that it must differ from both apoptosis and necrosis (22). Unfortunately, this concept is largely based on observations made using PMA-stimulated neutrophils without careful time-dependent analysis of NET formation and death-related events. In addition,

live-cell microscopic analysis revealed diffuse DNA release from neutrophils after PMA stimulation alone, and the generation of DNA-containing fibers was not demonstrated (21). Furthermore, the time window between potential NET formation and cell death following PMA stimulation is rather small, explaining, at least partially, why the concept of NETosis which has evolved might be misleading. Taken together, the observations show that NETs can be formed by viable neutrophils. Cell death may subsequently occur, but has never been convincingly demonstrated as being a requisite for NET formation. Some authors use the term 'NETosis' to describe the process of NET formation that, however, does not seem to add more clarity in the nomenclature regarding potential mechanisms of DNA release.

Other controversial findings previously reported are related to the origin of the DNA found within NETs. Clearly, if cell death is hypothesized as a requirement for DNA release, one would assume that NETs consist of nuclear DNA following disintegration of nuclei. However, by a combination of microscopic and molecular biological techniques, it has been demonstrated that NET-DNA is of mitochondrial origin (17). Recently, another rapid mechanism of DNA release was reported that involves vesicles containing nuclear DNA but without cell death (19). Regardless of its origin, the DNA within the NETs, although itself unlikely to be toxic, appears to be essential for extracellular killing of bacteria (1, 17). Moreover, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity was required for DNA release, as has been demonstrated by pharmacological and genetic approaches (1, 17). Neutrophils had to be sufficiently mature in order to express the required machinery for transmembrane signaling events (18).

Eosinophil extracellular traps

Eosinophils are found in hematopoietic and lymphatic organs as well as in the gastrointestinal tract, where they physiologically reside in the stomach, small intestine, and colon in man (23). Eosinophils are believed to be involved in host defense against helminth and bacterial infection, particularly by releasing cationic granule proteins (24). In addition, eosinophils have been demonstrated to express multiple cytokines under *in vivo* conditions, suggesting that they play important immunoregulatory roles (25). Recently, eosinophils were reported to be able to kill bacteria in the extracellular space by releasing mitochondrial DNA together with granule proteins to form net-like structures called eosinophil extracellular traps (EETs) (2). Colocalization of released DNA with granule proteins, such as eosinophil cationic protein (ECP) and major basic protein (MBP), has been observed both *in vitro* and *in vivo* (2, 26–28). As shown with pharmacological blocking experiments, the underlying intracellular mechanisms leading to ejection of mitochondrial DNA on the one hand and piecemeal degranulation of granule proteins on the other seem to be at least partially different (2).

Isolated eosinophils require priming with either IL-5 or IFN- γ and subsequent brief stimulation with lipopolysaccharide (LPS), eotaxin, or complement factor 5a (C5a) for EET formation (2). Recently, it has been reported that

thymic stromal lymphopoietin (TSLP) and *Staphylococcus aureus* serve as strong stimuli for EET generation in the context of eosinophil adhesion (29). Like NETs, EETs and their ability to kill bacteria could be destroyed by deoxyribonuclease (DNase) (2). Evidence that mitochondrial and not nuclear DNA is released by eosinophils was obtained from live-cell imaging analyses showing that the DNA was derived from extranuclear cell structures, as well as from several molecular biological techniques (2). The DNA release took place in viable eosinophils in a catapult-like manner in less than 1 s. Neither in tissue nor in isolated blood eosinophils was EET production seen in association with eosinophil cell death, suggesting that the release of mitochondrial DNA and granule proteins is an active process by viable cells (2, 29).

Mast cell extracellular traps

Mast cells are tissue-dwelling cells found at various surfaces of the body, such as skin and mucosa of the respiratory and gastrointestinal tract. They have been recognized as effector cells in IgE-mediated allergic diseases (29), and in addition, there is increasing evidence that mast cells play a role in innate immune defense (30). For instance, mast cells were able to kill opsonized bacteria upon binding to complement or IgG Fc receptors, and endocytosis was observed *via* both oxidative and nonoxidative pathways (30). Furthermore, it has been reported that mast cells prestore and release pro-inflammatory cytokines and compounds with antimicrobial activity from their granules upon contact with pathogens (30). Like eosinophils and neutrophils that are able to form EETs and NETs, respectively, mast cells also generate extracellular structures composed of DNA and granule proteins such as cathelicidin LL-37 and tryptase (3) that have been called mast cell extracellular traps (MCETs). The bacteria were shown to be entrapped by MCETs, and, as with NETs and EETs, digestion of the DNA within MCETs by DNase abolished the extracellular bacterial killing effect (3). MCET formation was NADPH oxidase dependent and occurred within 15 min after exposure of mast cells to *Staphylococcus pyogenes*. If the exposure to *S. pyogenes* was prolonged over hours, some mast cell death occurred and MCETs acquired histone proteins in addition to DNA and granule proteins (3).

Monocyte extracellular traps

Monocytes are active innate immune effector cells responding to pathogenic organisms in the blood, mucosal surfaces, and tissues (31). They produce inflammatory cytokines and are able to take up cells and toxic molecules (30). During inflammation, they may differentiate to macrophages or dendritic cells (31). Following phagocytosis, monocytes rapidly die, undergoing either classical apoptosis or nonapoptotic cell death depending on the type of bacteria to which they had been exposed (4). In response to *Escherichia coli* or *Klebsiella pneumoniae*, monocytes were observed to form extracellular DNA traps within 1 h postexposure (4). These structures were shown to contain histones in addition to DNA (4). Furthermore, DNA release was associated with caspase-1

activation; its pharmacological inhibition significantly reduced the number of monocytes releasing DNA (4). It should be noted that extracellular DNA release in association with histones and cathelicidins from PMA-stimulated RAW 264.7 macrophages has also been reported (32). Recently, it was shown that bovine macrophages can produce DNA traps upon stimulation with *Mannheimia haemolytica* bacteria (33).

Extracellular DNA traps in infectious diseases

Extracellular DNA trap formation has been related to host defense in both animals and plants (Fig. 2). Gram-positive and gram-negative bacteria have been demonstrated entrapped by the extracellular DNA structures generated by neutrophils (1), eosinophils (2), mast cells (3), monocytes (4), and plant root tips (11). Attacking and killing bacteria require both DNA and antimicrobial proteins, as either disintegrating the traps with DNase or blocking of antimicrobial proteins abolished the bactericidal activity (1–3). The first evidence that extracellular DNA traps play a role *in vivo* came from the observation that NETs could be detected during *Shigella* infection of rabbits as well as in spontaneous human appendicitis (1). In addition, EETs were shown to be present in the gut of patients suffering from *Spirochetosis* or *Schistosoma* infection (2). EETs were also present in infectious skin diseases, such as creeping disease and scabies (26). It has been hypothesized that the formation of extracellular DNA structures allows a targeted delivery of antimicrobial components to the invading pathogens and/or limits local immunopathology (1, 2).

Besides such descriptive data, functional observations demonstrating antibacterial activity of extracellular DNA traps have also been reported using experimental *in vivo* models. For instance, in experimental sepsis, it could be shown that TLR4-activated platelets induce platelet–neutrophil interactions, resulting in NET formation in blood vessels, especially in liver sinusoids and pulmonary capillaries. These NETs were able to entrap gram-negative bacteria (20). On the other hand, in this sepsis model, platelet-mediated NET formation was associated with increased endothelial and tissue damage, suggesting that NETs might also be relevant for multiorgan dysfunction in sepsis patients (20). The antibacterial role of eosinophils in association with EET formation has been demonstrated in IL-5-transgenic mice (2).

The formation of extracellular DNA traps appears to be particularly important for the defense against pathogens that are too large to be phagocytosed, such as fungi, for example *Candida albicans* or *Nectria haematococca* (5, 11). Moreover,

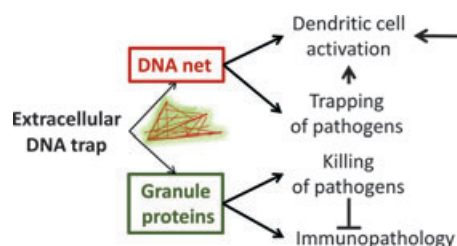


Figure 2 Potential roles of extracellular traps in host defense.

in a mouse model of infection with the protozoan *Toxoplasma gondii*, NETs have been demonstrated to limit parasitic infection (34). In addition, *Leishmania promastigotes* induced NETs, which subsequently captured and killed the pathogen, a mechanism that seems to be important in human cutaneous *Leishmania* infections (35).

In subgroups of patients with immune deficiencies, defects in the production of extracellular DNA traps have been identified. Neutrophils from patients completely deficient in MPO were unable to produce NETs and, thus, failed to inhibit *Candida albicans* growth (15). In addition, both neutrophils and eosinophils from patients with chronic granulomatous disease (CGD) were unable to generate extracellular DNA traps due to an impaired NADPH oxidase function (2, 17, 21). Following gene therapy for CGD, the ability of neutrophils to produce NETs was restored, resulting in rapid cure of a patient suffering from invasive aspergillosis (36). Moreover, the protective effect of statins on pneumonia infections was found to be due to enhanced NET formation, resulting in efficient bacterial clearance (32). Sputum of patients with cystic fibrosis contains abundant NETs (37). Nuclease therapy leads to degradation of DNA structures and subsequent increase in neutrophil elastase proteolytic activity (37).

Meanwhile, a number of mechanisms allowing bacteria to escape DNA trapping have been recognized. For instance, the DNases of invasive group A *staphylococci* and *pneumococci* (*Streptococcus pneumoniae*) are able to degrade the extracellular DNA and thereby destroy NETs, resulting in severe infection, such as necrotizing fasciitis and pneumonia, respectively (38–40).

Extracellular DNA traps in allergic diseases

In the pathogenesis of allergic diseases, eosinophils play an important role, as they are known to release a broad spectrum of cytokines and mediators that may lead to tissue damage, enhancement of inflammation, and remodeling (25). Eosinophils are frequently found in asthmatic airways and the numbers increase upon allergen challenge and asthma exacerbations (41). In one subgroup of asthma patients, a predominantly neutrophil inflammation was observed (41). Recently, the question has been addressed, whether eosinophils and neutrophils infiltrating the airways produce extracellular DNA traps in human asthmatics (27). In bronchial biopsies taken from allergic asthmatics, the number of EETs consisting of DNA and colocalizing MPB correlated with the number of infiltrating eosinophils (27). In patients with a high number of infiltrating neutrophils, formation of NETs was observed (27). Interestingly, allergen challenge was not followed by a detectable increase in extracellular eosinophil or neutrophil DNA trap formation. What might be the function of EETs and NETs in asthma? Clearly, DNA traps might be produced as efficient tools to fight against infectious agents potentially invading bronchi after epithelial cell damage. On the other hand, it is still possible that the DNA traps themselves contribute to the epithelial cell damage characteristic for asthma. Finally, a DNA trap-mediated mechanism programmed to limit tissue damage, for example by preventing the wide distribution of cytotoxic proteins, can also

not be excluded. Thus, currently, the role of EETs and NETs in asthma remains unclear.

Besides asthma, EETs have also been detected in acute atopic dermatitis, allergic contact dermatitis, and drug hypersensitivity reactions using immunofluorescence staining techniques and subsequent confocal microscopic analysis of biopsy specimens (26). Z-stack analysis of the slides revealed the three-dimensional structures of EETs composed of DNA and ECPs in eosinophilic skin tissues (26). Although the overall number of eosinophils producing EETs in these tissues was rather small, aggregates of EETs around clusters of eosinophils have been detected, for example in allergic contact dermatitis, where up to 30% of eosinophils had carried out EET formation (26). Similar to the situation with asthma, at present, we can only speculate on the pathogenic role of these EETs in allergic skin diseases. Evidence that EETs might cause tissue damage was obtained by an observation made in bullous pemphigoid, an autoimmune skin disease with subepidermal blister formation. In prebullous stages, EET-producing eosinophils were seen lining up along the cells of the dermal–epidermal junction. In the epidermis covering these areas, the basal cell layer showed signs of apoptosis. These findings suggest that the EETs might contribute to blister formation (26). Moreover, the presence of EETs in bullous delayed pressure urticaria also points to the possibility that EETs participate in blister formation (28).

Extracellular DNA traps in autoimmune diseases

Psoriasis is considered an autoimmune disease exhibiting a predominantly pathogenic role of IL-17 and IL-23 (42). Among the inflammatory cells, increased numbers of mast cells are present in the papillary dermis. Neutrophils infiltrate both dermis and epidermis, where they form microabscesses. Recently, it has been reported that both neutrophils and mast cells produce extracellular DNA traps, containing MPO or tryptase and chymase, respectively (43). Interestingly, both cell types also released IL-17, which seemed partially to associate with the DNA-containing fibers of the traps. When skin explants were treated with IL-1 β , IL-23, or both for 3 days, degranulating and MCET-producing mast cells were observed (43). Although the functional role of the extracellular DNA traps released in association with IL-17 in psoriasis remains to be investigated, it is possible that the antimicrobial peptide LL-37 converts the DNA of the traps into a potent activator of plasmacytoid dendritic cells to produce IFN- α *via* Toll-like receptor (TLR) 9 activation (44), thus contributing to the predominantly Th1 differentiation in this disease (Fig. 3).

Systemic lupus erythematosus (SLE) is a life-threatening autoimmune disease affecting multiple organs, such as kidney, blood, and brain (45). Characteristic immunological features of SLE are the autoantibody production by B cells against nuclear self-antigens, for example, DNA and histones, immune complex formation, as well as chronic activation of plasmacytoid dendritic cells (pDC) (46). Furthermore, a deficiency in the clearance of apoptotic cells, which leak autoantigens and consequently stimulate autoantibody production, has been recognized as the crucial pathogenic mechanism (47). Furthermore, it has become evident that

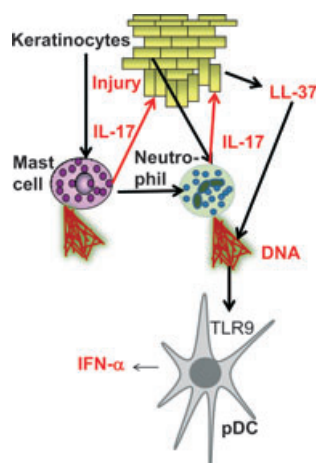


Figure 3 Extracellular DNA traps activate plasmacytoid dendritic cells (pDC). Upon keratinocyte injury, neutrophils and mast cells are activated, resulting in IL-17 release and extracellular DNA trap formation. IL-17 leads to LL-37 production by keratinocytes that, together with the extracellular DNA, is able to stimulate pDC via TLR9 to secrete IFN- α . Such a mechanism has been inferred in the pathogenesis of psoriasis.

NETs provide the link between autoantigen release, pDC, B cells, and autoantibodies in SLE (10, 44). Upon priming with type I interferons and exposure to autoantibodies, for example antiribonucleoprotein antibody, neutrophils produced NETs (10). As has been shown for psoriasis (44), LL-37 was essential for the TLR9-mediated activation of pDC by self-DNA to release IFN- α (10). NET components may also activate B cells via B-cell receptor and TLR9 to produce autoantibodies against DNA and LL-37 (44). Anti-DNA as well as anti-LL-37 autoantibodies can be abundantly detected in the blood of SLE patients (44). Moreover, these autoantibodies may bind to DNA and LL-37 in NETs forming antibody-DNA/LL-37-complexes that can be recognized by both pDC and B cells and thus further perpetuate an autoimmune inflammation (10) (Fig. 4).

In addition to the increased formation of NETs (48), an impaired degradation of NETs has been observed in SLE patients in association with a kidney involvement that may contribute to the presentation of self-antigens (49). The deficiency in NET clearance is due to either inhibitors or antibodies that protect NETs from DNase 1, which is responsible for NET degradation (49).

Extracellular DNA traps promote vasculitis and thrombosis

Evidence that NETs can damage the endothelium of blood vessels and thus contribute to disease pathogenesis came from research with sepsis (20) and SLE (46). *In vitro* experiments demonstrated that activated endothelial cells can directly stimulate neutrophils to generate NETs (50). After prolonged coculture with neutrophils, the endothelial cells underwent cell death, which could be prevented by DNase,

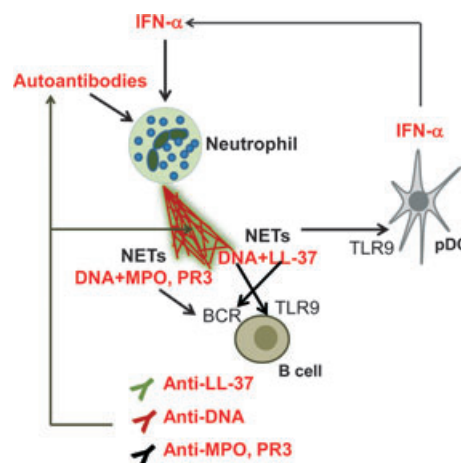


Figure 4 Neutrophil extracellular traps (NETs) maintain autoimmune inflammation. Extracellular DNA together with LL-37 stimulates pDC to release IFN- α , thus priming neutrophils for NET production. NETs contain besides DNA several granule proteins such as LL-37, MPO, and PR3. The granule proteins may activate B cells via the B-cell receptor (BCR) to produce autoantibodies, whereas the DNA in association with LL-37 is able to stimulate pDCs and B cells via TLR9. Autoantibodies produced by B cells might further activate neutrophils to produce NETs and, additionally, to bind to NETs. The resulting immune complexes may then contribute to pDC and B-cell activation. Such a pathogenic scenario has been implicated in SLE.

suggesting that the endothelial cell injury is mediated by NETs (50). Antineutrophil cytoplasm antibodies (ANCA) are the characteristic clinical findings correlating with disease activity in patients with small vessel vasculitis (51). ANCA are directed against the granule proteins of neutrophils, such as proteinase-3 (PR3) and MPO, and they may activate primed neutrophils to release lytic enzymes and ROS, leading to detachment and lysis of endothelial cells (51). Recently, it has been reported that ANCA stimulate the formation of NETs (8). In agreement with previous observations that LL-37 may convert self-DNA into an activator of pDC (44), an increased local IFN- α production in kidney biopsy specimens from patients with active SLE has been observed (8). Moreover, infiltration of neutrophils was associated with NETs containing PR3, MPO, and LL-37 (8).

As discussed earlier, NETs can be formed by platelet-neutrophil interactions in vessels (19). On the other hand, NETs have been shown to play a pathogenic role in thrombus formation, an observation that might explain the link between thrombosis and infection (Fig. 5). In experimental models of deep vein thrombosis, extracellular DNA was observed in association with histones (52, 53); thus, a key role for NETs in thrombus formation was postulated (54). Infusion with DNase protected mice from thrombosis (53). NETs perfused with blood promoted platelet adhesion and aggregation, recruited red blood cells into the web, and bound plasma proteins that are important for thrombus stability (52). In case of blood stasis, monocytes and neutrophils

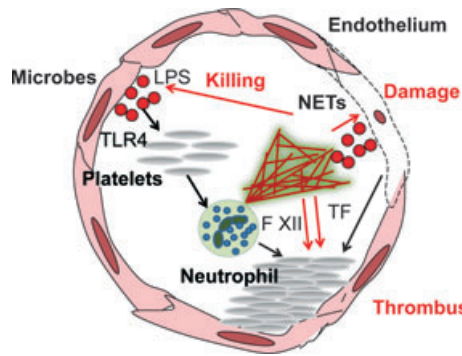


Figure 5 Neutrophil extracellular traps (NETs) link infections with thrombosis. In sepsis, platelets recognize LPS via TLR4 and activate neutrophils to generate NETs associated with tissue factor (TF). This is followed by damage of endothelial cells, which furthermore release TF causing additional thrombocyte activation. The NETs activate factor XII (F XII)-dependent coagulation, resulting in intravascular thrombus formation. Therefore, NETs not only kill microbes, but also seem to be involved in endothelial injury and thrombus formation. NET-induced thrombosis may play a role in the pathogenesis of sepsis, but also in any other pathological condition associated with reduced blood flow.

ils adhere to venous endothelial cells and release tissue factor (TF), resulting in intraluminal fibrin formation. Neutrophils present in the thrombi bind and activate factor XII by generating NETs composed of DNA, MPO, neutrophil elastase, histones, and, notably, disulfide isomerase, an enzyme that activates factor XII (54). Furthermore, platelets not only supported leukocyte recruitment, but also stimulated NET formation, resulting in factor XII-dependent coagulation (54). Interestingly, heparin was shown to exhibit antithrombotic activity by reducing NET formation (54) in addition to its action on NET degradation (52). As thrombotic microangiopathy associated with degranulated eosinophils has been observed in patients with hypereosinophilic

syndromes (55, 56), one can speculate that EETs may also contribute to the thrombus formation.

Conclusions and future directions

The production of extracellular DNA traps is part of the innate immune response, which is known to exert both beneficial and unwanted effects on the host. Therefore, under conditions of chronic inflammatory responses, it might make sense to prevent the formation of DNA traps in order to interrupt the inflammatory cascade, thus blocking associated diseases such as thrombosis. On the other hand, extracellular DNA traps are very efficient tools to bind and kill pathogens, and a general therapeutic inhibition of their production might cause a severe immunodeficiency.

Several research questions appear to be open. For example, what are the molecular mechanisms of DNA release? This question also includes the question of the origin of the DNA: Is it mitochondrial or nuclear DNA or both? Under which conditions does cell death play a role (if any)? Are there additional cell types able to produce extracellular DNA traps? Moreover, other potential physiological agonists are clearly of further interest. What is the overall contribution to antimicrobial defense by extracellular DNA traps? Do extracellular DNA traps play a role in cancer? These are just some of the obvious questions, which will hopefully be answered in the near future.

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Conflict of interest

The authors declare no conflict of interest related to this manuscript.

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