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The NET, the trap and the pathogen: neutrophil extracellular traps in cutaneous immunity

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Abstract: Neutrophil extracellular traps (NETs), large chromatin structures casted with various proteins, are externalized by neutrophils upon induction by both self- and non-self-stimuli. It has become clear that NETs are potent triggers of inflammation in autoimmune skin diseases. Moreover, the ability of NETs to trap pathogens suggests a crucial role in innate host defense. However, the outcome of the encounter between pathogens and NETs remains highly controversial. Here, we discuss recent insights into the morphology and formation of NETs, their role in skin inflammation and how NETs might contribute to host protection in skin infection.

Key words: autoimmunity – bacterial trapping – cutaneous immunity – neutrophil extracellular traps

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Introduction

As part of the innate host response to pathogens neutrophils are the first leucocytes recruited to the site of infection, for instance to human skin. To be able to rapidly infiltrate infected tissues in high numbers, neutrophils continuously differentiate from myeloid precursors in the bone marrow and enter into the blood stream (1–3). Under steady-state conditions, human blood granulocytes have an average half-life of 6.6 h (4) resulting in a turnover of ~1 × 10^9 cells/kg per day (5,6). However, during an inflammatory response, release of leucocytes from the bone marrow is enhanced (7,8), the neutrophil half-life increases by several fold (9), and chemotactic factors guide neutrophils to the site of inflammation (10–13). At inflammatory sites, neutrophils secrete *de novo* synthetized, as well as prestored cytokines to attract other immune cells [reviewed by Sheshachalam et al. (14) and Amulic et al. (15)]. Moreover, neutrophils provide antimicrobial activity against pathogens at least in three different modes of action. First, during degranulation neutrophils secrete granule-stored antibacterial proteins to fight extracellular pathogens [reviewed in detail by Kolaczkowska and Kubes (16)]. Second, neutrophils phagocytize pathogens and kill them intracellularly via granule-derived antibacterial proteins or reactive oxygen species (16). Third, as part of the host response neutrophils form and release neutrophil extracellular traps (NETs) (17), a mechanism, which was only identified within the last decade.

NET morphology

In 2004, Brinkmann and colleagues discovered a novel pathway in human neutrophils (17), termed NETosis (18). Originally characterized as a cell death pathway (17), it was distinct from apoptosis and necrosis (19,20). NETosis results in the formation of extracellular structures composed of chromatin and a set of proteins (17). The backbone of these fractal-like structures (21), termed NETs, is composed of decondensed chromatin fibres, which are casted with granular proteins, such as neutrophil elastase (NE) and myeloperoxidase (MPO) (17), the antimicrobial peptide cathelicidin (22–24) (Fig. 1), as well as cytoplasmic and cytoskeletal proteins (25). Of note, the use of host-derived nucleic acids as a mechanism of innate immunity might be evolutionarily interesting, as it was also reported in invertebrate hosts (26).

Formation of NETs

In the last years, much progress has been made in understanding the organized mechanisms of NET formation. In brief, inflammatory stimuli result in generation of ROS, regulate autophagy and mobilize Ca^{2+}. However, whether these events are part of a single pathway or rather represent parallel pathways downstream of the activation by different stimuli remains unclear. In any case, these events lead to chromatin decondensation, which is critically mediated by degradation of histones, as well as histone citrullination (Fig. 2a). Subsequently, the nuclear and granular membranes disintegrate, allowing mixture of nuclear, granular and cytosolic components, which is followed by rupture of the cell membrane and exposure of extracellular traps to the outside (Fig. 2b). The following discusses crucial steps of NETosis in more detail.

NET inducers

Various stimuli, including bacteria, fungi, viruses and parasites, induce NETosis [in detailed reviewed by Brinkmann and Zychlinsky (27)]. A recent study found that induction of NETosis depends on the size of the pathogen, because large pathogens as well as aggregates of *Mycobacterium bovis*, but not single bacteria, promoted NET formation (28). Furthermore, NET formation is regulated by signals from the extracellular matrix. In this regard, costimulation of the Mac-1 integrin adhesion receptor supported NET formation (29,30). Moreover, the ubiquitous matrix component fibronectin significantly promoted NET release induced by fungal pathogen-associated molecular patterns (31). Noteworthy, in experimental setups, NETs are most often induced via stimulation of neutrophils with phorbol myristate acetate (PMA) (17) or calcium ionophore A23187 (32).

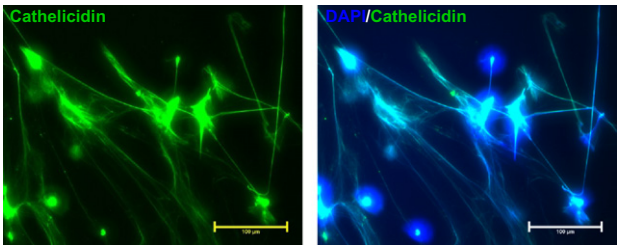

Figure 1. Neutrophils forming NETs. Primary human neutrophils were activated with calcium ionophore (A23187) for 1.5 h. Subsequently, cells were fixed, DNA was visualized with DAPI (blue), and cathelicidin was detected by a monoclonal antibody (green).

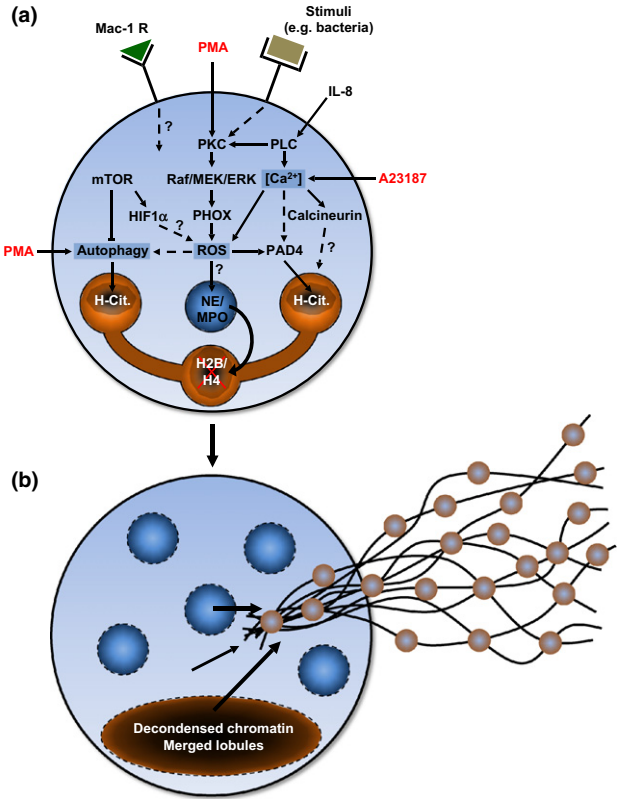

Figure 2. Model of known and proposed mechanisms of NET formation. (a) Generation of reactive oxygen species (ROS) via the phagocytic oxidase complex (PHOX) is the best-studied pathway of NET induction. Moreover, induction of autophagy and Ca^{2+} influx from extra- and intracellular stores contributes to NETosis. However, whether these pathways represent independent modes of activation, dependent on the stimuli, or are part of a single network, requires further research. (b) Downstream, both histone citrullination (H-Cit.) and degradation of histones result in chromatin decondensation. Subsequently, the nuclear lobules merge and the nuclear and granular membranes disintegrate, allowing mixture of nuclear, cytoplasmic and granular components. Finally, the plasma membrane ruptures and NETs are exposed to the extracellular space. Arrows represent known pathways. Dotted arrows represent predicted or proposed pathways. Question marks represent unknown modes of action. The two non-physiological inducers of NET formation PMA and A23187 are shown in red.

ROS-dependent NET formation

A number of studies have shown that NET formation requires production of reactive oxygen species (ROS) by the NADPH oxidase enzyme complex, also known as phagocytic oxidase (PHOX) (19,27,33). First, neutrophils isolated from patients with chronic granulomatous disease (CGD), which carry a mutation in the NADPH oxidase and thus are unable to produce ROS, fail to form NETs upon induction with bacteria or PMA (19,33). Strikingly, restoration of a functional NADPH oxidase by gene therapy in a patient with CDG restored the ability to generate NETs (33). Second, in mice carrying a specific knockout for Rac2, a member of the Rho family of small GTPases regulating ROS generation via PHOX, NET formation and nitric oxide (NO) production were significantly impaired (34). Consistently, Patel et al. (35) have demonstrated that NO donors induce NET formation. Furthermore, PMA-induced NETosis is dependent on the activation of protein kinase C (PKC) (20). Yet, whether PKC is required for NET formation upon stimulation with physiological stimuli remains unclear. For instance, *Helicobacter pylori* induced NETosis independently of signalling via PKC (20). Moreover, generation of NETs seems to be mediated via the Raf-MEK-ERK pathway (20). Given that chemical inhibitors targeting this pathway block ROS production, it seems that PKC signalling and the Raf-MEK-ERK pathway are upstream of PHOX (20) (Fig. 2a).

ROS-independent NET formation

Several studies provide evidence that NETs are also formed via ROS-independent mechanisms. For example, uric acid induced NET formation in a NADPH oxidase-independent manner (36). Likewise, stimulation of neutrophils with ionomycin resulted in NADPH oxidase-independent formation of NETs, whereas NADPH oxidase was required upon induction via PMA or bacteria (37). Therefore, Arai et al. conclude that requirement of NADPH oxidase in NETosis may depend on the stimuli.

NET formation and autophagy

Additional information regarding the induction of NETosis was provided by studies linking autophagy to NET formation (38–44). In PMA-induced neutrophils, both autophagy and production of ROS was required for NET formation (38). Interestingly, ROS production and autophagy occurred independently of each other; however, activation of both pathways was required for efficient chromatin decondensation. Additionally, pharmacological inhibition of the mTOR pathway enhanced autophagy and accelerated NET formation in neutrophils (39). A recent study shows that mTOR signalling results in upregulation of HIF-1α protein expression and regulates NET formation (45). In turn, blockade of mTOR activity with rapamycin significantly reduced HIF-1α protein expression and NET formation. This is contrary to the above-described results showing that inhibition of mTOR causes enhanced NET formation and, thus, requires further investigation. Of note, HIF regulates ROS production in cancer; however, whether this is also the case in neutrophils is not known (46).

The role of calcium in NET formation

Mobilization of extra- and intracellular Ca^{2+} is required for IL-8-mediated NET formation and links the calcineurin pathway with NETosis (47). The pathway is induced by IL-8-mediated activation of phospholipase C (47). Pharmacological inhibition of calcineurin resulted in reduced NET formation; however, the precise mechanism how calcineurin contributes to NET formation is not known. Moreover, a recent study demonstrated that *Mycobacterium tuberculosis* stimulated the production of NETs via an increase in intracellular Ca^{2+} (48).

Decondensation of chromatin during NETosis

A key event in NET formation is the decondensation of chromatin, which initially involves merging of the neutrophil-specific

nuclear lobules (19). Subsequently, the nuclear membrane disintegrates into vesicles, whereas the granules disappear, allowing contact of nuclear, granular and cytosolic components (19,27). However, the precise mechanism how ROS production results in chromatin decondensation is incompletely understood. It has been shown that hypercitrullination of histones contributes to chromatin decondensation during NET formation (29,49) (50). Histone citrullination is mainly mediated via the action of PAD4, which in turn is activated by both NADPH oxidase and an increase in intracellular Ca^{2+} (29,47,49–51) (Fig. 2a). Additionally, it has been proposed that microtubuli and actin filaments mediate histone deimination and NET release (29). A model established by Papayannopoulos and colleagues states that chromatin decondensation is driven by neutrophil elastase (NE) and myeloperoxidase (MPO) (52,53). In this model, NE and MPO are released from azurophilic granules by an unknown process downstream of ROS production. Subsequently, NE enters the nucleus and digests H2B and H4 resulting in chromatin decondensation (52). This process is supported by the action of MPO, which does not contribute to histone degradation, yet dramatically enhances the effect of NE in chromatin decondensation (52) (Fig. 2a). Noteworthy, ROS and MPO are required for the release of NE from azurophilic granules (54). Together, it seems likely that chromatin decondensation during NETosis is driven by a combination of histone citrullination as well as degradation of H2B and H4.

NET release

The mechanism of NET release into the extracellular space that follows chromatin decondensation is not well understood. Live cell imaging analyses revealed that cells round up approximately 80 min after PMA treatment, while the cells are still alive (19). This process is immediately followed by rupture of the cell membrane and release of the specific components into the extracellular space resulting in NET exposure (19,27). At this late phase of NETosis, the cytoskeleton seems to mediate the release of chromatin (29). Interestingly, the interaction of neutrophils with the Mac-1 integrin adhesion receptor might guide a polarized release of NETs towards the source of the stimulus (29).

NETs and autoimmunity

NET formation has been linked to the pathophysiology of a broad spectrum of diseases, including rheumatoid arthritis (55), systemic inflammatory response syndrome (56), sepsis (57–59), endothelial injury (23), venous thrombosis (60,61) and small-vessel vasculitis (62). Moreover, NETs play a critical role in the pathophysiology of dermatomyositis, psoriasis and systemic lupus erythematosus (SLE) (20,23,24,63,64). In the epidermis of psoriatic skin lesions, extracellular traps produced by neutrophils and mast cells have been identified as carriers for mast cell- and neutrophil-derived IL-17 (64,65), a key pro-inflammatory cytokine in driving inflammation in this disease. Skrzeczynska-Moncznik et al. (66) have identified the serine proteinase inhibitor SLPI as component of NETs in lesional skin of patients with psoriasis. Importantly, NE is the enzymatic target of SLPI, and NETs positive for SLPI and NE colocalized with pDCs in psoriatic skin lesions. Strikingly, *in vitro* generated complexes of SLPI with NE and DNA induced production of type I IFN by cultured pDCs. Therefore, the authors proposed a role of SLPI as part of NETs in initiation and/or augmentation of psoriasis via activation of pDCs.

Within the blood mononuclear cell fraction of psoriasis as well as SLE patients, a distinct population of neutrophils, termed 'low-density granulocytes' (LDGs), has been identified (64,67–70). In SLE, LDGs synthetize type I interferons and induce endothelial cell cytotoxicity (70). LDGs in the blood of patients with SLE, as well as patients with psoriasis, are prone to NETosis (23,64). Interestingly, Villanueva et al. (23) have shown that LDGs in SLE expose more NET-associated IL-17 through enhanced NETosis as compared to peripheral blood neutrophils. Moreover, skin biopsies from patients with SLE stained positive for NETs; however, only a small portion was positive for IL-17. The authors suggest a model, in which neutrophils release most of their IL-17 before extravasation and migration into tissues (23). Using a human pDC cell line, the authors have further demonstrated that stimulation of pDCs with supernatants from lupus neutrophils and LDGs induces IFN-α synthesis. This process was inhibited upon treatment with micrococcal nuclease, indicating that NETs trigger pDC activation. As LDGs induce cytotoxicity of endothelial cells (70), Villanueva et al. (23) have postulated that NETs formed by lupus LDGs are the bearer of endothelial cell cytotoxicity. Indeed, coculture of human umbilical vein endothelial cells with lupus LDGs resulted in enhanced endothelial cell cytotoxicity as compared to neutrophils from healthy donors and lupus neutrophils. Given that addition of micrococcal nuclease significantly decreased cytotoxicity, the authors concluded that NETs, at least in part, mediated the observed effect.

In a study published in 2011, Lande et al. (24) isolated immune complexes, composed of DNA and anti-DNA antibodies, which contain the neutrophil antimicrobial peptides cathelicidin and human neutrophil peptide (HNP), from patients with SLE. Stimulation of pDCs with these complexes resulted in strong production of IFN-α. Moreover, *in vitro* generation of such complexes and subsequent incubation with pDCs resulted in TLR9-mediated activation of pDCs and production of IFN-α. Of note, this TLR9-mediated activation of pDCs by DNA–cathelicidin complexes was also reported as a central mechanism to trigger IFN-α production in psoriasis (71). Cathelicidin was required for the activation of pDC via isolated immune DNA complexes or via *in vitro* generated DNA–antimicrobial peptide complexes, whereas HNP supported the action of cathelicidin. Of interest, DNA–antimicrobial peptide complexes, which trigger pDC activation, can originate from NETs (24). Thus, NETs are considered as a source for DNA–antimicrobial peptide complexes that trigger chronic activation of pDCs in SLE (24). Importantly, cathelicidin as part of DNA containing immune complexes induces aggregation into insoluble particles, which are inaccessible for nuclease degradation. In addition, anti-NET antibodies as well as NET–cathelicidin were found to prevent access of nucleases to NETs (72,73). Interestingly, a subpopulation of patients with SLE, suffering from renal involvement, poorly degraded NETs *in vitro* (72). This was associated with the presence of DNaseI-specific inhibitors in the sera of these patients. In conclusion, these findings suggest that enhanced NETosis or a reduced ability to degrade NETs via DNases plays a key role in initiating and/or sustaining inflammation in SLE. Interestingly, DNA also shuttled bound cathelicidin into monocytes and triggered IFNα production; however, in contrast to pDCs, this process was Toll-like receptor independent (74).

NETs trap, but do they kill?

The trapping and killing of pathogens has been considered a crucial function of NET formation since its discovery in 2004. However, William Nauseef has recently stated that '. . .there are at present limited, if any, data directly or causally linking NET formation to host defense' (75). NETs are generally considered structures that immobilize a broad range of pathogens; however, whether immobilized pathogens are killed remains highly controversial. Several studies have indicated that NETs kill a broad variety of Gram-positive and Gram-negative bacteria (17,19), as well as fungi (76). Moreover, NETs were linked to antiviral defense, when Saitho et al. (77) reported that NETs capture human immunodeficiency virus (HIV)-1 and promote HIV-1 elimination through myeloperoxidase and α-defensin. However, *Mycobacterium tuberculosis* or *Streptococcus pneumoniae* were trapped in NETs; however, killing was not observed (78,79). Recently, the experimental approach used to measure killing of microbes in NETs has been questioned (80). The commonly used experimental setup to analyse NET-mediated killing was to induce NET formation, for instance with PMA (19,78), IL-8 (17) or preinfection with *M. tuberculosis* (78). Next, activated neutrophils were incubated with fresh medium with or without addition of DNaseI and cytochalasin D (17), which degrade NETs (17,78) and inhibit phagocytosis, respectively (81,82). Bacteria were added, followed by centrifugation and a further incubation step to allow trapping of bacteria in NETs. Subsequently, the culture media was collected and bacterial viability measured by colony-forming unit (CFU) assays (17,19,78). A reduced number of bacteria found in the supernatant of netting neutrophils was attributed to killing in NETs, when the observed effect was inhibited by the DNase treatment, but not by blocking of phagocytosis with cytochalasin D (17,80). However, Menegazzi and colleagues have pointed out that there is no formal proof that bacteria immobilized in NETs are necessarily dead. Strikingly, the authors have shown in their experiments that addition of DNaseI to release *Staphylococcus aureus* or *Candida albicans* from NETs shortly before pathogen recovery for CFU assays – in initially not DNase-treated conditions – partially restored pathogen growth. This indicates that bacteria associated with NETs might be trapped, but not dead (80). Interestingly, Parker et al. (83) demonstrated that *Staphylococcus aureus* was released from NETs by DNase digestion and therefore not killed in NETs, which were isolated form neutrophil cell bodies in the experimental approach. However, addition of H_2O_2 *in vitro* resulted in MPO-mediated killing of the trapped bacteria. *In vivo*, H_2O_2 might be provided by surrounding cells (83). In summary, it seems clear that NETs trap bacteria and at least temporarily inhibit some bacterial species in their growth. However, whether immobilized bacteria are killed, thus dead, requires further investigation. Furthermore, some bacteria can even evade NET-mediated growth inhibition/killing [in detail reviewed by Hahn et al. (84)], for instance by degradation of NETs (79,85,86). Another controversy regarding the role of NETs in host defense came from the report on a patient, who suffered from Papillon–Lefèvre syndrome (PLS) due to a missense mutation cathepsin C gene. The patient's neutrophils did not produce NETs, yet clinically, only a very mild phenotype and no increased frequency of infections was reported (87).

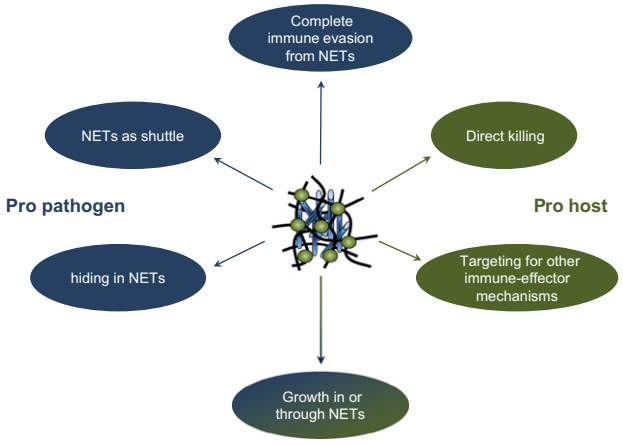

Figure 3. Hypothetical fates of NET-trapped pathogens. Blue rots represent pathogens, black lines illustrate NET DNA, and NET proteins are shown in green.

NETs in skin infection

To date, two central immune aspects of NET formation in disease have been discussed: trapping of pathogens and formation of immune complexes that act as inflammatory triggers in autoimmunity. With respect to skin disease much focus has been drawn to the function of NETs in autoimmunity, and yet, little is known about NETs in cutaneous host defense. Nevertheless, given that neutrophil infiltration is a hallmark of many cutaneous infectious, it is likely that NETs play an important role in the immune response to skin infections. In this regard, a study published in 2012 reported age-dependent formation of NETs at the site of skin infection upon staphylococcal inoculation in a murine model (88). Only in infected skin from young mice, but not in infected skin of old mice, NET formation was observed. Likewise, cultured neutrophils isolated form aged mice formed NETs poorly upon induction with PMA. Additionally, a mutant strain of group A *streptococcus* (GAS), unable to produce extracellular DNases, was cleared significantly faster from the infected murine skin as compared to wild-type streptococci, which produced extracellular DNases (89). As mentioned above, a number of bacteria express extracellular DNases, and a role of nucleases in bacterial NET evasion has been demonstrated (47,79,85,86,90). Indeed, in skin biopsies of mice infected with a mutant GAS strain unable to produce extracellular DNase, NET formation was observed in abscess exudates. In contrast, no formation of NETs was observed in skin infected with wild-type GAS (85). One study examined NET formation upon skin infection using a live imaging mouse model *in vivo* (91). NET release was observed during Gram-positive skin infections; however, NET formation occurred much faster as commonly observed *in vitro* and seemed, intriguingly, not to be associated with neutrophil cell death. In 2010, the same group reported a novel form of extracellular trap formation upon induction by *Staphylococcus aureus,* which did not result in neutrophil cell lysis

Table 1. Central open questions regarding the role of neutrophil extracellular traps (NETs) in host defense

Do NETs kill bacteria?
If NETs do not kill bacteria, what is the fate of NET-trapped pathogens?
Is there a level of specificity in pathogen trapping?
What is the *in vivo* role of NETs?
Do NETs contribute to acquired host defense?

or death (92). Thus, the authors of this study suggest the presence of at least two forms of NETosis: fast, vesicle-mediated release of NETs without rupture of the cell membrane, referred to as 'vital NETosis', and slower release connected with membrane rupture and cell death, termed 'suicidal NETosis' (59,92–94). However, unlike recently reported in eosinophils, which can form extracellular traps via fast release of mitochondrial DNA (95), the traps observed in this study seemed to be mainly composed of nuclear DNA. Thus, Pilsczek et al. speculated that neutrophils, which do not divide and have a short half-life, can fulfil antimicrobial activities even after extrusion of their DNA (92). Of note, in line with the hypothesis that both inflammatory triggers, as well as stimuli from the extracellular matrix cooperatively induce NET formation, Yipp et al. (91) reported that formation of NETs in their *in vivo* study was dependent on both Toll-like receptor 2 (TLR2) activation and the presence of complement factor C3.

In humans, only few studies have linked NETosis to cutaneous host defense. The *in vivo* role of NETs in this regard might possibly be suggested by the fact that neutrophils isolated from patients with chronic granulomatous disease (CGD), which are prone to infections including skin (96,97), were severely defective in NETosis (19). Moreover, in biopsies of human cutaneous leishmaniasis, complexes of DNA and elastase were observed (98), indicating the *in vivo* presence of NETs in leishmania infection.

In conclusion, it seems clear that NETs trap pathogens, but the outcome of the encounter between a NET and a pathogen remains unclear. Hypothetically, several fates of NET-trapped pathogens are possible (Fig. 3), and this should also depend on the nature of the pathogen. On one hand, NETs might provide direct antimicrobial activity or NETs might immobilize pathogens for attack by other immune effector mechanisms. The latter concept is supported by *in vitro* experiments showing complement activation by NETs in SLE (99). Noteworthy, in SLE, this mechanism has a potential role in the failure of NET degradation, because binding of complement factor C1q to NETs inhibited DNaseI-mediated NET destruction. On the other hand, NETs might not harm pathogens, pathogens might simply grow in or through NETs, or pathogens might be capable to completely evade from NETs. Moreover, it is possible that microbes exploit NETs to hide from the host immune mechanisms or subvert NET-mediated host defense mechanisms. In this context, a recently published study shows that *Staphylococcus aureus* produces deoxyadenosine from NETs, thereby turning NETs into toxic agents for immune cells (100). Finally, pathogens could hijack NETs as shuttles for dissemination in the host. This concept could be supported by two findings. First, it was shown that Neisseria use intact neutrophils for dissemination in the host (101). Second, NETs were found to sequester circulating tumor cells and promote metastasis (102).

For sure, NETosis and its role in infections is a very rapidly growing field generating many controversies, yet also exciting new insights into neutrophil-mediated host protection. It is obvious that future research is urgently needed to clearly decipher the role of NETs in host defense (Table 1 states central open questions in NET research). In this regard, we think that dermatological research will significantly contribute to this exciting field by studies *in vitro*, *in vivo* animal models, and also by analysing NETs in biopsies of human skin infections.

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Conflict of interests

The authors have declared no conflicting interests.

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