REVIEW

Neutrophil extracellular traps (NET): not only antimicrobial but also modulators of innate and adaptive immunities in inflammatory autoimmune diseases

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ABSTRACT
Polymorphonuclear neutrophils (PMN) represent one of the first lines of defence against invading pathogens and are the most abundant leucocytes in the circulation. Generally described as pro-inflammatory cells, recent data suggest that PMN also have immunomodulatory capacities. In response to certain stimuli, activated PMN expel neutrophil extracellular traps (NET), structures made of DNA and associated proteins. Although originally described as an innate immune mechanism fighting bacterial infection, NET formation (or probably rather an excess of NET together with impaired clearance of NET) may be deleterious. Indeed, NET have been implicated in the development of several inflammatory and autoimmune diseases as rheumatoid arthritis or systemic lupus erythematosus, as well as fibrosis or cancer. They have been suggested as a source of (neo)autoantigens or regulatory proteins like proteases or to act as a physical barrier. Different mechanisms of NET formation have been described, leading to PMN death or not, depending on the stimulus. Interestingly, NET may be both pro-inflammatory and anti-inflammatory and this probably partly depends on the mechanism, and thus the stimulus, triggering NET formation. Within this review, we will describe the pro-inflammatory and anti-inflammatory activities of NET and especially how NET may modulate immune responses.

INTRODUCTION
Polymorphonuclear neutrophils (PMN) are classically defined as terminally differentiated, non-dividing and short-lived cells dying after a few hours. They represent more than 50% of blood leucocytes in humans and are described as typical pro-inflammatory cells. They are among the first cells recruited at inflammatory sites. Traditionally, PMN are thought to carry out their functions through elementary mechanisms, namely phagocytosis, production of reactive oxygen and nitrogen species or release of granules containing proteases and antimicrobial peptides. They secrete chemokines and pro-inflammatory cytokines as well, like IL-8 and TNF. Although they represent key innate immune cells involved in response to infections, PMN are also activated during sterile inflammation, for example, in response to endogenous ligands and especially damage-associated molecular patterns (DAMP). Therefore, they can exert beneficial or detrimental and even pathogenic roles. Surprisingly, although PMN are described as pro-inflammatory cells, they were until recently relatively sparsely studied in inflammatory diseases like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), on which we will focus in this review. Actually, PMN have gained more interest since the discovery of neutrophil extracellular traps (NET). Indeed, PMN and especially NET may represent a source of both autoantigens and DAMP. Here we will discuss the immunomodulatory activities as well as the pathogenicity of NET, with a special emphasis on these two diseases. Although
the aim of the present review is not to detail the different signalling pathways leading to NET formation, we will discuss critical points to avoid confusion with other biological processes.

PMN: MORE SOPHISTICATED THAN BELIEVED

Because distinct PMN sub-populations may differ in their capacity to produce NET or may form NET of variable composition (see below), we will shortly emphasise PMN heterogeneity and plasticity. Vision on PMN has evolved during recent years. It was thought classically that circulating PMN have a relatively short half-life, but an in vivo lifespan of 5.4 days has been reported for human blood PMN, and PMN survival is believed to be increased under inflammatory conditions.

Moreover, non-classical functions of PMN have been identified. Indeed, and as recently reviewed, PMN may behave as antigen-presenting cells, produce type I interferon (IFN-I), communicate with several immune cell types (such as natural killer cells, dendritic cells (DC), pro-inflammatory Th17 lymphocytes, macrophages/macrophages but also regulatory T lymphocytes) and display also immunomodulatory functions, like secretion of soluble CEACAM8, IL-10 production or even B cell-helper PMN. The latter was especially reported in the mouse. All those data suggest that PMN link innate and adaptive immunities.

In addition to non-classical functions, neutrophil heterogeneity has become apparent with the description of PMN sub-populations in blood and tissues. Heterogeneity of human blood PMN was very recently confirmed by single-cell RNA-sequencing analysis. Particularly interesting in the context of RA and SLE, pro-angiogenic PMN (recruited by vascular endothelial growth factor (VEGF)-A) have been reported in hypoxic tissues whereas low-density granulocytes (LDG) are enriched in the circulation of patients with diverse inflammatory diseases (detailed below).

Interestingly, particular functions or subsets have been identified in inflammatory autoimmune diseases. Thus, RA PMN differentiate into DC-like cells and express RANKL which plays a key role in osteoclastogenesis. Moreover, PMN from patients with RA express B-cell activating factor and IFN-α. Synovial fluid PMN from patients with RA have a higher capacity to express cell surface MHC II and to induce T-cell proliferation and are more sensitive to non-apoptotic cell death triggered by Siglec-9. Particularly, RA synovial fluid is anti-apoptotic for PMN cultured under hypoxia, mimicking the in vivo situation within joints, whereas RA synovial fluid PMN express higher levels of chemokines than autologous RA peripheral blood PMN. Regarding subsets, LDG with pro-inflammatory properties were first observed in SLE and shown to activate T lymphocytes. LDG are also present in patients with RA and present different characteristics compared with autologous classical RA PMN, for example, altered transcriptome and lower NET formation in response to phorbol myristate acetate (PMA).

Among the new neutrophil functions described during the last two decades, the formation of NET is particularly intriguing and exciting. It became a topic with intense research and is of particular interest in the field of inflammatory autoimmune diseases. It has also generated debates and controversies, especially potential NET triggers as well as mechanisms leading to NET formation and pathways involved, or methods to identify, characterise and analyse NET. The implication of NET in pathological conditions has shed new light on PMN, especially in SLE and RA.

UNDERSTANDING PROPERLY NET FORMATION IN THE CONTEXT OF RA AND SLE

Because NET composition may vary depending on the stimulus or the mechanism/pathway triggered during NET formation (see below) and may influence NET immunological properties, a brief overview of NET formation mechanisms is presented. NET were first discovered in 2004 by Brinkmann and colleagues. They have shown that stimulation of neutrophils with PMA, lipopolysaccharides (LPS) or IL-8 induces the release in the extracellular environment of structures composed of nuclear DNA filaments decorated with histones (namely chromatin) and granule proteins but devoid of membrane. Numerous proteins have been shown associated with NET, including some cytoplasmic proteins. Importantly, these fibres are capable of trapping and killing gram-positive and gram-negative bacteria, defining a new approach to neutralise and remove pathogenic bacteria by PMN.

Since Brinkmann’s work, the composition and role of NET have received a lot of interest and have been better characterised, without reaching a consensus. In addition to bacteria like Staphylococcus aureus and their products such as LPS, NET are induced by or even involved in host defence against fungal and parasitic infections. They also display antiviral properties. Additional NET-inducing stimuli include activated platelets and, especially interesting in RA and SLE, immobilised immune complexes or cytokines. Particularly, NET may form in response to a variety of DAMP or alarmins during sterile inflammation (eg. HMGB1 or LL-37), a situation where NET are potentially pathogenic.

After NET extrusion in Brinkmann’s original model, neutrophils were shown to die in 2–4 hours by a process which differs from apoptosis or necrosis and named NETosis. This is the canonical mechanism and is sometimes named suicidal NETosis. However, it is known now that several mechanisms and pathways are involved in NET formation, depending on the stimulus, and not all of them lead to cell death. The latter alternative mechanisms are named vital (or live) NETosis. Thus, the term ‘NETosis’ should only be used when NET extrusion is accompanied with neutrophil death, or at least ‘suicidal’
or ‘vital’ should be included. Otherwise, the correct term is ‘NET formation’. 20

However, the most used in vitro stimulus is PMA, which strongly induces classical NETosis through activation of the multimeric NADPH oxidase 2 (NOX2) complex. 36 NOX2 produces reactive oxygen species (ROS) which are required for NETosis. 21 ROS primarily trigger activation of neutrophil elastase (NE). Myeloperoxidase (MPO) acts synergistically with NE to enhance chromatin decondensation. 37 38 Some NET inducers activate peptidyl-arginine deiminase 4 (PAD4), which citrullinates, for example, histones, favouring chromatin decondensation. 39 In response to PMA, histone citrullination is very low, but detectable in primary human PMN. All these molecular events lead to disintegration of the nuclear envelope. As a result, the cytoplasm and karyoplasm become intertwined, the plasma membrane ruptures and NET are released into the extracellular space. 21

Regarding non-suicidal pathways, formation of NET containing genomic DNA has also been described. Indeed, activation of PMN by S. aureus in vivo and in vitro results in early nuclear condensation which is followed by the separation of inner and outer membranes of nucleus. Subsequently, transport vesicles containing nuclear DNA are formed and burgeon through the plasma membrane into the extracellular space, without breach of the plasma membrane in rapid kinetics (5–60 min). Once in the external environment, the vesicles rupture and NET are released. 40 41 This mechanism is NOX-independent and bactericidal. Intact anuclear PMN or cytoplasts have been observed in vivo and may retain some functions, such as chemotaxis and phagocytosis. This mechanism was later on named vital NETosis. 42

In addition, NET formation by viable PMN can be accomplished by rapidly (1 hour) ejecting mitochondrial (instead of nuclear) DNA bound to granule proteins in a ROS-dependent manner. 43 These NET do not contain histones but do contain granule proteins. Production of NET containing mitochondrial DNA is generally observed after priming PMN with GM-CSF, followed by stimulation with LPS or complement factor 5a (C5a), and preserves membrane integrity. This process does not require ATG5-dependent autophagy. 44 These NET were subsequently shown to kill bacteria. 45 46

However, citrullination levels were not evaluated in the latter two models. The function of PAD4 in NET production is the subject of much discussion, especially in the field of RA because citrullinated proteins are targeted by autoantibodies. Several studies suggested the need for PAD4 to induce NET formation in response to specific stimuli. 47 48 However, other reports highlight that NET can be formed in the absence of functional PAD4. 39 The latter study suggests that citrullination occurs during NET formation but that PAD activity is not necessary. Furthermore, the presence of citrullinated histones on NET may be the result of extracellular citrullination by PAD enzymes, which are released when neutrophils are activated. 50

Thus, several mechanisms of NET formation, triggered by different pathways, exist and lead to PMN death or not. Depending on the stimulus, the composition of NET differs (nuclear vs mitochondrial DNA, containing histones or not, possibly enriched in some post-translational modifications). A minimal common definition for NET may be complexes made of DNA and proteins from granules (e.g., NE, MPO), possibly with other associated proteins.

One point has to be discussed in the context of RA and SLE. It has been proposed that NET-like structures might result from other processes confused with NETosis, namely leucotytic hypercitrullination or defective mitophagy. 31 Leucotytic hypercitrullination would be triggered by pore-forming proteins like immune proteins, bacterial toxins, or calcium ionophores. However, Parker et al. 44 have shown that ionomycin induces the release of DNA associated with MPO. This is the definition of NET. More recently, Kenny et al. 32 reported that the calcium ionophore A23187 triggers the release of structure made of genomic DNA and NE, with bactericidal activity and leading to PMN death, namely NETosis. Thus, both studies reported NET formation and one confirmed the involvement of NETosis, although additional pathways might be involved. Importantly, NET induced by calcium ionophores are highly citrullinated, suggesting that NET induced by pore-forming stimuli might be a source of citrullinated autoantigens in RA. Regarding defective mitophagy, it is a normal PMN process. Most cells eliminate damaged mitochondria via mitophagy. 52 In contrast, mitophagy is defective in PMN. 53 Instead, they release mitochondrial content into the extracellular space, like mitochondrial DNA–proteins complexes. If mitochondrial DNA is oxidised, it is redirected to lysosomes for degradation. 53 However, SLE PMN or IFN-α-primed healthy PMN exposed to lupus anti-ribonucleoprotein (RNP) autoantibodies extrude oxidised mitochondrial DNA–proteins complexes rather than routing mitochondrial DNA to lysosomes. 53 Similarly, SLE PMN or healthy PMN exposed to RNP-containing immune complexes release extracellular oxidised mitochondrial DNA which is interferogenic. 34 It is however unclear whether oxidised mitochondrial DNA released in response to the latter stimuli is physically associated with granule proteins and whether these mitochondrial DNA–proteins complexes are antibacterial, and thus whether these structures strictly comply with the definition of NET. In contrast, and as described above, GM-CSF+LPS/C5a stimulation induces NET made of mitochondrial DNA and granule proteins with antimicrobial activity. Nevertheless, NET induced by calcium ionophores or anti-RNP autoantibodies/immune complexes are highly relevant in the context of SLE and RA as (1) oxidised mitochondrial DNA is pro-inflammatory, (2) anti-oxidised mitochondrial DNA autoantibodies are present in a fraction of patients with SLE and (3) citrullinated proteins are the targets of the
RA-specific anti-citrullinated protein antibodies (ACPAs), autoantibodies present in about 70% of patients.

**PHYSIOLOGICAL FUNCTIONS AND ANTIMICROBIAL ACTIVITY OF NET**

NET react with and/or are induced by a variety of pathogens. Particularly, NET are protective in response to a range of bacterial infections.

To support a physiological role of NET, the existence of NET in vivo was first demonstrated both in experimental shigellosis in rabbits and in spontaneous human appendicitis. Moreover, NET were shown to enhance bacteria trapping in vivo in mice on cooperation with platelets activated through Toll-like receptor (TLR) 4. Then, vital vesicular NET formation was confirmed in vivo during Gram-positive skin infections in mice and humans.

Interestingly, NET have been observed in saliva and blood PMN exposed to saliva undergo NET formation. Those NET display high resistance to deoxyribonucleases (DNase) and high capacity to kill bacteria, suggesting their involvement in the antimicrobial defence and/or tissue homeostasis at the oral mucosa.

NET have been shown to bind to bacteria, either Gram-positive or Gram-negative ones. They degrade bacterial virulence factors, for example, IpaB from *Shigella flexneri* or α-hemolysin from *S. aureus*, via serine proteases. Particularly, NET display an extracellular bactericidal activity, as demonstrated by the extracellular bacterial killing, which is reduced after digestion of NET with nucleases. NET-associated factors contribute to their antimicrobial activity and the presence of antimicrobial molecules on NET may be a way to locally increase their concentrations. Actually, NET-bound cathepsin G and NE process and activate the pro-inflammatory cytokines IL-1α and IL-36, amplifying the inflammatory response to fight pathogens. Interestingly, the antimicrobial effect mediated by NET passed especially through histones, cathelicidin (LL-37 in humans) as well as calprotectin (S100A8/A9). It should be noted that histones have been shown in the past to be bactericidal.

Some of these proteins kill microbes by forming membrane pores. Likewise, an antimicrobial activity has been reported for DNA. Moreover, it has been proposed that PMN release NET only in response to pathogens too large to be phagocytosed in order to selectively neutralise them, whereas phagocytosis inhibits NET release.

NET also act as a physical barrier to limit bacterial biofilm dissemination. During keratitis induced by *Pseudomonas aeruginosa*, PMN are recruited to the bacterial biofilm formed on the cornea and form NET at the base of the biofilm. In mice, NET create a ‘dead zone’ barrier which impedes bacterial dissemination into the brain. However, NET formation in turn amplifies biofilm formation, promoting ocular pathology. Thus, a tight regulation of NET formation and its intensity is required.

As a defence mechanism, bacteria have developed evading strategies against NET, for example, via the secretion of nucleases degrading NET or virulence factors inhibiting the activity of antimicrobial peptides. Some bacteria express a surface nuclease degrading NET to escape killing by NET. To evade NET, *Neisseria meningitides* is able to modify the lipid A moiety of its LPS or to upregulate its zinc uptake receptors, whereas *Streptococcus pneumoniae* modifies its surface charge or produces a capsule. Reciprocally, the antimicrobial peptide LL-37 present in NET confers resistance of NET against bacterial nucleases.

NET were also observed in the cerebrospinal fluid of patients with pneumococcal meningitis. However, NET formation appears deleterious in this context, as in a rat model of meningitis NET hinder bacterial clearance in the central nervous system. This result exemplifies the dual activity of NET and the required balance between beneficial versus pathological effects of NET.

Although NET are usually beneficial in response to infections, NET may become pathogenic in some particular cases, when NET formation is (locally) too intense or when NET are not efficiently cleared or do not form aggregated NET (aggregates formed at high PMN density; see ‘Beneficial activities of NET in diseases’ section). Pathogenic NET may also form during sterile inflammation in response to injury or disease-associated triggers, some of them being endogenous self-molecules such as DAMP or cytokines.

**IMPACT OF NET ON THE REGULATION OF IMMUNE RESPONSES**

For the reasons mentioned above, we focused here on studies either using classical NET inducers (essentially PMA, LPS, crystals, autoantibodies/immune complexes, cytokines but also calcium ionophores) or depicting well characterised NET containing DNA (genomic or mitochondrial) and proteins and not on studies dealing only with extracellular DNA release. Due to their potential pathogenic activity in SLE, complexes made of oxidised mitochondrial DNA in response to anti-RNP autoantibodies/RNP-containing immune complexes will also be mentioned. Moreover, we focused on data generated with primary cells and not cell lines, either for generating NET or for cells targeted by NET. The data described below refer to the immunomodulatory activities of NET rather than their role as autoantigens.

Besides the physiological and protective effects of NET in response to invading pathogens, NET have been described in several diseases, especially in inflammatory and/or autoimmune diseases (table 1) and fibrosis. NET can be beneficial and protective, for example, through anti-inflammatory activity, but in most cases they have a detrimental and pathogenic role in these diseases where they stimulate immune responses through their pro-inflammatory, antigenic (which is particularly true in RA and SLE) and immunogenic activities. NET work as a source of autoantigens and DAMP or may even be considered as DAMP themselves. As such, they may be key endogenous ligands involved in sterile inflammation.
Connective tissue diseases

Physiological modulatory activities of NET

Using a short differentiation protocol (3 days with M-CSF), NET have been shown to be taken up by human monocyte-derived macrophages. Healthy donor NET did not induce (either pro-inflammatory or anti-inflammatory) cytokine secretion by resting macrophages from healthy donors, although a slight but significant induction of IFN-α was observed (table 2), and this was thus described as a silent process (figure 1). Nevertheless, concentrations of NET used to stimulate macrophages were not indicated. Interestingly, NET-mediated induction of IFN-α was also reported for healthy donor plasmacytoid DC (pDC). Likewise, stimulation of healthy donor monocyte-derived DC with NET from healthy donors was not associated with DC activation as estimated by HLA-DR and CD80/83/86 expression (figure 1).

On the contrary, using a 6-day differentiation protocol, we have shown that NET from healthy donors induce the secretion of pro-inflammatory cytokines, but not immunomodulatory IL-10, by resting M-CSF-differentiated monocyte-derived macrophages. Activation was associated with HLA class I and class II as well as CD86 upregulation. A similar pro-inflammatory response was triggered by healthy donor NET on unprimed healthy donor PMN (figure 1). One plausible hypothesis to explain the difference with above results is that highly mature or differentiated macrophages are more responsive to NET. Similarly, macrophages have been shown to phagocytose NET and to express IFN-I after cGAS activation. In agreement with our results and a pro-inflammatory response induced by NET, although NET do not trigger cytokine secretion by healthy macrophages or DC, they potentiate IL-1β, TNF and IL-6 secretion by LPS-stimulated macrophages (3 day differentiation protocol) as well as IL-1β and IFN-γ secretion by LPS-stimulated DC. Interestingly, NET also influence cells from adaptive immunity (figure 2A).

In healthy individuals, NET can directly prime resting CD4+ T lymphocytes. NET-induced activation is TCR-dependent but does not trigger T-cell proliferation. In coculture, NET induce cluster formation, upregulation of the activation markers CD25 and CD69, and phosphorylation of the TCR-associated signalling kinase ZAP70 in CD4+ T cells. Moreover, NET increase T-cell responses to specific antigens, making T cells capable of being activated by sub-optimal stimuli. Although NET-primed CD4+ T lymphocytes do not proliferate, they do proliferate and secrete IFN-γ in the presence of resting DC and without specific antigens (a suboptimal stimulus). Importantly, this study also confirms indirectly the stimulatory

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<td>Source of autoantigens</td>
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<td>Nucleosomes</td>
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<td>Rheumatoid arthritis (RA)</td>
<td>Autoantibodies</td>
<td>Pathogenic</td>
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<tr>
<td>Autoimmune small-vascular</td>
<td>Autoantibodies</td>
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<td>Psoriasis</td>
<td>?</td>
<td>Pathogenic</td>
<td>IL-17 exposure</td>
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ANCA, anti-neutrophil cytoplasmic antibodies; DC, dendritic cells; IFN, interferon; NET, neutrophil extracellular traps.
potential of NET on DC. Indeed, when cocultured with DC and only in the presence of NET, both unprimed purified CD4+ and CD8+ T cells were activated. Regarding CD4+ lymphocytes, NET trigger activation of both naïve and memory cells in the presence of DC.75 Similarly, NET are able to trigger polyclonal activation of memory B lymphocytes from healthy individuals and to induce total IgG secretion.76 Using complexes made of purified DNA and granule proteins (especially LL-37) to mimic NET, authors have shown that these structures are internalised and activate B lymphocytes partly in a TLR9-dependent manner.

### Beneficial activities of NET in diseases

In gout, monosodium urate crystals induce NET formation77 which limits inflammation by degrading cytokines (especially pro-inflammatory cytokines, but also IL-10) and chemokines.78 This anti-inflammatory property of NET may happen only at sites where PMN are highly concentrated, generating aggregated NET, as observed in gout tophi.78 In addition, inflammation may lead to the release of extracellular histones and the latter contribute to endothelial dysfunction.79 Interestingly, at high concentrations, NET have been shown to proteolytically degrade extracellular free histones (not NET-bound histones), at least in vitro, resulting in attenuated histone-mediated cytotoxicity in cell cultures.80 In patients suffering from chronic granulomatous disease, characterised by NOX2 mutations, ROS-dependent NET formation is impaired and results in recurrent bacterial and fungal infections; in these immunodeficient patients,

### Table 2  Involvement of NET in the regulation of human immune responses

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<th>Mechanisms reported</th>
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<td></td>
<td>Enhanced activation of LPS-primed macrophages</td>
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<td>IL-1β and IFN-γ secretion74</td>
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<td>Activation of the complement system</td>
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<td>In type 1 diabetes</td>
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Direct effects of NET on immune cells or molecules are shown. NET-containing immune-complexes are not presented here. Consequences on B and T cells are shown in figure 2. ANCA, anti-neutrophil cytoplasmic antibodies; DC, dendritic cells; IFN, interferon; LPS, lipopolysaccharides; NET, neutrophil extracellular traps; PMN, polymorphonuclear neutrophils; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus.
NET formation and protection against *Aspergillus* infection can be restored by gene therapy targeting NOX.\(^{23}\) Also, NET have been suggested to form a physical barrier during acute necrotising pancreatitis and peritonitis in order to isolate necrotic areas from surrounding healthy tissues, limiting spreading of necrosis-associated pro-inflammatory mediators.\(^{81}\)

**Pathological NET-mediated mechanisms in inflammatory autoimmune diseases**

On the contrary, a pathogenic role of NET has been reported in several inflammatory conditions, like acute pancreatitis, sterile lung inflammation, vascular occlusion during severe bacterial infection, chorioamnionitis, atherosclerosis, fibrosis, thrombosis and cancer. Regarding inflammatory autoimmune diseases (table 1), NET are present in skin biopsies from psoriatic patients and contain the pro-inflammatory cytokine IL-17A, which is involved in psoriasis pathogenesis.\(^{82}\) Similarly, NET were observed in situ in lesional skin biopsy of patients with bullous pemphigoid and blister fluids from patients induce NET formation ex vivo.\(^{83}\) Interestingly, in autoimmune small-vessel vasculitis, anti-neutrophil cytoplasmic antibodies (ANCA) trigger in vitro formation of NET which are found deposited in inflamed kidneys of patients.\(^{84}\) Moreover, injection of NET-loaded DC induces ANCA production in mice.\(^{85}\) In addition, NET may participate in the severity of COVID-19 and reciprocally SARS-CoV-2 infection has been suggested as a trigger of autoimmunity.\(^{86}\) Actually, SARS-CoV-2 triggers the release of NET\(^{87}\) which are detected in lung microthrombi of patients\(^{88}\) and COVID-19 adult patients display a reduced capacity to degrade NET.\(^{89}\)

**RA and SLE**

Particularly, it is now believed that NET may also play a pathogenic role in SLE or RA, as a result of increased NET formation, decreased NET clearance and/or altered composition.\(^{2,14}\)

In SLE, a PMN sub-population (LDG) has a higher capacity to spontaneously form NET in vitro and NET are detected in skin and kidney lesions from patients.\(^{90}\) The same group suggested that IL-18 induced NET release by LDG.\(^{91}\) Moreover, sera from a subgroup of lupus patients have an impaired NET-degrading activity, which correlates with high titers of anti-double stranded DNA autoantibodies (precisely a lupus marker).\(^{92}\) These patients develop NET-binding antibodies. It was recently shown that AIM2 and IFI16 are autoantigens binding NET, protecting them from DNase1 degradation.\(^{93}\) Circulating nucleosomes, a DAMP and classical lupus autoantigen, triggers NET formation,\(^{94}\) leading to an
Figure 2  Direct and indirect effects of NET on B and T lymphocytes. (A) NET effects in a physiological context. On the left part (red arrows), NET inhibit myeloid DC activation in response to LPS, leading to impaired T-cell response. On the right panel (green arrows), NET directly activate memory B lymphocytes to secrete total IgG. NET can also prime resting CD4+ T cells, leading to CD25 and CD69 upregulation, as well as ZAP70 phosphorylation, without requiring dendritic cells. Likewise, NET stimulate resting DC to activate resting CD4+ T cells, favouring a Th1-like response. Data are pooled from studies with NET and target cells from healthy individuals. (B) NET effects in a pathological context. In type 1 diabetes (green background), NET stimulate myeloid dendritic cells to activate Th1 lymphocytes. In SLE (pink background), NET directly activate memory B lymphocytes to secrete ANCA. Finally, in RA (blue background), NET activate fibroblast-like synoviocytes on internalisation and NET peptides are presented to antigen-specific CD4+ T cells, leading to their activation. NET also activate myeloid dendritic cells, which potentially (dotted line) stimulate T lymphocytes. Effects of NET-containing immune complexes are not depicted in this figure. ANCA, anti-neutrophil cytoplasmic antibodies; DC, dendritic cells; FLS, fibroblast-like synoviocytes; LPS, lipopolysaccharides; mDC, myeloid DC; NET, neutrophil extracellular traps; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; T1D, type 1 diabetes.
amplification loop of the inflammatory process. Likewise, plasmas from patients with SLE induce a stronger release of NET by normal PMN than plasma from healthy volunteers and high levels of NET formation are associated with increased plasma levels of antinuclear antibodies, anti-double stranded DNA antibodies and a high IFN signature in patients with SLE. Regarding the pathogenic mechanisms triggered (table 2), lupus NET spontaneously produced by LDG stimulate IL-1β and IL-18 secretion by LPS-primed macrophages from healthy individuals. Mature (cleaved) IL-1β was secreted and associated with caspase-1 activation, indicating inflammasome involvement. In addition, NET induced in PMN from healthy donors and patients with SLE increase calcium flux in macrophages from healthy donors and patients with SLE, whereas NET spontaneously formed by lupus LDG were less efficient (figure 1 and table 2). This increased calcium flux may be partly triggered by activation of CXCR4 on macrophages by ubiquitinated proteins present in NET. NET induced in classical SLE PMN also trigger TNF and IL-6 secretion by lupus macrophages; especially TNF secretion, but also IL-10, was higher in lupus macrophages versus normal macrophages in response to NET. In support of a pathogenic role of NET in SLE, LDG-derived NET impair endothelium-dependent vasorelaxation in vitro. Actually, these NET extrude RNA and induce IFN-I-stimulated genes once internalised by endothelial cells. In SLE, IL-33 is complexed with NET and the amount of NET-IL-33 complexes is correlated with disease activity. These complexes trigger IFN-α (a key lupus cytokine) production by pDC in an IL-33 receptor-dependent manner. In addition, lupus anti-RNP autoantibodies induce formation of NET containing LL-37, a DNA-binding protein facilitating DNA uptake by pDC, in SLE but not healthy PMN. Those anti-RNP-induced lupus NET trigger the activation of healthy pDC and the secretion of IFN-α in a TLR9-dependent manner. Likewise, PMN stimulated with RNP-containing immune complexes release oxidised mitochondrial DNA inducing IFN-α production in PBMC in a Stimulator of Interferon Genes-dependent manner. Such complexes are also spontaneously released by lupus LDG. Lupus patients produce also circulating ANCA, for example, anti-LL-37 antibodies. Importantly, in patients with ANCA-positive SLE but not in healthy donors, anti-LL-37-induced NET (which also contain LL-37) trigger the production of ANCA, and especially anti-LL-37 antibodies, by memory SLE B cells in an antigen-dependent manner (figure 2B).

As observed in SLE, RA PMN (both blood and synovial fluid PMN) produce more NET in vitro than PMN from healthy individuals, either spontaneously or in response to LPS or PMA, and NET are present in both rheumatoid nodules and the synovial fluid of patients with RA. Actually, RA serum and RA synovial fluid increase NET formation by healthy PMN. Similar results were obtained in the mouse collagen-induced arthritis model in which bone marrow PMN from diseased mice produce more NET in vitro than PMN from normal mice. In another RA mouse model, NET have been suggested to mediate joint hyperalgesia. Interestingly, PMA-stimulated PMN release NET containing alarmin S100A11 (calgizzarin), which behaves as a DAMP in RA. In addition, we and others have shown that ACPA-rich IgG purified from patients with RA bind to NET or even induce NET formation. NET binding and NET induction were confirmed with purified ACPA. Likewise, RA sera recognise activated PMN and NET and monoclonal antibodies generated from RA synovial B lymphocytes have a strong reactivity against citrullinated histones and PMA-induced NET. As NET formation can be associated with citrullination (at different levels and in response to some stimuli), NET may be a source of RA autoantigens and either the targets of ACPA or even the true autoantigen triggering ACPA production. Moreover, NET formation is associated with the release of active PAD2 and PAD4 in the extracellular milieu, either free or bound to NET, which is potentially associated with the citrullination of extracellular autoantigens locally in affected tissues. NET formation in RA joints may thus be linked to citrullination and potentially to recognition by ACPA or even induction of ACPA production, triggering pathogenic mechanisms such as immune complex deposition in tissues and complement activation. Interestingly, in an RA model consisting of HLA-DR4-transgenic mice in which fibroblast-like synoviocytes loaded with RA NET were injected in the synovial space, animals develop ACPA and show impaired cartilage integrity, without however overt arthritis. In addition to ACPA, patients with RA develop antibodies against carbamylated proteins, which predict a more severe disease, and NET externalise carbamylated proteins such as carbamylated LL-37. These NET are thus also the targets of RA autoantibodies, including anti-carbamylated LL-37 antibodies. Recently, anti-NET antibodies have been reported in RA, SLE, Sjögren’s syndrome and scleroderma. Regarding the mechanism involved, we have recently shown that RA NET are more efficient in activating macrophages and PMN than healthy donor NET, leading to the secretion of the pro-inflammatory cytokines IL-8, IL-6 and the key RA cytokine TNF, with minimal induction of IL-10 (figure 1). These data indicate that, in RA, both NET formation and the pro-inflammatory activity of NET are increased in comparison to healthy individuals (when activity is defined by the capacity of NET to trigger in target cells a cytokine secretion profile in favour of pro-inflammatory vs anti-inflammatory cytokines). Actually, we have recently demonstrated that NET from patients with RA activate several myeloid cell sub-populations towards a pro-inflammatory profile (Seninet et al, submitted). The fact that RA NET are more efficient in inducing pro-inflammatory cytokines might be explained by a slightly different NET composition. Proteomic analyses revealed that the nature of the stimulus or origin of the PMN (healthy individuals vs autoimmune patients) may influence the composition of NET. Similarly, NET from...
patients with RA have been shown to activate (although moderately) myeloid DC from healthy volunteers, and with a higher activity than healthy donor NET, as shown by HLA-DR/CD86 upregulation and IL-6/TNF secretion.102 NET are also directly involved in arthritic cartilage damage in RA by disrupting the cartilage matrix in a NE-dependent manner.115 In RA, autoantibodies induce NET formation in vitro and RA NET stimulate fibroblast-like synoviocytes (the cells invading cartilage in RA) to secrete IL-6 and IL-8101 and to upregulate MCH class II on internalisation of NET105; NET indirectly lead to T-cell activation via presentation of NET peptides (figure 2B). In turn, RA synovial fluid CD8+T cells induce NET formation.116 Similarly, in an in vitro mouse cell culture model, ovalbumin-loaded DC have a higher capacity to stimulate ovalbumin-specific CD4+T cells from OT-II mice when DC are treated with NET from collagen-induced arthritis mice as compared with NET from control mice, as shown by the increased frequency of IFN-γ-producing Th1 cells,102 demonstrating increased antigen-presenting cell functions after NET treatment. Recently, carbamylated NET (as those observed in patients with RA) have been shown to directly trigger monocyte differentiation into osteoclasts, supporting a role of NET in bone erosion.117

However, in different experimental lupus models, using NOX2-deficient or PAD4-deficient mice, NET have been suggested to be protective or without influence118–120; nevertheless NOX2 and PAD4 are also involved in cellular processes other than NET formation and might play different roles at different stages of the disease. As mentioned above, NOX-independent NET formation has been reported, whereas PAD activity may be involved but not required for NET formation. Interestingly, recent data suggest that NET formation partly occurs in a NOX-independent manner in vivo in patients with SLE.121 One should also take into account that the PMN blood frequency is much lower in mice than in humans, probably affecting NET impact. On the opposite, PAD inhibition protects from lupus manifestations in the MRL/lpr mouse model,122 whereas disease severity is reduced in PAD4-deficient animals in the TNF-α-transgenic mouse model of RA.123 In a recently described new RA mouse model (collagen-induced arthritis with G-CSF administration), PAD4-deficient animals present lower levels of citrullinated histones in the blood and the synovial tissue and develop a less severe disease124; in this model, oral administration of a PAD4 inhibitor able to block NET formation in vitro reduces arthritis development.125 Interestingly, NET have been suggested to contribute to lupus nephritis in MPO-deficient mice; these mice developed more severe nephritis with increased deposition of NET in glomeruli than wild-type mice.126 The latter result indicates that effects of MPO and NET are not necessarily linked, which makes sense as MPO is also present in monocytes. Moreover, some stimuli do not require ROS to induce NET formation and even PMN from patients with inactive NOX may produce NET; similar observations were reported regarding MPO.34 35

Finally, the stimuli triggering NET formation in vivo are unknown and therefore it is tricky to speculate on the NET mechanism/pathway involved in these autoimmune patients or mice.

It should be noted that physiological NET clearance mechanisms are impaired in patients with SLE and RA (as recently reviewed5), amplifying NET-mediated pathological mechanisms in patients. Thus, both in patients with RA and SLE, defective NET clearance may result in activation of the complement system on deposition of residual complement factor 1q (C1q) on NET,127 as well as enhanced release of autoantigens and DAMP.

**Diabetes**

NET also impair wound healing, especially in diabetes,128 where PMN from patients (with either type 1 or type 2 diabetes) extruded more DNA spontaneously or after ionomycin stimulation than PMN from healthy controls. Likewise, PMN from healthy individuals extruded more DNA in response to PMA or ionomycin when exposed to high glucose concentration. In addition to priming, it should be noted that glucose alone also stimulated DNA release.129 Thus, altered metabolic regulation, for example, via the glycolytic pathway, may predispose PMN to form NET. Furthermore, energy metabolism and mitochondrial involvement in NET release is supported by the fact that glycolytic ATP production is required for NET formation after GM-CSF priming and subsequent C5a stimulation.46 Indeed, PMN lacking the mitochondrial protein OPA1 have a decreased ATP production through glycolysis and fail to release NET. Potential pathogenic consequences of NET release have been described in type 1 diabetes. In monocyte-derived DC cultures with autologous NET, patient NET contribute to a significant increase in DC maturation markers and inflammatory cytokine production as compared with normal NET-DC cultures. DC capacity to induce IFN-γ-producing T lymphocytes (both Th1 and CD8+ cells) was enhanced in DC-T cell cocultures with patient NET and this does not result from the direct effect of NET on T cells (figure 2B). Using RNA-seq analysis, patient NET were shown to downregulate TGF-β while upregulating IFN-α in healthy donor DC.129

All the results described in that section suggest that NET behave as a source of DAMP as well as autoantigens and we suggest they may also behave as an adjuvant, influencing both innate and adaptive immunities. They can trigger directly or indirectly pro-inflammatory and antigen-specific responses. NET may also contribute to sustained inflammation by inhibiting efferocytosis. Impaired efferocytosis is a hallmark of several autoimmune diseases and is involved in delayed resolution of inflammation. Indeed, extracellular cold-inducible RNA-binding protein (a DAMP present at high concentration in the blood and especially in the synovial fluid of patients with RA130) induces NET that inhibit efferocytosis.131
In addition to direct effects on cells, NET can also act indirectly via immune complexes. NET are targets of autoantibodies in several diseases (e.g., ACPA in RA, NET-binding antibodies in SLE, ANCA in patients with vasculitis) although the fine specificity of those antibodies is not always characterised. For example, what is recognised in NET by lupus autoantibodies is not clear (DNA, maybe only partly? Individual NET components? Or are they true ‘anti-NET’ antibodies recognising tertiary NET structure, that is, NET-restricted antibodies?). In RA, there are probably also non-ACPA antibodies binding to NET, for example, anti-carbamylated protein antibodies. Among classical ANCA, anti-proteinase 3 and anti-MPO are the most frequent autoantibodies. The resulting immune complexes may become pathogenic on recognition through Fc receptors. NET can also become more active in immune complexes. In SLE, anti-LL-37 antibodies induce formation of NET containing LL-37. Although NET alone stimulate pDC to secrete IFN-α, this secretion is strongly enhanced in the presence of anti-LL-37 antibody, which is abrogated by a TLR9 inhibitor. As other immune complexes, NET-containing immune complexes may bind and activate the complement system and be even more strongly recognised by cells expressing complement receptors. NET-containing immune complexes may be more efficient stimuli after internalisation and activation of intracellular receptors (e.g., endosomal TLR9 recognising DNA in NET). Indeed, although mammalian self DNA normally poorly stimulates TLR9, it may activate TLR9 when it reaches this receptor or on enforced translocation to endosomes.129-134

**NET and the regulation of inflammation**

On the contrary, NET may be anti-inflammatory or may at least limit inflammation in some circumstances (figure 1 and table 2). Indeed, we have shown that NET inhibit the response of LPS-stimulated macrophages, leading to reduced IL-6 secretion, whereas IL-10 secretion was enhanced. This was observed with both NET from healthy individuals or patients with RA and on both macrophages from healthy individuals or patients with RA. Interestingly, although NET activate also resting PMN, LPS-stimulated PMN are not responsive to inhibition by NET. Similarly, NET from healthy individuals partly inhibit LPS-induced maturation of normal myeloid DC, as shown by reduced HLA-DR, CD40, CD80 and CD86 upregulation as well as reduced secretion of both pro-inflammatory (e.g., TNF or IL-8) and immunomodulatory cytokines (IL-10). This was associated with a lower ability of DC to stimulate CD4+ T lymphocyte proliferation and altered CD4+ T lymphocyte polarisation (reduced IL-10 and Th1/Th17 cytokine secretion, increased Th2 cytokines; figure 2A). NET-mediated inhibition of DC maturation in the presence of LPS was confirmed in healthy subjects, showing in addition inhibition of IL-12 secretion. Such inhibitory activities of NET on macrophages were not observed by Farrera et al, especially not the impaired IL-6 secretion by LPS-stimulated macrophages. This might be explained by the different protocol used to differentiate macrophages from monocytes in that study, harvesting macrophages after 3 days instead of 6 days, which probably generates less differentiated cells.

Finally, NET are immunomodulatory by targeting directly key immune proteins (table 2). Free NET (not only NET-containing immune complexes) bind C1q and activate the complement cascade, leading to the production of the C5a anaphylatoxin. Thus, non-degraded NET may participate in complement consumption and inflammation in SLE. These NET-complement complexes may also be recognised by cells expressing complement receptors. On the opposite, NET may directly degrade some PMN-derived cytokines, through NET-associated serine proteases, to resolve inflammation. Interestingly, patients affected by Papillon-Lefèvre syndrome are characterised by nonfunctional PMN serine proteases and intense periodontal inflammation; although PMN from these patients have been reported to display impaired canonical NET formation, they aberrantly release some NET-like DNA structures nearly devoid of NE and unable to degrade inflammatory mediators. However, NET-bound proteases can also process and activate pro-inflammatory cytokines. Therefore, excessive NET formation and activation of such cytokines may convert a protective response in a detrimental pro-inflammatory and tissue-damaging reaction, both in the context of infection and sterile injury.

**WHAT HAVE WE LEARNED FROM IN VITRO STUDIES?**

The present review clearly indicates that NET are extracellular structures composed of DNA and a set of proteins that may vary depending on the stimuli, the physiological or pathological context and, accordingly, to the pathways and/or the mechanisms triggered during NET formation, although many of these proteins are overlapping. Some of them may be modified or altered, either by post-translational modifications (e.g., citrullination), partial degradation or cleavage as observed with histones. Therefore, we believe that the simple measure of cell-free DNA, without at least testing whether DNA forms complexes with proteins and testing known NET proteins (or even better characterising associated proteins) should not be used as a surrogate marker to estimate the presence of NET in biological fluids or cell culture supernatants. Likewise, citrullinated histone H3 is found during NET formation, but not in response to all stimuli and at different levels, and therefore is not a clear NET marker. Moreover, citrullination of histones is not only and specifically induced during NET formation and thus additional tools have to be used simultaneously to refer to NET. As an alternative, NET-detecting sandwich ELISA have been developed that measure extracellular complexes made of DNA and MPO or NE. This approach is more NET-associated but not specific; it might also only partly reflect the extent of NET formation. Indeed, these
ELISA may only detect NET induced by some stimuli and they assume that such complexes are only released during NET formation and not during other cellular processes or by monocytes which also express MPO and also release extracellular traps. The best NET characterisation remains thus the visualisation of DNA–proteins complexes using a combination of stainings for both DNA and multiple proteins, for example, by fluorescence microscopy.

Regarding in vitro cell activation assays with NET prepared from isolated neutrophils, we recommend to use NET obtained from adherent activated PMN (detached by vigorous pipetting or mild nuclease digestion) rather than NET-containing cell culture supernatants to avoid transferring on target cells the stimulus or, for example, cytokines induced by that stimulus. Likewise, adding target cells directly on adherent NET or, for example, cytokines induced by that stimulus would be important for a better understanding. However, those NET have to be better characterised, cell activation mechanisms differ from those triggered by cell-free DNA.

In all cases, we suggest using primary PMN instead of cell lines and, when working with mouse PMN, Ly-6G+ and not Gr-1+ cells should be used. For cell activation assays, NET prepared from highly purified PMN should be used.

Although PMA is the best characterised inducer of NET, using more physiological stimuli to analyse the impact of NET in different mechanisms and pathologies would be important for a better understanding. However, PMA is also one of the most often used inducers because it triggers high levels of NET formation; anyway, PMA allows measuring the capacity of PMN to release NET. Actually, there are not so many natural and disease-specific or even disease-associated NET stimuli described. In SLE, stimulation of PMN with anti-RNP antibodies or RNP-containing immune complexes leads to the release of NET made of oxidised mitochondrial DNA which are interferogenic. However, those NET have to be better characterised. In RA, some cytokines or autoantibodies have been reported to induce NET, however often at low levels.

PERSPECTIVES

The data described here raise several questions. Other cell types (eosinophils, basophils, mast cells, and more recently monocytes and even lymphocytes) have been reported to release extracellular traps or DNA. In the latter case, these structures do not contain antibacterial proteins. As their composition may differ from NET, their capacity to modulate immune cell responses and their role in inflammatory and/or autoimmune diseases should be tested. Likewise, as PMN subpopulations have been described in blood and tissues, it would be interesting to compare activities of NET prepared from these different PMN subtypes, first in healthy individuals, and then in patients suffering from different inflammatory and/or autoimmune diseases. Activities of NET are probably influenced by their composition. NET content and ET-associated proteins were originally characterised by immunofluorescence in NET induced in vitro with normal PMN. NET composition was confirmed on PMA-stimulated healthy PMN by mass spectrometry analysis and proteins were quantified by immunoblotting. Then, proteomic analyses on total blood PMN revealed that NET composition differs in healthy individuals according to the stimulus used (PMA vs calcium ionophore). Similar results were obtained with normal PMN stimulated with PMA, calcium ionophore or LPS but showing in addition that post-translational modifications of NET proteins are influenced by the stimulus. Moreover, NET composition also slightly varies according to diseases, as shown with NET induced in vitro by PMA or calcium ionophore on RA versus SLE PMN. Likewise, in vitro, RA-associated stimuli as TNF, rheumatoid factor and RA IgG induce NET with different compositions in PMN from healthy individuals. In addition, IgM rheumatoid factor induce NET containing citrullinated proteins in control PMN. In SLE, NET induced in vitro by LPS stimulation contain lower amounts of ubiquitinated proteins. Similarly, using PMA-stimulated PMN, it has been shown that NET composition is modified in patients with SLE as compared with healthy individuals and even characterises different SLE subsets, especially those with severe disease i.e. with nephritis. particularly, NET from patients with active SLE are enriched in IL-17A and tissue factor, as evidenced by immunofluorescence and immunoblotting. Replicating proteomic analyses with NET induced by physiological and disease-related stimuli would support hypotheses on NET functions. These data suggest also that epigenetic modifications might control NET activities. Although additional studies will be required to support this hypothesis, NET have been suggested to trigger macrophage activation through the binding of NET ubiquitinated proteins (potentially histones) to CXCR4. In addition, carbamylated NET from patients with RA are particularly efficient in activating fibroblast-like synoviocytes. Likewise, NET induced by particular lupus immune complexes are enriched in oxidised mitochondrial DNA. Additional studies support disease-associated differences in NET composition. Compared with NET from healthy individuals, SLE NET contain increased amounts of acetylated and methylated histones. Some of these post-translational modifications are targets of lupus IgG autoantibodies, as acetylated histone H4. Finally, proteomic analyses
revealed the presence of several post-translational modifications in NET, which vary according to the stimulus, and are potentially different between healthy individuals and autoimmune patients.\textsuperscript{112,113}

CONCLUSIONS
NET are not only antimicrobial but also antigenic, immunogenic and pro-inflammatory/anti-inflammatory by exposing immunomodulatory molecules, and may behave as a DAMP depending on the microenvironment. This dual pro-inflammatory/anti-inflammatory activity was also observed with aggregated NET. Indeed, aggregated NET may also become pro-inflammatory; they are produced in pancreatic ducts in response to pancreatic juice and promote pancreatic inflammation by occluding pancreatic ducts.\textsuperscript{115} Although NET directly act on different immune cell types, resulting in enhanced or impaired cell activity, those effects can be modulated by cofactors able to bind NET, such as Clq and LL-37 which recognise DNA, provided the target cells express cell surface receptors for Clq or LL-37.\textsuperscript{72} Further studies will be required to determine to which extent inhibiting NET formation and/or accumulation could be a therapeutic option in autoimmune patients. For example, stimulating signalling via the inhibitory receptor SIRL-1 in PMN using specific antibodies inhibits NET formation in vitro.\textsuperscript{146} Finally, as different antibodies recognise NET (eg, ACPA, ANCA, anti-camlylated protein antibodies), we wonder whether some of them belong to the anti-chromatin antibodies family first described in SLE or whether it is time to define a new anti-NET antibodies family.

Interestingly, similar properties have been reported for NET and extracellular chromat in patients with SLE and RA, especially their DAMP activity, for example, PMN activation.\textsuperscript{34} Nucleosomes represent a key lupus autoantigen. They are present at higher concentrations in the circulation of patients as compared with healthy individuals\textsuperscript{147,148} and deposit in kidneys and skin. Similarly, chromatin is present in the synovial fluid of inflamed joints in patients with RA\textsuperscript{149} and deposits in affected joints.\textsuperscript{150} Part of circulating nucleosomes might derive from NET due to their auto-catabolic activity,\textsuperscript{151} especially from the smooth stretches which are probably only composed of histones and DNA and have dimensions similar to nucleosomes.\textsuperscript{19} Because it might influence their activity, it would be interesting to determine the cell and tissue origin of circulating nucleosomes and/or NET by analysing nucleosome positioning.\textsuperscript{152}

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