Neutrophil extracellular traps in immunity and disease

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Abstract | Neutrophils are innate immune phagocytes that have a central role in immune defence. Our understanding of the role of neutrophils in pathogen clearance, immune regulation and disease pathology has advanced dramatically in recent years. Web-like chromatin structures known as neutrophil extracellular traps (NETs) have been at the forefront of this renewed interest in neutrophil biology. The identification of molecules that modulate the release of NETs has helped to refine our view of the role of NETs in immune protection, inflammatory and autoimmune diseases and cancer. Here, I discuss the key findings and concepts that have thus far shaped the field of NET biology.

Neutrophil elastase
(NE). A neutrophil-specific antimicrobial serine protease stored in azurophilic granules.

Myeloperoxidase
(MPO). A haem-containing enzyme that reacts with hydrogen peroxide to generate hypochlorite and other halide oxidants.

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Neutrophils are the most abundant innate immune effector cells of the human immune system. They are armed with broadly effective antimicrobials that are stored predominately in specialized granules. Given that this neutrophil arsenal can also damage host tissues, its deployment is tightly regulated through three major strategies: phagocytosis, degranulation and the release of neutrophil extracellular traps (NETs). NETs are large, extracellular, web-like structures composed of cytosolic and granule proteins that are assembled on a scaffold of decondensed chromatin[1]. Although the majority of DNA in NETs originates from the nucleus, these structures also contain mitochondrial DNA[2]. NETs trap, neutralize and kill bacteria[1], fungi[3], viruses[4] and parasites[5] and are thought to prevent bacterial and fungal dissemination[6,7]. However, if dysregulated, NETs can contribute to the pathogenesis of immune-related diseases.

Initially, 24 proteins were identified in NETs formed by stimulation of neutrophils with phorbol 12-myristate 13-acetate (PMA), a molecule that activates protein kinase C (PKC) and triggers the production of reactive oxygen species (ROS). Among these proteins were histones, the serine protease neutrophil elastase (NE; also known as ELANE), myeloperoxidase (MPO), calprotectin, cathelicidins, defensins and actin[8]. Subsequent studies have extended this list, suggesting that the composition of NETs varies depending on the stimulus. For example, different *Pseudomonas aeruginosa* mucoid and non-mucoid strains induce the formation of NETs containing 33 common proteins and up to 50 variable proteins[9]. Whether and how differences in NET composition impact NET function remains to be investigated.

NET release occurs primarily through a cell death process termed NETosis[10]. To initiate this process, neutrophils arrest their actin dynamics and depolarize[11]. Next, the nuclear envelope disassembles, and nuclear chromatin decondenses into the cytoplasm of intact cells, mixing with cytoplasmic and granule components[10] (FIG. 1). The plasma membrane then permeabilizes, and NETs expand into the extracellular space 3–8 hours after neutrophil activation. An alternative mechanism termed non-lytic NETosis leads to the rapid release of NETs within minutes of exposure to *Staphylococcus aureus* via the secretion of chromatin and granule contents[12,13] and in the absence of cell death. This phenomenon has been observed by intravital microscopy in a small fraction of neutrophils during systemic *S. aureus* infection and generates NETs and anucleated cytoplasts that crawl and phagocytose bacteria[13]. This multitasking non-lytic rapid response is mounted by the first neutrophils to arrive at sites of infection.

The mechanisms that clear NETs are less well understood. During infection, NETs persist for several days[7] and are thought to be dismantled by the secreted plasma nuclease DNase I (REF. 14). Injection of this enzyme during *S. aureus* infection leads to rapid degradation of NET-associated DNA[15], but the dynamics of NET clearance by endogenous enzymes are unknown. Strikingly, NET proteins persist long after DNA degradation[15], suggesting that they are cleared via additional mechanisms. These mechanisms might involve macrophage scavenging, as DNase I facilitates the ingestion of NETs by macrophages *in vitro*[16].

Here, I provide an overview of the mechanisms that regulate NET formation and clearance and describe recent advances in our understanding of how NETs protect against infection and cause pathology associated with several diseases. These topics are organized in a conceptual manner according to the immunological function of NETs. I pay attention to key findings, highlight open questions and discuss the controversies in the field.

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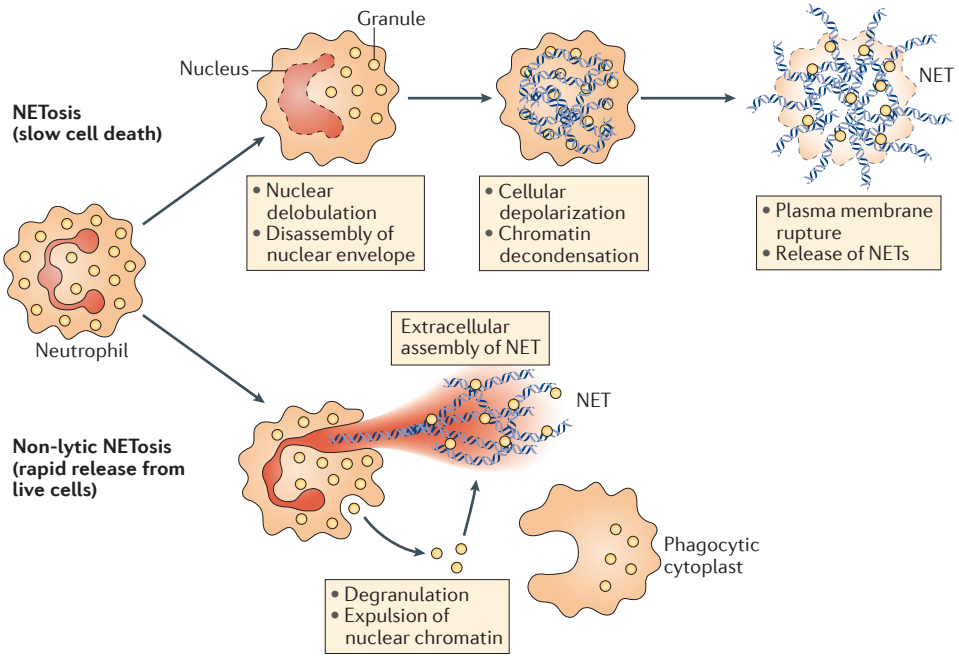

Figure 1 | **NET formation pathways.** Neutrophil extracellular traps (NETs) form via two pathways. The first is a cell death pathway termed NETosis that begins with nuclear delobulation and the disassembly of the nuclear envelope and continues with loss of cellular polarization, chromatin decondensation and plasma membrane rupture. The second is a non-lytic form of NETosis that can occur independently of cell death and involves the secreted expulsion of nuclear chromatin that is accompanied by the release of granule proteins through degranulation. These components assemble extracellularly and leave behind active anucleated cytoplasts that continue to ingest microorganisms.

NADPH oxidase
A membrane-associated complex of proteins that transfer electrons from NADPH to molecular oxygen to generate the oxygen radical superoxide.

Azurophilic granules
A subset of neutrophil granules that contain antimicrobials such as myeloperoxidase and neutrophil elastase. Within the granule membranes, a complex of eight antimicrobial proteins forms the azurosome.

Chronic granulomatous disease
(CGD). An inherited X-linked immune deficiency caused by genetic mutations that disrupt the activity of NADPH oxidase. It is associated with hyperinflammation and increased susceptibility to bacterial and fungal infections.

Thrombosis
Formation of a blood clot (thrombus) in blood vessels, resulting in partial or complete vessel occlusion.

DEK
A DNA-binding protein that alters DNA structures and is involved in DNA repair.

Mechanisms of NET formation

From ROS to chromatin decondensation. Two enzymes in the ROS pathway have critical roles in NETosis. ROS generated by NADPH oxidase stimulate MPO to trigger the activation and translocation of NE from azurophilic granules to the nucleus, where NE proteolytically processes histones to disrupt chromatin packaging[17]. Subsequently, MPO binds chromatin and synergizes with NE in decondensing chromatin independently of its enzymatic activity[17] (FIG. 2). NADPH oxidase activity can be redundant in response to some stimuli, such as immune complexes in which mitochondrial ROS are sufficient to drive NETosis[2].

NE release from azurophilic granules does not require membrane rupture or fusion. In resting neutrophils, a fraction of MPO is bound to NE as part of a complex called the azurosome, which spans granule membranes[11]. Hydrogen peroxide selectively releases NE into the cytosol in an MPO-dependent manner (FIG. 2). It is important to clarify that inhibition of the enzymatic activity of MPO does not block but only delays NETosis[18], potentially owing to the role of MPO in activating the proteolytic activity of NE against large protein substrates. This oxidative activation is important because NE binds to F-actin filaments in the cytoplasm and must degrade them in order to enter the nucleus[11]. NE is sufficient to decondense nuclei *in vitro*[17], but unknown mechanisms may help to disassemble the nuclear envelope in neutrophils.

This MPO–NE pathway is induced by many NET stimuli, such as fungi and crystals[19,20], and its role is supported by studies of neutrophils from patients with chronic granulomatous disease (CGD)[10] and with complete MPO deficiency[18], as well as by studies using NE-deficient mice or NE inhibitors in mouse models of sepsis, cancer and pulmonary infection[7,15,17,21]. NET release is also abrogated in NADPH oxidase-deficient mice during pulmonary fungal infection, which stimulates robust NET release[22]. Similarly, NETosis is defective in neutrophils from patients with Papillon–Lefèvre syndrome caused by mutations in the cysteine protease cathepsin C (CTSC), which processes NE into its mature form[23,24]. Mice lacking CTSC fail to form NETs upon pulmonary Sendai virus infection[25] and in aortic aneurism models[26]. Moreover, isolated CTSC-deficient neutrophils exhibit defects in NETosis, although the impairment is less striking than that observed after pharmacological NE inhibition.

One study challenged the requirement of NE in NETosis[27] on the basis of experiments with PMA-induced mouse neutrophils that yielded low levels of NETs. By contrast, NE deficiency and inhibitors attenuated NETosis upon stimulation with the Ca^{2+} ionophore ionomycin, which induced a robust response[27]. In the same study, NE deficiency did not reduce NET-mediated thrombosis *in vivo*[27], but this result contradicts prior literature[28].

Another nuclear chromatin-binding protein that has recently been implicated in NETosis is DEK. NETosis is defective in *Dek*-deficient neutrophils and can be rescued by addition of exogenous recombinant DEK protein, which suggests that DEK binding promotes chromatin decondensation in a similar manner to MPO[29].

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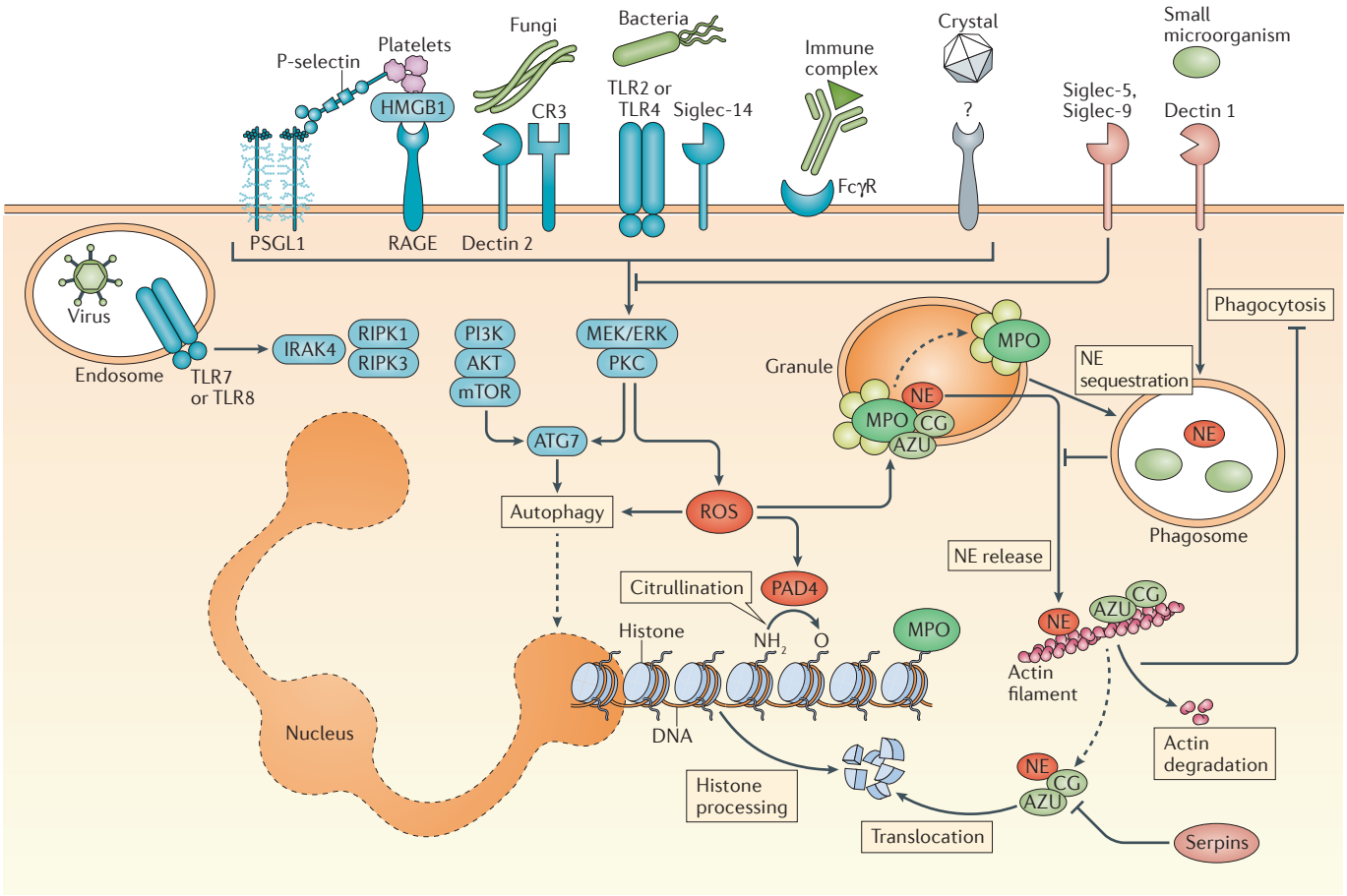

Figure 2 | **Molecular mechanisms regulating NETosis.** The formation of neutrophil extracellular traps (NETs) — known as NETosis — can be triggered by microorganisms and endogenous stimuli, such as damage-associated molecular patterns and immune complexes. Ligation of a number of receptors by bacteria, fungi, viruses, immune complexes and crystals activates NETosis through various downstream effector proteins. Activated platelets can also trigger NETosis via high mobility group protein B1 (HMGB1)–receptor for advanced glycation end products (RAGE) and P-selectin–P-selectin glycoprotein ligand 1 (PSGL1) interactions. The induction of reactive oxygen species (ROS) via MEK–extracellular-signal-regulated kinase (ERK) signalling triggers a myeloperoxidase (MPO) pathway. In this pathway, MPO-mediated oxidative activation of neutrophil elastase (NE) is required for NE to degrade the actin cytoskeleton in the cytoplasm to block phagocytosis. NE then translocates to the nucleus to drive chromatin decondensation by processing histones. Chromatin decondensation is also promoted by MPO and DEK (not shown) binding and the activation of protein-arginine deiminase type 4 (PAD4), which citrullinates histones. Autophagy is also thought to have a role in NET formation. Phagocytic receptors such as dectin 1 inhibit NETosis in response to small microorganisms by promoting phagosome formation that sequesters NE away from the nucleus. Siglec-5 and Siglec-9 suppress NETosis by limiting neutrophil activation and ROS generation. Endogenous serpin protease inhibitors block NETosis by inhibiting NE. ATG7, autophagy-related protein 7; AZU, azurophilic granule; CG, cathepsin G; CR3, complement receptor 3; IRAK, IL-1 receptor-associated kinase; MEK, MAPK/ERK kinase; mTOR, mechanistic target of rapamycin; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; RIPK1, receptor-interacting serine/threonine-protein kinase 1; TLR, Toll-like receptor.

As mentioned above, some NET stimuli, such as immune complexes, ionomycin and nicotine, have been proposed to trigger NETosis independently of NADPH oxidase, relying instead on mitochondrial ROS[2,30,31]. Non-lytic NETosis is also thought to occur independently of ROS[12]. It is therefore important to consider the effects of ROS-blocking compounds on ROS generated by both the NADPH oxidase and the mitochondria. ROS do not only trigger chromatin decondensation. Chlorinated polyamines generated upon reaction with hypochlorous acid, produced by MPO, crosslink NET proteins, increasing NET stability and integrity and potentiating the capture of micro-organisms[32]. This crosslinking reaction might explain why NET proteins persist longer than DNA following DNase I administration *in vivo*[15]. Interestingly, glycans in saliva induce NETs via an unknown mechanism that does not involve ROS or NE. These NETs are more resistant to nucleases and kill microorganisms more effectively than NETs generated with PMA[33]. Therefore, different pathways may generate NETs with different functional attributes.

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Another chromatin modification that is implicated in chromatin decondensation is histone deamination or citrullination, which is driven by protein-arginine deiminase type 4 (PAD4), a nuclear enzyme that citrullinates arginine residues, converting amine groups to ketones[34,35] (FIG. 2). Despite evidence that PAD4 activity requires a reducing environment[36], inhibition of NADPH oxidase decreases citrullination. Moreover, hydrogen peroxide is sufficient to activate PAD4 (REFS 37,38), which requires calcium[39] and is activated by PKCζ[40,41], a kinase that is implicated in the ROS burst. Together, these observations suggest that PAD4 lies downstream of ROS and calcium signalling during NETosis. The degree and specificity of citrullination seems to vary depending on the stimulus owing to the activation of different PKC isoforms that activate or suppress PAD4 (REFS 41–43). Physiological stimuli such as fungi and crystals induce histone citrullination during NETosis[7,20]. However, the contribution of citrullination to chromatin decondensation has been more difficult to evaluate[38,44,45]. Experiments with cell lines treated with PAD4 inhibitors or with mouse neutrophils derived from PAD4-deficient mice were initially difficult to interpret owing to low NET yields[38,46]. Mixed results have been reported with pharmacological PAD4 inhibition with Cl-amidine in human neutrophils known to be robust NET producers. For example, PAD4 inhibition blocks NETosis induced by nicotine but does not interfere in the formation of NETs induced by cholesterol crystals[20,31]. One complicating issue is that histone citrullination is often used as the sole marker to detect NETs in PAD4-deficient or PAD4-inhibited mice[47,48]. However, recent studies using multiple NET markers showed that PAD4 inhibition blocks NET release in mouse models of sepsis and cancer[15,21]. Moreover, PAD4-deficient mouse neutrophils fail to release NETs upon stimulation with lipopolysaccharide (LPS) and tumour necrosis factor (TNF)[43].

Whether histone citrullination is sufficient to promote chromatin decondensation in the absence of NE activity is unclear. NE inhibitors block chromatin decondensation during pulmonary fungal infection without interfering with histone H3 citrullination[7], suggesting that histone citrullination occurs independently of NE activity, but histone citrullination might not be sufficient to drive chromatin decondensation. Interestingly, recent findings suggest that the repertoire of citrullinated proteins in NETosis induced by microorganisms or PMA is dominated by histones and is distinct from extensive protein hyper-citrullination associated with stress inducers such as ionomycin, pore-forming toxins and immune complexes[42,49]. Therefore, different NET-inducing stimuli might engage PAD enzymes in diverse ways, and the pattern of citrullinated substrates could help to determine the relevant immunopathogenic mechanisms *in vivo*.

In summary, these pathways are implicated in NETosis, and their pharmacological inhibition blocks chromatin decondensation in a variety of scenarios. Nevertheless, examples of alternative mechanisms are also emerging. Furthermore, PMA and ionomycin are useful for mechanistic studies, but data obtained with these non-physiological stimuli should be viewed with caution until validated with physiological stimuli. PMA and fungi elicit common pathways downstream of ROS (TABLE 1). The ionomycin-induced pathway that involves calcium signalling and small conductance calcium-activated potassium channel protein 3 (SK3) may share features with pathways induced by immune complexes, platelets and other stimuli that elicit a faster mitochondrial ROS-dependent response with varying degrees of citrullination[30,41].

Upstream signalling pathways. The pathways that promote NETosis upstream of ROS are incompletely understood. A number of ROS-inducing receptors (BOX 1) and kinases, such as MEK (MAPK/ERK kinase), extracellular-signal-regulated kinase (ERK), IL-1 receptor-associated kinase (IRAK), PKC, phosphoinositide 3-kinase (PI3K) and AKT, have been linked to NETosis in response to PMA, microorganisms, parasites and immobilized immune complexes[4,40,50–53] (FIG. 2; TABLE 1). The requirement for PI3K in NETosis has also implicated a role for autophagy, which also depends on this enzyme[54]. Consistent with this, promyelocytes that lack the autophagy-associated protein ATG7 exhibit a modest decrease in NET release[55]. By contrast, a requirement for mechanistic target of rapamycin (mTOR), which suppresses autophagy, has also been reported in NETosis[56]. Nevertheless, LC3B$^+$ vacuoles that resemble autophagosomes have been observed in neutrophils undergoing NETosis[54,55,57]. Finally, ROS are known to induce autophagy[58], which in turn is required to sustain the ROS burst[59] and might also help to tolerate ROS-induced stress.

During NETosis, plasma membrane permeabilization occurs in a programmed manner and not as a consequence of physical disruption by the expanding chromatin[11]. This observation suggests that NETosis involves programmed cell death. Consistent with this observation, NET inducers, such as monosodium urate (MSU) crystals, promote necroptosis[60], and neutrophils lacking receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and RIPK3, two kinases involved in necroptosis, fail to form NETs without altering their ROS burst, which indicates that these enzymes act downstream or in parallel with the ROS pathway[61]. However, the role of these kinases in NETosis has been challenged by others[62], and more evidence is needed to confirm their role and mode of action.

Regulation of NETosis. NETosis must be tightly regulated to prevent pathology. The size of microorganisms is one of several factors that influence NETosis. The sensing of pathogen size depends on the competition between NETosis and phagocytosis for access to NE. This mechanism enables neutrophils to preferentially deploy NETs against large microorganisms. Small microorganisms are taken up into phagosomes that fuse with azurophilic granules, sequestering NE away from the nucleus and blocking chromatin decondensation[7] (FIG. 2). The absence of phagosomes in neutrophils that

Autophagy
An evolutionarily conserved process, in which acidic double-membrane vacuoles sequester intracellular contents (such as damaged organelles and macromolecules) and target them for degradation and recycling, through fusion with lysosomes.

Necroptosis
A form of programmed necrosis that is initiated by the kinases receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and RIPK3 in response to external signals, in conditions in which caspase 8 activity is compromised.

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Table 1 | **Cellular pathways involved in NETosis**

Stimulus	Activating receptors	Repressing receptors	Signalling intermediates I	Signalling intermediates II	Independent of	Refs
PMA	NA	NA	MEK, ERK, AKT, PI3K, mTOR, ATG7, RIPK1, RIPK3	NOX2, MPO, NE, DEK	PAD4	10,11,17,18,29,37, 40,41,45, 50,53,55,56,60
Ionomycin	ND	ND	SK3, PKCζ	mitoROS, NE, PAD4	ERK, NOX2	27,30,37,41
Fungi	Dectin 2, CR3	Dectin 1	ND	NOX2, MPO, NE	ND	7,11,17,18,22,176,177
Bacteria	TLR4, Siglec-14	Siglec-5, Siglec-9, SIRL1	ND	NOX2, MPO, NE, PAD4	ND	10,15,38,85,172
Immune complexes	FcγRIIIb	SIRL1	ND	mitoROS	NOX2	52,84,181
Crystals	ND	SIRL1	RIPK1, RIPK3	NOX2, MPO, NE	PAD4	19,20,60,61,84,85
Parasites	TLR2, TLR4	ND	ND	ND	NOX2	51,178
Viruses	TLR7, TLR8	IL-10R	ND	ND	ND	4,94
LPS and/or platelets	TLR2, TLR4, PSGL1, RAGE	ND	HMGB1	NE	NOX2	118,120,172,173
Non-lytic NETosis	ND	ND	ND	ND	NOX2	12,13

ATG7, autophagy-related protein 7; CR3, complement receptor 3; ERK, extracellular-signal-regulated kinase; HMGB1, high mobility group protein B1; LPS, lipopolysaccharide; MEK, MAPK/ERK kinase; mitoROS, mitochondrial reactive oxygen species; MPO, myeloperoxidase; mTOR, mechanistic target of rapamycin; NA, not applicable; ND, not determined; NE, neutrophil elastase; NOX2, NADPH oxidase 2; PAD4, protein-arginine deiminase type 4; PI3K, phosphoinositide 3-kinase; PKCζ, protein kinase C ζ; PMA, phorbol 12-myristate 13-acetate; PSGL1, P-selectin glycoprotein ligand 1; RAGE, receptor for advanced glycosylation end products; RIPK, receptor-interacting serine/threonine-protein kinase; SIRL1, signal inhibitory receptor on leukocytes 1; SK3, small conductance calcium-activated potassium channel protein 3; TLR, Toll-like receptor.

engage microorganisms that are too large to be ingested allows NE to translocate to the nucleus via the slower azurosome pathway and to drive NETosis. Furthermore, NE release into the cytosol promotes actin cytoskeleton degradation, blocking phagocytosis and committing cells to NETosis[11] (FIG. 2). The influence of particle size on NETosis also applies to sterile stimuli. Larger, needle-shaped urate crystals trigger NETosis more potently than urate microaggregates that are small enough to be ingested[63]. The selective induction of NETosis limits unnecessary tissue damage during infection by pathogens that are small enough to be killed intracellularly. Accordingly, mice that lack the antifungal phagocytic receptor dectin 1 are unable to selectively suppress NETosis and are susceptible to NET-mediated pathology in response to small microorganisms[7].

However, NETosis induced by small bacteria has been widely reported. Other studies even report an increase in both phagocytosis and NET formation upon bacterial opsonization by IgA[64] or disruption of the bacterial capsule[65]. Nevertheless, many of these microorganisms can survive and escape phagosomes[66]. It is therefore possible that NETosis is reserved for small virulent microorganisms that interfere with phagosomal killing. Consistent with this idea, virulent enteropathogenic bacteria induce NET formation, whereas non-virulent probiotic bacteria do not[67]. One strategy for small microorganisms to evade phagocytosis is aggregation. Large aggregates of *Mycobacterium bovis* Bacillus Calmette–Guérin drive NETosis in a microorganism size-dependent manner[7]. Similarly, *S. aureus*, which has been shown to stimulate NETosis in mouse models of sepsis[12,13], forms large abscesses and aggregates upon exposure to plasma[68,69]. Aggregation might also explain early observations of pulmonary NET induction following infection with clumps of *Klebsiella pneumoniae* grown in solid phase[17].

Alternatively, microbial interference with phagosome maturation may also enable small microorganisms to induce NETosis. *Neisseria gonorrhoeae* delays the fusion of the phagosome with azurophilic granules and induces NETosis[70]. Virulence mechanisms are also involved in the ability of *P. aeruginosa* to induce NETosis, which depends on expression of a motile flagellum[71]. Bacteria that lack flagella fail to elicit a potent ROS burst and NETosis, but flagella alone are not sufficient to induce NETosis. These findings appear to contradict the size-dependence principle. However, flagella are also known to alter host cell biology[72,73], and it will be interesting to investigate whether and how they might potentiate the translocation of NE to the nucleus. Several findings suggest that by altering neutrophil cell biology, microbial virulence factors affect NETosis[74]. Many virulent *S. aureus* serotypes kill neutrophils[75] and might promote the association of NET components by physical lysis of cellular membranes. For example, the *S. aureus* pore-forming toxin leukotoxin GH is sufficient to drive NETosis, but it is unclear whether it is required for NET induction by bacteria[76]. Moreover, expression of invasin, an adhesin that binds β-integrins, potentiates the ROS burst to induce NETosis in response to *Yersinia pseudotuberculosis*[77]. Finally, the observation that *Porphyromonas gingivalis* mutants that lack a phagocytosis-promoting protease drive NETosis[78] is also consistent with the ability of phagocytosis to regulate NETosis.

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Box 1 | **Receptors that trigger the release of NETs**

During sepsis, lipopolysaccharide (LPS) triggers platelet activation through Toll-like receptor 4 (TLR4), which promotes the association of platelets with neutrophils and drives the formation of neutrophil extracellular traps (NETs)[173]. Neutrophil–platelet interactions are mediated by P-selectin[118] and allow platelet-derived high mobility group protein B1 (HMGB1)[174] to stimulate NETosis[110,173] through binding to receptor for advanced glycation end products (RAGE)[174] — a pathway that has also been implicated in vascular inflammation and damage[175]. Interestingly, HMGB1 also signals through TLR2 and TLR4 (REFS 110,176). In contrast to dectin 1, which suppresses NETosis, dectin 2, which binds fungal cell wall mannan, promotes the association of neutrophils with fungal hyphae and the release of NETs[177]. Whether NETosis is activated by dectin 2 signalling through its Fc co-receptor or whether it acts indirectly by enhancing the activation of other receptors remains to be investigated. Although the fungal molecules and receptors that activate NETosis have not yet been identified, experiments with surfaces coated with the fungal cell wall components β-glucan and fibrinogen implicate a role for complement receptor 3 (CR3) in dectin 2-induced NETosis[178].

A role for TLR2 and TLR4 in NETosis stems from studies with the parasite *Trypanosoma cruzi* and its secreted compounds[179]. In addition, the endosomal receptors TLR7 and TLR8 mediate NETosis triggered by HIV[4]. In macrophages, cholesterol crystals activate the inflammasome through the receptor CD36 (REF. 180), which mediates apoptosis in neutrophils[181] and might be implicated in NETosis.

Immune complexes activate NETosis by engaging FcγRIIIb. By contrast, antibody-mediated FcγRIIb or integrin receptor signalling does not induce NETosis[52,182]. Less is known about the importance of antibodies in microorganism-induced NETosis. Opsonization is not necessary in fungus-induced NETosis[7], but coating *Staphylococcus aureus* or beads with IgA promotes NETosis through FcαRI signalling[64].

In addition, microorganisms attenuate NETosis by engaging host receptors that suppress neutrophil activation. Both group A streptococci (GAS) and group B streptococci (GBS) deploy molecules that resemble sialic acids to dampen the ROS burst and reduce NETosis[79,80] (FIG. 3; TABLE 1). Similarly, *P. aeruginosa* and GAS suppress NETosis through Siglec-9 by coating themselves with host sialylated glycoproteins[80,81]. Moreover, β-protein from GBS suppresses NETosis by binding to Siglec-5. However, engagement of Siglec-14 by β-protein antagonizes the repressive effects of Siglec-5 by activating mitogen-activated protein kinase (MAPK) signalling, which explains why polymorphisms that disrupt Siglec-14 increase host susceptibility to GBS[82]. Ligation of signal inhibitory receptor on leukocytes 1 (SIRL1) also attenuates NETosis by downregulating ROS production in response to *S. aureus* or MSU crystals[83–85], but the physiological role of this pathway is unclear. In a similar manner, whereas bacterial biofilms induce NET formation[86], fungal biofilms suppress NETosis by blocking ROS generation and increasing resistance to neutrophil killing[87]. The suppression of NETosis depends on mannosylation enzymes, but these enzymes are also important for fungal cell wall integrity, thereby making it difficult to attribute the virulence of these fungi solely to the suppression of NETosis[87]. Finally, the induction of immunosuppressive cytokines such as IL-10 can also inhibit NET release[4].

In summary, microorganisms modulate NETosis through diverse mechanisms, depending on their size and the expression of virulence factors.

NETs in host defence

Given that most of the proteins that are implicated in NETosis are also important for phagocytosis and cytokine regulation, it has been difficult to define the specific contribution of NETs to immune defence. The dependence of NETosis on microorganism size enabled us to study the role of NETs independently of phagocytosis. In humans, complete MPO deficiency leads predominately to recurrent fungal infections[88]. Experiments with MPO-deficient mice are also consistent with a crucial role for NETs against pathogens that are too large to be killed intracellularly, such as fungal hyphae[7]. The importance of NETs in clearing systemic fungal infection is also supported by the restoration of NETosis in a patient with CGD following gene therapy[89].

Consistent with a selective antimicrobial role for NETs, only a small number of NET-deficient patients with Papillon–Lefèvre syndrome show susceptibility to pyogenic infections, and their neutrophils have no defects in bacterial killing[23]. Moreover, the reported lack of NETosis in PAD4-deficient mice does not affect bacteraemia and survival in polymicrobial sepsis[90] or *Burkholderia pseudomallei*-induced sepsis[91]. Likewise, in the original study implicating NETs in protection against bacterial sepsis, NET degradation with DNase yielded only a twofold increase in bacteraemia following *S. aureus* skin infection[13] and reduced bacterial load at the primary site of skin infection, which was interpreted as an increase in dissemination[13]. However, in most cases, dissemination is accompanied by uncontrolled growth at the primary site of infection. However, a reduction of skin bacteria upon DNase treatment may also be caused by biofilm breakdown or disruption of NET-mediated immune evasion mechanisms of *S. aureus*[92]. By contrast, impaired killing of *Shigella flexneri* and GAS has been reported in PAD4-deficient neutrophils alongside larger lesions in a model of GAS-induced necrotizing fasciitis[38], which was attributed to defects in NETosis. Finally, many parasites trigger NETosis *in vitro* (reviewed in REF. 93), but it is unclear whether NETs offer protection against these pathogens. These studies suggest that NETs play a critical role against fungal infections and virulent bacteria that can subvert other neutrophil antimicrobial strategies.

NET release has also been observed in response to viruses such as HIV[4] and respiratory syncytial virus or syncytial viral proteins[94]. NETs trap and reduce the infectivity of HIV virions[4], but evidence for an antiviral role for NETs *in vivo* is lacking[95]. Notably, NETs were absent in mild infection with influenza virus or co-infection with *S. pneumoniae* in wild-type mice[96]. Consistently, PAD4-deficient animals do not exhibit increased susceptibility to influenza virus[95]. On the contrary, NETs are thought to mediate pathology during severe influenza virus infection in mice deficient in viral sensing pathways[97]. Under these conditions, virus-induced tissue damage results in bacterial overgrowth associated with NET release and pathology. In this study, antibiotics, DNase treatment, neutrophil depletion and inhibition of neutrophil recruitment rescued mortality. However, given that the effect of antibiotics on NETosis was not examined, it is unclear whether NETs are

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triggered directly by the microbiota or in response to host damage-associated molecular patterns (DAMPs), because alveolar epithelial cells isolated from influenza virus-infected lungs stimulate NETosis[98]. How NETs cause pathology in severe flu infection is unknown, but NETs have been associated with increased inflammation in pulmonary Sendai virus infections[25].

It is therefore evident that although NETs may be critical against specific infections, NET-driven pathology affects host survival. The molecular basis for the antimicrobial capacity of NETs is not well understood but is summarized along with several microbial NET countermeasures in BOX 2 and FIG. 3.

NETs in disease

In addition to recent advances that point to a specialized immune-protective function of NETs, the list of conditions in which NETs cause pathology is continuously expanding. This section focuses on the mechanisms of NET-mediated pathology and the medical conditions in which they are implicated (FIG. 4).

NETs damage tissues. The ability of NETs to damage tissues is well documented in infection and sterile disease. NETs directly kill epithelial and endothelial cells[99,100], and excessive NETosis damages the epithelium in pulmonary fungal infection[7] and the endothelium in transfusion-related acute lung injury[101]. NETs are also linked to hepatic damage during sepsis with methicillin-resistant *S. aureus,* and this damage can be averted by NE or PAD4 deficiency[15]. Together with the poor protection NETs offer, these observations suggest that NETs have a predominately detrimental role in bacterial sepsis[13].

In sepsis and acute injury, free circulating histones are cytotoxic, owing to their ability to compromise cell membrane integrity[102,103]. Antibody-mediated neutralization studies suggest that NET-bound histones have a central role in NET-mediated cytotoxicity[99], although neutrophils and NETs do not seem to be a direct source of free histones in sepsis[104]. Other NET proteins, such as defensins, permeabilize eukaryotic cells[105,106], and NE targets extracellular matrix proteins that disrupt cell junctions[107]. DNA binding downregulates the proteolytic activity of NE, but it also shields the enzyme from complete inhibition by endogenous serpin protease inhibitors[17,108]. Therefore, the interaction with DNA may alter the properties of these factors and should be taken into account when considering the effects of therapeutic DNases.

Interestingly, DNase treatment abrogates NET-mediated cytotoxicity of epithelial and endothelial cells *in vitro*[99]. However, *in vivo*, DNase fails to immediately dislodge NET proteins and is less effective in blocking acute tissue pathology during systemic infection[15]. By contrast, DNase is effective in blocking NET pathology during chronic inflammation[20]. Likewise, DNase treatment and, to a similar extent, PAD4 inhibition reduce NET-associated citrullinated histones and minimize immune cell recruitment[109] and liver damage associated with ischaemia–reperfusion injury[110].

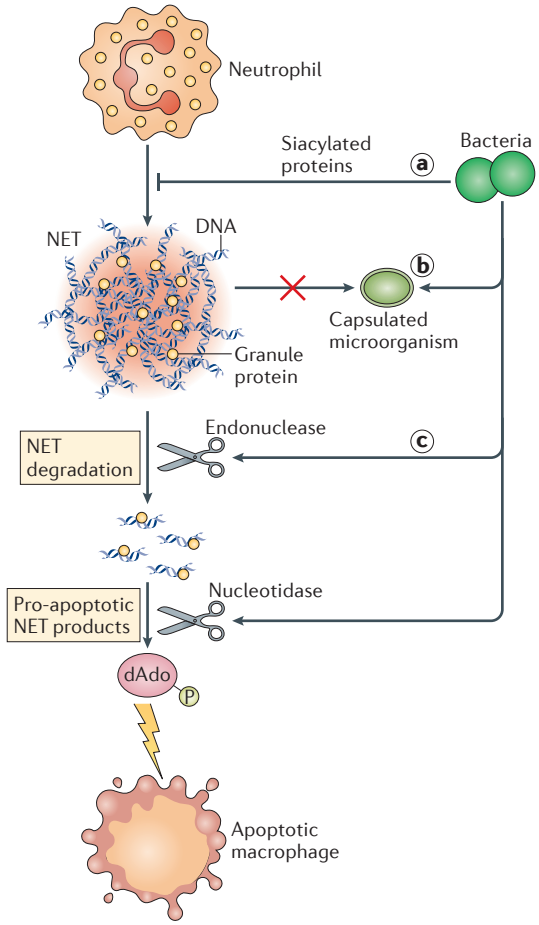

Figure 3 | **NET evasion mechanisms.** Microorganisms can evade neutrophil extracellular traps (NETs) through three known mechanisms: by inhibiting NET formation (part **a**), by coating themselves with a capsule that reduces their avidity to NETs and makes them more resistant to NET-mediated killing (part **b**) and by secreting endonucleases that degrade NETs (part **c**). Specialized microbial enzymes can also convert the NET-derived products into cytotoxic molecules that kill immune cells. *Staphylococcus aureus* adenosine synthase (a 5′, 3′-nucleotidase) converts NET-derived nucleotides into deoxyadenosine (dAdo), which triggers apoptosis, eliminating macrophages in close proximity to abscesses.

The ability of NETs to damage tissues may explain the role of PAD4 in promoting age-related fibrosis[111]. Furthermore, NET-mediated damage might enhance rather than limit certain infections during chronic inflammation. NETs are prominent in the sputum of patients with cystic fibrosis[112]. The lungs of patients with cystic fibrosis are often colonized with *P. aeruginosa,* a microorganism that has evolved sophisticated strategies to overcome lung barrier function. It is not known what triggers NET formation in cystic fibrosis, but *P. aeruginosa* is a potent inducer of NETosis, and although NETs eliminate laboratory strains, clinical isolates of this pathogen are resistant to NET-mediated killing[74,113]. These bacteria might benefit from the destructive capacity of NETs, which might contribute to the generation of microbial niches in fibrotic areas of the lungs.

Damage-associated molecular patterns
(DAMPs). Conserved mammalian motifs, recognized by pattern recognition receptors, that are broadly upregulated in response to cellular stress and that trigger an innate immune response. Examples include heat shock proteins, high mobility group protein B1 (HMGB1), DNA-binding proteins and uric acid.

Cystic fibrosis
An autosomal recessive genetic condition secondary to mutations in the cystic fibrosis transmembrane conductance regulator (a chloride channel), causing lung, gastrointestinal, endocrine and fertility complications. Chronic infection of the lungs is associated with sputum that is rich in neutrophil proteins and DNA..

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Box 2 | **NET-mediated control of microorganisms and microorganism evasion strategies**

Neutrophil extracellular traps (NETs) kill or suppress fungal and bacterial proliferation, but the underlying mechanisms are poorly understood. Histones, defensins and cathelicidins are potent antimicrobials in NETs, but their role in NET-mediated microbial killing has not yet been defined[106,183]. NETosis is also likely to be the major route for the release of calprotectin[8], a metal chelator protein that protects against fungal infection. Neutralization of calprotectin attenuates the antifungal activity of NETs *in vitro*[184], but whether it protects against other microorganisms and synergizes with other antimicrobials is unclear.

The physical sequestration of microorganisms by NETs is also thought to prevent systemic dissemination[6,7,173]. In myeloperoxidase-deficient mice, the absence of NETs is associated with fungal dissemination, a phenotype that is not observed upon infection with yeast-locked mutants that can be killed by phagocytosis. Further support that NETs block microbial dissemination is based on the finding that bacterial strains with mutations in a NET-degrading nuclease are unable to disseminate[6], but it is difficult to rule out an alternative mechanism that involves the degradation of bacterial biofilms. Bacterial endonucleases degrade preformed NETs, and the bacterial capsule reduces NET trapping *in vitro*, but these results may have a different aetiology if NETs are not strongly induced by these microorganisms *in vivo*[6,185–188].

Surprisingly, bacteria may even use NETs to their own advantage. For example, *Staphylococcus aureus* expresses two nucleases that convert NETs into pro-apoptotic nucleotides and promote macrophage killing around abscesses[92]. This is an intriguing hypothesis, but evidence of NETs forming around these abscesses is lacking.

The tissue-damaging capacity of NETs explains why their release is likely to be restricted to infections that cannot be cleared through less harmful strategies.

NETs promote vaso-occlusion. Another detrimental function of NETs is occlusion of the vasculature. NETs that form in the circulation provide a scaffold that promotes deep vein thrombosis (DVT)[114], a condition that is more prominent in patients with cancer and obesity. A functional role for NETs in thrombosis is supported by the finding that DNase treatment and PAD4 inhibitors block DVT in mice[115,116]. Also consistent with a role for NETs in thrombosis are the observations that patients with acute thrombosis exhibit lower levels of plasma DNase I activity[117] and that thrombosis in mouse models is attenuated in NE-deficient mice[28].

NET formation during thrombosis is thought to be initiated by hypoxia-induced release of von Willebrand factor (VWF) and P-selectin from the endothelium that recruits and activates neutrophils for NETosis[114,118]. Neutrophils accumulate in the vasculature in a P-selectin-dependent manner, and this is followed by platelet recruitment. Neutrophils promote thromboxane A2 production by platelets, which induces endothelial cell expression of intercellular adhesion molecule 1 (ICAM1) to strengthen neutrophil interactions with the endothelium[119]. This process triggers NETosis through a mechanism involving platelet-derived high mobility group protein B1 (HMGB1), ROS and integrins[118,120].

In addition to providing a scaffold, NETs contribute to thrombosis through other means. NETs recruit Factor XIIa, a protein that promotes coagulation and mobilizes endothelial cell granules known as Weibel–Palade bodies that contain VWF, P-selectin[121] and Factor XIIa[120]. Extracellular NET histones bind VWF and fibrin[122] to recruit platelets and red blood cells[114,115]. In addition, NET-bound NE cleaves tissue factor pathway inhibitor (TFPI), a factor that inhibits coagulation[28], and proteolytically activates platelet receptors to increase platelet accumulation[123]. However, a recent study contradicts these findings by showing that NE inhibitors and NE deficiency failed to reduce DVT[27]. Therefore, this issue remains unresolved.

Moreover, NETs released through alternative mechanisms promote vascular pathology in other conditions. For example, NETs form in response to the build-up of bicarbonate salts and occlude pancreatic ducts to drive pancreatitis[124]. Likewise, NETosis in response to free haem may contribute to vaso-occlusion in sickle cell disease[125].

NETs modulate sterile inflammation. In addition to their antimicrobial capacity, NETs regulate inflammatory cytokines directly or indirectly by modulating other immune cells. During the early inflammatory stages of atherosclerosis, microscopic cholesterol crystals[126] induce NETs that turn on the transcription of genes encoding IL-6 and pro-IL-1β in macrophages, predominately via Toll-like receptor 2 (TLR2) and TLR4 (REF. 20). The upregulation of these cytokines augments T helper 17 cell differentiation and increases myeloid cell recruitment to atherosclerotic lesions[20]. Accordingly, mice deficient in the neutrophil proteases required for NETosis or treated with PAD4 inhibitors have lower inflammation and develop smaller atherosclerotic lesions[20,48]. Neutrophil serine proteases are also known to process pro-IL-1β into its active form[127]. However, caspase 1-mediated cytokine processing is critical in atherosclerosis[126], and protease deficiency or the administration of DNase attenuates cytokine transcription in lesions, suggesting that these proteases regulate inflammation transcriptionally in a NET-dependent way[20]. MPO-deficient individuals are also protected against atherosclerosis[88], but MPO is known to promote disease via low-density lipoprotein (LDL) oxidation and other mechanisms.

The pro-inflammatory role of NETs has also been documented in a mouse model of ischaemia–reperfusion injury in which NETs amplify inflammation and liver damage that can be markedly reduced with DNase treatment or PAD4 inhibitors[110]. NETs are thought to form in response to extracellular HMGB1 and histones in a

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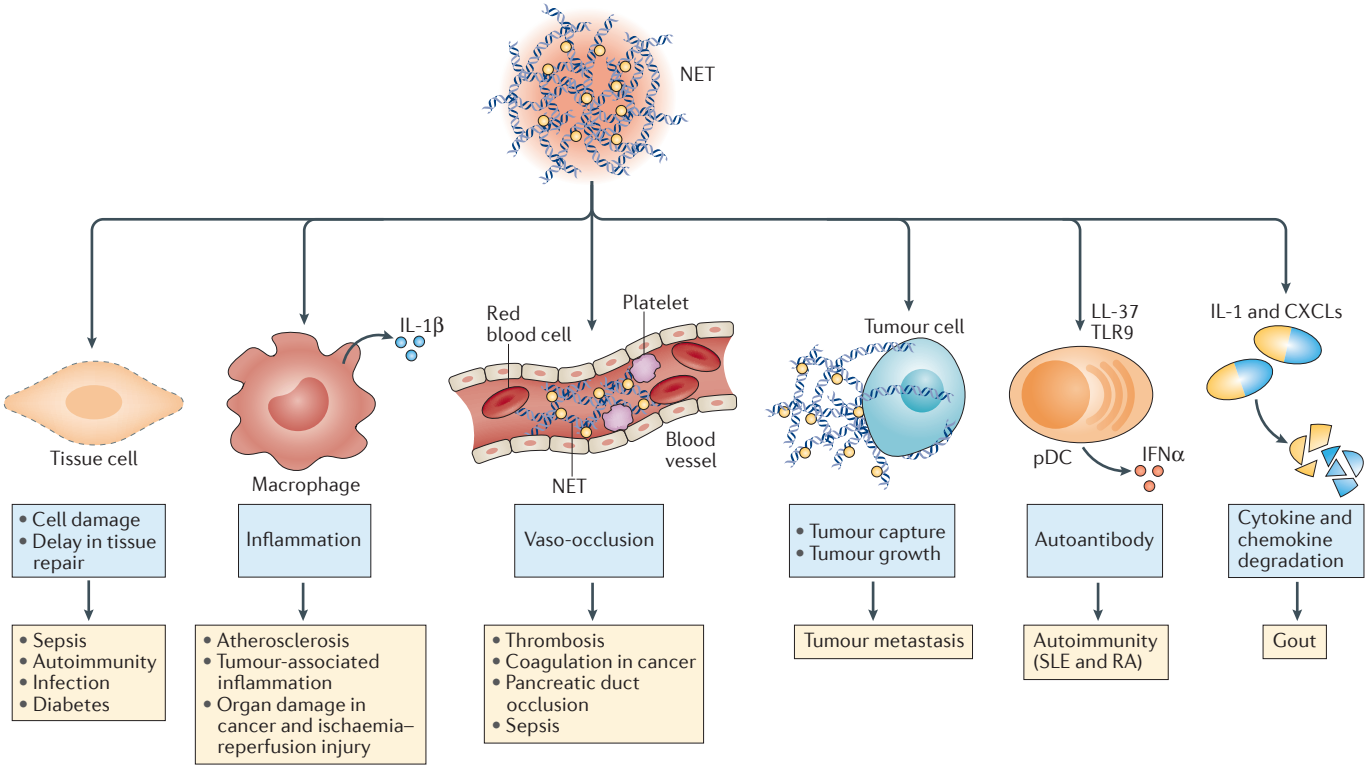

Figure 4 | **Mechanisms of NET-mediated pathology.** Neutrophil extracellular traps (NETs) cause pathology in a number of conditions through several mechanisms. Direct cell damage is implicated in infection, sepsis, autoimmunity and diabetes. By licensing macrophages for inflammation, NETs drive atherosclerosis. The increased propensity for NETosis promotes inflammation and organ damage in cancer and ischaemia–reperfusion injury. NET formation in the circulation promotes coagulation, vascular occlusion and thrombosis. NETs in capillaries can also capture and, potentially through other mechanisms, promote tumour metastasis. Finally, although NETs can promote inflammation, an accumulation of NETs promotes the resolution of inflammation through the degradation of cytokines and chemokines. CXCLs, CXC-chemokine ligands; IFNα, interferon-α; pDC, plasmacytoid dendritic cell; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; TLR9, Toll-like receptor 9.

TLR4- and TLR9-dependent manner, and mice deficient in these receptors have reduced liver pathology. Therefore, by inducing NETosis, endogenous danger signals are sufficient to initiate inflammation in the absence of microbial priming cues.

NETs and neutrophil-derived IL-17 have also been observed in the brain in mouse models of Alzheimer disease in which neutrophil depletion or blockade of neutrophil recruitment improved cognitive performance[128]. However, whether and how NETs contribute to neuronal degeneration has to be further evaluated.

In contrast to this pathogenic role, NETs were suggested to have an anti-inflammatory role in mouse models of gout induced by MSU crystals[19]. The functional significance of NETs was demonstrated by showing that ROS-deficient mice that do not release NETs exhibit more inflammation and gouty arthritis. This phenotype was rescued by injecting *in vitro*-generated preparations of aggregated NETs that degrade pro-inflammatory cytokines and chemokines through NET-bound proteases[19]. On the basis of these findings, the authors proposed that the local concentration of NETs might influence their effect on inflammation, with a high density of NETs suppressing inflammation. This idea requires further validation. It is important to remember that ROS directly suppress inflammation independently of their role in NETosis[129], and it is unclear whether sufficient NET concentrations are achieved to suppress inflammation under physiological conditions. Moreover, a recent report confirmed that NETs from human but not mouse neutrophils degrade pro-inflammatory cytokines, but there was no evidence for increased inflammation upon neutrophil depletion in a mouse model of gouty arthritis[130]. However, the method used to deplete neutrophils in that study (GR1-specific antibody) also targets other myeloid cells and may cancel out competing responses. The species-specific differences might reflect known differences in protease specificity. Moreover, the suppressive association with DNA might explain the need for high NET concentrations. Nevertheless, studies examining NETosis in response to synthetic nanoparticles are consistent with a dynamic process in which NETs promote the onset of inflammation but also speed up its resolution in arthritis and air pouch models[131]. Therefore, it is possible that NETs initiate inflammation and, as they build up over time, potentiate its resolution.

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Given that the acute phase of inflammation suppresses tissue repair, the ability of NETs to promote inflammation and tissue destruction may explain their ability to delay wound healing in patients with diabetes[47]. Glucose is required for NETosis, and neutrophils from patients with diabetes release NETs more readily[47,132,133]. This is likely to be linked to an increase in the ROS burst caused by the effects of elevated glucose on NADPH oxidase and the mitochondria[134,135]. The role of glucose in NETosis is also consistent with a possible contribution of the mTOR pathway[56]. However, others reported that higher glucose levels suppress NETosis[136]. Likewise, both higher and lower serum concentrations of NET proteases have been associated with type 1 diabetes[137,138]. Nevertheless, skin injury triggers neutrophil infiltration[139] and NETosis through unknown mechanisms. The increase in NET deposition in skin wounds of diabetic mice reduces healing rates and normal healing rates can be restored by PAD4 deficiency[47]. How NETs obstruct wound healing is unknown, but the process might involve tissue damage or the modulation of inflammation and the downregulation of tissue repair mechanisms[140]. Finally, NET-driven inflammation may also contribute to type 1 diabetes and obesity. NETs were recently detected in adipose tissues of obese mice, and although PAD4 inhibition did not affect inflammation[141], NE deficiency is associated with lower inflammation and lower insulin resistance in mice fed a high-fat diet[142].

It is therefore important to remember that neutrophils can modulate inflammation through NET-dependent and NET-independent mechanisms in a context-specific manner. NET-independent mechanisms seem to be more prevalent during infection[143], whereas with few exceptions[144], NET-dependent regulation of inflammation has so far been documented predominately in sterile conditions of inflammation.

NETs in autoimmunity. Following their discovery, NETs were proposed to serve as a source of self-antigen in autoimmune diseases, particularly those associated with autoantibodies against neutrophil-derived proteins. Evidence of NET deposition was first reported in kidney biopsy samples from patients with antineutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis, who generate antibodies against NET components such as MPO and proteinase 3 (REFS 57,145). Similarly, NET components have been detected in the synovial fluid of patients with rheumatoid arthritis (RA) in which antibodies against citrullinated proteins are prevalent[146]. In many autoimmune conditions, a small population of circulating low-density granulocytes (LDGs) releases NETs spontaneously, as first shown in systemic lupus erythematosus (SLE) and RA[146,147]. Even neutrophils with normal density produce NETs in response to sera from these patients or purified SLE antiribonucleoprotein antibodies in a manner that requires priming by type I interferons (IFNs) and depends on ROS[147]. Consistently, a type I IFN-driven gene expression signature is a hallmark of SLE neutrophils[147,148]. NETs induced by these autoimmune stimuli activate plasmacytoid dendritic cells (pDCs) via TLR9 and TLR7 signalling, which promotes type I IFN expression and drives autoimmune pathology in animal models[148,149]. When pDCs are loaded with NETs and injected into mice, they induce the generation of neutrophil-specific autoantibodies and renal damage[150]. The ability of NETs to activate pDCs is attributed to the association of DNA with the antimicrobial peptide LL-37, which potentiates the activation of the DNA receptor TLR9 (REF. 151). The feedback between NETs and type I IFNs provides a model to explain how NETs exacerbate autoimmune pathology. Therefore, it would be interesting to examine whether spontaneous NETosis is abrogated in ongoing clinical trials that target type I IFN signalling. Moreover, patients with severe SLE symptoms tend to have defects in degrading NETs, owing to either low activity of DNase I or the presence of other factors such as antibodies that protect NETs from degradation[14]. However, these mechanisms might also reduce the clearance of non-NET DNA.

The role of NETs in autoimmunity was challenged by the observation of exacerbated symptoms in NADPH oxidase-deficient mice. These data highlight the importance of NET-independent roles of NOX2-derived ROS in immune suppression[152,153]. This inconsistency was resolved by recent work showing that NETosis triggered by ribonucleoprotein-containing immune complexes depends on mitochondrial ROS rather than ROS generated by NADPH oxidase[2]. Mitochondrial ROS oxidize NET DNA to increase its ability to activate the stimulator of interferon genes (STING) pathway and trigger IFN production by pDCs[2,154]. Compared with reduced DNA, oxidized DNA is also more resilient to nuclease degradation[154]. Consistent with this, NETs that are generated spontaneously by neutrophils from patients with SLE are more oxidized and more immunogenic than NETs from healthy neutrophils. Furthermore, blocking mitochondrial ROS with mitoTEMPO or PAD4 inhibitors ameliorated autoimmunity and vascular complications in the lupus-prone MRL/lpr and New Zealand mixed 2328 mouse models[155,156]. Nevertheless, strong evidence for NET deposition in these mice is still lacking.

Furthermore, the evidence that links NET immunogenicity exclusively to oxidized DNA of mitochondrial origin is inconclusive, as mitochondrial ROS also oxidize the much more abundant NET genomic DNA[2]. Genomic and mitochondrial DNA oxidation was compared at a single locus, overestimating the amount of oxidized mitochondrial DNA[2]. Nevertheless, DNA oxidation is an important modification in NETs that potentiates type I IFN induction and autoimmunity with a surprising specificity with regard to the source of the DNA and ROS. Moreover, the release of NET-associated DNA during rhinovirus infection was recently shown to potentiate type 2 T cell responses and contribute to the exacerbation of allergic asthma[144]. Rhinovirus does not infect neutrophils, suggesting that NETs are released via indirect mechanisms in response to infection, as seen during acute infection with influenza virus[97].

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Additional evidence for the role of NETs in RA stems from studies that blocked NETosis and protein citrullination by *Dek* deficiency or by treatment with DEK-targeting DNA aptamers. These interventions reduced inflammation and symptoms in the joints of mice with zymozan-induced arthritis[29]. Similarly, compared with wild-type mice, PAD4-deficient mice exhibit reduced arthritis severity, autoantibody titres and inflammation in RA induced by immunization with glucose-6-phosphate isomerase (GPI)[157]. Furthermore, PAD4 inhibition ameliorates symptoms in collagen-induced arthritis[158]. By contrast, PAD4 deficiency did not ameliorate spontaneous arthritis in the K/BxN mouse model, which is neutrophil-dependent and mediated by antibodies against GPI[159], suggesting that these models involve different effector mechanisms. Interestingly, the synovial fluid of human arthritic joints contains a large number of citrullinated proteins, which is consistent with the extensive citrullination observed in neutrophils treated with T cell-derived pore-forming toxins rather than the predominant histone citrullination associated with NETosis[42]. Moreover, protein citrullination is mediated by PAD2 rather than PAD4 in a TNF-induced model of arthritis. These data point to distinct pathogenic mechanisms that involve NET-dependent and NET-independent pathways driving different mouse models of arthritis and potentially clinical arthritis.

Neutrophils are also prominent in psoriatic skin lesions, but their contribution to disease is poorly understood. Interestingly, sera from patients with psoriasis trigger NETosis, and NETs have been observed in imiquimod-induced mouse models of this disease[160]. Psoriatic symptoms are exacerbated in the absence of secretory leukocyte protease inhibitor (SLPI), which inhibits NETosis, suggesting that NETs contribute to pathology possibly by amplifying inflammation and damaging tissues[108]. However, SLPI may also act through NET-independent mechanisms by inhibiting proteolytic cytokine maturation[161,162].

Therefore, neutrophils modulate T cell responses through NETs in varying contexts. It is still possible that genetic and pharmacological interventions that link NETs to autoimmunity in animal models function through NET-independent mechanisms. Although there is mounting evidence to implicate NETs in autoimmunity, determining the importance of these NET-mediated mechanisms in human disease will likely require clinical studies on specific NET-blocking therapies.

NETs in cancer. Neutrophils affect cancer through multiple mechanisms, and evidence for a role of NETs is also emerging. Tumours have systemic effects that modulate NETosis, causing NET-associated complications in cancer. Granulocyte colony-stimulating factor (G-CSF) expression is upregulated in many cancers, and this in turn increases systemic NETosis[163]. Furthermore, intestinal tumours disrupt the intestinal barrier, allowing LPS to leak into the circulation. By activating the alternative complement pathway, LPS drives the conversion of neutrophils into NETotic LDGs that promote coagulation[164]. These findings provide a mechanistic link between cancer and the risk of thrombosis. The association of neutrophils with platelets in the kidneys of mammary carcinoma-bearing MMTV–PyMT mice has also been proposed to drive NETosis and IL-1β-mediated inflammation causing kidney failure[165]. Administration of DNase reduced the association of neutrophils with platelets, but evidence for NETs in the damaged kidneys is weak. However, NETs have been detected in pancreatic carcinomas, and NET deposition can be prevented by blocking neutrophil infiltration through deficiency in receptor for advanced glycation end products (RAGE), which mediates platelet–neutrophil interactions[166], or by administration of the autophagy inhibitor chloroquine[165]. Consistently, DNase I treatment can reduce inflammation and pancreatic tumour growth[167].

NETs that form in the necrotic core of subcutaneous G-CSF-secreting Lewis lung carcinoma tumours might also promote tumour growth, as these tumours grow slower in PAD4-deficient mice[168]. NE also promotes adenocarcinoma growth in a mouse lung cancer model, but thus far, this has been attributed to a NET-independent mechanism[169].

Finally, neutrophils promote metastasis through NET-independent mechanisms[170,171], but NETs have also been implicated in this process. Notably, mammary tumour cell lines trigger NETosis *in vitro*, but strong evidence for NET formation in these tumours *in vivo* is still lacking[172]. However, the administration of DNase I crosslinked to nanoparticles to prolong its half-life inhibits lung metastasis of aggressive mammary tumours[172]. One underlying mechanism for this inhibition is thought to be the NET-mediated capture of migrating tumour cells, especially at sites of inflammation, which can be blocked with NE and PAD4 inhibitors[21]. Therefore, targeting NETs through these avenues may be a promising therapeutic option to treat cancer.

Conclusion

Early on, the ability of NETs to trap microorganisms generated much enthusiasm, but it is their pathogenic potential that has attracted recent attention. However, it might turn out that their immune-modulatory properties have yet unknown beneficial roles in immune defence. Several factors determine whether NETs are beneficial or detrimental. Constitutive activation, dysregulation of suppressive mechanisms and excess NET yield are prominent pathogenic mechanisms that are likely to contribute to disease. Therefore, dose is a critical factor, as is the temporal regulation of NET release and clearance. In autoimmunity, aberrant NETosis may result from a breakdown in adaptive tolerance, but NETs seem to play a primary role in inflammatory disease pathogenesis. The capacity of NETs to potentiate or suppress inflammation may have a beneficial function in sterile disease and other yet unknown circumstances. Given the multitude of NET proteins, novel NET functions are likely to emerge. A better understanding of the functions and impact of NETs on health will enable the suppression of detrimental attributes without interfering with beneficial ones and ultimately allow us to exploit NETs to treat disease.

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