Neutrophil extracellular traps and their role in health and disease

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The human innate immune system is indispensable for protection against potentially invasive microbial and viral pathogens, either neutralising them or containing their spread until effective mobilisation of the slower, adaptive (specific), immune response. Until fairly recently, it was believed that the human innate immune system possessed minimal discriminatory activity in the setting of a rather limited range of microbicidal or virucidal mechanisms. However, recent discoveries have revealed that the innate immune system possesses an array of novel pathogen recognition mechanisms, as well as a resourceful and effective alternative mechanism of phagocyte (predominantly neutrophil)-mediated, anti-infective activity known as NETosis. The process of NETosis involves an unusual type of programmed, purposeful cell death, resulting in the extracellular release of a web of chromatin heavily impregnated with antimicrobial proteins. These structures, known as neutrophil extracellular traps (NETs), immobilise and contribute to the eradication of microbial pathogens, ensuring that the anti-infective potential of neutrophils is sustained beyond the lifespan of these cells. The current review is focused on the mechanisms of NETosis and the role of this process in host defence. Other topics reviewed include the potential threats to human health posed by poorly controlled, excessive formation of NETs, specifically in relation to development of autoimmune and cardiovascular diseases, as well as exacerbation of acute and chronic inflammatory disorders of the airways.

Introduction

Until fairly recently, the protective activities of the human innate immune system, which are highly conserved throughout evolution, were thought to be achieved by a limited range of mechanisms with minimal discriminatory potential. Predominant amongst these mechanisms are the physical barriers presented by the skin and mucous membranes, engulfment and destruction of microbial and viral pathogens by resident and migratory phagocytes, and the non-specific antimicrobial activity of various blood and tissue proteins. These mechanisms either prevent infection or, in the case of a breach by a pathogen, contain the infection until adaptive (specific) host defences are effectively mobilised. Over the last decade, however, a number of significant discoveries have revealed that the human innate immune system not only possesses a level of discrimination previously considered improbable, but also includes additional, resourceful mechanisms of phagocyte-mediated antimicrobial and antiviral activity.

With respect to pathogen detection, cells of the innate immune system (phagocytes, mast cells, basophils and dendritic cells), as well as epithelial cells, have been found to possess various types of pathogen recognition receptors which recognise conserved molecular structures broadly expressed on or in microbial and viral pathogens. These receptors include the Toll-like receptors, the nucleotide oligomerisation domain-like receptors, and the abundant cytosolic microbial and viral nucleic acid sensors, activation of which initiates a potentially protective inflammatory response. These receptors have been the subject of several recent reviews.[1,2]

In 2004, Brinkmann et al.[3] described an unusual mechanism by which human blood neutrophils immobilise pathogens extracellularly, exposing them to a highly concentrated array of anti-infective proteins. Neutrophils (also known as granulocytes or polymorphonuclear leucocytes) are the predominant small circulating phagocytes. These cells have an estimated lifespan of 5.4 days in the circulation,[4] which is longer when they are exposed to anti-apoptotic cytokines. These cells exit the circulation via transendothelial migration and chemotaxis to sites of microbial and viral infection where they phagocytose and destroy pathogens via intracellular exposure to microbicidal and virucidal reactive oxygen species (ROS), proteases and proteins.

The studies of Brinkmann et al.[3] enhanced the body of knowledge on neutrophil function through the discovery that these cells also respond to infectious challenges via the formation of neutrophil extracellular traps (NETs). NETs are web-like structures composed of decondensed chromatin heavily impregnated with different antimicrobial granular proteins which capture, neutralise and kill a variety of pathogens. NETs are produced predominantly by neutrophils, but also by other cell types of the innate immune system such as monocytes and macrophages, eosinophils, basophils and mast cells, in which the process is termed ETosis. Phylogenetic studies have revealed that ETosis is a highly conserved 'ancient defence weapon, predating the evolution of the coelom' operative in haemocytes, the phagocytic cells of invertebrates.[5]

NETs form large extracellular barriers to bacterial dissemination, and provide a mechanism for localised concentration of effector molecules. Importantly, NET formation has been demonstrated in both the clinical and experimental infection settings using immunohistochemistry and spinning disc vital microscopy.[6] Several strategies to measure NET formation in vitro have also been described, including immunofluorescence and electron microscopic procedures, as well as spectrofluorimetric and other methods which detect extracellular DNA and associated granule proteins.[3] Human neutrophils undergoing NETosis in vitro are shown in Figure 1.

NETs are the topic of this review, which is focused on mechanisms of NETosis and the role of this process in host defence, as well as on the potentially harmful consequences of excessive NETosis for the host and possible pharmacological control strategies.

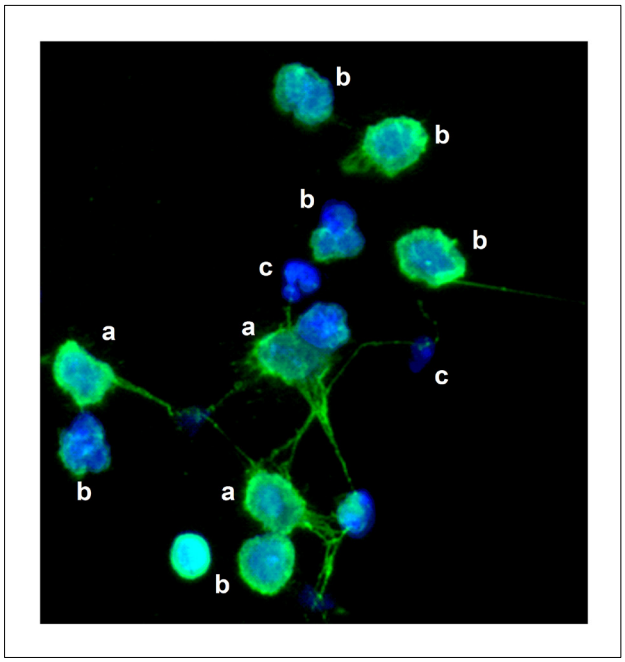

Figure 1: Fluorescence micrograph showing isolated human neutrophils undergoing NETosis. Following activation with phorbol myristate acetate (6.25 ng/mL), a potent stimulator of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and the production of reactive oxygen species, adherent neutrophils were exposed, sequentially, to unlabelled polyclonal rabbit antibodies, to citrullinated histone H3 residues and to Alexa Fluor 488 labelled goat anti-rabbit antibodies, before DNA was stained with a nuclear dye (DAPI). DNA is stained blue, while neutrophil extracellular traps (NETs), containing both DNA and citrullinated histone residues, are stained green. Neutrophils which have undergone NETosis, cells in the early stages of NETosis, and a non-NETotic neutrophil showing intact, multi-lobed nuclear morphology (stained blue), are labelled a, b and c, respectively.

Mechanisms of NET formation

Although the exact molecular and biochemical mechanisms involved in the formation and release of NETs are incompletely understood, three different types of NETotic pathway have been described. The best characterised of these pathways is slow and leads to lytic cell death over the course of 2–3 h. The second pathway has been described as a rapid mechanism (vital), independent of cell lysis, which requires the rapid (within minutes) vesicular release of neutrophil nuclear contents.[6,7] Unlike the first two mechanisms of NETosis which involve release of nuclear DNA, the third mechanism, which is also non-lytic, involves the release of mitochondrial DNA.[8]

Lytic NETosis, also known as suicidal NETosis, is an active cell death related process distinct from either necrosis or apoptosis.[9] This process requires chromatin decondensation, nuclear envelope disintegration and a mixing of nucleic acids and granule proteins within an intracellular vacuole. Subsequent release of vacuole contents into the extracellular domain is preceded by plasma membrane perforation or lysis. Various stages in the process constituting lytic NETosis have been identified. The interaction of pathogens and their products with their counter-receptors on or in neutrophils includes, in addition to the pathogen recognition receptors mentioned above, the opsonin receptors FcR (receptor for pathogen-bound immunoglobulin G) and CR3 (receptors for pathogen-bound complement components C3b and C3bi), which promote adherence of pathogens to neutrophils. This interaction, in turn, leads to a series of pathogen-activated intracellular signalling events, most importantly: (1) activation of the receptor-linked signalling complex protein kinase C(PKC)-raf/-MEK-ERK which mediates activation of the neutrophil membrane-associated, electron-transporting, ROS-generating system, NADPH oxidase[10]; (2) ROS-mediated activation of intracellular signalling pathways converging on the cytosolic transcription factor, nuclear factor kappa B (NFκB), which, following nuclear translocation, has been proposed to promote NETosis by initiating transcription of the peptidylarginine deiminase 4 (PAD4) gene, as well as by blocking apoptosis, an alternative pathway of programmed cell death[11]; and (3) receptor-mediated increases in cytosolic Ca^{2+} via activation of phospholipase C, which, in turn, leads to Ca^{2+}-dependent activation of PAD4. ROS also promote collapse of both the cytoplasmic granule and nuclear membranes, enabling access of cytoplasmic PAD4 and granule proteins to chromatin. PAD4 mediates histone hypercitrullination (conversion of protein arginine residues to citrulline), a key event in chromatin decondensation[12,13], which is facilitated by the limited proteolysis of nuclear histones mediated by granule enzymes – neutrophil elastase and myeloperoxidase (MPO) – operating in unison[3,14]. While ROS appear to promote the rupture of both the cytosolic granule and nuclear membranes, a recent study has also implicated the involvement of the granule antimicrobial polypeptide LL-37 (which consists of 2 N-terminal leucines and a total of 37 amino acids).[15] LL-37 is a cationic amphiphilic polypeptide of the cathelicidin family which binds to anionic membrane phospholipids promoting membrane disruption. The nuclear membrane appears particularly vulnerable to the disruptive actions of LL-37. In addition, the cationic properties of LL-37 also promote binding of the polypeptide to neutrophil DNA, increasing resistance to degradation of NETs by microbial nucleases.[16]

The proposed mechanism of lytic NET formation based on current knowledge is shown in Figure 2.

The concept that alternative NETosis pathways exist, in addition to the lytic-cell death pathway, has been advanced by several groups.[6,8,17,18] One such pathway is vital NETosis. Vital NETosis is a rapid process whereby cell viability and function are retained in the context of controlled, incremental discharge of nuclear material, following exposure of neutrophils to various microorganisms and their products, and appears to be a generalised response against various classes of microbial pathogens.[18,19] In this setting, neutrophils are stimulated to 'release NETs via nuclear envelope bleb formation and vesicular exportation, preserving the integrity of the plasma membrane'.[6,20] Importantly, and also in contradistinction to lytic NETosis, the requirement for involvement of NADPH oxidase in vital NETosis is variable, with increases in cytosolic calcium seemingly adequate in the case of some stimuli such as bacterial pore-forming toxins[6] and calcium ionophores[7], or alternatively via ROS-independent activation of NFκB[21]. Unlike lytic NETosis, only 20–25% of the neutrophil population undergoes NETosis on exposure to microbial pathogens or their products in vitro.[20] This observation is not only consistent with the existence of a sub-population of neutrophils highly specialised for the performance of vital NETosis, but also suggests that vital, as opposed to lytic, NETosis is the more physiologically relevant of the two processes.[20]

The formation of mitochondrial-derived NETs is also a type of vital NETosis with variable dependence on activation of NADPH oxidase according to the nature of the cell activator. Although the existence of mitochondrial DNA-containing NETs has been demonstrated in vitro, less is known about its role in host defence which is likely to be limited by the absence of histones.[22,23] Interestingly, mitochondrial DNA has been found to induce NET formation, consistent with a role in the amplification of NETosis.[22]

NET constituents

The DNA scaffolding of NETs is provided by decondensed nuclear chromatin. A variety of NET-associated proteins, predominantly highly negatively charged histones, is arranged on this scaffolding. Via their strong positive charge, histones facilitate the adhesion to, as well as the sequestration of, microbial and viral pathogens[24] and also confer bactericidal activity, as does DNA.[25] The remaining NET-associated proteins comprise granule, cytoplasmic and cytoskeletal proteins, as well as metabolic enzymes.[24] The concept of a core NET-associated proteome that remains constant regardless of the specific agent responsible for NET induction, has been proposed by Rahman and Gadjeva[26]. The core

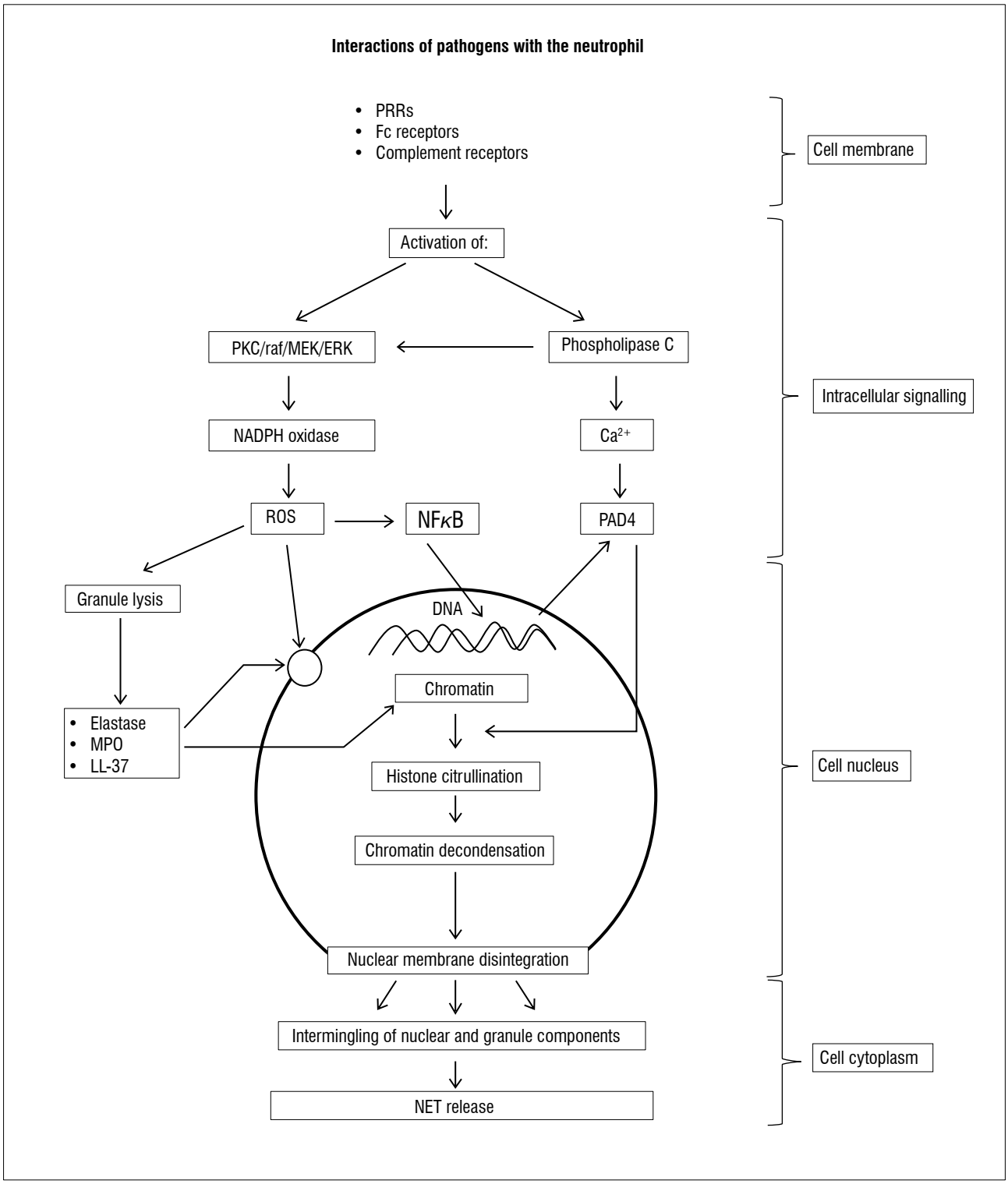

Figure 2: Basic mechanism of lytic NETosis. Exposure of neutrophils to pathogens or their cell-wall and intracellular components bound to pathogen recognition receptors (PRRs) or receptors for antibody (Fc) or complement components is linked to activation of: (1) the protein kinase C (PKC)/raf/erk kinase (MEK)/extracellular signal regulated kinase (ERK) intracellular signalling axis, which, in turn, activates the membrane-bound, electron-transporting, reactive oxygen species (ROS)-generating complex, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; and (2) the enzyme phospholipase C which cleaves membrane phosphatidylinositol to generate diacylglycerol (which also activates NADPH oxidase) and inositol triphosphate, which mobilises calcium (Ca^{2+}) from intracellular stores. ROS initiate activation of the latent cytosolic transcription factor, nuclear factor kappa B (NFκB), which translocates to the nucleus and induces, amongst others, the gene encoding the Ca^{2+}-dependent, pro-NETotic enzyme peptidylarginine deiminase 4 (PAD4). ROS also promote disruption of the membranes of cytosolic granules leading to release of granule antimicrobial proteins and enzymes, including LL-37, which act in concert with ROS to augment nuclear membrane disintegration, while elastase and myeloperoxidase (MPO) also mediate chromatin decondensation. Nuclear membrane disruption enables PAD4 to access the nucleus, which, in turn, triggers the series of events culminating in neutrophil extracellular trap (NET) formation. This involves PAD4-mediated citrullination of nuclear histones followed by chromatin decondensation, and intermingling of nuclear and granule antimicrobial components in the cytoplasm to form a matrix which is released extracellularly as NETs.

Table 1: A summary of the major antimicrobial constituents of neutrophil extracellular traps

Constituent	Origin	Anti-infective spectrum
DNA	Cell nucleus	Broad-spectrum activity
Histones	Cell nucleus	Broad-spectrum activity
Myeloperoxidase (MPO)	Neutrophil primary granules	Broad-spectrum when combined with hydrogen peroxide
Neutrophil elastase (NE)	Neutrophil primary granules	Serine protease with broad-spectrum activity
Proteinase 3 (PR3)	Neutrophil primary granules	Serine proteinase with broad-spectrum activity
Cathepsin G	Neutrophil primary granules	Serine proteinase with broad-spectrum activity
α-Defensins	Neutrophil primary granules	Broad-spectrum activity; also known as human neutrophil peptides 1–4 (Hnp 1–4)
Azurocidin (cationic antimicrobial peptide-37)	Neutrophil primary granules	Broad-spectrum antimicrobial polypeptide, also known as cationic antimicrobial peptide-37
Bactericidal permeability-increasing protein (BPI)	Neutrophil primary granules	Selective activity against Gram-negative bacterial pathogens
Lysozyme	Neutrophil primary, secondary and tertiary granules	Selective activity against Gram-positive bacterial pathogens
LL-37 (cathelicidin)	Neutrophil secondary granules	Broad-spectrum activity
Lactoferrin	Neutrophil secondary granules	Broad-spectrum activity
Calprotectin	Neutrophil cytosol	Selective activity against the yeast *Candida albicans* and *Aspergillus* fungal species

Sources[16,23-25,37]

NET-associated proteome is made up of a combination of 19 constituent proteins, arranged in association with a variety of decorative proteins. The fact that the protein structure of NETs exhibits a constant element to its composition supports the hypothesis that NET formation is indeed an innate immune system response which is non-specific.[26] The major antimicrobial components of NETs are summarised in Table 1.

Beneficial effects of NETosis

Role in host defence

NETs have been shown to degrade microbial and viral virulence factors and to restrict pathogens by forming a physical barrier that prevents dissemination. Examples of bacterial, fungal and protozoal parasitic pathogens which are ensnared in NETs are shown in Table 2. While some bacterial pathogens such as *Pseudomonas aeruginosa*[27], *Borrelia burgdorferi*[28] and *Burkholderi pseudomallei*[29] are killed following entrapment in NETs, others appear less vulnerable, probably as a result of the production of anti-adhesive surface structures such as polysaccharide capsules and/or NET-degrading nucleases, as indicated in Table 2.[24,27-47]

Neutrophils appear to be particularly adept at sensing microbial size, with large microorganisms the most effective inducers of NETosis.[31] For example, in the case of *Candida albicans*, the yeast responsible for most fungal infections in humans, NETs are effectively induced by this pathogen in both the yeast and hyphal forms.[48] Protozoa such as *Toxoplasma gondii, Plasmodium falciparum* and *Leishmania* spp. have also been shown to possess the requisite signals to trigger NET formation, which, in some cases, may lead to the death of entrapped parasites, as well as interference with the invasion of host cells. Some microbial pathogens, such as the opportunistic Gram-negative bacterium *Acinetobacter baumannii* and the fungus *Cryptococcus neoformans,* do not appear to activate NETosis, which in the case of the latter has been attributed predominantly to the ability of the polysaccharide capsule to prevent the requisite signalling mechanisms.[49,50]

Based on observations of pathogens such as *Staphylococcus aureus* and *Streptococcus pneumoniae,* which withstand NETs, it has been proposed that the primary function of NETs is to immobilise, weaken and expose infective agents to other antimicrobial cellular and humoral components of the innate immune system, including tissue macrophages and the proteins of the complement system respectively.[51-53] An example of this type of cooperation involves the interaction of neutrophils and macrophages in the eradication of the bacterial pathogen *Mycobacterium tuberculosis*. This pathogen has also been reported to induce NETs in which it is trapped extracellularly, but remains viable. Entrapped bacilli are then engulfed by alveolar macrophages, the primary cell type involved in the eradication of *M. tuberculosis.*[30]

With respect to the role of NETs in antiviral host defence, neutrophils have been shown to detect HIV-1 via interaction with pathogen recognition receptors which recognise viral RNA. This detection in turn triggers NET formation, leading to NET-mediated inactivation of HIV-1, resulting from exposure to MPO-derived oxidants and α-defensins.[54] This response may, however, be attenuated via the release of the anti-inflammatory cytokine, interleukin-10, from bystander HIV-infected dendritic cells, which, in turn, inhibits NET formation.[54]

Table 2: Examples of microbial pathogens which induce NETosis and their escape strategies

Pathogen	Type	Protective actions of neutrophil extracellular traps (NETs)	Escape/survival mechanisms
Pseudomonas aeruginosa	Bacterium	Entrapment and killing of some strains, others escape	Shedding of outer membrane vesicles which compete with NET binding sites, as well as acquired resistance
Borrelia burgdorferi	Bacterium	Entrapment and killing	None evident
Burkholderia pseudomallei	Bacterium	Entrapment and killing of strains with low capsule expression	Evasion by capsular polysaccharides
Staphylococcus aureus	Bacterium	Entrapment only	Subversion of macrophage-mediated uptake and killing of NET-associated bacteria by production of pro-apoptotic deoxyadenosine
Streptococcus pneumoniae and other streptococci species	Bacterium	Entrapment of strains with low capsule expression	Capsule polysaccharide-mediated interference with binding to NETs and escape as a result of production of microbial nucleases
Neisseria meningitidis	Bacterium	Entrapment resulting in decreased proliferation	Shedding of competitive outer membrane vesicles and adaptive cell surface modifications
Neisseria gonorrhoea	Bacterium	Entrapment and limited bactericidal activity	Escape as a result of subsequent production of a thermonuclease and inhibition of production of pro-NETotic reactive oxygen species by neutrophils
Vibrio cholera	Bacterium	Entrapment and limited bactericidal activity	Escape as a result of subsequent production of two microbial nucleases
Mycobacterium tuberculosis	Bacterium	Entrapment without killing	Resistance to killing mediated by the thick, waxy outer coat of the pathogen
Candida albicans	Yeast	Entrapment and killing	Escape reported, but mechanisms unknown
Aspergillus species	Fungus	Entrapment and killing	No escape mechanisms described
Leishmania species	Protozoal parasite	Entrapment and limited killing	Escape mediated by release of 3'-nucleotidase/ nuclease activity
Toxoplasma gondii	Protozoal parasite	Entrapment and killing	Escape mechanisms not yet identified
Plasmodium falciparum	Protozoal parasite	Entrapment and limited killing	Escape mechanisms not yet identified

Sources[24,27-47]

Although the exact role of NETs in the host response to infection remains to be convincingly elucidated, the increased susceptibility of patients with chronic granulomatous disease (CGD) to infection is noteworthy in this context. This condition is an inherited primary immunodeficiency disorder caused by a complete absence of NADPH oxidase and failure of phagocytes to generate ROS, and consequently NETs. The severe impairment of neutrophil protective activity as a result of the combined absence of production of antimicrobial ROS and NETs causes the patients to suffer from severe and often life-threatening infections.[9] However, NET production by neutrophils in chronic granulomatous disease is responsive to activation by ROS-independent pro-NETotic mechanisms.[21] In addition, transient and acquired abnormalities of NET formation have also been demonstrated in human neonates and the elderly, respectively – a previously unrecognised deficit in extracellular bacterial killing which may underpin age-associated vulnerability to microbial and viral infection.[55,56]

In addition, the increased susceptibility of humans with stable or transient severe neutropenia for development of disseminated fungal infections has been attributed to attenuation of 'trapping' by NETs.[20]

Evasion of NETs by pathogens

Several strategies have been described that enable pathogens to evade NET-mediated immobilisation and/or killing by NET-associated proteins, many of which are listed in Table 2. These strategies include the production of nuclease enzymes that degrade the DNA backbone of the NET structures by various types of microbial pathogens, including, but not limited to, pathogens of the *Streptococcus* and *Staphylococcus* genera. In addition, the acquisition by microorganisms and viruses of molecular patterns that interfere with pathogen recognition by pathogen recognition receptors has also been reported to attenuate NET formation.[34-36] The acquisition of a cell capsule, for example, is of particular benefit in evading NET-mediated trapping of organisms, by altering the surface charge of bacteria to neutral and thereby negating the electrostatic attraction posed by positively charged NET fibres and histone residues.[36]

The involvement of NETs in the pathophysiology of disease

Despite beneficial effects in host defence, NETosis may occur at the expense of injury to the host.[57] Inappropriate and/or excessive NET

formation has been documented in the following autoimmune, cardiovascular and pulmonary diseases.

The role of NETs in autoimmune diseases

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a complex and heterogeneous disease, with patients displaying a variety of symptoms of which glomerulonephritis is particularly serious.[57,58] This condition has a definite female preponderance and a prevalence which seemingly varies according to race. The hallmark of SLE is the overproduction of autoantibodies against a range of nuclear antigens including, not only DNA and histones, but also neutrophil granule proteins.[57] These autoantibodies are believed to contribute significantly to disease pathogenesis. In this context, several studies have reported that the ability to degrade NETs was reduced in a subset of patients with a severe form of SLE which was associated with both glomerulonephritis and the presence of circulating autoantibodies reactive with various constituents of NETs.[59,60] Mechanistically, disassembly of NETs in the physiological setting is mediated by the enzyme serum endonuclease DNase1, interference with which is likely to favour persistence and exaggerated immunogenicity of NETs.[60] In this setting, binding of anti-NET antibodies to NETs has been reported to prevent access of DNase I to NETs. The consequence is impairment of DNase1 function, resulting in failure to dismantle NETs, correlating with renal involvement in SLE.[60] As alluded to earlier, it appears that NETosis in SLE also involves a subset of neutrophils known as low density granulocyes.[61]

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, systemic autoinflammatory disease which primarily affects the synovial joints. It occurs at high frequency (1–3%) in the general population with a female preponderance and, like SLE, is associated with high morbidity and mortality. The majority of RA patients present with high levels of circulating antibodies to citrullinated proteins known as anti-citrullinated peptide antibodies (ACPAs) which are serodiagnostic for RA. Although a clear mechanistic relationship between dysregulation of NETosis and production of ACPAs remains to be established, it is noteworthy that neutrophils from patients with RA exhibit exaggerated NETosis in the circulation, the skin and rheumatoid joint when compared with neutrophils from healthy controls and patients with osteoarthritis.[62,63] Other supporting evidence includes: (1) the finding of a significant positive correlation between NET formation and serum levels of anti-citrullinated peptide antibodies, as well as with other circulating biomarkers of inflammation and neutrophil activation; (2) a report that citrullinated histone 4, a component of NETs, is reactive with ACPAs; and (3) the finding that NETs act as strong stimulants of fibroblast-like synoviocytes (cells that invade cartilage in RA).[62] Taken together, these findings appear to implicate dysregulation of NETosis in the pathogenesis of RA which, in turn, may lead to the identification of novel targets for the treatment of this and other diseases.[62]

Small vessel vasculitis

Small vessel vasculitis is a chronic autoinflammatory condition in which small blood vessels show necrotic inflammation. The condition is associated with the presence of anti-neutrophil cytoplasmic autoantibodies (ANCAs). The main targets for ANCAs are the granule enzymes MPO and PR3 (proteinase 3).[64] Kessenbrock et al.[65] observed that the binding of ANCAs to neutrophils resulted in activation of NETosis. They also demonstrated typical components of NETs present in kidney biopsies of patients with small vessel vasculitis. The NETs were decorated with the autoantigens MPO and PR3. Deposition of NETs in inflamed kidneys suggests that NET formation plays a pathogenic role in autoimmune small vessel vasculitis by presenting autoantigens to the immune system with resultant vascular damage.[65,66]

The role of NETs in deep vein thrombosis

Deep vein thrombosis (DVT) is the formation of a blood clot (or thrombus) in a deep vein, predominantly in the legs. DVT can be triggered by disturbances in venous blood flow, activation or dysfunction of the vascular endothelium, and hypercoagulability. NETs provide a new link between innate immunity and hypercoagulability,[67] stimulating the coagulation process by activating platelets, the coagulation cascade and the vascular endothelium.[68] NETs provide a scaffold for platelet and red blood cell adhesion and also concentrate effector proteins involved in thrombosis.[68] It is speculated that NET-associated enzymes may enhance coagulation indirectly through proteolytic degradation of tissue factor pathway inhibitor, the major trigger protein in the onset of blood clotting.[69] Histones have also been shown to increase thrombin generation, causing platelet activation and coagulation.[70] In the experimental setting, administration of DNase1 to mice was found to promote disassembly of NETs with resultant suppression of DVT enlargement,[71,72] underscoring the importance of NETS in the pathogenesis of DVT.[67] The presence of NETs, according to the detection of citrullinated histone H3 positive cells, has also been described in human venous thrombi.[73]

NETs in lung disease

NETs may also contribute to the pathogenesis and severity of several inflammatory lung conditions, including cystic fibrosis[74], acute lung injury and acute respiratory distress syndrome[75], severe asthma[76] and chronic obstructive pulmonary disease[77].

Acute lung injury and acute respiratory distress syndrome

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) represent a spectrum of lung diseases resulting from direct and indirect insults to the lung. These insults may be a result of infectious or sterile causes.[78] The disease process is characterised by a disruption of the endothelial-epithelial barriers, alveolar damage, pulmonary oedema and various degrees of respiratory failure.[78] ALI/ARDS is characterised by an influx of neutrophils into the pulmonary capillaries, with retention of hyperreactive neutrophils in the damaged vasculature.[79] NETs are able to contribute directly to the pathology of ALI/ARDS by inducing lung epithelial cell death.[66]

NETs in cystic fibrosis

Cystic fibrosis (CF) is a lifelong inherited condition primarily affecting the lungs and digestive tract, with prevalence varying according to race, and seemingly more common in those of North European descent. CF patients develop chronic lung infections associated with airway obstruction mediated by viscous and insoluble mucus secretions.[80] In such patients, chronic bacterial colonisation of the airways develops, usually with the intransigent bacterial pathogens *S. aureus* and *P. aeruginosa*. Sputum viscosity is caused by extracellular DNA released from invading inflammatory cells, much of which is believed to originate from NETosis. This contention is supported by the observation that neutrophil elastase and MPO, which are found in high concentration in CF sputum, are bound to DNA, a key molecular signature of NETs.[81] If detached from NETs, neutrophil-derived proteolytic enzymes may also damage components of pulmonary connective tissue, especially elastin, compromising airway elasticity and function which may underpin the correlation between the magnitude of NET formation in the airways of CF patients and the degree of impairment of lung function.[74,82]

Pharmacological control of NETosis

Given the apparent involvement of excessive NETosis in the development of autoimmune and cardiovascular diseases, as well as in exacerbation of CF, pharmacological regulation of aberrant NETosis has definite therapeutic potential. This potential has yet to be realised, however, due in large part to the relatively recent discovery of NETosis, as well as the current limited insights into the diversity of the molecular mechanisms underpinning this process. Possible strategies include: (1) inhibition of the generation of pro-NETotic ROS, or, alternatively, neutralisation of ROS using oxidant-scavengers such as N-acetylcysteine or ascorbic acid;[11] (2) inhibitors of the activation of NFκB such as ascorbic acid and acetylsalicylic acid (aspirin), both of which have shown promise in experimental animal models of excessive NET formation[11,83]; and (3) inhibitors of PAD4 which are currently in pre-clinical development[84].

With respect to alternative therapies, inhaled recombinant human DNase is widely used in the treatment of CF, primarily as a strategy to degrade neutrophil-derived DNA, a significant contributor to the viscosity of airway mucus.[85]

Conclusions

NETs appear to increase the versatility and potency of the anti-infective armamentarium of neutrophils, as well as several other cell types of the innate immune system, possibly prolonging protective activity beyond cell death, thereby ensuring maximal utilisation of antimicrobial granule proteins. However, several important questions relating to the exact role of NETs in host defence remain incompletely understood. Remaining avenues for exploration include: (1) determining the biological relevance of the various types of NETosis; (2) unravelling the precise molecular and biochemical mechanisms underpinning these processes; and (3) characterising cooperative, beneficial interactions of NETosis with other cellular and humoral components of the innate and adaptive immune systems. As with other indiscriminate phagocyte-derived antimicrobial systems, such as the generation of tissue damaging and carcinogenic ROS, the extracellular release of nuclear material and enzymes such as elastase and MPO during NETosis presents the potential threat of development of autoimmune, cardiovascular and other disorders. Balancing NETosis in favour of host defence using pharmacological and other strategies represents an ongoing challenge.

Authors' contributions

All the authors contributed equally to the compilation of the manuscript, as well as to the coordination of the final version.

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