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Neutrophil extracellular traps as a new paradigm in innate immunity: friend or foe?

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Neutrophilic polymorphonuclear leukocytes, also referred to as neutrophils or polymorphonuclear leukocytes (Fig. 1), are terminally differentiated killer cells vital to both the innate and acquired immune systems. They constitute 60% of all leukocytes (8) and they act as primary effector cells against microbes, a function mediated via the binding of their pattern recognition receptors (e.g. toll-like receptors) to microbe-associated molecular patterns (e.g. lipoteichoic acids or lipopolysaccharides). They are also terminal effector cells of antibody-mediated humoral immunity, acting via Fc gamma-receptor binding to the Fc component of immunoglobulins (mainly IgG).

Key components of neutrophil biology

Neutrophilic polymorphonuclear leukocytes are derived from multipotent stem cells of the hemopoietic lineage (13) and differentiate sequentially into myelo-monocytic progenitor cells, myeloblasts, promyelocytes, myelocytes, band cells and ultimately into mature peripheral blood neutrophils (Fig. 2). Approximately $1–2 \times 10^{11}$ neutrophils are produced daily and their release from bone marrow appears to be regulated by an increase in cell-surface expression of C-X-C chemokine receptor type 4 relative to expression of C-X-C chemokine receptor type 2 (32). Neutrophils are granulocytes, which by definition contain 'granules' or lysosomes, and the neutrophil possesses three principal types of granule:

- Primary (azurophilic) granules (containing myeloperoxidase and neutrophil elastase).
- Secondary (specific) granules (containing lactoferrin, matrix metalloproteinase-8 and pentraxin-3).
- Tertiary (gelatinase) granules (containing gelatinase, matrix metalloproteinase-9 and MT-6 matrix metalloproteinase).

There are, however, other important neutrophil enzymes that are not granule specific and that are synthesized by the neutrophil after its exit from the bone marrow. These include lysozyme and a member of the cathelicidin antimicrobial peptide family called hCAP18, the C-terminus of which is currently known as LL37 (121).

Mature neutrophils are a first line of defence at exposed epithelial (including mucosal) surfaces where they enter secretory fluids, such as alveolar lining fluids in the lungs and gingival crevicular fluid in the periodontium, to challenge colonizing microorganisms and maintain a 'health-promoting resident microflora' (79). They are also recruited by tissue macrophages (Langerhans' cells) and epithelial cells in response to successful bacterial perturbation of the body's physical barriers to tissue entry. Macrophage/epithelial-derived signaling molecules activate vascular endothelial receptors for complementary neutrophil surface ligands in order to effect neutrophil endothelial transmigration (diapdesis) and, via generation of chemotactic signals (e.g. interleukin-8, complement C5a, N-formyl-methionine-leucine-phenylalanine and lipopolysaccharide), facilitate neutrophil homing (chemotaxis) to sites of infection (Fig. 3). The characteristics and efficiency of neutrophil directional movement (i.e. the velocity and persistence of directional motion) is known as 'chemotaxis', as opposed to the speed of movement itself, which may be inefficient and poorly targeted, is referred to as 'chemokinesis' (116) and is critical for nonhost destructive neutrophil activity. Inefficient, misdirected or, indeed, excessive chemotaxis increases tissue-transit times alongside the extracellular release

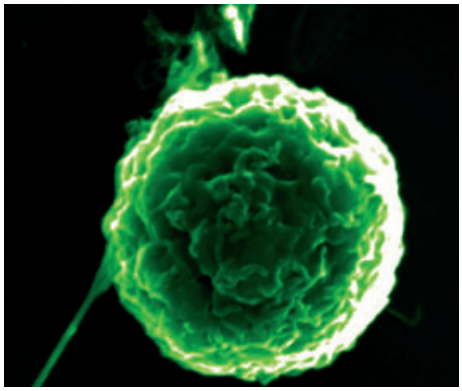

Fig. 1. Scanning electron micrograph of a neutrophilic polymorphonuclear leukocyte (neutrophil/polymorphonuclear leukocyte) with associated fibers from an extruded neutrophil extracellular trap.

of neutrophil elastase, matrix metalloproteinases and reactive oxygen species, and causes significant collateral host tissue damage (21, 72).

Once in position, the neutrophil phagocytoses accessible microbes in a process mediated by surface pattern recognition receptors (e.g. toll-like receptors) and/or IgG receptors (Fc gamma-RII and Fc gamma-RIII) into a phagosome, one that involves the actin cytoskeleton to direct phagosome formation. Once formed, the phagocytic vacuole receives a lethal payload of lysosomal (granule-derived) enzymes, cathelicidin antimicrobial peptides and reactive oxygen species to effect microbial destruction. This process arises within the safe confines of the vacuolar membrane and the neutrophil then enters an apoptotic state, in conjunction with the expression of 'death signals', such as phosphatidylserine surface moieties (53), which attract macrophages to remove the potentially destructive apoptotic bodies through a process of 'efferocytosis'. Rapid and efficient neutrophil efferocytosis is also an essential homeostatic mechanism that prevents leakage of cytotoxic (e.g. neutrophil elastase and reactive oxygen species) and/or immuno-antigenic (neutrophil DNA or extractable nuclear antigens) contents into the surrounding tissues. When apoptosis is disturbed, or when neutrophils degranulate their payload extracellularly, significant collateral host tissue damage arises and the resulting 'damage associated molecular species' further propagate inflammatory responses (104), contributing substantially to chronic neutrophilic inflammopathies such as chronic obstructive pulmonary disease and periodontitis.

The emergence of neutrophils into the gingival crevice following transit through the junctional epithelium facilitates reasonably safe extracellular killing of plaque biofilm species as a result of the relative distance from the gingival soft tissues. However, the closer to the epithelium that such reactive species are released by the neutrophil, and indeed the closer to alveolar bone that they accumulate, the more likely they are to cause unintentional host tissue damage (41). This is important to periodontitis pathogenesis

Fig. 2. Maturation cycle of neutrophils from multipotent stem cells to mature granulocytes.

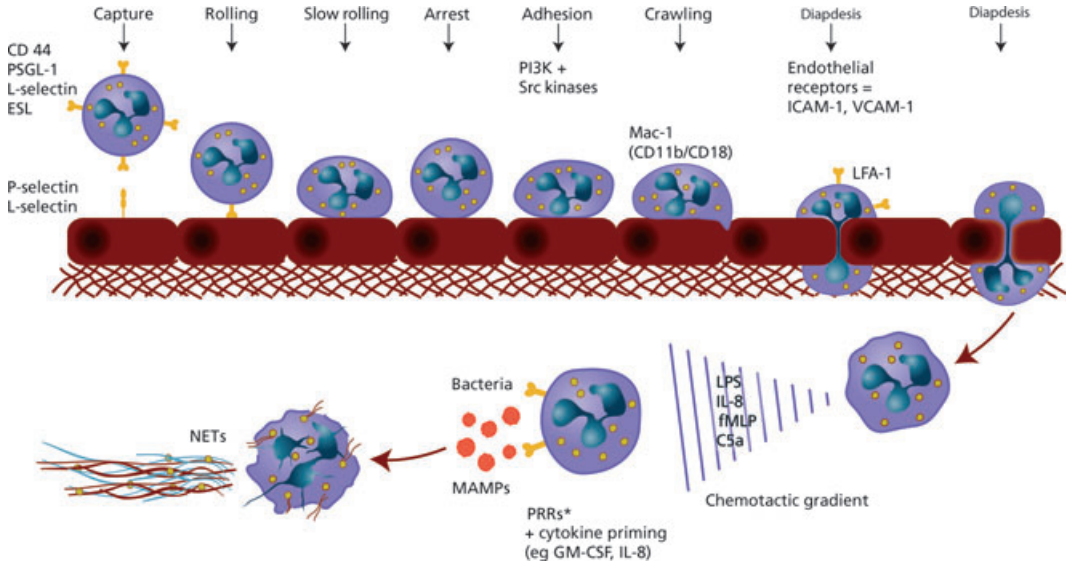

Fig. 3. Neutrophil vascular immobilization (capture, rolling and arrest), endothelial margination (adhesion and crawling), diapedesis, transmigration and chemotactic trafficking into tissues in response to prokaryotic stimuli and eukaryotic 'help' signals. E- and P-selectin receptors are activated on vascular endothelium by complement C5a, leukotriene B4, tumor necrosis factor-alpha, interleukin-1, lipopolysaccharide, interleukin-17 and histamine, and start to make contact with complementary neutrophil surface proteins such as L-selectin. This process pulls the neutrophil out of midstream blood flow where it contacts the endothelial cells, rolls along the endothelium and eventually arrests its motion. The adhesion is strengthened by enzymatic activity involving phosphatidylinositol 3-kinase (PI3K) and Akt/protein kinase B. The neutrophil then moves between the endothelial cells mediated by binding of intergins on its cell surface (LFA-1, Mac-1 and VLA-4) to intercellular adhesion molecules (intercellular adhesion molecule-1 and intercellular adhesion molecule-2) on the endothelial cells, which allow the neutrophil to move across the basement membrane and into the tissues. The neutrophil then moves along a chemotactic gradient (chemotaxins include IL-8, fMLP, C5a and LPS) towards the site of infection. C5a, complement component 5a; LPS, lipopolysaccharide; fMLP, N-formyl-methionine-leucine-phenylalanine; GM-CSF, granulocyte–macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; IL-8, interleukin 8; LFA-1, lymphocyte function-associated antigen-1; LPS, lipopolysaccharide (also known as endotoxin); Mac-1, macrophage-1 antigen (comprising CD11b and CD18, and complement receptor); MAMPs, microbe-associated molecular patterns; NETs, neutrophil extracellular traps; PRRs, pattern recognition receptors; PSGL-1, P-selectin glycoprotein ligand 1; VCAM-1, vascular cell adhesion molecule 1; VLA-4, very late antigen-4 (also called intergrin α4β1, comprising CD49d and CD29).

because peripheral blood neutrophils from patients with periodontitis have been shown to be both hyperreactive to a microbial stimulus (82) and also hyperactive in the absence of such a stimulus (83) with respect to the release of extracellular reactive oxygen species (in particular, hypochlorous acid release). Whilst there is evidence for the presence of sufficient levels of granulocyte–macrophage colony-stimulating factor, interleukin-8 and interferon-alpha within the plasma of patients with periodontitis to stimulate the release of reactive oxygen species from neutrophils (unlike control plasma), interferon-alpha also appears to be capable of priming peripheral blood neutrophils to enhance their release of reactive oxygen species when exposed to a secondary bacterial stimulus, such as when present in periodontal tissues (27). Thus, neutrophil hyperactivity with respect to reactive oxygen species release may be induced by interleukin-8, interferon-alpha and granulocyte–macrophage colony-stimulating factor, and interferon-alpha also appears to be capable of generating neutrophil hyper-reactivity (27). Interestingly, Wright et al. (141) demonstrated a strong type-1 interferon gene-expression signature of in peripheral blood neutrophils from periodontitis patients with such a 'hyperactive' neutrophil phenotype, and also showed that the concentrations of plasma interferon-alpha in patients with periodontitis were capable of reproducing the same neutrophil phenotype when added to peripheral blood neutrophils as obtained from periodontally healthy control patients. Therefore, interferon-alpha appears to be strongly implicated in the excessive release of reactive oxygen species by neutrophils, in particular in periodontitis. Further work by the same group (84, 85) demonstrated that the water-soluble components of cigarette smoke, other than nicotine or cotinine, are capable of antagonizing the production of protective neutrophil reactive oxygen species against periodontal pathogens (via the Fc gamma-receptor), whilst at the same time directly inducing the release

of damaging extracellular reactive oxygen species in unstimulated neutrophils. These findings suggest 'potential neutrophil-mediated mechanisms by which smoking may initiate and maintain oxidative stress at periodontally healthy sites and participate in disease progression, by reducing acquired immune responses'. The relevance here is that excess reactive oxygen species release generates oxidative stress (21), and oxidative stress manifested along with elevated cytokine levels in close proximity to alveolar bone can activate forkhead box O3 and Wnt signaling pathways, which in turn trigger RANKL-mediated bone resorption (38).

Evidence demonstrates that neutrophils are the predominant effector cells in periodontal innate/acquired immunity and are capable of generating substantial periodontal tissue damage when their contents are released extracellularly within tissues. Reactive oxygen species release is indeed enhanced in peripheral blood neutrophils from patients with periodontitis. Neutrophil interferon-alpha reactivity (interferon-alpha is probably released from dendritic and other tissue immune cells) appears to play a significant role in the resulting release of reactive oxygen species and oxidative stress, and any process that subverts neutrophil chemotaxis and/or apoptosis is likely to enhance this destructive process in susceptible patients. Neutrophil phagocytosis of opsonized or unopsonized bacteria facilitates their destruction within the safe confines of the neutrophil phagolysosome and plasma membrane before apoptosis and subsequent efferocytosis by macrophages. Under normal physiological conditions, the macrophages appear to be phenotypically 'switched' into a nonphlogistic (nondestructive) phenotype in response to the release of lipoxins by the neutrophils themselves, facilitating safe neutrophil tissue clearance by phagocytosis (40, 130). It appears that neutophils govern their own physiological fate by switching their own phenotype from one of 'proinflammatory' lipid mediator production (e.g. prostaglandin E_2 and leukotriene B4) to one of 'proinflammation-resolving' lipid mediator production. These proresolving lipids are derived from polyunsaturated fatty acids, which may be endogenous to the cell (arachadonic acid-derived lipoxins) or exogenously obtained from the diet (resolvins and protectins derived from omega-3 fatty acids) (117). The trigger for the neutrophil to switch into 'proresolution' mode from 'proinflammatory' mode may paradoxically be high surrounding tissue levels of prostaglandin E_2 (70) generated by the acute inflammatory response. In this way the protective role of neutrophils in generating an acute inflammatory response to invading microorganisms is self-redirected to resolve the acute response. Perturbation or dysregulation of that phenotypic switch appears to generate a 'nonresolving' chronic inflammatory response, which is more typically associated with host tissue damage via some of the mechanisms previously described.

Neutrophil extracellular traps: a new paradigm in immunity

For many years, scientists researching neutrophil biology had observed unusual extracellular fiber-like structures microscopically and regarded them as processing artefacts. However, in 2004, Brinkmann et al. (14) documented narratively and visually, through an elegant series of static and video microscopic studies, a hitherto-unrecognized and powerful method of neutrophil-mediated microbial killing – the release of neutrophil extracellular traps. When challenged by certain signals, including interleukin-8 and lipopolysaccharide (but usually multiple simultaneous stimulations are necessary – see later), the neutrophil undergoes a programmed sequence of events that lead to the release of its entire nuclear chromatin (DNA and associated histone-rich protein backbone), which, having mixed with, and then embedded, granular cathelicidin antimicrobial peptides into its structure before extracellular release, is actively extruded by the neutrophil as a type of biologic 'spiders web' into the extracellular space or tissue (Fig. 4).

Neutrophil extracellular traps can only be generated by mature neutrophils because immature cells have not yet developed the molecular machinery to transduce extracellular signals via membrane receptors and second messengers to trigger the enzyme systems necessary for their production (80). Neutro-

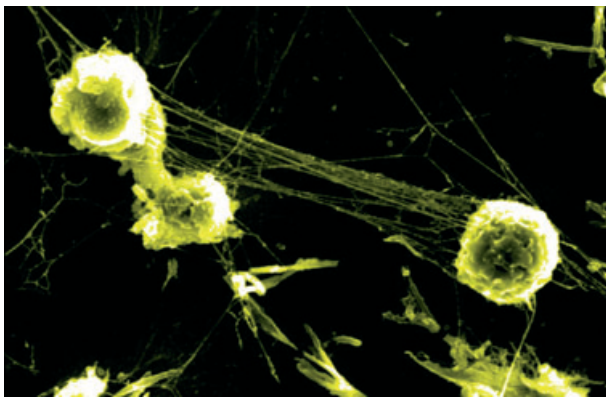

Fig. 4. Scanning electron micrograph showing neutrophil extracellular traposis induced by *Tannerella forsythia*.

phil extracellular traps have been shown to snare gram-positive bacteria and also have cationic properties enabling adhesion to gram-negative bacteria, as well as possessing the ability to bind fungi (33) and other microorganisms to kill them *in-situ* independently of phagocytosis. Notably, the inhibition of the actin cytoskeleton using cytochalasin D prevents phagocytosis and therefore when bacteria such as *Staphylococcus aureus* are included with cytochalasin D following the production of neutrophil extracellular traps, the neutrophil extracellular trap structures kill ~30% of the bacteria independently of phagocytosis (14). The same group demonstrated that neutrophil extracellular traps are capable of deactivating certain microbial virulence factors, presumably through granule protease activity, derived from the granular peptides embedded within the structure of the neutrophil extracellular trap. Interestingly, within a transiently resident tissue population of neutrophils, only a small percentage of cells release neutrophil extracellular traps; our own studies indicate ~30% (L.J. Palmer, P.R. Cooper, I.L.C. Chapple, unpublished data in Palmer (99)), with evidence existing that the presence of viable neutrophils is also necessary for this process to occur (90). This novel mechanism has been claimed to be as powerful an antimicrobial strategy as phagocytosis itself (36). In this review, we provide an overview of the current state of neutrophil extracellular trap biology, areas of consensus and controversy, and we propose that nuclear DNA-derived neutrophil extracellular traps, when formed in excess within gingival and periodontal tissues, not only contribute to periodontal pathogenesis through autoimmune processes, but also provide a vital potential causal link between periodontitis and systemic autoimmune diseases such as rheumatoid arthritis (26).

Current thinking in neutrophil extracellular trap biology

Despite an explosion in neutrophil extracellular trap-related publications in the last 5 years, there are large gaps in our knowledge of neutrophil extracellular trap biophysiology, and some areas of controversy still remain. There is consensus that neutrophil extracellular trap release is an active process and does not simply result from neutrophil necrosis because lactate dehydrogenase release is not a feature of the process (at least up to 2 h after neutrophil extracellular trap stimulation) (14). Whilst aged neutrophils can also undergo caspase-dependent (34) and -independent apoptosis (75), there is no evidence for neutrophil extracellular trap release being a classical form of apoptosis. However, it remains likely that such a highly orchestrated process is, at least under certain circumstances, a form of programmed cell death (14). What remains controversial is whether neutrophil extracellular traps are predominantly released as part of a novel programmed cell-death process now termed 'neutrophil extracellular traposis', or whether neutrophil extracellular traposis is just one of several mechanisms through which neutrophil extracellular traps are released, some of which are not associated with cell death. There is good evidence that neutrophil extracellular traps can be released from viable neutrophils following priming with granulocyte–macrophage colony-stimulating factor and a relatively short (15 min) stimulation with lipopolysaccharide (via toll-like receptor-4) or complement C5a, where, remarkably, 80% of neutrophils produced neutrophil extracellular traps (144). Eosinophils have also been shown, by the same group, to release neutrophil extracellular traps in a rapid 'catapult-like' manner, following interleukin-5 or interferon-gamma priming and subsequent activation by gram-negative bacteria (143). Such neutrophil extracellular trap complexes contain mitochondrial DNA rather than nuclear DNA (144) and lack the nuclear (e.g. lamin B, poly-ADP-ribose polymerase) and cytoplasmic (e.g. beta-catechin, cytochrome *c*) proteins reported to be present when nuclear DNA neutrophil extracellular traps are released following a more aggressive or prolonged stimulation. As the NADPH-oxidase inhibitor, diphenyl iodonium completely blocked mitochondrial DNA (neutrophil extracellular trap) release, Yousefi et al. (144) concluded that reactive oxygen species generation is also essential for mitochondrial neutrophil extracellular trap release. However, key to this issue is the incorrect assumption that the actions of diphenyl iodonium are limited to the NADPH-oxidase and somehow activation of the latter is necessary for mitochondrial neutrophil extracellular trap release. Yousefi et al. did not state this and indeed the actions of diphenyl iodonium are not limited to NADPH-oxidase inhibition. Diphenyl iodonium was first demonstrated as an NADPH-oxidase-independent inhibitor of mitochondrial respiration (55), by blocking flavin components of the electron transport chain. Bulua et al. (18) demonstrated that diphenyl iodonium blocks mitochondrial respiration (and reactive oxygen species production), independently of NADPH-oxidase, and further showed that activation of key proinflammatory cytokines via the NOD-like receptor family, pyrin domain containing 3 inflammasome may be dependent upon mitochondrial reactive oxygen species rather than on NADPH-oxidase-dependent reactive

oxygen species. Therefore, it is most likely that mitochondrial reactive oxygen species generated from viable granulocytes (neutrophils and eosinophils) are responsible for rapid mitochondrial DNA-neutrophil extracellular trap release as a key antimicrobial strategy within tissues. Moreover, the generation of reactive oxygen species during neutrophil extracellular trap production activates proinflammatory cytokines such as interleukin-1beta via the NLRP-3 inflammasome, and, as discussed later, the resulting neutrophil extracellular traps appear to be capable of interacting with platelets to contribute to the extracellular inflammatory milieu.

Neutrophils have traditionally been regarded as terminally differentiated suicide effector cells, with a very short half-life of up to 24 h within the bloodstream. However, more recently, evidence from *in-vivo* life-expectancy studies has challenged this paradigm and demonstrated half-lives as long as 5.4 days (107), indicating that neutrophils probably possess far more sophisticated and diverse properties than the indiscriminate lethality with which they are associated. The latter include the ability to present antigens (92) and to communicate with macrophages (92), lymphocytes (T-cells) (6) and dendritic cells (9). One intriguing remaining possibility, which we believe has significant biological plausibility, is that low-level stimulation of mitochondrial neutrophil extracellular trap release from viable cells may constitute an effective and relatively safe antimicrobial strategy within tissues. In contrast, neutrophil extracellular traposis (large-scale nuclear DNA neutrophil extracellular trap release) into tissues rather than the extracellular space (such as gingival crevice or alveolar lining fluids), a process in which neutrophil extracellular traps also associated with released neutrophil nuclear and cytoplasmic peptides, probably results from a more catastrophic neutrophil challenge and is more likely to induce various pathogenic sequelae.

Neutrophil extracellular trap structure

Nuclear chromatin is a complex structure, comprising double-stranded DNA wrapped tightly around a histone protein-rich backbone within a double-helix structure forming nucleosomes. Several histones wrap up into fibers of ~30 nm. When genes are transcribed, the local region of chromatin unwraps to a looser structure that associates with RNA polymerases, called 'euchromatin', and nontranscribing regions are more tightly packed and are referred to as 'heterochromatin'. Neutrophil extracellular traps released during neutrophil extracellular traposis consist of nuclear DNA and various histones and, most importantly, high-resolution scanning electron microscopy demonstrated that they are studded or 'decorated' with globuli of 30–50 nm in diameter (15) that contain the multiple cathelicidin antimicrobial peptides which originate within the neutrophil granules (lysosomes) and which co-localize into the web-like mesh that forms (Fig. 5). The co-localization of these granular proteins/enzymes, or indeed of histones (e.g. H1, H2A, H2B, H3, H4 or a complex of H2A–H2B), with the DNA is critical in discriminating DNA released during cell necrosis from that specific to neutrophil extracellular trap formation. Therefore, the demonstration of myleloperoxidase and/or elastase co-located with the DNA is important to verify the identity of structures within tissues as true neutrophil extracellular traps. This was elegantly demonstrated by Brinkmann et al. (14) in their original description, where importantly they verified that neutrophil extracellular trap fibers were DNA structures rather than proteins, because by using DNases they dismantled and dispersed the fiber-like structures, whereas proteases had no such effect. The fiber-like structures were examined using high-resolution scanning electron microscopy (Fig. 6) and demonstrated strands of varying diameter and length, some of which formed 'cable-like' structures (15). When produced in multiwell plates *in vitro*, neutrophil extracellular traps float within the fluid medium, rather like a spider's web does in moving air. It is easy to understand how, given their ultrastructure, the fact that they are 'sticky' as a result of their electrostatic charged nature and that they extend over areas of several microns, they are very effective at trapping microorganisms moving in the vicinity (Fig. 4). Figure 7 is, to our

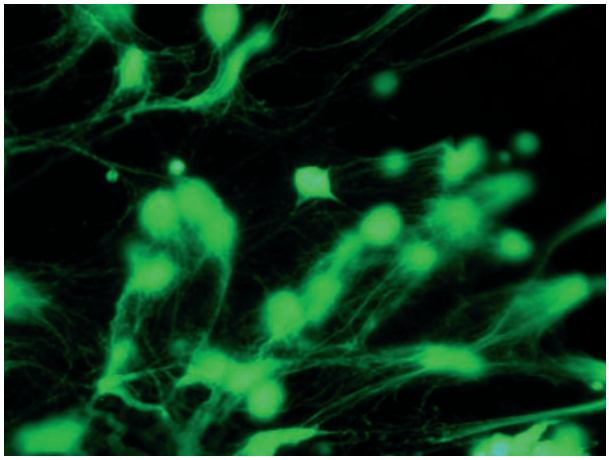

Fig. 5. Fluorescence microscopy of Sytox® green-stained neutrophil extracellular trap DNA structures following extrusion by neutrophils to form a web-like trap.

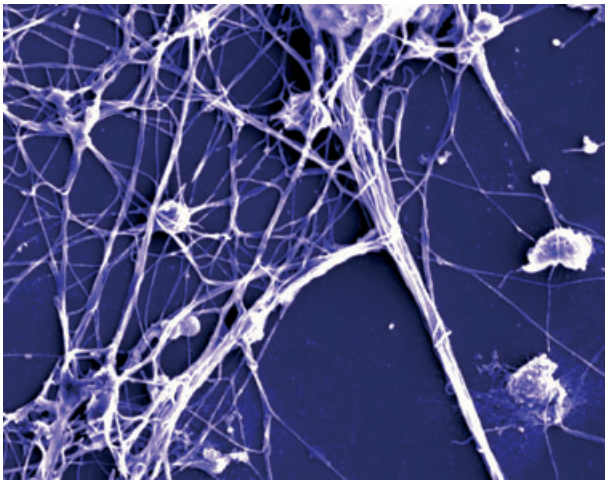

Fig. 6. Scanning electron micrograph of neutrophil extracellular trap structure demonstrating fibers and 'cables' of varying diameter and length.

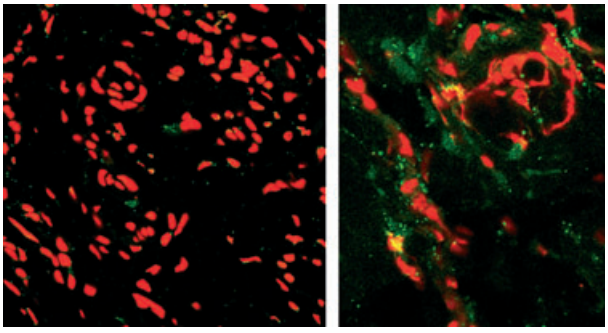

Fig. 7. The first demonstration of neutrophil extracellular trap structures within gingival connective tissues from a patient with gingivitis (right panel) compared with a healthy control (left panel). Confocal images were taken by Dr Shida Yousefi (University of Berne) and demonstrate the co-localization of myeloperoxidase (in green) with DNA (in red) in gingivitis tissues where neutrophil extracellular trap-like structures are evident. In contol tissues there are no neutrophil extracellular traps visible, simply nuclear DNA.

knowledge, the first demonstration of neutrophil extracellular traps within the center of gingival tissues obtained from an area of gingivitis compared with viable neutrophils from an area of gingival health.

Importantly, neutrophil extracellular traps produced from mitochondrial DNA release have a slightly different structure compared with those derived from nuclear DNA. Yousefi et al. (144) demonstrated co-localization of neutrophil granule proteins (elastase and myeloperoxidase) with mitochondrial DNA in their *ex-vivo* short-term-stimulation neutrophil extracellular trap studies, but an absence of the nuclear proteins lamin B, nuclear matrix protein-45 and poly-ADP-ribose polymerase. They also reported an absence of cytoplasmic caspase-3, beta-actin, mitochondrial cytochrome *c* and the membrane markers CD15 and CD16. Therefore, it seems likely that neutrophil extracellular traps derived from mitochondrial DNA interact with the host tissues and the immune system in a different manner compared with those derived from nuclear DNA.

Requirements for neutrophil extracellular trap release

Summary of events encompassing neutrophil extracellular trap release

The classical process of neutrophil extracellular trap release, as iteratively described in a series of publications by Zychlinsky, Brinkmann, Fuchs et al., is summarized below, and the key roles of the granule enzyme peptidyl arginine deiminase-4, the NADPH-oxidase/downstream reactive oxygen species, the actin cytoskeleton and microtubular organelles, and the different priming and stimulating agents, described to date, are subsequently discussed in more detail. In summary, Fig. 8 illustrates the complex series of orchestrated events that follow cell stimulation and which define classical neutrophil extracellular traposis.

Stage 1 of neutrophil extracellular trap release involves the generation of reactive oxygen species via activation of the NADPH-oxidase membrane complex, following cell-surface receptor–ligand binding and second messenger activity. NADPH-oxidase activation generates the superoxide anion, the primary oxygen radical created during the 'respiratory burst'. A series of downstream enzymes convert the superoxide to a variety of other reactive oxygen species, as illustrated in Fig. 9. This appears to be the major intracellular event that triggers neutrophil extracellular trap production, although it is recognized that superoxide can also be generated by mitochondrial leakage as a side effect of ATP production (reviewed by Chapple & Matthews; 21) and such events may well explain how viable cells also appear to be capable of neutrophil extracellular trap release, involving mitochondrial DNA rather than nuclear DNA (144).

Stage 2 involves the activation of an enzyme called peptidyl arginine deiminase-4 (46). Peptidyl arginine deiminase-4 is known to hypercitrullinate the condensed nuclear chromatin, replacing charged arginine amino-acid residues with uncharged citrulline residues, thus effecting rapid and large-scale decondensation (unfolding) of the nuclear chromatin within the nuclear membrane. This process is also

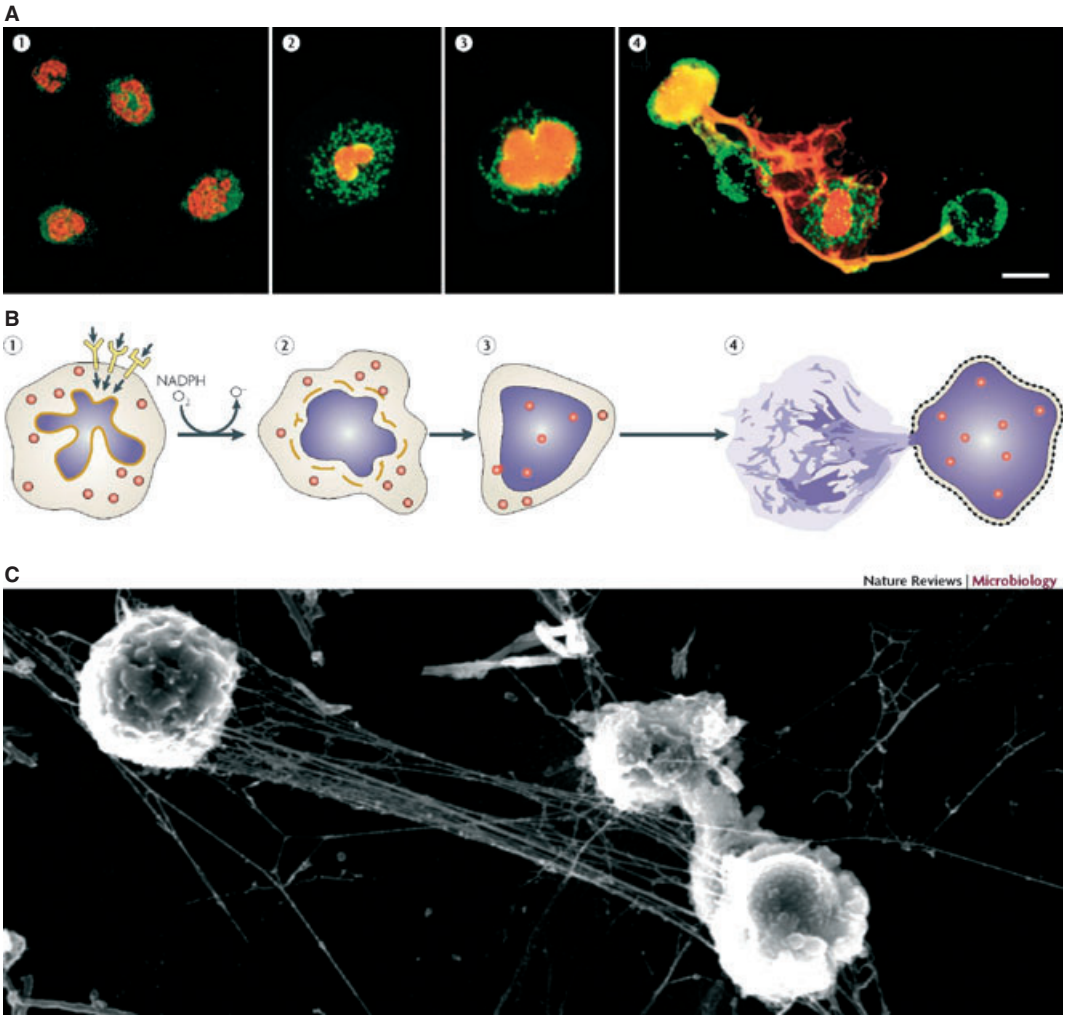

Fig. 8. Sequence of events leading to, and following, neutrophil extracellular trap release. (A) Fluorescence photomicrographs and (B) schematic maps of the sequence of these events. (C) Scanning electron micrograph from our own work demonstrating the events at higher magnification. Stages 1–4 are described in detail in the main text. The exquisite fluorescence micrographs shown in panel A were reproduced with kind permission from Brinkmann & Zychlinsky (15).

referred to in the biochemistry literature as 'deimination' (replacement of the amino-acid arginine with the amino-acid citrulline). The rapidly unfolding DNA/chromatin complex expands to the inner margins of the nuclear membrane. Papayannopoulos et al. (102) demonstrated that neutrophil elastase is also involved in the processing of histones in preparation for chromatin decondensation during neutrophil extracellular traposis.

During stage 3 of neutrophil extracellular trap release, the space between the inner and outer nuclear membrane increases and eventually forms distinct vesicles. Neutrophil granule membranes are lost, which allows the release of granule proteins, specifically neutrophil elastase, which then co-localize with the nuclear chromatin. The nuclear envelope disintegrates, allowing the nuclear DNA/histone complex to mix with granular cathelicidin antimicrobial peptides such as elastase, LL37 and myeloperoxidase, subsequently filling the cytoplasmic space.

Stage 4 was illustrated by Fuchs et al. (36), using vital cell dyes. They employed live-cell imaging to follow neutrophil extracellular trap production whilst simultaneously monitoring cell viability. They observed that upon stimulation with phorbol 12-myristate 13-acetate, neutrophils flattened and their nuclei lost their characteristic lobular morphology and filled the majority of the intracellular cytoplasmic space. During this time the chromatin began to decondense so the distinction between euchromatin and heterochromatin was lost. After mixing of the nuclear and granule contents, the cells started to lose the vital dye calcein blue, indicating loss of membrane integrity and subsequent loss of cytoplasm. At the same time, neutrophil extracellular traps were first visualized and the cells became annexin V-positive by

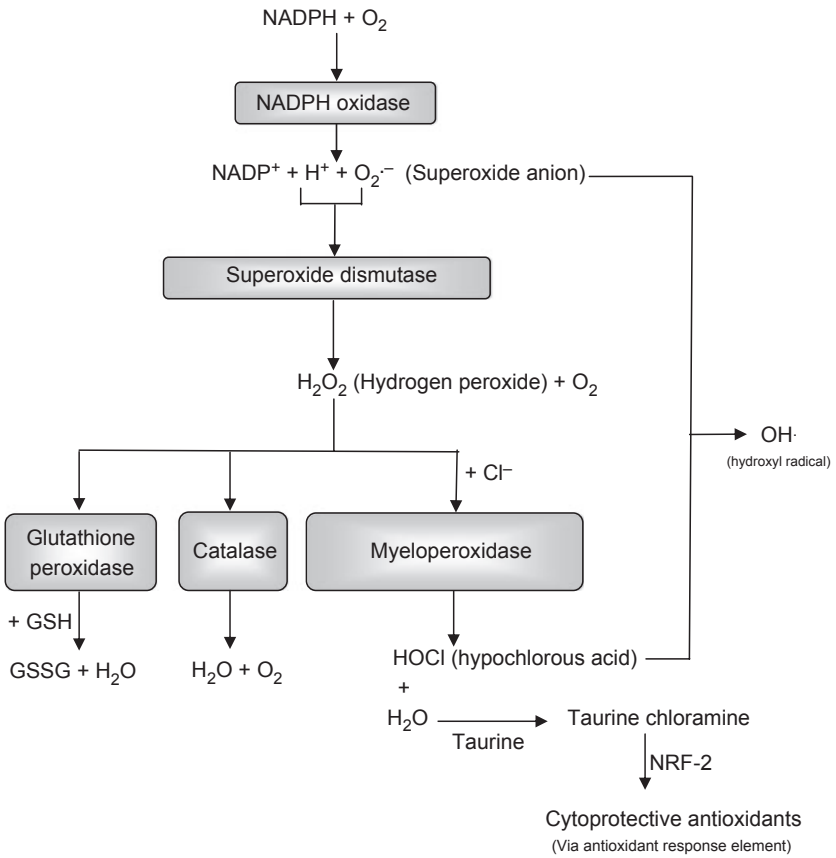

Fig. 9. Generation of superoxide by the NADPH-oxidase and downstream reactive oxygen species generated by various enzyme systems which act sequentially to effect single-electron reductions of superoxide to eventually form water after four single-electron reductions. GSH, reduced glutathione; GSSG, oxidized glutathione.

binding to phosphatidylserine on the inner leaflet of the cell membrane. As the cells became annexin V-positive, they lost vital dye simultaneously, indicating a rupture of the neutrophil cell membrane. Notably, if the cells had been apoptotic they would have become annexin V-positive before calcein release, and hence Fuchs et al. (36) concluded that neutrophil extracellular traps were formed during a novel cell-death program that was quite distinct from apoptosis and necrosis. The differences from necrosis were observed visually in that the stimuli used to induce necrosis (secreted pore-forming toxins from *S. aureus*) did not result in extracellular DNA release and, although similar in the loss of nuclear lobules and loss of differentiation between euchromatin and heterochromatin, the nuclear and granule membranes remained intact only during necrosis.

Stage 5 involves the actin cytoskeleton of the neutrophil and the microtubular complex in neutrophil extracellular trap deployment (94) and active extrusion of the DNA/histone/cathelicidin antimicrobial peptide cocktail into the extracellular space. Neeli et al. (93) employed cytochalasin D to inhibit actin cytoskeleton formation and nocodazole, which inhibits tubulin polymerization (and thus the microtubular system that transports cell organelles and granules towards vacuolar and cell-membrane fusion), and demonstrated reduced formation of neutrophil extracellular traps, suggesting an important role for the actin cytoskeleton and for microtubules in neutrophil extracellular trap formation.

The neutrophil extracellular trap structures that form are understood to function by immobilizing microorganisms (Fig. 10) (thus preventing their dissemination within the tissues), neutralizing virulence factors by protease degradation and finally killing through a battery of cathelicidin antimicrobial peptides embedded within, and associated with, the neutrophil extracellular trap structure. However, as previously discussed, excess neutrophil extracellular trap formation, or the release of nuclear-DNA-containing neutrophil extracellular traps may have a pathogenic role.

Neutrophil priming and stimulation

The point at which neutrophil extracellular traps are released from neutrophils is critical because by their

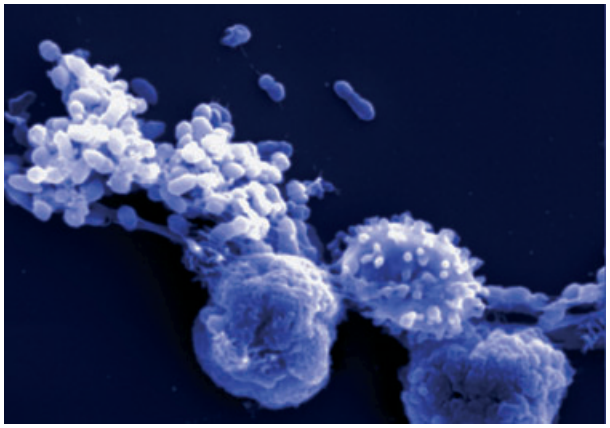

Fig. 10. Scanning electron micrograph of *Streptococcus constellatus* trapped within neutrophil extracellular traps released *in vitro* by peripheral blood neutrophils.

very nature, as 'traps', they will impede other functions of the neutrophil (e.g. chemotaxis toward the pathogenic stimulus, recruitment of other leukocytes and, to a certain extent, phagocytosis). The individual neutrophil may or may not die at the point of neutrophil extracellular trap release, depending upon the nature, magnitude and chronicity of the stimulus, but following neutrophil extracellular traposis it may not be capable of further functionality itself. Currently recognized stimuli for neutrophil extracellular trap release include:

- Nitric oxide (60).
- Cytokines (14).
- Microbes and their products, including bacteria (36), bacterial endotoxins (lipopolysaccharide) (14, 66, 111), other bacterial toxins (e.g. autolysin from *S. aureus (*108) and alpha-enolase from *Streptococcus pneumoniae* (89)), yeasts (128) and protozoa/parasites (1, 7, 44).
- Antimicrobial peptides such as human beta-defensins (platelet-derived) (62).
- Antibodies such as anti-LL37 and anti-human neutrophil peptide (65); anti-HNA-3a antibodies (neutrophil alloantigen-3a) (126); and anti-neutrophil cytoplasm antibodies implicated in the pathogenesis of small-vessel vasculitis (61).
- Platelets activated by lipopolysaccharide or collagen (20, 81) and platelet toll-like receptor-4 (23). Platelet-activated neutrophil extracellular traps also appear to be a fundamental trigger for subsequent deep-venous thrombosis formation (134).
- Statins – whilst paradoxically antagonizing the respiratory burst and also phagocytosis itself (22).

Various signaling pathways have been described which ultimately converge on the activation of the NADPH-oxidase, but importantly several other cellular processes appear to be involved in a complex sequence of events that lead to neutrophil extracellular trap release, the temporality of which remains unknown (Table 1).

The majority of authors researching neutrophil extracellular trap biology use phorbol 12-myristate 13-acetate as a positive-control stimulus for NADPH-oxidase activation and subsequent neutrophil extracellular trap formation. Phorbol 12-myristate 13-acetate is structurally and functionally similar to diacylglycerol, which is the natural intracellular second-messenger lipid-signaling molecule that directly activates protein kinase C physiologically. Protein kinase C then orchestrates assembly of the NADPH-oxidase subunits at the vacuolar membrane and starts generating superoxide (Fig. 11). Phorbol 12-myristate 13-acetate has the advantage that it directly activates protein kinase C, bypassing receptor–ligand binding on the neutrophil surface and also avoiding phagocytosis. It has been reported that approximately one-third of phorbol 12-myristate 13-acetate-activated neutrophils isolated from human venous blood make neutrophil extracellular traps *in vitro* (36), data consistent with our own studies employing anti-neutrophil cytoplasm antibody stimulation of neutrophil extracellular trap release (unpublished data), and in contrast to 80% of neutrophils exposed to granulocyte–macrophage colony-stimulating factor priming and a short subsequent stimulus with C5a or lipopolysaccharide (144). However, it is important to remember that phorbol 12-myristate 13-acetate is not a physiologically relevant stimulus and studies should also employ unopsonized or opsonized bacteria and/or other physiologically relevant agonists (e.g. lipopolysaccharide and other bacterial components).

Keshari et al. (60) demonstrated neutrophil extracellular trap formation by neutrophils following nitric oxide generation using the nitric oxide donor DETA-NONOate. Interestingly, they found both mitochondrial and nuclear DNA within the neutrophil extracellular traps and employed diphenyl iodonium to inhibit NADPH-oxidase and 4-amino-benzoic acid hydrazide to prevent myeloperoxidase and neutrophil extracellular trap formation, demonstrating a role for both enzymes in neutrophil extracellular trap production. These authors also demonstrated that incubation of neutrophil extracellular traps with platelets or the THP-1 monocyte cell line led to elevated release of interleukin-1beta, interleukin-8 and tumor necrosis factor-alpha (with THP-1), providing further evidence of a role for neutrophil extracellular traps in augmenting local cytokine networks within tissues. Brinkmann et al. (14) exposed neutrophils to the

Table 1. Activation of neutrophil extracellular trap release

Pathway component	Evidence	Stimuli used	Effect on neutrophil extracellular trap release	References
Protein kinase C	Staurosporine (a protein kinase inhibitor)	Phorbol 12-myristate 13-acetate	↓	(49)
		1-2-dioctanoyl-*sn*-glycerol (diacylglycerol analog)	↓	
		Platelet-activating factor	↓	
		Helicobacter pylori	=	
ERK pathway activation	MEK1/2 inhibitor	Phorbol 12-myristate 13-acetate, *H. pylori* or platelet-activating factor	↓	(49)
		Parasite (*Toxoplasma*)	↓(early time points) =(later time points)	(1)
		Activated platelets	↓	(20)
	c-Raf kinase (MEK–ERK signal transduction) inhibitor	Phorbol 12-myristate 13-acetate, *H. pylori* or platelet-activating factor	↓	(49)
	ERK inhibitory peptide	Phorbol 12-myristate 13-acetate, *H. pylori* or platelet-activating factor	↓	(49)
Reactive oxygen species	Diphenyl iodonium (NADPH oxidase inhibitor)	*Staphylococcus aureus* or phorbol 12-myristate 13-acetate	↓	(36)
		Eimeria bovis sporozoite or phorbol 12-myristate 13-acetate	↓	(7)
		Lipopolysaccharide	↓	(142)
		Nitric oxide donors	↓	(106)
		Phorbol 12-myristate 13-acetate, 1-2-dioctanoyl-*sn*-glycerol (diacylglycerol analog), *H. pylori* or platelet-activating factor	↓	(49)
		Phorbol 12-myristate 13-acetate	↓	(100)
		Mannheimia haemolytica leukotoxin	↓	(4)
	3-Amino triazole (catalase inhibitor)	Phorbol 12-myristate 13-acetate	↓	(100)
	Hydrogen peroxide		↑	(36, 93, 100)
	CGD neutrophils	*S. aureus* or phorbol 12-myristate 13-acetate	↓	(36)
		Glucose oxidase	↑	
		Phorbol 12-myristate 13-acetate	↓ (although cells did flatten, indicating activation)	(12)
		Phorbol 12-myristate 13-acetate after gene-therapy replacement of $gp91^{phox}$	↑	
		Phorbol 12-myristate 13-acetate	↓	(96)
		Singlet oxygen (generated by photofrin and irradiation)	↑	
		Phorbol 12-myristate 13-acetate (X-CGD PLB-985 cell line)	↓	(77)

Table 1. (*Continued*)

Pathway component	Evidence	Stimuli used	Effect on neutrophil extracellular trap release	References
		Hydrogen peroxide (X-CGD PLB-985 cell line)	↑ (but still lower than healthy cells)	
	Antioxidants			
	N-acetyl-L-cysteine	Nitric oxide donors Phorbol 12-myristate 13-acetate	↓ ↓	(106) (100)
	4-Amino-benzoic acid hydrazide	Nitric oxide donors Phorbol 12-myristate 13-acetate	↓ ↓	(106) (100)
	Taurine	Phorbol 12-myristate 13-acetate or hypochlorous acid	↓	(100)
	Edaravone (free-radical scavenger)	Phorbol 12-myristate 13-acetate	↓	(96)
	α-Phenyl-*N*-*tert*-butyl nitrone (singlet-oxygen scavenger)	Phorbol 12-myristate 13-acetate	↓	
	Enzymes			
	Myeloperoxidase	Phorbol 12-myristate 13-acetate	↑	(100)
	Superoxide dismutase	Phorbol 12-myristate 13-acetate	↑	
Peptidyl arginine deiminase (PAD)	Cl-amidine (PAD4 inhibitor)	Calcium ionophore	↓	(137)
		Interleukin-8 and *Shigella flexneri*	↓	
		Lipopolysaccharide	↓	(71)
	PAD4 knockout mouse	Lipopolysaccharide, phorbol 12-myristate 13-acetate or hydrogen peroxide	↓	(71)
		Lipopolysaccharide	↓	(52)
		Serum transfer-induced model of arthritis	↓	(112)
Actin cytoskeleton	Cytochalasin D (actin polymerization inhibitor)	Interleukin-8 then *S. flexneri* or *S. aureus*	=	(14)
		Lipopolysaccharide	↓ (although early stages of neutrophil extracellular trap activation were seen)	(94)
		Group A streptococci	=	(67)
		Phorbol 12-myristate 13-acetate	=	(44)
		M. haemolytica leukotoxin or proleukotoxin	↓	(4)
		Phorbol 12-myristate 13-acetate or IgG-opsonized *S. aureus* (cytochalasin B)	↓	(100)
		Toxoplasma	=	(1)
CD18 pathway	CD18 blocking antibody	Leukotoxin	↓	(4)

↓, decreased; =, unchanged; ↑, increased.
Not all studies listed used human neutrophils; Wang et al. (137) used the dHL60 'neutrophil-like' cell line. Aulik et al. (14) used bovine neutrophils and Li et al. (71) used murine cells.

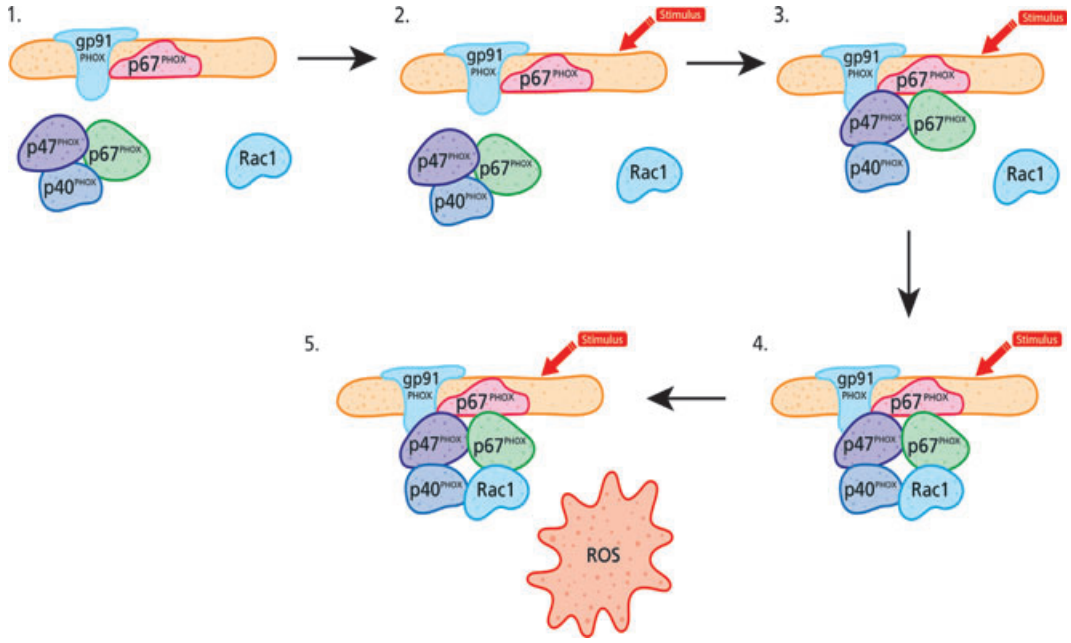

Fig. 11. Assembly of PHOX proteins following neutrophil stimulation. The cytoplasmic components p47^{PHOX}, p67^{PHOX} and p40^{PHOX} migrate to the vacuolar membrane and assemble with gp91^{PHOX} and p22^{PHOX}. The addition of Rac1 completes the NADPH (also referred to as NOX) enzyme complex, which then generates superoxide. ROS, reactive oxygen species.

cytokine interleukin-8 for 30–60 min, as well as to phorbol 12-myristate 13-acetate and lipopolysaccharide. Interleukin-8 is produced by macrophages, endothelial cells and epithelial cells and induces neutrophil chemotaxis, whilst lipopolysaccharide is a component of the outer membrane of gram-negative bacteria and acts via toll-like receptor-4 ligation. It has, however, been demonstrated that whole bacteria induce neutrophil extracellular traps more efficiently than do interleukin-8 or lipopolysaccharide alone, suggesting that activation of multiple receptors is required for optimal stimulation of neutrophil extracellular traps (36). Indeed, our own studies, employing the physiologically and periodontally relevant stimuli *Fusobacterium nucleatum* (heat killed for toll-like receptor-2, -4 and -9 stimulation) and opsonized *S. aureus* (for Fc gamma-receptor stimulation) corroborate these findings (100). In several investigations cytokine priming (e.g. with granulocyte–macrophage colony-stimulating factor) has been necessary before neutrophil extracellular trap release is elicited (144). Interleukin-1-mediated transmigration of neutrophils across the brain endothelium has also been reported, in a murine model, to generate neurotoxicity, the neuronal death being induced by neutrophil extracellular trap release during experimentally induced neuroinflammatory disease (3).

Guimarães-Costa et al. (44) found that the parasite *Leishmania amazonensis* was capable of stimulating neutrophil extracellular trap release, but pretreatment of the neutrophils with phorbol 12-myristate 13-acetate resulted in a 53% increase in promastigote (the flagellate form of the parasite) killing. The purified promastigote membrane component lipophosphoglycan was also able to elicit neutrophil extracellular trap release, but to a lesser extent than the whole organism. Additionally, dead sporozoites of the bovine parasite *Eimeria bovis* were able to elicit neutrophil extracellular trap release upon direct contact with neutrophils, but the cellular reaction was significantly stronger with live sporozoites (7).

Yeasts such as *Candida albicans* (128), *Aspergillus nidulans* (12) and *Aspergillus fumigatus* (16) are known to induce neutrophil extracellular traps, although in the case of *A. nidulans* this effect was shown to be greatly increased by the addition of phorbol 12-myristate 13-acetate. It has also been shown that in mouse bone marrow-derived neutrophils challenged with *C. albicans*, that the hyphal form is more effective at inducing neutrophil extracellular trap release than the spore form (33).

In studies by Fuchs et al. (36), whilst it was acknowledged that microorganisms themselves were directly capable of stimulating neutrophil extracellular trap release, phorbol 12-myristate 13-acetate and glucose oxidase were also used to pre-activate/prime the neutrophils before stimulation with *S. aureus*. Glucose oxidase is an exogenous enzyme that promotes hydrogen peroxide synthesis within cells, in addition to hydrogen peroxide formation by dismuta-

tion of superoxide radicals produced by the endogenous NADPH oxidase. Notably, high concentrations of serum in the culture medium have been shown to inhibit neutrophil extracellular trap formation. Whilst the mechanism of this inhibition is currently unknown, it is most likely to be related to serum protein antioxidants scavenging hydrogen peroxide released by viable neutrophils, and preventing the hydrogen peroxide from crossing the cell membranes of other neutrophils or formation of other reactive oxygen species downstream of hydrogen peroxide to trigger neutrophil extracellular trap production.

Kraemer et al. (62) identified the presence of human beta defensin 1 within platelets and found that platelet stimulation by the alpha-toxin of *S. aureus* triggered the release of human beta defensin 10 and the defensin then powerfully triggered neutrophil extracellular trap formation, perhaps explaining one mechanism through which activated platelets stimulate neutrophil extracellular traps (23).

The extracellular signal-related kinase pathway is also implicated in neutrophil extracellular trap release. Hakkim et al. (49) demonstrated that inhibition of raf-MEK-extracellular signal-related kinase (as well as diacylglycerol and protein kinase C inhibition) blocked phosphorylation of the $p47^{PHOX}$ component of the NADPH-oxidase and subsequent reactive oxygen species generation and neutrophil extracellular trap release. Furthermore, Abi Abdullah et al. demonstrated that *Toxoplasma gondii* triggered extracellular signal-related kinase1/2 mitogen-activated kinase in both human and murine neutrophils to release neutrophil extracellular traps. Inhibition of this pathway abrogated neutrophil extracellular trap formation.

NADPH oxidase activation

Assembly of the NADPH oxidase responsible for the generation of reactive oxygen species during the 'respiratory burst' requires phosphorylation of the four cytosolic subunits (p47-phox, p40-phox, p67-phox and Rac) to enable their combination with cytochrome b_{558} (comprised of membrane-bound gp91-phox and p22-phox) (Fig. 11). Once the NADPH oxidase complex is fully assembled at the vacuolar membrane, it generates superoxide anions (O_2^-) by the single-electron reduction of molecular oxygen. The superoxide then dismutates to hydrogen peroxide, either spontaneously or via the antioxidant enzyme superoxide dismutase. Hydrogen peroxide is capable of diffusing across cell and nuclear membranes where it may form more toxic reactive oxygen species, such as the hydroxyl radical, adjacent to DNA and other vital intracellular and extracellular molecules and structures, causing structural and functional damage (reviewed by Chapple & Matthews) (21). Therefore, the cell possesses several protective antioxidant enzyme systems (including glutathione peroxidase and catalase) to further reduce the hydrogen peroxide into the less toxic species water and oxygen (Fig. 9). However, another hydrogen peroxide-dependent enzyme – myeloperoxidase – is also capable of generating the microbicidal species, hypochlorous acid. Hypochlorous acid functions by chlorinating bacterial targets such as the origin of the replication site for DNA synthesis and membrane proteins, to bring about bacterial death. As discussed later, this sequential four-electron reduction of molecular oxygen to superoxide first, then to hydrogen peroxide, hydroxyl radicals and ultimately to water, can be exploited in order to determine the specific reactive oxygen species trigger for neutrophil extracellular trap release.

The importance of reactive oxygen species for the function of neutrophils is demonstrated in dysfunctional or nonfunctional NADPH oxidase activity, and thus in failure to generate reactive oxygen species. Indeed, individuals with this disorder are highly susceptible to frequent and life-threatening bacterial and fungal infections (28). In a logical, sequential series of studies employing different agonists and antagonists of hydrogen peroxide production, Fuchs et al. (36) made major inroads into elucidating the reactive oxygen species trigger for neutrophil extracellular traps. Using the NADPH-oxidase inhibitor diphenyl iodonium, they first of all demonstrated a significant inhibition of neutrophil extracellular trap production. They then demonstrated that neutrophils from patients with chronic granulomatous disease were unable to generate neutrophil extracellular traps because of the deficient NADPH oxidase enzyme system, but that their ability to produce neutrophil extracellular traps could be restored by the addition of glucose oxidase. This enzyme can substitute for the dysfunctional NADPH oxidase to generate hydrogen peroxide. Glucose oxidase was also used with neutrophils from healthy subjects and was shown to significantly increase and restore neutrophil extracellular trap production following addition of the NADPH-oxidase inhibitor, diphenyl iodonium. In further investigations, catalase was used to remove hydrogen peroxide from the system and a reduction in neutrophil extracellular traps was observed. Conversely, the introduction of 3-amino-1,2,4-triazole to inhibit endogenous catalase activity caused an increase in neutrophil extracellular trap production (36). This work demonstrated that neutrophil extracellular trap production

was not dependent only on reactive oxygen species production but specifically on hydrogen peroxide, which has since been used directly as a stimulus to induce neutrophil extracellular trap release (94). As previously discussed, diphenyl iodonium has also since been used by multiple research groups as a negative control for neutrophil extracellular trap release (7, 44, 135). Significantly, a study utilizing neutrophils derived from patients with X-linked $gp91^{phox}$-deficient chronic granulomatous disease was able to demonstrate that, using gene therapy, replacement of the absent $gp91^{phox}$ subunit could restore the capacity for neutrophil extracellular trap production (12). After 6 weeks the percentage of neutrophils expressing $gp91^{phox}$ had reached 26–29% and the yeast infections (caused by *A. nidulans* conidia) from which patients had been suffering were resolved. This correlated with the ability of patient's neutrophils to produce neutrophil extracellular traps when challenged with *A. nidulans* and phorbol 12-myristate 13-acetate and which were subsequently effective in inhibiting the growth of conidia and hyphae. This effect was abrogated when micrococcal nuclease was introduced (micrococcal nuclease digests the DNA backbone of neutrophil extracellular traps), indicating that the fungicidal activity was caused by neutrophil extracellular trap-DNA release and not by another killing mechanism, such as phagocytosis. In studies utilizing murine neutrophil extracellular traps, a gp91 knockout mouse ($gp91^{-/-}$) was used as a model of chronic granulomatous disease (33). Upon stimulation with 100 nM phorbol 12-myristate 13-acetate the bone marrow-derived mature neutrophils from these mice were incapable of producing reactive oxygen species or neutrophil extracellular traps, although the cells exhibited a flattened appearance, as observed in the early stages of neutrophil extracellular trap production. In fact, phorbol 12-myristate 13-acetate stimulation promoted cell survival during the 16-h observation period in this knockout strain.

Given the ubiquitous nature of hydrogen peroxide we postulated that the major trigger for neutrophil extracellular traposis was likely to reside downstream of hydrogen peroxide within the reactive oxygen species generation chain (99). In work published in early 2012 (100), and using the scheme of inhibitors and agonists of NADPH-oxidase and associated downstream species illustrated in Fig. 9, we confirmed that:

- Diphenyl iodonium did indeed inhibit neutrophil extracellular trap formation.
- Superoxide dismutase enhanced neutrophil extracellular trap release by increased formation of hydrogen peroxide.
- Activating the glutathione peroxidase system by supplementation with N-acetyl cysteine (which increases intracellular reduced glutathione/GSH levels) reduced neutrophil extracellular trap formation.
- Catalase addition also reduced neutrophil extracellular trap formation.

However, we were unable to reproduce the results of Fuchs et al. (36) with the catalase inhibitor, 3-amino-1,2,4-triazole, using our experimental systems. Indeed, we found that 3-amino-1,2,4-triazole inhibited, rather than enhanced, neutrophil extracellular trap release and we postulated that 3-amino-1,2,4-triazole had a broader spectrum of activity than just catalase inhibition, and that 3-amino-1,2,4-triazole also inhibited myeloperoxidase. We demonstrated significant and substantial knock down of myeloperoxidase generation by peripheral blood neutrophils and proposed hypochlorous acid, which is the product of myeloperoxidase activity on hydrogen peroxide, as the major trigger for neutrophil extracellular traposis. Indeed, we were able to demonstrate that hypochlorous acid stimulated dramatic neutrophil extracellular trap release in as short a time as 60–70 min (threefold higher than stimulation with phorbol 12-myristate 13-acetate) and that a more specific inhibitor of myeloperoxidase, 4-amino-benzoic acid hydrazide, significantly reduced neutrophil extracellular trap formation. We were able to induce neutrophil extracellular traps from neutrophils derived from patients with chronic granulomatous disease (with no active NADPH-oxidase), using physiologically relevant concentrations of hypochlorous acid, proving an important role for hypochlorous acid in neutrophil extracellular traposis (Fig. 12). Finally, the hypochlorous acid scavenger taurine (present in millimolar concentrations in neutrophils) inhibited hypochlorous acid-stimulated neutrophil extracellular traps completely after 20 min, and also inhibited phorbol 12-myristate 13-acetate-stimulated neutrophil extracellular traps after 200 min and rescued the neutrophils from programmed cell death (100). Interestingly, at the same time as our studies, Metzler et al. (88) also demonstrated the requirement for myeloperoxidase in neutrophil extracellular trap formation, but did not continue to the level of hypochlorous acid activity.

Peptidyl arginine deiminase activation

Activation of peptidyl arginine deiminase-4 is necessary for production of neutrophil extracellular traps (36) and decondenses the nuclear chromatin by replacing positively charged arginine and methylarginine amino acids in the histone protein backbone

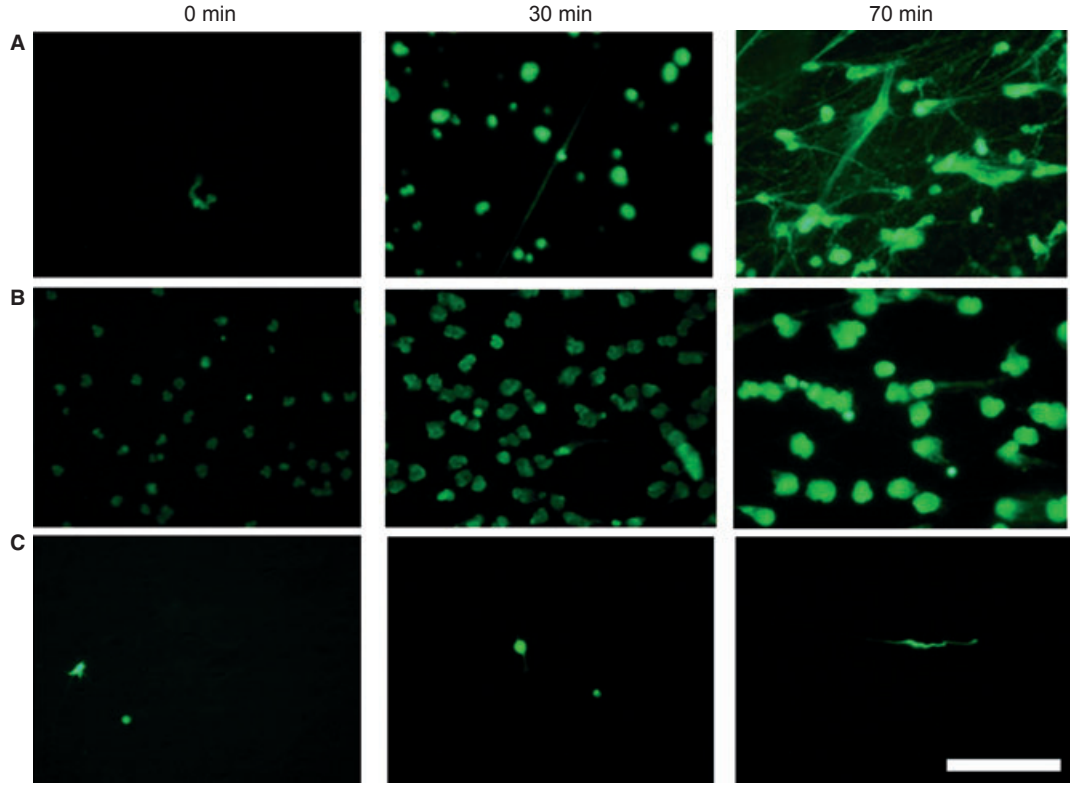

Fig. 12. Time-course fluorescence photomicrographs demonstrating Sytox® Green-stained DNA from neutrophils stimulated with (A) 0.75 mM hypochlorous acid, (B) 1% Triton X-100 (to induce necrosis) or (C) phosphate-buffered saline (negative control). Triton X-100 lysed cells immediately at time 0 and the resulting DNA released by neutrophils demonstrated no neutrophil extracellular trap-like structures. However, hypochlorous acid produced typical neutrophil extracellular traps in under 70 min. Phosphate-buffered saline had no effect. Based on Palmer et al. (100).

with neutrally charged citrulline residues. The process is called 'deimination' or 'citrullination', and the loss of charge facilitates the unfolding of the chromatin. Peptidyl arginine deiminases are a family of Ca^{2+}-dependent enzymes of which there are five: peptidyl arginine deiminases 1–4 and peptidyl arginine deiminase-6. Peptidyl arginine deiminase-4 is also referred to as peptidyl arginine deiminase-I (the gene encoding peptidyl arginine deiminase-4) or peptidyl arginine deiminase-V (91) as it originally appeared to be different from the peptidyl arginine deiminase-4 of rats. Peptidyl arginine deiminase-1 is found in epidermis and uterus; peptidyl arginine deiminase-2 is found in many tissues, including epithelial tissue, muscle, brain and hemopoietic cells; peptidyl arginine deiminase-3 is found in hair follicles and epidermis; and peptidyl arginine deiminase-4 is found in granuloctyes. Peptidyl arginine deiminase-4 is thought to localize to the tertiary granules of neutrophils (125) and effects large-scale histone deimination by acting predominantly on histones H3 and H4 (137), the presence of DNA-associated H3 and H4 associated with extracellular DNA being pathognomonic of neutrophil extracellular trap release. Indeed, Neeli et al. (93) reported that histone H3 is the major product of peptidyl arginine deiminase-4 activation in neutrophils and is not associated with apoptosis, more with neutrophil activation by inflammatory stimuli, probably via the nuclear factor-kappaB pathway. Peptidyl arginine deiminase-4 activation was shown to be essential for neutrophil extracellular trap release in calcium ionophore-stimulated neutrophil-like HL60 cells, as pretreatment with Cl-amidine, a peptidyl arginine deiminase-4 inhibitor, eradicated all neutrophil extracellular trap production (137). However, the role of peptidyl arginine deiminase-4 in primary human neutrophils remains to be fully tested and Papayannopoulos et al. (102) proposed an alternative: peptidyl arginine deiminase-independent mechanism of neutrophil extracellular trap formation. They demonstrated that primary granule-derived neutrophil elastase enters the nucleus during neutrophil extracellular trap formation and partially degrades certain histones, and then at a later stage myeloperoxidase derived from the same granules binds to chromatin and further decondenses the chromatin, independently of its enzymatic behavior. This series of events is proposed to be triggered by reactive oxygen species generation.

Actin cytoskeletal and microtubule function

Recent investigations have examined the role of actin and microtubules in neutrophil extracellular trap release (94). Pretreatment with the tubulin polymerization inhibitor 'nocodazole' resulted in a reduction in lipopolysaccharide-stimulated neutrophil extracellular trap release, from 30% to 5%. Pretreatment with the actin polymerization inhibitor, cytochalasin D, did not inhibit the disintegration and mixing of the nuclear and granule compartments, but only 9% of cells extruded neutrophil extracellular traps. Notably, some of the remaining cells swelled to twice their normal diameter, indicating that actin may be necessary for neutrophil extracellular trap extrusion. As integrins are receptors responsible for transmitting extracellular signals to the cytoskeleton, the Mac-1 integrin (on the neutrophil cell membrane; Fig. 3) was also inhibited using a monoclonal antibody. Data indicated that this inhibition resulted in a significant decrease in neutrophil extracellular trap production and in a 51% reduction in histone citrullination (94), and it was proposed that the actin cytoskeleton may transmit signals from the neutrophil cell surface to the nucleus to effect histone deimination. As for the role of the microtubules, it seems plausible that they transport vital granular proteins, including peptidyl arginine deiminase-4, neutrophil elastase and myeloperoxidase, to the nucleus, a process mediated by reactive oxygen species production, and either peptidyl arginine deiminase-4 and/or elastase and myeloperoxidase conspire to decondense the nuclear histones preneutrophil extracellular trap release following nuclear membrane disintegration. Neeli et al. (94) proposed that the cytoskeleton positions the nucleus relative to the plasma membrane, and that a co-ordinated interplay between the cytoskeleton and microtubules may play a role in the temporal and spatial dynamics that are necessary for neutrophil extracellular trap release.

Functional roles of neutrophil extracellular traps

DNA neutrophil extracellular trap-like defences in plants: an evolutionary tale

There is evidence that the protective antimicrobial role of DNA is an ancient and highly conserved strategy based on studies of plant root-tip pathology. The root tip is vital to health as it derives nutrients and water from the soil to support the life cycle of the plant. However, root-tip infections are rare, and this is attributed, in part, to the production of a mucilaginous or 'slime-like' matrix around the root cap. In a fascinating study carried out by Wen et al. (140) extracellular DNA was found to be a major component of that root-cap slime, and its digestion by DNases resulted in infection of the root tip with an inoculated fungal pathogen. Prior to DNase digestion of the root-tip slime, fungal organisms were unable to contact the root tip and a zone of inhibition of fungal growth was observed. This was lost upon DNase treatment, resulting in root-tip infection. In a recent review, Hawes et al. (50) discuss how the release of extracellular DNA from root tips may form an innate immune process and it may therefore be that neutrophil extracellular traps have a long-standing and highly conserved evolutionary base within plant immunobiology.

Extracellular microbial killing by neutrophil extracellular traps

The most obvious *in-vivo* role of neutrophil extracellular traps in higher organisms is to combat microbial pathogens. Indeed, neutrophil extracellular traps were originally shown to be effective against gram-negative *Escherichia coli* (42) and *Shigella flexneri* and against gram-positive *S. aureus* (14), and have since been shown to be effective in combating a full range of pathogens, including intracellular parasites such as *T. gondii* (1) and fungi such as *C. albicans* (128). As neutrophil extracellular traps are large extracellular structures it is logical that they are effective in attacking pathogens several times larger than the neutrophil itself, such as fungal hyphae. The antimicrobial actions of neutrophil extracellular traps comprise two main phases, which include:

- Trapping and immobilizing of pathogens to prevent tissue and systemic spread.
- Pathogen killing by neutrophil extracellular trap-embedded cathelicidin antimicrobial peptides.

Those pathogens that stimulate neutrophil extracellular trap release but remain viable whilst trapped (Figs. 4 and 10) include the intracellular bacterium *Mycobacterium tuberculosis* (109), the intracellular parasite *E. bovis* (7), blastospores of the fungus *C. albicans* and the bacterium *S. aureus* (87). In these infections, the neutrophil extracellular trap functions to prevent the spread of the invading pathogen, even though no cytotoxic effect is observed (7). Clearly this immobilization could subsequently facilitate killing by other immune cells, such as macrophages.

Pathogens reported to be destroyed extracellularly within neutrophil extracellular traps include the parasite *T. gondii* (1), the hyphal and yeast forms of *C. albicans* (128), and the bacteria *S. aureus*, *S. flexneri* and Group A streptococcus (14, 67). Some doubt has, however, been cast with regard to the reliability of these reports by Menegazzi et al. (87) and Parker et al. (105), as it was reported that viability was unaffected after 90 min of exposure to neutrophil extracellular traps. In their studies DNase was used to digest the neutrophil extracellular traps and free the pathogens, and Menegazzi et al. (87) implied that the mechanical disruption and washing utilized in most other studies was inappropriate. Whilst it is possible that neutrophil extracellular trap fragments remaining around the microorganisms are sufficient to inhibit growth of certain pathogens under certain conditions, for other pathogens, such as *L. amazonensis*, neutrophil extracellular trap fragments have been proven to be as cytotoxic as intact neutrophil extracellular traps (44). Furthermore, both *M. tuberculosis* and *Listeria monocytogenes* were trapped within neutrophil extracellular traps, but when assayed in parallel using identical experimental protocols only *L. monocytogenes* was killed (109).

Further studies have shown that certain pathogens are susceptible to killing by isolated components of neutrophil extracellular traps and better characterization of neutrophil extracellular trap components may identify novel targeted therapeutic antimicrobials. Indeed, *C. albicans* has now been shown to be killed within neutrophil extracellular traps by calprotectin derived from neutrophil granule extracts (129). When investigating the precise agents responsible for the killing of neutrophil extracellular trap-entrapped pathogens, Parker et al. (105) also demonstrated that *S. aureus* was killed by myeloperoxidase-generated hypochlorous acid, but that it required the addition of the myeloperoxidase substrate, hydrogen peroxide. They recorded the proportion of myeloperoxidase released from 'neutrophil extracellular trapping'neutrophils to be ~30% of the total myeloperoxidase content of the neutrophil and determined almost all of the extracellular myeloperoxidase to be bound to neutrophil extracellular traps and in an active form. Notably, the substrate for myeloperoxidase, hydrogen peroxide, appeared to be lacking in *in-vitro* experiments but it is understood that *in-vivo* hydrogen peroxide would be generated by ongoing neutrophil activation. One report has, however, described the presence of NADPH oxidase within neutrophil extracellular traps (90), but agreed that it was unlikely to be functional as the enzyme requires a cross-membrane potential (25). The authors did, however, provide some evidence of extracellular myeloperoxidase activity which was partially DNase sensitive, suggesting that this may be acting whilst situated within neutrophil extracellular trap structures, citing a subpopulation of neutrophils that had not formed neutrophil extracellular traps as a possible source of hydrogen peroxide. Interestingly, the antimicrobial proteins, especially LL37, that adhere to neutrophil extracellular trap strands also appear to serve a secondary function in protecting the DNA from degradation by DNase (65).

Antimicrobial activity of histones

It has been proposed that histones, which are a core component of the neutrophil extracellular traps (along with the DNA strands), accounting for 70% of its total protein content (129), can exert antimicrobial effects (54) alongside the cathelicidin antimicrobial peptides that also localize within neutrophil extracellular trap structures. As described previously, the primary function of histones is to package DNA as compactly as possible (i.e. to enable 2 m of DNA to be packaged within a 5-μm-diameter nucleus) but during the process of neutrophil extracellular trap release the chromatin decondensation (36) allows greater exposure of the histone proteins (71). Antibody neutralization of the histone component of neutrophil extracellular traps has been shown to improve recovery of viable *S. aureus* and *S. flexneri* from neutrophil extracellular traps. This effect may be in part a result of reduced bacterial trapping by histone interactions but it is also likely to be a result of reduced bacterial killing as (i) purified histone was demonstrated to be cytotoxic on its own (14) and (ii) antibody neutralization of purified histone has been shown to negate its cytotoxic effect (44). One of the four core histone subunits, purified H2A, has previously found to be effective at killing both gram-negative (*S. flexneri* and *Salmonella typhimurium*) and gram-positive (*S. aureus*) bacteria at a relatively low concentration of 2 μg/ml (14). In addition, our recent data have revealed purified mixed histone to be effective in reducing the growth of over two-thirds of periodontal bacterial species tested (99). Indeed, 62% of *L. amazonensis* promastigotes (the form in which the parasite is transferred to the host by the insect vector and responsible for leishmaniasis), are also killed by purified histone H2A at 20 μg/ml (44). Furthermore, in the context of neutrophil extracellular traps, when histones were neutralized by an anti-histone antibody, the survival of *L. amazonensis* increased by 42%. Even when the neutrophil extracellular traps

were digested using DNase, the fragments were still effective at killing the promastigotes, and addition of anti-histone Ig increased promastigote survival 7.5-fold, indicating that, again, the effect was probably mediated by the antimicrobial actions of histones within the remaining DNA fragments.

Further highlighting the pathogen specificity of the antimicrobials associated with neutrophil extracellular traps, it has been demonstrated that *C. albicans* was resistant to both purified histone H2A and mixed histone at concentrations as high as 200 μg/ml in both its yeast and hyphal forms (128). As a positive control the histone used was proven to be effective against *S. flexneri* and *S. aureus*, as previously reported by Brinkmann et al. (14). Those data indicate that although histones are highly conserved proteins with a broad cytotoxic range, the role of histones within neutrophil extracellular traps is pathogen specific.

The cytotoxic effect of neutrophil extracellular traps is not limited to foreign pathogens as the host's own endothelial and epithelial cells have been shown to be susceptible to neutrophil extracellular traps and their DNase-generated neutrophil extracellular trap fragments (115). This damaging effect of neutrophil extracellular traps has been at least partially attributed to histones through experiments using anti-histone blocking antibodies and by neutralization using the negatively charged histone-binding glycan, polysialic acid (115).

Microbial evasion of neutrophil extracellular traps

For every protective immune process, as a result of the host–pathogen co-evolutionary arms race, pathogens develop virulence traits to facilitate their evasion of host-defense mechanisms. Neutrophil extracellular traps are no exception to this phenomenon, as detailed in Table 2.

Microbial surface modifications

A relatively simple method employed by pathogens to subvert neutrophil extracellular trap-killing is to ensure that they stimulate the release of fewer neutrophil extracellular traps. Group B streptococci have been found to utilize molecular mimicry to reduce the degree to which neutrophils are activated, including limiting their stimulation of neutrophil extracellular trap release. Sialylated polysaccharides are subsequently presented on the bacterial capsule to mimic the host's own sialic acids and engage Siglec-9 on the neutrophil surface to dampen the immune response, thereby improving their chances of survival (19). Hydrophobin protein 'HypA' expression is also expressed on the surface of the fungus *Arthroderma benhamiae* and this has been shown to modulate neutrophil extracellular trap release as well as to reduce dendritic cell stimulation and cytokine release. Ultimately this trait has been shown to confer increased survival *in vitro* at least (51).

Some bacteria appear to be more efficient at stimulating neutrophil extracellular trap release; however, these same bacteria demonstrate increased survival within the neutrophil extracellular traps (77, 115, 131). It is possible that in these instances the detrimental effect of excess neutrophil extracellular traps upon the host or on co-infecting microbes may provide benefit in terms of growth advantage for the pathogen. An example of this is group A streptococci, which express the M1 virulence factor protein that stimulates a significantly higher level of neutrophil extracellular traps compared with M1 knockout strains (67). However, this same protein also protects the bacteria from extracellular LL37-associated neutrophil extracellular trap killing by binding and sequestrating LL37 via its N-terminal domain. A further example of microbes increasing neutrophil extracellular trap stimulation, but exhibiting decreased neutrophil extracellular trap killing, is demonstrated by the tropical yeast *Cryptococcus gattii*, which expresses extracellular fibrils in its encapsulated form in order to do this (122). Avoidance of trapping by neutrophil extracellular traps is a further strategy employed by pneumococci and conferred by the expression of a polysaccharide capsule (139). This highlights a novel function of a well-established virulence trait, as encapsulated pneumococci demonstrate between 4- and 12-fold less neutrophil extracellular trap trapping than do nonencapsulated strains. D-alanylation of lipoteichoic acids on cell walls is a further adaptation of the microbial surface by gram-positive bacteria to evade neutrophil extracellular trap killing. This process is encoded by the *dlt* operon and is demonstrated to increase the virulence of *S. pneumoniae* (139) as a result of the positive charge introduced by incorporation of D-alanine. Although *S. pneumoniae* appeared to be resistant to killing within neutrophil extracellular traps, regardless of capsule expression, deletion of the *dltA* operon, and therefore the capacity for D-alanylation of lipoteichoic acids, resulted in them being out-competed by the wild-type strain in a mouse model of pneumococcal pneumonia.

Table 2. Neutrophil extracellular trap evasion mechanisms

Neutrophil extracellular trap evasion mechanism	Example	Microbial species	Effect	References
Surface modification	D-alanylated lipoteichoic acid	*S. pneumoniae*	↑survival	(139)
	Lipooligosaccharide moieties promote biofilm formation	*H. influenzae*	↑survival	(56)
	Hydrophobin on surface	*A. benhamiae* conidia (filamentous fungi)	↓neutrophil extracellular trap induction and ↑survival	(51)
Surface structures	Capsule	*S. pneumoniae*	↓trapping	(139)
	Extracellular fibrils	*C. gattii* (yeast)	↑neutrophil extracellular trap induction but ↑survival	(122)
Molecular mimicry	Sialylated capsular polysaccharide engages neutrophil Siglec9	Group B *Streptococcus*	↓neutrophil extracellular trap induction	(19)
Inhibition of antimicrobial peptides	M1 protein protects against cathelicidin LL37	M1 group A streptococci	↑neutrophil extracellular trap induction but ↑survival	(67)
Extracellular DNase	Spd, spd3 and particularly sdaD2 secretion	M1 group A streptococci	↑survival (*in vitro*) and ↑virulence (*in vivo*)	(123)
	Sda1 secretion	Group A streptococci	↓neutrophil extracellular trap integrity and ↑survival (*in vitro*) ↑virulence (*in vivo*)	(17)
	EndA surface expression	*S. pneumoniae*	↓trapping (*in vitro*) ↑dissemination (*in vivo*)	(8)
	Sda1 secretion	M1T1 group A streptococci	↓neutrophil extracellular trap integrity (*in vitro*) and ↑virulence (*in vivo*)	(136)
	Nuclease secretion	*S. aureus*	↓trapping (*in vitro*) ↑*S. aureus* in lung and ↑rate of mortality (*in vivo*)	(10)
	Secreted and surface expression of nuclease	Periodontal bacteria	↓neutrophil extracellular trap integrity	(101)

The gram-negative nontypable bacteria *Haemophilus influenzae*, a common cause of otitis media, was also shown to be able to escape killing by phorbol 12-myristate 13-acetate- or nontypable *H. influenzae*-induced neutrophil extracellular traps. Notably, mutants that exhibited alterations in the lipooligosaccharide cell-wall component were found to be more susceptible to killing (56, 58). While neutrophil extracellular trap survival was associated with the ability of *H. influenzae* to exist in a biofilm in a chinchilla model of otitis media, the same mutations of the lipooligosaccharides that rendered the bacteria susceptible to neutrophil extracellular trap killing also impaired biofilm formation (124). This suggests that there may be an important balance between avoidance of host-immune responses and the population of the ecological niche.

Extracellular DNase production in neutrophil extracellular trap evasion

Although the cathelicidin antimicrobial peptides of neutrophil extracellular traps undoubtedly serve an important function, it is the DNA component that forms the structural backbone, and neutrophil extracellular traps can therefore be destroyed/dismantled by DNAses. Although bacteria have previously been reported to express DNases (54), this function was

never properly appreciated until more recently. Since the discovery of neutrophil extracellular traps, several bacteria have been found to express extracellular DNases, which are effective against the decondensed mammalian chromatin of neutrophil extracellular traps, including many periodontal pathogens (101). Unsurprisingly, bacteria expressing DNases demonstrate increased neutrophil extracellular trap survival *in vitro,* which also associates with increased virulence and disease severity *in vivo* (8, 10, 17). For example, EndA is a DNase expressed on the surface of *S. pneumoniae,* which is the primary cause of community-acquired pneumonia (8). While mice infected in the upper respiratory tract with EndA-expressing wild-type bacteria had a 20% lower survival rate 65-h postinfection than did those infected with the EndA deletion mutant, the EndA deletion mutant of strain TIGR4 (serotype 4), unlike the wild-type strain, failed to disseminate into the lungs and bloodstream. This data indicated that the neutrophil extracellular traps were important in limiting bacterial dissemination. Buchanan et al. (17) have examined the DNase Sda1 expressed by M1 serotype group A streptococci. The Sda1 deletion mutant exhibited increased susceptibility to neutrophil killing *in vitro*, and in an *in-vivo* mouse model of necrotizing fasciitis subsequently demonstrated significantly less virulence than the wild-type strain. Notably, the addition of G-actin to inhibit DNase expression in the wild-type strain also reduced its virulence in the mouse model. Significantly, exudates of abscesses from the infected mice showed higher levels of neutrophil extracellular traps remaining in infections with the Sda1 mutant compared with infections with the wild-type strain. Walker et al. (136), also studying group A streptococci, found that expression of Sda1 by the rare, but invasive, M1T1 serotype paradoxically provided a selection pressure for a shift in the bacterial population to the highly invasive phenotype. Natural selection resulted in those bacteria expressing the protease SpeB, which degrades the DNase Sda1 that is trapped in neutrophil extracellular traps, whereas those bacteria able to evade neutrophil extracellular traps and increase in number were found not to express SpeB. Indeed, decreased SpeB expression is a mechanism known to correlate with invasive disease and part of the shift to a highly invasive phenotype. In a further study examining the neutrophil extracellular trap-related survival advantage of DNase expression by M1 serotype group A streptococci, all three putative secreted DNase-encoding genes (one chromosomal and two prophage encoded) were targeted for deletion to create a range of mutants (123). The varying degrees of skin-lesion frequency and severity resultant from these mutants in the mouse model further demonstrated the importance of DNase expression in contributing to bacterial virulence. In agreement with this was the increased extracellular killing by neutrophils (assumed to occur through neutrophil extracellular trap release) observed in an *in-vitro* assay using the mutant strains compared with the wild-type strain.

In-vivo studies using a mouse model intranasally infected with *S. aureus* demonstrated that the expression of DNase was associated with a 10-fold increase in bacterial load within lung tissues 24 h after administration of a sublethal dose and with an increased rate of mortality after administration of a lethal dose. Further analysis demonstrated that the wild-type DNase producing strain was significantly more resistant to extracellular killing by activated neutrophils compared with the knockout strain owing to decreased trapping (10), further demonstrating the importance of expression of bacterial DNase in disease pathogenesis.

Neutrophil extracellular trap functions in innate and adaptive immune responses

While neutrophil extracellular traps are understood to be key to localizing and concentrating the neutrophils' own cathelicidin antimicrobial peptides released from their granules, neutrophil extracellular traps have also been reported to aid the antimicrobial activity of other neutrophils not undergoing neutrophil extracellular traposis (e.g. by the destruction of microbial virulence factors) (14). Indeed, certain reports have proposed that neutrophil extracellular traps are also an adjuvant to phagocytosis, which is a primary role of neutrophils. Brinkmann et al. (14) found that the invasion plasmid antigen B of *S. flexneri*, a virulence factor that enables bacterial escape from the phagosome (43), was degraded within neutrophil extracellular traps by neutrophil elastase before phagocytosis. Under certain circumstances it has also been proposed that neutrophil extracellular trap release acts as an alternative to phagocytosis because milk proteins have been reported to inhibit this process in bovine neutrophils while neutrophil extracellular trap release remained unaffected (73).

The direct extracellular destruction of the bacterial virulence factor, alpha-toxin, an exotoxin causing host-cell lysis that is produced by *S. aureus* (11), has also been demonstrated by neutrophil extracellular

trap-associated proteases (14). *In vivo* this mechanism could function to enable other neutrophils to migrate to the site of infection and destroy the bacteria without the risk of being lysed. In one of the most surprising examples of host–microbe co-operation involving one bacterial species against another, neutrophil extracellular traps have also been found to be stimulated by, and also to provide a surface for, the binding of σ-toxin, an antimicrobial agent secreted by *Staphylococcus epidermidis*, and thus allow more efficient killing of group A streptococci (24). An alternative interpretation is that *S. epidermidis* employs this strategy to confer a colonization advantage over group A staphylococci, using neutrophil extracellular traps as a mechanism to achieve this.

Neutrophil extracellular traps also interact with the complement system, which comprises a series of serum proteins that function to opsonize foreign pathogens and to bring about their cell lysis via the membrane attack complex. Neutrophil extracellular traps activate complement *in vitro* via the binding of C1q to neutrophil extracellular trap DNA, which initiates activation of the classical pathway. Activation was measured by the consumption of C3 and C4, the generation of C5a and decreased hemolytic activity of sera after neutrophil extracellular trap release (69). With regards to the specific/acquired immune system, neutrophil extracellular traps have also been shown to prime T-cells (127). Indeed both $CD4^+$ and $CD8^+$ T-cells exposed to neutrophil extracellular traps showed increased proliferation and cytokine (interferon-gamma) release when co-cultured with dendritic cells, a stimulus that would not usually evoke any response. Further analysis indicated that this response required cell-to-cell contact and was not mediated by soluble factors. Even in the absence of any other stimuli, neutrophil extracellular trap-primed $CD4^+$ T-cells also showed signs of activation by clustering and up-regulation of surface markers, such as CD25. Neutrophil extracellular trap-priming of T-cells also appeared to occur via the T-cell receptor, as inhibition of T-cell receptor-mediated signaling significantly reduced the priming effect.

Neutrophil extracellular traps in health and disease

Table 3 highlights a range of diseases and physiological processes that have been associated with neutrophil extracellular trap activity. Current research has identified direct clinical links between neutrophil extracellular traps and certain pathologies; however, for other diseases the research is at a much earlier exploratory stage with reports only highlighting studies in *in-vitro* or animal models. In general, diseases that associate with neutrophil extracellular traps are a result of either the interaction of neutrophil extracellular traps with bacteria, some of which we have described above, or the relationship between neutrophil extracellular traps and other arms of the host immune response, which potentially lead to an aberrant chronic inflammatory response. Below we will discuss some of the neutrophil extracellular trap-associated diseases highlighted in Table 3 and what is currently known about the potential mechanisms involved in the pathogenic process.

From the data discussed above (and presented in Tables 2 and 3) in relation to how bacteria may evade capture and killing by neutrophil extracellular traps through surface modifications or DNase expression, it is quite clear that appropriate functioning of neutrophil extracellular traps is an important facet of the immune response. Indeed, in further support of this, while previously the lack of reactive oxygen species was believed to be responsible for susceptibility to infection in patients with chronic granulomatous disease, our recent work (100), and that of others, now also implicates defective neutrophil extracellular trap production as being involved in infection susceptibility in these patients. In addition, recent work has also demonstrated that neutrophil extracellular trap formation is impaired in neonates and this may also predispose newborn infants to infection (142). While the decoration of neutrophil extracellular traps with granule proteins is essential to this main function of bacterial destruction, it is not difficult to foresee how this concentration of cytotoxic proteins could cause host collateral tissue damage, in particular if these structures are not cleared efficiently. Indeed, the cytotoxic effect of neutrophil extracellular traps has already been demonstrated against both epithelial and endothelial cells (115, 131), and neutrophil extracellular traps have been found to damage the actin cytoskeleton of enterocyte-like cells (77). The extracellular presence of large amounts of neutrophil cellular contents, including nuclear DNA, are also reported as being immunogenic and therefore have the potential to drive chronic inflammatory diseases. Recent studies have also now shown that mitochondrial DNA, which is also reported to exist in some forms of neutrophil extracellular traps (as described above), can lead to chronic inflammation, resulting in significant tissue damage and ultimately leading to organ failure (78). Indeed, neutrophil extracellular traps are also known to be a rich source of autoantigens, including

Table 3. Neutrophil extracellular trap-associated conditions

Location/condition	Pathogen	Species	*In vitro*/*in vivo*	References
Experimental dysentery	*Shigella*	Rabbit	*In vivo*	(14)
Spontaneous appendicitis		Human	*In vivo*	(14)
Pre-eclampsia		Human		(45)
Insemination (reproductive tract)		Equine	*In vitro*	(2)
Necrotizing fasciitis	Group A streptococci	Murine		(17)
Mastitis		Bovine		(73)
Blood sepsis		Human	*In vitro*	(23)
Kidney		Zebra fish		(98)
Malaria	*P. falciparum*	Human		(5)
Tuberculosis	*M. tuberculosis*	Human		(109)
Septic arthritis (synovial fluid)		Human	*In vivo*	(74)
Leishmaniasis	*L. amazonensis* (parasite)	Human	*In vivo* and *in vitro*	(44)
Small-vessel vasculitis (kidney)		Human	*In vivo* kidney and *in vitro*	(61)
Periodontitis		Human	*In vivo*	(132)
Aspergillosis	*A. nidulans* conidia (fungi)	Human	*In vitro*	(12)
Otitis media	*S. pneumoniae*	Chinchilla	*In vivo*	(110)
	H. influenzae	Chinchilla	*In vivo*	(36)
Feline leukemia virus	*L. amazonensis*	Feline	*In vitro*	(138)
Eimeria bovis parasitosis	*E. bovis* sporozoites	Bovine	*In vitro*	(7)
Repiratory disease	*M. haemolytica*	Bovine	*In vivo*	(4)
Deep-vein thombosis		Baboon	*In vivo*	(37)
		Murine	*In vivo*	(134)
Systemic lupus erythematosus		Human	*In vitro*	(39)
Lung inflammation	Influenza A	Murine	*In vivo*	(52)
	Lipopolysaccharide-induced	Murine	*In vivo*	(115)
Pneumonia	*P. aeruginosa*	Murine	*In vivo*	(30)
Cystic fibrosis		Human	*In vivo*	(76)
		Human	*In vivo*	(103)
Transfusion-related acute lung inflammation	Lipopolysaccharide then major histocompatibility complex class I antibody	Murine	*In vivo*	(126)
	Activated platelets	Human	*In vitro*	(20)
Cerebrovascular inflammation		Murine	*In vitro*	(3)
Rheumatoid arthritis		Murine	*In vivo*	(126)
		Murine	*In vivo*	(112)
Atherosclerosis		Murine	*In vivo*	(29)
Enterocyte damage	Entervirulent *E. coli*	Human	*In vitro*	(77)

proteinase-3 and myeloperoxidase, and peptidyl arginine deiminase enzymes can also be released during neutrophil extracellular traposis (as described earlier). Peptidyl arginine deiminase enzymes have the potential to generate additional deiminated autoantigens (e.g. histones) (31, 61). The failure to effectively clear neutrophil extracellular traps, or their excessive production (68), regardless of whether they are nuclear or mitochondrial derived, could clearly therefore have significant detrimental effects on human health.

Leffler et al. (69) recently reported that increased levels of anti-DNA and anti-histone auto-antibodies in the sera of patients with systemic lupus erythematosis, which is characterized by high levels of extracellular self-DNA, are proposed to originate from neutrophil extracellular traps (113). This hypothesis was further supported by the release of elevated levels of resting and stimulated neutrophil extracellular traps from the neutrophils of patients with systemic lupus erythematosis (39, 65). The clinical relevance of this finding was also demonstrated by the correlation between an impaired ability of sera to degrade neutrophil extracellular traps with periods of active disease (48). The mechanism of this impaired DNase activity was further explored and was found to be a combination of both autoantibodies and complement C1q binding of neutrophil extracellular traps, which subsequently blocked the action of DNase. Notably, DNase I activity has been identified in healthy human serum and has been shown to be capable of efficient degradation of neutrophil extracellular traps, which was inhibited by G-actin, EDTA (which chelates the required DNase I cofactor, Ca^{2+}) and also by a DNase-binding antibody (49). The impaired DNase activity in sera from patients with systemic lupus erythematosis was only partially restored by the addition of exogenous DNase, indicating that this deficiency was also caused by the presence of serum inhibitors. To explore, in greater detail, the effect of uncleared neutrophil extracellular traps in this context, antigen-presenting plasmacytoid dendritic cells were exposed to these neutrophil extracellular traps. In this respect, either a primary human plasmacytoid dendritic cell line and analysis of supernatant cytokine levels (39, 65), or a plasmacytoid dendritic cell line and detection of mRNA levels (131) (where a significant elevated production of interferon-alpha was detected), were used. Dendritic cell interferon-alpha production is known to contribute to systemic lupus erythematosis pathogenesis (113), and interferon-alpha is known to prime for neutrophil extracellular trap production (80). These cyclic interactions could therefore explain the chronicity observed in this autoimmune disorder. In another autoimmune disorder, psoriasis, host DNA has been shown to be more readily taken up by dendritic cells when in complex with the granule protein, LL37, resulting in the stimulation and the release of greater levels of interferon-alpha compared with exposure to either LL37 or DNA alone (64). Neutrophil extracellular traps also significantly stimulated dendritic cell interferon-alpha via intracellular toll-like receptor-9 activation (65), and other neutrophil granule proteins were shown to be able to amplify the effect of LL37 on DNA entry into dendritic cells but were ineffective when used in isolation. These findings help inform a hypothesis whereby periodontitis and rheumatoid arthritis may be causally linked (Fig. 13) and will be discussed later.

DNase activity has indeed been demonstrated as being beneficial in several pathological and physiological processes. In cystic fibrosis, uncleared neutrophil extracellular trap DNA is believed to significantly underpin the gel-like excess mucous and elastase-containing structures present within the airways (76). In a clinical trial examining the daily administration of aerosolized recombinant human DNase, a reduction in symptoms was observed (103). Indeed, it is thought that the webs formed by neutrophil extracellular traps impair the ciliary clearance of mucous from the airways, and treatment with DNase reduced the viscocity of the mucous, enabling its removal and providing relief of patients' symptoms. Studies in horses have demonstrated that insemination evokes an acute inflammatory-like response in the female reproductive tract, including the release of neutrophil extracellular traps in response to spermatozoa (2). Subsequently, DNase was identified in equine seminal plasma, which reduced the number of neutrophil extracellular traps *in vitro* and improved spermatozoa motility, which was subsequently assumed to improve fertility. Seminal plasma was also found to degrade neutrophil extracellular traps induced by spermatozoa, but did not affect neutrophil clearance of bacteria, and these data indicated that there may be specificity that enhances fertility without compromising defence against infection. DNase activity has subsequently been detected in swine and human seminal plasma at similar levels to those found in horses, further supporting the proposal that the host's capacity to clear neutrophil extracellular traps from the reproductive tract is important in fertility.

The role of neutrophil extracellular traps in facilitating other immune processes may also play a significant role in disease pathogenesis. The contact system comprises a set of serine proteases and a bradykinin precursor, which require a negatively

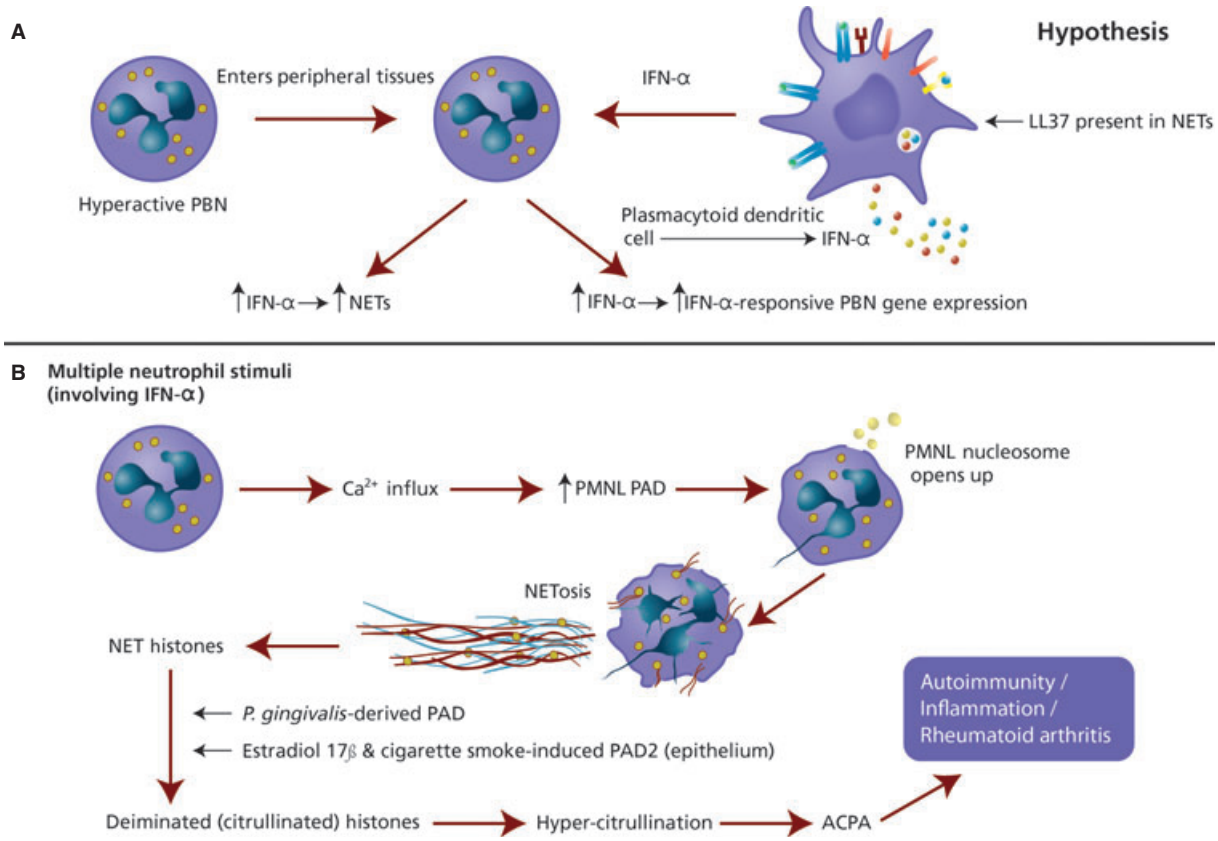

Fig. 13. (A) **Role of interferon-alpha when released by dendritic cells in response to LL37 (present within neutrophil extracellular trap structures) in neutrophil extracellular trap release. Interferon-alpha primes neutrophils for further release of neutrophil extracellular traps upon stimulation with microbial products, cytokines, reactive oxygen species or advanced glycation end-products present within periodontal tissues. (B) Hypothesis whereby citrullinated histones released during neutrophil extracellular traposis may be hypercitrullinated by *Porphyromonas gingivalis* peptidyl arginine deiminase or by the increased expression/release of peptidyl arginine deiminases induced by estradiol-17β or cigarette smoke. Such hypercitrullination generates autoantibodies to citrullinated proteins, or molecular mimicry by *P. gingivalis* proteins that have been autocitrullinated by peptidyl arginine deiminase generates anti-citrullinated protein antibodies, which in turn drive a breach in immune tolerance and trigger rheumatoid arthritis (adapted from de Pablo et al.; 26). ACPA, anti-citrullinated protein antibody; IFN-α, interferon-alpha; NET, neutrophil extracellular trap; PAD, peptidyl arginine deiminase; PBN, peripheral blood neutrophil; PMNL, polymorphonuclear leukocyte.**

charged surface for activation and which results primarily in coagulation activation via the intrinsic pathway (35). The negatively charged DNA strands of neutrophil extracellular traps were found to provide a suitable surface for the binding of two contact system components – high-molecular-weight kininogen and factor XII – resulting in their subsequent activation (97). As controls, DNase-treated neutrophil extracellular traps or intact resting neutrophils displayed no contact-system activation. Apart from coagulation, activation of the contact system generates bradykinin (35), a proinflammatory mediator, and therefore the presence of neutrophil extracellular traps can function to amplify innate immune responses. In support of this, neutrophil extracellular traps were shown to promote thrombus formation *in vitro* (37) and also in a venous thrombosis mouse model (134). Further to their role in thrombus formation, activated platelets can also stimulate neutrophil extracellular trap release (20, 23, 81, 134). This interaction may explain why large amounts of intravascular neutrophil extracellular traps were visualized *in vivo* in a mouse model 3 h after the initiation of venous thrombosis following blood-flow restriction (134). The associated neutrophil extracellular traps were found to be decorated with tissue factor, the main trigger of the extrinsic coagulation pathway and of thrombus formation, and also provided a surface for factor XII binding and activation, as previously reported, supporting a key role for neutrophil extracellular traps in venous thrombosis pathogenesis (97). In addition, *in-vitro*-generated neutrophil extracellular traps were found to provide a scaffold for the binding of serum proteins, such as von Willebrand factor, fibronectin and

fibrinogen, which are involved in thrombus formation (37). Further analysis has demonstrated that the role of neutrophil extracellular traps in venous thrombosis is significant because application of DNase I prevented thrombus formation in the *in-vitro* model (37) and limited venous-thrombosis growth in the mouse model (134). Interestingly, not only were neutrophil extracellular traps stimulated by activated platelets, but platelets were activated by perfusion over neutrophil extracellular traps, suggesting a feedback mechanism that may contribute to venous thrombosis pathogenesis.

Another disease in which platelet-driven neutrophil extracellular trap release is documented as a causative factor is transfusion-related acute lung injury. In mouse models, neutrophil extracellular traps were identified in lung alveoli (126) and treatment with aspirin or a glycoprotein IIb/IIIa inhibitor to reduce platelet activation, and which reduced neutrophil extracellular trap release locally, resulted in decreased lung damage (20). Notably, after treatment with DNase I, mice with transfusion-related acute lung injury exhibited reduced neutrophil extracellular trap formation and tissue damage, and subsequently exhibited improved symptoms (20, 126). With regards to transfusion-related acute lung injury in humans, the neutrophil extracellular trap biomarkers of DNA and nucleosomes were detected in the peripheral blood of patients (126).

Potential role of neutrophil extracellular traps in periodontal disease pathogenesis

Periodontitis arises because of an aberrant host response to a pathogenic biofilm, manifest in susceptible individuals, and neutrophils are the major immune-cell type involved in the periodontal inflammatory response. It is therefore reasonable to speculate that perturbations in neutrophil function may determine a patient's periodontal state. Indeed, our research has already demonstrated that peripheral blood neutrophils in periodontitis show both hyperreactivity to plaque organisms (82) and hyperactivity in terms of reactive oxygen species release (83). As neutrophil extracellular trap release is known to be dependent upon the production of reactive oxygen species (Fig. 9) (36, 100), when combined, this data would support the contention that periodontal disease may associate with an excessive production of neutrophil extracellular traps (82). Potentially, neutrophils from patients with periodontitis could either exhibit constitutive hyperactivity and a raised baseline level of neutrophil extracellular trap production or they could be hyper-reactive, resulting in excessive neutrophil extracellular trap production in response to periodontal bacteria and local proinflammatory mediators. In either scenario the implication is that degradative enzymes and autoantigenic components are concentrated within the abundant neutrophil extracellular traps present within the tissue in a process triggered initially by the response of neutrophils to plaque bacteria. As previously highlighted, such high and concentrated levels of neutrophil extracellular trap-associated molecules could lead to a localized chronic inflammatory response, potentially with an autoimmune component, leading to significant gingival tissue damage (86).

In further support of this hypothesis is the fact that interferon-alpha primes neutrophil extracellular trap production (80) and recently we have demonstrated elevated peripheral type-I interferon levels in patients with periodontitis (141). It is conceivable therefore that a potential etiological factor, such as viral infection, which is known to raise type-I interferon levels (47), could subsequently lead to periods of disease activity. Indeed, several studies have implicated herpesviruses in the pathogenesis of periodontitis, with infected tissues harboring elevated levels of periodontopathogenic bacteria, including *Porphyromonas gingivalis*, *Tannerella forsythia* and *Prevotella intermedia* (119). As the majority of adults are herpesvirus carriers, it is also possible that following periods of latency, subsequent activation and replication of the virus, triggered by immunosuppression, stress, trauma or other common viral infections (e.g. influenza), could raise the levels of type I interferon (59), initiating neutrophil extracellular trap-mediated disease activity in periodontitis-susceptible individuals (Fig. 13A).

Currently, existing data on the association of neutrophil extracellular traps with periodontitis pathogenesis is limited, although recently, neutrophil extracellular traps have been visualized in purulent exudates from the gingiva (122) and in gingival crevicular fluid from patients with chronic periodontitis (63). These data are comparable with previous results that demonstrated neutrophil extracellular traps in abscess exudates from group A streptococcal infections of mice (17) and in human mixed bacterial appendicitis infections (14). Vitkov et al. (132) also observed that all 22 of the gingival purulent exudate samples collected showed significantly high levels of neutrophil extracellular traps and that in seven samples trapped bacteria were associated with the

neutrophil extracellular traps. Notably, transmission electron microscopic analysis of pocket epithelium biopsies from patients with chronic periodontitis has also indicated the presence of neutrophil extracellular traps (133). Additionally, our preliminary work has shown that neutrophil extracellular trap structures are apparent within the gingival connective tissues at sites of inflammation and are less easily detected in healthy gingival tissue as analyzed using immunofluorescence staining (Fig. 7).

The associations between periodontitis and other chronic inflammatory conditions, including rheumatoid arthritis, are now well documented (26). While several biologically causal mechanisms might account for this association, there is some evidence to suggest that periodontitis could lead to the initiation and maintenance of the autoimmune inflammatory response that occurs in rheumatoid arthritis. Recently, studies have implicated the potential of anti-citrullinated protein antibodies to provide a mechanistic link between the two diseases because these molecules, while well characterized in rheumatoid arthritis pathogenesis, have now been identified within the periodontium (95). As peptidyl arginine deiminase enzymes (as discussed earlier) are not only central to neutrophil extracellular trap production (peptidyl arginine deiminase-4), but are necessary for the formation of anti-citrullinated protein antibodies, it is interesting to speculate that their activity may link the two diseases. Notably, the periodontopathogen, *P. gingivalis*, is currently the only known bacterial species to express a peptidyl arginine deiminase enzyme and this has led to speculation that the microbial-derived enzyme (known as peptidyl arginine deiminase enzyme of *P. gingivalis*) may provide a source of anti-(citrullinated protein) Igs or may even stimulate neutrophil extracellular trap production processes that could potentially provide the mechanistic link between rheumatoid arthritis and periodontitis (26, 114).

Conversely, it is also reasonable to speculate, based on data from other diseases involving neutrophil extracellular trap–bacterial interactions (as described earlier), that ineffective neutrophil extracellular trap activity could contribute to periodontitis pathogenesis. This could result from hypo-active neutrophil extracellular trap production or by rendering neutrophil extracellular traps ineffective by either complete degradation via DNase activity or evasion of trapping by capsule expression or membrane charge modification. The result of ineffective neutrophil extracellular trap function would result in bacterial infiltration into the periodontal tissues, evoking a more widespread inflammatory response and culminating in periodontal tissue destruction. In addition, we have characterized membrane-bound and secreted bacterial DNase in periodontal bacteria and have demonstrated that ~80% of the 34 complex-associated periodontal bacteria analyzed (120) exhibited DNase activity (101). DNase expression is likely to not only confer neutrophil extracellular trap-evasion ability to the expressing bacteria but it may also be advantageous to the bacterial community in which it exists. It is therefore conceivable that pathogenic biofilms, which are able to stimulate chronic inflammation while evading the bacterial-killing mechanism of neutrophil extracellular traps, may develop within periodontitis. Interestingly, however, it has recently been proposed that neutrophil extracellular traps can become incorporated as part of the biofilm structure (56), and, in addition, bacterial nucleic acids can also form the framework for the bacterial community within plaque (57). These data appear to be consistent with the previously observed variable and regulated expression of DNase in periodontal bacteria and which appeared to be dependent on growth conditions (101) as clearly constitutive expression may result in break down of the biofilm. Indeed, exogenous addition of DNases potentially has therapeutic application for disrupting pathogenic biofilms (118) and may therefore have utility in periodontitis management. These results also further support the importance of the host's response to DNA, regardless of whether it is bacterially or host derived. Indeed, in systemic lupus erythematosis as we have previously discussed above, lack of removal of host DNA contributes to pathogenesis. Therefore, in our preliminary studies we analyzed DNase levels in both plaque and gingival crevicular fluid and demonstrated that the levels increase over the duration of a 21-day model of plaque-induced gingival inflammation (M. Cross, L.J. Palmer, P.R. Cooper, M.R. Ling, I.L.C. Chapple, unpublished). These studies were performed in healthy volunteers, demonstrating a physiological response to plaque accumulation and, surprisingly, DNase activity was more readily detected within gingival crevicular fluid than within plaque, potentially indicating its host derivation. It is therefore conceivable that if DNase activity was impaired, and therefore nucleic acids derived from biofilms or neutrophil extracellular traps were not removed efficiently, chronic inflammation would ensue.

Currently, the role of neutrophil extracellular traps in the pathogenesis of periodontitis is at an early stage of research and clearly more studies characterizing their involvement are required. While we have

highlighted potentially opposing mechanisms (i.e. hypo- or hyper-neutrophil extracellular trap production) that may contribute to periodontitis pathogenesis, the situation is probably more complex. Indeed, it is possible to envisage a scenario which unifies the two hypotheses, which would include the possibility that the degradation and evasion of neutrophil extracellular traps by virulent periodontal pathogens may cause neutrophils to respond by up-regulating the release of neutrophil extracellular traps. Such a response may not result in trapping and clearance of bacteria but instead may lead to the immobilization and localization of neutrophils responsible for periodontal tissue destruction. Therefore, the factors determining why one individual develops inflammatory periodontitis, whilst another does not, may be determined by (i) the type of bacteria inhabiting the gingival crevice, (ii) whether these bacteria possess virulence factors for neutrophil extracellular trap evasion and (iii) the individual's innate ability for neutrophil extracellular trap production. Indeed, in support of biological variation with regard to neutrophil extracellular trap production and its potential to contribute to disease, data from Fuchs et al. (36) demonstrate a significant range in healthy individuals. Future studies may therefore identify novel mechanisms involving neutrophil extracellular traps in periodontitis pathogenesis which subsequently lead to new treatment regimes.

Summary, conclusions and hypotheses

Neutrophil extracellular traps represent a novel, recently discovered, innate immune strategy for microbial containment and killing. Evidence indicates that neutrophil extracellular traps can be released from viable cells and from those undergoing 'neutrophil extracellular traposis', the former comprising largely mitochondrial DNA and the latter comprising largely nuclear DNA and chromatin. Mitochondrial neutrophil extracellular trap release appears effective as an antimicrobial strategy and has the advantage that the responding cells remain viable and nuclear contents do not enter the extracellular tissues, as the release of nuclear DNA within neutrophil extracellular traps has broad implications. We propose that the catastrophic neutrophil extracellular trap release from neutrophils that results from strong and multiple receptor stimulations (neutrophil extracellular traposis), and which results in nuclear DNA release in conjunction with histone proteins and other nuclear and cytoplasmic components, is more likely to be pathogenic through the generation of autoantibodies to cytoplasmic elements and nuclear antigens (e.g. extractable nuclear antigens) from neutrophils released into tissues during neutrophil extracellular traposis. Thus, neutrophil extracellular traposis, like any 'last-resort' strategy designed to cause maximal microbial damage, may well also be responsible for substantial collateral tissue damage and lead to chronic infammation.

However, it is also plausible that, given the common evolutionary status of human mitochondrial DNA with that of microbial DNA, and its structural similarities to that derived from prokarytotic cells, mitochondrial DNA may also generate an autoimmune/inflammatory response when released into the tissues of susceptible patients. Indeed, recent evidence for the role of mitochondrial DNA in human inflammopathies is now starting to emerge.

Whatever the role of neutrophil extracellular traps in periodontal pathophysiology, considerable attention is needed within the periodontal science community to explore the characteristics of neutrophil extracellular trap release in response to periodontal bacteria, their composition and functional relevance. The role of both host DNA and microbial DNA production is probably crucial in neutrophil extracellular trap evasion strategies and also in neutrophil extracellular trap retention within tissues, and the potential pathological consequences. Given the hyperactivity and hyper-reactivity of neutrophils from periodontitis patients in terms of reactive oxygen species release, and the role of reactive oxygen species in neutrophil extracellular trap production, it seems likely that neutrophil extracellular trap biology plays a significant role in periodontal health and disease. Moreover, the production of peptidyl arginine deiminase by *P. gingivalis* provides a biologically plausible role for this organism in the breach of immune tolerance that characterizes the pathogenesis of rheumatoid arthritis. The study of autoantibodies to citrullinated proteins (anti-(citrullinated protein) Igs; which are diagnostic of seropositive rheumatoid arthritis) within the plasma of patients with periodontitis and unaffected controls is overdue and may provide vital evidence for autoimmune mechanisms originating within periodontal tissues that contribute to rheumatoid arthritis.

This exciting new area of innate immunity is likely to add considerable knowledge and understanding to the pathogenesis of periodontitis and also to its association with other chronic inflammatory diseases such as rheumatoid arthritis.

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