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Review

Neutrophil Extracellular Traps: The Biology of Chromatin Externalization

Gabriel Sollberger,[1,2] Dorothea Ogmore Tilley,[1,2] and Arturo Zychlinsky[1,2,*]
[1]Department of Cellular Microbiology, Max Planck Institute for Infection Biology, Charitéplatz 1, Campus Charité Mitte, 10117 Berlin, Germany
[2]These authors contributed equally
*Correspondence: zychlinsky@mpiib-berlin.mpg.de
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Neutrophils are essential to the homeostatic mission of safeguarding host tissues, responding rapidly and diversely to breaches of the host's barriers to infection, and returning tissues to a sterile state. In response to specific stimuli, neutrophils extrude modified chromatin structures decorated with specific cytoplasmic and granular proteins called neutrophil extracellular traps (NETs). Several pathways lead to this unique form of cell death (NETosis). Extracellular chromatin may have evolved to defend eukaryotic organisms against infection, and its release has at least three functions: trapping and killing of microbes, amplifying immune responses, and inducing coagulation. Here we review neutrophil development and heterogeneity with a focus on NETs, NET formation, and their relevance in host defense and disease.

Introduction

Neutrophils are the most abundant white blood cell in circulation and are essential in maintaining health. Neutrophils differentiate from hematopoietic stem cells and leave the bone marrow when terminally differentiated. They spend their short life circulating in the bloodstream, patrolling for signs of infection and disturbed homeostasis. Aged neutrophils either return to the bone marrow where they are phagocytosed by resident macrophages (Casanova-Acebes et al., 2013) or undergo apoptosis in peripheral tissues. Apoptotic neutrophils are eaten by macrophages, triggering a feedback loop that controls the production of new neutrophils (Stark et al., 2005). In an immune challenge, neutrophils are the first cells to exit the circulation and migrate to the area under attack. There, neutrophils kill microbes, communicate the damage status to other immune cells, and initiate healing. Neutrophils are recognized by their characteristic lobulated nucleus, earning them the moniker of polymorphonuclear cells (PMNs). A second characteristic of neutrophils is the abundance of granules in their cytoplasm. Indeed, in the late nineteenth century granular staining with neutral dyes led Paul Ehrlich to call these cells neutrophils.

The antimicrobial activity of neutrophils is their best understood function. Neutrophils are professional phagocytes and carry a potent antimicrobial arsenal in their granules that, together with the production of reactive oxygen species (ROS), kills microbes inside the phagosome. These antimicrobial proteins are also released, at least *in vitro*, through "degranulation." A third antimicrobial mechanism is the release of neutrophil extracellular traps (NETs). NETs are released upon neutrophil death and consist of modified chromatin and antimicrobial proteins from both the cytoplasm and granules of the cell. Here, we first describe the origin and diversity of neutrophils and then review how NETs are made, and discuss their function.

Neutrophil Development

Granulopoiesis describes the development of neutrophils and other cells of this lineage that are rich in secretory granules. It depends on key transcription factors, environmental cues, and the host's inflammatory state. In this section, we review granulopoiesis during homeostasis and infection/inflammation, and discuss how neutrophil development might lead to different subpopulations.

Homeostatic Development

During homeostasis, granulopoiesis produces up to 2×10^{11} neutrophils daily. These cells develop from hematopoietic stem cells that give rise to granulocyte-macrophage precursors (GMPs). Terminal neutrophil differentiation begins with the transition from GMPs to myeloblasts and then further differentiation into promyelocytes, myelocytes, metamyelocytes, band cells, and, finally, mature neutrophils (Figure 1). This process takes approximately 2 weeks in steady-state development. At the promyelocyte stage, neutrophil precursors stop dividing, exit the cell cycle, and differentiate into non-cycling, terminally differentiated cells. During terminal differentiation, neutrophils acquire their characteristic granules. Granules are subdivided into primary (azurophilic), secondary (specific), and tertiary (gelatinase) granules, and secretory vesicles according to their cargo. Notably, protein loading into granules does not require any specific signal peptides. Therefore, the cargo of a granule subtype reflects the transcriptional/translational program of the neutrophil precursor at the time of granule loading (Borregaard, 2010).

Granulopoiesis is a complex process regulated by a core set of transcription factors and cytokines. Upstream of terminal neutrophil differentiation, GMP development requires high levels of the transcription factor PU.1. However, prolonged expression of PU.1 favors macrophage generation; therefore, another transcription factor, CCAAT/enhancer-binding protein alpha (C/EBP-α), antagonizes PU.1, allowing neutrophil terminal differentiation (reviewed by Ostuni et al., 2016). Indeed, C/EBP-α-deficient mice lack mature neutrophils (Zhang et al., 1997). Granulocyte-colony stimulating factor (G-CSF), an essential cytokine in granulopoiesis, is a target of C/EBP-α. Like C/EBP-α-deficient animals, mice lacking G-CSF, or its receptor, are almost completely devoid of neutrophils (Lieschke et al., 1994; Liu et al., 1996). G-CSF directs GMPs down the neutrophil lineage (Rieger et al., 2009). However, some neutrophils still develop

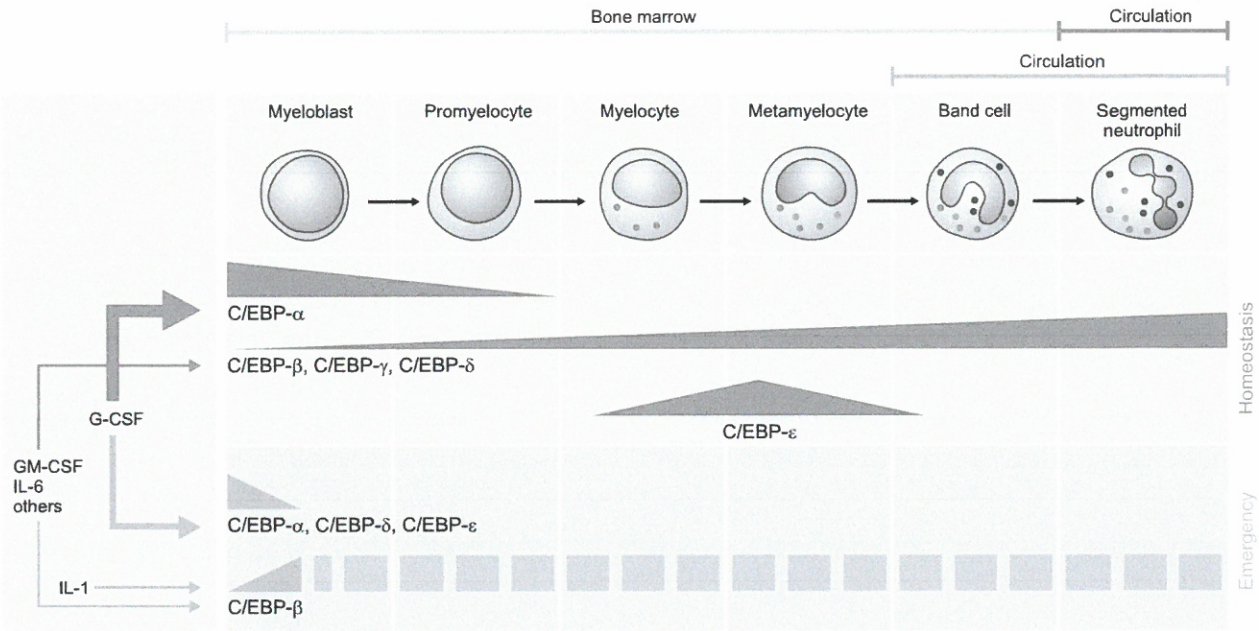

Figure 1. Granulopoiesis during Homeostasis and Emergency
Neutrophil differentiation begins with the development of myeloblasts to promyelocytes, which then further differentiate into myelocytes, metamyelocytes, band cells, and segmented neutrophils. This process depends on various cytokines and transcription factors (here focused on the C/EBP transcription factor family). Granule formation starts at the myelocyte stage and granule cargo depends on the time of their synthesis. Differentiated mature neutrophils leave the bone marrow and circulate in the host's bloodstream. In the case of severe infections, homeostatic granulopoiesis (blue) shifts to emergency granulopoiesis (orange). Emergency granulopoiesis depends on different subsets of cytokines and transcription factors and can lead to the premature release of band cell-like neutrophils into the circulation. C/EBP-β is supposed to be expressed throughout emergency granulopoiesis as indicated by the dashed line.

in the absence of G-CSF, demonstrating that other cytokines, including granulocyte-macrophage-colony stimulating factor (GM-CSF) and interleukin-6 (IL-6), can partially compensate for the loss of G-CSF signaling (Liu et al., 1997; Seymour et al., 1997).

Different physiological and environmental cues regulate granulopoiesis. In peripheral tissues, a feedback loop exists in which macrophage phagocytosis of apoptotic neutrophils dampens the production of IL-23/IL-17, and subsequently G-CSF, leading to less granulopoiesis. A lack of neutrophils in the periphery consequently boosts neutrophil production in the bone marrow via IL-23/IL-17 and G-CSF (Stark et al., 2005). In the circulation, neutrophil numbers fluctuate in a circadian rhythm, entering the bloodstream during the animal's active period and returning to the bone marrow toward the end of the resting period, as aged cells. Aged neutrophils shed the surface marker CD62L and upregulate CXCR4, favoring homing to the bone marrow. There, macrophages phagocytose old neutrophils, triggering the release of freshly matured cells (Casanova-Acebes et al., 2013). Furthermore, the microbiota is important in neutrophil development. Microbial cues signal via TLR4/TRIF, initiating granulopoiesis. Consequently, germ-free mice are severely neutropenic (Bugl et al., 2013), even more so than G-CSF-deficient mice.

Emergency Granulopoiesis

Microbe-derived signals influence granulopoiesis, not only in homeostasis but also during infection when neutrophils are in demand. This is particularly evident during severe systemic infections when neutrophils leave the bone marrow at higher rates than during homeostasis. This process is called emergency granulopoiesis (reviewed by Manz and Boettcher, 2014) and also depends on C/EBP transcription factors. However, C/EBP-β has a key role in mounting an emergency granulopoiesis response to cytokines or infections with the fungal pathogen *Candida albicans* (Hirai et al., 2006). It is not yet clear how the switch from C/EBP-α to C/EBP-β occurs. Notably, G-CSF is not essential for emergency granulopoiesis (Basu et al., 2000; Lieschke et al., 1994). Furthermore, in mice, lipopolysaccharide injection enhances G-CSF production by endothelial cells, driving neutrophil development. The involvement of other cytokines, such as IL-1, is context dependent (Boettcher et al., 2014; Ueda et al., 2009). Hence, there are different, and probably redundant, ways to induce emergency granulopoiesis.

Neutrophils also appear to develop at the site of infection, ensuring that mature cells reach the site where they are needed. These neutrophils develop from either residing hematopoietic stem cells (Granick et al., 2013) or immature granulocyte precursors (Deniset et al., 2017).

Neutrophil Heterogeneity

An ideal immune system mounts specific responses to all pathogenic events endangering the host, thus requiring complexity and plasticity. Adaptive immune cells clonally expand upon antigen recognition and develop into different subsets depending on environmental cues. In contrast, there are different pre-existing subsets of innate immune cells. For example, macrophages respond differently to signals depending on their origin or the

tissue in which they are residing (Okabe and Medzhitov, 2016). As terminally differentiated and short-lived cells, neutrophils cannot clonally expand or further differentiate in response to danger signals. There are, however, descriptions of tissue-resident neutrophils. Marginated neutrophils in the lung are mobilized upon infection (Devi et al., 2013). Neutrophils also reside in lymphoid tissues (Beauvillain et al., 2011; Nauseef and Borregaard, 2014). Two subsets of neutrophils reside in the red pulp of the spleen: a mature mobile population that scans the tissue and an immature (band cell-like) immobile population that proliferates in infection and becomes mobile mature cells (Deniset et al., 2017). Additionally, neutrophils can leave the inflammatory site and re-enter the circulation, which changes their properties (Mathias et al., 2006). The assumption that neutrophils are a homogeneous population of circulating microbe killers is an oversimplification that, with new single-cell technologies, is being critically revisited.

The best described neutrophil subpopulation are low-density granulocytes (LDGs) which were first found in patients with systemic lupus erythematosus (SLE) and rheumatic disease (Hacbarth and Kajdacsy-Balla, 1986). LDGs have neutrophil morphology but separate with the peripheral blood mononuclear cell fraction during density centrifugation. LDGs could be mature neutrophils that have partially degranulated or immature "band cell-like" cells that left the bone marrow in response to high cytokine levels. Interestingly, for the purpose of this review, LDGs from SLE patients spontaneously form NETs *in vitro* (Villanueva et al., 2011).

Many studies report differences in surface markers in neutrophil subpopulations. Examples include CD62L, which is shed by aged neutrophils and upon activation (Casanova-Acebes et al., 2013; Zhang et al., 2015), and degranulation or maturation markers (reviewed in detail by Garley and Jablonska, 2017). Recently, CD10 emerged as a surface marker that discriminates between mature (CD10 positive) and immature (CD10 negative) LDGs found in G-CSF-injected individuals. Furthermore, CD10-expressing cells suppress T cells in contrast to CD10-negative cells (Marini et al., 2017).

With the exception of LDGs, the ability of neutrophil subpopulations to make NETs is understudied. While neutrophil transmigration through the endothelium enhances NET formation, this is likely due to activation rather than the action of a distinct subpopulation (Allen et al., 2012). Interestingly, a small proportion of murine neutrophils can release NETs without undergoing cell death (Yipp et al., 2012), raising the question of whether this is a NET-prone subpopulation. Notably, in inflammatory conditions aged neutrophils release NETs more readily, and this is regulated by the microbiota via the TLR/MyD88 axis (Zhang et al., 2015). Using bromodeoxyuridine labeling to determine the neutrophil's age, a study found that aged cells infiltrate inflammatory sites earlier and are more phagocytic than younger cells *in vivo* (Uhl et al., 2016). However, their capacity to make NETs remains to be determined. Neutrophil aging correlates with CD62L shedding, and $CD16^{high}/CD62L^{dim}$ neutrophils are associated with better prognosis in squamous cell carcinomas patients (Millrud et al., 2017). Another subpopulation of human neutrophils expresses olfactomedin-4 (OLFM4), a protein in the specific granules (Clemmensen et al., 2012). Thus, depending on its expression, neutrophils form OLFM4-positive or -negative NETs (Welin et al., 2013). OLFM4 expression by neutrophils favors autoimmunity and correlates with a poor prognosis during septic shock (Alder et al., 2017; Amirbeagi et al., 2015), although the role of NETs in these conditions is not yet known.

Taken together, neutrophils are more heterogeneous than previously anticipated. Whether this heterogeneity reflects different activation states or "true" subpopulations is unclear. The capacity of neutrophil subsets to execute typical neutrophil behaviors, e.g., phagocytosis, degranulation, or NET formation, will need to be analyzed. As NETs are both beneficial and pathogenic, it will be important to investigate whether certain subpopulations can be targeted to inhibit the adverse effects of NETs while leaving their beneficial properties intact.

Neutrophil Extracellular Traps

NETs are a modified form of chromatin and retain the periodicity of nucleosomal spacing (Urban et al., 2009). Cytoplasmic (e.g., calprotectin) and granular proteins (e.g., myeloperoxidase [MPO] and neutrophil elastase [NE]) bind in globular patterns to NETs (Brinkmann et al., 2004). Animals, plants, and even unicellular eukaryotes (Zhang et al., 2016) use chromatin in immunity. Interestingly, elegant work by the Hawes lab shows pathogenic fungi provoke chromatin release from plant roots and these extracellular structures are essential for defense (Hawes et al., 2016). These observations suggest that the use of chromatin in antimicrobial defense emerged early in eukaryotic evolution. It is interesting to speculate that the evolutionary forces that shaped the highly conserved histone sequence and structure were determined, at least in part, by their function in immunity.

In vitro, NETs are fragile structures that float and aggregate with slight disturbances of the medium. They are identified as structures containing DNA, histones (often citrullinated), and at least one neutrophil cytoplasmic or granular marker. *In vivo*, NETs are found at inflammatory sites with high cellularity. NET formation follows a well-orchestrated cell death program called NETosis. Eventually, NETs are dismantled by serum DNases (Hakkim et al., 2010; Jimenez-Alcazar et al., 2017b), although they may persist differently in some infections (Kolaczkowska et al., 2015). NETs are observed by intravital microscopy using DNA dyes and neutrophil markers (Yipp et al., 2012). It is challenging to generate animals with labeled granular proteins since they are synthesized as pre-pro-enzymes, but these constructs will help elucidate NETosis *in vivo*.

During NETosis, the nucleus first delobulates while the granules disappear, followed by membrane vesiculation. After nuclear disintegration and before cell rupture, the chromatin expands, allowing contact between granular and cellular components. Finally, the cytoplasmic membrane breaks, releasing a NET into the extracellular space (Figure 2). Different stimuli initiate alternative signal transduction pathways leading to NETosis, although these pathways are still poorly understood. Interestingly, despite temporal and molecular characteristics specific to each pathway, the intracellular membrane reorganization that allows the coupling of proteins and chromatin to construct NETs appears to be common to all forms of NETosis.

Mechanisms of NET Formation

Neutrophils are challenging cells to work with. Some key executioners of their functions are only transcribed during

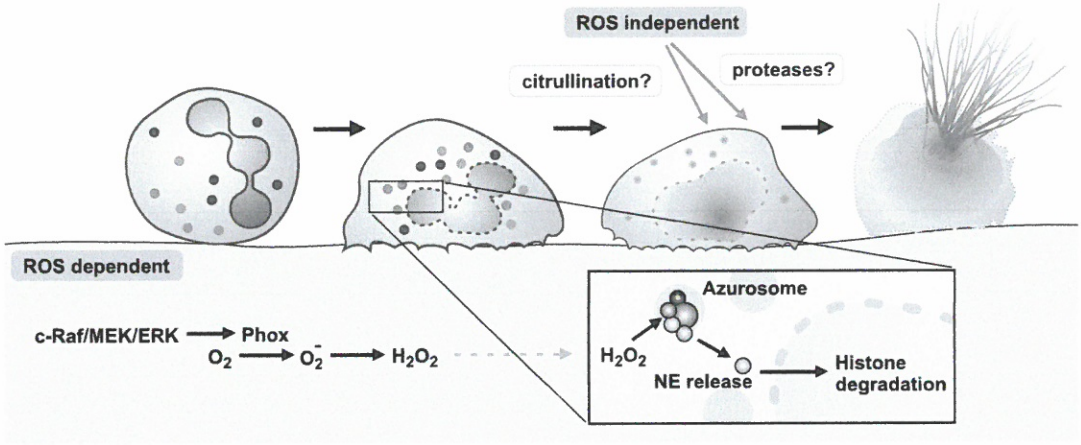

Figure 2. ROS-dependent and ROS-independent NET Formation
Upon activation, receptors on the neutrophil surface initiate a cascade resulting in the assembly of NADPH oxidase (Phox) and superoxide production. Superoxide dismutates to hydrogen peroxide, which is sensed by the azurosome. This complex is composed of several proteins including MPO which converts hydrogen peroxide to halic acids. This conversion liberates neutrophil elastase (NE) and other serine proteases from the azurosome into the cytoplasm. These proteases move to the nucleus and cleave histones. In parallel, the nucleus delobulates and the nuclear membrane vesiculates, allowing the contact of chromatin with cytoplasmic and granular proteins. Eventually, the cytoplasmic membrane ruptures to release NETs. ROS-independent NET formation occurs in the absence of Phox and MPO activity and does not require NE. There may be other proteases and/or histone citrullination that facilitate chromatin decondensation. The final step, similar to ROS-dependent NET formation, is the rupture of the plasma membrane and the release of NETs into the extracellular space.

development, precluding gene-silencing experiments. Additionally, neutrophil-specific promoters, allowing specific gene modifications in mice, are only now emerging (Hasenberg et al., 2015) and may be complicated by late expression of drivers in neutrophil development. Moreover, human and murine neutrophils differ, complicating data interpretation. Lastly, cell lines poorly mimic neutrophil behavior. Thus, probing the mechanism(s) of NET formation in human cells has so far relied on chemical inhibitors and access to samples from rare inherited immunodeficiencies, as described below. We will focus on two main pathways, namely nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent and NADPH oxidase-independent NETosis.

NADPH Oxidase-dependent NETosis. Antibodies (Behnen et al., 2014; Garcia-Romo et al., 2011), microbes, cholesterol (Warnatsch et al., 2015), and mitogenic stimuli, including phorbol 12-myristate 13-acetate (PMA) and concanavalin A, induce NADPH oxidase-dependent NETosis (Amulic et al., 2017). These stimuli trigger activation of c-Raf, MEK, Akt, ERK, and protein kinase C (PKC) that in turn activate NADPH oxidase (Hakkim et al., 2011).

Specific receptor activation can block NETosis. Prostaglandin E_2 inhibits NETosis through its cognate G-protein-coupled receptors, increasing intracellular cyclic AMP and inhibiting PKC and downstream events leading to NET formation (Shishikura et al., 2016). Activated protein C also inhibits NETosis through activation of its cognate receptor or cooperative interaction of protease-activated receptor 3 (PAR3) and integrins CD11b/CD18 (Mac-1) (Healy et al., 2017). This suggests that NETosis is regulated by both inflammatory and anti-inflammatory mediators.

O_2, CO_2, bicarbonate levels, and pH modulate NETosis. PMA, but not *Staphylococcus aureus*, requires normoxia to induce NETs (Branitzki-Heinemann et al., 2016). Surprisingly, the normoxia requirement is not regulated by hypoxia-inducing factor 1α (HIF1α). Hypoxia increases the membrane's cholesterol content which might affect cell signaling loci, lipid microdomains (Brown and London, 2000), and membrane rigidity (Khatibzadeh et al., 2013). Furthermore, neutrophils might respond to CO_2, HCO_3^-, and pH at the geographical edge of inflamed tissue, making NETs more readily in the alkaline periphery (Maueroder et al., 2016) than in the center of an inflammatory site. Indeed, NETs formed in the periphery might seal off the affected area. Consistently, an acidic environment decreases NETosis, possibly through reduced glycolysis (Behnen et al., 2017). Glucose metabolism may affect NET formation on different levels; the pentose phosphate pathway affects NETosis by providing NADPH (Azevedo et al., 2015; Siler et al., 2017).

After activation, NADPH oxidase converts molecular oxygen to superoxide. Pharmacological inhibition of NADPH oxidase or ROS scavengers block NET formation with certain stimuli (Hakkim et al., 2011). More importantly, neutrophils from patients with mutations in NADPH oxidase subunits fail to produce NETs in response to mitogens and microbes, confirming genetically the relevance of ROS (Bianchi et al., 2009). Superoxide dismutates to hydrogen peroxide which MPO then converts to halic acids. Consistently, inhibitors of MPO also block NETosis and patients with complete MPO deficiencies do not form NETs (Metzler et al., 2011).

MPO is part of a protein complex, the "azurosome," which resides in azurophilic granules and contains eight proteins, including three highly homologous serine proteases: NE, cathepsin G, and azurocidin. Hydrogen peroxide triggers azurosome dissociation and this is proposed to cause leakage of serine proteases into the cytoplasm where they migrate to the nucleus by an unknown mechanism (Metzler et al., 2014). These proteases lack nuclear localization signals but are small enough to passively diffuse through the nuclear pore. In the nucleus, the serine proteases clip histones, facilitating chromatin relaxation.

NADPH oxidase-dependent NETosis requires activation of cyclin-dependent kinases (CDKs) which attempt to pull neutrophils out of G_0 and back into the cell cycle (Amulic et al., 2017). As terminally differentiated cells, the requirement for a cell cycle protein is surprising. Neutrophils undergoing NETosis express the proliferation marker Ki-67, and CDK6 is required for NETosis. Consistently, CDK6-deficient mice are more susceptible to infection. During NET formation, CDK6 phosphorylates its cell cycle substrate, retinoblastoma protein. However, S-phase events, including DNA synthesis and transcription of histone genes, do not happen during NETosis. Surprisingly, the M-phase events, lamin phosphorylation and centrosome separation, are part of NET formation. These findings suggest the neutrophil uses part of the cell cycle machinery to disassemble the nuclear membrane.

NADPH Oxidase-independent NETosis. Two other NET inducers, calcium ionophore A23187, produced during the growth of *Streptomyces chartreusensis*, and the potassium ionophore nigericin, derived from the bacteria *Streptomyces hygroscopicus*, do not require NADPH oxidase or MPO activity (Kenny et al., 2017; Neeli and Radic, 2013). ROS-independent NETosis does not require NE, and histone H3 is often citrullinated. The mechanism that leads to NET release is not understood. Neither the NADPH oxidase-dependent nor -independent pathways require *de novo* gene expression, showing that the executioners are present in neutrophils (Kenny et al., 2017; Sollberger et al., 2016). Importantly, like the canonical form, the alternative NETosis is distinct from necroptosis (Kenny et al., 2017), apoptosis (Remijsen et al., 2011), and other forms of cell death. Notably, NET formation can depend on mitochondrial (Lood et al., 2016; van der Linden et al., 2017) or pathogen (Kenny et al., 2017) derived ROS through a pathway that remains to be described in detail.

Histones. Histones are short, basic proteins that package DNA into chromatin. They are also potent antimicrobials and toxic to animal cells. The antimicrobial activity, of both the holo-protein and cleaved peptides, was first described in the 1940s (Miller et al., 1942). The cytotoxicity of histones is well described; anti-histone antibodies prevent pathogenesis in various disease models (Wildhagen et al., 2014; Xu et al., 2009). NETs release large amounts of histones into tissues where they can target microbes but also cause tissue damage. Indeed, the antimicrobial function of NETs might be, at least partially, due to histones. Histones also likely contribute to the pathogenic effect of NETs, as described later in this review.

Post-translational histone modifications regulate gene expression and chromatin structure (reviewed by Bannister and Kouzarides, 2011). One histone modification already mentioned in NETosis is "clipping" by serine proteases which might facilitate chromatin decondensation. Histone clipping is a drastic and irreversible event. Histones are also citrullinated in NETs (Dwivedi et al., 2014). Citrullination is the conversion of arginine to citrulline (a non-encoded amino acid) by peptidylarginine deiminases (PADs). PAD4 is implicated in histone citrullination during NETosis *in vitro*, and chemical or genetic ablation of PAD4 diminishes NET formation (Knight et al., 2013, 2015; Lewis et al., 2015; Li et al., 2010). However, the question remains as to whether PAD4 is required for NETosis.

Pharmacological or genetic inhibition of PAD4 reduces pathology in several diseases models where NETs are implicated, including rheumatoid arthritis (RA), SLE, atherosclerosis (Knight et al., 2014), and ischemia/reperfusion injury (Savchenko et al., 2014). Importantly, PAD4 also regulates the transcriptional response to hormones (Cuthbert et al., 2004) and is essential to maintain the undifferentiated state of pluripotent stem cells (Christophorou et al., 2014). Indeed, PAD4 knockout mice are born in lower numbers than expected by Mendelian inheritance (Li et al., 2010). Additionally PAD4 may citrullinate other proteins and their relevance during NETosis is not yet known. Thus, the effects of PAD4 inhibition in NETosis could be, at least in part, independent of histone citrullination. Neutrophil-specific gene ablation of PAD4 will help clarify this issue. Nevertheless, in combination with other markers (e.g., NE or MPO), citrullinated H3 helps to identify NETs.

NETosis and Other Forms of Cell Death. NET formation is different from other forms of cell death. While neutrophils undergo canonical apoptosis, necroptosis, and necrosis to appropriate stimuli (Kenny et al., 2017) with morphology comparable with that of other cells, nuclear delobulation and expansion is unique to NETosis. Molecularly, as expected, neutrophil apoptosis and necroptosis is blocked by caspase inhibitors and necrostatin or MLKL inhibitors, respectively. However, while these inhibitors do not block NETosis, there is some debate as to the extent that RIPK3- and MLKL-dependent processes are involved in NET formation (Amini et al., 2016; Desai et al., 2016, 2017; Schreiber et al., 2017). Conflicting data are also reported regarding the relevance of autophagy in NETosis: unspecific phosphoinositide 3-kinase inhibitors block NET formation (Germic et al., 2017), while biochemical or genetic ablation of ATG5 does not. Notably, although most NET inducers culminate in neutrophil death, under some conditions neutrophils release NETs and remain viable, indicating that this fascinating process has many facets (Yipp et al., 2012).

NETs in Host Defense and Disease

The biological relevance of NETosis is evident from studying diseases where NETs are dysregulated. The earliest evidence for the role of NETs in host defense was in a chronic granulomatous disease patient with invasive aspergillosis who failed to clear the fungal infection or produce NETs, both of which were recovered with gene therapy (Bianchi et al., 2009). Indeed, further work showed that neutrophils make NETs when they encounter a foreign body or pathogen that they cannot phagocytose (Branzk et al., 2014). While killing of microbes by NETs is limited, and sometimes controversial (Kenny et al., 2017; Menegazzi et al., 2012), it likely depends on the pathogen; many express virulence factors to avoid capture by NETs (recently reviewed by Storisteanu et al., 2017). More generally, NETs prevent dissemination of infection, trapping microbes and facilitating their killing by antimicrobial proteins and professional phagocytes. However, inappropriate NET production causes pathogenic inflammation, autoimmunity, and vessel occlusion. This can occur when NETs are overproduced or when mechanisms to resolve NETs, e.g., DNases, are lacking.

Evidence for the role of NETs in host defense and disease comes from the detection of NETs in tissues or biological fluids using immunofluorescent microscopy or ELISAs. In contrast, the combined detection of individual NET components—cell-free DNA, histones, NE, and MPO—in separate assays

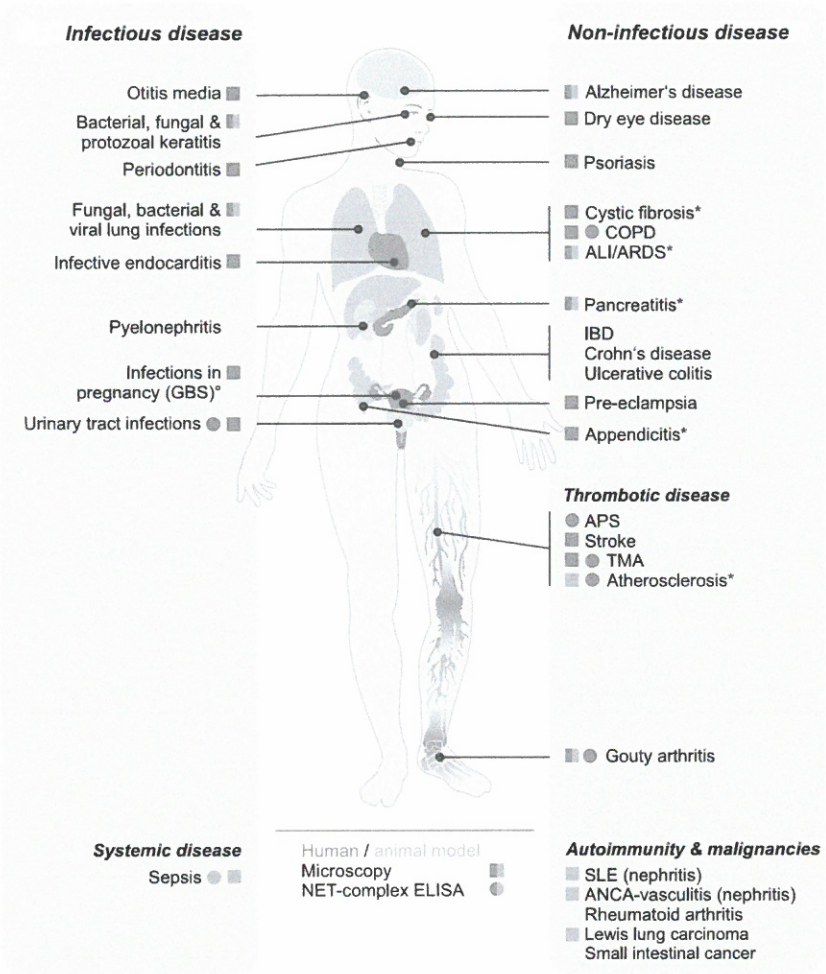

Figure 3. NETs in Human Disease
NETs are implicated in multiple infectious and non-infectious diseases in humans. Direct evidence of NETs in disease comes from the observation or detection of NET complexes in blood and peripheral tissues. This has been achieved using immunofluorescent microscopy (square) and co-localization of DNA with a neutrophil cytoplasmic or granule marker, or detection of soluble NET complexes—DNA and MPO/NE—by sandwich ELISA (circle). Supporting evidence comes from animal models of disease. ANCA, anti-neutrophil cytoplasmic antibody; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; APS, antiphospholipid syndrome; COPD, chronic obstructive pulmonary disease; GBS, group B *Streptococcus*; IBD, irritable bowel disease; SLE, systemic lupus erythematosus; TMA, thrombotic microangiopathy. *Disease is also associated with infection. °Observed only in a non-human primate model.

only suggests the presence of NETs. Further indirect evidence for NET function is inferred from (1) the application of anti-NET agents, including recombinant DNases or anti-histone antibodies, or (2) genetic or chemical inhibition of components required for NETosis, e.g., NE or NADPH oxidase. In the following section, we discuss the contribution of NETs to host defense and pathogenesis in humans. Figure 3 lists diseases in which NETs are detected and/or are proposed to be pathogenic.

NETs in Respiratory Disease

In respiratory diseases, NETs contribute to the viscosity of mucus effusions that combat infection and are found in the sputum and lung secretions of bacterial, fungal, and viral infections (Bianchi et al., 2009; Cortjens et al., 2016; Hamaguchi et al., 2012). NETs are essential to clear fungi from the lung (Bianchi et al., 2009). In chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF), NETs contribute to the reduction in pulmonary function and airway blockade. In COPD, the amount of NETs in patient sputum correlates with disease severity and microflora (Dicker et al., 2017). Inhaled recombinant DNase treatment improves lung function in CF. Interestingly, NE processes histones, helping to solubilize sputum by making it more accessible to DNases (Papayannopoulos et al., 2011).

Existing inflammatory conditions can be aggravated by NETs. Rhinovirus infections in asthmatic patients increase DNA and NE in nasal secretions, suggesting the presence of NETs. This produces a type 2 inflammatory response that is recapitulated in mice and is attenuated by DNase treatment or blocking NET formation by pharmacological inhibition of NE, thus demonstrating that NETs contribute to amplification of immune responses (Toussaint et al., 2017).

Acute lung injury (ALI), and the more severe acute respiratory distress syndrome (ARDS), involve rapid and acute pulmonary failure with diverse etiology. In humans, NETs are observed in transfusion related ALI (Caudrillier et al., 2012). Mouse models indicate that histones contribute to the pathology of this form of ALI (Caudrillier et al., 2012; Thomas et al., 2012) but also in malaria (Sercundes et al., 2016) and endotoxemia-related ALI (Saffarzadeh et al., 2012). Furthermore, DNase or anti-histone antibodies are therapeutic in these models.

NETs at the Microbial-Epithelial Interface

Microbes constantly challenge the host's epithelial barriers, inducing NETs, particularly in the eye, oral mucosa, and skin. However, NET production and degradation must be tightly regulated to prevent pathogenic inflammation. Divergent antimicrobial and pathogenic effects of NETs are documented in several diseases.

On the cornea, NETs combat bacterial and fungal eye infections (keratitis) but can also exacerbate pathology as shown in *Pseudomonas aeruginosa* keratitis (Shan et al., 2014). In contrast, in fungal keratitis patients, NET load is inversely correlated with disease duration and treatment response (Jin et al., 2016). In sterile inflammation of the cornea, e.g., in dry eye disease, hyperosmotic secretions might induce NETs (Sonawane et al., 2012), and DNase treatment alleviates discomfort (Tibrewal et al., 2014).

The saliva and dental plaque of healthy and diseased individuals contains NETs (Hirschfeld et al., 2015). Consistent with the antimicrobial activity of NETs, periodontitis is common in patients with neutrophil defects including congenital and cyclic neutropenia (Chen et al., 2013) and Papillon-Lefèvre syndrome, a genetic disease whereby patients lack functional serine proteases required for NETosis (Roberts et al., 2016). In contrast, periodontal patients, with healthy neutrophils, have reduced circulating DNase I and a reduced ability to degrade NETs *in vitro* (White et al., 2016).

In staphylococcal skin infections, NETs prevent bacterial dissemination into the bloodstream (Yipp et al., 2012). In addition, NET production decreases with age, resulting in bacteremia in older mice (Tseng et al., 2012). This deterioration in NET function with aging provides insight into the increased incidence of bacteremia in the elderly. Psoriatic skin lesions also contain NETs and neutrophils from these patients make NETs spontaneously, correlating with disease severity (Hu et al., 2016; Lin et al., 2011). Additionally, serum from these patients stimulates NET formation *in vitro* and NETs can directly stimulate keratinocytes to produce β-defensin 2, a hallmark of psoriasis (Hu et al., 2016).

NETs in Vessel and Ductal Occlusion

NETs contribute to the formation of occlusive bodies that block circulation and secretion processes in the body.

NETs are important in thrombosis, as reviewed by Martinod and Wagner (2014). NETs induce platelet activation, coagulation, and thrombus formation (Fuchs et al., 2010; Kambas et al., 2012). It is not yet known whether NET-induced thrombosis/coagulation plays a role in innate immune defense, contributes to normal hemostatic function, or is simply a pathological consequence of inappropriate NET formation. However, DNase treatment consistently reduces vessel occlusion in multiple models of thrombotic disease (Brill et al., 2012; Chen et al., 2014; De Meyer et al., 2012; Savchenko et al., 2014; von Bruhl et al., 2012). Indeed, a recent review of cardiovascular disease in humans indicates that prognosis and disease severity is correlated with cell-free DNA (Jimenez-Alcazar et al., 2017a). In humans, NETs are observed in stroke (Hirose et al., 2014), thrombotic microangiopathies (Arai et al., 2013; Ramos et al., 2016), atherosclerosis (Borissoff et al., 2013), and antiphospholipid syndrome (Yalavarthi et al., 2015). While it is unclear whether NETs contribute to pathogenesis in these diseases, in an atherosclerosis model, neutrophil serine protease deficiency or DNase treatment reduced atherosclerotic plaque size (Warnatsch et al., 2015).

In pancreatitis, aggregated NETs are formed upon the accumulation of bicarbonate ions, carbonate crystals, and altered pH, leading to occlusion of the pancreatic duct (Leppkes et al., 2016). DNase treatment in a pancreatic mouse model alleviates disease (Merza et al., 2015). While not involved in occlusive body formation, gout also results from accumulation of crystals—monosodium urate (MSU) crystals—causing temporary arthritis. MSU crystals induce aggregated NETs with local high protease concentrations which degrade proinflammatory cytokines and chemokines (Schauer et al., 2014). In gout, NETs contribute to resolution of these events, controlling inflammation and preventing chronic disease (Schauer et al., 2014).

Cancer

Cancer is a heterogeneous disease and defining the role of NETs depends on the malignancy type. In breast cancer models, NETs may facilitate metastasis as it is prevented by DNase treatment (Park et al., 2016). NET-induced coagulation is considered a sequela in some malignancies (Levi, 2016) and in an intestinal cancer model, DNase reduced coagulation and tumorigenesis (Guglietta et al., 2016). In contrast, therapeutic viral infection of tumors induces neutrophil-dependent intratumoral coagulation and tumor cell killing, but it remains to be seen whether this is NET mediated (Breitbach et al., 2011).

Autoimmunity

NETs are relevant in autoimmunity, as reviewed by Gupta and Kaplan (2016). Patients with SLE, RA, and anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV) develop autoantibodies that recognize NET components: double-stranded DNA (Wu et al., 2006), citrullinated proteins (Pratesi et al., 2014), and azurophilic granule components (Falk and Jennette, 1988; Niles et al., 1989), respectively. These antibodies may originate from prolonged exposure to NETs due to excessive production or a reduced ability to degrade NETs. Indeed, inherited deficiencies in DNases lead to pediatric SLE (Al-Mayouf et al., 2011; Yasutomo et al., 2001), Additionally, NET uptake by antigen-presenting cells drives autoimmunity in an RA model where NET-loaded fibroblasts stimulated anti-citrullinated peptide antibody production (Carmona-Rivera et al., 2017). Anti-NET autoantibodies also inhibit degradation of NETs, exacerbating disease (Hakkim et al., 2010). As disease progresses, immune complexes with NET components are found in glomerulonephritis, a common sequela of SLE and AAV (Hakkim et al., 2010; O'Sullivan et al., 2015). Furthermore, anti-histone antibodies protected mice from developing kidney lesions in a necrotizing glomerulonephritis model (Kumar et al., 2015). Oxidized neutrophil DNA, of both genomic and mitochondrial origin, induces type 1 interferon responses, *in vitro* and in SLE models, suggesting that NET formation, with concomitant ROS production, drives inflammation (Gehrke et al., 2013; Lood et al., 2016). Interestingly, NOX2 deficiencies, which restrict the membrane but not mitochondrial ROS, exacerbate disease in RA and SLE models (Campbell et al., 2012; Jacob et al., 2017; Kienhofer et al., 2017; Maicas et al., 2011). Similarly, chemical activation of NOX2 ameliorates SLE in a murine model (Kienhofer et al., 2017). Together, these observations suggest the mechanism of NETosis and the type of NET generated may be important in initiating and perpetuating autoimmunity.

Sepsis

Sepsis is an acute complication of severe infection with high morbidity and mortality. The pathology of this systemic disease is complex, but it appears that NETs may promote survival. Neutrophils from patients who survive sepsis produce more NETs *in vitro* than neutrophils from patients who succumb to the disease (Park et al., 2017). This may be due, in part, to the antimicrobial function of NETs in early infection. Indeed, in a polymicrobial mouse model, DNase treatment accelerated the onset of sepsis (Meng et al., 2012). However, as disease progresses, NETs may damage the lungs (ALI and ARDS) and liver (Saffarzadeh et al., 2012; Weber, 2015). Interestingly, direct injection of histones mimics this pathology, and antihistone antibodies protect mice in multiple infection models (Xu et al.,

2009). Thrombosis also contributes to organ failure in sepsis and, as for thrombotic disease, circulating cell-free DNA correlates with disease severity in septic patients and organ damage in animal models (Czaikoski et al., 2016). Consistently, DNase treatment reduces organ damage and improves survival, but only when provided after the establishment of infection and in combination with antibiotics (Czaikoski et al., 2016). This highlights the dual role of NETs in early infection and the pathogenesis of organ damage in sepsis.

Conclusions

Neutrophils differentiate in the bone marrow and contain the machinery to quickly deploy chromatin in host defense. Different, and still poorly understood, pathways lead to NETosis. Microbes and mitogens elicit a mechanism that requires the formation of ROS, allowing the translocation of proteases to the nucleus where they process histones to allow chromatin decondensation. In addition, the neutrophil uses mechanisms similar to mitosis to dismantle the nuclear membrane.

NETs are instrumental in the immune defense against acute infection, since these structures trap and kill microbes. This innate immune function likely drove the evolution of mechanisms to externalize chromatin early in eukaryotic history. However, like all host responses, NETs are pathogenic if uncontrolled. Inappropriate NET formation, or a failure to resolve NETs, is a risk factor for autoimmunity and coagulation diseases. NETs continue to be identified in a growing number of infectious and non-infectious diseases in diverse anatomical sites, from the urine in urinary tract infections (Yu et al., 2017) to the brain parenchyma in Alzheimer's patients (Zenaro et al., 2015), raising the possibility of yet more NET functions. Continued investigations into the relevance of NETs in disease will reveal new functions for NETs and shed further light on the role(s) of extracellular chromatin. NETs demonstrate that chromatin and its components have an extracellular function, and continued research on NETs is starting to shed light on this unprecedented notion.

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