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STATE-OF-THE-ART REVIEW

NET formation – mechanisms and how they relate to other cell death pathways

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Cell death is an integral part of both infectious and sterile inflammatory reactions. Many cell death pathways cause the dying cell to lyse, thereby amplifying inflammation. A special form of lytic cell death is the formation of neutrophil extracellular traps (NETs), large structures of chromatin and antimicrobial proteins, which are released by dying neutrophils to capture extracellular pathogens and limit the spread of infections. The molecular mechanisms of NET formation remain incompletely understood. Recent research demonstrated substantial crosstalk between different cell death pathways, most notably between apoptosis, pyroptosis and necroptosis. Here, we review suicidal and vital NET formation and discuss potential crosstalk of their mechanisms of release with other forms of cell death.

Neutrophils: an introduction

Neutrophils, the most abundant human leucocytes, are essential for defence against a variety of infections. Together with eosinophils and basophils, they form the granulocyte family of immune cells. As this name implies, neutrophils carry a characteristic arsenal of granules, small organelle-like structures containing a diverse set of proteins. Granules can fuse with intracellular membranes or the plasma membrane, delivering signalling molecules or antimicrobial proteins to phagosomes or to the extracellular space. This enables neutrophils to pursue various antimicrobial functions. Granules develop during granulopoiesis, the differentiation of neutrophils from committed precursor cells in the bone marrow [1]. Granulopoiesis occurs

Abbreviations
ANCA, antineutrophil cytoplasmic antibodies; ASC, apoptosis-associated speck-like protein containing a CARD; ATG5/ATG7, autophagy related 5/7; BMAL1, brain and muscle ARNT-like 1; C5a, complement component 5a; CDK6, cyclin-dependent kinase 6; CGD, chronic granulomatous disease; CTSG, cathepsin G; CXCR2/CXCR4, C-X-C chemokine receptor type 2/4; DNASE1L3, deoxyribonuclease 1 like 3; ESCRT, endosomal sorting complexes required for transport; G-CSF/GM-CSF, granulocyte colony-stimulating factor/ granulocyte-macrophage colony-stimulating factor; GSDMD, gasdermin D; GSDME/DFNA5, gasdermin E/ deafness, autosomal dominant, 5; HBSS, Hanks' balanced salt solution; IL-1β, interleukin-1beta; LPS, lipopolysaccharide; MLKL, mixed lineage kinase domain like pseudokinase; MPO, myeloperoxidase; mtDNA, mitochondrial DNA; NADPH, nicotinamide adenine dinucleotide phosphate; NE, neutrophil elastase; NETs, neutrophil extracellular traps; NLR, nod-like receptor; NLRP3, nlr family pyrin domain containing 3; PAD4, peptidyl arginine deaminase 4; PAMP/DAMP, pathogen/danger-associated molecular pattern; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PRTN3, proteinase 3; RIPK1/RIPK3, receptor-interacting serine/threonine kinase 1/3; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; WDFY3, WD repeat and FYVE domain containing 3; xCT, glutamate/cystine xCT antiporter; XIAP, X-linked inhibitor of apoptosis.

at high rates (an adult human being produces up to 2×10^{11} neutrophils per day) to replenish the pool of circulating mature neutrophils, which are short-lived cells [2].

During their maturation, neutrophils also develop their unique lobular nuclear morphology. It is not entirely clear why neutrophils present with this nuclear shape, but one explanation is that the lobulation of the nucleus and/or the composition of the nuclear membrane helps the cells to migrate through narrow pores [3–6]. As various migrating cell types seem to use their nucleus as a sensor for pore size [7], having a defined number of small lobules might help the cell to migrate quickly through space-restrained sites.

Mature neutrophils are terminally differentiated, and once they enter the bloodstream, they spend their short lifespan patrolling the host's circulation. Neutrophils undergo ageing in circulation, and this process depends on the circadian clock [8]. The clock component BMAL1 induces upregulation of the chemokine receptor CXCR2, which favours ageing, whereas expression of CXCR4 antagonizes it [8]. Depending on their ageing status and on whether they sense any signs of infection, neutrophils can leave the bloodstream and enter various tissues [8,9]. There, they follow chemokine gradients to find pathogenic invaders or areas of tissue damage. If not activated in tissues, neutrophils undergo apoptosis. Their subsequent phagocytosis by tissue-resident macrophages is part of a feedback loop that ensures appropriate production of new neutrophils in the bone marrow [9,10]. If activated by infectious agents or signs of damage, neutrophil lifespan extends as they commit to fighting potentially pathogenic microorganisms using various effector functions. Neutrophils are very active phagocytes, able to engulf bacterial or fungal species and to kill them within the hostile environment of their phagosomes [11]. As mentioned above, neutrophils can also release antimicrobial proteins into the extracellular space to target extracellular pathogens through degranulation, a process in which granules fuse with the plasma membrane [12,13]. Neutrophils also produce various cytokines and chemokines to amplify inflammatory responses and to recruit other immune cells. Even though neutrophils produce and release less cytokine per cell than other immune cells (such as dendritic cells, macrophages or innate lymphoid cells), the sheer number of neutrophils at an inflammatory site can result in substantial cytokine production, reshaping the inflammatory response [12,14,15].

In addition to the aforementioned strategies to cope with infectious challenges, neutrophils are able to release their chromatin, decorated with granule proteins, into the extracellular space via a specialized cell death pathway. These web-like structures are called neutrophil extracellular traps (NETs) and are used to contain pathogens and limit the spread of infection [16]. Since the discovery of NETs in 2004, much research has focused on the mechanisms of their formation. Here, we review these mechanisms, compare NET formation with other forms of cell death and discuss whether NET formation always requires neutrophil death.

NETs

Morphology and importance of NETs

NETs are large structures (originally described as long (> 500 μm) fibres with diameters up to 50 nm [16]) composed of chromatin, mitochondrial DNA and mostly granule-derived proteins. They are generally considered to be an antimicrobial defence strategy, containing infectious microorganisms to prevent their dissemination [17–19]. In contrast to their beneficial role during infection, overproduction of NETs or a failure to degrade extracellular chromatin also drives various tissue pathologies. Diseases with a pathological implication of NETs include malaria [20], thrombosis [21,22], autoimmune diseases [23–25] and cancer [26–28]. Given this broad involvement in health and disease, it is imperative to understand the molecular pathways leading to NET release. However, due to the fragile and short-lived nature of neutrophils, we still have many open questions regarding these mechanisms.

Strong activation of neutrophils induces NET formation via various mechanisms, with seemingly different kinetics, efficiency and morphological features of the resulting NETs, as discussed below. Contributing to these variations are differences in the species and origin of neutrophils used for experiments (murine neutrophils derived from bone marrow versus human neutrophils isolated from circulation), the amount of preactivation of the cells (isolation methods, homeostatic neutrophils vs patient neutrophils, time of day) or the experimental conditions themselves (different tissue culture media, different supplements, different sources of serum, different cytokine priming steps). Many of the controversies on the use of various buffers, the question about preactivation of cells during isolation or the species differences, are highlighted in ref. [29]. As an example, extracellular acidification to pH 6.7 enhanced ROS production in human neutrophils cultivated in bicarbonate-buffered RPMI and stimulated with formylated peptides, immune

complexes or zymosan [30]. However, using human neutrophils in bicarbonate-buffered RPMI and acidification to pH 6.5–5.5 decreased ROS production and NET formation in response to immune complexes and PMA [31].Despite the considerable number of confounding variables, after over a decade of intensive research there is some consensus on the molecular pathways leading to NET formation.

Most inducers of NET formation will cause the death of the neutrophil casting the NET. These suicidal NET formation pathways can be broadly classified into two groups, according to their requirement for ROS production via NADPH oxidase. The nuclear membrane of neutrophils activated to form NETs disintegrates, leading to chromatin expansion, the mixing of chromatin with granule contents inside the cell and eventually cell lysis and NET release. It is important to point out that due to the highly diverse nature of NET inducers, the involvement of different proteins might be context-dependent. As discussed above, there are many confounding factors prohibiting direct comparison of studies. Consequently, the results of a recent survey within the NET field showed that there is a strong need for systematic and comparative studies to investigate the involvement of specific proteins in NET formation [29]. Such studies are complicated by the fact that human primary neutrophils are not amenable to genetic manipulation. Nevertheless, with research aiming to overcome these limitations, for example by using neutrophil-like cell lines with genetic modifications [32–34], future studies will provide more detail to help us understand the NET formation pathways, enabling us to investigate whether and how certain proteins are involved.

NADPH oxidase-dependent NET formation

The mitogen phorbol 12-myristate 13-acetate (PMA) is the best studied (and a very robust) inducer of NADPH oxidase-dependent NET formation. PMA treatment of human and murine neutrophils activates protein kinase C (PKC) [35], which induces downstream activation of the Raf-Mek-Erk pathway [36], ROS production through NADPH oxidase [37–39], activation of myeloperoxidase (MPO) [40] and release of neutrophil proteases such as neutrophil elastase (NE) from granules into the cytoplasm [41,42]. NE then migrates to the nucleus where it cleaves histones [41,42]. This is followed by nuclear membrane disintegration, chromatin expansion and eventual cell lysis and NET release [37] (Fig. 1). Importantly, more physiological inducers including sterile (cholesterol crystals, immune complexes) or infectious (fungi, bacteria) stimulants follow roughly the same line of events, both in human and murine neutrophils and in various cell culture media spanning RPMI, DMEM or HBSS [43–47]. Fungal species such as *Candida albicans* (*C. albicans*) hyphae or *Aspergillus* species, for example, also trigger a ROS-dependent pathway to NET formation [48,49]. The importance of NAPDH oxidase in antifungal defence is further emphasized by findings in patients suffering from chronic granulomatous disease (CGD), caused by inactivating mutations in NADPH oxidase components. These patients are highly susceptible to bacterial and fungal infections, and CGD neutrophils do not form NETs in response to NADPH oxidase-activating stimuli [37,48]. Interestingly, *C.albicans* also seems able to induce NETs via an NADPH oxidase-independent pathway [50]. These discrepancies between studies could be explained by the observation that opsonized *C. albicans* bind to different receptors on neutrophils than unopsonized fungi, and therefore, they activate different signalling pathways [50].

It is not entirely clear whether NADPH oxidase-derived ROS are directly killing the cell, whether they act as a signalling intermediate or whether the energy-consuming process of replenishing NADPH is responsible for cell death. NADPH is replenished through the pentose phosphate pathway, and blocking this pathway inhibited NET formation in human neutrophils [51,52], suggesting that a persistent ROS burst is required for the pathway. Additionally, there is evidence that NET formation requires ROS and not simply activation of NADPH oxidase. ROS scavengers block NET formation in response to PMA and *C. albicans* [48]. Furthermore, exogenous ROS production via the enzyme glucose oxidase induces NET formation, even in neutrophils treated with NADPH oxidase inhibitors or derived from CGD patients [37]. A downstream product of NADPH oxidase activation, H_2O_2, is the substrate of MPO, and neutrophils derived from MPO-deficient patients or treated with MPO inhibitors fail to produce NETs in response to PMA or *C. albicans* [40,47].

Further players involved in the NADPH oxidase-dependent pathway

The NADPH oxidase-dependent pathway involves neutrophil serine proteases (NE, cathepsin G [CTSG] and proteinase 3 [PRTN3]). NE, CTSG and PRTN3 reside in primary granules, and induction of NET formation via the NADPH oxidase-dependent pathway results in release of NE from granules into the cytoplasm [42]. Upon its release from granules, NE migrates to the nucleus where it clips histones, which

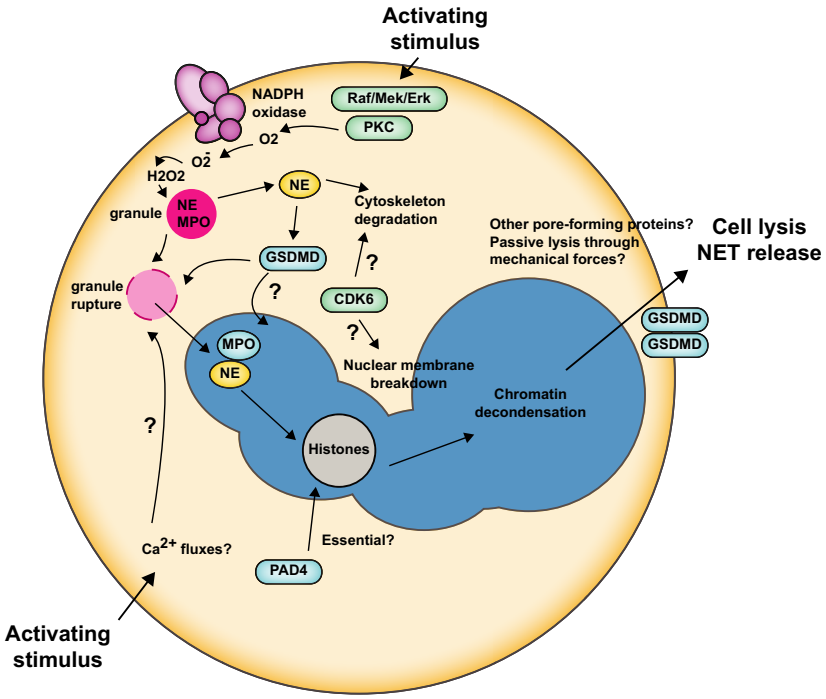

Fig. 1. NET formation pathways. NET formation pathways can be divided into NADPH oxidase-dependent (upper half of the figure) and NADPH oxidase-independent (lower half of the figure). NADPH oxidase-dependent NET formation: Stimulation of neutrophils leads to activation of the Raf-Mek-Erk pathway, of protein kinase C (PKC) and of NADPH oxidase, which converts O_2 to superoxide (O_2^-). The downstream product hydrogen peroxide (H_2O_2) triggers release of neutrophil elastase (NE) from granules. NE degrades the actin cytoskeleton and activates the protein gasdermin D (GSDMD). GSDMD acts in a feed-forward loop to allow more NE release from granules. The kinase CDK6 plays an important role in NADPH oxidase-dependent NET formation, but it is unclear what its substrates are. NE migrates to the nucleus and cleaves histones to allow chromatin expansion. As the nuclear membrane breaks down, chromatin fills the cell and is released as a NET upon cell lysis. This lysis event likely depends on pore-forming proteins (such as GSDMD), but might also occur passively due to forces generated during chromatin swelling. NADPH oxidase-independent NET formation: Activation of neutrophils likely causes Ca^{2+} fluxes. Ca^{2+} is essential for activation of the enzyme PAD4, which converts arginine to citrulline. Histone H3 is citrullinated in NETs, but it is unclear whether this citrullination event is necessary for NET formation to occur.

assists the decondensation of chromatin [41]. Incubation of granules with H_2O_2 is sufficient to induce NE leakage [42], suggesting that ROS mediate granule rupture. However, there might be more factors leading to protein release from granules (Fig. 1). For example, the pore-forming protein gasdermin D (GSDMD) can attack plasma membranes and cause cell lysis [53–56], but a recent study proposed that it also mediates granule permeabilization during NET formation, initiating a positive feedback loop of NE release and further GSDMD activation by NE [57]. We currently lack mechanistical insight into how exactly GSDMD permeabilizes granules, but another study confirmed GSDMD-mediated granule rupture through this positive feedback loop, both in human and in murine neutrophils [58]. GSDMD also localizes to the plasma membrane of PMA-stimulated neutrophils [57], suggesting that it mediates lysis of neutrophils during NET formation. However, decondensation and swelling of chromatin inside the cell exerts a force on the plasma membrane and could therefore lead to lysis in the absence of any regulatory proteins [59].

Many of the processes in NET formation pathways (such as receptor-mediated pathogen sensing, NADPH oxidase activation or nuclear membrane breakdown) involve kinase signalling. The Raf-Mek-Erk pathway and PKC are involved in the upstream events leading to NADPH oxidase activation and the oxidative burst in human neutrophils [35,36]. Other studies found involvement of PI3K and JNK in human neutrophils stimulated to form NETs with pyocyanin (a toxin produced by *Pseudomonas aeruginosa*) [60] and of Src/Syk, PI3K, and ERK, Akt and p38 in NET formation of human neutrophils stimulated by immune complexes [45]. Still, we do not fully understand which kinases or signalling pathways are involved at which part of NET formation, particulary in response to various physiological inducers under standardized

conditions. As discussed above, some pathogens might trigger different receptors and kinases, depending on their growth/metabolic state, on their opsonization or on other parameters.

A surprising addition to the panel of kinases with roles in NET formation came with the discovery that cyclin-dependent kinase 6 (CDK6), an enzyme involved in mitosis and proliferation, mediated NET formation, in response to PMA but also upon *C. albicans* infection [44]. Inhibition of CDK6 blocks translocation of NE to the nucleus, and while the exact substrate(s) of CDK6 are unknown, the study shows that NET formation 'hijacks' enzymes from other pathways, such as mitosis. More such examples, focusing on proteins from other cell death pathways, will be discussed below.

NADPH oxidase-independent NET formation

Some inducers of NETs, including calcium ionophores (e.g. A23187, derived from *Streptomyces chartreusensis*) or the potassium ionophore nigericin (derived from *Streptomyces hygroscopicus*), stimulate NET formation in the absence of a functional NADPH oxidase [49,61] (Fig. 1). We still have many open questions regarding this pathway (or these pathways). For example, it is unclear whether it is truly ROS-independent or merely independent of NADPH oxidase while ROS are provided by other means such as mitochondria [34]. Along the same line, if ROS are involved in granule disintegration, it is unclear what would allow protease release in the case of truly ROS-independent NETs. It has been suggested that NADPH oxidase-independent forms of NET formation proceed in the absence of protease activity, for example upon nigericin stimulation [49]. However, it is unclear how it is possible to overcome the requirement for protease activity in nuclear expansion and chromatin swelling. One explanation is that calcium fluxes, which are a prominent feature of NADPH oxidase-independent NET formation, induce activation of the enzyme peptidyl arginine deaminase 4 (PAD4), which citrullinates arginines on various proteins, including histones. Citrullination of histones removes a positive charge from the proteins. The hypothesis that histone citrullination leads to chromatin decondensation and NET formation by reducing charge–charge interactions of histones and DNA is appealing, and histone citrullination is a good marker to detect NETs *in vitro* and *in vivo* both in murine and in human cells or tissues [62–65]. However, several studies have questioned whether PAD4 is essential for NET formation [47,49,66]. The above-mentioned survey confirmed that the debate about involvement of PAD4 in NET formation is one of the most controversial aspects in the field [29]. Interestingly, a recent study shed some light on the involvement of PAD4 in ionomycin-induced NET formation, using mouse and human neutrophils in HBSS for stimulations. The Ca^{2+} fluxes induced by ionomycin activate both PAD4 and the protease calpain, and the concerted action of the two enzymes was necessary and sufficient to allow nuclear expansion and chromatin decondensation [67]. The finding that calpain can promote chromatin decondensation in the presence of citrullination could also explain why stimuli such as nigericin are able to induce NET formation in the absence of NE activity.

Whether citrullination is essential for NET formation or a bystander effect of the pathway, it is clear that NETs contain citrullinated proteins. A fascinating question is therefore whether citrullination of NET-binding proteins alters their inflammatory potential *in vivo*. Only one study so far specifically addressed the impact of citrullination on the function of NETs, and found that citrullination enhanced the ability of NETs to signal via TLR4 [66]. It will be interesting to follow up on these experiments, to define the 'citrullinome' of NETs and to investigate how this modification of proteins affects their behaviour.

NETs and other cell death pathways

The original classification of cell death into two pathways, apoptosis (active and programmed) and necrosis (passive), has tremendously expanded within the last decades. We now know that cells die in many different ways, most with at least some regulated components. It has also become clear that there is substantial crosstalk between these pathways. This demonstrates the importance of cell death, especially in inflammatory or infectious settings. Once a cell initiates a death pathway, there will be multiple backups to ensure that death occurs even if pathogens interfere with one of the mechanisms, as discussed in more detail below.

As NET formation results, in most cases, in neutrophil cell death, two main questions arise: Firstly, 'Can NET formation pathways also crosstalk to other forms of cell death by using the same initiator or executioner proteins?'; and secondly 'Does canonical induction of these cell death pathways cause NET formation as a consequence in neutrophils?' We will discuss these questions below by focusing mostly on apoptosis, necroptosis and pyroptosis.

NETs and apoptosis

Apoptosis, the 'clean form' of programmed cell death, follows a series of events to activate executioner caspases, which subsequently digest a variety of substrates, leading to a loss of cell viability [68]. Apoptotic cells communicate their death to the environment and expose 'eat me' signals on their surface, allowing the phagocytosis of apoptotic corpses (a process called efferocytosis), ensuring that apoptotic cell death does not cause an inflammatory response. Apoptosis is strictly dependent on caspases, and there is good evidence that NET formation in response to a broad variety of stimuli occurs independently of apoptotic caspases [37,38,49,57,69]. NETs therefore result from a nonapoptotic process.

However, an interesting question is what happens when apoptotic neutrophils are not adequately removed by efferocytosis. In the absence of efferocytosis, apoptotic cells can undergo a process of secondary necrosis. Recent studies showed that secondary necrosis can involve a pore-forming protein of the gasdermin family, GSDME (DFNA5) [70–72]. Even though it is tempting to speculate that such secondary necrotic events might favour chromatin release, it is unclear whether the released chromatin would behave like NET chromatin. Additionally, GSDME-driven secondary necrosis does not necessarily happen in every cell type and immune cells seem particularly resistant to this type of lysis (reviewed in ref. [73]). In addition, apoptosis leads to chromatin condensation and eventually degradation [68], making it unlikely that a failure to clear apoptotic neutrophils results in bona fide NETS. Chromatin from apoptotic cells is usually released in microparticles [74] and degraded by serum DNases. Interestingly, mice deficient for the serum DNases DNASE1 [75] or DNASE1L3 [74] develop autoimmune disease with autoantibodies against chromatin and a phenotypic similarity to systemic lupus erythematosus (SLE). Likewise, defective efferocytosis correlates with the occurrence of SLE-like symptoms in mice [76–78]. It would be interesting to investigate whether, especially in situations with excess dying neutrophils, secondary necrosis contributes to extracellular chromatin with NET features in inflamed tissues.

Efferocytosis of neutrophils in tissues is part of an important feed-forward loop to regulate granulopoiesis [10]. Efferocytosis by macrophages dampens the production of the cytokine granulocyte colony-stimulating factor (G-CSF), the master regulator of granulopoiesis. If efferocytosis fails, there will be enhanced G-CSF production and, consequently, enhanced granulopoiesis [10]. In such cases, we speculate that enhanced and prolonged G-CSF production could lead to the release of various neutrophils subpopulations (or slightly immature neutrophils) with different abilities to respond to stimulation, including NET-inducing agents. Therefore, even though mechanistically NET formation does not depend on apoptotic processes, disturbance of apoptosis or clearance of apoptotic cells might still influence NET release *in vivo*.

NETs and necroptosis

Necroptosis is a necrotic form of programmed cell death, causing cell lysis and resulting in an inflammatory response. Induction of necroptosis usually requires absence or inhibition of caspase-8 [79]. Cells receiving an apoptotic signal while failing to activate caspase-8 can initiate phosphorylation events dependant on receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and receptor-interacting serine/threonine-protein kinase 3 (RIPK3), culminating in phosphorylation of mixed lineage kinase domain like pseudokinase (MLKL). Phosphorylated MLKL will then multimerize, form pores in the plasma membrane and cause cell lysis [79] (Fig. 2). Necroptosis can therefore be considered as a backup programme to allow cell death when apoptosis is inhibited. Necroptosis is tightly linked to other cell death pathways, particularly apoptosis or pyroptosis, and these different pathways can compensate for each other on multiple levels (for an excellent review, see ref. [80]). This makes sense since necroptosis and pyroptosis often occur in the context of infection. An infected cell has to make sure that the death signal is transmitted, even if pathogens interfere with one of the cell death pathways.

As there is substantial crosstalk of necroptosis with other cell death pathways, several studies investigated whether induction of NET formation by established stimuli requires components of the necroptosis machinery or whether necroptosis in neutrophils could have NET release as a consequence.

For the former, it has been suggested that NET induction by PMA and crystalline particles [69,81] by activated platelets [82] or by antineutrophil cytoplasmic antibodies (ANCA) [83] involve activity of RIPK1 and RIPK3. However, contradicting studies found that NET induction by PMA, LPS or complement component 5a [84], as well as by PMA, *C. albicans*, nigericin, group B streptococci or the calcium ionophore A23187 [49], did not require RIPK1 or RIPK3. In a similar contradiction, induction of necroptosis via classical stimuli was both shown to result in the release of NETs [85] or kill neutrophils in the absence of NET release [49]. Further complication arises from the

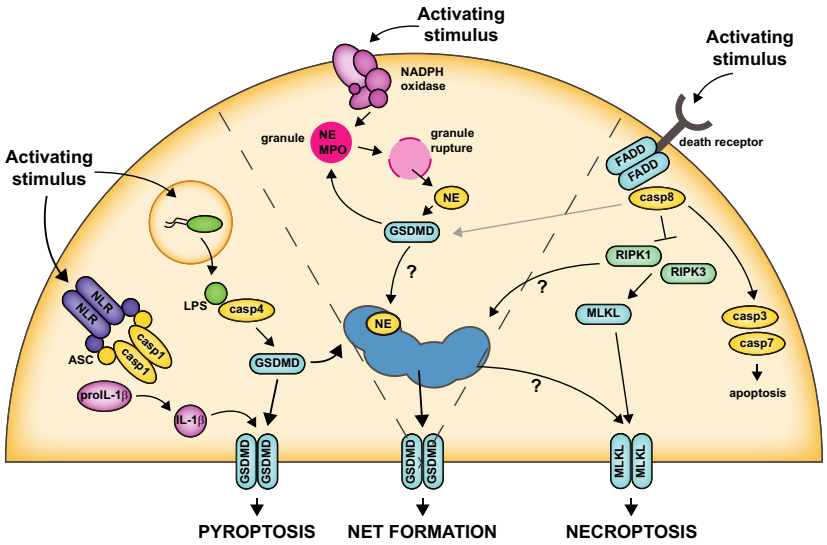

Fig. 2. Crosstalk of NET formation with other cell death pathways. Pyroptosis (left panel), NET formation (middle panel) and necroptosis (right panel) are lytic forms of cell death, causing inflammation, and it is likely that they share some of their molecular machinery. Pyroptosis occurs downstream of the activation of inflammatory caspases (casp1/casp4). Casp1 is activated through inflammasome complexes with a Nod-like receptor (NLR) sensing PAMPs or DAMPs, leading to inflammasome formation, caspase recruitment via the bridging protein ASC and caspase activation by multimerization. Casp1 then activates the cytokines interleukin(IL)-1β and IL-18 (not shown). Casp4 is activated upon sensing of cytoplasmic lipopolysaccharide (LPS). Both caspases cleave and activate GSDMD, which subsequently forms pores in the plasma membrane leading to lysis. Casp4-activated GSDMD in neutrophils plays an important role in NET formation by allowing nuclear expansion, lysis and NET release. Necroptosis requires activation of the kinases receptor-interacting protein kinase (RIPK) 1 and 3. The protease caspase-8 (casp8) inhibits this activation. Casp8 activity can also promote GSDMD processing and lysis via pyroptosis. When casp8 itself is inhibited, activation of death receptors allows RIPK1 and RIPK3 activation, subsequent phosphorylation of the pseudokinase MLKL and MLKL-mediated plasma membrane pores. It is unclear whether necroptosis proteins are involved in NET formation.

interesting finding that at least mouse neutrophils are rather resistant against the induction of necroptosis. The expected shift from apoptotic to necroptotic death upon inhibition of caspases was only seen in cells deficient for X-linked inhibitor of apoptosis (XIAP), whereas wild-type cells did not undergo necroptosis [86]. Therefore, while the concept that MLKL pores contribute to the lytic events seen during NET formation is appealing, there is no consensus so far whether necroptosis is truly involved in NET release. Given the facts that necroptosis is activated under conditions of protease (or at least caspase) inhibition, that caspase-8 can activate GSDMD to promote lysis [87] (Fig. 2) and that NET formation is in many cases a process relying on high protease activity, involvement of the necroptotic machinery might be highly context-dependent.

NETs and pyroptosis

Pyroptosis is a programmed form of necrotic cell death induced by inflammasome and caspase-1 or caspase-4/-11 activation [88,89]. It is associated with release of the proinflammatory cytokines IL-1β and IL-18 and with cell lysis through gasdermin D (GSDMD) pore formation [53–56] (Fig. 2). Canonical inflammasome activation occurs through sensing of pathogen-associated molecular patterns (PAMPS) or danger-associated molecular patterns (DAMPs) by various Nod-like receptors (NLRs). Upon sensing PAMPs and DAMPs, NLRs recruit caspase-1 via the adaptor protein ASC, leading to caspase-1 multimerization, IL-1β processing and pyroptosis [89]. Non-canonical inflammasome activation occurs upon detection of cytoplasmic lipopolysaccharide (LPS) by caspase-4 [90,91], driving its activation, GSDMD pore formation and caspase-1 activation through the NLRP3 inflammasome. Pyroptosis is best described in macrophages, and we do not fully understand whether pyroptotic events in neutrophils lead to NET release.

Noncanonical inflammasome stimulation by delivery of LPS to the neutrophil cytoplasm leads to caspase-4/-11 activation and NET release in a GSDMD-dependent manner [92], showing that neutrophils undergoing

pyroptosis are able to release NETs. However, three enzymes characterized in more canonical NET formation (NE, MPO and PAD4) are dispensable for NET extrusion during caspase-4/-11 activation [92]. Thus, the mechanism of NET release during pyroptosis differs from other pathways and seems to be driven mainly by GSDMD (Fig. 2). GSDMD also plays an important role in more canonical pathways of NET formation. In PMA-treated human neutrophils, GSDMD is cleaved by NE and affects nuclear expansion during NET formation, likely via disruption of granules [57]. Other studies also demonstrate activation of GSDMD by neutrophil proteases [93,94] and involvement of GSDMD in granule rupture [58]. GSDMD could potentially link pyroptosis and more canonical NET formation in neutrophils, but it also affects NET-independent processes in neutrophils [58,93,94]. We require more research into neutrophil-specific functions of gasdermins.

In contrast to the above-mentioned findings, murine neutrophils are resistant to pyroptosis downstream of canonical inflammasome activation. While these cells express inflammasome components and are able to release active IL-1β, unlike macrophages they do not die by pyroptosis [92,95–97]. Several mechanisms could contribute to this resistance. A recent study described a hyperactive state of activation in macrophages following canonical inflammasome activation, where IL-1β was released through GSDMD pores without pyroptosis [98]. There could be a similar mechanism in neutrophils that allows them to resist pyroptosis while secreting IL-1β through GSDMD pores. Alternatively, neutrophils might engage efficient membrane repair mechanisms. Sublytic GSDMD pores engage a membrane repair mechanism in macrophages (depending on proteins of the endosomal sorting complexes required for transport [ESCRT] family), which restricts pyroptotic lysis [99]. It is tempting to speculate that neutrophils use similar mechanisms to resist pyroptosis upon inflammasome activation. However, ESCRT-dependent membrane repair reduced pyroptotic lysis both upon canonical and noncanonical inflammasome activation [99]. Neutrophils preferentially resist pyroptosis in the context of canonical inflammasome activation [92,95–97], and it is not clear how ESCRT-mediated repair would discriminate between the two activation pathways in neutrophils but not in macrophages. Neutrophils might express less caspase-4/-11 than macrophages, and therefore have a reduced potential to activate GSDMD activity or neutrophil caspase-1 might be less efficient in processing GSDMD than caspase-4/-11 [92]. However, a recent report showed that even with efficient caspase-1-mediated GSDMD processing, murine neutrophils remained resistant to pyroptosis, whereas caspase-11 readily activated the cells to die [97]. Discussing previous reports showing GSDMD integration into neutrophil granules [58], the authors suggested that such events would prevent plasma membrane pore formation and lysis. However, as GSDMD pores in granule membranes cause release of proteases such as NE [57,58], GSDMD-mediated granule rupture might, depending on the context, still cause cell death.

Interestingly, in addition to the neutrophil's ability to resist pyroptosis upon canonical inflammasome activation, the cells also seem to respond differently to these canonical inducers than macrophages do. The NLRP3 inflammasome is the most promiscuous inflammasome, it responds to a broad variety of stimuli ranging from crystalline particles to ionophores and to various infectious agents. Murine neutrophils, however, did only activate the NLRP3 inflammasome in response to soluble inducers but not upon stimulation with crystals [96]. Furthermore, while murine neutrophils readily release mature IL-1β, the picture of human neutrophil-derived IL-1β is less consistent. Depending on the study, human neutrophils released no detectable IL-1β [57], very low levels [100] or a broad spectrum of different levels depending on the donor [58,101].

Taken together, it appears that pyroptosis is most efficiently induced by noncanonical inflammasome activation in neutrophils and this was also the condition where NET formation was observed. An important open question is how the findings regarding inflammasome activation, cytokine release and resistance to pyroptosis in murine neutrophils translate to human cells.

NETs and other necrotic cell death pathways

NET formation, necroptosis and pyroptosis are not the only regulated necrotic pathways. Many different pathways have been described within the last years, some of them more restricted to certain cell types or stimuli than others [102]. The information about whether these pathways might crosstalk to NET formation is extremely limited. Of interest, ferroptosis is a form of cell death induced by lipid peroxidation [102], and since many inducers of NETs act via a massive oxidative burst, it is tempting to speculate that components of the ferroptosis machinery could be involved in NET formation. However, although one study identified an inducer of ferroptosis to accelerate NET formation, the authors concluded that this was independent of the cystine transporter

component xCT, a key component of ferroptosis [103].

NETs and autophagy

Autophagy is a process of cells engulfing cytoplasmic macromolecules or organelles in a double-membraned compartment called an autophagosome and subsequently degrading them via lysosomal pathways. This process provides material to generate new proteins or targets damaged molecules and organelles. Therefore, autophagy is usually considered to be protective rather than a cell death pathway. Although autophagy can lead to a rather unspecific degradation of cytoplasmic material, it can also be selective, for example when targeting pathogenic microorganisms [104].

Autophagy affects immunity and, more specifically, neutrophils in different ways. It is beyond the scope of our review to cover all of these aspects; therefore, we refer the readers to other reviews describing how autophagy influences the immune system and neutrophils [104,105]. It is, however, important to keep in mind that any effects of autophagy on NET formation could be mediated indirectly via other pathways. For example, deletion of ATG7 (and thereby blocking autophagy) in mice inhibits appropriate maturation of neutrophils [106]. Autophagy is required during neutrophil maturation to provide free fatty acids that fuel mitochondrial respiration [106]. Deletion of ATG7 in murine neutrophils also leads to reduced ROS production through NADPH oxidase and to reduced degranulation in response to formylated peptides [107], and it could be that such changes in neutrophil function are at least in part caused by disturbances in maturation.

The role of autophagy in NET formation is controversially discussed. While treatment with rather unspecific PI3K inhibitors, such as wortmannin or 3-MA, reduced NET formation in a number of studies and in response to various stimulations [38,108–112], genetic deletion of ATG5 did not affect the ability of murine neutrophils to form NETs [108]. On the other hand, deletion of WDFY3, a regulator of selective autophagy, led to a reduction in ROS production and NET formation in murine neutrophils [113]. We need more research using specific/genetic tools to elucidate how and under which conditions autophagy affects NET formation pathways. A further interesting question will be to define the proteins bound to NETs if autophagy is active or inhibited. As it is a degradation pathway, the protein content of NETs might differ significantly in the presence or absence of autophagy (also reviewed in ref. [105]), which might alter the effects of NETs on microbes or host tissue.

Vital NET release

While the focus of this review is on NET formation as a cell death pathway, NET structures can be released by live neutrophils through two distinct nonlytic processes. The first enables neutrophils to release chromatin without lysing, generating intact anuclear neutrophils. Although this has been referred to as 'vital NETosis' [114], we will avoid this contradictory term in favour of 'vital chromatin release'. The second pathway involves the release of mitochondrial DNA (mtDNA). While it is not unusual for NETs to contain both mitochondrial and nuclear DNA [49], neutrophils can also release mtDNA through a nonlytic process, which may offer an alternative source of DNA for NET formation [115]. We will refer to this process as 'vital mtDNA release'.

Vital chromatin release

Human neutrophils exposed *in vitro* to nonopsonized *Staphylococcus aureus (S. aureus)* undergo a rapid NADPH oxidase-independent process that contains chromatin within vesicles. This chromatin is released into the extracellular environment to trap and kill *S. aureus* without rupturing the cell (Fig. 3). These nonlytic NETs had detectable NE activity, although at lower levels than NETs formed by neutrophil lysis after a longer incubation with *S. aureus* [116]. Further research studying *S. aureus* infection in mouse skin observed mouse and human neutrophils releasing chromatin while moving, and described intact anuclear neutrophils. The addition of DNase to the site of infection promoted the spread of *S. aureus* from the wound into the blood, indicating that the released chromatin forms NETs and traps pathogens without requiring neutrophil lysis [117]. Vital chromatin release has also been observed after LPS stimulation of neutrophils in the presence of platelets. The released DNA colocalized with MPO and NE, but the NE activity was much lower than the activity detected in NETs formed by neutrophil lysis in the absence of platelets [118].

While the viability of anuclear neutrophils has been questioned [119], advocates of vital NET release point to artificially created anuclear neutrophils, which remain motile and phagocytically active [114,120]. If vital chromatin release enables neutrophils to contain pathogens through phagocytosis while also releasing NETs, one could postulate that such a pathway is more useful *in vivo* than lytic NET release [121]. This ability will need to be verified through further experiments, since these anuclear neutrophils may not be as

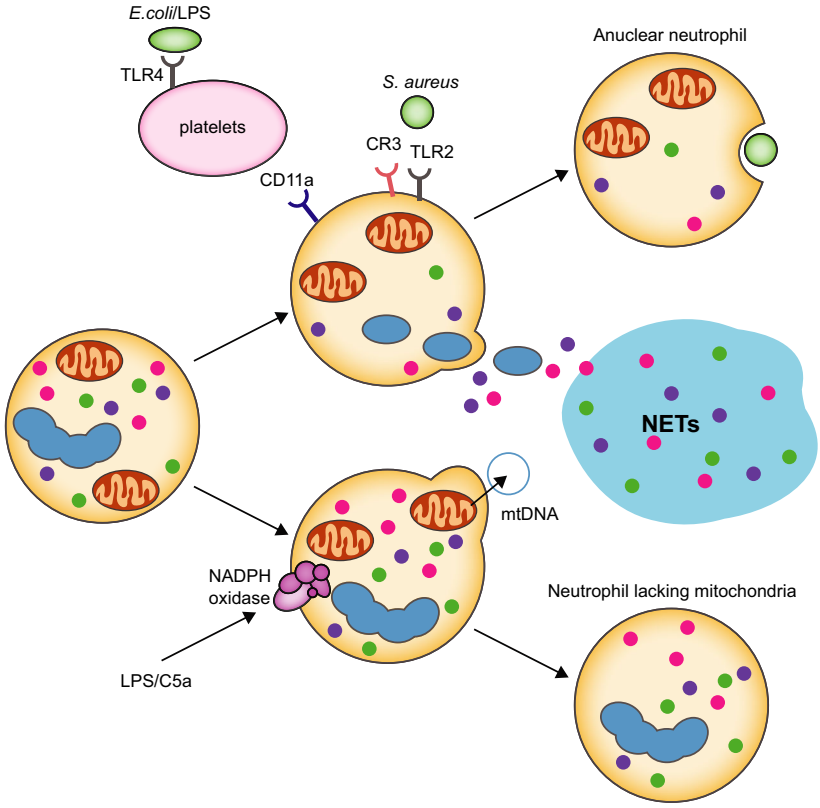

Fig. 3. Vital NET formation. NET formation in the absence of cell lysis involves either chromatin release (upper panel) or the release of mitochondrial DNA (lower panel). Chromatin release is induced by the Gram-positive bacterium *S. aureus* via Toll-like receptor 2 (TLR2) and a complement receptor (CR3, also called Mac-1). Chromatin is released in vesicles without neutrophil lysis, and anuclear neutrophils remain motile and capable of phagocytosis. Mitochondrial DNA (mtDNA) release occurs upon stimulation with lipopolysaccharide (LPS) or the complement component C5a. Neutrophils release mtDNA by an unknown mechanism, which involves activation of NADPH oxidase, but not cell lysis.

capable as the artificially created anuclear neutrophils described in earlier research.

Anuclear neutrophils after NET formation have so far only been observed in the presence of *S. aureus,* a pathogen that releases calcium channel agonists and pore-forming leukotoxins [122], which can induce NET release even in the absence of *S. aureus* bacteria [123]. Vital chromatin release could be a side effect of an abnormal calcium influx caused by these toxins [124], although this does not rule out a role in containing *S. aureus*. Further research is needed to investigate the diversity of stimuli leading to vital chromatin release from neutrophils. Is this a phenomenon that occurs upon infection with very specific pathogens (such as *S. aureus*), or is vital chromatin release a hallmark of a broader set of infectious agents?

In addition, there is a need for more studies on non-DNA components of NETs released by viable neutrophils, since the consistent detection of lower NE activity [116,118] suggests that they will be less effective at killing bacteria than lytic NETs. The intriguing hypothesis that anuclear neutrophils formed by vital chromatin release remain capable of phagocytosis [114] needs to be experimentally tested, since they may not be as capable as artificially generated anuclear neutrophils.

Vital mtDNA release

Structures comprised of mtDNA, NE and MPO were first described in GM-CSF primed neutrophils stimulated *in vitro* with LPS or C5a. This release of mtDNA relies on NADPH oxidase but does not require neutrophil lysis [125] (Fig. 3). It has been proposed that *in vivo* NETs could exclusively form from mtDNA, since this process would not require neutrophil lysis and so would enable phagocytosis to continue [114], although the observation that mtDNA is able to induce suicidal NET release challenges this hypothesis [126]. While it is unclear how the mitochondrial membranes would be breached to release mtDNA in the absence of cell death, the required force could be generated by the unravelling of the supercoiled mitochondrial nucleoid after a double-stranded DNA break [127].

Extracellular mtDNA can be detected *in vivo* [128] and has been linked to the autoimmune disease SLE [129], especially when the mitochondrial DNA is oxidized [130]. However, this mtDNA could be a component of NETs formed primarily from nuclear DNA, or could be released as a side effect of defective mitophagy [124]. Ejection of mtDNA is not unique to neutrophils and has been observed in eosinophils [131],

basophils [132,133] and B lymphocytes [134]. While antibacterial effects have been reported [131,133], the main function appears to be proinflammatory [134]. While mtDNA lacks the antimicrobial properties of histones, mitochondria contain n-formylated peptides and oxidized mtDNA with unmethylated CpG sequences, which act as potent proinflammatory signals [119]. Further research is needed to determine how and why these various immune cells release mtDNA, and to test whether mtDNA released by neutrophils can contain and kill pathogens, in addition to acting as a proinflammatory signal. Future studies should also assess the oxidation state of the mtDNA, since oxidized DNA is another proinflammatory signal that could be released from mitochondria [98].

Concluding remarks

Extensive research efforts aiming to elucidate the molecular pathways of NET formation have allowed us to understand important aspect of these pathways. We learned that most of the pathways leading to NET release require protease activity to allow cytoskeleton degradation, nuclear membrane breakdown and chromatin expansion, all key processes in NET formation. It is unlikely that deficiency in only one of the several highly active neutrophil proteases will be sufficient to block all of these processes. Furthermore, as discussed above, depending on the context, phosphorylation events (such as the phosphorylation of lamins that assists nuclear membrane disintegration [44]) or other post-translational modifications (such as citrullination [62–65]) contribute to protease release from granules or their activation, to weakening of the nuclear lamina, to expansion of chromatin or to neutrophil lysis. Future, systematic and comparative studies will determine which proteins are involved in these events and define whether they are crucial to NET formation or merely accelerate the process. NET formation appears to be a multilayered process with context-dependent regulatory events (sometimes even allowing chromatin release from viable cells), and we are only just beginning to understand how all these regulations and molecular processes interact.

It has become clear that cell death pathways overlap and crosstalk on many levels, ensuring that once a cell is committed to death, it will do so even if infectious or pathogenic agents interfere with specific pathways. While NET formation is clearly different from apoptosis, there is evidence that neutrophils use components of the pyroptotic machinery (such as caspase-4 and GSDMD) to drive NET release, at least under certain conditions. It is interesting that at least murine neutrophils are resistant to canonical pyroptosis, but induce NET formation when they detect cytoplasmic LPS, suggesting that the cells rely on phagosomal killing of pathogens and will only trigger the rather extreme defence mechanism of NET formation once bacteria breach the phagosome.

There is conflicting information about the crosstalk of NET formation pathways and necroptosis. However, it seems that a requirement for necroptotic proteins, if it exists at all, is highly context-dependent and will not be detected in all forms of NET release.

In conclusion, 'NET formation' summarizes various processes, which lead to chromatin release from neutrophils. While most canonical forms described feature unique events separating them from other pathways, specific contexts likely allow crosstalk with other forms of cell death.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

GS conceptualized the review. TR, JW and GS searched the literature, wrote the manuscript and created the figures. TR and JW contributed equally and are listed in alphabetical order.

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