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Cellular Mechanisms of NETosis

Hawa Racine Thiam[1], **Siu Ling Wong**[2], **Denisa D. Wagner**[3,4,5], **Clare M. Waterman**[1]

[1]Cell and Developmental Biology Center, National Heart, Lung, and Blood Institute (NHLBI), National Institutes of Health, Bethesda, Maryland 20892, USA

[2]Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore 308232

[3]Program in Cellular and Molecular Medicine, Boston Children's Hospital, Boston, Massachusetts 02115, USA

[4]Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115, USA

[5]Division of Hematology/Oncology, Boston Children's Hospital, Boston, Massachusetts 02115, USA

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Neutrophils are critical to innate immunity, including host defense against bacterial and fungal infections. They achieve their host defense role by phagocytosing pathogens, secreting their granules full of cytotoxic enzymes, or expelling neutrophil extracellular traps (NETs) during the process of NETosis. NETs are weblike DNA structures decorated with histones and antimicrobial proteins released by activated neutrophils. Initially described as a means for neutrophils to neutralize pathogens, NET release also occurs in sterile inflammation, promotes thrombosis, and can mediate tissue damage. To effectively manipulate this double-edged sword to fight a particular disease, researchers must work toward understanding the mechanisms driving NETosis. Such understanding would allow the generation of new drugs to promote or prevent NETosis as needed. While knowledge regarding the (patho)physiological roles of NETosis is accumulating, little is known about the cellular and biophysical bases of this process. In this review, we describe and discuss our current knowledge of the molecular, cellular, and biophysical mechanisms mediating NET release as well as open questions in the field.

1. INTRODUCTION

Neutrophils are crucial to host defense. For instance, patients with a low number of neutrophils have recurrent bacterial and fungal infections and defects in clearing

watermancm@nhlbi.nih.gov, hawa.racinethiam@nih.gov.

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such infections (Dale & Hammond 1988). Differentiated in the bone marrow from the granulocyte–monocyte progenitor cells (Akashi et al. 2000, Görgens et al. 2013), mature neutrophils are characterized by the presence of granules in their cytosol and a multilobulated nucleus (Campbell et al. 1995). Neutrophils are released into the circulation, where they can be recruited to sites of both sterile and pathogen-induced injury by activated endothelial cells, resident immune cells, and injured epithelial cells (de Oliveira et al. 2016). Once they reach the site of injury, engagement of neutrophil surface receptors with proinflammatory signals, such as bacterial components, fungal β-glucan, or cytokines, induces a signaling cascade resulting in enhanced neutrophil effector functions (i.e., activation) (Mayadas et al. 2014). Activated neutrophils can neutralize invaders by releasing their granules by degranulation, internalizing and degrading pathogens by phagocytosis, and releasing neutrophil extracellular traps (NETs). The mechanisms of neutrophil differentiation, migration to injury sites, degranulation, and phagocytosis have been extensively reviewed (de Oliveira et al. 2016, Hidalgo et al. 2019, Ley et al. 2018). Here we focus on the more recently characterized process of the release of NETs.

1.1. Neutrophil Extracellular Traps

NETs are made of decondensed chromatin that forms weblike DNA structures with approximately 200-nm pores (Pires et al. 2016). They are coated with nuclear proteins including histones, granule proteins (such as neutrophil elastase and myeloperoxidase), and cytosolic proteins (such as S100 calcium-binding proteins A8, A9, and A12, as well as actin and α-actinin) (Chapman et al. 2019, Petretto et al. 2019, Urban et al. 2009). In addition to neutrophils, eosinophils (Yousefi et al. 2008), basophils (Morshed et al. 2014), mast cells (von Köckritz-Blickwede et al. 2008), and macrophages (Chow et al. 2010) have all been observed to release extracellular DNA upon stimulation. Initially described as a programmed cell death pathway distinct from apoptosis and necrosis, the release of extracellular traps is also thought to occur without cell lysis in a process termed vital NETosis (Adrover et al. 2020, Clark et al. 2007, Pilsczek et al. 2010, Yipp & Kubes 2013, Yipp et al. 2012).

1.2. NETosis Is a Double-Edged Sword

The seminal work of Brinkmann and colleagues (2004) first reported the ability of activated neutrophils to release nuclear DNA into the extracellular environment, where it can trap and neutralize pathogens in a process termed NETosis. The ability of various pathogens to induce NETosis (Abi Abdallah et al. 2012, Brinkmann et al. 2004, Saitoh et al. 2012, Urban et al. 2006), together with the demonstration that NETs can trap and kill bacteria (Berends et al. 2010, Brinkmann et al. 2004, Lappann et al. 2013, Pilsczek et al. 2010) and fungi (Urban et al. 2006) as well as limit HIV-1 virus infectivity (Saitoh et al. 2012), suggests that NETosis could be important for innate immunity. However, NETs also form in sterile inflammation (Sorvillo et al. 2019, Wong & Wagner 2018). Moreover, the ability of the cytotoxic proteins associated with NETs to damage host cells (Clark et al. 2007) and activate platelets (Fuchs et al. 2010), together with the presence of autoantibodies against proteins released during NETosis in patients with autoimmune diseases (Hakkim et al. 2010, Jorch & Kubes 2017), illustrates the double-edged sword effect of NETosis (Thanabalasuriar et al. 2019).

While the (patho)physiological relevance of NETosis has been demonstrated, the cellular mechanisms of NETosis have just begun to be revealed. For decondensed DNA decorated with nuclear, granular, and cytosolic contents to be released extracellularly, the process has to be initiated either extracellularly or intracellularly; the chromatin has to be decondensed and released from the nucleus; and the cytoskeleton, organelles, and intracellular and nuclear as well as plasma membranes must be remodeled. In the following, we review our current knowledge of these cellular steps.

2. NETOSIS INITIATION

While the primary signal that initiates NETosis instead of other neutrophil responses remains unknown, NET release starts with the activation of surface receptors followed by changes in intracellular calcium concentration, activation of kinase signaling cascades, and production of reactive oxygen species (ROS). These first signaling events translate into morphological changes, particularly an increase in cell spreading and changes in cell shape. In the following, we discuss these early signaling and cellular events.

2.1. Neutrophil Activation Is a Prerequisite for NETosis

NETosis requires neutrophil activation. Therefore, resting neutrophils in noninflammatory conditions do not undergo NETosis. Moreover, neutrophils of mice deficient in Toll-like receptor 2 (TLR2) or complement component 3 and, more generally, mice defective in interleukin-1 (IL-1)-receptor/TLR signaling [MyD88 knockout (KO)] do not release NETs upon *Staphylococcus aureus* stimulation (Yipp et al. 2012). Ligands of G-protein-coupled receptors (GPCRs) (Gupta et al. 2014, Rossaint et al. 2014), tumor necrosis factor (TNF) (Keshari et al. 2012), and Fc (Rossaint et al. 2014) receptors have been reported to induce NETosis, suggesting that these receptors might be involved in NETosis initiation. Although CD18 (β2 integrin) is dispensable for NETosis induced by calcium influx (Wong et al. 2015), phorbol 12-myristate 13-acetate (PMA), saliva mucin (Mohanty et al. 2015), or exogenous H_2O_2 (Raftery et al. 2014), it is required for activated-platelet- (McDonald et al. 2012, Rossaint et al. 2014), *S. aureus*– (Mohanty et al. 2015), and hantavirus-induced NET release (Raftery et al. 2014). In addition to plasma membrane surface receptors, NETosis can be triggered by activation of the nucleotide oligomerization domain (NOD)-like receptor protein 3 (P. Munzer, R. Negro, S. Fukui, L. di Meglio, C. Cherpokova, N. Sorvillo, L. Shi, V. Giri Magupalli, S. Gutch, L. Chu, R. Goldman, K. Ridge, R. Scharf, C. Waterman, H. Wu & D. Wagner, unpublished manuscript). Bacterial toxins such as ionomycin (Liu & Hermann 1978, Wang et al. 2009) and nigericin (Kenny et al. 2017, Lardy et al. 1958), as well as reactive oxygen species (ROS) (Fuchs et al. 2007), can induce NETosis, presumably without activating surface receptors. Thus, neutrophil activation, via either bacterial toxins or surface receptor engagement, initiates NETosis.

2.2. Calcium Spikes in NETosis

Neutrophil activation leads to an increase in the intracellular calcium concentration (Krause et al. 1990). For instance, ligand binding on GPCRs, Fcγ receptors, TLR4, and complement receptors (β_2 integrin) triggers a release of endoplasmic reticulum (ER)-stored calcium followed by the opening of plasma membrane calcium channels (Dixit & Simon 2012,

Gennaro et al. 1984, Hellberg et al. 1996, Immler et al. 2018, Kandasamy et al. 2013, Schappe et al. 2018, Schorr et al. 1999). In the context of NETosis, an increase in intracellular calcium was observed in neutrophils stimulated by lipopolysaccharides (LPSs), IL-8, and PMA, and treatment with the calcium ionophores ionomycin and A23187 (de Bont et al. 2018, Gupta et al. 2014) leads to NET release (Wang et al. 2009). More importantly, extracellular calcium chelation inhibits IL-8-, PMA-, *Pseudomonas aeruginosa*–, ionomycin-, and nigericin-stimulated NETosis (Gupta et al. 2014, Kenny et al. 2017, Parker et al. 2012), while intracellular calcium chelation impairs IL-8-, PMA-, and nigericin-induced but not *Candida albicans*– or Group B *Streptococcus*–induced NETosis (Kenny et al. 2017, van der Linden et al. 2017). Thus, an increase in intracellular calcium, via either release of intracellular stores or influx from the extracellular environment, is important for NETosis. However, the cellular processes requiring calcium during NETosis are poorly understood. One pathway known to be mediated by calcium is via peptidyl arginine deiminase 4 (PAD4), an enzyme critical for NETosis (Li et al. 2010, Wong & Wagner 2018) that requires a high concentration of calcium for its activation (Kearney et al. 2005). Thus, further identification of the mechanisms of calcium increase and the cellular targets of calcium during NETosis will be critical for understanding how calcium regulates NET release.

2.3. The Role of Kinases in NETosis

Both kinases activated downstream of calcium influx or cytokine engagement and regulators of the cell cycle have been implicated in NETosis. The phospholipid-dependent, phorbol ester- and calcium-activated key cell cycle regulator kinase, protein kinase C (PKC), in particular PKCα, PKCβ1, and PKCζ, mediates PMA-, ionomycin-, IL-8-, platelet-activating factor–, *C. albicans*–, and Group B *Streptococcus*–induced NETosis (Gupta et al. 2014, Hakkim et al. 2011, Kenny et al. 2017, Radic & Neeli 2013). Cyclin-dependent kinase 6, which regulates the G_1/S transition of the cell cycle, and the Raf-MEK-ERK MAP kinase pathway are critical to PMA-induced NETosis (Amulic et al. 2017), while the SYK-PI3K-mTorc2 pathway mediates monosodium urate crystal– and *S. aureus*–induced NETosis (van der Linden et al. 2017). Finally, the nonreceptor tyrosine kinase Janus kinase 2 (JAK2), which transduces cytokine-mediated signaling and controls cell proliferation, has recently been implicated in NETosis. Indeed, an activating mutation in JAK2 that causes cancer also enhances the propensity for NETosis (Wolach et al. 2018). However, similar to calcium, the requirement of PKC, the Raf-MEK-ERK and SYK-PI3K-mTorc2 pathways depend on the NETosis stimulant (Hakkim et al. 2011, van der Linden et al. 2017). Thus, downstream of receptor activation and/or calcium influx, several kinases implicated in cell cycle regulation mediate NETosis. Determination of their downstream effectors will be crucial for designing drugs for manipulating NETosis.

2.4. NADPH Oxidase: The Role of Reactive Oxygen Species in NETosis

Although the production of ROS has traditionally been regarded as a direct mechanism of killing pathogens via oxidative damage (Nguyen et al. 2017), ROS are also central to the signaling implicated in NETosis. The two main sources of ROS in neutrophils are NADPH oxidase and mitochondria. Stimulation of neutrophils with PMA, A23187, *C. albicans*, *S. aureus*, or Group B *Streptococcus* leads to a rapid (within 20 min) increase in ROS

production. Treatment of neutrophils with ROS scavengers inhibits PMA- and *C. albicans*–induced NETosis (Fuchs et al. 2007, Kenny et al. 2017). Importantly, neutrophils from chronic granulomatous disease patients that lack functional NADPH oxidase fail to undergo NETosis upon *S. aureus* or PMA stimulation (Bianchi et al. 2009). However, NADPH oxidase–mediated ROS are not required for NETosis stimulated by *Leishmania donovani* (Gabriel et al. 2010), *C. albicans* (Byrd et al. 2013), *Paracoccidioides brasiliensis* (Mejía et al. 2015), soluble immune complexes (Chen et al. 2012), A23187, the potassium ionophore nigericin, Group B *Streptococcus* (Kenny et al. 2017), *S. aureus*, or monosodium urate crystals (van der Linden et al. 2017). Thus, similar to the requirement for calcium and specific kinases, the importance of ROS and NADPH oxidase in NETosis seems to depend on the stimulus. The exact mechanism by which NADPH oxidase drives NET release remains unclear.

2.5. Cell Morphodynamics on Initiation of NETosis: Increased Cell–Extracellular Matrix Adhesion and Shedding of Plasma Membrane Microvesicles

The initiation of NETosis is accompanied by profound cellular rearrangements. Upon stimulation of NETosis in vitro, neutrophils spread on substrates before shedding plasma membrane microvesicles and rounding up (Neubert et al. 2018, Thiam et al. 2020). The initial increase in cell spreading suggests activation of cell surface extracellular matrix (ECM) receptors such as integrins. While integrin activation occurs during neutrophil activation and promotes NETosis, depending on the stimulus (Byrd et al. 2013, 2016; McDonald et al. 2012; Mohanty et al. 2015; Rossaint et al. 2014), neutrophils deposited on surfaces lacking integrin ligands still undergo NETosis upon PMA stimulation (Neubert et al. 2018). This suggests that cell spreading and integrin engagement might not be required for NETosis per se.

After spreading, NETosis in adherent cells proceeds by shedding of annexin V–positive plasma membrane microvesicles (Figure 1) containing cytosolic components including granules (Thiam et al. 2020). In addition to microvesicle shedding, neutrophils also round up after their initial spreading (Neubert et al. 2018), similar to adherent cells undergoing mitosis. While plasma membrane microvesicle shedding and rounding up could lead to a decrease in effective cell surface area, whether these morphological changes are required for NETosis progression remains unclear. Nevertheless, the microvesicles that are shed at NETosis onset are of great interest: They could be messengers that induce systemic effects, such as promoting thrombosis (Hrachovinová et al. 2003). In addition, annexin V–positive microvesicles could be subjected to the same fate as annexin-positive apoptotic cells and be internalized by macrophages without inducing inflammation (Gordon & Plüddemann 2018). It is also possible that granule proteins in these microvesicles could exert toxic effects on both pathogens and host cells. Neutrophil microvesicles have been shown to limit bacterial growth (Timár et al. 2013), induce cytokine secretion from endothelial cells (Mesri & Altieri 1998), activate platelets (Pluskota et al. 2008), and downmodulate macrophage activation (Gasser et al. 2003), although whether such neutrophil-derived vesicles arise via NETosis is unclear. However, it is tempting to speculate that microvesicle shedding at NETosis onset may participate in the bactericidal function of NETosis while dampening inflammation. Further studies will be required to reveal the roles of microvesicles formed in NETosis

under infection and sterile inflammation and to shed light on the molecular mechanisms underlying their release.

2.6. NETosis Initiation: Open Questions

It remains unclear why activation of the same surface receptors that trigger phagocytosis or degranulation also induces NETosis. The size of the pathogen has been proposed as a key determinant in NETosis decision-making: When the pathogen is too large to engulf, neutrophils release NETs (Branzk et al. 2014). However, a multitude of small extracellular or even intracellular pathogens, including viruses, can also induce NETosis (Delgado-Rizo et al. 2017). Indeed, *C. albicans* can trigger NETosis after it has been phagocytosed by a neutrophil (Thiam et al. 2020). It has recently been proposed that the higher granule count in younger neutrophils makes them more prone to NETosis than their older counterparts that have fewer granules (Adrover et al. 2020), suggesting that the number of granules might determine neutrophils' susceptibility to undergoing NETosis. However, this does not agree with the observation that aged neutrophils in the circulation are more prone to form NETs (Zhang et al. 2015). Moreover, low-density granulocytes found in patients with systemic lupus erythematosus (SLE) or psoriasis are characterized by their low granule content but spontaneously undergo NETosis (Lood et al. 2016). Thus, the number of granules might not be a key factor for NETosis decision-making. Investigations into what determines when NETosis occurs in response to stimulation, whether the decision depends on the concentration of the stimulus or the activation status of the cells, would provide insights that would allow us to better define the physiological significance of NETosis.

3. MECHANISM OF DNA DECONDENSATION DURING NETOSIS

Chromatin decondensation is the main defining feature of NETosis compared with other cell death processes such as apoptosis, necrosis, or pyroptosis, in which chromatin either is not changed or becomes condensed (de Vasconcelos et al. 2019, Goldmann & Medina 2013). Chromatin decondensation during NETosis is characterized by the loss of chromatin heterogeneity (Figure 2; see also Figure 1), suggesting a loss in heterochromatin, as well as by an increase in the cellular space occupied by DNA. Similar to chromatin decondensation for DNA transcription, chromatin decondensation in NETosis is thought to be mediated by histone posttranslational modifications. Although recent evidence suggests the involvement of acetylation (Hamam et al. 2019), the best-characterized histone modifications during NETosis are citrullination mediated by PAD4 (Wang et al. 2009) and cleavage mediated by serine proteases (Papayannopoulos et al. 2010). In the following, we discuss these unusual histone modifications and how they could drive chromatin decondensation and NETosis.

3.1. PAD4, Histone Citrullination, Chromatin Decondensation, and NETosis

PAD4 is currently thought to drive NETosis by citrullinating histones. This induces chromatin decondensation by decreasing the electrostatic interaction between histone and DNA.

3.1.1. PAD4 and histone citrullination.—Citrullination is a posttranslational modification that converts an arginine to a citrulline, resulting in the loss of a positive

charge, a small decrease in mass (<1 Da), and the release of ammonia (Smith & Denu 2009). PADs constitute a family of proteins that catalyze citrullination (Thompson & Fast 2006). Among the five types of PADs expressed in mammals (van Beers et al. 2013), PAD4 is mainly expressed in granulocytes (Nakashima et al. 1999) and is the only type that contains a conventional nuclear localization signal (Nakashima et al. 2002). Although PAD4 cytosolic activity has been reported (Sun et al. 2017), direct visualization has shown that PAD4 primarily localizes to the nucleus in resting neutrophils (Nakashima et al. 2002, Thiam et al. 2020). PAD4 mediates citrullination of the nucleosome histones H3 at arginines 2, 8, 17, and 26 (Cuthbert et al. 2004) and H4 and H2A at arginine 3 (Hagiwara et al. 2005), as well as the linker histone H1 at arginine 54 (Christophorou et al. 2014). The ability of PAD4 to citrullinate histone and to modify histone–DNA interactions suggested its role in transcription regulation (Thompson & Fast 2006). As such, PAD4 was reported to associate with promoters (Cuthbert et al. 2004), and its expression level correlates with the transcription of several key pluripotency genes, including *Tcl1* and *Nanog* in mouse embryonic stem cells (mESCs) (Christophorou et al. 2014). The ability of PAD4 to citrullinate histones and its specific expression in granulocytes make it a perfect candidate for mediating chromatin decondensation during NETosis.

3.1.2. The role of PAD4 in chromatin decondensation and NETosis.—The findings of various studies converge toward PAD4 having a critical role in NETosis (Wong & Wagner 2018). Neutrophils from PAD4 KO mice do not show citrullinated histone H3, nor do they undergo NETosis induced by PMA, LPSs, *Shigella flexneri* (Li et al. 2010), calcium ionophores (Martinod et al. 2013), methicillin-resistant *S. aureus* infection (Kolaczkowska et al. 2015), or exposure to *P. aeruginosa* biofilms (Thanabalasuriar et al. 2019). PAD4 was also shown to be required for NETosis during sterile inflammation, such as deep vein thrombosis and cancer (Demers et al. 2012, Martinod et al. 2013), and its expression level is upregulated in chronic diseases such as diabetes (Wong et al. 2015). Pharmacological inhibition of PADs abrogates histone citrullination and diminishes NET release in unopsonized *C. albicans* or ionomycin-stimulated mouse neutrophils (Lewis et al. 2015, Wu et al. 2019), nicotine, fMLP, granulocyte–macrophage colony-stimulating factor (GM-CSF), TNF-α, and PMA-stimulated primary human neutrophils (Hosseinzadeh et al. 2016, Tatsiy & McDonald 2018), as well as ionomycin-stimulated neutrophil-like HL60 cells (HL60-derived neutrophils) (Wang et al. 2009). However, the specificity of these inhibitors is subject to question (Jones et al. 2012, Muth et al. 2017). More recently, it was shown that HL60-derived neutrophils with a disrupted *PAD4* gene failed to rapidly decondense their chromatin or release NETs upon ionomycin stimulation (Thiam et al. 2020), indicating that PAD4 may be crucial to NETosis in humans as well as mice.

Although much evidence implicates PAD4 in NETosis, the idea that it is a universal requirement has been challenged. PMA-induced NETosis has been reported to occur without histone H3 citrullination (Kenny et al. 2017, Radic & Neeli 2013). In addition, pharmacological inhibition of PADs has been reported to have no effect on NETosis upon PMA, A23187, nigericin, *C. albicans*, or Group B *Streptococcus* stimulation (Kenny et al. 2017). And while opsonized *C. albicans*–induced NETosis correlates with histone

citrullination, it still occurs in PAD4 KO mice (Guiducci et al. 2018). These data suggest that the requirement of PAD4 for NETosis may vary depending on the NETosis stimulus.

3.1.2.1. How is PAD4 activated during NETosis?: PAD4 is a calcium-specific enzyme containing five calcium-binding sites; its activation in vitro requires high calcium concentration (>100 μM to mM range) at the optimal pH of 7.6 (Kearney et al. 2005, Nakayama-Hamada et al. 2005). However, the concentration of intracellular calcium in activated neutrophils was reported to be in the low micromolar range (0.7 μM) (Krause et al. 1990), much below the level needed for in vitro PAD4 activation, suggesting that either NETosis-specific mechanisms for increasing intracellular calcium or alternative PAD4 activation pathways must exist. ROS were proposed to activate PAD4 (Neeli et al. 2008); however, direct evidence is lacking. Thus, the understanding of PAD4 activation during NETosis requires better insight into the cellular mechanisms of calcium regulation in neutrophils.

3.1.2.2. How does PAD4 mediate chromatin decondensation and NETosis?: The current model in the field is that a reduction in histone positive charge induced by PAD4-mediated citrullination decreases the affinity between histones and the negatively charged DNA, resulting in histone dissociation from DNA and leading to the loss of the compacted chromatin structure and decondensation (Wang et al. 2009). In line with this concept, PAD4-deficient neutrophils are less efficient at decondensing their chromatin (Thiam et al. 2020). Rescue experiments showed that PAD4 nuclear localization and enzymatic activity are required for efficient chromatin decondensation, indicating that PAD4 citrullination activity in the nucleus mediates NETosis. Several lines of evidence indicate that PAD4 can directly decondense chromatin. DNA from PAD4-activated cells is degraded faster by micrococcal nuclease, suggesting that the linker DNA is more accessible for cleavage and, thus, chromatin is less condensed (Wang et al. 2009). PAD4-citrullinated histone H1 in mESCs is dissociated from chromatin and affects chromatin condensation (Christophorou et al. 2014). These data suggest that PAD4 mediates chromatin decondensation by inhibiting linker histone–mediated compaction. Linker histone citrullination has been demonstrated in mESCs (Christophorou et al. 2014), but whether it occurs during NETosis requires further investigation. More importantly, experiments with non-citrullinatable histone mutants are required to directly demonstrate that histone citrullination mediates chromatin decondensation during NETosis.

Although PAD4 appears to be crucial for chromatin decondensation, other factors may contribute to achieving complete chromatin decondensation during NETosis. While heterologous expression of PAD4 in non-neutrophil cell types causes them to expel decondensed chromatin upon ionomycin stimulation (Leshner et al. 2012), isolated neutrophil nuclei treated with PAD4 protein exhibited only a mild (20%) increase in nuclear area (Gößwein et al. 2019). In addition, chromatin decondensation is delayed but not inhibited in PAD4-deficient neutrophil-like HL60 cells (Thiam et al. 2020). This suggests that additional factors may be required to achieve the extent of chromatin decondensation observed during NETosis. Thus, in human neutrophils PAD4 may not be fully sufficient for

chromatin decondensation or may be required for a specific phase of decondensation, such as initiation, with complete decondensation requiring cooperation with other cellular factors.

3.1.2.3. PAD4 in NETosis: open questions.: The significance of PAD4 in NETosis is now well recognized in both mouse and human cells; however, many questions remain. How is PAD4 activated during NETosis in (patho)physiological conditions *in vivo*? What is the required calcium concentration and its mechanism of production for PAD4 activation *in vivo*? Does PAD4 undergo posttranslational modifications to regulate its threshold of activation? Does PAD4 drive chromatin decondensation via histone citrullination alone, or do other PAD4 substrates contribute to the process? Answers to these questions will not only shed light on the process of chromatin decondensation during NETosis but also reveal novel insights into the fundamental mechanisms of the regulation of histone–DNA interaction.

3.2. The Role of Proteases in Chromatin Decondensation During NETosis

Proteases are central to neutrophil function. For instance, they mediate bacterial killing both inside the phagosome and in the extracellular environment, and they facilitate neutrophil migration by cleaving the ECM (Belaaouaj et al. 1998, Okada 2017, Weinrauch et al. 2002). The proteases neutrophil elastase, proteinase 3 (PR3), and cathepsin G (which are all contained in primary granules), as well as calpain (a cytosolic protease), have all been implicated in NETosis.

3.2.1. The role of primary granule resident proteases.—Papayannopoulos and colleagues (2010) were the first to propose that primary granule-resident serine proteases are critical for NETosis. Indeed, they showed that neutrophil elastase and PR3 can induce chromatin decondensation in isolated nuclei, as assessed by the increase in DNA surface area. Inhibition of neutrophil elastase (with a nonselective inhibitor that impacts PR3 as well) impairs the release of extracellular traps upon PMA or *C. albicans* stimulation. The importance of proteases in NETosis was supported by the finding that neutrophils from mice deficient in the serine protease inhibitor SerpinB1 release more NETs upon PMA, platelet-activating factor, chemokine ligand 2 (cxcl2), LPS, or *P. aeruginosa* stimulation (Farley et al. 2012). Additionally, neutrophils from neutrophil elastase KO mice were shown to be deficient in NETosis upon *Klebsiella pneumoniae* (Papayannopoulos et al. 2010) or methicillin-resistant *S. aureus* infection (Kolaczkowska et al. 2015) or exposure to *P. aeruginosa* biofilm (Thanabalasuriar et al. 2019).

While the requirement for serine proteases in NETosis is well accepted, the specific requirement of neutrophil elastase for chromatin decondensation is controversial. Indeed, ionophore- or saliva mucin–induced NETosis proceeds without neutrophil elastase activity (Kenny et al. 2017, Mohanty et al. 2015). Moreover, neutrophils from neutrophil elastase–deficient mice still undergo NETosis upon PMA or platelet-activating factor stimulation (Martinod et al. 2016).

3.2.1.1. How do primary granule proteases mediate chromatin decondensation in NETosis?: Proteases are proposed to mediate chromatin decondensation and NETosis via histone cleavage, thus releasing DNA from histones (Papayannopoulos et al. 2010). Indeed,

histones H1, H2A, H2B, H3, and H4 can be degraded in vitro by neutrophil elastase and PR3. In addition, degradation products of H2B and H4 were observed in neutrophils stimulated with PMA, and inhibition of neutrophil elastase prevents H4 degradation in PMA-stimulated neutrophils (Papayannopoulos et al. 2010). This suggests that H4 cleavage by neutrophil elastase is critical for chromatin decondensation during NETosis.

Because neutrophil elastase, PR3, and cathepsin G localize to membrane-bound granules in the cytoplasm, the mechanism by which they obtain access to histones inside the nucleus during NETosis remains unclear. Transient activity of neutrophil elastase and cathepsin G but not PR3 is detected in the cytosol of neutrophils 30 min after PMA stimulation, indicating that these proteases are released intracellularly from granules in their active form (Metzler et al. 2014). This finding led to the hypothesis that active neutrophil elastase and cathepsin G are first released to the cytosol before translocating to the nucleus during NETosis. How do proteases leave granules? Myeloperoxidase (MPO) and hydrogen peroxide (H_2O_2) have been proposed to mediate the release of proteolytically active neutrophil elastase from intact granules (Metzler et al. 2014); however, the associated mechanism still needs to be revealed. The pore-forming protein gasdermin D has been implicated in neutrophil elastase nuclear translocation (Sollberger et al. 2018). Whether gasdermin D mediates neutrophil elastase exit from granules or entry into the nucleus remains to be determined. Thus, the cellular mechanism by which serine proteases mediate chromatin decondensation during NETosis remains to be investigated.

3.2.1.2. Primary granule resident proteases in NETosis: open questions.: Although evidence implicates proteases in NETosis, much is still unknown. How and when do neutrophil elastase and cathepsin G obtain access to chromatin during NETosis? Do they contain a nuclear localization sequence that allows them to enter the nucleus? Do they enter the nucleus before or after nuclear envelope permeabilization or rupture? More importantly, neutrophil elastase has a strong affinity for DNA, which was shown to inhibit its protease activity (Duranton et al. 2000). This begs the question, How does this protease mediate H4 cleavage in the presence of DNA? Live-cell imaging of neutrophil elastase, PR3, and cathepsin G during NETosis could shed light on their roles during this process.

3.2.2. Calpain: the new player in chromatin decondensation.—An interesting emerging concept is that chromatin decondensation during NETosis could result from the combinatorial effect of PAD4-mediated citrullination and protease-mediated cleavage. As such, the calcium-activated serine protease calpain was shown to enhance chromatin decondensation in the presence of PAD4 but not by itself. Moreover, ionomycin-induced NETosis was impaired upon calpain inhibition (Gößwein et al. 2019). It is thus tempting to speculate that PAD4-mediated citrullination could target nuclear proteins for calpain-mediated degradation, thus driving chromatin decondensation. However, further studies are required to understand this synergistic effect.

3.3. The Role of Myeloperoxidase in Chromatin Decondensation and NETosis

MPO, a primary granule resident protein that catalyzes chloride oxidation into hydrochloric acid in the presence of hydrogen peroxide, is implicated in chromatin decondensation during

NETosis. Neutrophils from MPO-deficient patients fail to release NETs upon PMA, *C. albicans*, or Group B *Streptococcus* stimulation (Kenny et al. 2017, Metzler et al. 2011). Interestingly, pharmacological inhibition of MPO only delays NETosis, suggesting that limited MPO activity is sufficient for NETosis (Metzler et al. 2011). While MPO does not directly decondense chromatin in isolated nuclei or degrade histones in vitro, it was shown to enhance neutrophil elastase–mediated chromatin decondensation (Papayannopoulos et al. 2010). How does MPO facilitate chromatin decondensation? Does it mediate oxidation of DNA and block its inhibitory effect on neutrophil elastase? Live-cell imaging of MPO-deficient neutrophils stimulated for NETosis would help identify which NETosis step is inhibited and hence the role of MPO in NET release.

4. DNA RELEASE FROM THE NUCLEUS TO THE CYTOSOL

For decondensed DNA to be released extracellularly, it must first escape from the nucleus (Fuchs et al. 2007). Chromatin in the nucleus is isolated from the cytosol by the nuclear membrane, which is made up of two lipid bilayers connected by multiprotein complexes including the nuclear pore complex (NPC) and the linker of nucleoskeleton and cytoskeleton (LINC) complex (Janota et al. 2017). Underneath the inner nuclear membrane (INM) lies a meshwork of lamin intermediate filaments composed of lamin A/C, B1, and B2. Lamins play major roles in gene expression and have also been described as molecular shock absorbers (Dahl et al. 2004), suggesting that they can dampen mechanical forces generated outside the nucleus before they reach the chromatin. Shielding the chromatin from nucleases and mechanical forces, the nuclear envelope was long thought to be very stable except during mitosis, when the combined actions of mitotic kinase–mediated lamin disassembly and pulling forces from microtubule (MT) motors drive its rupture and subsequent release of compacted chromatin into the cytosol (Güttinger et al. 2009). However, mechanical forces alone were reported to be sufficient for nuclear envelope rupture during immune and cancer cell migration under confinement (Denais et al. 2016, Raab et al. 2016).

Similar to the rearrangement described during cell division and migration, DNA release into the cytosol during NETosis was shown to involve lamin remodeling as well as formation of holes in the nuclear envelope prior to its rupture (Chen et al. 2018, Fuchs et al. 2007, Gößwein et al. 2019, Li et al. 2019, Neubert et al. 2018, Thiam et al. 2020). Interestingly, vital NETosis is proposed to occur without nuclear membrane disruption with decondensed DNA leaving the nucleus in vesicles (Pilsczek et al. 2010). In the following, we describe the molecular players involved in DNA release in the cytosol and discuss how the mechanics of the chromatin could drive this process.

4.1. Lamin Dynamics During NETosis

Dramatic changes in the lamin meshwork have been observed during NETosis (Figures 1 and 2). The appearance of local discontinuities in the lamin B1 and B2 networks has been reported in human and mouse neutrophils stimulated with PMA or ionomycin (Li et al. 2019, Neubert et al. 2018, Thiam et al. 2020). Using high-resolution live-cell imaging of HL60-derived neutrophils stimulated with ionomycin, Thiam and colleagues (2020) found that decondensed DNA extruded from these lamin discontinuities.

How does the lamin meshwork disassemble during NETosis? The formation of meshwork discontinuities could be the result of lamin posttranslational modifications. As such, lamin A/C (Amulic et al. 2017) and PKCα-mediated lamin B1 (Li et al. 2019) phosphorylation was reported in PMA-induced NETosis. Indeed, mutation of the PKC phosphorylation sites of lamin B1 impairs extracellular trap release in RAW264.7 macrophages (Li et al. 2019). Additionally, lamin B1 degradation has been observed during ionomycin-induced NETosis (Gößwein et al. 2019), and PAD4 deficiency has been shown to delay the formation of lamin discontinuities and to diminish lamin B1 degradation in ionomycin-stimulated neutrophils (Gößwein et al. 2019, Thiam et al. 2020). This suggests that PKC-mediated lamin phosphorylation and/or PAD4-mediated lamin degradation could drive lamin disassembly during NETosis. Whether PKC phosphorylates lamin B1 in neutrophils and how PAD4 drives lamin B1 degradation await further studies.

4.2. Nuclear Membrane Dynamics During NETosis

The nuclear envelope has been proposed to vesiculate during both suicidal (Fuchs et al. 2007) and vital NETosis (Pilsczek et al. 2010). Indeed, using electron microscopy, Fuchs and colleagues (2007) detected vesicles containing the lamin B receptor (LBR) that presumably emanated from the nuclear membrane. This led to the hypothesis that vesiculation of the nuclear envelope mediates its disintegration, thus allowing chromatin release into the cytosol during PMA-induced NETosis. But these results are open to interpretation due to thin-section artifacts that cannot distinguish between continuous and discontinuous membrane networks. Moreover, LBRs are also present in the ER (Ellenberg et al. 1997) that has been observed by live-cell microscopy to vesiculate during NETosis (Thiam et al. 2020), raising the possibility that the observed LBR-positive vesicles might originate from the ER. During vital NETosis, the nuclear envelope is thought to vesiculate but not to rupture. Indeed, an electron microscopy study by Pilsczek and colleagues (2010) detected vesicles containing small amounts of material reminiscent of strands of DNA with nucleosomes that were present in the cytosol of neutrophils stimulated with *S. aureus*. They proposed that those vesicles form by budding off of the outer nuclear membrane (ONM) and are then exocytosed at the plasma membrane, leaving both the nuclear envelope and the plasma membrane intact during NET release. However, it is unclear how a vesicle containing nuclear DNA would form from the ONM without either breaking the inner nuclear envelope or forming a double-membrane vesicle. High-resolution, live-cell imaging of the INMs and ONMs of human, mouse, and HL60-derived neutrophils stimulated with ionomycin showed that the nuclear membrane does not disintegrate by vesiculation but rather ruptures at multiple points, from which the chromatin is released into the cytosol (Figures 1 and 2) (Thiam et al. 2020). This rupture of the INMs and ONMs occurs within a minute after the appearance of lamin discontinuities, and no subsequent nuclear envelope resealing was observed. However, these studies were performed on isolated cells in vitro, and how DNA is released from the nucleus in a physiological setting remains to be determined.

4.2.1. How does the nuclear envelope rupture during NETosis?—

As discussed earlier, the nuclear envelope can rupture by forces generated either by MT motors during mitosis or in the extracellular environment during cell migration under tight confinement. In contrast, in neutrophils undergoing NETosis, the nuclear envelope ruptures after complete

disassembly of the cytoskeleton (Thiam et al. 2020) in the absence of cell confinement. Thus, alternative mechanisms must be at play during NETosis to drive nuclear envelope rupture. PAD4 deficiency was shown to impair nuclear envelope rupture (Thiam et al. 2020). The dependence of both chromatin decondensation and nuclear envelope rupture on PAD4 suggests that rupture could be driven by decondensation. Whether entropic (Neubert et al. 2018) or osmotic pressure generated by chromatin decondensation could be sufficient for bursting the nuclear envelope remains to be determined.

4.2.2. Gasdermin D–mediated nuclear envelope permeabilization.—Gasdermin D is a pore-forming protein that can be cleaved by caspases 1, 4, 5, and 11 (in mice) (Shi et al. 2015) to release the N-terminal domain (gasdermin N) that can insert in membranes and form 20-nm pores by oligomerization (Ruan et al. 2018, Sborgi et al. 2016). Gasdermin D is proposed to be cleaved by caspase 11 during cytosolic LPS-induced NETosis (Chen et al. 2018) and by neutrophil elastase during PMA-induced NETosis (Sollberger et al. 2018). Under both stimuli, neutrophils from gasdermin D–null mice failed to extrude their DNA into the cytosol (Chen et al. 2018, Sollberger et al. 2018), suggesting a defect in nuclear envelope rupture. Nuclear envelope permeabilization prior to DNA release into the cytosol was observed by live imaging (Thiam et al. 2020). Thus, it is tempting to speculate that during NETosis, gasdermin D cleavage mediates nuclear membrane permeabilization, allowing proteases to obtain access to histones and promote subsequent chromatin decondensation. Gasdermin D preferentially inserts into phosphorylated phosphatidylinositol- and phosphatidylserine-positive membranes (Ding et al. 2016, Sborgi et al. 2016). Although these lipids, particularly phosphatidylinositol(4,5)bisphosphate [PtdIns(4,5)P_2], were reported at the nuclear membrane (Fiume et al. 2012, Kleinig 1970, Smith & Wells 1983), whether they are present in the INM or ONM, or both, is unclear. Thus, whether and how gasdermin D pores form at the nuclear envelope during NETosis require further investigation.

4.2.3. DNA release into the cytosol: open questions.—Although it is well accepted that for NETosis to occur, decondensed chromatin must breach the lamin meshwork and the nuclear membrane, not much is known about the mechanisms involved in this process. What mediates the appearance of discontinuities in the lamin meshwork during NETosis? If they are the result of local disassembly, what enzymes mediate the posttranslational modifications necessary for lamin meshwork disassembly during NETosis? Could mechanical forces generated by chromatin decondensation mediate the rupture of both the lamin meshwork and the nuclear envelope? What are the dynamics of the INM and ONM and of the lamin network during vital NETosis? If decondensed chromatin is packed into vesicles emanating from the nuclear envelope, what is the structure of those vesicles? And if these vesicles contain both the INMs and ONMs, how would they fuse with the single membrane of the plasma membrane? High-resolution live imaging of cells undergoing vital NETosis will be critical to understanding the mechanism of DNA release during this process.

5. REMODELING OF CYTOSKELETAL AND MEMBRANOUS ORGANELLES DURING NETOSIS

Once decondensed chromatin is released from the nucleus into the cytosol, it must breach the crowded and interconnected network of the cytoskeletons and membranous organelles before reaching the plasma membrane. The cytoskeletons and membranous organelles undergo drastic remodeling during lytic NETosis (Figures 1 and 2). Indeed, the actin filaments, MTs, and vimentin intermediate filaments disassemble prior to extracellular DNA release (Amulic et al. 2017, Metzler et al. 2014, Neubert et al. 2018, Thiam et al. 2020). Similarly, the ER was shown to vesiculate (Thiam et al. 2020), granules are proposed to disintegrate (Brinkmann et al. 2004, Metzler et al. 2014, Okubo et al. 2016, Papayannopoulos et al. 2010), and mitochondria may release their DNA (Caielli et al. 2016, Lood et al. 2016, McIlroy et al. 2014, Wang et al. 2015, Yousefi et al. 2019).

Mitosis is another cellular process during which similar drastic remodeling of the cytoskeleton and membranous organelles occurs. Formation of the mitotic spindle first requires disassembly of the preexisting interphase MT array (Belmont et al. 1990). Similarly, remodeling of the interphase actin (Kunda et al. 2008) and vimentin intermediate filament (Duarte et al. 2019) networks is critical to the formation of the stiff cortex in metaphase. Golgi apparatus vesiculation (Guizzunti & Seemann 2016) and mitochondrial fission (Mishra & Chan 2014) during mitosis ensure equal distribution of these organelles into daughter cells. Therefore, in addition to nuclear envelope rupture, cytoskeletal and membranous organelle disassembly is a common step between mitosis and NETosis, although these structures do not reform in the latter.

5.1. Cytoskeletal Disassembly During NETosis

The three cytoskeletal systems of the cell form a dense, interconnected network that confers and maintains the physical integrity of the cell. The stiff MT network regulates the transport and distribution of membranous organelles throughout the cell, the dynamic actin cytoskeleton at the cortex sets the plasma membrane tension and drives cell shape changes, and the dense network of intermediate filaments allows cells to resist large deformations. Intriguingly, all of these cytoskeletal networks rapidly disassemble during NETosis.

5.1.1. Actin disassembly: an early NETosis event.—Among the three cytoskeletal networks, the actin cytoskeleton has been the most studied during NETosis. It is proposed to be degraded during *C. albicans*–induced NETosis (Metzler et al. 2014). However, the absence of fluorescent phalloidin staining (Sollberger et al. 2018) and the solubilization of both monomeric and filamentous actin probes (Neubert et al. 2018, Thiam et al. 2020) have been reported in PMA-, ionomycin-, and LPS-induced NETosis, suggesting that the actin cytoskeleton disassembles by filament depolymerization rather than by degradation. The disassembly of the actin cytoskeleton is one of the earliest events in PMA-, ionomycin-, and LPS-induced NETosis, occurring within minutes after NETosis stimulation (Neubert et al. 2018, Thiam et al. 2020). More importantly, stabilization of the actin cytoskeleton using jasplakinolide impairs extracellular trap formation (Neubert et al. 2018, Thiam et al. 2020). However, Neubert and colleagues (2018) reported that the early addition of actin

filament depolymerization drugs after PMA stimulation also impairs NETosis, suggesting that a dynamic actin network is important for NETosis onset and that actin filament depolymerization is temporally regulated.

5.1.1.1. Possible mechanisms of actin disassembly during NETosis.: As actin disassembly is required for efficient NETosis, understanding this mechanism is important. The intracellular calcium concentration increases during NETosis, and several actin-depolymerization factors including myosin II and gelsolin are calcium sensitive (Downey et al. 1990, Larson et al. 2005, Yoshida et al. 1984). Thus, intracellular calcium influx could be a main driver of actin disassembly during NETosis. Actin oxidation by proteins from the molecule interacting with CasL (MICAL) family drives filament disassembly and prevents its repolymerization (Hung et al. 2010, 2011). Considering that ROS increase rapidly at NETosis onset and that oxidized actin is present at high levels in calcium ionophore–induced NETosis (Petretto et al. 2019), MICAL is also a good candidate for mediating actin disassembly during NETosis. Because actin filament disassembly is rapid and global during NETosis, it is likely to be caused by a combinatorial effect of several actin-disassembling factors.

5.1.1.2. Possible roles of actin disassembly during NETosis.: How does actin cytoskeleton disassembly contribute to NETosis? Actin degradation has been proposed to mediate neutrophil elastase translocation to the nucleus (Metzler et al. 2014). However, this concept has not been tested by disrupting actin degradation, and a role for actin in regulating nuclear import has not been established. Live-cell imaging of jasplakinolide-treated neutrophils stimulated for NETosis showed that actin filament stabilization impairs extracellular DNA release without affecting DNA release from the nucleus into the cytosol. Moreover, in cases where NETosis did occur in the presence of jasplakinolide, decondensed chromatin was released locally through holes in the actin cortex (Thiam et al. 2020). These data suggest that the cortical actin filament network acts as a physical impediment to plasma membrane rupture and DNA release into the extracellular environment, and early disassembly of the actin cytoskeleton acts to remove this impediment. Whether actin filament disassembly facilitates plasma membrane rupture by modifying the cell membrane composition and/or decreasing the required plasma membrane rupture force warrants investigation.

5.1.2. Microtubule and vimentin intermediate filament disassembly.—In addition to actin depolymerization, NETosis is accompanied by a drastic decrease in polymerized MTs and vimentin intermediate filaments. Neutrophils undergoing NETosis disassemble their MT networks, as reflected by the decrease in α- and β-tubulin immunostaining (Amulic et al. 2017, Neubert et al. 2018) and rapid solubilization of MT markers during live imaging (Thiam et al. 2020). Similarly, the vimentin intermediate filament network is remodeled during NETosis as assessed by a decrease in vimentin immunostaining and solubilization of vimentin-enhanced green fluorescent protein (eGFP) in live cells (Thiam et al. 2020). Interestingly, immunostaining suggests that peripheral vimentin disassembles more than the perinuclear filaments, implying that vimentin disassembly during NETosis may be a spatially regulated process.

5.1.2.1. Possible mechanisms and roles of microtubule disassembly during NETosis.:

Current data suggest that disassembling or stabilizing MTs pharmacologically does not impair NETosis (Neubert et al. 2018, Thiam et al. 2020), indicating that neither an intact MT network nor its disassembly is required for NETosis. However, the rapid and dramatic loss of MTs is nonetheless interesting. MTs are well known to be calcium labile (Weisenberg & Deery 1981) and have been shown to disassemble in cells at calcium concentrations of 1 to 4 μM (Schliwa et al. 1981). The intracellular calcium concentration in activated neutrophils is in the micromolar range (Krause et al. 1990). Thus, the intracellular calcium concentration in neutrophils undergoing NETosis may be sufficient to induce MT disassembly. The mechanism of MT disassembly during NETosis and the possible role of microtubule regulatory proteins await further study.

5.1.2.2. Possible mechanisms and roles of vimentin remodeling during NETosis.:

Similar to the disassembly of actin filaments, the loss of vimentin networks could act to clear the way for chromatin to mediate its more efficient release from the cell, which makes the mechanism of vimentin remodeling of interest. PAD4 deficiency delays vimentin remodeling during NETosis, suggesting that this process involves PAD4 functions (Thiam et al. 2020). In addition, a recent report has shown that neutrophils from vimentin-null mice release fewer NETs upon ionomycin and nigericin stimulation (P. Munzer, R. Negro, S. Fukui, L. di Meglio, C. Cherpokova, N. Sorvillo, L. Shi, V. Giri Magupalli, S. Gutch, L. Chu, R. Goldman, K. Ridge, R. Scharf, C. Waterman, H. Wu & D. Wagner, unpublished manuscript). Interestingly, vimentin is citrullinated during NETosis (Khandpur et al. 2013), and vimentin citrullination in calcium ionophore–stimulated macrophages is thought to induce vimentin redistribution around the nucleus (Asaga et al. 1998). Thus, vimentin remodeling during NETosis could be induced by citrullination. Citrullinated vimentin is found in the synovial fluid of rheumatoid arthritis (RA) patients and is involved in RA pathogenesis (Van Steendam et al. 2011). Moreover, antibodies against citrullinated vimentin can induce NETosis (Khandpur et al. 2013), suggesting that citrullinated vimentin might contribute to the detrimental effects of NETosis. However, similar to the actin cytoskeleton, kinases and proteases that regulate the disassembly or degradation of vimentin intermediate filaments are also sensitive to intracellular calcium (Ando et al. 1991, Nelson & Traub 1982, Spruill et al. 1983, Yoshida et al. 1984), suggesting an alternative mechanism to PAD4. Further studies are required to decipher the contribution of citrullination and calcium in vimentin disassembly during NETosis.

5.1.3. Cytoskeletal disassembly: open questions.—

In addition to its requirement for the structural integrity of cells, the cytoskeleton is critical for signaling, including mechanotransduction, cell polarity, and transport of organelles, as well as force generation. Thus, the extent of cytoskeletal disassembly during NETosis raises several questions: Is the plasma membrane of cells undergoing NETosis polarized? How are organelles positioned during NETosis? How are the forces necessary for NETosis completion generated? How would NETosis proceed in physiological environments in which cells have to integrate ECM stiffness and topology? Alternative thinking and physical models for force generation will be critical to understanding how NETosis occurs in vivo.

5.2. Membranous Organelles

The cytosol is crowded with not only the cytoskeleton but also organelles including granules, ER, Golgi apparatus, endosomes, lysosomes, mitochondria, and ribosomes, among others. Although many organelle systems have not been thoroughly examined during NETosis, there is evidence that the ER, granules, and mitochondria undergo major changes during NETosis.

5.2.1. Endoplasmic reticulum vesiculation and granule disintegration.—The ER was shown to vesiculate within minutes following the disassembly of the cytoskeleton during NETosis stimulated by ionomycin and LPS (Thiam et al. 2020). Interestingly, ER vesiculation occurred much earlier than nuclear membrane rupture, suggesting a loss of continuity between these two organelles. Calcium ionophores are known to be inducers of ER vesiculation (Koch et al. 1988, Wilson et al. 1998), suggesting that the increase in intracellular calcium might mediate ER vesiculation during NETosis.

Neutrophil granule resident proteins, including neutrophil elastase, MPO, PR3, cathepsin G, and lactoferrin (Brinkmann et al. 2004, Metzler et al. 2014, Okubo et al. 2016, Papayannopoulos et al. 2010), are found on NETs. While the release of neutrophil elastase and cathepsin G from primary granules occurs without granule lysis, the mechanism by which MPO, PR3, or lactoferrin localizes to NETs is unknown. Interestingly, while MPO shows a diffuse intracellular localization on DNA before NET release (Papayannopoulos et al. 2010), lactoferrin has been reported to localize to the plasma membrane (Okubo et al. 2016), suggesting that specific granular components may be targeted to specific cellular compartments during NETosis. Whether neutrophil granules become permeabilized or rupture during NETosis to allow the release of granule-resident proteins on NETs awaits further study.

5.2.2. The role of mitochondria during NETosis.—Mitochondria play two roles in NETosis. First, mitochondria can mediate ROS production, and second, mitochondrial DNA (mtDNA) can be released extracellularly.

An increase in mitochondrial ROS production was shown to be required for calcium ionophore– or ribonucleoprotein immune complex (RNP IC)–induced NETosis as well as spontaneous NETosis by low-density granulocytes from SLE patients (Douda et al. 2015, Lood et al. 2016). Thus, in addition to NADPH oxidase, ROS can be released during NETosis by mitochondria.

Several studies have shown that mtDNA can be released extracellularly and can associate with nuclear DNA on NETs (Caielli et al. 2016, Lood et al. 2016, McIlroy et al. 2014, Wang et al. 2015). However, the underlying mechanism remains unclear. Lood and colleagues (2016) proposed that during RNP IC–induced NETosis, mitochondria localize to the plasma membrane, where they release oxidized mtDNA. However, mitochondria have two membranes; thus, how this organelle would fuse with the single membrane of the plasma membrane and release DNA is unclear. Is mtDNA first released in the cytosol by mitochondrial rupture? Do mitochondria fuse with the plasma membrane? These questions remain to be answered by careful analysis of mitochondrial membrane and DNA dynamics.

6. EXTRACELLULAR DNA RELEASE: BREACHING THE PLASMA MEMBRANE

NETs are frequently observed as extracellular DNA stained with membrane-impermeable DNA dyes (Sytox green or DAPI) (Brinkmann et al. 2004, van der Linden et al. 2017), a finding indicative of DNA access to the extracellular milieu. For DNA to be released extracellularly, decondensed chromatin must breach the plasma membrane. Other than vital NETosis, during which decondensed DNA is thought to be released extracellularly via exocytosis (Pilsczek et al. 2010), most of the current evidence indicates that extracellular DNA released during lytic NETosis occurs following plasma membrane lysis. However, the mechanisms underlying the changes in the plasma membrane during vital or lytic NETosis remain largely unknown. Recent evidence indicates that breach of the plasma membrane may be a multistep process during NETosis in which the permeability of the plasma membrane increases prior to its rupture and extracellular DNA release (Figures 1 and 2).

6.1. Increase in Permeability of the Plasma Membrane During NETosis

During NETosis, the plasma membrane becomes permeable to increasingly larger molecules over time after stimulation yet retains the ability to contain chromatin expanding from the ruptured nucleus into the cytoplasm (Thiam et al. 2020). The plasma membrane was shown to first become permeable to calcein, then to 10 kDa dextran, and finally to 70 kDa dextran before exhibiting a large hole and releasing DNA extracellularly (Thiam et al. 2020). The progressive permeabilization of the plasma membrane without drastic cellular swelling or rapid bursting after cells become permeable to small molecules (calcein) suggests that several classes of membrane pores with various pore sizes may be forming and/or sealing over time.

Neutrophils express several types of pore-forming proteins; among these, gasdermin D has been implicated in NETosis (Chen et al. 2018, Sollberger et al. 2018). Is plasma membrane permeabilization during NETosis mediated by gasdermin D? The N-terminal domain of gasdermin D has been shown to form pores of about 20 nm in diameter (Sborgi et al. 2016). As calcein (0.6 kDa) and dextran 10 kDa and 70 kDa have hydrodynamic radii of <1 nm, 1.9 nm, and 6.8 nm, respectively (Armstrong et al. 2004, Chouinard-Pelletier et al. 2012, Guo & Santschi 2007), they would be able to fit through 20-nm gasdermin D pores all at once rather than progressively. Thus, gasdermin D pores might form only during the later stages of plasma membrane permeability. Bacterial toxins, such as the one released by *S. aureus*, have been shown to disrupt plasma membrane permeability during NETosis (van der Linden et al. 2017). However, progressive plasma membrane permeabilization during NETosis has been observed in the absence of toxins, ruling out their requirement. Thus, how and why plasma membrane permeability gradually increases during NETosis await further study.

6.2. Plasma Membrane Rupture Driven by Chromatin Swelling

Live-cell imaging of neutrophils stained with a plasma membrane dye or expressing fluorescent plasma membrane markers has shown that the release of extracellular DNA occurs locally at sites of plasma membrane rupture (Neubert et al. 2018, Thiam et al. 2020).

Importantly, such membrane rupture occurs minutes to hours after a gradual increase in its permeability (Thiam et al. 2020), suggesting that it is not merely a consequence of cell swelling and bursting. The molecular mechanism of plasma membrane rupture during NETosis remains to be revealed.

Neubert and colleagues (2018) proposed a biophysical mechanism for plasma membrane rupture in NETosis. They showed that extracellular DNA release at the end of NETosis is a passive process independent of glycolysis, ATP, metabolism, and MPO. Estimating the entropic pressure generated by the fully unraveled genomic DNA to be 100–200 Pa and the membrane pressure at the end of NETosis to be about 20 Pa, they proposed that chromatin swelling could mechanically rupture the plasma membrane (Neubert et al. 2018). This concept brings a novel perspective to understanding the biophysics of NETosis; however, it is challenged by other existing data. For instance, if chromatin swelling is a point of no return sufficient for plasma membrane rupture, why would some cells release their DNA only in the cytosol but not extracellularly even hours after induction for NETosis (Thiam et al. 2020)? Thus, further studies will be required to understand the molecular and physical mechanisms of plasma membrane rupture during NETosis.

7. CONCLUSION AND PERSPECTIVES

The field of NETosis is in an exciting stage in which evidence of the (patho)physiological relevance of NET release has been accumulating but the molecular, cellular, and biophysical mechanisms driving this process have just begun to be revealed. Most of the current literature focuses on which stimuli can induce NETosis and which proteins can inhibit NET release without well-defined insights into how these factors influence the cellular events of NETosis. As NETosis proceeds via a specific sequence of events (Figures 1 and 2), determining how proteins that inhibit NETosis perturb this sequence will help us to understand the basic mechanisms of NETosis and will inform us of new targets to modulate NETosis in diseases. Moreover, studying this unusual cellular process may provide insights into other cellular mechanisms, including chromatin decompaction for transcription, as well as into diseases such as laminopathies. Among all the questions that have been raised in this review, several may merit particular attention to significantly further our understanding of the cell biology and biophysics of NETosis (see Future Issues).

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FUTURE ISSUES

1. How is NETosis initiated?
2. What makes a neutrophil undergo NETosis versus other forms of host defense?
3. What are the mechanisms and roles of cytoskeletal and organelle remodeling?
4. How are the necessary forces required for NETosis generated?
5. What is the mechanism of vital NETosis?
6. How do neutrophils integrate the molecular and physical complexity of the *in vivo* environment while undergoing NETosis?

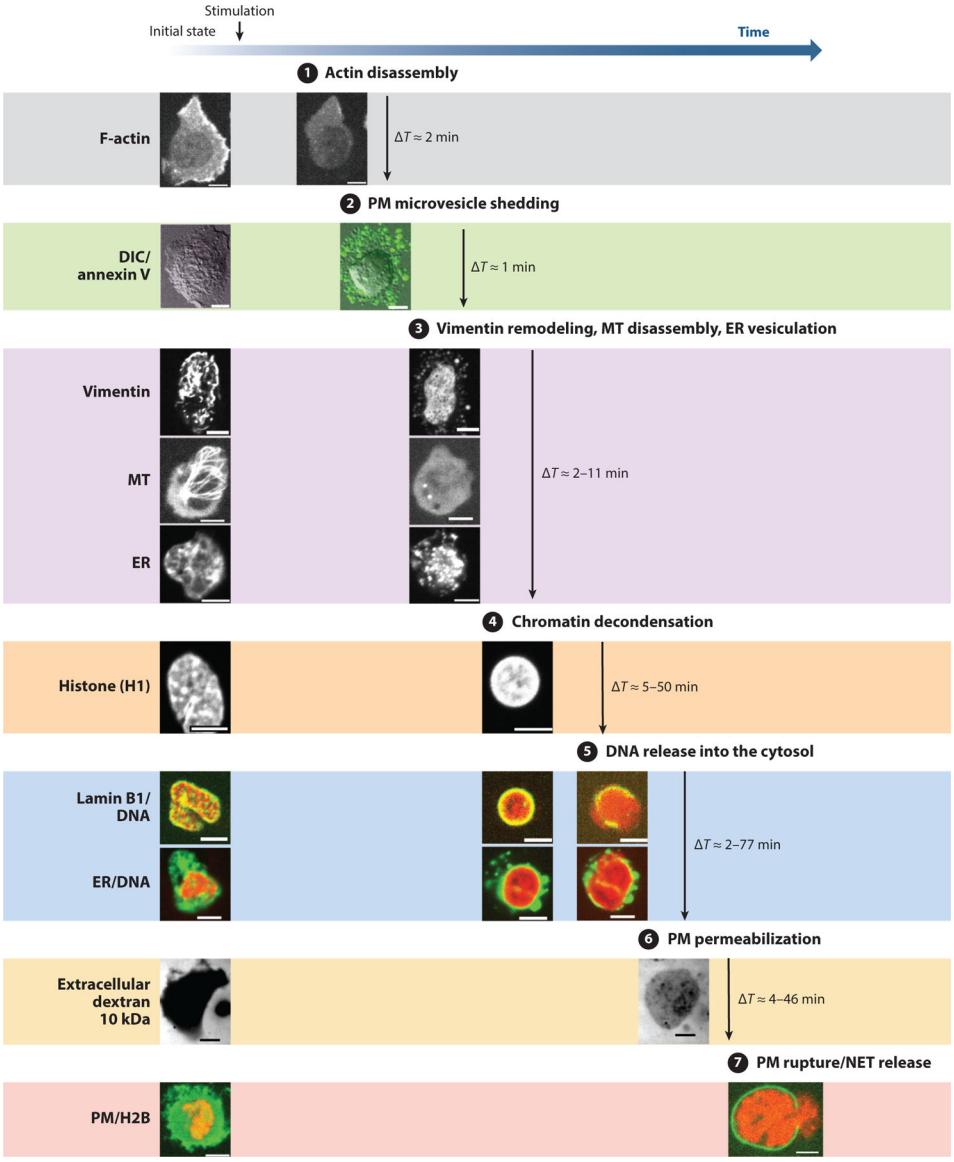

Figure 1.

NETosis proceeds via a well-defined sequence of cellular events. Representative images are shown of living neutrophil-like HL60 cells expressing fluorescent protein–tagged cytoskeletal (actin, MT, and vimentin), nuclear [histones (H1 and H2B) and lamin B1], and membranous (ER and PM) marker probes, before (initial state) and after NETosis stimulation. Fluorescently tagged annexin V and 10-kDa dextran were added to the imaging media before NETosis stimulation. DNA was stained with SiR-DNA. (❶) Actin disassembly. The fluorescent intensity of the F-actin probe (F-tractin-mApple) decreases. (❷) PM microvesicle shedding. Shows the appearance of annexin V–positive microvesicles (*green* meaning exposed phosphatidylserine) while the cell body remains annexin V negative. (❸) Vimentin remodeling, MT disassembly, and ER vesiculation. Illustrates the solubilization of the vimentin intermediate filament probe (vimentin eGFP) and then the MT probe (ensconsin MT-binding domain fused to eGFP). Finally, the tubular ER

network is lost and vesiculates, as assessed by the ER probe (ER-5-mEmerald: KDEL sequence combined with the ER-retention signal sequence from calreticulin fused to mEmerald). (❹) Chromatin decondensation. Histone (H1-mEmerald) and DNA fluorescent signal heterogeneity decreases. (❺) DNA release into the cytosol. Shows the appearance of discontinuities in the lamin B1 (lamin B1–mApple) and outer nuclear membrane (visualized with ER-5-mEmerald) from where the DNA is released. (❻) PM permeabilization. The intracellular fluorescent intensity of membrane-impermeable, 10-kDa dextran from the extracellular media increases. This increase corresponds with a contrast decrease shown by DIC microscopy at the cell periphery. (❼) PM rupture/NET release. Illustrates the appearance of discontinuities in the plasma membrane (CAAX-mApple) from where the DNA is released. All scale bars are 5 μm. ΔT represents the time between two consecutive events. Abbreviations: DIC, differential interference contrast; eGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; MT, microtubule; NET, neutrophil extracellular trap; PM, plasma membrane.

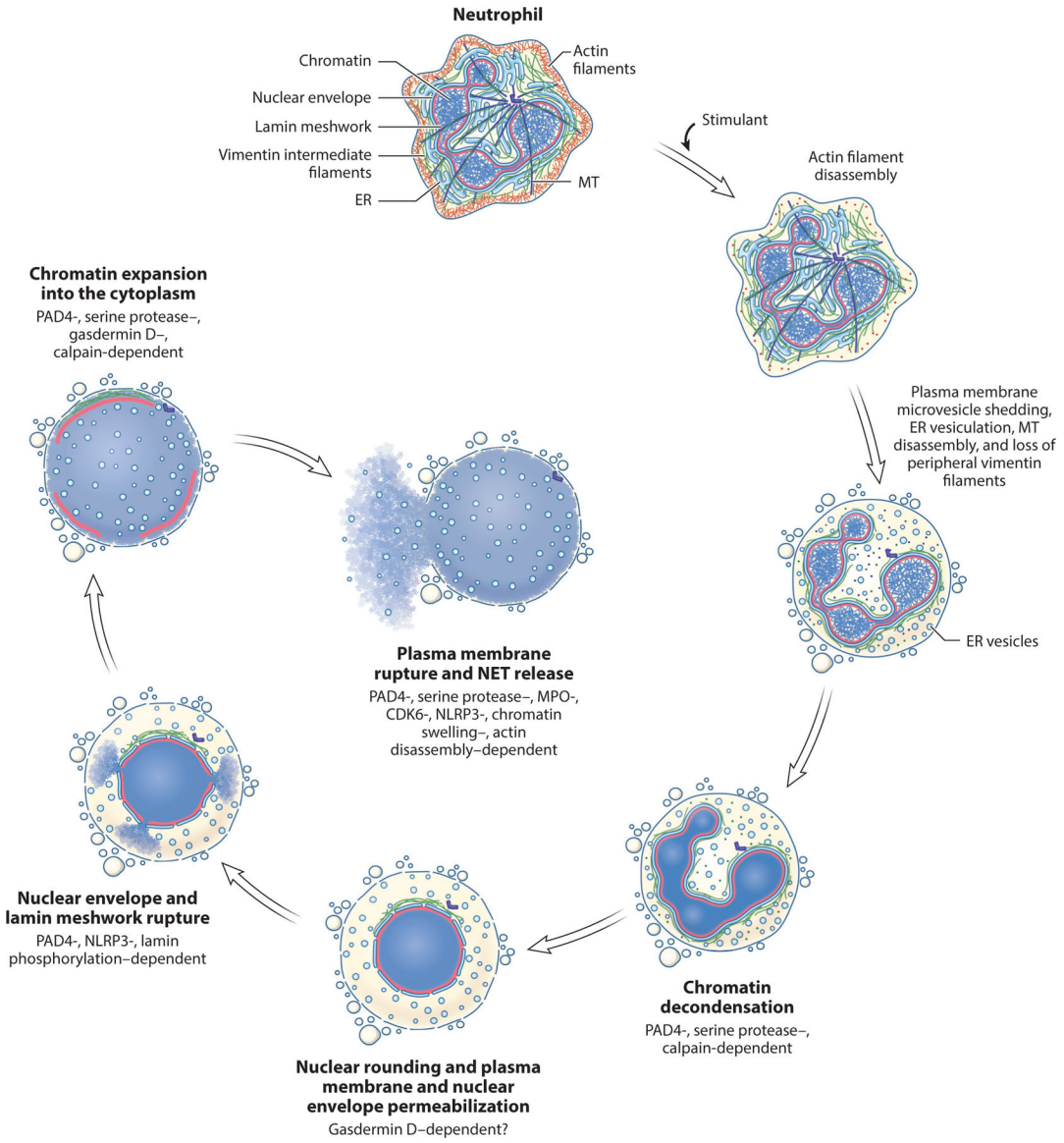

Figure 2.

Model of the cellular events driving NETosis and their molecular regulators. Figure adapted from Thiam et al. (2020). Abbreviations: CDK6, cyclin-dependent kinase 6; ER, endoplasmic reticulum; MPO, myeloperoxidase; MT, microtubule; NET, neutrophil extracellular trap; NLRP3, nucleotide oligomerization domain (NOD)-like receptor protein 3; PAD4, peptidyl arginine deiminase 4.