Neutrophil Extracellular Traps in Host Defense

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Neutrophils are produced in the bone marrow and then patrol blood vessels from which they can be rapidly recruited to a site of infection. Neutrophils bind, engulf, and efficiently kill invading microbes via a suite of defense mechanisms. Diverse extracellular and intracellular microbes induce neutrophils to extrude neutrophil extracellular traps (NETs) through the process of NETosis. Here, we review the signaling mechanisms and cell biology underpinning the key NETosis pathways during infection and the antimicrobial functions of NETs in host defense.

In 1880, Paul Ehrlich described cells with granules and a lobulated nucleus, and called these cells polymorphonuclear leukocytes. Polymorphonuclear leukocytes were then further classified into eosinophils, basophils, and neutrophils. Neutrophils are now recognized as essential effector cells of innate immunity and key regulators of both innate and adaptive immune responses. Neutrophils are produced in the bone marrow and then patrol blood vessels, from which they can be rapidly recruited to a site of infection. Neutrophil activation by pathogen products or the local inflammatory milieu prolongs neutrophil life span and arms these cells with antimicrobial effector functions (Dale et al. 2008; Nauseef and Borregaard 2014).

Neutrophils combat microbes using a suite of defense mechanisms. Neutrophils internalize microbes through the process of phagocytosis after which the antimicrobial arsenal of the neutrophil granules is delivered to the phagosome to mediate microbial killing. Neutrophils can also

externalize their granule content to neutralize extracellular microbes in a process called degranulation. The key granule proteins involved in microbial killing are α -defensins, lysozymes, and the three neutrophil serine proteases, neutrophil elastase (NE), cathepsin G (CatG), and proteinase-3 (PR-3). Phagocytosis and degranulation also induce the assembly of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. NADPH oxidase converts oxygen into superoxide, which can be further catalyzed to H_2O_2 , hydroxyl anion, or peroxynitrite anion, to produce an array of reactive oxygen species (ROS) (Faurischou and Borregaard 2003; Borregaard 2014).

Although neutrophils have evolved multiple strategies to inactivate and eliminate invading microbes, pathogens have also developed strategies to escape the neutrophil immune defense machinery described above. Neutrophils can, thus, exert an additional antimicrobial defense program when they encounter microbes that are

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S.S. Burgener and K. Schroder

either too large for phagocytosis, or have evaded phagosomal destruction by escaping into the cytosol. Here, neutrophils extrude a physical barrier to pathogen dissemination, called a neutrophil extracellular trap (NET) (Amulic et al. 2012; Papayannopoulos 2018). This review summarizes the major pathways that lead to the extrusion of NETs during infection and their functions in host defense.

NEUTROPHIL EXTRACELLULAR TRAPS

NETs are webs of neutrophil DNA coated with histones and antimicrobial proteins that entrap microbes. Neutrophils cast their NETs in a multistep process called NETosis (Brinkmann et al. 2004; Fuchs et al. 2007; Papayannopoulos et al. 2010). NETosis is a distinct cellular program from apoptosis or necroptosis, and true NET structures are not usually cast during these latter forms of cell death. NETosis involves several distinct and sequential morphological changes in the neutrophil (Fig. 1; Fuchs et al. 2007; Yipp and Kubes 2013): (1) The characteristic lobulated architecture of the nucleus is lost, (2) the nuclear and granular membranes become permeable, (3) histone inactivation leads to chromatin expansion into the cytosol, (4) chromatin mixes with granule content, (5) the loss of internal membranes leads to the disappearance of cytosolic organelles, and (6) the plasma membrane becomes permeable and the NETs are released into the extracellular space.

Within NETs, the DNA is decorated with a conserved set of nuclear, granular, and cytoplasmic proteins. The key antimicrobial effector proteins of NETs are histones (H2A, H2B, H3, and H4) and granule proteases (such as NE, CatG, and PR-3), as well as myeloperoxidase (MPO) and lactotransferrin. The proteases remain enzymatically active even when the NET is exposed to endogenous protease inhibitors. Other granule proteins, such as azurocidin, lysozyme C, and α -defensins, are also reported within NETs (Dwyer et al. 2014). NETs also contain cytoplasmic and cytoskeleton proteins such as S100 calcium-binding proteins, actin, myosin, and cytokeratin (Urban et al. 2009; O'Donoghue et al. 2013; Dwyer et al. 2014). Proteomics stud-

ies identified 24 proteins within PMA-induced NETs (Urban et al. 2009; O'Donoghue et al. 2013), and 80 proteins within NETs induced by nonmucoid and mucoid strains of *Pseudomonas aeruginosa* (Dwyer et al. 2014). It is, thus, possible that the composition of the NET is influenced by its eliciting stimulus.

THE NEUTROPHIL'S DECISION TO CAST A NET IS INFORMED BY MICROBE SIZE AND LOCATION

When encountering a microbe, the neutrophil needs to determine which antimicrobial defense function to deploy. A microbe size-sensing mechanism allows neutrophils to decide whether or not to launch a NET in response to extracellular pathogens (Branzk et al. 2014). Small microbes that are readily taken up by phagocytosis are poor NET stimulants because the phagosome fuses with neutrophil azurophil granules, and neutrophil granule proteases are, thus, unavailable for initiating NETosis (Branzk et al. 2014). Phagosome formation, hence, serves as a checkpoint to prevent NETosis. Some microbes are too large to be engulfed by phagocytosis, for example, the fungus *Candida albicans* that forms large filamentous hyphae, or aggregates of *Mycobacterium bovis*. If a neutrophil encounters these large microbes, the azurophil granule protein NE is released into the cytoplasm to initiate NET formation. Neutrophils deficient in dectin-1, a receptor that mediates phagocytosis of fungi, are hindered in their ability to determine which antimicrobial defense mechanism to apply, resulting in uncontrolled NET release (Branzk et al. 2014). Microbe size and location also affect other neutrophil antimicrobial functions. Neutrophil interaction with large microbes, such as *C. albicans* hyphae, leads to the extracellular deployment of ROS and strong interleukin (IL)-1 β secretion with resultant neutrophil clustering wherein about eight neutrophils engage with one 100- μ m hyphal filament. In contrast, small phagocytosed microbes trigger the intracellular deployment of ROS, suppressing cytokine release and neutrophil clustering, and thereby preventing neutro-

Figure 1. NETosis is a distinct cellular program in neutrophils. Neutrophil NETosis involves sequential morphological changes: (1) The characteristic lobulated architecture of the nucleus (purple) is lost, (2) the membranes of the nucleus and granules (pink) become permeable, (3) histone inactivation by clipping and/or citrullination (Cit-histones) leads to chromatin expansion into the cytosol, (4) chromatin mixes with granule content, (5) the loss of internal membranes leads to the disappearance of cytosolic organelles, and (6) the plasma membrane breaks and neutrophil extracellular traps (NETs) are released into the extracellular space.

S.S. Burgener and K. Schroder

phil accumulation and tissue damage during microbial clearance (Warnatsch et al. 2017).

Microbe size may not be the only factor that determines the antimicrobial response of neutrophils. Some microbes can manipulate the neutrophil response by evading phagocytosis, and may thereby induce NET formation (Sumbly et al. 2005; Döhrmann et al. 2014; Eby et al. 2014; Storisteanu et al. 2017; Eisenbeis et al. 2018). For example, Gram-negative bacteria that escape the phagosome to translocate to the cytosol induce NETs in a newly described pathway that involves the noncanonical inflammasome machinery (Chen et al. 2018).

In the 15 years since their discovery, NETs have been an area of intense research, which has provided important insights into this novel antimicrobial mechanism, and revealed several distinct pathways for trap release. In this review, we describe three key mechanisms by which neutrophils extrude NETs in host defense through the process of suicidal NETosis, noncanonical NETosis, and vital NETosis. Whereas all of these NETosis pathways ultimately lead to neutrophil death, NET extrusion occurs during cell rupture in suicidal and noncanonical NETosis while NETs are expelled from neutrophils that can continue to perform cell functions (e.g., migration) in vital NETosis.

SUICIDAL NETosis

The first NETosis pathway was described in 2004, in which NET extrusion and cell death (suicidal NETosis) was observed in response to high doses of phorbol-12-myristate-13-acetate (PMA) (Brinkmann et al. 2004). The strong and robust NET responses induced by PMA have led to the adoption of PMA as a key in vitro stimulant for the study of NETosis. PMA is a potent activator of several signaling pathways within neutrophils such as protein kinase C (PKC) (Steinberg 2008). PMA mediates suicidal NETosis by inducing the assembly of NADPH oxidase, which triggers ROS production, MPO activation, and the cytoplasmic release of NE, and leads to chromatin decondensation in the nucleus (Fig. 2). Following this, the nuclear membrane breaks down, resulting in the release

of the decondensed chromatin into the cytosol. Chromatin is then expelled into the extracellular space via GSDMD pores or GSDMD-driven membrane tears, and results in neutrophil death (Brinkmann et al. 2004; Fuchs et al. 2007; Sollberger et al. 2018). Below, we will focus on this well-characterized pathway of NE-, MPO-, and ROS-driven NETosis as an exemplar for suicidal NETosis. However, ionophores (e.g., A23187, nigericin) also trigger suicidal NETosis through a poorly characterized mechanism that is independent of NE, MPO, and ROS (Neeli and Radic 2013; Kenny et al. 2017), highlighting the complexity of NETosis pathways.

NADPH Oxidase

On activation by PMA, neutrophils up-regulate glycolysis (Rodríguez-Espinosa et al. 2015), and this is required for NET expulsion (Rodríguez-Espinosa et al. 2015; Siler et al. 2017). Glucose uptake induces signaling by extracellular signal-regulated kinase (ERK) (Rodríguez-Espinosa et al. 2015), leading to the phosphorylation of one of the three cytoplasmic regulatory subunits of the NADPH oxidase, $p47^{phox}$ (El Benna et al. 1996; Hakkim et al. 2011). On phosphorylation, $p47^{phox}$ is recruited to the granule and plasma membranes, in which it interacts with other subunits to assemble the NADPH oxidase complex and generate superoxide (Dang et al. 1999; Lopes et al. 1999; Kleniewska et al. 2012; Nunes et al. 2013; Dinanuer 2016). Inhibitors of the ERK pathway, thus, block NADPH oxidase function and PMA-induced NET release (Hakkim et al. 2011). PMA- and microbe-induced NET release is also suppressed in neutrophils deficient in Rac2, a small GTPase required for NADPH oxidase function (Lim et al. 2011; Gavillet et al. 2018), and in neutrophils from chronic granulomatous disease (CGD) patients that are deficient in NADPH oxidase function (Bianchi et al. 2009; Röhm et al. 2014). Gene therapy restored NADPH oxidase function in a CGD patient, and consequently reinstated the capacity of their neutrophils to produce NETs, which were able to inhibit *Aspergillus nidulans* growth (Bianchi et al. 2009). As invasive aspergillosis is a leading cause of death in CGD patients, this suggests

Figure 2. Suicidal NETosis. Phorbol-12-myristate-13-acetate (PMA) or extracellular microbes induce suicidal NETosis by inducing the extracellular signal-regulated kinase (ERK)-dependent assembly of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which generates reactive oxygen species (ROS) and the cytoplasmic release of granule proteins, and leads to chromatin decondensation in the nucleus. Chromatin is then expelled into the extracellular space via gasdermin D (GSDMD) pores or GSDMD-driven membrane tears, resulting in neutrophil death. (NE) neutrophil elastase, (MPO) myeloperoxidase, (NET) neutrophil extracellular trap.

that ROS-dependent NETs may be critical for host defense against *Aspergilli* species.

Granule Proteases

Under resting conditions, the neutrophil's antimicrobial arsenal is stored in granules. ROS generation by NADPH oxidase triggers the release of these proteins into the cytosol. The protease NE is particularly critical for suicidal NETosis, as murine NE deficiency prevents NET formation during in vivo challenge with *Klebsiella pneumoniae* (Papayannopoulos et al. 2010).

The exact mechanism by which NE is released into the cytosol to initiate NETosis is unclear, but it is proposed to occur without granule rupture (Metzler et al. 2014) and involve co-

operation between granule proteins (O'Donoghue et al. 2013). In resting neutrophils, NE clusters together with MPO, azurocidin, CatG, defensin-1, lysozyme, and lactoferrin to form a complex called an "azurosome" in the azurophil granule (Brinkmann et al. 2004; Fuchs et al. 2007; Papayannopoulos et al. 2010; Metzler et al. 2011). On neutrophil NADPH oxidase activation, ROS triggers MPO activation and the dissociation of MPO and NE from the azurosome (Metzler et al. 2011, 2014). This induces the release of NE into the cytosol through an unclear mechanism that requires MPO activity within the cell (Metzler et al. 2014). Human mutations in the *MPO* locus can decrease or ablate MPO function (Nauseef et al. 1996; Dinauer 2014). Whereas neutrophils from patients with a

S.S. Burgener and K. Schroder

partial MPO deficiency can still form NETs on exposure to *C. albicans*, those from MPO-deficient patients cannot (Metzler et al. 2011).

In the cytoplasm, NE binds to the actin cytoskeleton where it degrades F-actin, leading to actin disassembly and neutrophil immobilization (Metzler et al. 2014). In the nucleus, NE cleaves and inactivates histones such as H4 and H2B, leading to chromatin relaxation and DNA decondensation (Papayannopoulos et al. 2010). MPO functions synergistically with NE to mediate chromatin decondensation during NETosis (Papayannopoulos et al. 2010). The exact requirement for MPO in this process is unclear, but may simply involve NE liberation from granules as described above.

PAD4-Dependent Histone Citrullination

Suicidal NETosis is often accompanied by histone citrullination by peptidylarginine deiminase type 4 (PAD4), which can contribute to chromatin relaxation. PAD4 is activated by a cytosolic spike in calcium as is induced by ionophores (Neeli et al. 2008) but not by PMA (Neeli and Radic 2013; Damgaard et al. 2016). Whereas PAD4-dependent histone citrullination often accompanies suicidal NETosis, it is often dispensable for NET extrusion via this pathway (Claushuis et al. 2018; Guiducci et al. 2018), perhaps because histones are additionally inactivated by NE-dependent cleavage, providing redundancy. PAD4 is, however, crucial for chromatin decondensation during vital NETosis.

Gasdermin D: A NETosis Executioner

Gasdermin D (GSDMD) was recently identified as an executioner of suicidal and noncanonical NETosis (Chen et al. 2018; Sollberger et al. 2018). GSDMD usually resides in the cytosol of resting cells. On cleavage by specific proteases, a pore-forming fragment (GSDMD-p30) is generated. During PMA-induced suicidal NETosis, NE cleaves GSDMD to generate GSDMD-p30 pores in the plasma and granule membranes, leading to rupture of these membranes during NETosis (Sollberger et al. 2018). GSDMD pores permeabilize the nuclear mem-

brane during noncanonical NETosis (Chen et al. 2018) and may similarly contribute to nuclear permeabilization during suicidal NETosis. A GSDMD inhibitor blocked PMA-induced NETosis without affecting the function of NADPH oxidase, NE or MPO, highlighting the requirement for membrane permeabilization in this pathway (Sollberger et al. 2018).

Model for Suicidal NETosis

The above studies together suggest the following model for suicidal NETosis (Fig. 2). On neutrophil activation, ROS triggers the activation of MPO and azurosome dissociation within azurophil granules, thereby facilitating the MPO-driven release of NE into the cytoplasm. This small amount of NE appears to be sufficient to cleave GSDMD to GSDMD-p30. This, in turn, induces the formation of GSDMD pores within the granule membrane, leading to granule rupture, further NE release into the cytoplasm, and further GSDMD cleavage in a feedforward loop. NE then translocates into the nucleus, where it cleaves and inactivates histones, leading to chromatin expansion and mixing with cytoplasmic and granule contents. GSDMD-p30 pores then induce plasma membrane rupture to enable NET release (Sollberger et al. 2018).

NONCANONICAL NETosis

We recently described a new pathway of suicidal NETosis, which we here propose to term noncanonical NETosis, which is deployed when neutrophils detect Gram-negative bacteria in their cytosol. This pathway relies on bacterial sensing by the noncanonical inflammasome (Fig. 3), leading to caspase-4/11- and GSDMD-driven NET extrusion (Chen et al. 2018) through a signaling pathway that resembles cell lysis by pyroptosis but results in the morphological features of neutrophil death by NETosis.

Signaling by the Noncanonical Inflammasome

The prefix “noncanonical” in this NETosis pathway indicates the involvement of the noncanonical inflammasome. Inflammasomes can be

Figure 3. Noncanonical NETosis. The lipopolysaccharide (LPS) of cytosolic Gram-negative bacteria triggers the assembly of the noncanonical inflammasome, leading to caspase-4/11 activation and gasdermin D (GSDMD) cleavage to p30. GSDMD-p30 pores rupture the nuclear and granule membranes and, eventually, the plasma membrane. Caspase-11 gains access to the nucleus, where it cleaves histones to mediate DNA decondensation. Chromatin is then expelled into the extracellular space during plasma membrane rupture, resulting in neutrophil death. During NETosis, GSDMD-driven NLRP3 inflammasome activation triggers the release of interleukin (IL)-1 β to induce neutrophil recruitment, activation, and killing of the neutrophil extracellular trap (NET)-entrapped bacterium.

broadly categorized into those that activate caspase-1 (“canonical inflammasomes”) and those that activate caspase-11 in mouse or caspase-4/5 in humans (“noncanonical inflammasomes”). The noncanonical inflammasomes are sensors for cytosolic Gram-negative bacteria. On detection of bacterial lipopolysaccharide (LPS), these inflammasomes assemble to activate caspase-4/5 in humans and caspase-11 in mice. Signaling by both canonical and noncanonical inflammasomes leads to caspase-1- or caspase-4/5/11-dependent cleavage of GSDMD to trigger GSDMD-p30 plasma membrane pores, the release of proinflammatory cytokines (e.g., IL-1 β and IL-18), and cell lysis by pyroptosis (Kayagaki et al. 2015; Shi et al. 2015; Liu et al. 2016). Pyroptosis has been intensely studied in macrophages but is less characterized in other cell types.

NET Extrusion via the Noncanonical Inflammasome

Compared to macrophages, neutrophils signal via specialized inflammasome pathways that induce distinct outcomes for cell death or viability. We, and others, previously showed that neutrophils do not undergo caspase-1-dependent pyroptosis during canonical inflammasome signaling (Chen et al. 2014, 2018; Karmakar et al. 2015; Monteleone et al. 2018). Neutrophils do, however, die on activation of the noncanonical inflammasome pathway (Chen et al. 2018). Human and murine neutrophils infected with Gram-negative bacteria that access the cytosol (Δ sifA *Salmonella enterica*, *Citrobacter rodentium*) died and extruded NETs through a cell death pathway displaying the hallmark features of NETosis, including nuclear delobulation,

S.S. Burgener and K. Schroder

DNA extrusion, DNA-MPO colocalization, and histone citrullination (Chen et al. 2018). Neutrophil NET extrusion required the pyroptotic machinery, caspase-4/11, and GSDMD, but proceeded independently of NE or MPO. PAD4-dependent histone citrullination accompanied noncanonical NETosis but was not required for death or NET extrusion. Mechanistically, GSDMD-p30 pores target several neutrophil membranes to mediate permeabilization of the granule and nuclear membranes and, eventually, rupture of the plasma membrane (Chen et al. 2018; Sollberger et al. 2018). GSDMD-p30 nuclear pores appear to enable caspase-11 to access the chromatin, in which caspase-11 performs an analogous function to NE to degrade histones and thereby relax chromatin (Chen et al. 2018).

Noncanonical NETosis suppresses bacterial residence in the neutrophil cytosol and prevents in vivo microbial dissemination. During in vitro infection, the noncanonical NETosis pathway suppressed neutrophil cytosolic infection with *ΔsifA Salmonella* (Chen et al. 2018), presumably by inducing the death of the infected neutrophil. Murine deficiency in *Casp11* or *Gsdmd* promoted microbial dissemination to a secondary infection site during murine in vivo challenge with *ΔsifA Salmonella* (Chen et al. 2018), confirming previous reports that the noncanonical inflammasome pathway mediates host defense (Aachoui et al. 2013; Storek and Monack 2015; Thurston et al. 2016). Interestingly, when NETs were dismantled at a primary infection site by applying DNase I, this promoted bacterial dissemination in wild-type (WT) but not *Casp11*^{-/-} or *Gsdmd*^{-/-} mice (Chen et al. 2018). The NETs themselves thus mediate host defense, likely via immobilizing bacteria to enable their destruction by newly recruited neutrophils.

VITAL NETosis

In contrast to the above, relatively slow, pathway of suicidal NETosis, vital NETosis involves the rapid release of NETs on neutrophil stimulation with specific bacteria, bacterial products, Toll-like receptor (TLR) 4-activated platelets or complement proteins (Fig. 4). Vital NETosis, also called “leukotoxic hypercitrullination” (Konig

and Andrade 2016), mimics features of suicidal NETosis but does not immediately induce neutrophil death. During vital NETosis, neutrophils are still capable of functions such as migration, phagocytosis, and killing of bacteria while releasing their NET (Clark et al. 2007; Pilsczek et al. 2010; Yipp et al. 2012). The key features of vital NETosis are rapid histone citrullination, nuclear blebbing, and the vesicular transport of nuclear blebs to the plasma membrane, in which chromatin is externalized without permeabilizing the plasma membrane.

Vital NETosis is often observed during thrombosis and autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and autoimmune vasculitis (Clark et al. 2007; Kessenbrock et al. 2009; Fuchs et al. 2010; Pilsczek et al. 2010; Yipp et al. 2012; Martinod et al. 2016). Here, we will focus on neutrophil vital NETosis during interaction with microbes, and summarize the key unique features of vital NETosis that are not observed in suicidal or noncanonical NETosis.

Receptor-Mediated Rapid Vital NETosis

Several surface receptors appear to be required for vital NETosis. TLR2 was required for vital NETosis during murine in vivo skin infection with *Staphylococcus aureus* (Yipp et al. 2012), and TLR2 or TLR4 receptor blockade suppressed NET formation induced by in vitro exposure to *S. aureus* (Wan et al. 2017). Neutrophil exposure to bacterial LPS in whole blood triggers vital NETosis. Here, NET extrusion requires TLR4 expression on platelets and platelet-neutrophil interaction via P-selectin (Pieterse et al. 2016). The complement system is also implicated in vital NETosis. When immobilized by the extracellular matrix component fibronectin, the β -glucan of *C. albicans* is recognized by complement receptor 3, inducing robust NET formation (Byrd et al. 2013). These findings highlight the complexity of host-pathogen interaction during a coordinated immune response.

PAD4 Functions in Vital NETosis

Calcium signaling, PAD4 activation, and histone citrullination appear to be common re-

Figure 4. Vital NETosis. Microbial products or activated platelets stimulate vital NETosis through cell-surface receptors (e.g., TLR2, TLR4, complement receptor-3 [CR3]). The resulting spike in cytosolic calcium activates neutrophil PAD4, triggering histone citrullination and DNA decondensation. Chromatin derived from nuclear blebs or the mitochondria is expelled to generate a neutrophil extracellular trap (NET) while the neutrophil is still able to perform cellular functions such as cell migration.

quirements for vital NETosis. Whereas most stimulators of suicidal NETosis require NADPH oxidase function and induce NE-dependent histone clipping, NADPH oxidase function is dispensable for vital NETosis (Pilszczek et al. 2010; Doua et al. 2015), which proceeds without apparent histone clipping (Pieterse et al. 2018). Ionophores, bacterial products, or an increase in extracellular pH trigger the generation of mitochondrial ROS, calcium influx, and calcium-activated PAD4 enzymatic function in neutrophils (Doua et al. 2015; Naffah de Souza et al. 2017). PAD4 can then citrullinate histones to mediate DNA decondensation. PAD4 deficiency in *Shigella flexneri*-infected neutrophils rendered these cells unable to citrullinate histones, undergo vital NETosis, or mediate bacterial killing (Li et al. 2010). In line with an essential function for PAD4 in this pathway, a selective PAD4 inhibitor abrogated vital NETosis induced by ionomycin or *S. aureus* (Lewis et al. 2015).

DNA Source during Vital NETosis

Whereas suicidal NETosis clearly involves the expulsion of nuclear DNA, the source of extruded DNA during vital NETosis is less clear. A number of studies report nuclear blebbing leading to the release of histone-rich nuclear DNA (Pilszczek et al. 2010; Yipp et al. 2012; Gupta et al. 2018). Other studies suggest that NETs contain mitochondrial DNA (Yousefi et al. 2009; Keshari et al. 2012; Itagaki et al. 2015; Wang et al. 2015; Lood et al. 2016). Several studies indicate that NETs containing mitochondrial DNA elicit proinflammatory cytokine production from immune cells (Keshari et al. 2012; Wang et al. 2015; Lood et al. 2016).

MICROBIAL TRIGGERS OF NETosis

A wide variety of microbes are reported to induce NETosis. NET-inducing bacteria include *S. flexneri* (Brinkmann et al. 2004), *Escherichia*

S.S. Burgener and K. Schroder

coli (Grinberg et al. 2008), *Streptococcus pneumoniae* (Beiter et al. 2006), *Streptococcus pyogenes* (Sumbly et al. 2005), and *Mycobacterium tuberculosis* (Ramos-Kichik et al. 2009). Fungi such as *C. albicans* (Urban et al. 2006, 2009) and *Aspergillus fumigatus* (Bruns et al. 2010) are well characterized to induce NETosis. Even parasites are reported to induce NETosis, including *Leishmania* spp. (Guimaraes-Costa et al. 2009), *Plasmodium falciparum* (Baker et al. 2008), and *Toxoplasma gondii* (Abi Abdallah and Denkers 2012; Abi Abdallah et al. 2012). NET extrusion thus appears to be a conserved immune defense strategy in response to many different microbes.

FUNCTIONS FOR NETS IN ANTIMICROBIAL DEFENSE

Most NET studies agree that these structures trap extracellular microbes such as *C. albicans* or *T. gondii* (Urban et al. 2006, 2009). Imaging techniques that use flow chamber systems or intravital microscopy elegantly showed *E. coli* capture by NETs in vitro and in vivo (Clark et al. 2007). For example, confocal intravital microscopy imaged NETs capturing circulating *E. coli* in the hepatic sinusoids (McDonald et al. 2012).

There is growing evidence that NETs can also directly kill some pathogens such as *E. coli* (Grinberg et al. 2008) or *Leishmania* spp. (Guimaraes-Costa et al. 2009). The direct antimicrobial properties of NETs remain a topic of debate, and it is possible that NETs exert variable efficiency for killing different microbial species. NETs contain a selection of proteins with known antimicrobial properties such as histones (Hirsch 1958), and granule-resident antimicrobial proteins such as NE and MPO. NE kills bacteria by cleaving proteins on the bacterial outer membrane and targeting virulence factors of colonic enterobacteria, such as *Shigella* or *Yersinia* (Belaouaj et al. 1998, 2000; Weinrauch et al. 2002), and is crucial in host defense against Gram-negative bacteria such as *E. coli* or *K. pneumoniae* (Belaouaj et al. 1998). MPO remains active upon the extruded NET, where it generates ROS, such as hypochlorous acid from H_2O_2 derived from NADPH oxidase or

the bacterium itself, to mediate bacteria killing (Allen and Stephens 2011; Parker et al. 2012a,b).

GSDMD pores may be a key antimicrobial weapon generated during noncanonical and suicidal NETosis (Chen et al. 2018; Sollberger et al. 2018). Both forms of NETosis trigger the cleavage of GSDMD to its pore-forming p30 fragment, which is poorly soluble, and immediately associates with lipids such as cardiolipin, phosphatidylinositol phosphates, and phosphatidylserine (Ding et al. 2016; Liu et al. 2016; Sborgi et al. 2016). GSDMD-p30 is reported to target bacterial membranes to induce the killing of extracellular *E. coli* and *S. aureus* (Ding et al. 2016; Liu et al. 2016) and cytosolic *L. monocytogenes* (Liu et al. 2016). GSDMD-p30 pores in the host cell plasma membrane are also crucial for its antimicrobial functions. Here, GSDMD-p30 pores facilitate plasma membrane rupture to enable NET release, and also the secretion of IL-1 β to recruit neutrophils to a site of infection (Monteleone et al. 2018). At an infection site, IL-1 β and other factors activate neutrophils and extend their life span to ensure they are able to mediate host-defensive functions (Miller et al. 2007; Rider et al. 2011; Biondo et al. 2014; Chen et al. 2014). Thus, GSDMD may serve several functions in the host defense, in which GSDMD-driven NETs first immobilize the pathogen, GSDMD pores directly target the bacterial membrane, and GSDMD-dependent IL-1 β serves as a call-to-arms to recruit circulating neutrophils to kill the entrapped microbe.

MICROBIAL EVASION OF NETs

The effectiveness of NETs in mediating host defense is highlighted by the observation that many microbes have developed NET-evasion mechanisms. Some microbes suppress NETs by blocking the upstream signaling pathways, whereas others encode DNases to degrade the NET or have developed resistance to antimicrobial proteins upon the NET (Sumbly et al. 2005; Döhrmann et al. 2014; Eby et al. 2014; Storisteanu et al. 2017; Eisenbeis et al. 2018). For example, *Bordetella pertussis* encodes an adenylate cyclase toxin (ACT) to produce intracellular cAMP to suppress the neutrophil ERK-depen-

dent oxidative burst and, hence, NETosis (Eby et al. 2014). Group A *Streptococcus* has developed several strategies to evade NET-dependent host defense, including the expression of nucleases to degrade NETs (Sumbly et al. 2005; Walker et al. 2007), and suppressing MPO release via the M1T1 serotype *streptococcal* collagen-like protein 1 (Scl-1) (Döhrmann et al. 2014). The *Staphylococcal* protein A (SpA) is a virulence factor that is secreted into the extrabacterial space to suppress neutrophil phagocytosis of *S. aureus*, but in doing so, promotes NETosis (Hoppenbrouwers et al. 2018). Other *S. aureus* virulence factors, such as the DNA-binding protein extracellular adherence protein (Eap), suppress NET formation and function (Eisenbeis et al. 2018). Such microbial evasion strategies highlight the requirement for neutrophils to have a multitude of microbial killing mechanisms at their disposal for many and varied host defense strategies.

CONCLUDING REMARKS

Diverse extracellular and intracellular microbes induce neutrophils to trigger NETs by suicidal, noncanonical, and vital NETosis. By entrapping microbes, NETs prevent microbial dissemination to a secondary infection site and, in some cases, NETs exert direct antimicrobial functions. In addition to these antimicrobial functions, emerging literature documents pathological roles for NETs in autoimmune diseases. It remains to be elucidated whether distinct NETosis pathways engender differences in immunomodulatory or antimicrobial properties of the resulting NET. The recent discovery that the suicidal and noncanonical NETosis pathways converge on a single cell death executioner protein, GSDMD, suggests that GSDMD inhibitors may have therapeutic potential in the treatment of NET-associated diseases such as thrombosis, rheumatoid arthritis, systemic lupus erythematosus, and autoimmune vasculitis. Further research to elucidate the molecular and cellular mechanisms by which neutrophils cast their NETs will be critical for understanding this important host defense program and the design of new autoimmune disease therapies.

Neutrophil Extracellular Traps in Host Defense

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Mechanism and Regulation of Gasdermin-Mediated Cell Death

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Cell Death and Neurodegeneration

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The Killer Pseudokinase Mixed Lineage Kinase Domain-Like Protein (MLKL)

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Neutrophil Extracellular Traps in Host Defense

Sabrina Sofia Burgener and Kate Schroder

Cell-Cycle Cross Talk with Caspases and Their Substrates

Patrick Connolly, Irmina Garcia-Carpio and Andreas Villunger

Cracking the Cell Death Code

Carla V. Rothlin and Sourav Ghosh

BAX, BAK, and BOK: A Coming of Age for the BCL-2 Family Effector Proteins

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