PERSPECTIVE

Evasion of Neutrophil Extracellular Traps by Respiratory Pathogens

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Abstract

The release of neutrophil extracellular traps (NETs) is a major immune mechanism intended to capture pathogens. These histone- and protease-coated DNA structures are released by neutrophils in response to a variety of stimuli, including respiratory pathogens, and have been identified in the airways of patients with respiratory infection, cystic fibrosis, acute lung injury, primary graft dysfunction, and chronic obstructive pulmonary disease. NET production has been demonstrated in the lungs of mice infected with *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Aspergillus fumigatus*. Since the discovery of NETs over a decade ago, evidence that "NET evasion" might act as an immune protection strategy among respiratory pathogens, including group A *Streptococcus*, *Bordetella pertussis*, and *Haemophilus influenzae*, has been growing, with the majority of these studies being published in the past 2 years. Evasion strategies fall into three main categories: inhibition of NET release by down-regulating host inflammatory responses; degradation of NETs using pathogen-derived DNases; and resistance to the microbicidal components of NETs, which involves a variety of mechanisms, including encapsulation. Hence, the evasion of NETs appears to be a widespread strategy to allow pathogen proliferation and dissemination, and is currently a topic of intense research interest. This article outlines the evidence supporting the three main strategies of NET evasion—inhibition, degradation, and resistance—with particular reference to common respiratory pathogens.

Keywords: neutrophil extracellular traps; immune evasion; deoxyribonuclease; *Streptococcus*

Clinical Relevance

The importance of neutrophil extracellular traps (NETs) in pathogen killing has received considerable attention, and it is now increasingly clear that many pathogens have evolved mechanisms to evade NET-mediated entrapment and killing. This article highlights this important and rapidly evolving field, and aims to alert respiratory scientists and clinicians to therapeutic opportunities to modulate pathogen–NET interactions.

What Are Neutrophil Extracellular Traps?

Neutrophil granulocytes, comprising over half of all circulating white blood cells, are terminally differentiated phagocytes that form a key first line of defense against invading microbes. Neutrophils engage invading microbes through a multitude of surface receptors, including antibody Fc receptors, complement receptors, and Toll-like receptors (TLRs), which recognize and respond to pathogen-associated molecular patterns (1). Once activated, neutrophils are responsible for phagocytic clearance, and can elaborate an arsenal of antimicrobial peptides, proteases, and reactive oxygen species (ROS) to achieve pathogen killing.

Our understanding of neutrophil "antipathogenic" mechanisms was broadened a decade ago with the finding that neutrophils can release extracellular networks of chromatin coated with granule proteins (2); this response could be triggered by a variety of infectious agents, including bacteria, viruses and parasites, host-derived cytokines, inflammatory mediators, and chemical factors, such as phorbol myristate acetate (PMA; Figure 1). The discovery of these so-called "neutrophil extracellular traps" (NETs) has catalyzed a growing body of research, and the ability of NETs to capture pathogens

1
TRIGGERS

Inflammatory cytokines
Activated platelets
Bacteria
Fungi
Viruses

2
CONSTITUENTS

Chromatin
Histones
Myeloperoxidase
Neutrophil elastase

3
EFFECTS

Antimicrobial
Proinflammatory:
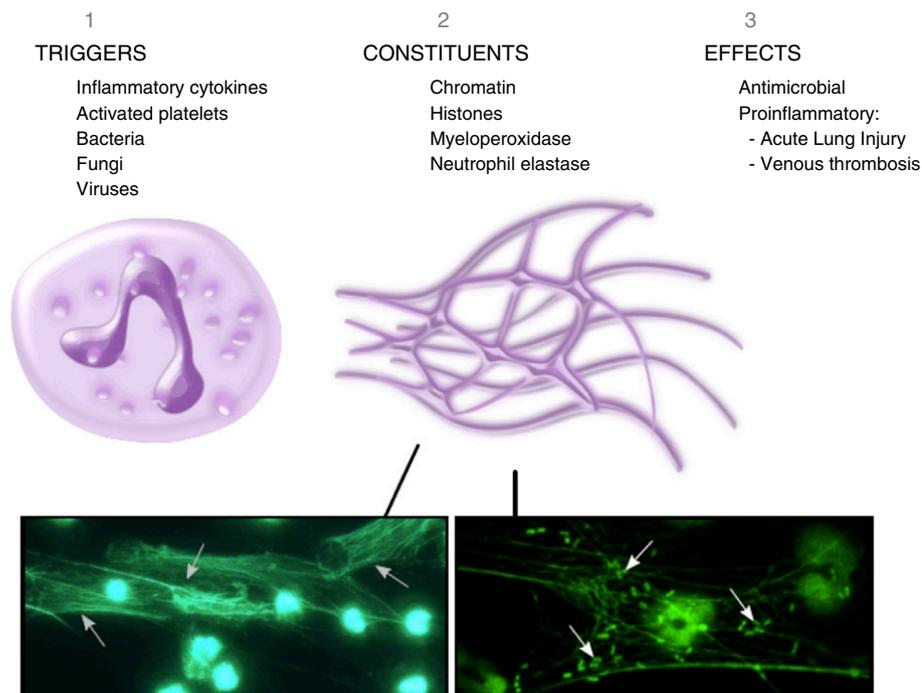- Acute Lung Injury
- Venous thrombosis

Figure 1. Neutrophil extracellular trap (NET) release. (*1*) Upon stimulation by a variety of triggers, including many pathogens, neutrophils release NETs into the extracellular space. (*2*) These structures are composed of DNA coated with histones and chromatin, and trap microbes in a concentrated environment of antimicrobial proteins, such as neutrophil elastase and myeloperoxidase. *Insets* are images showing (*left*) human NETs released by phorbol myristate acetate–stimulated neutrophils by fluorescence microscopy using Sytox Green (*arrows*), and (*right*) green-stained *Salmonella typhimurium* bacteria (*arrows*) trapped in murine NETs. (*3*) As well as exerting antimicrobial effects, NETs may play a proinflammatory role contributing to the pathogenesis of conditions, such as acute lung injury and venous thromboembolism.

has been demonstrated in many *in vivo* contexts, restricting their movement and surrounding them in a microenvironment of concentrated antimicrobial agents (3). The classically described NET pathway commits neutrophils to a specialized form of cell death, "NETosis," although some pathogens appear to induce a "vital NETosis," where certain neutrophil chemotactic and phagocytic functions remain temporarily intact, even after the cell becomes anuclear (3).

In the classical "suicidal pathway" of NETosis, agonists, such as PMA or IL-8, activate the extracellular signal–regulated kinase (ERK) cascade, which stimulates the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase to produce ROS, which, in turn, triggers granule membrane disintegration and release of neutrophil elastase (NE) and myeloperoxidase.

More remains to be discovered about the signaling events that trigger NET formation, as this response is neither uniform nor observed in every situation where NADPH is activated. Nevertheless, when NE translocates to the nucleus, it

acts to cleave histones, and, along with myeloperoxidase, decondenses chromatin and degrades the nuclear membrane. This process extrudes DNA, histones, and proteases into the cytosol, where they combine to form NETs, with eventual plasma membrane rupture and NET expulsion from the cell (3). This extracellular release of DNA occurs typically 2–3 hours after stimulation.

A second, NADPH oxidase–independent, calcium-mediated NETosis pathway has also been described, involving the enzyme, protein arginine deiminase 4 (PAD4) (4). Upon the increase of intracellular calcium in response to agents such as calcium ionophores, PAD4 binds calcium, crosses into the nucleus, and deiminates positively charged arginine residues to generate uncharged citrullin on histones H3 and H4. This loss of positive charge leads to the dissociation of the histones from negatively charged DNA, resulting in decondensation of the nucleosome. Citrullination of histones has been shown to be involved in

calcium-dependent, but not NADPH oxidase–dependent, NETosis (4). It was demonstrated that PAD4 deficiency or use of Cl-amidine to chemically inhibit PAD4 activity causes a reduction of histone decondensation and NET formation (5). Other studies suggest that PAD4-deficient mice and wild-type mice clear infections equally well (6); therefore, the role of PAD4 in infection-related NETosis is uncertain.

In contrast, vital NETosis appears to be a rapid event seen in response to a more limited group of bacteria (3). Hence, with infection with gram-negative bacteria, such as *Escherichia coli*, TLR4-activated platelets can stimulate NETs through a direct CD11a interaction with neutrophils, and gram-positive bacteria, such as *Staphylococcus aureus*, activate neutrophil TLR2 and complement receptor 3, leading to induction of vital NETosis. Much remains to be elucidated regarding the mechanisms underlying this latter process, but fluorescence and electron micrographs show intact plasma membranes despite the release of NETs.

The electrostatic charge of nucleic acid (3) and the presence of pathogen-binding molecules, such as surfactant protein D (7), combine to support bacterial–NET interactions. Intravital microscopy has demonstrated the binding of *E. coli* to NETs released in the liver microcirculation during sepsis, with inhibition of NET formation resulting in increased bacterial dissemination to distant organs (8). It appears that neutrophils can spread NETs over wide distances *in vivo*, and Yipp and Kubes (3) suggested that DNA-binding sites on interstitial fibronectin may explain this remarkable ability to disperse NETs over large areas.

A growing body of evidence supports the ability of NETs to kill pathogens. For example, neutrophils treated with cytochalasin D, which inhibits phagocytosis and thus most traditional neutrophil-killing mechanisms, are still bactericidal (2), and treatment of these neutrophils with DNase or anti-histone antibodies diminishes this killing. NETs have also been shown to kill *Aspergillus* (9), group A *Streptococcus* (GAS) (10), and yeast and hyphal forms of *Candida albicans* (11). However, some other studies have failed to show NETs killing, including reports where a subset of *S. aureus* trapped by NETs continued to proliferate after DNase-induced release (12). What is clear, however, is that the trapping of bacteria by NETs acts to reduce proliferation and prevent bacterial

dissemination from certain organs, including the lung (13).

Beyond trapping and killing pathogens, other suggested antipathogenic effects of NETs include their ability to neutralize virulence factors, such as IpaB from *Shigella flexneri* (2), functional opsonization of *Aspergillus fumigatus*, and inhibition of microbial growth via calprotectin-mediated chelation of zinc during *Aspergillus* infection (9).

Pathogen Evasion of NETs

Although the host immune system has evolved numerous strategies to neutralize pathogens, invading microbes have also developed various mechanisms to avoid being attacked by the immune system; examples include inhibiting host inflammatory responses, degrading antimicrobial molecules with catalytic enzymes, and avoiding or resisting host proteases. Research over the past decade has now shown that three comparable mechanisms are also employed by pathogenic bacteria, viruses, and parasites: inhibition of NETs release, degradation of NETs, and resistance to NETs killing (Figure 2). Perhaps the best example of this is GAS, which has evolved mechanisms to evade NETs using all three of these strategies (10, 14–17).

Inhibition of NETs

The induction of many neutrophil effector functions, including NETs, relies on the generation of ROS. Many pathogens inhibit oxidative pathways to evade immune function, and a handful of studies have

shown that this capability may also down-regulate NET induction. ROS inhibition may occur through interference with ERK phosphorylation upstream of NADPH oxidase (18), or by pathogen induction of the NET-suppressive cytokine, IL-10, to block TLR-induced ROS generation (15). Many pathogens are able to induce cellular IL-10, whereas some viruses encode their own IL-10 homologs (19). Although induction of this immunosuppressive cytokine likely benefits infecting pathogens in several ways, the importance of its ability to down-regulate NET induction has yet to be determined. It is likely, however, that attenuation of ROS generation will prove to be a broad NET-evasion strategy employed by a variety of pathogens.

Degradation of NETs

Studies investigating the production of NET-degrading factors by pathogens have focused largely, but not exclusively, on the nucleases produced by common gram-positive respiratory pathogens, including GAS, *Streptococcus pneumoniae*, and *S. aureus* (Table 1). Soon after its discovery, the evasion of NETs was suggested as a potential function of GAS nucleases (14), and, in the following year, two groups demonstrated that the degradation of NETs by Streptococcal nucleases resulted in increased bacterial dissemination and pathogenicity (10, 13). Of interest, it was later reported that *S. aureus* not only degrades NETs, but that the degraded products even support disease progression (20). Most of these studies used NETs induced either by PMA or the pathogen,

and to prevent confounding by phagocytosis, the actin-polymerization inhibitor, cytochalasin D, was often added to neutrophils before infection.

Recently, we found evidence of a host strategy to attenuate pathogen degradation of NETs: the antimicrobial peptide, LL-37 (cathelicidin), is constitutively expressed in neutrophils and present at high concentrations in NETs; however, it loses its microbicidal activity when bound to DNA. We found that purified neutrophil DNA and NETs released by PMA-treated neutrophils, when supplemented with LL-37, were resistant to degradation by *S. aureus*, *S. pneumoniae*, and GAS nucleases (21). These results were recapitulated using other cationic AMPs, with the cationicity of the agents correlating with the degree of resistance to degradation.

Resistance to NETs

In addition to the prevention of NET formation and the degradation of NETs, certain respiratory pathogens appear to resist NET trapping and killing by other mechanisms, including biofilm formation and electrochemical modification of the bacterial surface. Modification or masking of the negatively charged bacterial surface by bacterial enzymes or a capsule exploits the cationic nature of most NET components, preventing the electrostatic attraction thought to be responsible for bacterial adherence (17). Furthermore, certain bacterial virulence factors have been shown to confer resistance to the antimicrobial peptides present in NETs (22).

Respiratory Pathogens that Employ NET Evasion

GAS: NET Inhibition, Degradation, and Resistance

GAS employs multiple NET evasion mechanisms, including the ability to prevent NET formation, to digest preformed NETs, and to resist killing by microbicidal NET components. First, GAS inhibits NET formation by multiple mechanisms. The GAS pore-forming cytolysin streptolysin O blocks neutrophil ROS generation and, consequently, the generation of NETs (23). GAS also produces a peptide, S. pyogenes cell envelope protease, that cleaves IL-8, a potent neutrophil chemoattractant and activator, resulting in lower levels of neutrophil transmigration as well as reduced NET
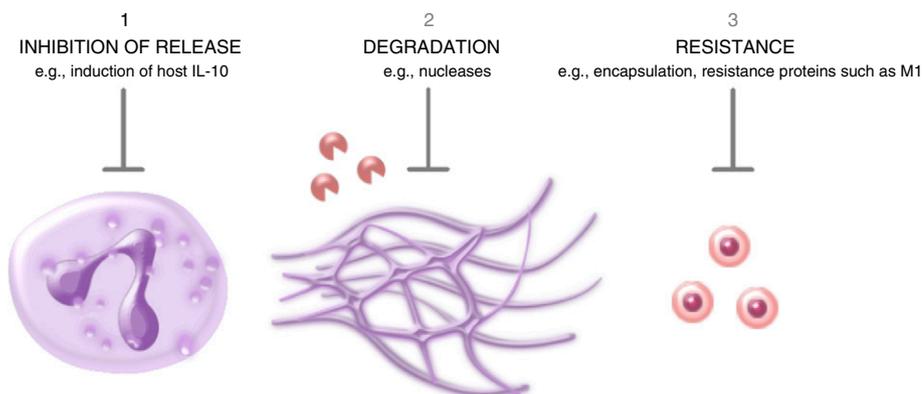

Figure 2. The three main NET evasion strategies. Certain respiratory pathogens have evolved the ability to evade the antimicrobial effects of NETs, using three main mechanisms: inhibition of the release of NETs; degradation of NETs by the production of pathogen nucleases; and resistance to the effects of the antimicrobial proteins that are enmeshed within NETs.

1
INHIBITION OF RELEASE
e.g., induction of host IL-10

2
DEGRADATION
e.g., nucleases

3
RESISTANCE
e.g., encapsulation, resistance proteins such as M1

Table 1. Properties of Neutrophil Extracellular Trap–Degrading Pathogen Nucleases

	Pathogen Species (Ref. No.)									
	Staphylococcus aureus (20, 28)	*Streptococcus sanguinis* (32)	*Streptococcus suis* (30, 31)	*Streptococcus pyogenes* (GAS) (10, 14)	*Streptococcus pneumoniae* (13)	*Streptococcus agalactiae* (GBS) (15, 8)	*Vibrio cholerae* (38)	*Neisseria gonorrheae* (39)	*Leishmania infantum* (40)	*Lutzomyia longipalpis* (41)
Nuclease	Nuc and AdsA	SWAN	SsnA and EndAsuis	Sda1/SdaD2	EndA	NucA	Dns and Xds	Nuc	3′ NT/NU	Lundep
Location	Secreted	Cell wall anchored	Cell wall–anchored and secreted (SsnA), membrane bound (EndAsuis)	Secreted	Membrane bound	Secreted	Extracellular	Secreted	Membrane anchored	Secreted
Pathogen induces NETs	Yes	Not known	Yes	NET induction inhibited by additional mechanism	Yes	NET induction inhibited by additional mechanism	Yes	Yes	Yes	N/A
Pathogen trapped/killed	Trapped and killed	Not killed	Trapped not killed	Not killed	Trapped not killed	Not killed	Trapped and killed	Killed	Trapped and killed	N/A
Other nuclease function	Biofilm dispersal; NET degradation products trigger macrophage apoptosis	Postulated to be able to bind integrins for a role in attachment	Potential role in nutrition (oligonucleotide production)	Prevents macrophage TLR9 activation; potential role in dissemination by liquefying pus	Necessary for DNA uptake in genetic transformation	Not known	Biofilm formation	Biofilm remodeling	Parasite nutrition (oligonucleotide production)	Lowering blood viscosity; facilitates sandfly feeding

Definition of abbreviations: AdsA, adenosine synthase A; Dns, deoxyribonuclease; EndA, endonuclease A; EndAsuis, endonuclease A suis; GAS, group A *Streptococcus*; GBS, group B *Streptococcus*; Lundep, *Lutzomyia* NET destroying protein; N/A, not applicable; NET, neutrophil extracellular trap; 3′NT/NU, 3′-nucleotidase/nuclease; Nuc, nuclease; Sda, streptodornase; SsnA, *Streptococcus suis*-secreted nuclease A; SWAN, Streptococcal wall anchored nuclease; TLR, Toll-like receptor.

induction (16). In addition, the GAS hyaluronic acid capsule binds to the inhibitory neutrophil receptor, Sialic acid binding Ig-like lectin 9 (Siglec 9), down-regulating both ROS generation and NET formation (24).

Many gram-positive bacteria express DNases that are associated with virulence (13, 14, 22); however, the pathogenic benefit of these enzymes was unknown for many decades. Sumby and colleagues (14) have shown in murine models that GAS organisms deficient in specific DNases are more susceptible to extracellular killing and clearance compared with wild type. Virulence in the wild type was largely attributed to the activity of Streptodornase 1, the principal extracellular DNase in the M1 serotype GAS strain, which is associated with necrotizing fasciitis (14). The authors of this study were the first to speculate that the DNase may be important in the avoidance of NETs, which has since been confirmed by several reports documenting NET degradation by DNases.

Concrete evidence supporting the ability of pathogens to escape from NETs was first documented in our study on GAS (10), and another paper published concurrently on *S. pneumoniae* (13). We found that wild-type GAS survived coincubation with NETs significantly better than an Sda1-deficient mutant, and that nonpathogenic *Lactococcus lactis* heterologously expressing Sda1 on a plasmid vector showed significantly greater survival (10). Confocal microscopy confirmed that this survival was associated with the ability of the pathogen to degrade NETs. Expression of the DNase also made GAS significantly more virulent in a mouse model of necrotizing fasciitis with increased skin lesions and bacterial load at the site of injection. Administration of GAS together with G-actin, a naturally occurring inhibitor of type I family DNases, decreased wild-type bacterial survival to a level comparable to the Sda1-deficient mutant, and substantially decreased the lesion size. Every strain of GAS appears to express at least one DNase (25). Our subsequent study found that the heightened resistance to NET killing *in vivo* in GAS expressing Sda1 may serve as a selective force to generate hypervirulent bacterial variants (26).

Resistance to NET-associated antimicrobial peptides has also been shown to play an important role in the pathogenesis of invasive GAS infection. In 2009, a novel role for the virulence protein M1 was discovered, involving both stimulation of, and bacterial survival within, NET structures (27). Targeted mutagenesis of the *emm1* gene coding for the M1 protein revealed an absence of

GAS-induced NET generation in the mutant and an increased susceptibility to NET killing. Resistance could be restored by either an M1-expressing plasmid or recombinant M1 protein, and expression of the M1 gene in the M49 serotype or in *L. lactis* increased resistance to NET-mediated killing. The ΔM1 mutant was found to be susceptible to killing by the antimicrobial peptide, LL-37, and further investigation using purified recombinant M1 fragments demonstrated this effect to be mediated by sequestration by the M1 N-terminal hypervariable domain. The M1 protein serotype is linked to severe GAS disease, and clinical analysis of a large panel of GAS clinical isolates found all M1 isolates to be cathelicidin resistant, and demonstrated an association between cathelicidin resistance and invasive GAS disease (27).

S. aureus: NET Degradation

In 2012, the Rooijakkers group was the first to show that an *S. aureus* nuclease confers resistance to extracellular killing by neutrophils, and leads to enhanced disease *in vivo* (28). After this, an elegant study by Thammavongsa and colleagues (20) demonstrated that *S. aureus* takes evasion a step further by degrading NETs into a product toxic to macrophages. Lesions containing replicating bacteria surrounded by fibrin form the abscesses commonly found during *S. aureus* infection. Mice were infected with variant *S. aureus* with an assortment of silencing inserts in multiple known virulent genes. Whereas neutrophils infiltrated the abscess and formed NETs, macrophages were shown to be typically excluded. However, mice infected with a strain deficient in nuclease (*nuc*), a nuclease important in *S. aureus* biofilm dispersal (28), or adenosine synthase A (*AdsA*) showed increased macrophage penetration into the abscess. The authors found that trypan blue staining of macrophages, which indicates loss of viability, increased significantly in response to a product generated from exposing PMA-induced NETs to *S. aureus*. This response was attenuated when using the *nuc* or the *adsA* mutant, which also generated reduced 2′-deoxyadenosine (dAdo), a deoxyribonucleoside base. Treatment of NETs with purified versions of both *nuc* and *AdsA* was necessary to produce dAdo, and purified dAdo also induced caspase-3 activation and apoptosis in macrophages. Taken together, these data show that the *S. aureus* products, *nuc* and *AdsA*, are capable

of degrading NETs and producing a toxin, dAdo, which kills macrophages, preventing their infiltration into abscesses and thereby reducing their antimicrobial functions.

S. pneumoniae: NET Degradation and Resistance

Beiter and colleagues (13) were the first to demonstrate *in vitro* that *S. pneumoniae* is trapped rather than killed by NETs. The authors found that the pneumococcal strain, TIGR4 (serotype 4), was able to degrade and escape PMA-induced NET capture, whereas a mutant strain deficient in the membrane-bound nuclease *endA* was captured by intact NETs. Intranasal infection with wild-type and the *endA* mutant organisms induced neutrophil recruitment, NET release, and the induction of pneumonia in murine lungs, whereas mice infected with the *endA* mutant showed improved survival. After simultaneous infection with wild-type and mutant bacteria, equal numbers of the two strains were recovered from the upper respiratory tract; however, considerably more wild-type organisms were recovered in the lungs and bloodstream, demonstrating the importance of *endA* for bacterial invasion. Using the presence of NE in cell supernatants as a reporter of NET degradation, the authors also showed that six of seven pneumococcal strains were able to degrade NETs. These early observations of NET evasion by a pathogen, and its association with virulence, stressed the importance of NET formation as an antipathogenic mechanism, and highlight the therapeutic potential of agents that preserve NET integrity.

More recently, the pneumococcal bacterial capsule has also been shown to confer resistance to NET trapping and killing (17). Encapsulated *S. pneumoniae* strains show significantly reduced trapping by NETs compared with nonencapsulated strains, and the addition of a capsule was thought to mask the negatively charged bacterial surface, preventing binding to the mostly cationic antimicrobial peptides enmeshed in NETs. In addition, *S. pneumoniae* has developed the ability to modify its surface charge through the action of a set of enzymes, which incorporate D-alanine into surface lipoteichoic acids (LTAs); these are tethered to the bacterial membrane, and result in a positive charge, which further repels cationic peptides (17). Wartha and colleagues (17) showed that the encapsulation and D-alanylation of LTA is associated with resistance to NET-mediated killing *in vitro*,

and deletion of the *dlt* operon encoding the enzymes responsible for D-alanylation of LTA increases bacterial sensitivity to NET-mediated killing in nonencapsulated strains. The group went on to show, in a murine model of pneumococcal pneumonia, that mutant strains lacking functional *dltA* produce a ower bacterial burden *in vivo* than wild-type organisms.

Group B *Streptococcus*: NET Degradation and Inhibition

Another streptococcal nuclease, *nucA*, was found to be secreted by group B *Streptococcus* (GBS) and demonstrated to be capable of degrading NETs (29). As in the previous examples, the nuclease-deficient mutant was associated with higher bacterial clearance and lower mortality. The up-regulation of the NET-suppressing cytokine, IL-10, and subsequent attenuation of the respiratory burst was also demonstrated to be employed as a NET inhibition strategy by GBS (15). Recently, we found that the sialylated capsular polysaccharide of GBS acts as a mimic "self"-ligand to neutrophil Siglec-9, down-regulating effector responses (15). Blocking of Siglec-9 caused dampening of GBS-induced IL-10 up-regulation, resulting in a stronger oxidative burst, increased NET release and increased extracellular killing.

Other Streptococci: NET Degradation

Streptococcus suis, which induces and is trapped by porcine NETs, expresses *Streptococcus suis*-secreted nuclease A, a cell wall–anchored DNase that is partially released into the supernatant (30). In 2014, de Buhr and colleagues (30) demonstrated degradation of PMA-induced NETs by *S. suis*, but not by a mutant that lacked SsnA function, and that wild-type *S. suis* showed significantly higher viability after incubation with PMA and cytochalasin-treated neutrophils. A second *S. suis* nuclease homologous to pneumococcal *endonuclease A (EndA)*, designated *endonuclease A suis (EndAsuis)*, was described the following year (31), accounting for residual NET degradation seen in *ΔSsnA* mutants during the exponential growth phase. However, an *EndAsuis* mutant did not show increased susceptibility to NET-mediated killing, suggesting that *EndAsuis* was not as important as SsnA

for protection against NETs, and an alternative role was suggested for the second nuclease, potentially to degrade pathogen DNA, thus avoiding TLR9 stimulation.

Morita and colleagues (32) discovered in *Streptococcus sanguinis* (an organism that colonizes the surfaces of teeth), a cation-dependent nuclease with a cell wall sorting signal, which they termed *Streptococcal wall anchored nuclease (SWAN)*. A SWAN deletion mutant showed a 20% reduction in survival compared with wild type after incubation with PMA and cytochalasin D–treated neutrophils, suggesting a modest contribution to evasion from NETs. Furthermore, expression of *SWAN* in *L. lactis* significantly increases bacterial survival.

Pseudomonas aeruginosa: NET Inhibition and Resistance

P. aeruginosa, a significant pathogen in cystic fibrosis (CF), has evolved multiple mechanisms to attenuate NET production and resist NET-mediated killing. The pathogen has been shown to express surface sialic acids that are capable of binding and inducing signaling through neutrophil, Siglec-9, which suppresses the oxidative burst, and thus NET formation (33). The authors in this study showed that treatment with sialidase or use of *P. aeruginosa* strains lacking in sialic acids led to increased NET production compared with sialic acid–positive strains. Furthermore, paired *P. aeruginosa* strains isolated from patients with CF at early and late stages of disease showed that resistance to NET-mediated killing evolved over time, and correlated with the development of the mucoid phenotype characterized by excess alginate production (34). However, a mutant *P. aeruginosa* strain that overexpressed alginate did not show increased survival on incubation with PMA-treated neutrophils, suggesting that the acquired resistance of *P. aeruginosa* to NET-mediated killing in the CF airway was due to an as-yet unknown mechanism (34).

Haemophilus influenzae: NET Resistance

The respiratory pathogen, *H. influenzae*, evades NET killing through both the production of antioxidants and the formation of biofilms—surface-adherent bacterial communities that resist clearance by immune

cells and antimicrobials through encasement in a polymer matrix (22). Nontypeable *H. influenzae* (NT*Hi*), a leading cause of chronic otitis media, has been demonstrated to form a biofilm within the middle ear during infection, a process promoted by the production of lipo-oligosaccharides, which is thought to promote bacterial persistence. Biofilm presence was shown to correlate with bacterial load in an experimental model of otitis media (22), with microscopy revealing the presence of NT*Hi* communities with biofilm phenotypes within a DNA lattice containing neutrophils, elastase, and histones, consistent with the appearance of NETs. The bacteria were found to be resistant to phagocytosis and extracellular neutrophil killing *in vitro*, which was lipo-oligosaccharide dependent.

NT*Hi* also produces the antioxidants, peroxiredoxin–glutaredoxin and catalase, two molecules that confer resistance to hydrogen peroxide *in vitro*. Mutant strains with a deletion of the two genes encoding these factors, *hktE* and *pdgX*, displayed reduced survival in the presence of hydrogen peroxide, increased susceptibility to NET-mediated killing *in vitro*, and attenuated persistence in the middle ear (35). Susceptibility to NET-mediated killing was partially rescued by the addition of exogenous catalase, and is thought to be due to the antioxidants providing resistance against the microbicidal activity of ROS within NETs. The possibility that the antioxidants may, in fact, prevent NET induction by neutralization of ROS, which are required for NET release, is an intriguing one, although there is, as yet, no evidence supporting this in *H. influenzae* or other catalase-expressing bacteria.

Bordetella pertussis and *Cryptococcus neoformans*: NET Inhibition

B. pertussis (18) and the fungus, *C. neoformans* (36), both inhibit NET formation by the suppression of ROS generation. Wild-type strains do not induce NETs, but mutants of these two pathogens lacking ROS- and NET-suppressing agents can certainly induce NET release. NETs are inhibited by *C. neoformans* via its capsular polysaccharide glucuronoxylomannan, and in *B. pertussis* through generation of adenylyl cyclase toxin (ACT). ACT generates high levels of cyclic AMP, which

has a wide range of effects, including inhibition of ERK phosphorylation, thus suppressing ROS generation and NET formation. Intriguingly, convalescent-phase sera from patients with pertussis are able to reverse ACT-mediated inhibition of PMA-induced ROS and NETs (18).

Burkholderia pseudomallei: NET Inhibition

Evasion of NET killing has also been demonstrated by the gram-negative bacterium, *B. pseudomallei*, the causative agent of mellioidosis, an infection endemic to Southeast Asia, with multiple manifestations, including pneumonia. Neutrophils play a crucial role in controlling infection in murine models of the disease, and investigation of the response of human neutrophils to *B. pseudomallei* showed that the pathogen triggers the induction of NETs, which exert microbicidal activity (37).

Riyapa and colleagues (37) also showed that the bacteria modify the magnitude of NET formation through the action of two known virulence factors, the bacterial type III protein secretion system encoded by the *bsa* locus, including the components *bsaZ* and *bsaQ*, and capsular polysaccharide 1 encoded by the *wcb* operon. Mutant strains deficient in either of these components induce higher levels of NET production, which result in increased bacterial killing by NETs compared with wild-type bacteria (37). Increased NET production was mirrored by higher levels of phagocytosis of the mutants with an elevated oxidative burst, implying that the type III protein secretion system and capsular polysaccharide 1 attenuate NET release through inhibition of the NADPH oxidase pathway. These mechanisms are postulated to be responsible for the attenuated replication and plaque formation seen with *bsaZ* and *basQ* mutants *in vitro* and the reduced virulence of *wcb* mutants in murine models of disease.

Other Pathogens: NET Degradation

The degradation of NETs has also been suggested as a function of nucleases in gram-negative bacteria. Seper and colleagues (38) found that a *Vibrio cholerae* strain deficient for two extracellular nucleases, deoxyribonuclease and *xds*, was less

invasive than wild-type *V. cholerae* in immunocompetent mice, although no difference between strains was seen in neutropenic mice, and quantitative RT-PCR revealed that the presence of either NETs or DNA was able to up-regulate the expression of deoxyribonuclease and *xds* (38). *Neisseria gonorrheae* has also been shown to encode the heat-stable nuclease, *nuc*, thought to be important for remodeling *N. gonorrheae* biofilms (39). NETs were shown to be rapidly induced by *N. gonorrheae*, but thereafter NET integrity decreased over time in *nuc*-containing strains compared with a Δ*nuc* mutant, as measured by Sytox Green immunofluorescence. The presence of Nuc enhanced survival of the pathogen on exposure to PMA-stimulated neutrophils, implicating *nuc* as a virulence factor important for host immune evasion.

In 2014, two studies documented NETs degradation in biological kingdoms other than bacteria. The parasite *Leishmania infantum* was shown to express high levels of 3′-nucleotidase/nuclease when cultured in low-phosphate media, a phenotype that showed higher survival in NETs (40). A highly active nuclease is also present in the saliva of the parasite's sandfly carrier, *Lutzomyia longipalpis* (41), and a recombinant form of the nuclease, *Lutzomyia* NET destroying protein, was shown to degrade NETs and increase parasite survival when coincubated with neutrophils. Coinjection of the recombinant nuclease with *Leishmania* in mice increased lesion size and parasite load, suggesting that the nuclease may aid promastigote evasion of NETs as well as lower blood viscosity to facilitate sandfly feeding.

Conclusions

Broadly, there exist three main strategies by which respiratory pathogens counteract NETs: inhibition of NETs release, degradation of NETs by pathogen- or vector-encoded nucleases, and resistance to NET-mediated killing, for example, by production of inhibitors of antimicrobial peptides. These effects likely overlap significantly with other functions (40).

The prevalence of NET evasion mechanisms within respiratory pathogens supports the view that NETs are an important antipathogenic mechanism. The relevance of NET evasion by pathogens is further supported by evidence that the

body also appears to "fight back"—for example, high concentrations of the antimicrobial peptide, LL-37, within NETs increases resistance to the action of DNases (21), and the presence of anti-ACT in the sera of patients infected with *B. pertussis* indicates the participation of humoral responses in combating the specific pathways by which pathogens modify NETs.

This body of knowledge must, however, be interpreted acknowledging the limitations of many commonly used NETs assays. For example, although the use of PMA is convenient to assess the effect of a compound on already-released NETs, the physiological relevance of this chemical factor has been repeatedly questioned (42). There are stark differences between PMA-induced NETs and those induced by more physiological agonists; for example, PMA induces all neutrophils to release DNA, whereas only 25% of neutrophils do so with *S. aureus*. Furthermore, cytochalasin D is often used to inhibit phagocytosis to separate traditional neutrophil killing mechanisms from NET-associated effects; however, phagocytosis is integral to so many neutrophil functions that its inhibition may confound. Hence, Branzk and colleagues (43) show that phagocytosis via dectin-1 can inhibit the release of NETs as part of a neutrophil size–sensing mechanism that determines their antipathogenic response. Therefore, NET-mediated killing that is demonstrated in the presence of cytochalasin D could be misleading, if NET induction would have been inhibited by phagocytosis in its absence. These inherent caveats pertain to many NET studies, and can only be mitigated with the use of multiple complimentary techniques and the inclusion of physiological NET stimulants.

Future work is likely to involve translation of the advances in our understanding of pathogen evasion of NETs into pharmacological therapies. Development of a rapid technique to establish whether a pathogen is NET resistant, or a therapy counteracting common mechanisms of NET evasion by pathogens, could complement antimicrobial approaches. All three pathogen-evasion strategies may be targetable. Therapies aimed at neutralizing bacterial pathways that down-regulate NET induction may hold promise, including targeting the GAS IL-8 protease SpyCep or neutrophil Siglec-9, which is engaged by multiple pathogens to inhibit NETs induction (15). Pathogen-encoded nucleases are another attractive

target. G-actin, a natural pharmacologic inhibitor of type I DNases, has been shown to prevent Sda1-mediated degradation of NETs (10) and certain patients with systemic lupus erythematosus produce specific DNase1 inhibitors that act against endogenous serum DNase1 (44). Other therapeutic opportunities involve protecting NETs from degradation—high concentrations of LL-37 increase DNA resistance to pathogen DNases (21), and NETs are protected from degradation in systemic lupus erythematosus due to the deposition of autoantibodies, which are thought to shield them from serum DNase1 (44). Furthermore, targetable bacterial regulators of nuclease expression have been described, including period circadian protein homolog 1, which regulates GAS Sda1 expression (45), and the mgrA and Staphylococcal accessory regulator Z transcriptional regulators, which up-regulate *S. aureus nuc* expression (46). These are part of a wide family of bacterial peroxide-sensing transcriptional regulators, making this a promising area of research. Bacterial mechanisms of resistance to NETs may also be targeted, including modification of the protective capsule or inhibiting resistance genes, such as the GAS M1 protein.

Lastly, the above studies build a framework that may be used for assessing the many other bacterial species that contain nucleases, such as species of the bacterium *Mycoplasma*, which all contain surface nucleases. Investigation of their effects on NETs may reveal the evolutionary significance of such genes in evasion of the host innate immune response.

It is also important to recognize that certain immunosuppressive agents, such as the calcineurin inhibitors, have been shown to impair NET formation (47), which could further enhance susceptibility to opportunistic infections, whereas other drugs, such as statins and tamoxifen, appear to increase NET production and promote bacterial clearance both *in vitro* and *in vivo* (48). However, despite the positive role that NETs play in mediating infections, NET components have been shown to cause inflammation and tissue damage, and aberrant or excessive NETosis might play a role in inflammatory processes, including acute lung injury and venous thrombosis development (47). Excess NET formation damages the epithelium and leads to lung tissue damage, and has been reported in the lungs of patients with a wide range of pulmonary conditions that are associated with neutrophil infiltrates, including neutrophilic asthma, chronic obstructive pulmonary disease, CF, respiratory syncytial virus bronchiolitis, influenza, and tuberculosis (49). It is thought that NET-induced injury can be particularly severe in the lungs, due to the ability of NETs to expand easily in the alveolar space and to directly induce death in endothelial and epithelial cells by the action of histones. Excessive NET formation can also facilitate blood coagulation, thus disturbing the alveolar microcirculation (49). Furthermore, extracellular DNA has been recognized as a critical component of biofilms with the presence of neutrophil actin and DNA enhancing their formation (50), and NETs as a potential source for the formation of harmful biofilms is deserving of future research. Hence, extreme care will be needed in designing agents that modulate NET biology.

In conclusion, pathogens have evolved a range of mechanisms to evade NETs that can be categorized into three main categories: NET inhibition; NET degradation; and NET resistance. NET formation is now recognized as an important host mechanism to fight respiratory infections, and future research will involve identifying additional mechanisms to resist NETs as well as options to develop therapeutic interventions to counteract evasion. ■

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