Review

Staphylococcus aureus induces neutrophil extracellular traps (NETs) and neutralizes their bactericidal potential

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ABSTRACT

Neutrophil extracellular traps (NETs) are considered part of the innate human immune system because they are involved in host defense during bacterial infections. NETs are formed by activated neutrophils and consist of a DNA backbone combined with proteins with different biological functions. The activity of NETs can be significantly reduced by a *Staphylococcus aureus* DNase, which degrades the DNA backbone and enables the liberation of bacteria from NETs, and by Eap, a secreted protein which binds and aggregates linearized DNA, suppressing the formation of NETs. Furthermore, the pathogen can resist NET-mediated killing by expressing the surface protein FnBPB, which neutralizes the bactericidal activity of histones. Finally, the anti-staphylococcal activity of NETs is counteracted and blocked by *S. aureus* biofilm. Staphylococcal cells and several virulence factors such as protein A and phenol-soluble modulins can also elicit the formation of NETs which in turn can cause tissue injury, enhancing bacterial performance in host colonization. The identification of additional virulence factors involved in NET formation/neutralization could provide the basis for therapeutic interventions against this formidable pathogen.

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1. Introduction

The main mechanisms used by neutrophils to clear pathogens include a) phagocytosis, b) release of antimicrobial peptides, c) production of antimicrobial reactive oxygen and nitrogen species, and d) production of neutrophil extracellular traps (NETs) [1]. NETs are fragile structures composed of a branching network of extracellular DNA filaments decorated with cytoskeletal proteins and proteases mainly involved in host defense during bacterial, fungal and viral infections [2]. NETs not only contribute to pathogen elimination but in parallel can cause damage to bystander cells. In sepsis, a systemic acute infective disease with high morbidity and mortality, NETs may promote patient survival [3], although as the disease progresses, they may accumulate in organs and cause detrimental tissue damage [4,5]. Platelets and NETs cooperate to promote intravascular coagulation during sepsis in mice [6] and platelet-neutrophil interactions induce the release of intravascular NETs that in turn ensnare bacteria from the bloodstream and cause liver damage [7]. Moreover, as revealed by intravital imaging, during a bloodstream infection with methicillin-resistant *S. aureus*, neutrophils infiltrate the liver and release NETs into the vasculature. The NETs remain anchored to the vascular wall *via* von Willebrand factor and produce profound hepatic injury [8] (Fig. 1).

Importantly, NET formation can also occur in noninfectious chronic inflammation such as autoimmunity (for example, systemic lupus erythematosus and rheumatoid arthritis) [9], vasculitis [10,11], cancer [12,13], and atherosclerosis [14,15]. Moreover, NETs play an important role in thrombosis [16] and induce platelet activation, coagulation, and thrombus formation [17,18] (Fig. 1). Hence, due to the combination of various proteases, cytotoxic proteins, and enzymes bound to the NETs, the NETs can be a double-edged sword.

In the face of this potentially harmful arsenal of weapons associated with NETs, microorganisms are endowed with a variety of virulence factors that effectively contrast the action of these host structures.

Staphylococcus aureus is the etiological agent of diseases ranging from mild infections to severe diseases such as sepsis, pneumonia, endocarditis, and medical device-associated infections [19]. Among the variety of virulence factors to combat host defenses [20,21], *S. aureus* has evolved pathogenetic activities to escape NETs or induce NET formation to facilitate bacterial performance in the colonized host (Fig. 2). In this mini-review, we discuss the mechanisms of NET induction and formation by *S. aureus*, as well as the *S. aureus* virulence factors and the role they may have in counteracting the bactericidal activities of NETs.

2. NET formation

NET formation or NETosis can occur by two mechanisms:

2.1. Lytic or suicidal mechanism

Stimulation of neutrophils with phorbol myristate acetate (PMA), autoantibodies, and cholesterol crystals results in the activation of NADPH oxidase, *via* PKC and RAF-MEK-ERK
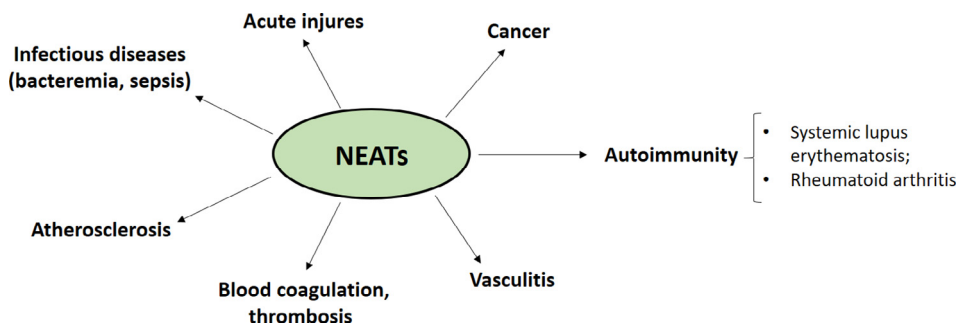

Fig. 1. Schematic representation of NET involvement in infectious and noninfectious diseases. NET formation induced by staphylococcal bacteremia and sepsis can cause tissue damage and enhance bacterial permeation of deeper structures in the body. NETosis also has a role in noninfectious disease such as autoimmunity (systemic lupus erythematosus and rheumatoid arthritis), atherosclerosis, vasculitis, and cancer.

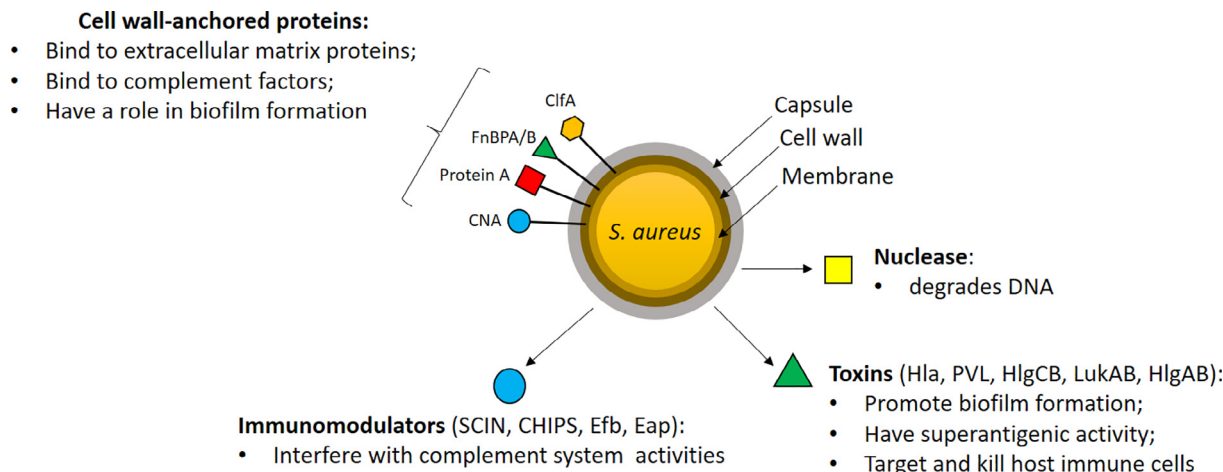**Fig. 2.** Representation of the *S. aureus* surface and secreted virulence factors. *S. aureus* is endowed with a number of surface-expressed and secreted virulence factors. The most known surface proteins are the cell wall-anchored proteins that promote adhesion to extracellular matrix proteins and are involved in biofilm formation. Secreted virulence factors include toxins, autolysins, and modulators of the complement pathways.
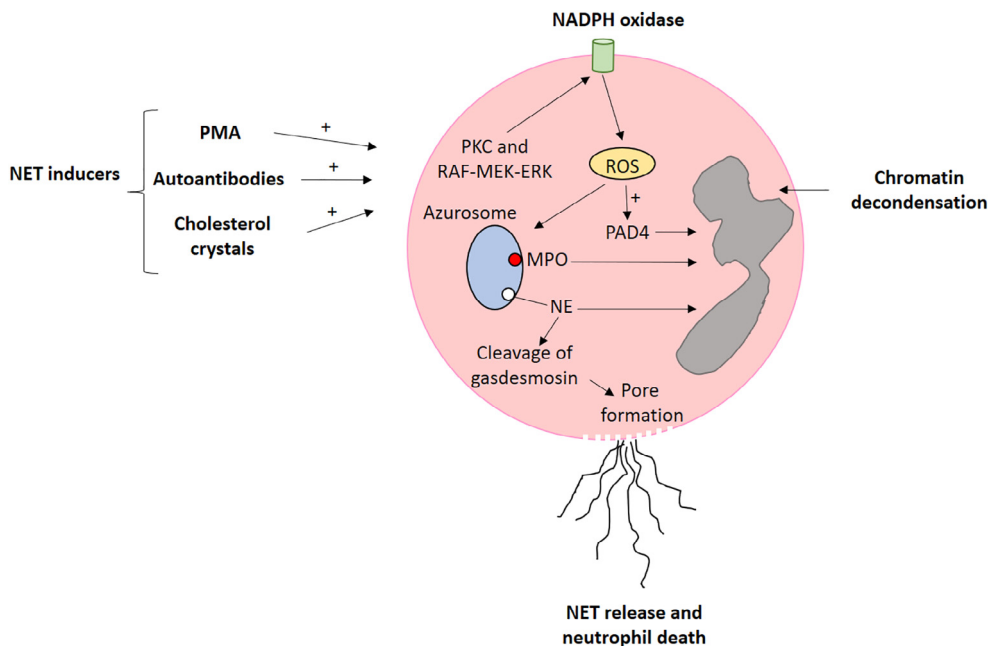
Fig. 3. Lytic NET formation. Different stimuli activate neutrophils *via* the activation of NADPH oxidase and induce consequent ROS formation. Then, PAD4 is activated and citrullinates histones in the nucleus, causing chromatin decondensation. At same time, myeloperoxidase and elastase translocate from cytosol to the nucleus where they contribute to further unfolding of chromatin. Elastase also activates gasdermin D, which forms pores in the nuclear and plasma membranes. Consequently, chromatin is released in the cytosol and mixed with cytosolic proteins forming NETs. After the secretion of NETs, neutrophils die.

signaling pathways and the consequent generation of reactive oxygen species (ROS). This activates peptidyl arginine deiminase (PAD4), which induces the conversion of arginine to citrulline (citrullination) in histones. Through citrullination, PAD4 modulates the conversion of positively charged arginine side chains into uncharged side chains of histones causing chromatin decondensation. The hydrogen peroxide is in turn consumed by MPO to produce hypochlorous acid and other oxidants and the generation of oxidants liberates neutrophil elastase from azurophilic granules, allowing it to translocate to the nucleus where it promotes the further unfolding of chromatin and nuclear membrane disruption. After nuclear membrane disintegration, chromatin is released into the cytosol where it associates with granular and cytosolic proteins [22,23]. NE also cleaves gasdermin D (GSDMD) in the cytosol to its active form (GSDMD-NT), which forms pores in the plasma membrane and granular membranes. Finally, NETs are released into the extracellular space and neutrophils die [24] (Fig. 3).

2.2. Nonlytic or vital NET release mechanism

Nonlytic NET formation is a rapid process induced by the recognition of stimuli through complement receptors and does not depend on NADPH activation. Vital NET formation is induced by *S. aureus* through both complement receptors and TLR-2 ligands, or by *Escherichia coli* directly *via* TLR-4 or indirectly *via* TLR-4 activated platelets. As reported for lytic NET formation, during nonlytic NET formation PAD4 and elastase are activated and translocate to the nucleus where they promote chromatin decondensation. The unfolded chromatin is released into the cytosol, becomes decorated with cytosolic proteins and is finally expelled *via* vesicular export into the extracellular environment [25]. Importantly, after the release of NETs, this pathway preserves the integrity of neutrophils' plasma membranes and the anucleated neutrophils named cytoplasts remain alive and retain the capacity to migrate and phagocytose [26,27] (Fig. 4).

3. NET composition, structure, and bactericidal function

The composition of NETs is critical for their pathological impact. Due to its structure and charge, DNA represents the structural unit around which the other components of NETs are assembled. The DNA backbone in NETs is coated with at least 20–30 different proteins including nuclear proteins (histones), granule proteins (NE (neutrophyl elastase), MPO (myeloperoxidase), lactoferrin, cathepsin G, proteinase-3) and cytosolic proteins (S100 calcium-binding proteins A8, A9, A12, as well as actin, α-actinin and calprotectin), which are attached to DNA by electrostatic forces [28–30].

The mechanisms by which NETs contrast and eventually kill microbial invaders remain controversial. Menegazzi *et al* showed that when captured by NETs, microorganisms such as *S. aureus* and *Candida albicans* are trapped but not killed by these structures [31]. On the other hand, several reports state that NETs efficiently kill bacteria. According to Halverson *et al*, DNA possesses a rapid bactericidal activity due to its ability to sequester surface bound cations, disrupt membrane integrity and lyse bacterial cells [32]. The cationic antimicrobial peptides such as cathelicidin LL-37 can protect neutrophil-derived DNA from bacterial nuclease degradation [33]. The antimicrobial function of NETs has also been attributed to NET-bound proteins including histones [34,35], the zinc-chelating protein calprotectin [30], and the granular serine protease cathepsin G [36]. Moreover, MPO associated with NETs exhibits a bactericidal activity needed to kill the pathogen in the presence of hydrogen peroxide [37]. The molecular details by which the different components of NETs interfere with the viability of the microorganisms remain to be elucidated.

4. *S. aureus* and its arsenal of virulence factors

The explanation for the high pathogenetic potential of *S. aureus* lies in the ability of the bacterium to express a large number of virulence factors and the capacity to colonize and infect host tissues
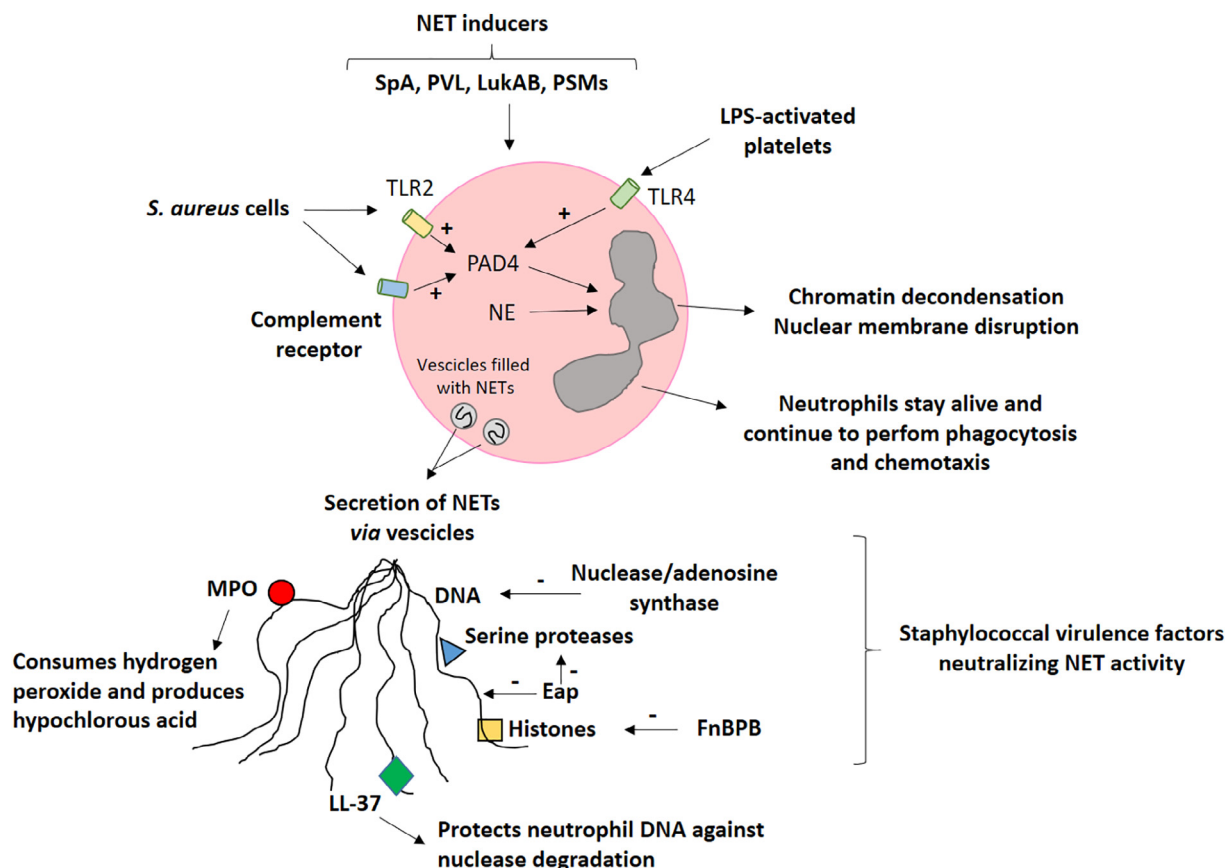
Fig. 4. Nonlytic NET formation promoted by *S. aureus* and *S. aureus* virulence factors. *S. aureus* cells activate complement receptor or TLR2 on neutrophils. Afterwards, PAD4 is activated and along with elastase translocates to the nucleus where they induce chromatin decondensation. Chromatin decorated with cytosolic proteins is expelled via vesicles without plasma membrane disruption. Loss of the nucleus does not compromise processes such as phagocytosis, chemotaxis and release of cytotoxic molecules. Paradoxically, *S. aureus* virulence factors such as nuclease, Eap, and FnBPB can interact with specific components of NETs and block their anti-bacterial activity.

and organs. The virulence factors are mostly surface-associated or secreted proteinaceous products. A significant number of cell wall-anchored (CWA) proteins act as receptors for extracellular matrix components (fibronectin, fibrinogen, collagen) and play additional roles in biofilm formation [38]. Biofilms are multicellular microbial communities formed on either biological or inorganic surfaces that are encased within a self-produced matrix. Fibronectin-binding proteins FnBPA and FnBPB and clumping factor A (ClfA) are the most relevant adhesins that bind to fibrinogen. Furthermore, as a result of their fibronectin-binding activity, FnBPA and FnBPB mediate *S. aureus* colonization of fibronectin rich-tissues and fibronectin-mediated host cell invasion. Protein A (SpA), another important CWA protein, binds to the Fc domain of IgG in an incorrect orientation which results in the protection of staphylococci from opsonophagocytosis and killing. SpA also binds to the Fab region of surface IgM located on B lymphocytes, triggering the proliferation and apoptotic collapse of adaptive immune responses [39–41] (Fig. 2).

S. aureus expresses more than 60 surface-exposed lipoproteins (Lpp), which are involved in a number of metabolic processes such as nutrient uptake (ion, sugar, and amino acids and oligopeptide transporters), enzymes and foldases [42].

S. aureus has also evolved a series of secreted proteins/peptides that functionally interfere with complement C3 and C5 convertase activities and reduce the chemotactic activity of neutrophils. For example, the extracellular adherence protein Eap specifically inhibits both the classical and lectin pathways, disrupting the formation of C4bC2 proconvertase. This results in the inhibition of C3b formation and consequent reduction of *S. aureus* phagocytosis

and killing by neutrophils. Moreover, *S. aureus* secretes a peptide named CHIPS (Chemotaxis Inhibitory Protein of *S. aureus*) that binds to formyl peptides and C5a receptors and this reduces neutrophil activation and migration to the site of infection [21]. To further contribute to pathogenesis, *S. aureus* secretes a vast group of cytotoxins, among them Hla, leukocidins and phenol-soluble modulins (PSMs). After binding to the host receptor ADAM-10, the Hla monomer oligomerizes to form heptameric pores in host cell membranes, causing the lysis and death of many cell types. Leukocidins are composed of two monomers, termed S- and F-subunits. The S-subunit recognizes a specific receptor on the plasma membrane and then dimerizes with the F-subunit. This is followed by oligomerization of three additional dimers to form a complete octameric pore in the plasma membrane of leukocytes. PSMs are cytotoxins belonging to a family of amphipathic peptides (20–25 amino acid residues) that have a variety of roles in *S. aureus* pathogenesis such as cell lysis, immune modulation and biofilm formation [43] (Fig. 2).

5. *S. aureus* induces NET formation by specific, secreted virulence factors

Nonlytic NET formation is caused primarily by intact cells of *S. aureus* (Fig. 2). NET formation is also promoted by released staphylococcal products. PVL (Panton-Valentine Leukocidin), but not the structurally similar leukotoxin gamma hemolysin CB (HlgCB), was reported as the dominant inducer of lytic NET formation [25,44]. There have been recent insights concerning the

mechanism of PVL-promoted NET formation [25]. The mechanism starts with PVL binding to its specific receptor, which in turn leads to its endocytosis by neutrophils. In parallel, cytosolic intracellular Ca²⁺ is rapidly mobilized from endoplasmic reticulum and a direct interaction of PVL with mitochondrial membranes occurs. The increase of intracellular calcium triggers the activation of small conductance potassium channels, SK and PVL binding to neutrophil mitochondria induces an increase of reactive oxygen species (ROS) and triggers the enzymatic activity of MPO. Finally, PVL promotes PAD4 activation and the consequent formation of citrullinated histone 3.

Importantly, PVL is also a potent cytotoxic factor and can induce rapid death in human and rabbit neutrophils [44]. We can hypothesize that the differential effects of the PVL action in the studies reported above (cell lysis and NETosis in one case and cell death in the second) may be explained, in part, by the experimental conditions used. Specifically, under a critical threshold of PVL concentration, NET formation and release might favorably prepare the host to resist infection.

Aside from PVL, other staphylococcal molecules contribute to NET formation. For example, leukotoxin LukGH (also named LukAB) promotes the release of NETs which, in turn, ensnare but do not kill *S. aureus* cells. It has been proposed that the ability of LukAB to promote the formation of NETs contributes to the inflammatory response and host defense against *S. aureus* infection [45]. Notably, LukAB released by staphylococcal biofilm contributes to the neutralization of NETs (see below) [46].

Moreover, phenol-soluble modulin α (PSMα) induces rapid formation of NETs through a ROS-independent pathway [47]. Finally, staphylococcal SpA has been shown to be involved in NET formation [48]. NET formation by *S. aureus* is facilitated by the cathelicidin LL37 released by epithelial cells and phagocytes upon infection [49] (Fig. 4).

It must be kept in mind that the biological activity of these staphylococcal virulence factors has been determined *in vitro* and in environmental conditions of pH and ionic strength that may differ from *in vivo* operational conditions. Moreover, it is not known whether the expression of each of the above factors is expressed in bloodstream and host tissues in sufficient amounts to induce NET formation. Therefore, to validate the supposed role of these NET inducers, more studies with animal models and the use of specific deletion mutants will be needed.

6. NET neutralization by *S. Aureus*

6.1. Degradation of NETs by staphylococcal nuclease

Several Gram-positive bacteria including *S. aureus* express 5′-nucleotidases (5′-NTs), enzymes that catalyze the hydrolysis of nucleoside monophosphates to produce nucleosides and phosphate [50]. In a study performed to examine the potential role of *S. aureus* nuclease in NET degradation and virulence in a murine respiratory tract infection model, Berends *et al* showed that an isogenic deficient-nuclease mutant lacked the ability to degrade NETs compared with the parental strain and consequently appeared to be more susceptible to extracellular killing by activated neutrophils. On the contrary, nuclease expression by *S. aureus* enhanced the escape of bacteria from NETs in an *in vivo* mouse model of *S. aureus* respiratory tract injection [51]. In a more detailed study Thammavongsa and colleagues found that *S. aureus* escapes host defenses by converting DNA in NETs to deoxyadenosine (dAdo) through the concerted action of two enzymes, nuclease and adenosine synthase (AdsA). dAdo in turn kills macrophages, preventing their infiltration into *S. aureus*-induced abscesses and thereby reducing their antimicrobial action [52]. Moreover, data

produced by Herzog *et al* demonstrates that high nuclease activity of *S. aureus* isolates correlates with long-term persistence and survival within the airways of cystic fibrosis patients due to the protection against NET-mediated killing [53] (Fig. 4). In summary, *S. aureus* nuclease exhibits a critical role in NET degradation *in vivo*. However, due to the presence of a number of DNase inhibitors in serum (for example, C1q of the complement system), it remains to be determined whether *S. aureus* nuclease keeps its activity intact in body fluids.

6.2. Eap protein and its effect on the bactericidal activity of NETs

The Extracellular Adherence Protein Eap blocks NETs formation and activities. By using atomic force microscopy, evidence has been provided that Eap can bind and aggregate linearized DNA. Consistent with this, Eap interferes with the formation of NETs, suggesting that it may protect bacteria from being trapped by structures such as microthrombi (Fig. 1) [54]. Notably, Eap and its homologues Ehp1 and Ehp2 potently inhibit the neutrophil serine proteases (NSPs) elastase (NE), proteinase 3, and cathepsin G [55]. Thus, Eap proteins could potentially block the enzymatic activities of NET proteases. However, considering that the NET-bound NSP could be inactivated by high concentrations of NSP inhibitors such as alpha1-proteinase inhibitor in serum [56], it is unclear whether the serine-protease inhibitory activity of Eap proteins plays an effective role in blocking the anti-bacterial activity of NETs (Fig. 4).

6.3. FnBPB confers resistance to bactericidal activity of NETs

As reported by several authors, histones are expressed and extruded in NETs in abundant amounts, estimated at 2.5 μg/10⁶ neutrophils, such that histones comprise more than two thirds of the total protein content within the NET structure. In a recent study it was discovered that fibronectin-binding protein B (FnBPB) is the main histone receptor and that histone H3 displays the highest affinity. Importantly, an FnBPB-deletion mutant bound less H3 and was more susceptible to histone bactericidal activity, whereas a mutant overexpressing FnBPB bound more H3 and was more resistant to killing by histone. This information raised the question whether inhibition by FnBPB of histone-mediated bacterial killing is biologically significant. As a matter of fact, in a bactericidal assay promoted by NETs it was shown that FnBPB protected staphylococci from killing by NETs, demonstrating that FnBPB-mediated resistance is important when histones are present in a biological relevant milieu [57] (Fig. 4). The histone-neutralizing activity of FnBPB is reminiscent of the behavior of the M1 protein, a classical *Streptococcus pyogenes* surface virulence factor, which also protects bacteria against released extracellular histones in NETs [58] (Fig. 4).

6.4. *S. aureus* biofilm induces NETosis and blocks their anti-microbial activity

When they encounter neutrophils, *S. aureus* biofilms release the leukocidins PVL and HlgAB and produce NETs and cytoplasts. The generated anuclear neutrophils, although still capable of permeating the biofilm structure and phagocytosing bacteria, were not effective at clearing the biofilms. Likewise, the induced NETs were not sufficient for clearing *S. aureus* biofilms. The inefficiency of these structures is attributed to the leukocidin LukAB, a toxin which promotes *S. aureus* survival during phagocytosis [46]. The persistence of biofilm bacteria trapped in NETs is also facilitated by *S. aureus* nuclease-mediated degradation of DNA in NETs, resulting in the dispersal of bacteria and persistence of the chronic infection [59].

7. Conclusions

Virulence factors of *S. aureus* and the role they play in the formation and destruction of NETs were examined here. Up to now, at least three staphylococcal factors have been identified which interfere with the anti-microbial activity of NETs: a) a nuclease that degrades DNA in NETs; b) the Eap protein that forms complexes with linear DNA and, possibly, stabilizes the DNA structure and protects the molecule from enzymatic attack; 3) FnBPB, a staphylococcal surface protein that neutralizes the antimicrobial activity of histones (Fig. 2). Due to the complexity of the structure of NETs, other staphylococcal factors may target constituents of NETs. Scl-1, a streptococcal collagen-like protein in M1T1 group A *Streptococcus*, interferes with MPO activity and mediates bacterial survival in NETs [60]. Additionally, the M1 protein allows the survival of *S. pyogenes* in phagocytic extracellular traps through LL-37 inhibition [61]. Thus, we speculate that unidentified surface or secreted factors of *S. aureus* can further neutralize the function of enzymes, antimicrobial peptides, or ion chelating agents of NETs.

S. aureus cells can also induce the formation of NETs (Fig. 2) [44]. Specific *S. aureus* factors such as SpA [62], PVL [25], LukAB [45] and PSMα [49] directly elicit NET formation. The induction of NETosis is promoted by the alpha enolase and pneumolysin from *Streptococcus pneumoniae* [49,63], by gingipains of *Porphyromonas gingivalis* [64], and by hydrogen peroxide produced by *Streptococcus sanguinis* [65]. Therefore, NET formation induced by bacterial species is a common event. How can we solve the apparent paradox that virulence factors produced to protect bacteria from the host defense mechanisms also promote the formation of anti-bacterial structures? As reported above, NETs may either induce bacterial killing or mediate tissue damage both during acute and chronic inflammation [9,66,67]. Thus, it is possible that under specific pathological circumstances, *S. aureus* (and other bacterial species) benefit more from inducing the formation of NETs and using these structures to damage host tissues than from blocking the antibacterial activity of NETs. This strategy could allow bacteria to have better access to metabolic resources, favor the colonization of deeper tissues and definitively ensure safer and optimal survival in the host.

A better understanding of the biochemical details of these alternative strategies and a clearer definition of the role played by new staphylococcal factors in NET formation and destruction *in vivo* is key for the development of novel therapeutic approaches to control and combat this formidable pathogen.

Author statement

P.S. and G.P. conceptualized and wrote the manuscript. P.S. and G.P. supervised the overall conceptualization and draft writing. The authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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