Arteriosclerosis, Thrombosis, and Vascular Biology

BRIEF REVIEW

Staphylococcus aureus and Neutrophil Extracellular Traps: The Master Manipulator Meets Its Match in Immunothrombosis

Severien Meyers, Marilena Crescente, Peter Verhamme, Kimberly Martinod

ABSTRACT: Over the past 10 years, neutrophil extracellular traps (NETs) have become widely accepted as an integral player in immunothrombosis, due to their complex interplay with both pathogens and components of the coagulation system. While the release of NETs is an attempt by neutrophils to trap pathogens and constrain infections, NETs can have bystander effects on the host by inducing uncontrolled thrombosis, inflammation, and tissue damage. From an evolutionary perspective, pathogens have adapted to bypass the host innate immune response. *Staphylococcus aureus* (*S. aureus*), in particular, proficiently overcomes NET formation using several virulence factors. Here we review mechanisms of NET formation and how these are intertwined with platelet activation, the release of endothelial von Willebrand factor, and the activation of the coagulation system. We discuss the unique ability of *S. aureus* to modulate NET formation and alter released NETs, which helps *S. aureus* to escape from the host's defense mechanisms. We then discuss how platelets and the coagulation system could play a role in NET formation in *S. aureus*-induced infective endocarditis, and we explain how targeting these complex cellular interactions could reveal novel therapies to treat this disease and other immunothrombotic disorders.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: endocarditis ■ extracellular trap ■ neutrophils ■ platelet activation ■ Staphylococcus ■ virulence factors

Neutrophils are one of the first immune cells to arrive at a site of infection, and they have a whole arsenal of tools to combat pathogens. The release of neutrophil extracellular traps (NETs) is one of the main mechanisms of immune response to pathogens and has been extensively studied over the past 15 years. NETs are extracellular DNA fibers bearing histones, granular proteins including MPO (myeloperoxidase), NE (neutrophil elastase), and defensins, and cytosolic proteins including calprotectin and cathelicidins.[1] Although neutrophil extracellular trap (NET) structures were first imaged in the 1980s[2,3] and likely even already described in the 1880s as a fibrous mass released from disappearing leukocytes,[4] it was not until 2004 that their biological function became evident when Brinkmann et al[1] revealed that these unique extracellular structures could not only capture but also kill both Gram-positive and Gram-negative bacteria. It is now widely appreciated that NETs are released in various infectious diseases, including bacterial, viral, parasitic, and fungal infections.[5–12] This has been further highlighted by the current coronavirus disease 2019 (COVID-19) pandemic, where NETs form in abundance and contribute to severe disease and immunothrombosis in the lung and other organs.[13–16] Recent observations highlight the complex induction mechanisms underlying NET formation during infection, including distinct pathways resulting in lytic or nonlytic NET release.[17] When released into the extracellular space, NETs combat infection by physically trapping pathogens and inducing their death via NET microbicidal components.[1,11,12,18–22] More specifically, this death is induced, among others, by histones that introduce damage to the bacterial membrane, by MPO in the presence of hydrogen peroxide and by NE that cleaves thrombin into bactericidal thrombin-derived C-terminal fragments and degrades certain bacterial virulence factors.[1,19–23] As

Correspondence to: Kimberly Martinod, PhD. Department of Cardiovascular Sciences, KU Leuven, 49 Herestraat, bus 911, 3000 Leuven, Belgium. Email kim.martinod@kuleuven.be

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Nonstandard Abbreviations and Acronyms

ADAMTS13	a disintegrin and metalloprotease with a thrombospondin type 1 motif, member 13
AdsA	adenosine synthase A
COVID-19	coronavirus disease 2019
DNase	deoxyribonuclease
Eap	extracellular adherence protein
FXII	Factor XII
GP	glycoprotein
HMBG1	high-mobility box group 1
ICAM2	intercellular adhesion molecule 2
IE	infective endocarditis
IL	interleukin
MPO	myeloperoxidase
NE	neutrophil elastase
NET	neutrophil extracellular trap
NOS	nitric oxide synthase
PAD4	peptidylarginine deiminase 4
PF4	platelet factor 4
PVL	Panton-Valentine leukocidin
SpA	*S. aureus* protein A
TF	tissue factor
TFPI	TF pathway inhibitor
TLR4	toll-like receptor 4
TNF	tumor necrosis factor
tPA	tissue-type plasminogen activator
uPAR	urokinase plasminogen activator receptor
VWbp	von Willebrand factor-binding protein
VWF	von Willebrand factor

a counter-attack mechanism, pathogens have adapted to be able to bypass this innate immune response and to eventually thrive in the host environment. *Staphylococcus aureus* (*S. aureus*), in particular, proficiently overcomes host defense mechanisms via an arsenal of virulence factors that manipulate both the coagulation cascade and innate and adaptive immunity,[24,25] thus representing one of the deadliest bacteria in the developed world.[26] The failure of NETs in killing pathogens can be made worse by the bystander effects that NETs have on host cells and tissues. For example, NET formation within blood vessels can contribute to uncontrolled thrombosis and subsequent tissue damage.[27] In this review, we summarize the evidence surrounding the interplay between NETs, platelets, the coagulation system, and VWF (von Willebrand factor), and how this can progress from protective immunothrombosis to pathology. We then discuss NET formation during *S. aureus* infection and how this is linked to infective endocarditis (IE), a devasting immunothrombotic disease. Finally, we highlight some promising therapeutic targets to potentially treat IE as well other *S. aureus*-related pathologies.

Highlights

- Neutrophil extracellular traps play an integral role in immunothrombosis by interacting with pathogens, platelets, the coagulation system and von Willebrand factor.
- *Staphylococcus aureus* proficiently overcomes the antimicrobial properties of neutrophil extracellular traps using several virulence factors.
- Neutrophil extracellular traps could play a role in the various stages of infective endocarditis, which is an infected blood clot attached to the cardiac valves.
- The different cellular interactions in immunothrombosis represent several pharmacological targets to treat infective endocarditis and other immunothrombotic diseases.

NETS AND (IMMUNO)THROMBOSIS

In the course of a microbial infection, innate immune cells, including monocytes and neutrophils, can orchestrate a response with thrombosis mediators leading to the formation of microthrombi. This form of thrombosis, designated as immunothrombosis,[28] can either be limited to selected microvessels and serve the purpose of suppressing the dissemination of pathogens, or can affect larger vascular beds and have a detrimental impact on the host. NETs are central contributors to immunothrombosis; in fact, they are commonly found inside human thrombi and in close contact with platelets, VWF, and coagulation factors, including TF (tissue factor), factor XII, and fibrin/fibrinogen.[5,29–32]

NETs and Platelets

Platelets are generally the first cellular effectors recruited to a site of vascular injury, where they bind to subendothelial collagen or to VWF released from activated endothelial cells. Upon interaction with the endothelium, platelets undergo various stages of activation, leading to further recruitment not only of other platelets, but also of circulating leukocytes including neutrophils. Platelet-neutrophil interaction can lead to the subsequent activation of neutrophils,[33–35] which is crucial for NET formation and for further platelet accumulation.[12,34,36]

The dynamic collaboration between platelets and neutrophils in the context of NET formation has been experimentally demonstrated both in in vitro and in vivo models. In vitro, activated platelets induce NET release under static or flow conditions.[11,12,37–41] Various platelet agonists, such as lipopolysaccharide, thrombin, ADP,

and collagen, are able to initiate this process, as well as platelets from patients with sepsis or treated with septic plasma or bacteria.[11,12,38–40,42] *S. aureus*, in particular, interacts with and activates platelets which in turn enhances neutrophil activation toward NET release.[38,40] Intravital microscopy revealed that platelet aggregates were present within and downstream of NETs in mice with sepsis,[36] while experimental platelet depletion prevented NET formation in a mouse model of endotoxemia.[12] Finally, the available evidence that antiplatelet therapies, such as aspirin, cilostazol, prostacyclin, and ticagrelor, reduce NET release in vitro and in infectious disease, reinforces the idea that platelets are important contributors to NET formation during systemic infection.[38,39,43–46]

Various molecular mechanisms have been described in platelet-induced NET formation (NETosis) (Figure 1). Direct interaction of platelets with neutrophils via P-selectin (CD62P) and P-selectin glycoprotein ligand 1 (PSGL-1), respectively, is a fundamental mechanism whereby platelets induce NET formation.[39,41] A key role has been also established for the platelet receptors glycoprotein Ibα (GPIbα) and integrin αIIbβ3 (also known as GPIIb/IIIa), which respectively interact with integrin αMβ2 and SLC44A2 (solute carrier family 44 member 2) on neutrophils.[35,47] Platelet ICAM2 (intercellular adhesion molecule 2) which associates with αLβ2 on neutrophils enhances NET formation[12] as well as the activation of platelet TLR4 (toll-like receptor 4), which accounts for approximately half of platelet-neutrophil interactions when neutrophils are incubated with platelets in the presence of septic plasma.[11] In addition to directly interacting with neutrophils, activated platelets also secrete several soluble factors that affect neutrophils. For example, the release of HMBG1 (high-mobility box group 1), which binds to the receptor for advanced glycation end products (RAGE) on the neutrophil surface, is crucial for NET formation.[34,48] Moreover, platelets secrete β-defensin 1, CD40L, PF4 (platelet factor 4), and chemokine ligand 5 (CCL5) that induce NET formation by binding to various neutrophil β2 integrins,[49–52]

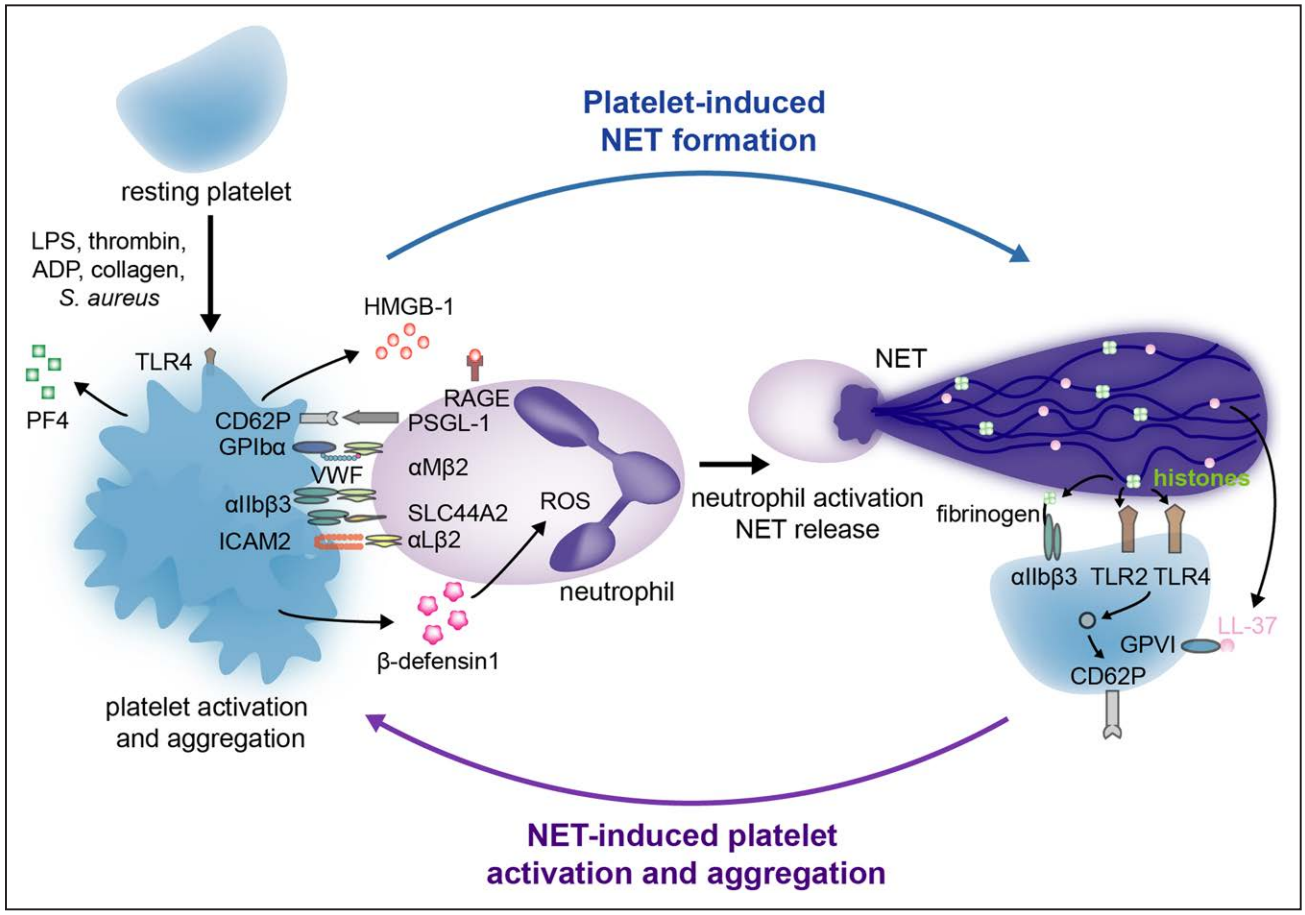

Figure 1. The dynamic interplay between platelets and neutrophil extracellular traps (NETs).
While some interactions between platelets and neutrophils trigger activated neutrophils to release NETs, others lead to platelet activation and aggregation. Via various receptors, secreted ligands, and specific components of the coagulation system, platelets can directly or indirectly induce NET formation (left). In return, specific components of NETs, more specifically histones and the cathelicidin LL-37, interact with several platelet receptors that result in platelet activation and aggregation (right). CD62P indicates P-selectin; GPIbα, glycoprotein Ibα; GPVI, glycoprotein VI; HMGB-1, high mobility box group 1; ICAM2, intercellular adhesion molecule 2; LPS, lipopolysaccharide; PF4, platelet factor 4; PSGL-1, P-selectin glycoprotein ligand 1; RAGE, receptor for advanced glycation end product; ROS, reactive oxygen species; SLC44A2, solute carrier family 44 member 2; TLR 2 or 4, toll-like receptor 2 or 4; and VWF, Von Willebrand factor.

as well as polyphosphates that promote NET formation through FXII (factor XII) activation.[53]

Reciprocal activation of platelets by NETs also occurs, thus generating a thromboinflammatory cycle (Figure 1). This was first shown by perfusing whole blood over NETs, which resulted in platelet adhesion, activation, and aggregation.[27] Isolated neutrophils from septic patients also cause platelet activation, which occurs in a P-selectin-dependent manner.[54] These effects were reduced in septic mice deficient in PAD4 (peptidylarginine deiminase 4), an enzyme crucial in NET formation[36,55] because it promotes chromatin decondensation by citrullinating histones H1, H3, and H4.[56] In addition to NETs themselves, histones are able to activate and aggregate platelets in a P-selectin and TLR2/4-dependent manner[27,57] or by associating with crosslinking molecules, such as fibrinogen, that in turn interact with platelets through the αIIbβ3 (GPIIb/IIIa) integrin.[27,58] This interaction of histones with TLR2 and TLR4 induces platelet-mediated thrombin generation, thus further supporting activation of the coagulation cascade.[57] Finally, NETs also activate platelets via the cathelicidin LL-37, which binds to GPVI on the platelet surface.[59]

NETs and Coagulation Factors

In addition to the reciprocal interaction with platelets, a dynamic interplay between NETs and several coagulation factors leads to enhanced fibrin formation,[27,36] (Figure 2). Neutrophil serine proteases, such as NE, impair the degradation of TF by the TFPI (TF pathway inhibitor), thus supporting the extrinsic pathway of coagulation.[60] As such, abundant amounts of TF have been found in the proximity of NETs formed in the liver microvasculature of mice with sepsis or vessel injury.[36,54,60] NETs also interfere with the intrinsic pathway of coagulation. In fact, in the presence of activated platelets, the negatively charged DNA backbone of NET can trigger the autoactivation of FXII.[53,61] This charge-driven interaction induces the generation of thrombin, an effect that disappears in FXII-deficient plasma or when FXII is blocked.[61–63] By enhancing thrombin generation, NETs are thus able to promote fibrin formation.[36] NETs can enhance thrombin formation via several other mechanisms that are either platelet dependent or independent. Intact NETs or histones H3 and H4 can interact with platelet TLR2 and TLR4 and sustain prothrombin cleavage to thrombin.[63] Histones can also prevent thrombomodulin from activating protein C,[64] which, in the presence of protein S, cleaves FVa and FVIIIa and limits thrombin production. Histone H4 has also been reported to directly bind to and autoactivate prothrombin.[65] The involvement of NETs in the generation of thrombin has been further demonstrated by reduced formation of thrombin-antithrombin complexes and reduced thrombin activity when NETs or NET formation were targeted using various strategies, including digestion by DNases (deoxyribonucleases), histone-neutralizing antibodies, PAD4-deficiency, or by neutrophil depletion.[36,55,62,66]

Different studies support the idea that NETs also promote coagulation by preventing fibrin degradation, as illustrated in Figure 2. tPA (tissue-type plasminogen activator) converts plasminogen into plasmin, an important enzyme that promotes fibrinolysis. Elastase bound to the NET DNA backbone degrades plasminogen, as evident by the high amount of NE-derived plasminogen fragments found in the plasma of septic shock patients.[67] Additionally, using clot lysis assays, it was found that cell-free DNA is able to bind plasmin and fibrin thereby impairing plasmin-mediated fibrinolysis.[68] To confirm these observations, treatment with DNase has been able to restore thrombolysis in different settings.[68,69] Moreover, histones have shown the ability to hamper tPA-driven fibrinolysis in in vitro assays.[69,70]

As depicted in Figure 2, coagulation factors, such as FXII and thrombin, are in turn able to promote additional NET formation (Figure 2). FXII induces NET formation via uPAR (urokinase plasminogen activator receptor)-induced pAkt2 signaling,[53] while thrombin activates protease activated receptors (PAR1/2/4) on platelets and potentiates platelet-induced NETosis.[71,72]

NETs and VWF

VWF is a multimeric protein released by activated platelets and by exocytosis of Weibel-Palade bodies from activated endothelial cells.[73] Once released in the vasculature, ultra-large VWF multimers elongate in response to shear stress from the flowing bloodstream and expose the A1 domain, which can recruit neutrophils by binding to their PSGL-1 and αmβ2 receptors[74] (Figure 3) but also directly binds to platelets and *S. aureus*.[27,75] VWF has also been found to colocalize with NETs within a thrombus.[27] Indeed, under flow conditions, histones and DNA from NETs bind the A1 domain of VWF (Figure 3), allowing them to stay in place and subsequently damage the vasculature.[76] In *S. aureus*-infected mice, NETs promote liver injury via VWF-mediated binding to the vasculature.[77] Histones themselves can provoke the release of Weibel-Palade bodies, thus further contributing to thrombus formation and immunothrombosis.[75,78] Typically, high ultra-large VWF is cleaved by the protease ADAMTS13 (A disintegrin and metalloprotease with a thrombospondin type 1 motif, member 13), endogenously active in circulation. In pathological conditions, however, this balance is often impaired.[79] PAD4, which is released on NETs, can citrullinate ADAMTS13 and subsequently abolish its activity, promoting ultra-large VWF-platelet string formation, such as in sepsis[80] (Figure 3). VWF, in turn, by binding to platelet GPIbα and neutrophil αMβ2, also facilitates platelet interaction with neutrophils and platelet-induced NET formation.[38]

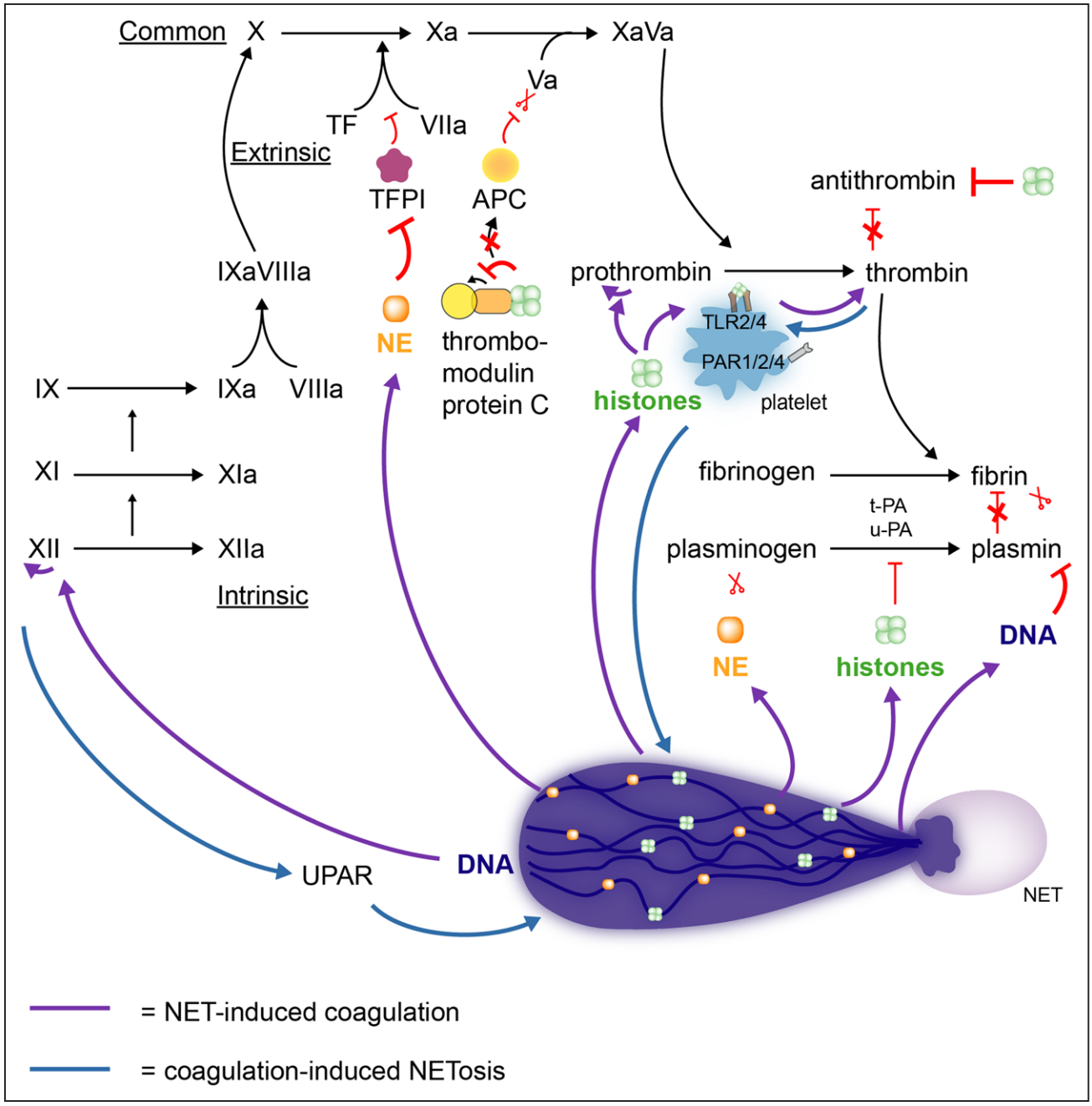

Figure 2. Neutrophil extracellular traps (NETs) promote fibrin formation by interacting with the coagulation system. NETs stimulate the formation of fibrin by triggering both the extrinsic (TF [tissue factor]) as well as intrinsic (FXII) pathway of the coagulation system (green arrows). In addition, histones are able to prevent the cleavage of FVa by APC (activated protein C). By the autoactivation of prothrombin or the interaction with TLR2 or 4 (toll-like receptor 2 or 4) on platelets, histones directly trigger thrombin generation (green arrows). Besides directly stimulating fibrin formation, NETs prevent its degradation by impairing t-PA (tissue-type plasminogen activator), u-PA (urokinase-type plasminogen activator), antithrombin, and plasmin-mediated lysis (green arrows). Not only are NETs able to interact with various components of the coagulation cascade, but these components (FXII and thrombin) can also prime for NETosis (blue arrows). UPAR indicates urokinase-type plasminogen activator receptor.

NETS AND *S. AUREUS*

S. aureus is the leading cause of several devastating infections, including sepsis, endocarditis, skin and soft tissue infections, osteoarticular, pleuropulmonary, and device-related infections.[26] One of the reasons why *S. aureus* is so proficient in overcoming the innate immune system is its ability to manipulate the coagulation cascade. *S. aureus* induces fibrin formation by activating prothrombin via the coagulases von Willebrand factor binding protein (VWbp) and staphylocoagulase (Coa).[81] Furthermore, *S. aureus* binds, activates, and aggregates platelets via its interaction with components of the coagulation system.[25,81] Altering *S. aureus*-induced coagulation improved

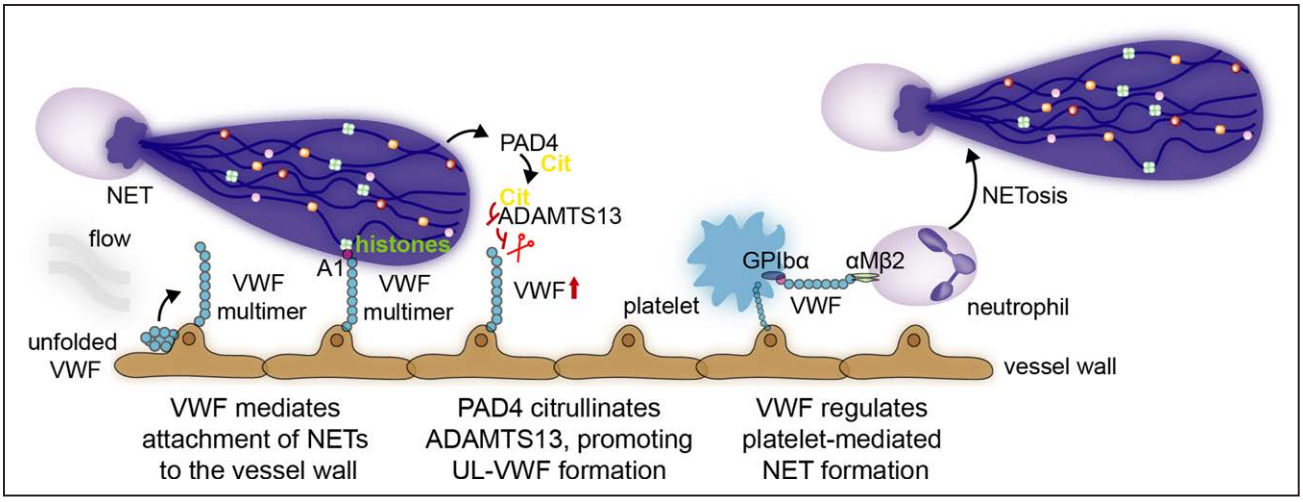

Figure 3. VWF (Von Willebrand factor) and neutrophil extracellular traps (NETs) interact via various mechanisms.
In response to shear stress of flowing blood, ultra-large VWF multimers elongate and expose the A1 domain. Via different mechanisms, NETs and neutrophils interact with these ultra-large VWF multimers. First, the A1 domain on VWF binds NET histones and DNA, thus allowing them to stay in place and damage the vasculature. Second, by releasing PAD4, NETs can promote ADAMTS13 (A disintegrin and metalloprotease with a thrombospondin type 1 motif, member 13) citrullination, a protease that cleaves VWF, and subsequently abolish its activity, promoting ultra-large VWF-platelet string formation. VWF, in turn, can regulate platelet-induced NET formation by acting on platelet GPIbα and neutrophil αMβ2.

outcome in various experimental models of bacteremia, sepsis, endocarditis, skin and soft tissue infections, and catheter-related infections.[82–87] The ability of *S. aureus* to induce fibrin via its coagulases distinguishes it from other staphylococci that are coagulase negative. Although the coagulase-negative strain *S. epidermidis* is also a frequent cause of medical device-related infections and prosthetic valve endocarditis, coagulase-negative staphylococci are less virulent than *S. aureus*.[88] In general, endocarditis and cardiac device-related infections induced by *S. aureus* are associated with high mortality and complications compared with coagulase negative staphylococci.[89,90] The only coagulase negative strain that mimics *S. aureus* is *S. lugdunensis*. It causes an infrequent but more aggressive form of native valve endocarditis, probably in virtue of the ability of *S. lugdunensis* to bind VWF.[91]

In addition to activating the coagulation system, *S. aureus* is a master in evading innate and adaptive host defenses.[24] In this section, we will specifically focus on *S. aureus* and its unique ability to manipulate neutrophils releasing NETs. By capturing and killing pathogens, NETs can serve as a mechanism of host defense from a variety of infections.[1,40,92] NETs are able to degrade *S. aureus* and its main virulence factor, α-toxin.[1] However, this deadly bacterium secretes or expresses a variety of virulence factors that both enhance or diminish NETosis to its advantage, as well as overcome NETs' microbicidal properties[24,25] (Figure 4). Whether this is a response of the host to constrain infection or a way for the bacteria to survive in the host environment is still unclear.[1,93]

S. aureus Promotes NETosis

In theory, when *S. aureus* enters the bloodstream, it can be rapidly captured and killed by neutrophils through the release of NETs. However, NETs can also work as a scaffold that allows bacteria to stay in place and grow,[94,95] contributing to a biofilm.[96] It has been hypothesized that neutrophils that are primed to form NETs are no longer able to kill *S. aureus* by phagocytosis and that this could be a strategy for *S. aureus* to circumvent the host's innate immune defense.[92,97,98] Nevertheless, it has been shown that NET-forming neutrophils can still be viable and retain their ability to crawl, transmigrate, and phagocytose as functional cytoplasts.[93,99]

One way by which *S. aureus* promotes NETosis is by activating platelets (Figure 4A). *S. aureus* induces platelet activation and aggregation by binding to platelet integrin αIIbβ3, the platelet immunoglobulin receptor FcγRIIa, or GPIbα.[100–104] *S. aureus* either binds directly to these receptors or via a variety of bridging molecules, such as fibrinogen, fibrin, fibronectin, or VWF. In particular, platelets can be affected by a variety of virulence factors, including: clumping factor A (ClfA), fibronectin binding protein A and B (FnBPA/B), Eap (extracellular adherence protein), SpA (*S. aureus* protein A), *S. aureus* iron-regulated surface determinant B (IsdB), Staphylococcal superantigen-like 5 (SSL5), and *S. aureus* toxin Panton-Valentine leukocidin (PVL).[100–106] However, it has not been established yet whether platelets activated by *S. aureus*-derived virulence factors induce NETosis. Nevertheless, a direct association between virulence factor-induced platelet activation and NET formation has been shown for α-toxin. This toxin causes the secretion of β-defensin 1 from human platelets, thereby inducing NET release in a reactive oxygen species-dependent manner.[49] Furthermore, antibodies against α-toxin reduced NET formation and bacterial load in diabetic mice with a *S. aureus*-infected wound.[107]

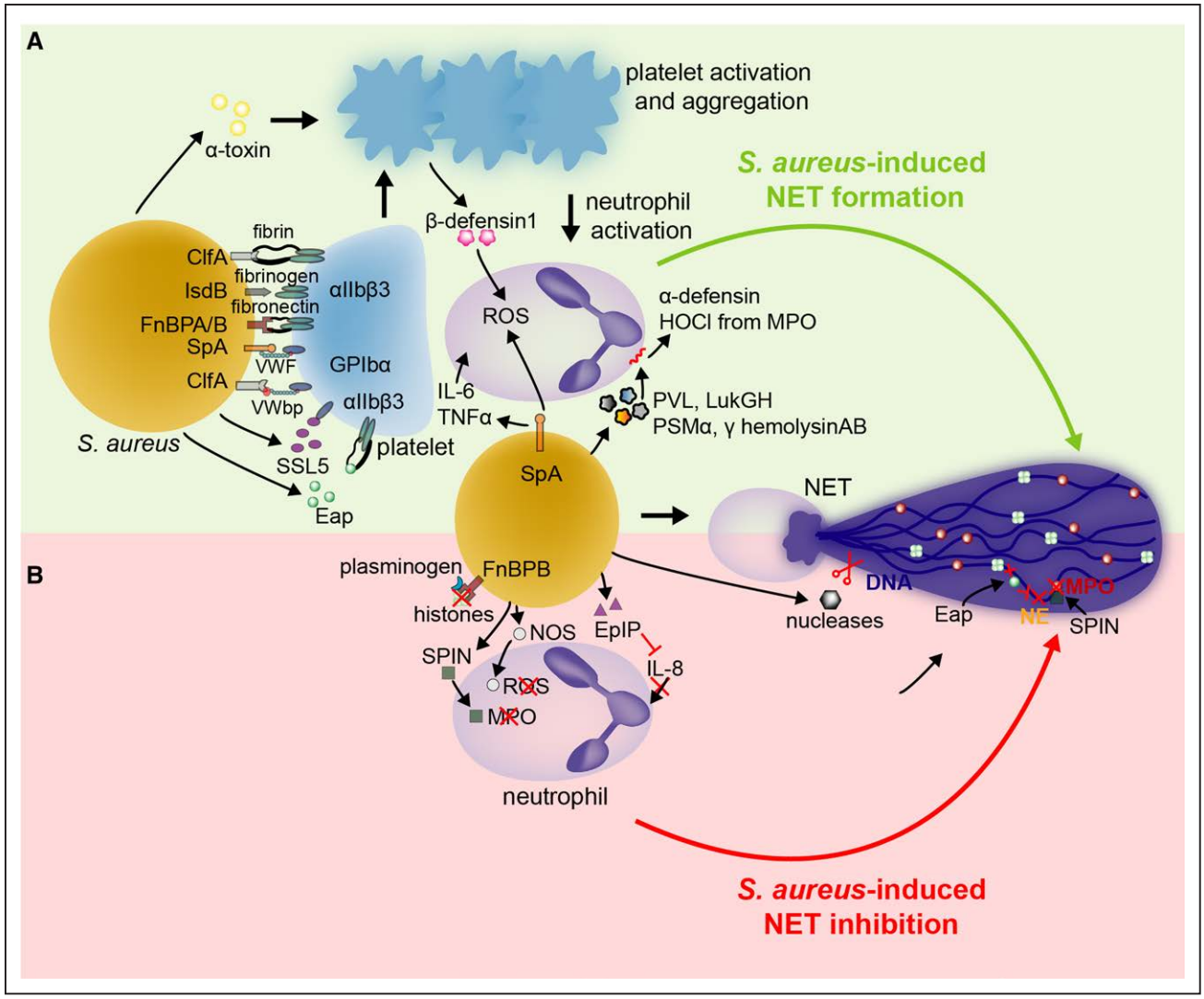

Figure 4. *Staphylococcus aureus* both promotes and hampers neutrophil extracellular trap (NET) formation via various virulence factors to survive in the host environment.

By interacting with platelet receptors, *S. aureus* activates and aggregates platelets and possibly promotes platelet-induced NET formation (**A**). Some *S. aureus* virulence factors can directly prime IL (interleukin)-6, TNFα (tumor necrosis factor α), reactive oxygen species (ROS), and damage-mediated NETosis (**A**). While enhancing NETosis could be a strategy of *S. aureus* to circumvent neutrophil-mediated killing, *S. aureus* is also equipped to diminish NET formation, destroy already formed NETs, or even to overcome their microbicidal properties (**B**). αIIbβ3 indicates glycoprotein GPIIb/IIIa; ClfA, clumping factor A; Eap, extracellular adherence protein; EpIP, epidermin leader peptide processing serine protease; FnBPA/B, fibronectin-binding protein A and B; GPIbα, glycoprotein Ib; IL-6/8, interleukin 6 or 8; IsdB, iron-regulated surface determinant B; MPO, myeloperoxidase; NE, neutrophil elastase; NOS, nitric oxide synthase; ROS, reactive oxygen species; SpA, *S. aureus* protein A; SPIN, staphylococcal peroxidase inhibitor; SSL5, Staphylococcal superantigen-like 5; TNFα, tumor necrosis factor α; VWbp, von Willebrand factor-binding protein; and VWF, Von Willebrand factor.

Although SpA and PVL-activated platelets likely do not stimulate NET formation directly, SpA and PVL are able to induce NETosis via other mechanisms (Figure 4A).[97,98] In vitro, SpA primes NET release via secretion of IL (interleukin)-6, TNF (tumor necrosis factor), and reactive oxygen species.[98] *S. aureus* also stimulates NETosis by forming pores on the neutrophils' nuclear membrane and inducing the release of DNA bearing histones and granular proteins including MPO and NE into the extracellular space. Leukotoxin GH (LukGH), phenol-soluble modulin α Peptide (PSMα), γ-hemolysin AB, and PVL are some of the pore-forming toxins that have been described to induce NET release.[92,94,97] These toxins have been most frequently found in community acquired MRSA strains as well in certain laboratory strains of *S. aureus* including USA300 LAC and Newman.[94,97,108] Incubation with PVL and γ-hemolysin mutants reduced the amount of killed neutrophils and bacterial load, thus revealing that in some conditions NETosis can allow bacteria to survive in the host. In addition, a rapid, nonlytic form of NETosis induced by PVL has also been observed.[93] Among different bacteria species, *S. aureus* is one of the most potent natural inducers of NET release.[93]

S. aureus Impairs NET Formation and Stability

As NETs and their components possess antimicrobial properties, it is more plausible that bacteria would promote their own survival by reducing NET formation rather than enhancing it. Indeed, *S. aureus* can reduce NET formation, destroy released NETs, and interact with NET microbicidal components to limit the antimicrobial potential of NETs (Figure 4B).

By virtue of its cationic properties, *S. aureus*-released Eap impairs NET formation by directly binding and aggregating the neutrophil DNA backbone, which was shown by atomic force microscopy.[109] Incubation of PMA-induced NETs with Eap resulted in a diminution of the DNA/histone H1 network. Furthermore, these DNA binding properties can also be used to promote biofilm formation.[110] Additionally, Eap and its homologs are potent inhibitors of NE and allow *S. aureus* to overcome NE's antimicrobial properties, reduce NET release, and thus survive in vivo.[111–114] Of note, however, elastase bound to the NET backbone is less sensitive to the inhibitory effect of Eap.[115] Complimentary to the effect of Eap, inhibiting NE using selective inhibitors or mice deficient in NE impaired bacterial clearance,[116,117] although this may also be due to NE's direct antimicrobial properties.

S. aureus possesses various other mechanisms to target NET formation or its microbicidal properties. IL-8 mediated NETosis may be reduced by epidermin leader peptide processing serine protease (EpiP), which is a homolog of the IL-8 protease SpyCEP from Group A Streptococci.[118] In addition to inhibiting NE, *S. aureus* inhibits MPO by secreting a specific virulence factor called staphylococcal peroxidase inhibitor (SPIN).[119] SPIN binds the active site of MPO and thereby prevents its interaction with hydrogen peroxide and the ability of MPO to kill bacteria. Via expression of NOS (nitric oxide synthase), *S. aureus* can also overcome the bacterial killing action of reactive oxygen species and cathelicidins such as LL-37.[120]

A final way for *S. aureus* to survive NETs is by destroying the extracellular chromatin structure and disarming its components, including histones. Under the regulation of the SaeR/S system, *S. aureus* produces 2 nucleases (Nuc and Nuc 2) that digest extracellular DNA and therefore also NETs.[96,121–124] Nuc is a secreted nuclease, whereas Nuc2 remains tethered to the *S. aureus* membrane[125]; this provides high local concentrations of nucleases in the vicinity of *S. aureus*. In mice with a respiratory tract infection, nuclease production diminished bacterial clearance and increased mortality.[121] Nucleases have been found in sputum from patients with cystic fibrosis. This explains why *S. aureus* persists for a long time in the airways of patients affected by this condition.[126] Moreover, the release of nucleases facilitates the escape of *S. aureus* from immunosurveillance by other cells, including macrophages. For example, the combined action of nuclease and AdsA (adenosine synthase A) converts NETs to deoxyadenosine and subsequently leads to caspase 3 activation, resulting in macrophage cell death.[124,127,128] In addition to degrading NETs, nucleases can disrupt the DNA matrix inside biofilms, thus promoting bacterial spread and remodeling of the biofilm.[96,122,123] Last, *S. aureus* is not only able to degrade the DNA backbone but also neutralize histones. In particular, its virulence factor FnBPB binds at the same time the histones released during the NETosis process and plasminogen, thus facilitating histone degradation by this enzymatic precursor.[129] Additional evidence that killing of neutrophils and degrading NETs and its microbicidal proteins is an important strategy of *S. aureus* to evade the immune system and promote disease has been recently demonstrated in a skin infection mouse model. In this model, *S. aureus* evaded the immune system via the ArlRS-MgrA cascade by regulating the expression of nuclease, SCIN (staphylococcal complement inhibitor), and leucocidins.[130]

These studies highlight the many facets by which *S. aureus* can manipulate the host response, including NET formation, and thus prolong its survival. The impact of this is clearly seen by *S. aureus*'s ability to cause potentially severe human disease, particularly when it is able to thrive in the bloodstream.

NETs and IE

There are few diseases that highlight the crucial interplay between coagulation, bacteria, and immunity better than IE.[131] IE is a condition defined by an infected blood clot or vegetation attached to the cardiac valves. The presence of bacteria, neutrophils, platelets, fibrinogen, and VWF in mice with an infected vegetation demonstrate the characteristic immunothrombotic properties of this disease (Figure 5). Immunohistochemical staining of 39 human IE valve samples revealed that extracellular DNA structures were commonly colocalized with MPO, suggesting the presence of NETs inside these vegetations.[5] Here elastase, extracellular MPO and cell-free DNA were more abundantly expressed than in normal valvular tissue, implying a major role of neutrophils and NETs in patients with IE.[5] However, the exact involvement of NETs in the pathophysiology of IE remains to be further elucidated. In Figure 6, we highlight the potential relevant players that could contribute to IE.

NETs could aid the very early steps of the disease by delivering *S. aureus* from a local infection site to the bloodstream. Once in circulation, this pathogen could then travel to the cardiac valves. On the contrary, NETs may also prevent dissemination of bacteria from a localized infection such as an abscess,[99] so the role of NETs in IE is complex. When reaching the valvular environment, *S. aureus* has to overcome its high shear rate by binding to platelets and/or VWF, and this enables it to colonize and finally form the infected vegetation characteristic of IE.[131,132] Similarly, to overcome the valvular shear stress and to reach the bacterial aggregates embedded within

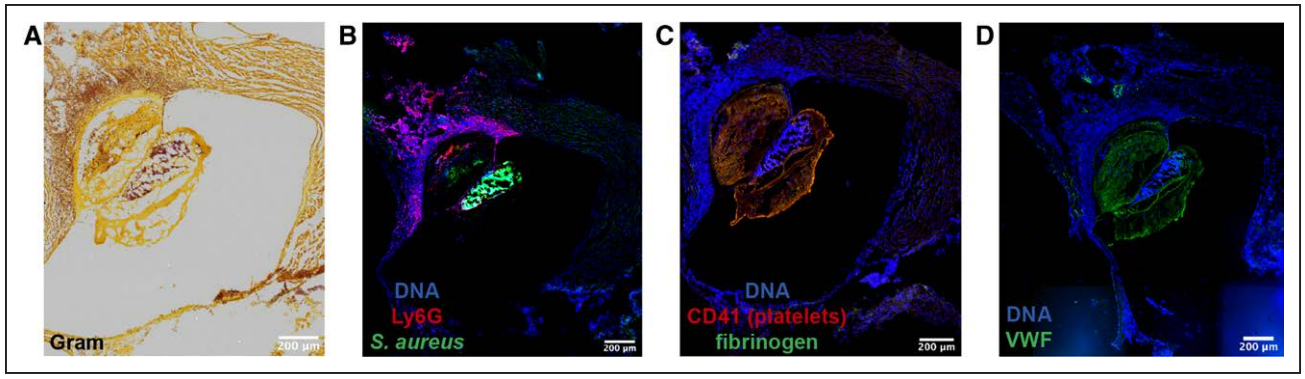

Figure 5. Immunostaining of a cardiac valve vegetation in a model of inflammation-induced IE.
IE vegetation was induced upon infection of mice with *S. aureus* and treatment with local histamine infusion via catheter instillation. **A**, Brightfield image of a Gram stain with bacteria seen in purple. **B–D**, Fluorescence images of the cardiac valve vegetation showing: neutrophil-specific marker Ly6G (red) and *S. aureus* (green) (**B**); platelet CD41 (red) and fibrinogen (green) (**C**); VWF (Von Willebrand Factor) in green (**D**). DNA is identified by Hoechst 33342 staining, depicted in blue. Images were acquired using a Zeiss Axioscan Z1 digital slide scanner or Zeiss Axiovert inverted microscope at the VIB-KU Leuven LiMoNe Bio Imaging Core and the KU Leuven Department of Cardiovascular Sciences Microscopy Core, respectively.

a lesion, neutrophils can bind to platelets, fibrin, and/or bacteria within the IE vegetation or can associate with VWF multimers released from endothelial cells.

Inside this infected thrombus, NETs may try to constrain the infection by trapping and eventually killing *S. aureus*.[1] Bacteria entrapped within NET-like structures have been identified inside IE vegetations in rats.[39,40,133] However, NETs may not effectively kill the bacteria, providing them a meshwork on which to easily grow and facilitate IE progression. Furthermore, NETs can induce platelet activation and promote coagulation, and this thrombotic environment in return promotes additional NET release that can further allow vegetation growth.[27] In a murine model of endocarditis, it has been recently reported that *S. aureus* induces coagulation within the vegetation by releasing two prothrombin activators, staphylocoagulase

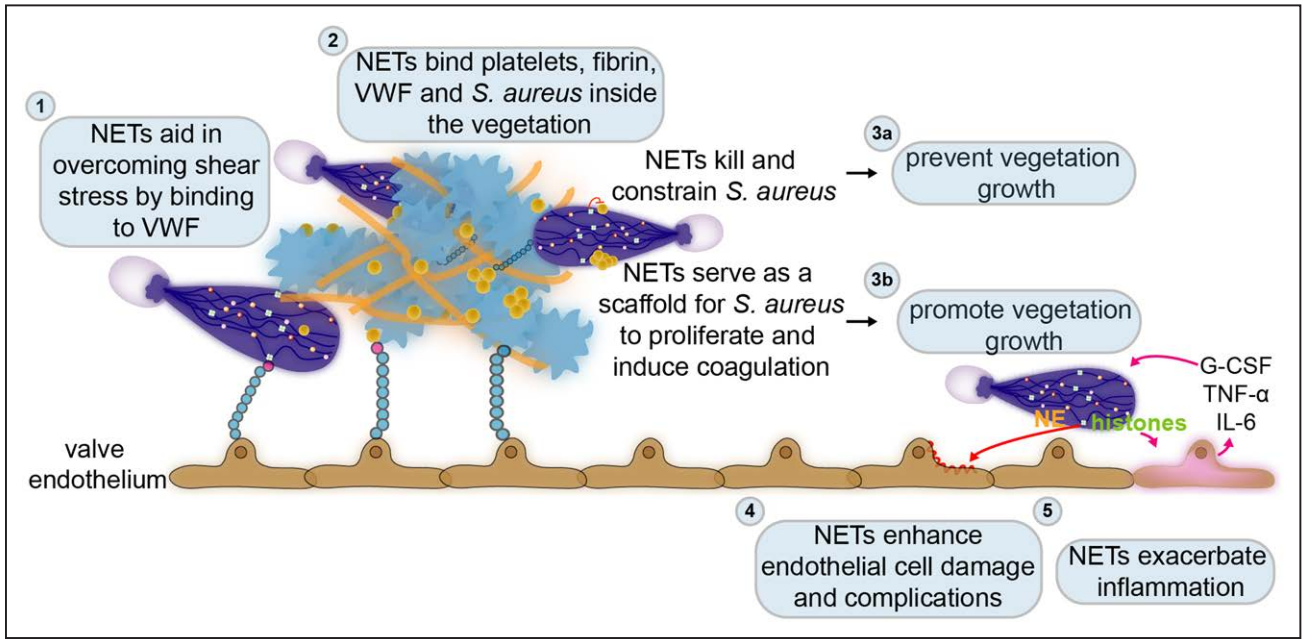

Figure 6. The dual role of neutrophil extracellular traps (NETs) in infective endocarditis.
Few diseases highlight the crucial interplay between coagulation, bacteria, and immunity better than infective endocarditis, which at its core is an infected blood clot attached to the cardiac valves. (1) Already at the very early stages of this disease, NETs can overcome the turbulent flow of the valves' environment by binding to the endothelium via VWF (von Willebrand factor). While attached to the endothelium, NETs provide a scaffold for binding of platelets, fibrin, and bacteria. (2) In the already formed vegetation or infected blood clot, NETs also bind platelets, fibrin, and *S. aureus*. (3) Inside the vegetation, NETs could either prevent its progression by killing and constraining *S. aureus* (3a) or enhance its formation by further stimulating coagulation or proliferation of *S. aureus* (3b). The infected blood clot or vegetation typically develops on damaged or inflamed cardiac valves. NETs may also induce further damage (4) and inflammation (5) in the valve endothelium. However, the exact role of NETs in infective endocarditis remains to be established. G-CSF indicates granulocyte colony-stimulating factor; IL-6, interleukin 6; and TNFα, tumor necrosis factor α.

(Coa) and VWbp (von Willebrand Factor-binding protein). The resulting bacteria-induced fibrin-rich layer serves as a shield against attack by myeloid cells. In particular, CD11b-positive cells accumulate outside the bacteria-rich environment.[86] In the presence of such a physical barrier, neutrophils and their released NETs may not be able to reach their target and carry out their bactericidal function. Additionally, DNase treatment in rats with endocarditis lesions reduced the size of the vegetation and the number of colonizing bacteria.[39,40] Whether this is an effect of NETs, however, still needs to be determined, as extracellular DNases can also degrade extracellular DNA from bacteria and thus affect the bacterial biofilm.

Typically, endocarditis develops on damaged or inflamed valves.[134] NET formation could aggravate the damage and inflammation of the valves' endothelium. For example, injecting mice with histones or citrullinated histone H3 results in endothelial cell damage.[135,136] In turn, the inflammatory response provoked by NETs could further prime NETosis.[137] Moreover, signs of proteolytic tissue damage and apoptosis linked to the activity of neutrophil proteases such as NEs were frequently present in vegetations isolated from rats and patients.[5,133] NE also induces apoptosis in myocardial cells ex vivo, thus possibly linking NETs to further complications such as heart failure.[133]

It is clear that additional studies on the involvement of NETs in the pathophysiology of IE are warranted. The detrimental or beneficial effects of NETs in endocarditis likely depend on several factors. First, there could be a time-dependent effect, where at an early stage NETs are protective, while later, they have more detrimental consequences. In various models of sepsis, NETs and their components promoted liver and renal damage after 8 to 24 hours; whereas NETs captured and killed bacteria effectively at earlier time points and thus had beneficial activity.[12,45,136,138] This could reflect the fact that when NETs fail to kill bacteria inside the vegetation, they instead promote vegetation growth by enhancing coagulation, inflammation, and valve damage. Another determining factor for the effect of NET formation in IE is the relative proportion of bacteria to NETs. If NETs are outnumbered, there is a higher chance for the bacteria to overcome NETs killing properties. Last, the virulence of the bacterium could also play a major role. More virulent strains likely express nucleases, which allow undisturbed bacteria growth and the release of fragments from the degraded NET backbone, which can have harmful consequences on the host.[115,121,122,126,137]

POSSIBLE THERAPEUTIC OPTIONS FOR PATHOLOGICAL IMMUNOTHROMBOSIS AND IE

The different cellular components involved in immunothrombosis represent several viable pharmacological targets to treat endocarditis or other immunothrombosis-related diseases. Here, we highlight the use of available or experimental drugs targeting platelets, coagulation, bacterial factors, and NETs as promising therapeutic approaches that could be applied in these disorders.

Targeting Platelets and Coagulation Factors

Antiplatelet and anticoagulant therapies, as well as strategies targeting VWF, have been shown to reduce NET formation in various animal models.[43,44,61,77] Both heparin and activated protein C affect histones and thrombus formation in models of DVT and *Escherichia. coli*–induced sepsis.[61,136] Combined treatment with tPA and DNase resulted in faster ex vivo thrombolysis of clots formed in vitro or collected from patients with acute ischemic stroke.[27,70,139] Although treatment with tPA can successfully reduce the extent of vegetation in infants affected by IE and degrade clots dissected from rabbits with IE,[140] the use of this drug is not recommended in patients with endocarditis due to the enhanced risk of bleeding.[141] Similarly, combined aspirin and ticlopidine prophylaxis has been shown to be beneficial in a rat model of *S. aureus* induced endocarditis,[142,143] but the use of these agents is currently not clinically supported.[141,144] Further investigation in the use of more targeted antiplatelet and antithrombotic agents to reduce thrombi and NET formation in IE is therefore warranted. The use of recombinant ADAMTS13, which targets UL-VWF but otherwise minimally impacts hemostasis,[145] may be a suitable approach to achieve anti-inflammatory and anti-NET efficacy.

However, platelets, fibrin, and VWF can constrain infection by capturing bacteria and preventing their dissemination. Platelets can also kill bacteria by releasing platelet microbicidal proteins.[146] As such, therapeutic strategies that interfere with immunothrombosis could potentially aggravate the infection. Indeed, antiplatelet treatment in mice with sepsis and peritonitis enhanced bacteremia.[12,44] However, while some authors have reported an increase in bacteria levels in the blood and various organs after anticoagulant and antiplatelet strategies, others could not show that these therapies promoted bacterial dissemination.[147] When evaluating new therapies that target immunothrombosis, also the impact on the proliferation and dissemination of the pathogen should be taken into consideration. New therapies should mitigate the detrimental effect of immunothrombosis and impair the growth and dissemination of the pathogen.

Targeting Bacterial Factors

Antibodies against SpA, PVL, γ-hemolysin AB, LukGH, and α-toxin have already been developed.[106,107,148–150] Nevertheless, until now, attempts to treat *S. aureus* infections by targeting these virulence factors have all clinically failed.[151,152] However, recent data suggest that

recombinant monoclonal antibodies against the bacterial factor SpA and α-toxin could be effective to treat *S. aureus* infections.[149,153,154] Interestingly, the antiplatelet agent ticagrelor has been recently found to have antibacterial properties by inhibiting α-toxin-mediated platelet injury *in vitro* and in a murine *S. aureus* bacteremia model.[155–157]

Targeting virulence factors involved in the degradation of NETs could be a valid therapeutic option to prevent *S. aureus* from growing and spreading in the host.[121,124,126] For example, cathelicidin LL-37, which binds to the negatively charged DNA backbone, could be used to prevent NET degradation.[158] Likewise, antibodies neutralizing nucleases could be promising to block NET degradation and bacterial growth. Finally, monoclonal antibodies against Coa and VWbp, which reduce the amount of fibrin surrounding the bacteria-rich environment and make bacteria more accessible to neutrophils, improved survival in mice with endocarditis.[86] Treatment with these antibodies also allowed neutrophils to better interact with *S. aureus* in a femoral artery vegetation model.[86]

Targeting NETs

A well-known agent that degrades the DNA backbone of NETs is DNase I, which is FDA-approved for the treatment of cystic fibrosis.[159] This drug suppressed thrombus growth and tissue damage in several infectious disease models.[27,36,39,40,61,160,161] Combining DNases with antibiotics may, however, be needed,[39,40,160] since nucleases also disrupt the extracellular DNA within biofilms and promote systemic bacteremia.[123,162] Elevated bacteremia levels have been shown after DNase treatment in animal models of endocarditis and sepsis,[12,39] although later treatment has a protective effect without decreased bacterial burden.[163] Combining DNase I with penicillin reduced the severity of bacteremia in rats with IE.[39] Inhibiting the protein components associated with NETs, that is, granular proteins and histones, may provide additional therapeutic opportunities. Indeed, inhibitors of NE and anti-histone antibodies reduced organ damage and lethality in mice with sepsis[136,164,165] but were also shown to impair bacterial clearance.[116,117] The detrimental outcomes of NETs can also be mitigated by preventing their formation. To do so, PAD4 can be targeted to prevent chromatin decondensation, loss of nuclear lamins, and cytoskeleton disassembly, all steps important for NETosis.[56,166,167] As such, PAD4-deficient mice with sepsis showed reduced intravascular coagulation and organ damage as compared with wild-type mice.[36,55,77,161] Similarly, administration of Cl-amidine, a pan-PAD inhibitor, effectively diminished NET release and progression of sepsis and wound infections in mice.[7,168,169]

On the other hand, LL-37, which is resistant to bacterial nucleases, promotes NETosis, but its activity suppresses the inflammatory response accompanying sepsis.[170] Pircher et al found that LL-37 is abundant in thrombi from patients with acute myocardial infarctions and the deletion of LL-37 homolog, CRAMP, in mice reduced platelet recruitment and thrombosis. Moreover, LL-37 or CRAMP promote platelet-neutrophil interactions.[59] Therefore, the use of LL-37 may promote unwanted thrombosis and the therapeutic use of LL-37 for the treatment of IE remains debatable.

A better understanding of the role of NETs in immunothrombosis is needed to find successful therapies for IE and, in general, diseases related to unregulated immunothrombosis. A fine tuning of NET formation and degradation might be required to allow NETs to remove bacteria from the circulation and reduce infection, without provoking untoward thrombotic complications in the host.

CONCLUSIONS

In summary, *S. aureus* can both diminish and enhance NET formation and can destroy released NETs and their associated components. Decreased NET formation can lead to *S.aureus* survival in the host and poor infection outcome, while enhanced NET formation, although trapping *S.aureus*, could also offer a scaffold for the formation of bacteria biofilm and growth. Moreover, enhanced NET formation could favor the development of uncontrolled immunothrombosis, consequent to the interaction of neutrophils and NETs with platelets, the endothelium and the coagulation system. In the context of IE caused by *S.aureus*, it remains unclear whether NETs prevent or promote vegetation growth. Targeting the cellular components involved in the pathophysiology of IE and immunothrombosis could represent valid approaches to treat these disorders. However, further basic and translational research is needed to validate the use of antiplatelet and anticoagulant drugs and of strategies affecting NET formation and stability in IE and other immunothrombotic diseases.

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Affiliations

Department of Cardiovascular Sciences, Center for Molecular and Vascular Biology, KU Leuven, Belgium (S.M., M.C., P.V., K.M.). Department of Life Sciences, Manchester Metropolitan University, United Kingdom (M.C.).

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