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REVIEW

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"The NET effect": Neutrophil extracellular traps—a potential key component of the dysregulated host immune response in sepsis

Andrew Retter[1,2,3*], Mervyn Singer[4] and Djillali Annane[5,6,7,8]

Abstract

Neutrophils release neutrophil extracellular traps (NETs) as part of a healthy host immune response. NETs physically trap and kill pathogens as well as activating and facilitating crosstalk between immune cells and complement. Excessive or inadequately resolved NETs are implicated in the underlying pathophysiology of sepsis and other inflammatory diseases, including amplification of the inflammatory response and inducing thrombotic complications. Here, we review the growing evidence implicating neutrophils and NETs as central players in the dysregulated host immune response. We discuss potential strategies for modifying NETs to improve patient outcomes and the need for careful patient selection.

Keywords Neutrophil extracellular traps (NETs), Sepsis, Immunothrombosis, Thromboinflammation, Histones, Complement, DAMPs (damage-associated molecular patterns), Coagulation, NETosis, Inflammation, Innate immune response, Host immune response, Thrombosis

Background

Sepsis is characterised by a dysregulated host response to infection, leading to life-threatening organ dysfunction [1, 2]. With an estimated global incidence of 49 million cases and 11 million deaths annually, sepsis represents a significant public health challenge [2, 3]. Recent advancements in our understanding of sepsis immunobiology have led to a more nuanced conceptualisation, incorporating immune-driven resistance, tolerance, resilience, resolution and repair [4].

Central to this evolving paradigm is the concept of immunothrombosis, a host defence mechanism that integrates the immune and coagulation systems to contain and eliminate pathogens in the bloodstream [5, 6]. This process involves the coordinated activation of multiple cellular and molecular components, including neutrophils, platelets, monocytes, the complement system, damage-associated molecular patterns (DAMPs),

*Correspondence:
Andrew Retter
Andrew.Retter@gstt.nhs.uk
[1] Critical Care, Guy's and St Thomas' NHS Foundation Trust, London, UK
[2] School of Immunology and Microbial Sciences, King's College, London, UK
[3] Volition, London, UK
[4] Bloomsbury Institute of Intensive Care Medicine, Division of Medicine, University College London, London, UK
[5] Department of Intensive Care, Raymond Poincaré Hospital, APHP University Versailles Saint Quentin–University Paris Saclay, INSERM, Garches, France
[6] IHU PROMETHEUS, Comprehensive Sepsis Center, Garches, France
[7] University Versailles Saint Quentin–University Paris Saclay, INSERM, Garches, France
[8] FHU SEPSIS (Saclay and Paris Seine Nord Endeavour to PerSonalize Interventions for Sepsis), Garches, France

and coagulation factors [7, 8]. While immunothrombosis serves a protective role under normal circumstances, excessive or uncontrolled activation can lead to widespread thromboinflammation [8–11]. In addition, the role of DAMPs, host cellular constituents released from damaged or stressed cells, act as danger signals that trigger inflammatory responses through pattern recognition receptors (PRRs), such as Toll-like receptors (TLR) and NOD-like receptors [12, 13]. However, excess DAMPs can also play an important role in amplifying and perpetuating the inflammatory response in sepsis and directly contributing to organ dysfunction [12, 14].

Neutrophil extracellular traps (NETs) composed of extruded nuclear chromatin decorated with histones and granular proteins, serve to trap and neutralise pathogens [15]. However, excessive NET formation and/or dysregulated clearance have been implicated in exacerbating sepsis pathophysiology by promoting inflammation, tissue damage and thrombosis [16, 17]. The intricate balance between protective and detrimental properties underscores the complexity of the host response in sepsis. Understanding these mechanisms is crucial for developing targeted immunomodulatory therapies to improve patient outcomes [18]. This narrative review aims to:

1. Describe the growing evidence implicating neutrophils and NETs as central players in both host defence and the dysregulated immune response in sepsis
2. Explore the role of NETs in immunothrombosis and thromboinflammation
3. Discuss potential strategies for modifying NETs to improve patient outcomes.

By elucidating these complex interactions, we aim to provide insights into novel therapeutic approaches that can modulate the immune response and potentially mitigate tissue damage in sepsis and other systemic inflammatory conditions.

The history and discovery of NETs

Neutrophils play a critical role in the innate immune response to any inflammatory insult. However, they present unique challenges to study due to their intrinsic characteristics and to technical issues related to their investigation [19]. Our understanding of neutrophil pathobiology consequently lags well behind that of other immune cells. Neutrophils have a short lifespan, typically surviving 5–7 days in circulation, that is reduced once activated [20]. This short lifespan is beneficial for supporting a rapid response to infection but presents a significant obstacle for researchers attempting to isolate cells and expand them in vitro. Once isolated from blood, neutrophils remain viable for only a few hours, limiting the window for experimental manipulation and analysis. The process of isolating neutrophils also inadvertently activates them, altering their physiology and potentially confounding results [21]. Neutrophil activation triggers a variety of processes including degranulation and production of reactive oxygen species (ROS) that can affect their function and interactions with other cell types [22, 23]. This sensitivity means that even minor changes in isolation and handling techniques can lead to significant variability in experimental outcomes. Neutrophils also require the presence of other cells, in particular monocytes and platelets, to facilitate their activation. This makes in vitro study more complicated and harder to achieve consistency in experiments [21, 24]. Neutrophils also exhibit a surprising degree of functional heterogeneity, with subsets displaying distinct phenotypic and functional profiles [25], the full extent of which is still being explored [23]. Such complexity adds another layer of investigative difficulty in view of the potential influence of these different subpopulations on experimental outcomes. Neutrophils perform many of their key functions within tissues and not just in the bloodstream. Studying these cells in their native context requires sophisticated techniques such as intravital microscopy that are not universally available [26]. Ethical and technical considerations also limit the extent to which invasive studies can be applied to humans to observe neutrophil behaviour under physiological and pathological conditions. Neutrophils only constitute 10–25% of circulating white cells in rodents [27], so direct transferability of findings is uncertain.

In 2004, Brinkmann et al. observed that activated neutrophils released their nuclear contents forming extracellular fibres that could trap and kill bacteria (Fig. 1) [15]. This observation was initially met with scepticism as it challenged the conventional view of neutrophils as short-lived, primarily killing pathogens through phagocytosis and degranulation [28]. In 2007, Fuchs et al. demonstrated that NET formation was triggered by a novel form of active cell death, NETosis, which required generation of ROS by nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase, NOX), a key enzyme in the neutrophil respiratory burst [29]. NET release is induced by a wide range of stimuli, for instance, bacteria, viruses, fungi and parasites; pro-inflammatory mediators such as interleukin (IL)−8, lymphotoxin-alpha and tumour necrosis factor alpha (TNFα); platelets, activated endothelial cells, and components of the complement system [24, 30, 31].

NETs are composed of a mix of chromatin, histones, nucleosomes and granular-derived components such as neutrophil elastase (NE), myeloperoxidase (MPO) and

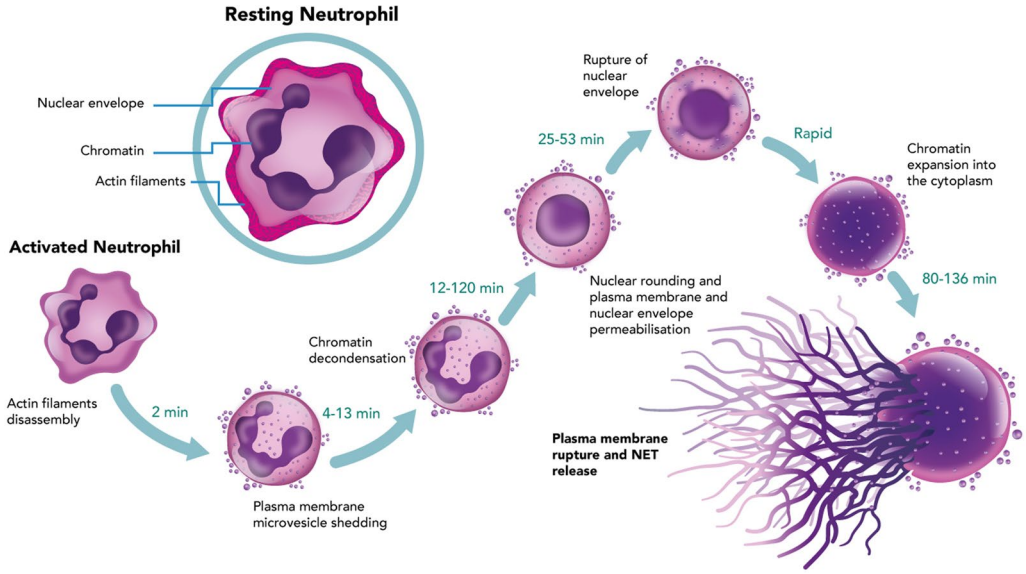

Fig. 1 Timeline showing the formation and release of NETs from an activated neutrophil. *Min* minute, *NET* neutrophil extracellular trap

cathepsin G [32, 33]. Both NE and cathepsin G are multifunctional neutrophil serine proteases with important roles in the inflammatory-immune response [33]. Their shared functions include: (i) antimicrobial properties that enable them to directly kill or inactivate pathogens [33, 34]; (ii) extracellular matrix degradation to facilitate cell migration and tissue remodelling during inflammation [33, 35]; (iii) neutrophil recruitment to sites of inflammation or infection by inducing neutrophil activation and chemotaxis; and (iv) proteolytical regulation of cytokines, chemokines and other inflammatory mediators [33, 36]. Cathepsin G also activates platelets, contributing to thrombosis [33, 36]. The heme-containing enzyme MPO is expressed by neutrophils and plays a crucial role in the innate immune response. It catalyses production of chlorinating oxidants, such as hypochlorous acid, facilitating oxidative killing of pathogens during phagocytosis [37, 38]. MPO can also modulate inflammation independent of its enzymatic properties by regulating neutrophil function and NET formation [37]. Extracellular histones can directly activate platelets, promoting their pro-inflammatory and pro-thrombotic functions [39]. In turn, activated platelets can trigger neutrophils to undergo NETosis [30], creating a positive feedback loop of inflammation and tissue damage.

The process of NETosis is pivotal for trapping and neutralising pathogens, preventing their spread, and facilitating their clearance [30]. However, dysregulated NET formation and clearance have been implicated in a broad spectrum of diseases, highlighting a paradoxical role in both defending against infection yet exacerbating disease pathology [40, 41]. Excessive or inadequately resolved NETs contribute to the pathology of chronic inflammatory and autoimmune diseases by promoting inflammation, tissue damage and thrombosis [42]. For example, high levels and increased activity of cathepsin G have been linked to the pathogenesis of rheumatoid arthritis and systemic lupus erythematosus (SLE) [36]; uncontrolled NET activity has been implicated in acute respiratory distress syndrome (ARDS) [43]; while elevated plasma levels of MPO are frequently detected in patients with sepsis [37]. In the context of sepsis, uncontrolled NET formation exacerbates endothelial damage and can promote microvascular thrombosis [5, 8]. The persistence of NET components can act as autoantigens, triggering autoimmune responses in susceptible individuals [44]. Targeting NETs and their regulatory mechanisms presents a promising therapeutic avenue to modulate immune responses, mitigate tissue damage, and improve outcomes in a range of inflammatory and autoimmune diseases [45].

Mechanisms of NETosis

NETosis is induced by three distinct mechanisms: suicidal, vital and mitochondrial NETosis (Fig. 2). Suicidal NETosis takes several hours to complete compared with the rapid mechanisms of vital and mitochondrial NETosis that do not involve cell death [31, 46]. The distinction between these mechanisms has implications in sepsis. Suicidal NETosis can contribute to the depletion of neutrophils and can potentially lead to immunosuppression, a frequent problem in late-stage sepsis. Conversely, vital and mitochondrial NETosis allow neutrophils to continue their antimicrobial functions even after NET

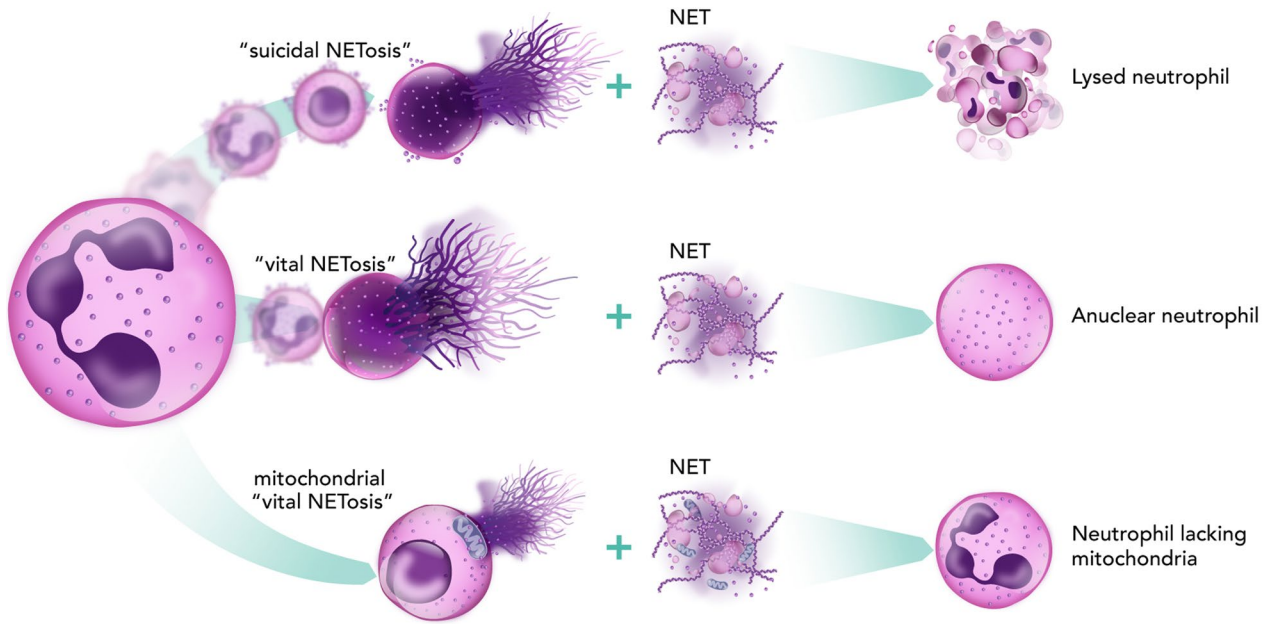

Fig. 2 Three different types of NETosis: suicidal, vital and mitochondrial. *NET* neutrophil extracellular trap. NETosis represents a specialised form of programmed cell death wherein neutrophils release NETs, complex structures composed of chromatin fibres, histones, and antimicrobial proteins. This process, first described by Brinkmann et al. [15], has emerged as a crucial mechanism in innate immunity and inflammation. Three distinct forms of NETosis have been identified: suicidal, vital, and mitochondrial NETosis. Suicidal NETosis, the classical pathway, involves a terminal process resulting in neutrophil lysis. This mechanism is characterised by chromatin decondensation, nuclear membrane breakdown, and mixing of nuclear contents with granular antimicrobial proteins [29]. The process typically requires 2–4 h and involves the generation of reactive oxygen species (ROS) through NADPH oxidase activation. Peptidylarginine deiminase 4 (PAD4) catalyses histone citrullination, facilitating chromatin decondensation. The final stage involves plasma membrane rupture and NET release, leaving behind a lysed neutrophil [40]. Vital NETosis, in contrast, represents a rapid response mechanism occurring within 30–60 min, wherein neutrophils remain viable and retain their antimicrobial functions after NET release [187]. This process, often triggered by bacterial components or inflammatory stimuli, involves the expulsion of nuclear DNA through vesicular transport while maintaining plasma membrane integrity. The resulting anuclear neutrophils continue to perform essential functions such as phagocytosis and chemotaxis, representing an evolutionary adaptation that preserves neutrophil functionality while deploying NETs [188]. Mitochondrial NETosis represents a distinct pathway characterised by the release of mitochondrial rather than nuclear DNA. This mechanism, first reported by in 2009 [189], occurs in response to specific stimuli and results in neutrophils lacking mitochondria but maintaining nuclear integrity. The process involves selective packaging of mitochondrial DNA with antimicrobial proteins, followed by their controlled release. This form of NETosis may represent a less destructive response mechanism compared with suicidal NETosis. The figure illustrates these three distinct NETosis pathways, highlighting their unique characteristics and outcomes. The top panel shows suicidal NETosis, resulting in complete cell lysis and NET release. The middle panel depicts vital NETosis, where the neutrophil remains intact as an anuclear cell following NET release. The bottom panel illustrates mitochondrial NETosis, showing the selective release of mitochondrial DNA while maintaining cellular integrity

release. Notably, mitochondrial NETosis is a major source of extracellular mitochondrial DNA in the plasma of patients with sepsis; levels correlate with disease severity and are associated with poor clinical outcomes [47]. Understanding these mechanisms will be critical in developing targeted therapies for sepsis, aiming to balance the beneficial antimicrobial effects of NETs with the potential for tissue damage and inflammation.

NETosis as a primary source of DAMPs

NETosis is a significant source of extracellular nucleosomes and histones in sepsis [15, 40]. Hypoxia, oxidative stress and direct injury can lead to cellular damage [48]. This may result in necrotic, pyroptotic, and/or apoptotic cell death, with release of intracellular contents, including DNA, nucleosomes and histones, directly into the extracellular space [49, 50]. Here they can exist freely, packaged in extracellular vesicles, or as part of NETs [15, 17, 40, 51, 52], and can by themselves act as DAMPs, exerting profound effects on the immune system and coagulation cascade [12, 14, 50, 51].

The overwhelming nature of sepsis may compromise the body's ability to clear apoptotic cells and cellular debris efficiently, leading to the accumulation of extracellular nucleosomes and histones. PRRs, particularly TLR2, TLR4 and TLR9, play a key role in recognising nucleosomes and histones as DAMPs [51, 53]. Extracellular histones directly bind to and activate TLR4 on immune cells, triggering nuclear factor kappa B-mediated signalling and production of pro-inflammatory cytokines,

TNF-α, IL-1β and IL-6 (Fig. 3) [54, 55]. Histones may act synergistically with other TLR ligands, such as lipopolysaccharide, to enhance inflammatory responses [56]. Histones can also induce activation of the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome, a macromolecular protein complex and key regulator of the innate immune response [57, 58]. Canonical NLRP3 inflammasome activation occurs in two stages [59, 60]. The initial priming signal can arise from DAMP-stimulated TLR-mediated signalling, inducing the upregulation of NLRP3 and pro-inflammatory cytokines [59, 61]. DAMPs can also trigger the second 'hit' required for NLRP3 inflammasome assembly and activation, leading to caspase-1 stimulation and IL-1β release, by activating multiple upstream events [59, 61, 62]. Release of IL-1β further amplifies the inflammatory response in sepsis, contributing to organ dysfunction.

Excessive NET formation with subsequent release of DAMPs can lead to tissue damage, organ dysfunction and the perpetuation of the inflammatory response, contributing to the pathogenesis of inflammatory and autoimmune diseases including sepsis, acute lung injury, and SLE [51, 63]. A recent study by Malamud et al. demonstrated that myeloid inhibitory C-type lectin-like receptor (MICL/CLEC12A) regulates NET formation by directly recognising NET-associated DNA. The researchers found that MICL deficiency or inhibition leads to uncontrolled NET formation through the ROS-PAD4 pathway, creating an auto-inflammatory feedback loop [64]. Loss of MICL functionality exacerbated joint inflammation in rheumatoid arthritis models. MICL acts as a PRR for NETs on neutrophils, inhibiting further neutrophil activation and NET formation upon recognition. Of note, the authors detected similarly inhibitory anti-MICL autoantibodies in patients with lupus and severe coronavirus disease 2019 (COVID-19).

NETs play a central role in the innate immune response and immunothrombosis

In addition to their crucial role in trapping and killing pathogens, NETs coordinate immune responses via their interactions with immune cells and mediators [40]. For example, NETs activate plasmacytoid dendritic cells by engaging TLR9 on the cell surface and stimulating production of type I interferons (IFNs). Type I IFNs are critical for activating the adaptive immune response,

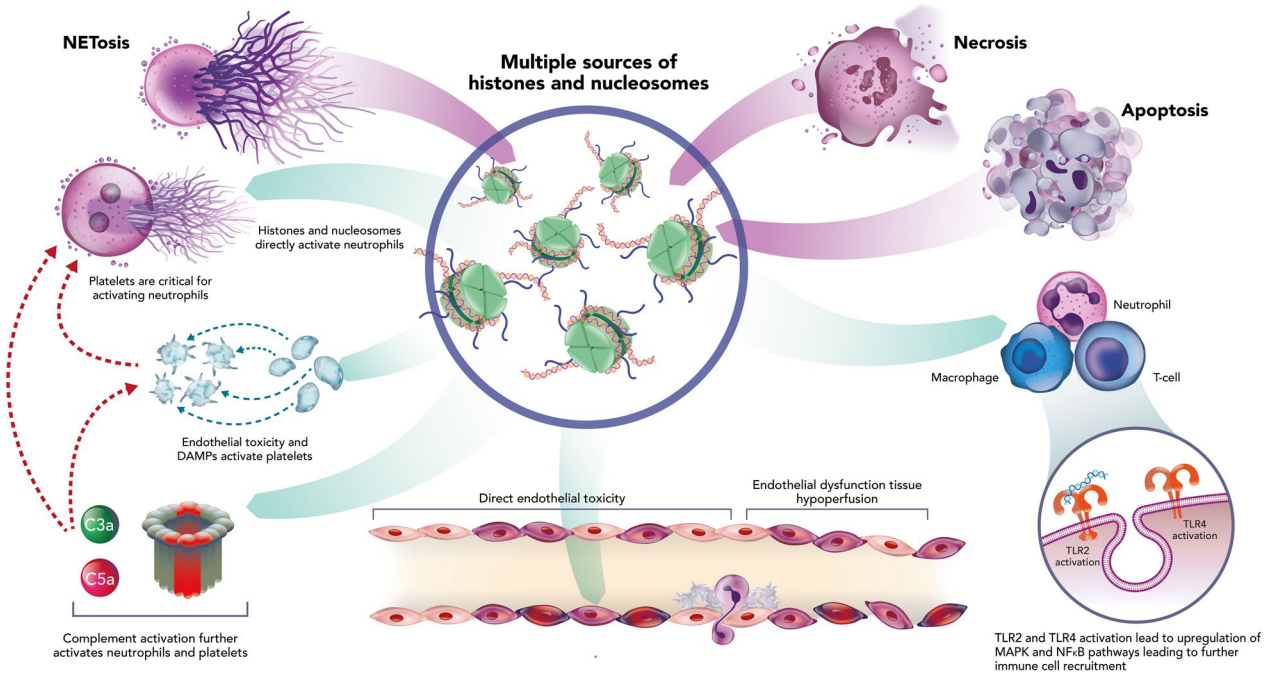

Fig. 3 The role of histones and nucleosomes in the dysregulated host immune response. Apoptosis, necrosis and NETosis are all sources of extracellular histones. During infection NETosis is the predominant source of histones and nucleosomes (> 80%). Histones are strongly cationic and directly toxic to cell membranes. This promotes endothelial dysfunction and activation of platelets and neutrophils. Neutrophils, platelets and complement are all directly activated by histones, leading to a forward positive feedback loop. Histones can also directly activate other immune cells through interactions with TLR2 and 4, activating the pro-inflammatory mitogen-activated protein kinase (MAPK) and NLRP3 inflammasome. This figure is a reproduction and adaption from [51] under the Creative Commons Attribution 4.0 International License, (https://creativecommons.org/licenses/by/4.0/) with permission from E Silk. *DAMPs* damage-associated molecular patterns, *MAPK* mitogen-activated protein kinase, *NF-κB* nuclear factor kappa B, *NLRP3* NOD-, LRR- and pyrin domain-containing protein 3, *TLR* toll-like receptor

including antigen-specific T- and B-cell responses [65, 66]. In vitro NETs activate T cells directly by lowering their activation threshold and inducing co-stimulatory signalling [67]. They also interact with macrophages, promoting cytokine release and amplifying the immune response [68]. These interactions highlight the central role of NETs in orchestrating the complex interplay between innate and adaptive immunity [69]. Considerable crosstalk exists between neutrophils and macrophages. In sepsis, macrophage pyroptosis, a highly inflammatory form of lytic-programmed cell death regulates NET formation. Pyroptotic macrophage-derived microvesicles cause tissue damage and can activate coagulation pathways [70]. While neutrophils can endocytose pyroptotic microvesicles containing macrophage mitochondria, this induces neutrophil mitochondrial dysfunction and further NET formation via the mitochondrial ROS/Gasdermin D axis [70]. Damage to the endothelium during sepsis may also be driven by the ability of activated endothelial cells to induce NET formation and their susceptibility to NET-mediated cell death [71]. This endothelial damage is a key component of thromboinflammation, contributing to microvascular dysfunction and organ failure in sepsis [72].

Another important aspect of NET function is their role in complement activation. Complement components such as properdin, C3 and factor B have been found deposited on NETs [7, 73, 74]. Together with NET-associated granular proteins and DAMPs, these are responsible for NET-mediated complement activation [7, 73, 74]. Extracellular histones and nucleosomes induce complement activation via interactions with PRRs on the surface of immune cells [51]. Additional NET formation can be triggered by C3a and C5b, propagating the complement pathway further [7, 75]. C1q, the recognition molecule of the classical complement pathway, can bind directly to NETs. This binding is facilitated by the positively charged globular heads of C1q interacting with the negatively charged DNA backbone of NETs. C1q-opsonised NETs are more efficiently phagocytosed by macrophages, aiding in the clearance of NETs and potential pathogens trapped within them, reducing the potential for NET-mediated tissue damage [76]. The interaction between NETs and complement proteins can create a positive feedback loop, enhancing local inflammation, immune responses and procoagulant activities.

NETs, complement and thrombosis

Through their dual role in pathogen capture and the promotion of thrombosis, NETs help to orchestrate immunothrombosis; a process that is both protective but potentially harmful in various disease states [5]. They contribute to thrombin generation through several mechanisms, including activation of the intrinsic coagulation pathway, inactivation of endogenous anticoagulants, and provision of a scaffold for the assembly of coagulation factors [77]. In addition, nucleosome and histone components also play a significant role in the dysregulated coagulation observed in sepsis.

Coagulation and thrombin formation can be promoted by the histone component of NETs, inducing platelet activation and aggregation via TLR2- and TLR4-mediated pathways [56]. Histone-induced platelet activation results in the exposure of phosphatidylserine on the platelet surface, providing a procoagulant surface [56, 78]. Increased platelet activation directly enhances and propagates thrombin generation, leading to a feed-forward loop supporting further platelet activation. Besides platelets, histones can induce surface expression of phosphatidylserine on red blood cells in vitro, supporting prothrombinase activity and shortening clotting times in plasma [79]. Histones also have the capacity to inactivate endogenous anticoagulants, such as tissue factor (TF) pathway inhibitor and thrombomodulin, promoting further thrombin generation and suppression of protein C activation [80, 81].

Excessive NET release triggers thrombin generation by activating the intrinsic coagulation pathway [82] through exposure of negative charges on damaged cells or foreign surfaces [83]. This excessive NET formation and subsequent activation of coagulation cascades is a hallmark of thromboinflammation, where the protective functions of immunothrombosis become dysregulated [10]. Circulating free nucleosomes, released during excessive NETosis provide the negative charge that can directly activate factor XII (FXII) [7, 84]. Activation of FXII by nucleosomes generates FXIIa which subsequently activates FXI (Factor XI) and FIX (Factor IX). Activation of FIX leads to formation of the tenase complex (FIXa-FVIIIa) which activates FX [85]. FXa then forms a prothrombinase complex with FVa, leading to thrombin generation. The negatively charged DNA backbone of NETs can therefore bind and concentrate coagulation factors, facilitating their activation and the formation of the tenase and prothrombinase complexes (Fig. 4) [86]. This NET-mediated assembly of coagulation factors contributing to thrombosis development occurs independently of the platelet or erythrocyte surface [87].

There is evidence that nucleosomes, NET-associated histones and free histones can initiate and propagate the extrinsic coagulation pathway. Extracellular histones can disrupt the integrity of the endothelial barrier, exposing the procoagulant subendothelial matrix to circulating factors [84, 88]. They can also induce endothelial expression of TF via TLR4- and TLR2-mediated signalling, leading to autoactivation of FVII-activating protease [89,

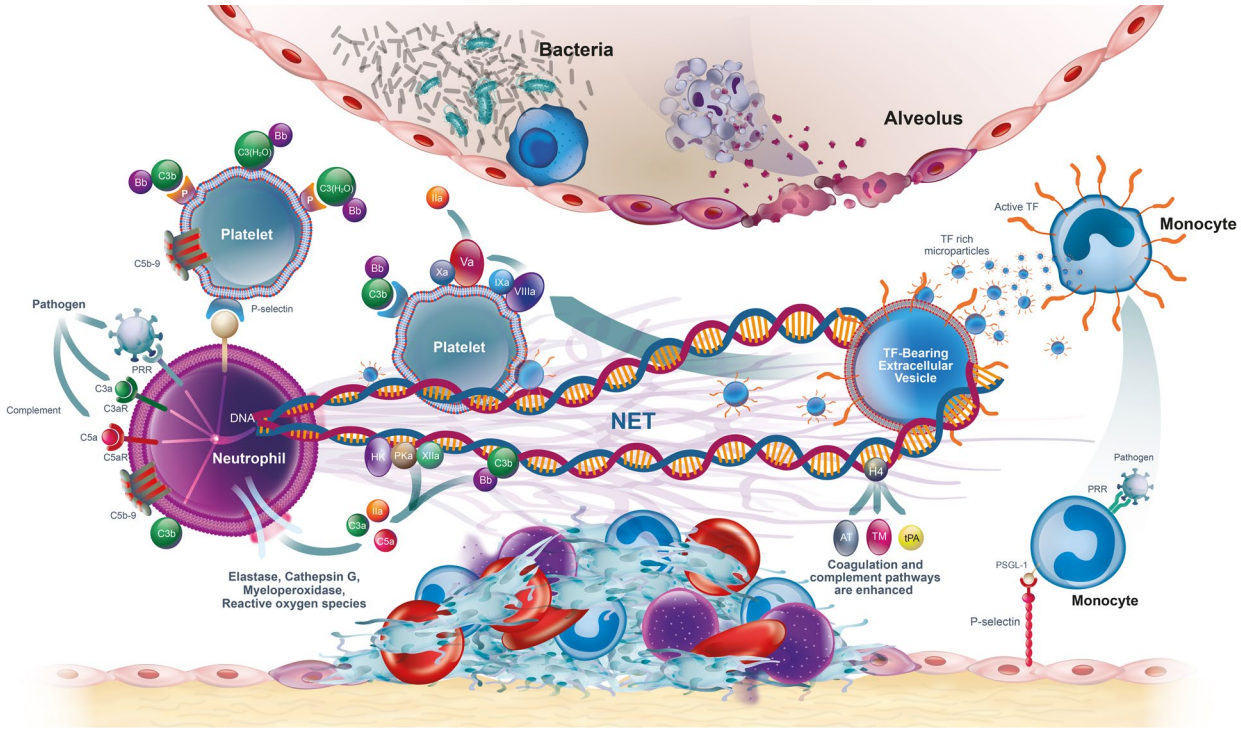

Fig. 4 Pathogenic invasion of a pulmonary capillary, illustrating the link between thrombin generation, complement and NETs. Infection of endothelial cells disrupts the basement membrane, stimulating cell-surface expression of TF and P-selectin and, therein, the recruitment of neutrophils and platelets. Activation of complement promotes further activation of platelets and neutrophils. Platelets are activated by both the classical and alternative complement pathways. C3b can directly bind to and activate neutrophils. Neutrophils have additional receptors for C5a and C3a. Platelets can activate neutrophils directly by P-selectin glycoprotein ligand-1 (PSGL-1). NETs catch and bind platelets providing a scaffold to further support thrombin generation and the recruitment of immune cells. Monocytes detect the invading pathogen and are recruited to the site of endothelial damage/disruption. TF-bearing microvesicles released by macrophages become ensnared in the NETs. The negative charge of the NETs activates FXII, initiating the intrinsic pathway of coagulation that results in further thrombin generation. Finally, histones in NETs antagonise the action of the natural anticoagulants, thrombomodulin and activated protein C. As NETs can extend over 50 µm, with the normal diameter of a pulmonary capillary being 20–30 µm, there is a potential risk of complete obstruction of the microcirculation. This figure is a reproduction and adaption from [7] under the Creative Commons Attribution 4.0 International License, (https://creativecommons.org/licenses/by/4.0/) with permission from E.L.G Pryzdial. *AT* antithrombin, *FXII* factor XII, *HK* high molecular weight kininogen, *NET* neutrophil extracellular trap, *P* properdin, *PKa* kallikrein, *PRR* pattern recognition receptor, *P-selectin* platelet selectin, *PSGL-1* P-selectin glycoprotein ligand-1, *TF* tissue factor, *TM* thrombomodulin, *tPA* tissue-type plasminogen activator

90]. Histone-induced endothelial activation also promotes the release of von Willebrand factor from endothelial cells, enhancing platelet adhesion and aggregation [91]. The involvement of NETs and associated DAMPs in both intrinsic and extrinsic coagulation pathways exemplifies how NETs serve as a critical link between innate immunity and coagulation in immunothrombosis [84, 86].

NETosis has been implicated in coagulopathies associated with conditions such as sepsis, COVID-19 and autoimmune disorders (SLE, antiphospholipid syndrome [APS]) [6, 41, 92]. These conditions represent states of excessive thromboinflammation [93]. In sepsis, excessive NET formation has been associated with the development of sepsis-induced coagulopathy and multiple organ failure [94, 95]. NETs contributed to the hypercoagulable state in patients with COVID-19, potentially explaining the high incidence of thrombotic complications in severe cases [96]. In SLE and APS, NETs are implicated in the development of thrombosis and cardiovascular disease in affected individuals [97]. Their role in cardiovascular disease has been reviewed in detail by Stark and Massberg [8]. The most extreme form of systemic coagulopathy, disseminated intravascular coagulation, is associated with severe bacterial infections [9, 98, 99]. Sepsis-induced coagulopathy develops in about 35% of sepsis cases [9]. Understanding the role of NETs in these pathological states of thromboinflammation is crucial for developing targeted therapies to modulate the immune response and improve patient outcomes [100].

The complement system and NETs engage in bidirectional interactions that can amplify inflammatory responses following trauma. Complement activation, particularly through C5a-C5aR signalling, triggers NET formation via NADPH oxidase-dependent ROS generation and peptidylarginine deiminase 4 (PAD4)-mediated histone citrullination [15, 87]. In turn, NETs activate complement through multiple mechanisms: extruded DNA and histones serve as platforms for C1q binding to initiate the classical pathway, while NET-associated proteases can directly cleave C3 and C5 to generate anaphylatoxins [74]. This creates a potentially harmful positive feedback loop in trauma patients, as NET-induced complement activation recruits additional neutrophils and promotes further NET formation, potentially contributing to organ dysfunction. Therapeutic strategies targeting this axis show promise—C5a inhibition reduces NET formation in experimental models, while DNase treatment both degrades NETs and limits complement activation [101, 102]. While this complement-NET interaction represents a key mechanism linking innate immunity and inflammation in trauma, further research is needed to fully characterise the spatial and temporal dynamics of these interactions and optimise therapeutic approaches.

Some microorganisms subvert the antimicrobial properties of NETs

While NETs play a crucial role in the innate immune response against invading microorganisms, some pathogens have evolved strategies that can manipulate NETs to enhance their survival and increase pathogenicity (Table 1) [4, 103–117]. These strategies include evading NET capture, resisting NET-mediated killing, degrading NETs, and exploiting NETs for enhanced virulence [111, 118–120]. To evade NET capture some pathogens have evolved surface modifications that reduce NET binding, allowing them to escape entrapment and destruction [111]. For example, *Streptococcus pneumoniae* has a negatively charged polysaccharide capsule that creates an electrostatic repulsion with the negatively charged DNA backbone of NETs, thereby preventing entrapment [112, 121]. Similarly, *Klebsiella pneumoniae* produces a capsule that inhibits NET formation and reduces bacterial capture [122]. Other bacteria modify their surface proteins to evade NET capture [103]. Microorganisms can also work cooperatively, forming biofilms that act as physical barriers, preventing neutrophil infiltration and NET formation [118, 120, 123]. Others have developed mechanisms to resist the antimicrobial effects of NETs, enabling them to survive and propagate within these structures [124]. Many bacteria produce nuclease enzymes or hijack host DNases to degrade and destroy the DNA backbone of NETs [104, 105, 112, 120, 125]. These include *Streptococcus pyogenes* which secretes DNase Sda1 and *Staphylococcus aureus* which induces release of host DNases [106, 120]. In contrast, LasR-deficient *Pseudomonas aeruginosa*, *Yersinia pestis*, and *Bordetella pertussis* strains reduce or prevent NET formation [116, 126, 127]. In *Mycobacterium tuberculosis* infections, NET formation in tuberculosis lesions may contribute to tissue damage and exacerbate inflammation, potentially facilitating bacterial dissemination and disease progression [127]. The mechanisms employed by microorganisms to manipulate NETs and their consequences for pathogenicity and survival are summarised in Table 1.

Future strategies to manipulate NETs and improve patient outcomes

The pathological impact of excessive or dysregulated NET formation offers potential therapeutic targets for a range of conditions such as sepsis, thrombosis and autoimmune disorders. Recognising that NETs and associated DAMPs are critical mediators of inflammation and coagulopathy in sepsis has opened new avenues for therapeutic interventions. Key therapeutic strategies include inhibition of NET formation, promotion of specific NET degradation and targeting specific NET components (Table 2) [128–146].

Inhibition of NET formation

NET formation can be inhibited by targeting molecular pathways underlying NETosis, but whether partial or full inhibition of NET formation should be targeted still needs to be considered. Potential inhibitors of NET formation include small molecules, peptides and antibodies. PAD4 is a key enzyme in the NETosis pathway that catalyses histone citrullination leading to chromatin decondensation and NET formation [128]. Inhibition of PAD4 reduced NET formation both in vitro and in vivo [147]. Cl-amidine, a small-molecule PAD4 inhibitor, attenuated NET formation and improved outcomes in animal models of sepsis, thrombosis and autoimmune diseases [129–131]. GSK484, another PAD4 inhibitor, was well tolerated in a phase 1 clinical trial in healthy volunteers [132]. However, the efficacy of PAD4 inhibitors in human disease remains to be determined. Long-term inhibition of PAD4 may also have unintended consequences on other cellular processes, such as gene regulation and cell differentiation [148].

NOX, a key enzyme in the generation of ROS, is essential for NET formation [29]. Inhibition of NOX reduced NET formation both in vitro and in vivo [149]. Diphenylene iodonium (DPI), a non-specific NOX inhibitor, inhibited NET formation in models of sepsis and lupus nephritis. However, DPI has off-target effects and

Table 1 Mechanisms employed by microorganisms to manipulate NETs and their consequences for pathogenicity and survival

Microorganism	Mechanism of NET manipulation	Consequence for pathogenicity and survival
S. aureus	• Secretion of Nuc and AdsA enzymes degrades surrounding NET DNA, reducing entrapment and generating deoxyadenosine [104, 107] • Deoxyadenosine triggers macrophage apoptosis and disrupts efferocytosis of apoptotic neutrophils and NETs [107] • *S. aureus* biofilms secrete leukocidins, Panton-Valentine leucocidin (PVL) and HIgAB to promote and inhibit NETosis allowing them to evade capture [108]	Facilitates persistence of infections [104, 107]
S. agalactiae (Group B Streptococcus, GBS)	• Some species of *S. agalactiae* secrete the nuclease, NucA, to degrade the DNA NET matrix enabling them to evade NET entrapment [105]	Promotes virulence and persisting infection [105]
S. pyogenes (Group A Streptococcus, GAS)	• Surface M protein binds fibrinogen forming a protective coat around the bacteria [103] • Secretion of DNase Sda1 facilitates bacterial survival and escape from NETs by degrading the DNA matrix [106] • DNase Sda1 activity may exert a selective pressure that increases virulence, shifting the infection from local to systemic [109]	Promotes bacterial survival and dissemination, increasing pathogenicity and facilitating tissue infiltration [106, 109]
N. meningitidis (meningococcus, Nm)	• Modification of lipid A of meningococcal lipopolysaccharide (LPS) with PEA protects the organism from bactericidal activity of NET-bound cathepsin G [110] • Expression of the high-affinity zinc uptake receptor ZnuD protects meningococci from NET-mediated nutritional immunity [110] • Spontaneously released outer membrane vesicles inhibit *N. meningitidis* binding to NETs by saturating meningococcal binding sites, neutralising NET action [110]	Promotes bacterial survival, dissemination and rapid progression of meningococcal disease [110]
S. pneumoniae (Pneumococcus)	*S. pneumoniae* has evolved several strategies to evade and exploit NETs Capsule protects against NET trapping: • The polysaccharide capsule of *S. pneumoniae* reduces bacterial trapping in NETs by 4–12 fold compared to non-encapsulated strains [111]. This allows encapsulated pneumococci to avoid confinement by NETS Resistance to NET-mediated killing: • Unlike many pathogens, encapsulated *S. pneumoniae* is not killed by antimicrobial components in NETs [112, 113]. This allows trapped bacteria to survive Endonuclease degrades NETs: • *S. pneumoniae* expresses a surface endonuclease called EndA that degrades the DNA scaffold of NETs, allowing bacteria to escape [112]. EndA-deficient mutants remain trapped in NETs and show reduced virulence in mouse models D-alanylation of lipoteichoic acids: • Modification of lipoteichoic acids with D-alanine introduces positive charge to the bacterial surface, reducing susceptibility to cationic antimicrobial peptides in NETs [111]. This promotes resistance to NET killing Pneumolysin activates NET formation: • The pneumococcal toxin pneumolysin induces NET formation [114]. While this may seem counterproductive, it may benefit the bacteria by depleting neutrophils Biofilm formation: • Pneumolysin promotes biofilm formation, which may help pneumococci evade capture by NETs [4]	Escaping NETs promotes spread of *Pneumococci* from the upper respiratory tract to the lung, and from lung to bloodstream during pneumonia

Table 1 (continued)

Microorganism	Mechanism of NET manipulation	Consequence for pathogenicity and survival
Y. pestis	• Platelets intoxicated by *Y. pestis* induced Type 3 secretion system expression, have a diminished response to prothrombotic stimuli. The T3SS allows *Y. pestis* to escape entrapment in platelet-neutrophil thrombi. Spinner et al. show that *Y. pestis* with Type 2 secretion system grown at 37°C inhibits neutrophil production of ROS, attenuating NET formation and reducing exposure to the antimicrobial effects of NETs [115, 116]	Facilitates bacterial survival and dissemination of infection by enabling *Y. pestis* to reduce NETosis in platelet thrombi [115, 116]
B. pertussis	• Secretion of adenylate cyclase toxin by *B. pertussis* generates supraphysiological levels of intracellular cAMP (Cyclic AMP), preventing the oxidative burst required for NETosis [117] • Adenylate cyclase toxin impairs neutrophil apoptosis and neutrophil-mediated phagocytosis [117]	Facilitates bacterial survival and dissemination; potentially influences local tissue damage during infection [117]
LasR-deficient Pseudomonas	• *LasR-deficient* strains alter both the quantity and quality of NETs, reducing overall NET formation while changing the spatial distribution of key antimicrobial proteins like BPI and neutrophil elastase on NET structures [126] • The reduction in NET formation occurs through LasR-regulated virulence factors (elastase LasB and protease LasA)	Up to 63% of chronically infected cystic fibrosis patients have *P. aeruginosa* with inactivating mutations in lasR, suggesting selective pressure for this adaptation [126] The ability to reduce NET formation represents a specific bacterial immune evasion strategy that may contribute to persistent infection in these patients

ACT adenylate cyclase toxin, *B. pertussis Bordetella pertussis*, *cAMP* cyclic AMP, *HlgAB* γ-haemolysin AB, *LPS* lipopolysaccharide, *N. meningitidis Neisseria meningitidis*, *NET* neutrophil extracellular trap, *PVL* Panton-Valentine leucocidin, *PEA* phosphoethanolamine, *ROS* reactive oxygen species, *S. agalactiae Streptococcus agalactaie*, *S. aureus Staphylococcus aureus*, *S. pneumoniae Streptococcus pneumoniae*, *S. pyogenes Streptococcus pyogenes*, *T3SS* type III secretion system, *Y. pestis Yersinia pestis*

Table 2 Potential anti-NET therapeutics

Therapeutic strategy	Agent or device	Description	References
Inhibition of NET formation	Cl-amidine	Small-molecule PAD4 inhibitor	[129–131]
	GSK484	Reversible, selective PAD4 inhibitor	[132]
	DPI	Small-molecule, non-specific NOX inhibitor	[133]
	GSK2795039	Small-molecule NOX 2 inhibitor	[134]
	APX-115	Specific NOX inhibitor	[135, 136]
Promotion of NET degradation	Dornase alfa (rhDNase I)	NET DNA	[137]
	C1q	DNase 1	[138]
Targeting NET components	AZD3241	Small-molecule selective and irreversible MPO inhibitor	[140]
	Sivelestat	Small-molecule NE inhibitor	[128, 141–143]
	3D2D-APC, 3D2D2A-APC	Rationally designed recombinant protein variants of APC	[174]
	M6229	Low-anticoagulant fraction of unfractionated heparin (UFH)	[139]
	NucleoCapture™	Therapeutic apheresis device that selectively removes NETs from blood	[144–146]

APC activated protein C, *DPI* diphenyleneiodonium, *MPO* myeloperoxidase, *NADPH* nicotinamide adenine dinucleotide phosphate, *NE* neutrophil elastase, *NET* neutrophil extracellular trap, *NOX* NADPH oxidase, *PAD4* peptidylarginine deiminase 4, *rhDNase I* recombinant human DNase I, *UFH* unfractionated heparin

can inhibit other flavoenzymes, limiting its therapeutic potential [133]. More specific NOX inhibitors, such as GSK2795039 and APX-115, show promise in preclinical studies [134–136]. Nevertheless, the potential risks of NOX inhibition should be considered, as ROS play important roles in host defence and cell signalling [150].

Promotion of NET degradation

Another therapeutic strategy is to promote NET degradation, either by enhancing activity of endogenous DNases or by administering such enzymes exogenously. Recombinant human DNase I (rhDNase I) degrades NETs [151]. Improved outcomes were seen in animal models of sepsis, acute lung injury and autoimmune diseases following administration of rhDNase I [151–153]. In humans, rhDNase I (dornase alfa) is approved for the treatment of cystic fibrosis where it reduces sputum viscoelasticity and improves lung function [137, 154, 155]. rhDNase I showed preclinical effectiveness against severe acute respiratory syndrome coronavirus 2 infection [151]. However, the efficacy of this therapy in other conditions associated with excessive NET formation remains to be established. Recent research has highlighted DNase's potential in treating thrombotic conditions, where NETs play a crucial role in promoting thrombosis [40]. Studies have demonstrated that NETs provide a scaffold for platelet adhesion and activation, contributing to thrombus formation. DNase has shown promise in treating thrombosis by degrading NET structures that promote blood clot formation. Clinical studies found DNase treatment reduced thrombus size and improved outcomes in conditions like deep vein thrombosis [156]. In experimental models, recombinant human DNase prevented thrombosis and reduced mortality [157, 158].

The therapeutic potential of DNase extends beyond its direct NET-degrading properties. Research suggests that DNase treatment may modulate inflammatory responses by reducing the availability of NET-associated DAMPs [159]. This reduction in inflammatory stimuli could contribute to breaking the cycle of chronic inflammation observed in many NET-associated pathologies. However, challenges remain in optimising DNase therapy [160]. The timing of administration, delivery methods, and potential combination with other therapeutic agents require further investigation. Potential risks of rhDNase I treatment, such as the development of anti-DNase antibodies and the impairment of host defence, need to be considered.

Endogenous DNases, such as DNase1 and DNase1-like 3, play a crucial role in NET degradation [161]. Deficiency or inhibition of these DNases is associated with accumulation of NETs and development of autoimmune diseases such as SLE [162]. Enhancing activity of endogenous DNases may represent another therapeutic strategy to promote NET degradation. C1q, a component of the complement system, enhances DNase1 activity and facilitates NET degradation [76]. Administration of C1q reduced NET accumulation and attenuated disease severity in animal models of SLE [138]. However, the therapeutic potential of C1q in human diseases remains to be investigated and the potential risks of modulating the complement system should be considered [163].

DNase has been administered through several routes across different clinical contexts. The most established approach is inhaled or nebulised DNase (dornase alfa/Pulmozyme), which is primarily used in respiratory conditions like cystic fibrosis, bronchiolitis and COVID-19, delivered via nebuliser directly to airways without

systemic absorption [164, 165]. In experimental settings, particularly in mouse models, DNase has been administered through intraperitoneal (IP) injection demonstrating that DNase at doses around 20 mg/kg could be effective, with timing being a crucial factor; administration at 4 or 6 h post-infection showed better outcomes than immediate treatment [152]. Intratracheal (IT) administration has also been studied in experimental models, with direct instillation into the trachea. Lefrançais et al. (2018) used this method in their research, administering doses of 2,000–4,000 units at regular intervals of 8–10 h [155]. The timing of administration appears crucial to therapeutic success, with evidence suggesting that delayed administration (4–6 h after infection) may be more beneficial than immediate treatment in some contexts [152]. While human trials have primarily focused on inhaled DNase, intravenous administration remains largely experimental, requiring further research to establish optimal protocols and safety parameters.

Targeting NET components

As described above, NETs comprise many components, including nucleosomes, histones, MPO and NE, which contribute to NET-mediated pathology [166]. Targeting these components may also be potentially advantageous, for example, histones exert cytotoxic and pro-inflammatory effects [53]. Neutralisation of histones with antibodies or small molecules (e.g., M6229, a low-anticoagulant fraction of unfractionated heparin) attenuated NET-mediated tissue damage and improved outcomes in animal models of sepsis, trauma and autoimmune diseases [139, 167]. A phase 1 study (NCT05208112) of M6229 in critically ill adults with sepsis has recently been completed [168]. The potential risks of modulating histone functions, such as impaired host defence and altered gene regulation, should be considered. MPO, a key enzyme in the generation of hypochlorous acid, is a potent oxidant [169], contributing to the antimicrobial and pro-inflammatory effects of NETs [170]. Inhibition of MPO reduced NET formation and attenuated NET-mediated tissue damage both in vitro and in vivo [171]. AZD3241, a small-molecule MPO inhibitor, has been evaluated in clinical trials for the treatment of neurodegenerative brain disorders [140]. However, their efficacy in NET-associated pathologies remains to be investigated and the potential risks of MPO inhibition, such as increased susceptibility to infection, should be considered [37]. Activated protein C (APC) can cleave histone H3, reducing their cytotoxicity [172]. However, due to the anticoagulant properties of APC and increased risk of bleeding, recombinant APC is no longer used therapeutically [173]. Two APC variants (3D2D-APC and 3D2D2A-APC) were designed with reduced anticoagulant activity and shown to have increased binding for H3 and proteolytic activity, reducing its cytotoxic effects on endothelial cells [174]. NE, a serine protease that contributes to the proteolytic activity of NETs, has been implicated in the pathogenesis of various inflammatory and autoimmune diseases [33]. Sivelestat, a small-molecule NE inhibitor, reduced NET formation and attenuated NET-mediated tissue damage both in vitro and in vivo [128, 141]. Sivelestat has been investigated clinically for treatment of acute lung injury and ARDS [142, 143] but its efficacy and potential risks, such as impaired host defence, in other NET-associated diseases remains to be determined [175]. The administration of DNase may reduce circulating nucleosome levels, and as discussed previously, has been shown to degrade NETs [151] and reduce organ damage in animal models of sepsis [152]. An alternative to a pharmacological approach is extracorporeal removal of NETs. NucleoCapture™ therapeutic plasmapheresis utilises histone H1.3 protein as a selective DNA adsorber to remove NETs from blood. It improved organ function and survival in a porcine model of sepsis [144, 145] and has been given to patients with sepsis in a pilot study (NCT04749238) [146]. The potential anti-NET therapeutics are summarised in Table 2.

Biomarkers of NETs

Prompt diagnosis and treatment are crucial for ensuring the best outcomes for patients with sepsis. As NETs are an important part of the immune response, care needs to be taken when selecting treatments, as blocking or disrupting low levels of NETs could, as with any immunomodulatory agent, have the potential for increased susceptibility to opportunistic infections. The 'current' recommended 'gold standard' for NET visualisation remains fluorescence microscopy utilising DNA stains combined with immunofluorescence staining for specific markers, including histone H3, neutrophil elastase, and myeloperoxidase [40]. The microscopic analysis should be complemented by cell-free DNA quantification and nucleosome detection [176]. The accurate, reproducible, quantifiable and translational diagnosis of NETosis is being worked on by the International Society of Thrombosis and Haemostasis. This is a critical step in informing and enabling the standardisation and comparison of further studies.

A range of tools can be used to detect and quantify NET formation and the most common techniques have been reviewed in detail by Stoimenou et al. [177]. These include immunoassays such as enzyme-linked immunosorbent assay (ELISA) and Western blot, flow cytometry-based techniques and microscopy methods. ELISA is the most used, objective and quantitative method for monitoring NETs, due to its low cost and simplicity, although

standardisation remains a challenge [177–179]. These tools are used in combination with NETs biomarkers such as, histones, NE, MPO, cell-free DNA, nucleosomes, and their complexes [178, 180]. However, discussion remains about the most suitable NET marker to use as a diagnostic, risk stratifier or prognostic tool. Several validated commercial ELISA tests and in-house protocols are currently available for detecting NETs, using serum or plasma levels of MPO-DNA complex, histones, and nucleosomes as surrogate markers [177–179, 181, 182]. Clinical data suggest that these circulating biomarkers may be associated with the presence and severity of thrombosis and sepsis, and correlate with hypercoagulability, mortality and organ damage [178, 180, 183, 184]. There is currently no consensus regarding biomarker thresholds for quantifying the presence of NETs in sepsis. Larger clinical trials are needed to confirm the utility of NETs biomarkers in clinical practice and specifically to guide interventions targeting NETs.

Conclusion

NETs and associated DAMPs are crucial players in immunothrombosis, a physiological process that links innate immunity with thrombosis to contain and eliminate pathogens [5, 6, 8]. When dysregulated, this process can lead to thromboinflammation, contributing to the pathogenesis of sepsis and other inflammatory conditions [5]. Excessive release or inadequate removal of NETs can lead to the development and progression of sepsis [10]. Therefore, manipulating NETs represents a promising therapeutic strategy for sepsis and other conditions associated with excessive or dysregulated NET formation. Importantly, modulating NET formation and function may also help balance the beneficial aspects of immunothrombosis with the detrimental effects of thromboinflammation [185]. Potential approaches include inhibiting NET formation, promoting NET degradation and targeting NET components. While these approaches have shown promise in preclinical studies, their clinical efficacy and safety need to be established in humans.

Recent research has focused on targeting specific pathways involved in immunothrombosis and thromboinflammation, such as the interaction between NETs and the complement system [186]. The development of NET-targeting therapies faces several challenges such as the heterogeneity of NETs, potential off-target effects of inhibitors, and the risk of impairing host defences. Therefore, a deeper understanding of the molecular mechanisms underlying NET formation and regulation, as well as the identification of specific targets and biomarkers, will be crucial for successful translation of NET-targeting therapies into clinical practice. Moreover, the potential risks and benefits of each approach should be carefully evaluated and guided by specific biomarkers of disease severity. Future research should aim to develop therapies that can selectively modulate NET function in thromboinflammation, potentially leading to more effective treatments for sepsis and related disorders.

Abbreviations

ACT	Adenylate cyclase toxin
APC	Activated protein C
ARDS	Acute respiratory distress syndrome
APS	Antiphospholipid syndrome
cAMP	Cyclic AMP
DAMP	Damage-associated molecular pattern
DPI	Diphenylene iodonium
ELISA	Enzyme-linked immunosorbent assay
FIX	Factor IX
FXII	Factor XII
GAS	Group A Streptococcus
GBS	Group B Streptococcus
HK	High molecular weight kininogen
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NE	Neutrophil elastase
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor kappa B
PAD4	Peptidylarginine deiminase 4
PRR	Pattern recognition receptor
PSGL-1	P-selectin glycoprotein ligand-1
PVL	Panton-Valentine leucocidin
rhDNase I	Recombinant human DNase I
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
TF	Tissue factor
TLR	Toll-like receptor
TM	Thrombomodulin
TNFα	Tumour necrosis factor alpha
tPA	Tissue-type plasminogen activator
UFH	Unfractionated heparin

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Author contributions

As lead author, I can confirm that this manuscript has not been previously published or simultaneously submitted elsewhere. All authors have fulfilled ICMJE criteria for authorship, made substantial contributions in drafting or revising the manuscript, and approved the final version for submission. The concept for the article was conceived by the three authors at an international meeting to discuss the role of NETs in sepsis. I wrote the first draft of the manuscript and constructed the figures. Professor Annane and Professor Singer both reviewed and substantially edited multiple drafts of the document.

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Declarations

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Consent for publication

Consent for publication is not applicable as this review article does not contain any individual person's data. All materials referenced are from previously published literature available in the public domain.

Availability of data and materials

Not applicable, as this is a narrative review article. All data referenced is from previously published studies cited in the references section and available through their respective publishers.

Competing interests

Professor Djillali Annane and Professor Mervyn Singer attend advisory board meetings for Volition, and I am currently an employee. Professor Djillali Annane is affiliated with the IHU PROMETHEUS Comprehensive Sepsis Center.

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