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A Review of Neutrophil Extracellular Traps (NETs) in Disease: Potential Anti-NETs Therapeutics

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Abstract

Activated neutrophils release neutrophil extracellular traps (NETs) in response to a variety of stimuli. NETosis is driven by protein-arginine deiminase type 4, with the release of intracellular granule components that function by capturing and destroying microbes, including viral, fungal, bacterial, and protozoal pathogens. The positive effects of pathogen control are countered by pro-inflammatory effects as demonstrated in a variety of diseases. Components of NETS are non-specific, and other than controlling microbes, they cause injury to surrounding tissue by themselves or by increasing the pro-inflammatory response. NETs can play a role in enhancement of the inflammation seen in autoimmune diseases including psoriasis, rheumatoid arthritis, and systemic lupus erythematosis. In addition, autoinflammatory diseases such as gout have been associated with NETosis. Inhibition of NETs may decrease the severity of many diseases improving survival. Herein, we describe NETosis in different diseases focusing on the detrimental effect of NETs and outline possible therapeutics that can be used to mitigate netosis. There is a need for more studies and clinical trials on these and other compounds that could prevent or destroy NETs, thereby decreasing damage to patients.

Keywords Neutrophil extracellular traps · NETs · Therapeutics · Anti-NETs · PAD4

Introduction

Neutrophil extracellular traps (NETs) were discovered in 1996 [1] and further detailed by Brinkmann et al. who termed the process NETosis [2–4]. Neutrophils are short-lived granulocytes that are the initial defense against invading pathogens. They achieve this through phagocytosis, degranulation, production of reactive oxygen species (ROS), and production of chemokines and cytokines to recruit other immune cells maximizing the host's immune response [5–7]. Neutrophils enhance their antimicrobial properties by releasing NETs, composed of extracellular chromatin decorated with histones and numerous granular proteins [3, 8] and were identified as part of innate immune response which can either be beneficial or pathological [2, 8, 9]. NET formation starts with the activation of neutrophils through the recognition of stimuli and activation of NADPH oxidase (NOX) complex through protein kinase C (PKC)-Raf/MERK/ERK [9–11] which in turn activate myeloperoxidase (MPO), neutrophil elastase (NE), and protein-arginine deiminase type 4 (PAD4) [12, 13]. PAD4 catalyzes citrullination of histones and promotes chromatin decondensation [14–16], while the ROS species promote NETosis by inducing gradual separation and loss of the nuclear membrane with the release of chromatin outside the cell through membrane pores. Cellular lysis with a final release of DNA, citrullinated histones (citH3), and other intracellular granules form the extracellular traps [10]. NETosis is induced in response to stimuli promoting pathogen clearance by trapping, and either killing through microbial toxicity or immobilizing microbes facilitating phagocytosis by other neutrophils and phagocytes [3, 15, 17, 18].

Due to the non-specific effects of the released enzymatic proteins, NETs may lead to uncontrolled inflammatory response causing tissue pathology. There is direct cell damage, recruitment of other pro-inflammatory cells and proteins, and formation of immune complexes that induce autoantibody production leading to tissue damage [19, 20]. NETs can capture metastatic tumors aggravating cancerous condition [21], and in diabetic cases, they lead to a delay in wound healing [22, 23]. Neutrophil can also form interactions with platelets

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mediated by P-selectin [24]. This leads to induction of platelet-derived high-mobility group protein B1 (HMGB1) [25] which stimulates NETs [24, 26] causing occlusion in the vasculature by promoting thrombosis and obstruction causing organ damage. Although NETs have been shown to promote inflammation, a study done by Christine et al. shows that an accumulation of NET aggregates can reduce inflammation in a mouse model of gout through the degeneration of cytokines and chemokines [27]. This means that there is still much about NETs we are not aware of, and thus the need for more studies to understand their specific mechanism and how to harness their benefits while limiting their negative effects.

There are certain compounds that have been identified in some studies to either inhibit or disrupt NETosis, but there is no available therapeutic that has been researched extensively or approved for human use. We propose that the limited therapeutics have been due to the different effects of NETs in different disease conditions; thus, identifying a stand-alone compound might be a challenge. In this review, we briefly mention NETosis in different diseases and try to reconcile different aspects of NET biology highlighting possible compounds that can be considered therapeutic. New approaches in therapeutic design and efficacy testing will have to be developed to find a truly efficacious treatment.

Effects Of NETs

Here is a brief discussion on the different researched effects of NETs.

Antimicrobial

NETs have been shown to have positive effects in controlling bacterial infections. They possess antimicrobial properties with components including histones, cathepsin G, NE, MPO, lactoferrin, antimicrobial peptide-LL37, pentraxin 3, gelatinase, proteinase 3, and peptidoglycan-binding proteins that are bactericidal [2, 28, 29]. NETs limit growth or kill bacterial as reviewed by Vidal Delgado-Rizo et al. which include *Shigella flexneri*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Shigella sonnei*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus albus*, *Staphylococcus aureus*, and *Propionibacterium* [8, 30].

In viral infections including influenza, HIV, and respiratory syncytial virus, there is an excessive neutrophil recruitment [31, 32]. These viruses stimulate NETosis through TLR 4, 7 and/or 8 with the release of ROS species and the NETs trap, contain, and eliminate viruses [32–34] or inhibit viral replication through the blockade of the PKC pathway. Histones are also important for viral aggregation and neutralization leading to a significant decrease in viral replication [35, 36].

Fungi like *Aspergillus nidulans*, *Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus* spp. induce NETosis through the recognition of β-glucan on hyphae by components of the extracellular matrix or activation of NOX [17, 37, 38]. NETs have been shown to be important in trapping and clearing large pathogens in vivo, thus being critical for antifungal defense [17, 39, 40].

In parasitic conditions including *Plasmodium falciparum* and *Toxoplasma gondii*, there is activation of platelets, monocytes, and neutrophils. NET formation, which is dependent on MEK–ERK pathway, limits the dissemination of the parasites by trapping and killing them [41, 42]. Histones reduce the replication of the *Leishmania* spp. [43] and together with other NETs-associated compounds, such as NE, MPO, and collagenase, were shown to kill these pathogens [41–44].

Most studies on NETs have been done in mice and in vitro, but there is still a gap in knowledge on the exact mechanism of NETs in vivo. This necessitates the need for more studies to clearly evaluate their effects in in vivo and in humans.

NETs as Biomarkers

The ability to detect NETs may be used as a prognostic tool for patients with conditions presenting with a higher rate of NET formation, facilitating clinicians to provide personalized treatment. For NETs to be used as screening tools, there has to be studies to standardize and define normal from abnormal levels. This could involve measurement of NET-associated products in the blood cfDNA, citH3, NE, and MPO. In colorectal and breast cancer patients, cfDNA has been quantified in serum samples via a simple nucleic acid–staining assay [45–48]. This can be used to classify the cancer; however, measuring circulating MPO/cfDNA conjugates and citH3 may be more specific for NET analysis than evaluation of cfDNA alone [49]. CitH3 is highly specific to NETosis making it a possible tool for understanding variances between NET levels [50]. Thalin observed that high plasma content of citH3 was a significant indicator of short-term mortality in some cancer patients [51], and some observational studies inform on the significance of NETs in progression of colorectal cancer [40]. Further human studies are needed to definitively quantify different levels of NETs and associate them with poor cancer/disease outcomes.

Negative Effects of NETs

Although NETs may protect the host against microbes, excessive NETosis can be detrimental to the host. Recent discoveries in in vitro experiments and animal models demonstrated the crucial role of NETs in the pathogenesis of some metabolic, autoimmune, and autoinflammatory diseases and certain septic conditions increasing morbidity and mortality.

Sepsis

Large amounts of circulating NETs demonstrated in septic patients are associated with poor outcome and multiple organ failure [50, 52, 53]. This could be due to increased NETosis, apoptosis, and necrosis or decreased clearance of extruded products with studies suggesting that cfDNA exacerbate inflammation by inducing TNF-α mRNA [54, 55]. Histones also function as damage-associated molecular patterns and can induce organ damage by promoting pro-inflammatory cytokine release causing endothelial dysfunction by inducing cytotoxicity and increasing ROS production [53, 56, 57].

Autoimmunity

NETs have been indicated in pathologic alterations in autoimmune and autoinflammatory diseases [58, 59]. Here, we discuss in brief a few of these diseases.

Psoriasis is a chronic immune-mediated disease characterized by demarcated erythematous plaques on the skin. Some patients may also suffer from psoriatic arthritis with joint pains and deformities [60–63]. Studies show that neutrophils are recruited to psoriasis lesions where they cluster to form spongiform pustules and Munro's microabscesses and produce pro-inflammatory cytokines including IL-6, IL-8, and IL-17s [60, 64]. IL-17 in keratinocytes increases the expression of LL37, a cathelicidin-derived antimicrobial peptide, and defensins which mediate NET formation in dermatological conditions [30, 65]. These inflammatory compounds have been shown to promote NETosis and pathology in the absence of infection [59] in these patients.

S*ystemic lupus erythematosus* (SLE) is an autoimmune disease characterized by immune complexes and high levels of IFN-α with the activation of autoreactive B cells [66, 67]. There is a possible production of autoantibodies against nucleic acids released by neutrophils undergoing NETosis [19, 68] with the generated immune complexes representing a source of self-antigens that enhance the autoimmune and inflammatory process. This in turn results in more injury and inflammation [20, 69].

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by persistent synovial inflammation that leads to cartilage and bone injury in the joints [70]. The synovial fluid at the synovial cavity of RA patients becomes infiltrated with neutrophils that readily form NETs [71, 72]. Studies have demonstrated that circulating neutrophils of RA patients are more easily stimulated to NETosis than those from healthy subjects [73, 74], and as in other autoimmune conditions, NETs act as a source of extracellular autoantigens leading to excessive innate and adaptive immune responses in the joints and subsequent tissue injury [73, 75].

Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by the destruction of β pancreatic cells leading to hyperglycemia [76]. This causes production of autoantigens that are recognized by immune cells with production of autoantibodies [30, 58]. T1DM patients are at a risk of developing neutropenia, and neutrophils can be found within infiltrates in pancreatic islets where elevated TNF-α induces formation of NETs [77]. Cytokines produced in this process lead to neutrophil recruitment to sites of inflammation, providing negative feedback and contributing to pathogenesis in autoimmune diabetes [58, 77].

Small vessel vasculitis (SVV) is a systemic disease of unknown etiology where the patients exhibit blood vessel inflammation, with necrotizing inflammation in small blood vessels potentially leading to organ damage [78–80]. These patients have been shown to have anti-neutrophil cytoplasmic antibodies (ANCAs) [7, 81]. Proteins released during NETosis are the main cause of ANCA production by activating the complement system resulting in endothelial damage [81, 82]. These studies have shown that α-PR3 and α-MPO ANCAs induce NETosis during active disease perpetuating a feedback loop [81].

Autoinflammatory Diseases

Gout is an autoinflammatory disease characterized by the deposition of monosodium urate (MSU) crystals in the joints, stimulating immune responses by attracting leukocytes and inducing NETs that promote inflammation [27, 72, 83–85].

Inflammatory bowel diseases (IBDs) are disease affecting the gastrointestinal tract characterized by chronic uncontrolled inflammation. The two major forms of IBD include ulcerative colitis (UC) and Crohn's disease (CD), which have different etiologies, pathogenesis, and diagnostic features with the differences not fully understood. CD clinically manifests as gastrointestinal disorders but is a systemic disease involving inflammation of the ileum and colon [86–88]. NET formation in CD has not been well-studied although studies indicate that ROS production is enhanced, which could promote NETosis [30, 89–91]. UC is also characterized by inflammation of the gastrointestinal tract mostly restricted to the colon with NETs observed in the colon accompanied by exacerbated inflammation [64]. There are a few studies looking into the mechanism of NETs in these conditions, but there are more studies needed to better inform on development of effective treatment options.

Metabolic Diseases

Metabolic diseases have been associated with chronic low-grade inflammation with activation of the innate immune response and recruitment of mononuclear and polymorphonuclear leukocytes increasing cellular dysfunction [92, 93]. This microenvironment favors NETosis linking it to immune

deregulation and hyperglycemia, oxidative stress, inflammation, and further complications of metabolic diseases.

Type 2 diabetes is a chronic metabolic condition characterized by glucose level build-up in the bloodstream, hyperglycemia, and cells unresponsive to insulin. Studies have shown that hyperglycemia predisposes neutrophils to release NETs. NET-related bioproducts (NE, MPO, and cfDNA) are increased compared with non-diabetic subjects and also positively correlate with increased glycated hemoglobin (HbA1c) levels [23, 77]. This suggests that the chronic pro-inflammatory conditions present during hyperglycemia promote NETosis in both the type 1 and type 2 diabetes [22, 94]; thus, NET formation is enhanced in hyperglycemic conditions independent of diabetes type and origin.

Obesity is a metabolic condition characterized by an excess of adipose tissue deposition, as a result of energy imbalance due to increased energy intake versus expenditure. Obesity is frequently associated with other chronic complications including cardiovascular disease and diabetes [95, 96]. Studies have shown an association between obesity and chronic inflammation with enhanced neutrophil activity, increased superoxide radicals, and NET formation [97, 98]. Moorthy et al. show that the neutrophils of mice fed with high-fat diet are more prone to spontaneous NET formation, compared with neutrophils derived from mice fed with low-fat diet [99]. The same is seen in mice fed with high-fat diet and infected with inlfuenza compared with the mice fed with low-fat diet [100]. There is an increase in obesity globally, necessitating more studies into this condition and the link to the other metabolic conditions. This will inform on management of conditions caused or exacerbated by obesity and how NETs play a role in this.

Potential Anti-NET Therapeutics

Although NETs can be beneficial, the detrimental effect of NETs can cause excessive tissue damage and pathology. There are studies evaluating the possible effects of certain compounds against NETs as illustrated in Table 1, and more studies need to be considered to mitigate the negative effects of NETosis.

Anti-Inflammatory/Immunomodulatory and NETs

Acetylsalicylic acid (Aspirin) is a non-steroidal drug with an antithrombotic and an anti-inflammatory effect used in the management of inflammatory symptoms. It functions through the irreversible acetylation of cyclooxygenase enzyme (COX), and suppresses prostaglandin generation [101, 224]. It is used as an antiplatelet agent for prevention of arterial thromboses as it inhibits thromboxane A2 [225, 226]. Thromboxane A2 is a vasoconstrictor that activates new platelets increasing platelet aggregation, an important function during tissue injury, inflammation, and healing([227]). Platelets are the primary effector cells of hemostasis [227], but recent evidence indicates that they play a direct role in innate immunity by interacting with pathogens or recognize pathogen-associated molecular patterns (PAMPs) [228, 229]. They facilitate innate immunity and activate NETosis via platelet-neutrophil interaction [51, 230]. Platelet activation through TLR2 and TLR4 leads to the expression of P-selectin which binds to neutrophil receptor (PSGL-1) inducing NETosis as demonstrated in mice [24, 26, 102]. NETosis is also mediated by the binding of αMβ2 (MAC-1) on neutrophils to glycoprotein 1bα (GP1bα) on platelets generating NETs in liver and lungs during endotoxemia and in septic conditions [231, 232]. In addition to LPS, platelets can be activated by thrombin and arachidonic acid to form NETs [103, 230]. Upon activation, platelets secrete soluble mediators including high-mobility group box-1 (HMBG-1) [25], platelet factor 4 (PF4), and CCL5 (RANTES) that induce NETosis via the neutrophil G protein coupled receptors [104]. The interactions between platelets and neutrophils mediate NETosis, and inhibition of this interaction using anti-platelet therapy has the potential to inhibit NET formation [105, 106]. In a study conducted on endotoxin-triggered acute lung injury, pretreatment of mice with aspirin showed a decreased intravascular NET formation and reduced degree of lung injury [103, 106]. In another study on effects of NETs on transplantation in mice, they discovered that platelet activation was also inhibited by aspirin [107]. Lapponi et al. conducted another study where they treated neutrophils with a steroidal immunomodulatory drug (dexamethasone) or aspirin, and discovered that dexamethasone had no effect, while aspirin prevented NET formation [108]. They demonstrated that aspirin functions by inhibiting NF-κB, an inflammatory transcriptional regulator, that promotes NETosis. These results show that aspirin could be a useful therapy in the management of pathologic NETosis induced by platelets, but we have to keep in mind the side effects of aspirin. Aspirin is a blood thinner and predisposes patients to stomach ulcers, so more studies are needed to find out which conditions could benefit from treatment with aspirin, without the excessive side effects.

Cyclosporine A is an immunosuppressant drug widely used in post-allogeneic organ transplant to reduce the activity of the patient's immune system, and therefore the risk of organ rejection [109, 110]. It causes reversible inhibition of immunocompetent lymphocytes and has been used to manage fungal infections, rheumatoid arthritis, asthma, dermatologic drug, and immunosuppressive agent [110, 111]. The mechanism of action of cyclosporine A involves binding to cytophilin, resulting in the downregulation of NFAT (nuclear factor of activated T cells) transcription factor and inhibiting the calcineurin pathway subsequently inhibiting NET formation [111, 112]. Gupta et al. analyzed the role of cyclosporine A, ascomycin (a macrolide with strong immunosuppressant

Table 1 Table representing potential anti-NETs therapeutics and mode of action

Anti-NET therapeutics	Pharmacological compounds	Target	Mode of action	Reference
Anti-inflammatory/immunomodulatory	Acetylsalicylic acid (Aspirin)	Cyclooxygenase enzyme (COX),	Inhibits thromboxane A2 inhibiting platelets	[98–105]
	Cyclosporine A	Cytophilin - downregulation of NFAT	Inhibiting the calcineurin pathway	[106–112]
	Chlor-amidine	PAD4	Inhibition of PAD4 required for NETosis	[113–118]
	PGE2	EP2 and EP4 receptors	Physiologic inhibition of NETosis through cAMP-PKA pathway and protein kinase A-dependent manner	[119–125]
	PA-dPEG24	Complement C1	Complement inhibition, blocking MPO pathway of NETosis	[126–128]
	Antibiotic (azithromycin, chloramphenicol, gentamicin)	Cytokines including- IL-6 and IL-8	Immunomodulating effects by influencing the activation and migration of neutrophils	[129–131]
Anti-thrombosis	Thrombomodulin	Protein C	Anticoagulant, inhibit NETosis	[132–135]
	Activated protein C (APC)	Protein C	Anticoagulant, disrupt extracellular histones	[136–142]
	Heparin	Antithrombin	Anticoagulant, suppresses histones	[143–148]
	Anti-high-mobility group box 1 (Anti-HMGB1)	HMGB1	Modulation of neutrophil chemotaxis, diminish NETOSIS	[25, 149–160]
	C1 esterase inhibitor (C1INH)	Complement factor (factor XIIa), kallikrein	Binds histones	[161–169]
NADPH/ROS inhibitors	Metformin	mTORC1, AMPK	Antidiabetic, inhibits mitochondrial ROS, inactivates the PKC-NOX pathway blunting NETosis	[170–175]
	Chloroquine/hydroxychloroquine	MMPs, TIMPs	Antimalarial, inhibits cytokine production, interferes with the stimulatory effect of platelet aggregation, maintaining extracellular matrix homeostasis	[176–185]
	Diphenyleneiodonium chloride (DPI)	NADPH	Inhibits NADPH oxidase ROS production	[186–189]
	N-Acetylcysteine (NAC)	ROS	Inhibits ROS production, prevents thrombus formation	[190–195]
Nucleases	Recombinant human DNase	DNA matrixes	Reduces neutrophil infiltration, cleaves DNA matrixes	[196–207]
	Staphylokinase	Plasminogen, alpha-defensins	Converting NETs to deoxyadenosine mediating death of immune cells	[208–211]
Notable compounds	Probiotics	PKC pathway	Dampening ROS production	[212–215]
	Vitamin D		Reduces endothelial damage, decreases cell apoptosis	[216–223]

PGE2 prostagladin E2, *NFAT* nuclear factor of activated T cells, *PAD4* protein-arginine deiminase 4, *ROS* reactive oxygen species, *PKC* protein kinase C, *MMPs* matrix metallopeptidase, *TIMPs* tissue inhibitors of metallopeptidase, *mTORC1* mechanistic target of rapamycin complex 1, *AMPK-5′* AMP-activated protein kinase.

properties), and rapamycin (a cell anti-proliferative and immunosuppressive agent), on both extra and intracellular calcium pools and their modulation in NETosis. Their data indicated that a combination of ascomycin and cyclosporine A reduced NETosis, but the same effect was not evident following treatment with rapamycin [113]. This opens up the possibility to therapeutically suppress or modulate NETosis using cyclosporine A or a combination therapy with ascomycin. There were no other studies we could find in this area, and this could be due to the fact that cyclosporine A and immunosuppressive drugs may impair normal host immune responses to microbes [114, 115], predisposing patients to frequent infections. Therefore, there is a need for more studies into how these drugs could be formulated to manage NETosis safely.

Chlor-amidine (Cl-amidine) is a compound designed to irreversibly inhibit protein-arginine deiminase (PAD) through covalent modification at the active site of the enzymes [116]. As described above, PAD4 is an enzyme involved in NETosis; thus, inhibition of PAD4 is a possible therapeutic target. Avin et al. run a study to evaluate the effect of inhibition of PAD4 in NETosis using an antagomiR-155, a pleiotropic microRNA important in the regulation of immune responses, demonstrating a decreased induction of PAD4 mRNA and subsequent reduced NETs in response to PMA challenge [13]. In a mouse model of lupus, systemic treatment with the PAD4 inhibitor (*BB-Cl-amidine)* showed protection of the mice from developing NET-mediated vascular damage, endothelial dysfunction, and kidney injury. The study indicated that PAD4 inhibition markedly downregulates the expression of type I interferon-regulated genes and reduces proteinuria and immune complex deposition in the kidneys, while also protecting against skin disease [117]. Another study found that PAD4-deficient mice (both diabetic and non-diabetic) possess faster wound healing and re-epithelization processes than their wild-type counterparts. This effect was independent of wound infection suggesting that NETosis could hinder wound healing by limiting keratinocyte migration and re-epithelization [118]. It is therefore possible to target inhibition of PAD4 to inhibit NET formation; however, it is important to note that PAD4 may have other important functions in immunity which may be impaired [119, 120]. In one study, they reported mixed results of pharmacological PAD4 inhibition using Cl-amidine in human neutrophils, where NETosis induced by smoking was blocked by inhibiting of PAD4, but NETosis induced by cholesterol crystals was not blocked [121]. With these results, developing a suitable targeted therapy for PAD4 may be challenging thus the need for more carefully considered human studies on the function of PAD4 before using its inhibition as a strategy for management of NETosis.

PGE2—Prostaglandins (PGs) are members of the eicosanoid family synthesized from arachidonic acid via COX enzymes and produced by nearly all cells within the body. PGE2 is the most abundant prostaglandin in the human body and has been shown to influence both inflammatory and in some cases anti-inflammatory effects [122, 123]. Shishikura et al. evaluated the effects of PGE2, agonists and antagonists of its receptors, and modulators of the cAMP-PKA pathway on the formation of NETs in vitro (in isolated neutrophils) and in vivo in a mouse model. They also discovered that PGE2 inhibited PMA-induced NET formation in vitro through EP2 and EP4. Exogenous PGE2 treatment limited NETosis of neutrophils collected from normal human volunteers and naive mice in an exchange protein activated by cAMP- and protein kinase A-dependent manner demonstrating a physiologic inhibition of NETosis. Incubation with a cell-permeable cAMP analogue, dibutyryl cAMP, or various inhibitors of a cAMP-degrading enzyme, rolipram (PDE4 inhibitor), and butaprost, (EP2 receptor agonist) also suppressed NET formation [124]. Interestingly, Domingo et al. conducted a study in murine bone marrow transplant mice (BMT) where neutrophils overexpress COX-2 and overproduce PGE2, leading to defective intracellular bacterial killing. They wanted to determine whether NETosis was defective after transplant and whether this was regulated by PGE2 signaling. Treatment of BMT neutrophils with rapamycin resulted in reduced NET formation relative to control cells while the EP2 receptor antagonist (PF-04418948) or the EP4 antagonist (AE3-208), Gαs-coupled receptors, restored NET formation suggesting that blocking PGE2-EP2 or EP4 signaling pathway restores NETosis [125]. These findings will contribute to the development of novel treatments for NETosis-related diseases although more studies need to be done to evaluate the effect of using PGE2 as a therapeutic. Although PGE2 is beneficial in management of SLE and other IFN-α-dependent, Th1-driven diseases [126], it could pose a challenge in conditions like arthritis [233, 234] associated with pain. PGE2 is known to contribute to pain as part of the inflammatory response, thus the need for more studies to evaluate its effects in different diseases compared with its benefits.

PA-dPEG24 is a peptide inhibitor of complement C1 (PIC1) which mitigates peroxidase activity of MPO, hemoglobin, and myoglobin through a reversible process [235]. Defective complement action caused by dysregulation and acute and chronic tissue damage or transplants can lead to host cell attack contributing to inflammatory conditions [236, 237]. This is more so in the kidney which has been shown to be particularly sensitive to complement-mediated damage [127, 128]. It is known that complement effectors including C5a and membrane attack complex (sC5b-9) interact with and can stimulate human neutrophils to generate NETs. Subsequently, products of NETosis can activate complements causing a destructive loop [129]. Therapeutic complement inhibition is successfully used in paroxysmal nocturnal hemoglobinuria showing a promise in its use in other clinical conditions [130, 131]. An article by Hair et al. demonstrated that

PIC1 showed dose-dependent antioxidant activity, acting via the single electron transport (SET) and hydrogen atom transfer (HAT) mechanisms interfering with oxidation of cysteine residues. They showed that PA-dPEG24 achieved complete inhibition with complement effector levels equivalent to background. PA-dPEG24 was also able to dose-dependently inhibit NET formation by human neutrophils, stimulated by PMA, MPO, or immune complex activated human sera [235]. Their results suggest that PA-dPEG24 inhibition of NETs occurs by blocking the MPO pathway of NET formation. This provides proof that peptides can potentially be developed to inhibit complement-induced NETosis and be used to manage conditions worsened by NETs, although since complements are important in immune response, this needs to be researched further.

Antibiotics have been used in the management of bacterial infections and also have immunomodulating effects by influencing the properties of numerous immune cells, including neutrophils [132, 133]. Bystrzycka et al. conducted a study to investigate the effects of *azithromycin* and *chloramphenicol* on degranulation, apoptosis, respiratory burst, and the release of NETs by neutrophils. Their study indicated that pretreatment of neutrophils with azithromycin and chloramphenicol decreases the release of NETs, with azithromycin showing a concentration-dependent effect on the respiratory burst in Bystrzycka et al.'s study [134]. Another article by Manda-Handzlik et al. looked into the effects of *cefotaxime* and *gentamicin* on NETs and discovered that gentamicin inhibits NET release by human neutrophils, while cefotaxime had no impact on this process [133]. The information that antibiotics can modulate NET release can be useful in the management of infectious diseases or patients suffering from NET-related diseases. Since different antibiotics have different effects, there is need for more studies on their mode of action. This will inform on a possible compound for use in the management of NETs without interfering with their antimicrobial function.

Anti-Thrombosis and NETs

Thrombomodulin is a protein cofactor expressed on endothelial cell surfaces that modifies the substrate specificity of thrombin by an allosteric mechanism [238]. Thrombin-thrombomodulin complex activates protein C, initiating an essential anticoagulant pathway [238–240]. Thrombosis is the formation of a blood clot within a blood vessel caused by cytokines and other inflammatory mediators produced during an injury, obesity, and in some cases drugs, e.g., estrogen pills [241]. Large amounts of circulating cfDNA, present in NETs, can influence thrombus formation by impairing fibrinolysis creating a scaffold for the binding of red blood cells, platelets, fibrin, and coagulation factors [129]. Besides cfDNA, other NET components also exert procoagulant properties with extracellular histones inducing platelet activation, aggregation, and thrombin generation [81, 229, 242]. Immuno-thrombosis-induced coagulopathy may contribute to hypercoagulability which increases occurrence of multiple organ dysfunction and mortality [135–137]. Helms et al. did a study where they looked into the use of a recombinant human thrombomodulin (rhTM) and found out that it can limit procoagulant responses. rhTM was also shown to fully inhibit NETosis in neutrophils cultured with platelets and in the presence of LPS [138]. There is not much research in effect of rhTM, but this provides a starting point for further research into its potential use to inhibit NETs.

Activated protein C (APC) is a multifunctional serine protease produced in blood by vitamin K–activating protein C. APC has anticoagulant, cytoprotective, and anti-inflammatory activities [139, 140]. Protein C has been shown to be an important prognostic indicator in patients with sepsis. During sepsis, there is a reduction in the conversion of protein C to its active form due to the downregulation of thrombomodulin by inflammatory cytokines [141]. The antithrombotic effects of activated protein C is mediated by its ability to inhibit the formation of clotting factors Va and VIIIa and disrupting extracellular histones [142, 243]. Healy et al. demonstrated that APC cleaves and detoxifies extracellular histones and prevents activated platelets from inducing NETosis. The pretreatment of neutrophils with APC before inducing NETosis inhibited platelet adhesion to NETs. They also used antibodies against the neutrophil receptors endothelial protein C receptor (EPCR), protease-activated receptor 3 (PAR3), and macrophage-1 antigen (Mac-1) which blocked APC inhibition of NETosis [244]. Another study demonstrated that the blockade of protein C activation lead to exacerbated sublethal LPS challenge to turn lethal, which was reversed by treatment with antibodies to histones [56, 245]. These findings suggest that the anti-inflammatory function of APC may include inhibition of NETosis. Drotrecogin alfa is a recombinant human activated protein C produced by Xigris approved for use in septic patients [143, 144, 246]. Studies should be done to evaluate if this and other similar compounds would be effective in minimizing NETosis in sepsis and other conditions with minimal side effects.

Heparin is a medication and naturally occurring glycosaminoglycan used as an anticoagulant (blood thinner). Specifically, it is used in the treatment of heart attacks and unstable angina, and also antagonizes the effects of histones [247, 248]. High levels of circulating histones have been positively correlated with disease severity in many disease conditions as they activate NF-κB pathway inducing the secretion of cytokines that amplify inflammation leading to organ damage [145–147]. Heparin has been shown to significantly suppress histone-induced disease [148, 149]. Studies have been done to evaluate the effect of unfractionated heparin, low molecular weight heparin, e.g., parnaparin and non-anticoagulant heparin [148, 150, 151]. These studies demonstrate that

heparin was able to protect mice from organ damage and death by antagonizing circulating histones attenuating tissue damage. Administration of heparin, especially the non-anticoagulant heparin, is a novel and promising approach that may be further developed to treat patients with high levels of circulating histones potentially inhibiting NETosis without increasing the risk of bleeding.

Anti-high-mobility group box 1 (HMGB1) is an abundant protein that regulates chromosome architecture and also functions as a damage-associated molecular pattern molecule [249–251]. It plays a beneficial role in microbial eradication through its pro-inflammatory actions and modulation of neutrophil chemotaxis [252–256]. Platelets are the major source of HMGB1 within the thrombi and present it to neutrophils promoting NETosis [152–154, 257]. Vogel et al. determined that platelet-derived HMGB1 is a critical mediator of thrombosis from their study using generated transgenic mice with platelet-specific deletion of HMGB1 [25]. These effects were mediated via TLR4-and MyD88-dependent recruitment of platelet guanylyl cyclase (GC) toward the plasma membrane, followed by MyD88/GC complex formation and activation of the cGMP-dependent protein kinase I [25, 155]. Mice lacking HMGB1 in platelets exhibited increased bleeding times as well as reduced thrombus formation, platelet aggregation, inflammation, and organ damage during experimental trauma/hemorrhagic shock [25, 156]. Exposure of neutrophils to HMGB1 resulted in enhanced formation of NETs in vitro through TLR4-dependent processes contributing to inflammatory processes and tissue injury [157–160, 257]. Studies show that the use of anti-HMGB1 antibodies may diminish NET formation, as seen in a reduction of histone 3 and free DNA in the BAL fluid of LPS-treated mice that received neutralizing antibodies to HMGB1 [159, 161]. However, decreased levels of cytokines in the lungs after administration of anti-HMGB1 antibodies to LPS-treated mice may not necessarily be a direct result of diminished NET formation but could reflect the effects of HMGB1 on other pro-inflammatory pathways [162, 163]. This is a promising area necessitating more research into how anti-HMGB1 interrupts NETosis, and possibly use it as a treatment option.

C1 esterase inhibitor (C1INH) is an acute phase protein found in blood and a serine protease inhibitor that targets the complement pathway, coagulation pathway (factor XIIa), and the contact system protease kallikrein. It is an endogenous inhibitor of C1 protein in the complement system [164, 258, 259]. C1-INH concentrates are approved for use in the management of hereditary angioedema (HAE), an autosomal-dominant disease caused by C1-INH deficiency due to a mutation in the C1-inhibitor gene [165, 166]. Studies have been done to evaluate whether CI-INH may protect from lung injury in vivo possibly explaining the underlying mechanisms mediating protection. These studies demonstrated that application of C1INH alleviates bleomycin-induced lung injury via direct interaction with extracellular histones [167, 168]. In vitro, C1INH was found to bind all histone types with the interaction being independent of its protease inhibitory activity, but dependent on its glycosylation status [169]. In vivo, histone-C1INH complexes were detected in bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome and multiple models of lung injury [170]. The reactive-center-cleaved C1INH attenuated pulmonary damage evoked by intravenous histones indicating that C1INH administration may provide a new therapeutic option for disorders associated with histone release [171]. Wygrecka and his colleagues tested C1INH for its ability to bind and neutralize histones and determined that C1INH can bind purified histones in vitro reducing epithelial cell death by blocking histone interactions with cell surface proteins [167]. In another study, there was evidence for active binding of the exogenous C1INH to extracellular and citrullinated histones released during NETosis suggesting an endogenous mechanism by which histones are potentially neutralized [172]. This mechanism could be exploited for therapeutic management of excessive NETosis in other conditions, but studies need to be done to evaluate the effectiveness of these and other compounds with similar effects.

NADPH/ROS Inhibitors and NETs

Metformin, a widely prescribed blood glucose–normalizing antidiabetic drug, suppresses immune responses. It mainly achieves this through the induction of AMP-activated protein kinase. AMPK is an enzyme that plays a role in cellular energy homeostasis, by activating glucose and fatty acid uptake and oxidation when cellular energy is low. Induction of AMPK subsequently inhibits the mammalian target of rapamycin (mTORC1), a pathway that regulates mammalian metabolism and physiology, by inhibiting mitochondrial ROS production [173]. This results in its direct effect on the cellular functions of various pro-inflammatory immune cells. Due to the ROS inhibitory effect of metformin, studies are underway to evaluate it as a drug for regulating autoimmune diseases, treating chronic autoimmune diseases and gero-protection [174, 175]. Menegazzo et al. investigated the effect of metformin against NETosis and discovered that compared with a placebo, it significantly reduced the concentrations of NETs in vitro. They showed a reduction in elastase, proteinase-3, histones, and cfDNA, whereas glucose control with insulin exerted no significant effect [176]. Metformin was shown to prevent membrane translocation of PKC-βII and activation of NOX in neutrophils altering pathologic changes in nuclear dynamics and DNA release [177, 178]. This resulted in a blunted NETosis in response to PMA and calcium influx. This provides information for a possible use of metformin on the PKC-NOX pathway as an anti-NETosis therapy.

Chloroquine/hydroxychloroquine is an antimalarial drug used to treat malaria and has effects on amoeba (a protozoa) and some viruses [179, 180]. Studies are currently underway to evaluate its effects on the novel corona-2 virus (COVID19) that is currently causing a pandemic [181, 182]. Hydroxychloroquine (HDQ) is a slightly less potent derivative of chloroquine that is used in the treatment of malaria and as an immunosuppressive drug for management autoimmune conditions including SLE and RA [183, 260]. It exerts its immunosuppressive effect through inhibition of cytokine production with modulation of co-stimulatory molecules and also inhibits leukocyte phagocytosis [261, 262]. HDQ interferes with the stimulatory effect of platelet aggregation even in the presence of a thrombin agonist [263]. MMPs are matrix metalloproteinases which are enzymes involved in extracellular matrix remodeling, and TIMPs are counter regulatory tissue inhibitors of MMPs [184, 264] that have been extensively studied in SLE [185, 265]. Research has shown that HDQ modulated MMPs-TIMPs interaction assisting in maintaining homeostasis of the extracellular matrix [266, 267] and may thus play a role in reducing NETs. In another study that looked at tumor-derived extracellular vesicle (EV), transportable vesicles important in the exchange of biological molecules between cells and induce formation of NETs [268, 269], HDQ was shown to inhibit neutrophil uptake of tumor-derived EVs, thus reducing NETosis [47, 186–188]. However, the precise mechanism of inhibiting the uptake is largely unknown.

Due to the associated complications of NETs in autoimmune conditions and cancer metastasis, it is important for future research efforts to focus on further investigation of these drugs and other new specific targets for prevention or control of the detrimental effects of NETs formation.

Diphenyleneiodonium chloride (DPI) is as a hypoglycemic agent able to block gluconeogenesis and respiration by inhibiting many enzymes; NADPH oxidase, nitric oxide synthase, xanthine oxidase, NADPH cytochrome P450 oxidoreductase and cholinesterase [189, 190]. DPI works by binding the heme group of NADPH oxidase, inhibiting of NADPH oxidase and thus inhibits ROS production [191]. Ostafin et al. evaluated the effect of DPI on ROS production in the context of NETs and discovered that addition of DPI to the sample led to a reduction of extracellular DNA release with the strongest inhibition noticed after adding 10 μM DPI. These findings confirmed that DPI is able to block NET creation. However, the addition of DPI together with PMA or the addition of inhibitor initially and then washing it out before stimulation resulted in different levels of NET formation [192]. These findings necessitate more studies to look into the mechanism of action of DPI under different conditions and in different diseases as a potential therapeutic for NETs.

N-acetylcysteine (NAC), also known as Acetylcysteine, is a medication used to treat acetaminophen overdose [193] and to loosen thick mucus in individuals with cystic fibrosis or chronic obstructive pulmonary disease [194]. It also functions as an antioxidant which helps mitigate symptoms for a variety of diseases exacerbated by ROS species [195, 196]. Zawrotniak and his team evaluated the effect of NAC, ketoprofen, and ethamsylate on NETosis and observed a reduction of ROS production in a dose-dependent manner. NAC inhibited netosis, but in the presence of hydrogen peroxide, this neutrophil ability was restored indicating that NAC influences NET formation by modulating ROS productivity [197]. The administration of ethamsylate led to a significant reduction in NET formation, but this effect was not restored by hydrogen peroxide suggesting an additional side effect of this drug. Ketoprofen seemed to promote ROS-independent NET release, simultaneously inhibiting ROS production [197]. Brianna et al. used an acute pulmonary thrombosis model in vivo where NAC reduced thrombus formation to a similar extent as the irreversible platelet inhibitor aspirin [198]. In vitro analysis of platelet activation revealed that NAC reduced thrombin-induced platelet-leukocyte aggregate formation in mice model of mutated Janus kinase 2, a common mutation found in patients with chronic hematologic malignancies (CHM), and reduced NET formation in primary human neutrophils from patients with CHM as well as healthy controls [198]. These results strongly suggest that the therapeutic strategies applied in many neutrophil-mediated diseases should take into account the NET-associated effects and that studies should look at the effect of these compounds in other diseases.

Nucleases and NETs

Recombinant human DNase, marketed as Pulmozyme (Dornase alfa) by Genentech, is a highly purified solution of recombinant human deoxyribonuclease I (rhDNase). This is an enzyme which selectively cleaves DNA and has been used to hydrolyze the DNA present in sputum/mucus of cystic fibrosis patients and reduces viscosity in the lungs promoting clearance of secretions [199]. Nucleases perform various functions like acquiring nucleotide nutrients, allowing or preventing uptake of foreign DNA, controlling biofilm formation/dispersal/architecture, aiding some pathogens in invading host by tissue damage, degrading DNA matrixes, and immunomodulating the host immune response [200–202]. Studies have demonstrated the destructive effect of DNase on DNA-nucleoprotein, and immune complexes, providing a rational way to interfere with the disease processes in SLE and lupus nephritis [203]. Numerous other studies have evaluated the effect of rhDNase on NETs, with results showing a reduction of NETosis with reduced neutrophil infiltration reducing the inflammatory response [204–206]. Albadawi et al. conducted a study where they observed reduced detection of extracellular traps in post-ischemic muscle but did not alter skeletal muscle fiber injury, levels of pro-inflammatory

molecules, or ATP level. RhDNase treatment enhanced post-ischemic hindlimb perfusion, decreased infiltrating inflammatory cells, and reduced the expression of thrombin-antithrombin III [207]. In addition, DNase I decreases tumor volume in rats when injected intramuscularly or intraperitoneally in conjunction with other proteases (papain, trypsin, and chymotrypsin) [208]; however, it is not known whether these effects are due primarily to NET inhibition, thus the need for more studies. Findings from a different study, showed that early and concurrent treatment with DNase I and antibiotics resulted in improved survival, reduced bacteremia, and organ dysfunction in septic conditions [209]) suggesting a possible combination therapy to control NETosis. Additionally, DNase I injection may have off-target effects that need to be considered in its use for control of NETs or they may fail to function as expected in vitro [210].

Staphylokinase is an exoprotein produced by *Staphylococcus aureus*, which activates host plasminogen [211]. It induces extracellular release of alpha-defensins from polymorphonuclear cells promoting a complex formation between alpha-defensins and staphylokinase. The effect of this interaction is an almost complete inhibition of the bactericidal effect of alpha-defensins [211]. Thammavongsa et al. reported that *S. aureus* escapes these defenses by converting NETs to deoxyadenosine, which triggers the caspase-3-mediated death of immune cells [212].Thus, the pathogenesis of *S. aureus* infections has evolved to anticipate host defenses and to repurpose them for the destruction of the immune system [213, 214]. Secretory nucleases also provide means of survival to other bacteria like iron-reducing Shewanella and such functions help them adapt and survive proficiently [200]. Other than their pro-pathogen roles in survival, nucleases can be used directly as therapeutics due to their biological functions and medical applications in diagnosis, immunoprophylaxis, and autoimmune therapy. In the future, these enzymes can impact human medicine positively by opening new avenues for therapeutics which have otherwise reached saturation due to multi-drug resistance.

Notable Compounds

*Probiotic*s are live microorganisms promoted with claims that they provide health benefits when consumed, generally by improving or restoring the gut flora [215, 216]. Probiotics are considered generally safe for consumption but may cause unwanted side effects and bacteria-host interactions in rare cases. Alterations in the gut microbiota, as well as the presence of local and systemic markers of inflammation, are strongly associated with the manifestation of a spectrum of intestinal disorders [217]. Linda et al. investigated the effects of a nonpathogenic, enteropathogenic, and probiotic bacteria on the dynamics of NET formation using murine bone marrow–derived neutrophils and the neutrophil-differentiated human myeloid cell line DHL-60. They demonstrate that the probiotic *Lactobacillus rhamnosus* strain GG (LGG) inhibits both PMA and *S. aureus* induced NETs by inhibiting PKC pathway and dampening ROS production disrupting NETosis supporting its antioxidative capacity [218]. Given the presence of NETs in inflamed intestine [91], it is possible that some of the beneficial effects of LGG are attributable to its action on local neutrophils. Probiotics have been shown to protect against bacterial-induced cytotoxicity, but more studies need to be done to highlights the dynamic interaction between beneficial bacteria and neutrophils to inform on the usefulness of probiotics as gut-protective and immunomodulatory compounds.

Vitamin D is a group of fat-soluble secosteroids important for increasing intestinal absorption minerals including calcium, magnesium, and phosphate. Vitamin D has other multiple biological effects including activating the innate immune system while dampening the adaptive immune systems [219–222]. In humans, vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol) are the most important [223]. There are suggestions indicating the benefits of vitamins D on various conditions, but evidence is lacking on whether supplementation of vitamin D helps to reduce the risk of these diseases including asthma, tuberculosis, irritable bowel disease, depression, and other conditions [270, 271]. In the case of NETosis, Handono et al. evaluated the effect of hypo-vitamin D on NETs in SLE patients [272]. They demonstrated a significant decrease in early apoptosis with a moderate positive correlation between NE externalizations with early apoptosis. They concluded that vitamin D could reduce endothelial damage by decreasing NETosis activity [272]. This result may reveal the possibility of vitamin D as supplementary therapy for SLE patients and other patients with hypo-vitamin D to prevent NETosis and endothelial damage.

Tools and Models to Investigate the Impact of Proposed Treatments on NETosis

Investigation of the impact of one or more of the therapeutics discussed above in modification of NET formation will vary depending upon the disease and drug of interest. Where there are animal models of a target disease (e.g., rheumatoid arthritis or psoriasis) administration of the therapeutic drug can be done in a placebo-controlled study evaluating different doses. Tools available include histological identification of NETs and comparison of NETs formed in placebo versus drug-treated animals manually or using available computer programs. One example from our own work involves using a bovine model of respiratory syncytial virus and examination of the role of ibuprofen, a cox-inhibitor which decreases pro-inflammatory prostaglandin production and thromboxane 2 [273]. Our theory is that ibuprofen would reduce NETosis

by decreasing neutrophil-activating cytokines and platelet activation. In our study, lungs are harvested at necropsy, fixed and stained with antibodies against citrullinated histones and neutrophil elastase, to delineate the presence of neutrophil NETs. Another model is the use of neutrophils incubated in vitro with the drug to be tested and staining to determine if there is an effect on NETosis under different drug doses.

Conclusion

NETs have been implicated in many disease processes, and although they have a positive effect by clearing pathogens, they are also destructive due to the release of enzymes and other proteins that cause tissue injury. Control of NETs is quickly becoming a target for therapeutics in the management of various disease, but it is clear to see that the different compounds that inhibit or clear NETs may have other unwanted effects on the immune system. This makes it challenging to conclude that one compound works better that the other and thus the need for more research. There is a possibility that the management of NETs may require using a combination therapy that incorporate conventional treatments such fluid therapy, antibiotics, antivirals, and NET-targeted drugs. To potentially optimize treatment efficacy and outcome in clinical patients, it is important we run more studies to evaluate the mode of action of these compounds to pick the actual effective component of these drugs, while evaluating the effect in the overall immune system to ensure there are no other detrimental effects.

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