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Inhibition of NETosis for treatment purposes: friend or foe?

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Abstract

Active neutrophils participate in innate and adaptive immune responses through various mechanisms, one of the most important of which is the formation and release of neutrophil extracellular traps (NETs). The NETs are composed of network-like structures made of histone proteins, DNA and other released antibacterial proteins by activated neutrophils, and evidence suggests that in addition to the innate defense against infections, NETosis plays an important role in the pathogenesis of several other non-infectious pathological states, such as autoimmune diseases and even cancer. Therefore, targeting NET has become one of the important therapeutic approaches and has been considered by researchers. NET inhibitors or other molecules involved in the NET formation, such as the protein arginine deiminase 4 (PAD4) enzyme, an arginine-to-citrulline converter, participate in chromatin condensation and NET formation, is the basis of this therapeutic approach. The important point is whether complete inhibition of NETosis can be helpful because by inhibiting this mechanism, the activity of neutrophils is suppressed. In this review, the biology of NETosis and its role in the pathogenesis of some important diseases have been summarized, and the consequences of treatment based on inhibition of NET formation have been discussed.

Keywords NETosis · Inflammation · Autoimmunity · Treatment · NET inhibition

Introduction

Neutrophil Extracellular Traps (NETs) were discovered in 2004, and studies of these networked structures consisting of DNA, histones, fiber, and other antimicrobial proteins show that they can kill bacteria, viruses, fungi, and protozoa [1]. Trap them and lead them to immobility and then remove these pathogens by releasing antimicrobial proteins. This event is known as a relatively new innate immune response to infections [2–5]. Evidence suggests that in addition to participating in the innate defense against pathogenic microorganisms, NET can contribute to the pathogenesis and induction of some diseases such as rheumatoid arthritis (RA), diabetes, cystic fibrosis (CF), systemic lupus erythematosus (SLE), psoriasis (PsO) and metastatic malignancies [6]. Traces of NET have also been observed in some pathological phenomena such as periodontitis, vasculitis, thrombosis, coagulation disorders, metastasis, and atherosclerosis [7–11].

Following excessive NET formation and release of autoantigen, plasmacytoid dendritic cells (pDCs) can recognize the exposed autoantigens leading to interferon and autoantibody production, which are accompanied by tissue damage [12]. Therefore, regarding the pathologic role of NET formation, it has been considered more by researchers as an attractive therapeutic target [13]. In this context, the use of NET inhibitors has been investigated in various studies [14–16]. NET can also be indirectly targeted and reserved by inhibition of protein arginine deiminase 4 (PAD4), Myeloperoxidase (MPO), and neutrophilic elastase [12]. Studies in animal models of inflammation as well as human respiratory disorders showed that inhibition of MPO and neutrophilic elastase significantly reduced neutrophil-mediated inflammation [17, 18].

Various inhibitors have been studied in the treatment of diseases, some of which, such as rituximab, indirectly inhibit NET and lead to a decrease in the autoantibody titer in autoimmune diseases, and others, such as F- and Cl-amide can directly inhibit NET formation, reducing destructive inflammatory response [19, 20]. Although the use of this therapeutic strategy has advantages in the treatment of some disorders that are Net-mediated, on the other hand, studies on animal models show that complete inhibition of NET

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can increase the susceptibility to infections and loss of neutrophil functions involved in innate immune responses [21, 22]. Therefore, this review summarized the various aspects of targeting NET as a therapeutic option as well as its advantages and disadvantages.

NETosis biology

NETosis is the extrude NETs by activated neutrophils, an innate defense against pathogens, and it has been revealed that a variety of signals and biological phenomenon can participate in NET formation [23–25]. Evidence suggests that the production of neutrophil elastase, MPO, reactive oxygen species (ROS), histone modification, and chromatin decondensation are the key events leading to NET formation [23, 26]. It has been demonstrated that following activation of NADPH oxidase 2 (NOX2), superoxide anions and hydrogen peroxide are produced, which are important in the NET formation. Hydrogen peroxide is considered a substrate for MPO, stimulating the production and release of elastase from neutrophil granules [27]. Consequently, the migration of elastase into neutrophil nuclei leads to chromatin decondensation. However, the exact mechanism has not yet been fully elucidated [28]. After decondensation and decomposition of chromatin, the integrity of the nuclear membrane is disturbed, leading to the formation of a high stability network containing chromatin, granular and cytosolic proteins, elastase and MPO, which are able to exert their antibacterial properties following rupture of the neutrophil membrane and excretion outside the cell [29, 30] (Fig. 1).

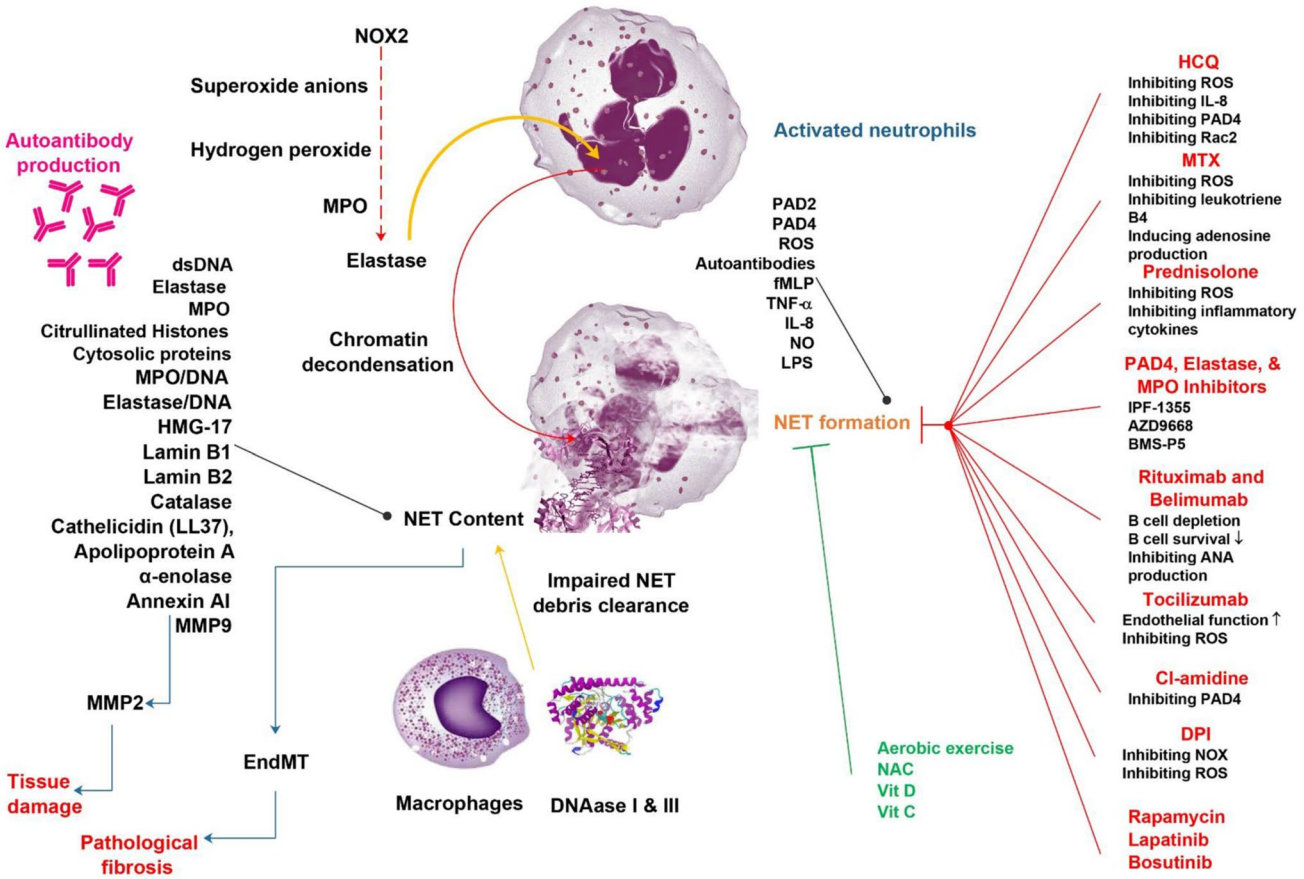

Fig. 1 NETosis consequences and NET inhibition. As the figure displays, many factors can induce. NET formation stimulators, including PADs, ROS, autoantibodies, fMLP, NO, LPS, IL-8, and TNF-α. Following these events, the contents of the NET are released, and due to the lack of proper clearance by macrophages as well as defects in DNase enzymes, the NET-derived debris (NET contents in the figure) are exposed to the immune system and are identified as autoantigens, resulting in autoantibody production, increased inflammation, tissue damage, and pathologic fibrosis. On the other hand, using NET formation inhibitors can be used to treat various NET-related diseases through various mechanisms such as inhibition of PADs, ROS production, and production of inflammatory mediators. *HCQ* hydroxychloroquine, *MTX* methotrexate, *ROS* reactive oxygen species, *PAD* protein arginine deiminase, *MPO* myeloperoxidase, *NOX* NADPH oxidase, *NET* neutrophil extracellular trap, *MMP* matrix metallopeptidase, *fMLP* N-formylmethionyl-leucyl-phenylalanine, *TNF* tumor necrosis factor, *IL* interleukin, *EndMT* endothelial-to-mesenchymal transition, *NO* nitric oxide, *LPS* Lipopolysaccharides

Studies show that due to differences between in vitro and in vivo conditions, NET formation is different in each of these conditions. For instance, the citrullination of histones by PAD4 and the subsequent unwrapping of the nucleosome are considered the NET formation's main events in vivo [31, 32]. In fact, NETosis is a type of programmed cell death different from necrosis and apoptosis, characterized by the release and exposure of the decondensed chromatin and the release of neutrophil granule content into the extracellular environment [33]. As discussed, various biological events play a role in NET formation via neutrophil activation. One of these events is the activation and function of the NOX2 enzyme to produce ROS or chromatin decondensation [34]. Citrullination of histone proteins by PAD4 is another vital bioevent that makes them suitable targets for treatment [35].

Moreover, post-translational modifications occurred in NET decorating proteins such as acetylation, citrullination, and methylation of histones. The mentioned modifications indicated that NETs could be a source of intracellular autoantigens in ADs [31, 36, 37]. Besides, due to the low number of mitochondria in neutrophils, NETs derived from mitochondrial DNA or mtDNA are probably very low [38, 39]. Based on studies in the field of NETosis, it has been determined that there are two types of NETosis, one is "suicide" or NOX2-dependent, which lasts about 3 h, and the other is "vital" or NOX2-independent that the formation of this antimicrobial network takes about 30 min and neutrophils can exert their antibacterial effects despite maintaining their structure. It has been demonstrated that calcium-activated vital NETosis is fast and mediated by mitochondrial ROS and calcium-activated small conductance potassium (SK) channel member SK3 [40]. Another difference between these two types of NETosis is that the vital type does not require NOX2 and MPO because only stimulation by the pathogenic bacterium is sufficient to induce this type of NETosis [41, 42]. It has been reported that several inflammatory mediators can stimulate NET release, including N-formylmethionyl-leucyl-phenylalanine (fMLP), tumor necrosis factor-α (TNFα), interleukin-8 (IL-8, CXCL8), lipopolysaccharides (LPS), and nitric oxide (NO) [43].

Physiologic and pathologic roles of NETosis

NETosis is an innate immune response against pathogenic microorganisms that eliminates pathogens via trapping and releasing antibacterial enzymes and free radicals [44]. As an innate immune cells, neutrophils are the first cells to migrate to the site of infection to control the spreading of the infectious agent through phagocytosis, secretion of inflammatory cytokines, and NET formation [41]. Studies show that other granulocytes such as macrophages can trap and immobilize microbes in addition to neutrophils by releasing decondensed chromatin [45]. In the homeostasis phase, clearance of the infection environment of cellular debris and NETs is vital [46]. Studies show that NETs are degraded by plasma DNA-degrading enzymes and then cleared by macrophages [47]. In mice, DNase (I and III) enzymes are deficient; following infection and activation of neutrophils, the animals die after a few days due to the deposition of NETs [48]. Furthermore, NETs are identified as intracellular autoantigens and recognized by the immune system, which is the basis of autoimmune diseases [49, 50]. In this section, the role of NETosis in some important diseases is reviewed.

Sepsis

Circulating NETs are also detected in patients with sepsis accompany poor multi-organ failure and poor clinical outcome [51–53]. Uncontrollable increase in NET formation, apoptosis, necrosis and, on the other hand, lack of clearance and degradation of cell death-derived debris and NET contents, including cell-free DNA, leads to increased expression of inflammatory mediators such as TNF-α in these patients. It has also been reported that NET-derived citrulline histones can act as damage-associated molecular patterns (DAMPs) by inducing the expression of inflammatory cytokines and disrupting endothelial cell function through cytotoxicity and increasing ROS production, resulting in multi-organ damage [54–56].

SLE

A study on RA and SLE patients reported that in 79% of the patients, NET-associated autoantigens including MPO/DNA and elastase/DNA complexes were detected, and the activated NETosis in these patients was detected NOX2-independent [57]. In patients with systemic lupus erythematosus (SLE), it has been reported that there is a significant association between disease severity and decreased DNases activity and the number of NET complexes (double-stranded DNA and post-translationally modified proteins) in the blood [58]. Following the exposure of NET-associated autoantigens, interferon (IFN)-producing pDCs activated, resulting in endothelial tissues and organs injury [59, 60]. Activated neutrophils that produce NETs are detected in SLE patients, and in vitro studies show that sera obtained from these patients can react with NET components including DNA, elastase, MPO, and citrullinated H3 histones, suggesting the presence of autoantibodies against the mentioned autoantigens [37, 61, 62]. NET components have also been observed in cutaneous lesions and kidneys of patients with SLE [60, 63]. As mentioned, autoantibodies play an important role in the pathogenesis of SLE and based on the laboratory findings, most SLE patients are positive for antinuclear antibodies (ANA) or anti-dsDNA [64].

Furthermore, anti-histone autoantibodies are also common in these patients [65]. In addition to anti-dsDNA and histone autoantibodies in SLE patients, autoantibodies against other NET components including high-mobility-group protein 17 (HMG-17), lamin B1, lamin B2, catalase, cathelicidin (LL37), apolipoprotein A, α-enolase, and annexin AI have been rarely detected [12, 66]. It is also noteworthy that NET proteins in SLE patients can contribute to tissue damage. One of the contents of NET is matrix metallopeptidase 9 (MMP9), which can induce vascular damage and endothelial cell apoptosis by MMP2 activation [67]. Excessive production of NET in glomeruli and lack of effective clearance of its residues also leads to vascular leakage and stimulation of the endothelial-to-mesenchymal transition (EndMT), which is involved in developing pathological fibrosis [68].

On the other hand, the deposition of the autoantibody/NET protein complexes and in the glomerulus of SLE patients leads to lupus nephritis [69]. These complexes can activate the complement system and lead to infiltrated leukocytes that express Fcγ and complement receptors. Activation of these leukocytes leads to ROS production and degranulation of proteases and tissue damage [70, 71].

RA

Evidence suggests that NET debris has been observed in RA patients' synovial fluid and serum [57, 72]. Synovial biopsy tissue staining also showed the presence of CD15, elastase, MPO and citrulline histone H3 as NET products in these patients [73, 74]. In RA, the enzymes PAD2 and PAD4 are responsible for the citrullination of a wide range of proteins detected by autoantibodies such as actin, histone H1.3, histone H3, vimentin, and α-enolase [66]. NET-derived neutrophil products including PAD2 and PAD4 along with MPO, neutrophil gelatinase-associated lipocalin (NGAL), annexin-A1, cathepsin G, and other citrulline proteins can be detected in the synovial fluid and also synovial necrotic regions of RA patients [75, 76]. Joint damage and increased disease activity due to NETosis in RA also occurred with different mechanisms. An animal study in transgenic HLA-DRB1*04:01 mice showed elastase could disrupt the cartilage matrix's biostructure and induce PAD2 production by fibroblast-like synoviocytes (FLS). FLS can internalize cartilage fragments and present them to T cells via major histocompatibility complex (MHC) class II.

Moreover, PAD2 is responsible for the citrullination of cartilage fragments. After recognizing the presented autoantigen, T cells help produce anti-citrullinated protein antibodies (ACPA) by autoreactive B cells [77, 78]. On the other hand, MMP8 and MMP9 as NET proteins produced in RA participate in the degradation of the cartilage matrix [79, 80]. Aggrecan is another major component of cartilage biostructure, and its degrading enzymes can be detected in NET contents [78]. Following citrullination of aggrecan fragments, they presented to autoreactive T cells, which induce the production of wind autoantibodies [78]. Furthermore, produced autoantibodies by synovial autoreactive B cells as well as NETs produced by blood and synovial neutrophils together lead to joint damage [81].

Cancer

Another pathological condition is malignancy, which according to studies in this field, the NET formation can also play a role in cancer pathogenesis [82, 83]. Evidence demonstrated that both animal cancer models and patients with malignancy had recognized NETs in the blood and tumor tissue. NETosis can play a pro-tumor or anti-tumor role, depending on the condition and the existing signals of the tumor microenvironment (TME) [25, 84]. In fact, according to previous studies, NETosis is associated with increased tumor cell proliferation, tumor progression, and metastasis due to the presence of various proteases as well as inducer signals in NET contents that stimulate tumor cells. On the other hand, tumor cells induce NET formation through the release of various mediators. In addition, the presence of tumor-associated neutrophils (TANs) in the TME can also be associated with tumor growth and development [6, 85]. In the TME, the release of IL-8 and granulocyte-colony-stimulating factor (G-CSF) produced by tumor cells can induce NETosis. G-CSF produced by cancer cells as a granulocyte growth factor increases neutrophils, followed by increased production of ROS and NETosis [85–88]. The outcomes of in vitro studies also show that the co-culture of neutrophil-activated endothelial cells increases NETosis via IL-8 produced by endothelial cells. The findings also show that these cancer cells increase NET formation by neutrophils by priming platelets in pancreatic cancer [89].

Heart failure and cardiomyopathy

It has been documented that several NET-related heart and systemic diseases including, atrial fibrillation, myocardial infarction (MI), hypertrophic cardiomyopathy, myocarditis, diabetes, chronic kidney disease, chronic obstructive pulmonary disease, and autoimmune diseases, could be cause heart failure [90]. Based on available knowledge, NETs may be accompanied by aseptic inflammation and microthrombosis, inducing myocardial ischemia–reperfusion (I/R) and MI [91]. Moreover, citrullinated histone H3, dsDNA, and neutrophil elastase levels increased in the culprit lesion site of patients with ST-segment elevation MI (STEMI) compared with the femoral site [92, 93]. These findings indicated that NET components are related to ventricular function, infarcted size, and clinical consequences in patients with

STEMI [69]. It has also been reported that patients with a high serum neutrophil-to-lymphocyte ratio (NLR) are significantly more susceptible to developing atrial fibrillation [94–96]. Pre-clinical and clinical studies demonstrated that neutrophil MPO is involved in the pathogenesis of atrial fibrillation. For instance, MPO deficient mice were protected from atrial fibrillation, and the protective condition was reversed following MPO restoration. These outcomes disclosed that MPO could be involved in myocardial remodeling, increasing susceptibility to atrial fibrillation [97]. Furthermore, a study on a hypertrophic and hypertensive cardiomyopathy mice model under treatment with transverse aortic constriction (TAC) showed that Wnt5a-mediated neutrophils infiltration into the heart of animals could lead to the NET formation, hyperinflammation and cardiac fibrosis and dysfunction [98]. Current studies reported that neutrophils might be involved in the pathogenesis of atherosclerosis and venous thromboembolism (VTE). However, the role of these cells and NETosis in atherosclerosis has been less studied, and most studies focused on macrophages and their involvement in vascular plaque destabilization. Recently, it has been revealed that NET formation may be involved in the development of atherosclerosis because, in patients with acute myocardial infarction, the presence of NET contents in the lumen of atherosclerotic arteries has been detected. The production of debris from various programmed cell death mechanisms in atherosclerosis, such as apoptosis, NETosis, and efferocytosis and lack of clearance and degradation of these fragments, may cause inflammation and disease progression [99]. Furthermore, NETs can tempt endothelial dysfunction and initiate inflammatory immune responses, resulting in venous thrombi and atherosclerotic plaques formation [100]. However, another experimental investigation in this field suggested that NET-derived PAD4 and bone marrow-derived cells cannot impact chronic atherogenesis, but it can contribute to the formation of acute thrombotic lesions [101].

Diabetes

NETosis is a major involved factor in the pathogenesis of various diabetes and diabetes-associated complications. In type 1 diabetes, the death of pancreatic beta cells leads to the infiltration of neutrophils into the pancreas, which leads to the activation of neutrophils and NET formation. In type 2 diabetes, an increase in the NET formation, followed by an increase in the release of NET content, has been observed. For example, dsDNA has been accompanying an increased susceptibility to cardiovascular disease and diabetic-related kidney disorders. NETosis has also been disclosed to be involved in diabetic retinopathy and impaired wound healing. The mechanism of inducing NETosis in diabetes is not yet fully understood, but hyperglycemia is an important trigger for NET formation. However, an increase in NETosis is also seen in patients with controlled glucose levels [102].

Periodontitis

In humans, periodontitis is one of the most common inflammatory, infectious diseases, often occurring in subgingival plaque due to uncontrolled responses of activated neutrophils to pathogenic bacteria. As a result, neutrophils are considered to be the main immune cells in the pathogenies of periodontitis. Once activated by pathogens, neutrophils can produce ROS, which stimulates NETosis. NET formation appears to act as a double-edged sword in periodontitis because it can control infection by trapping pathogenic bacteria and cause autoimmunity and destructive immune system responses through its autoantigens contents [10]. This suggests that adequate NET formation and its content are vital for periodontal health preservation [103].

Acute respiratory distress syndrome (ARDS)

Evidence suggests that neutrophil infiltration into the lungs and NET formation cause hyperinflammation in the lungs, and the rate of this infiltration and NET production is directly related to the severity of the disease [104]. Correspondingly, studies on animal models of acute lung injury (ALI) have shown that neutrophil depilation can have a protective effect against destructive neutrophil-mediated inflammatory responses [105]. In ventilator-induced lung injury (VILI), amplified levels of high-mobility-group box protein 1 (HMGB1) and IL-1β could induce NET formation [106, 107]. Mechanical deformation of pulmonary cells could stimulate phosphoinositide 3-kinase gamma (PI3Kγ)-mediated pathway, regulating pulmonary cells apoptosis and neutrophil-mediated inflammatory response via the phosphorylation of ERK1/2 and Akt [108]. Moreover, neutrophil-macrophage cooperation was detected in liver injury, atherosclerosis, kidney diseases, hematological disorders and inflammatory bowel disease (IBD) [109–112]. In addition, it has been shown that in ARDS, there is a significant association between infiltrated neutrophils, resident alveolar macrophages, and increase of caspase-1 levels as well as IL-1β production. In this context, it has been reported that pyroptotic alveolar macrophages are responsible for releasing caspase-1 and IL-1β and eventually neutrophil-derived NET and developing ARDS [113].

COVID-19

Recent studies on coronavirus disease 2019 (COVID-19) showed that viral pneumonia leads to respiratory failure and multi-organ failure due to dysregulated inflammation, complement activation, and thrombosis [114, 115]. Moreover,

Table 1 Potential anti NETs therapeutics and mechanism of action

Pharmacological compounds	Type of study	Target	Mechanism of action	References
Hydroxychloroquine	Pre-clinical	Inhibiting NET formation	Inhibiting TLR-9, ROS, and IL-8 production expression of PAD4 and Rac2	[16, 121]
Methotrexate	Pre-clinical/clinical	Inhibiting ROS/adenosine production	Indirectly inhibit NET production	[127–132]
Prednisolone	Pre-clinical	Inhibiting production of ROS and inflammatory mediators	Indirectly inhibit NET production	[134]
PF-1355	Pre-clinical	Inhibiting MPO	Inhibiting NET and IC formation	[17]
AZD9668	Clinical	Inhibiting neutrophil elastase/IL-1β IL-6, IL-8, TNFα	Indirectly inhibit NET production	[18]
BMS-P5	Pre-clinical	Inhibiting PAD	Indirectly inhibit NET production	[136, 137]
Rituximab and Belimumab	Clinical	Anti-CD20 and BlyS	B cells depletion, indirectly inhibit NET production	[15]
Tocilizumab	Pre-clinical/clinical	Anti-IL-6R	Indirectly inhibit NET production	[145]
Cl-amidine	Pre-clinical	Inhibiting PAD4	Indirectly inhibit NET production, decreasing atherosclerotic lesion area, reducing thrombosis	[146]
DPI	Pre-clinical	Inhibiting gluconeogenesis and cellular respiration enzymes, inhibiting ROS production	Indirectly inhibit NET production	[149, 150]
Recombinant human DNase	Pre-clinical	NET-derived DNA	Reduce their destructive effects of NET contents	[168, 169]
Azithromycin and chloramphenicol	Pre-clinical	Affect respiratory burst, apoptosis, degranulation of neutrophils	Indirectly inhibit NET production	[173]
THIQs	Pre-clinical	Neutrophils	Inhibiting different stages of NET formation without weakening neutrophil normal functions	[175]
Anthracyclines	Pre-clinical	Inhibiting of transcription initiation or DNA replication	Inhibiting NET formation	[177]

NETs neutrophil extracellular traps, *IL* Interleukin, *ROS* reactive oxygen spices, *TLR* toll-like receptor, *PAD* protein arginine deiminase 4, *BlyS* B lymphocyte stimulator, *THIQs* tetrahydroisoquinoline derivatives, *IL-6R* interleukin-6 receptor

active neutrophils play an important role in the pathogenesis of this disease, and an increase in the number of neutrophils along with a decrease in lymphocytes is evident in COVID-19 patients [115]. As discussed before, NET formation increases the inflammatory response and vascular micro-thrombosis due to the deposition of NET components, which in the lungs of patients leads to ARDS [116]. Studies have also reported that MPO/DNA complexes, cell-free DNA, and citrullinated histones, especially H3 as major NET components, can be detected in the serum of patients with COVID-19. On the other hand, in these patients, there is a positive and significant association between the levels of cell-free DNA and C-reactive protein (CRP), D-dimer, lactate dehydrogenase (LDH), as well as neutrophil count [117]. There is also a correlation between citrullinated H3 and platelet count, indicating the role of NETosis in thrombosis formation.

Furthermore, cell-free DNA and MPO/DNA levels were higher in patients under intensive care, and mechanical ventilation than in other patients admitted with breathing room air. As a result, NETosis may also be associated with disease severity in COVID-19. It has also revealed that COVID-19 patients' sera can activate neutrophils and NET formation in vitro [117].

NET inhibitors

One of the therapeutic approaches in autoimmune and inflammatory diseases, as well as metastatic cancers in which traces of NETosis have been found, is the use of NET inhibitors or enzymes involved in the NET formation such as PAD4 and MPO (Table 1). In this section, the most important NET inhibitors are briefly reviewed (Fig. 1).

Hydroxychloroquine

Hydroxychloroquine is an anti-malarial drug widely used to treat some diseases such as SLE, RA and COVID-19 [118–120]. This drug can inhibit the function of activated

neutrophils. Hydroxychloroquine has been shown to inhibit NET formation by inhibiting toll-like receptor-9 (TLR-9), inhibiting ROS, and preventing IL-8 production. Furthermore, hydroxychloroquine can inhibit the expression of PAD4 and Rac2 [16, 121]. In animal models of hepatic ischemia/reperfusion injury, treating animals with hydroxychloroquine could repress the production of NETs [16]. However, the results of some studies have been contradictory and have confirmed that hydroxychloroquine does not affect the expression of PAD4, MPO and neutrophilic elastase [122]. Several studies have proved that autophagy is closely connected to the platelet-induced NET formation, and the administration of hydroxychloroquine as an autophagy inhibitor in patients with COVID-19 could reduce immunothrombosis through inhibition NETosis [123–125].

Methotrexate

Methotrexate has been used in clinics for over 30 years as a first-line drug in the treatment of RA [126]. In addition to RA, it is also prescribed in SLE and can inhibit cytokine-delayed neutrophil apoptosis. It also can inhibit ROS production and leukotriene B4 synthesis [127–129]. As discussed, ROS is involved in the NET formation, so methotrexate can indirectly inhibit NET production by neutrophils. A recent study in patients with COVID19 showed that methotrexate could reduce inflammation by inducing adenosine production [130]. Adenosine is an immunomodulator that can also inhibit NETosis and thrombosis [131, 132].

Prednisolone

An active metabolite of prednisone known as prednisolone is widely used as a corticosteroid to treat inflammatory and autoimmune diseases. Following crossing the cell membrane and entering the cytoplasm, prednisolone can bind to glucocorticoid receptors and following this ligation, the complex can directly disrupt gene expression of enzymes and inflammatory cytokines. Furthermore, prednisone is able to rapidly inhibit the function of neutrophils, such as the production of ROS and inflammatory mediators, thereby disrupting NET formation [133, 134].

PAD4, elastase and MPO inhibitors

As mentioned in the mechanisms of NET formation, elastase and MPO enzymes are involved in the decoding of chromatin by neutrophils and their release. Therefore, inhibition of these enzymes can inhibit NETosis. An investigation on vasculitis mouse models using a 2-thiouracil mechanism-based MPO inhibitor (PF-1355 [2-(6-(2,5-dimethoxyphenyl)-4-oxo-2-thioxo-3,4-dihydropyrimidin-1(2H)-yl) acetamide) showed that MPO activity is involved in NETosis and immune complex (IC)-associated vasculitis. The findings revealed that PF-1355 could inhibit MPO and further NET and IC formation in vasculitis [17]. AZD9668 is known as an effective and reversible neutrophil elastase inhibitor [135]. A study on bronchiectasis patients showed that administration of AZD9668 could prevent inflammation by reducing the expression of elastase, IL-1β IL-6, IL-8, TNFα, resulting in improved lung function with minimum side effects [18]. However, due to the variability of the outcomes of studies in this field, enzyme inhibitors involved in NET formation need further studies. Moreover, it has been demonstrated that human and murine multiple myeloma cells can induce citrullination of histone H3 and NET formation. These occurrences could be inhibited through pharmacological targeting of PAD4 with BMS-P5, a novel and specific small PAD4 inhibitor molecule [136, 137].

Rituximab and belimumab

It has been reported that ICs in vitro can trigger NET formation whereas NET-derived DNA is considered as an autoantigen for ANAs in SLE [138]. In this context, rituximab (anti-CD20 mAb) depletes autoreactive B cells and inhibits further autoantibody production [139]. Moreover, belimumab (fully human IgG1λ recombinant mAB) can target BlyS and reduce autoreactive B cells' survival and autoantibodies production [140]. Interestingly, an investigation showed that a combination of rituximab and belimumab reduces the NET formation and ANA production in SLE patients [15]. Thus, the combination of these mAbs can inhibit NET formation in parallel with the depletion and inhibition of B cells, which play a major role in producing autoantibodies against NET-derived autoantigens.

Tocilizumab

Tocilizumab is a mAb against IL-6 receptor (IL-6R) widely used to treat inflammatory diseases such as RA, SLE, COVID-19, and even cancer [141–144]. A study on a total f twenty RA patients six months under treatment with162 mg per week subcutaneous tocilizumab showed that it could enhance endothelial function and decrease leukocytes-derived oxidative stress. Furthermore, tocilizumab decreased the frequency of low-density granulocytes and inhibited NET formation. These findings were also confirmed after treatment of neutrophils and monocytes with tocilizumab in vitro. This study also showed that pro-atherothrombotic complications might be reduced in RA patients following inhibition of NETosis. [145].

Cl-amidine

Inhibition of peptidyl arginine deiminase by Cl-amidine is a therapeutic approach that can reduce or inhibit NET formation in vivo. It has been reported that in murine models of atherosclerosis treated for 11 weeks with daily injections of Cl-amidine could inhibit NET formation by inhibiting PAD4 and decreasing atherosclerotic lesion area, and reducing thrombosis. These consequences support a role for abnormal NET formation in atherosclerosis pathogenesis [146].

Diphenyleneiodonium chloride (DPI)

DPI is a blood glucose control agent that exerts its effects by inhibiting gluconeogenesis and cellular respiration enzymes, including NO synthase, NOX, xanthine oxidase, cholinesterase, and NADPH cytochrome P450 oxidoreductase [147, 148]. DPI can bind to the heme group of the NOX enzyme and inhibit ROS production. A study in this field showed that DPI administration could inhibit ROS-induced NET formation and extracellular release of NET-derived DNA [149, 150].

Other studied NETosis inhibitors

In this section, other chemical drugs and natural compounds that inhibit NETosis are summarized. It has been reported that low doses of rapamycin and lapatinib can effectively inhibit NET formation, while high doses of these drugs induce NETosis. On the other hand, ponatinib, crizotinib, and bosutinib are identified as NET inducers and can be used in NET-deficient and CGD patients by targeting the NOX downstream intracellular molecules [34]. In contrast, another study showed that bosutinib could inhibit IC-stimulated NET formation but no other type of NETosis, which is receptor-independent and stimulated by the calcium ionophore ionomycin [151]. However, in periodontitis, a combination of bosutinib, ponatinib, and celastrol could reduce phorbol 12-myristate 13-acetate (PMA)-induced ROS production by hyperactive neutrophils, resulting in reducing NET formation [152, 153]. It appears that the dose of bosutinib, as well as combination with other drugs, can completely change the effect on NETosis and the reason for these discrepancies is probably these cases. Other NET inhibitor compounds such as erlotinib, carmustine, and lapatinib may be employed to treat SLE, CF, and RA as pathologic states in which aberrant NET formation is associated with disease progression [58, 73, 154].

As discussed earlier, ROS production is one of the most important inducers of NETosis, and any compound or substance that can inhibit ROS production, therefore, has the ability to inhibit NET formation by activated neutrophils. In this regard, a study reported that vitamin C and flavonoids such as catechin hydrate, rutin trihydrate, epicatechin are able to inhibit PMA-induced ROS production. Furthermore, pharmacological substances including 5-aminosalicylic acid (5-ASA) and *N*-acetyl-L-cysteine (NAC) can inhibit ROS and NET production. Therefore, many antioxidants can increase and decrease pathology by inhibiting the onset of NETosis. However, more studies are needed in this area [155]. Studies show that one of the most important laboratory findings in SLE patients is vitamin D deficiency. Vitamin D as an immunomodulator can modulate the destructive responses of the immune system and prevent autoimmunity. A study has reported that vitamin D can prevent endothelial cell damage by reducing NET formation. As a result, this vitamin can be used as a supplement to treat patients with autoimmune disorders such as SLE [156]. Surprisingly, it has been reported that aerobic exercise recovers lung inflammation in acute lung injury. Moreover, a positive association was observed between inflammatory alveolar macrophage polarization and NET formation. An investigation on LPS-induced acute lung injury mouse models showed that five weeks of aerobic treadmill running could alleviate acute lung injury by inhibiting the NET formation and alveolar macrophages pro-inflammatory phenotype polarization by suppressing ERK1/2 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways [157].

Advantages and disadvantages of NETosis inhibition

As the fallouts of the reviewed studies showed, NET formation is initially considered a kind of innate immune mechanism against pathogens that can control infections. However, when its production is dysregulated, it can be involved in the pathogenesis of several inflammatory disorders, tumors, and autoimmunity. As a result, inhibition of this phenomenon and its inducing factors can be an effective therapeutic approach to declared diseases. Like various therapeutic tactics, NETosis has its strengths and weaknesses, highlighted in this section.

According to various studies that have been performed so far, inhibition of PAD enzymes, which play a pivotal role in the NET formation, has been very useful. In various diseases, including RA, diabetic wound, multiple sclerosis (MS), colon cancer, atherosclerosis and spinal cord injury models, inhibition of PAD by various factors such as Cl-amidine has had satisfactory outcomes [26, 158–161]. Moreover, inhibition or knock-out of PAD enzymes can reduce the severity of the disease, reduce the citrulline of proteins, especially histones, and reduce inflammation and the production of destructive autoantibodies [162]. On the other hand, inhibition of PAD enzymes is associated with an alteration in differentiation from T helper (Th)1 and

Th17 to Th2, associated with a decrease in inflammatory responses mediated by Th1 and Th17 [163–165]. Investigations on human cancers showed that the inhibition of NETosis could also prevent tumor progression. However, combination therapy with NET inhibitors and checkpoint inhibitors, including anti- cytotoxic T-Lymphocyte associated protein 4 (CTLA4) and programmed death-1 (PD-1) mAbs, can increase the anti-tumor function of $CD8^+$ T cells. As a result, NET inhibitors can be used as adjuvants in combination immunotherapies, increasing the effectiveness of immune checkpoint blockers and other anti-cancer agents [166]. In patients with COVID-19, increased inflammation and thrombosis and multiple organ failure have been suggested as important disease features. In this regard, platelets can cause thrombosis by stimulating NETosis, and inhibition of NET in these patients can reduce thrombotic complications [124].

The abnormal and dysregulated NET formation has been reported in several lung diseases [154]. Chronic obstructive pulmonary disease (COPD), characterized by persistent respiratory symptoms and airway limitation due to airway or alveolar abnormalities, is one of these lung diseases [167, 168]. This disorder is most commonly realized in people who have been exposed to toxic gas particles. Evidence suggests that neutrophils are one of the major innate immune cells that infiltrate the lungs and can participate in the pathogenesis of COPD through NET production, inflammation, and direct induction of epithelial and endothelial cell death. As a result, inhibition of NETosis can be considered a suitable target for treating this type of lung disease. Studies showed that the use of recombinant human protease inhibitors and DNase could neutralize and degrade NET-derived DNA and proteins and reduce their destructive effects. Additionally, targeting the 2-chemokine receptor CXC (CXCR2) can inhibit neutrophil trafficking into the lung, reduces inflammation, decreases mucus production, and inhibits lung tissue destruction mediated by neutrophil proteinase [168]. A genetic defect in the cystic fibrosis transmembrane conductance regulator (CFTR) gene causes cystic fibrosis)CF(. Studies in infants with the CFTR mutation showed that peribronchial neutrophil infiltration occurred before the development of lung infection, leading to increased inflammatory response and involvement of the upper and lower airways. Increased mucosal viscosity and small and medium bronchioles obstruction also occurred following those as mentioned earlier infectious and inflammatory responses. Evidence also suggested that neutrophil cytotoxins, NETs, and extracellular DNA are associated with increased mucosal obstruction and lung tissue damage in CF patients.

Furthermore, neutrophil phenotypes, airway pH, and milieu salt concentrations can affect NETotic capacity and neutrophil survival. As a result, attenuation of NETs using different NET inhibitors such as DNAse can manage CF airways inflammation. However, the use of DNAse can lead to the release of cytotoxins from NETs and increase inflammation and even autoimmunity. As a result, further studies are needed to degrade NET residues with the maximum positive effect and minimum release of cytotoxic components [169].

Despite the positive consequences of NET inhibition and its inducing factors such as PAD, MPO, NOX enzymes, as well as inhibition of ROS production in various diseases related to NETosis, it can face some challenges. For example, PAD enzymes are also involved in various biological processes, including protective immunity, regulation of gene expression, and cell differentiation and using PAD inhibitors can impair these biological processes [21]. Studies have shown that PAD-deficient mice are more susceptible to bacterial infections than healthy mice, and this finding suggests that inhibition of PAD and subsequent inhibition of NET may make patients more susceptible to infection [170]. Therefore, the administration of NET inhibitors in immunocompromised patients should be more cautious. Moreover, cyclosporine as an immunosuppressive drug that can inhibit NETosis can disrupt the immune system and the required responses to pathogens clearance, leading to more recurrent infections in under treatment patients [159, 171]. Another study reported that PAD4 knock-out mice developed systemic inflammation and bacterial keratitis in the local cornea, where neutrophils activate through NETosis to protect against infection [22].

In addition to the antibacterial properties of antibiotics and their use in the management of bacterial infections, these drugs can have an immunomodulatory effect on the properties of immune cells such as neutrophils [172, 173]. In this regard, a study showed that azithromycin and chloramphenicol affect respiratory burst, apoptosis, degranulation of neutrophils as well as NET formation. Their study showed that pre-treatment of neutrophils with chloramphenicol and azithromycin decreased NET formation. Also, among gentamicin and cefotaxime, gentamicin can reduce NET release by neutrophils [173]. As a result, the use of antibiotics with NET formation inhibitory properties can be useful in combination therapies for dysregulated NETosis-mediated inflammatory diseases and management of probable further infection following NET inhibition without interfering with their antimicrobial function [174]. Because effector neutrophils are essential for preserving immune responses to infections, drugs and inhibitors that can selectively and without harm the normal function of neutrophils only inhibit or reduce NET overproduction are preferable to other NET inhibitors. Studies have shown that tetrahydroisoquinolines (THIQs) are a new class of NET formation inhibitors that, unlike the mechanisms as mentioned earlier, do not target the neutrophil granular proteins activity or the formation of ROS and

only reduce NET formation without weakening neutrophil normal functions [175].

Besides, analyses of transcriptomics disclosed that transcription initiated at multiple loci in all chromosomes prior to the rapid vital type of NETosis than Nox-dependent NETosis. NETosis-specific kinase cascades could activate transcription of different groups of genes in a different manner. Furthermore, transcription inhibitors can suppress vital and Nox-dependent NETosis types without affecting ROS production, essential for antibacterial neutrophil functions [176]. Another of these selective NET inhibitors is an anthracycline, which may reverse transcription initiation and inhibit NETosis. Inhibitory doses of anthracyclines have been shown to suppress ROS production, which is necessary for antimicrobial functions, and do not cause apoptotic cell death in neutrophils. In the case of combination therapy, an anthracycline with dexrazoxane can be used as a heart protective agent and limit the side effects of anthracyclines. Interestingly, dexrazoxane neither affects NETosis nor alters the NET inhibitory ability of anthracyclines. As a result, the correct doses of an anthracycline with dexrazoxane can be used as a highly effective treatment for inhibiting unsolicited NETosis in NET-mediated diseases [177].

On the other hand, some other studies acknowledge that mice and humans with NETosis deficiency who had defects in the MPO or PAD4 enzyme did not differ from healthy groups regarding susceptibility to infection. These findings indicate that using NET inhibitors will not increase the susceptibility to infection in treated patients [178, 179]. The reason for these discrepancies seems to be the function of the innate immune system in defense against pathogens in a compensatory manner. Because the compensatory mechanisms in cases of inherited or acquired immune response defects could be covered by other components of the immune system [180]. For example, in immunocompromised patients, recurrent infections are not always realized, which can confirm the immune system's network function in defense against pathogens [181].

Although NETs are effectively involved in various infectious diseases and pathogen clearance processes, they can also be destructive due to the release of enzymes and other cytotoxic proteins that cause tissue damage in other inflammatory and autoimmune disorders. Therefore, control and management of NET overproduction are quickly becoming a potential target for treatment, but it is important to note that different NET inhibitors may have other side effects, such as weakened immune systems and increased susceptibility to infections. Moreover, it is possible that optimizing the effectiveness and clinical outcomes of treatment and managing NETs may require the use of combination therapies with minimum impacts on weakening the immune system [174].

Concluding remarks

Based on the findings of recent studies on the role of NETosis in the host defense and innate immunity as well as the pathogenesis of various diseases, it appears that dysregulated NETosis can release a variety of intracellular genes out of the cell. Defects in the clearance and degradation of NET debris cause recognition by the immune system, failure of tolerance to local antigens, and activation of autoreactive immune cells, whose destructive responses cause tissue damage in autoimmune diseases. On the other hand, the use of NET inhibitors can effectively treat several NET-associated diseases, although this type of treatment should be accompanied by monitoring patients for control of recurrent infections, especially in immunocompromised patients. Therefore, NET inhibitor drug formulations or enzymes involved with minimal side effects can be useful in treating various NET-associated diseases. Further study and understanding of the regulation and balance of NET induction, inhibition, and degradation using NET inhibitors will be necessary without compromising the patient's immune defenses. In order to apply this type of treatment in the clinic, further investigations are needed to accurately understand the functions and effects of NETs on health and disease regarding the heterogeneous patterns of disease [182].

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