International Journal of Nanomedicine

Dovepress
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REVIEW

Role and Therapeutic Targeting Strategies of Neutrophil Extracellular Traps in Inflammation

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Abstract: Neutrophil extracellular traps (NETs) are large DNA reticular structures secreted by neutrophils and decorated with histones and antimicrobial proteins. As a key mechanism for neutrophils to resist microbial invasion, NETs play an important role in the killing of microorganisms (bacteria, fungi, and viruses). Although NETs are mostly known for mediating microbial killing, increasing evidence suggests that excessive NETs induced by stimulation of physical and chemical components, microorganisms, and pathological factors can exacerbate inflammation and organ damage. This review summarizes the induction and role of NETs in inflammation and focuses on the strategies of inhibiting NETosis and the mechanisms involved in pathogen evasion of NETs. Furthermore, herbal medicine inhibitors and nanodelivery strategies improve the efficiency of inhibition of excessive levels of NETs.
Keywords: neutrophil extracellular traps, inflammation, targeted inhibition, nanotherapy, herbal medicine

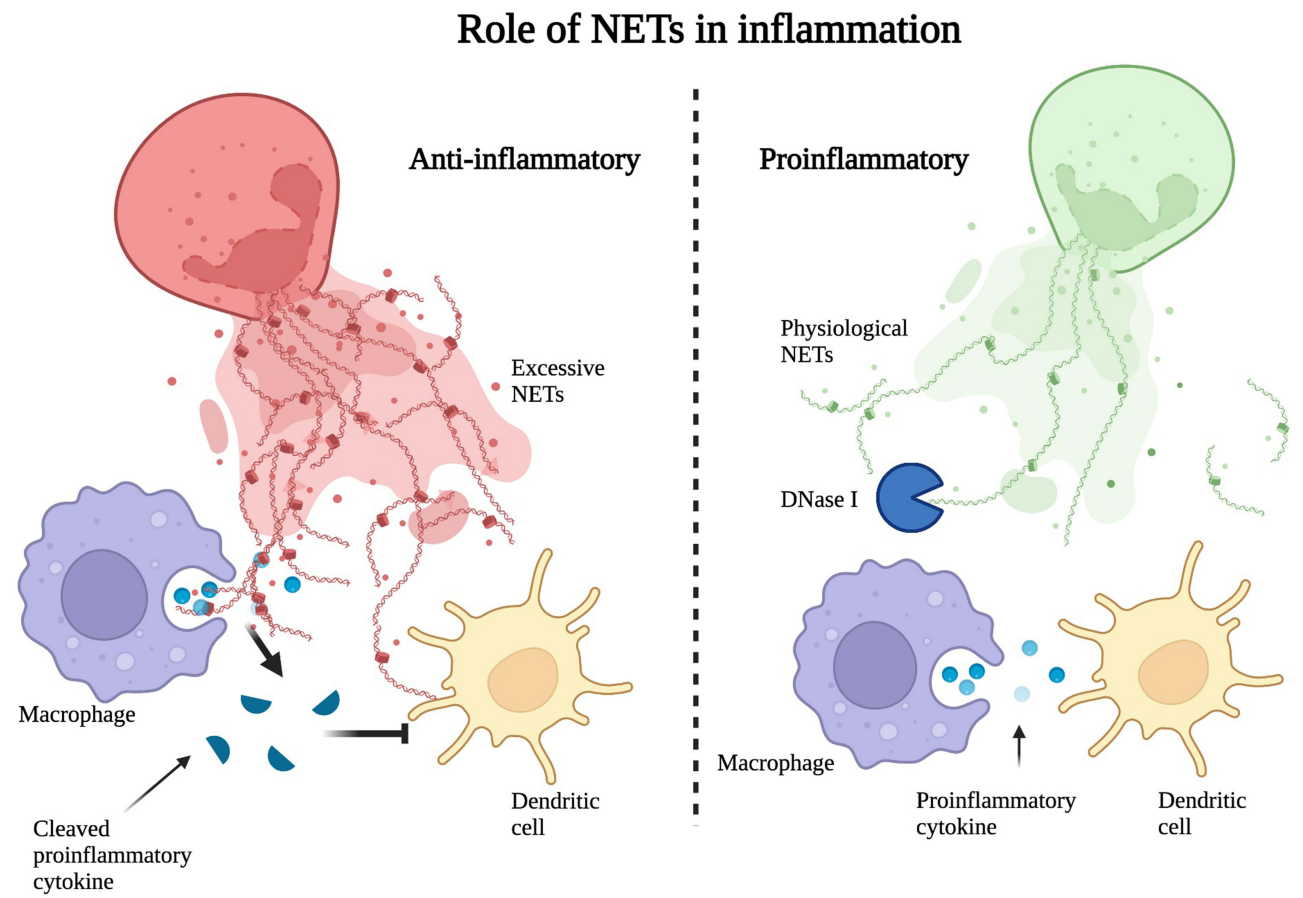
Introduction

The discovery of neutrophil extracellular traps (NETs) has opened a new chapter in our understanding of the nature and function of neutrophils. In addition to previously identified mechanisms of phagocytosis, degranulation, production of reactive oxygen species (ROS), and cytokines, neutrophils use an additional effector function—production of NETs—to modulate immune responses.[1] NETs were first discovered by Takei et al[2] in 1996 who determined that they were involved in a novel process of phorbol 12-myristate 13-acetate (PMA)-induced programmed neutrophil cell death, and activated a molecular pathway that differed from that of apoptosis and necrosis. In 2004, Brinkman et al[3] provided a more comprehensive description and named this process NETosis. In addition to chemical agents such as PMA, various stimuli in vivo or in vitro—including pathogens, inflammatory mediators, and cell damage products—have been found induce NETs formation through different effector mechanisms.[4] Despite the heterogeneity of activation of the NETosis signaling pathway by different stimuli, the stimulus ultimately leads to a general outcome involving chromatin and protein binding, reorganization of the intracellular membrane, and finally release of NETs into the extracellular space.[5,6] These stimuli activate NETosis by binding to pattern recognition receptors (such as Toll-like receptor [TLR], C-type lectin receptor, and nucleotide-binding oligomerization domain-like receptor), complement receptors, Fc receptors, chemokine receptors, and other receptors.[7,8]

NETosis mainly includes three pathways (Figure 1). The most widely characterized mechanism is the formation of suicidal NETs.[9,10] PMA stimulates the activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) and the production of ROS in neutrophils through the protein kinase C (PKC) and Raf-MEK-ERK signaling pathways.[11] The activation of myeloperoxidase (MPO) and ROS production promote the translocation of neutrophil elastase (NE) from azurophilic granules to the nucleus, and relocated NE initiates chromatin

Received: 22 April 2023
Accepted: 29 August 2023
Published: 18 September 2023

Graphical Abstract



decondensation in the nucleus.[12] Peptidylarginine deiminase IV (PAD4) promotes nucleosome histone cleavage and chromatin decondensation through citrullination.[13,14] PAD4 and cell cycle proteins jointly regulate nuclear membrane rupture,[15] and actin leads to plasma membrane rupture.[16,17] Finally, with fragmentation of the cell membrane, the decondensed chromatin is mixed with granule proteins, which are released into the extracellular space to form NETs.

Compared to the relatively lengthy period (3 to 4 hours) required for the formation of suicidal NETs, *Staphylococcus aureus* (*S. aureus*) rapidly stimulates neutrophil release of NETs in a very short time of 5 to 60 minutes. This rapid and novel mechanism of NETs formation is called "Vital NETosis".[18] In this pathway, NOX activity is not required and nuclear DNA-laden vesicles are extruded without disrupting the plasma membrane, which preserves neutrophil function.[18,19] A mechanism independent of ROS, NE, and PAD4 has been reported. Gram-negative bacteria and lipopolysaccharide (LPS) induce NETosis through the formation of gasdermin D (GSDMD) via a caspase-11-dependent mechanism.[20]

NETs are always accompanied by inflammation as a part of the immune response. Many stimuli that cause inflammation induce NETosis, whereas NETs promote the onset and worsening of inflammation, as seen in sepsis, diabetes, and rheumatoid arthritis (RA).[21–23] Therefore, the inhibition of NETosis is an important approach for the treatment of inflammatory diseases. Although many recent reviews have described the inhibition of NETs formation, an evaluation of their advantages and disadvantages and effective therapeutic targeting strategies is lacking.[7,24–27] To this

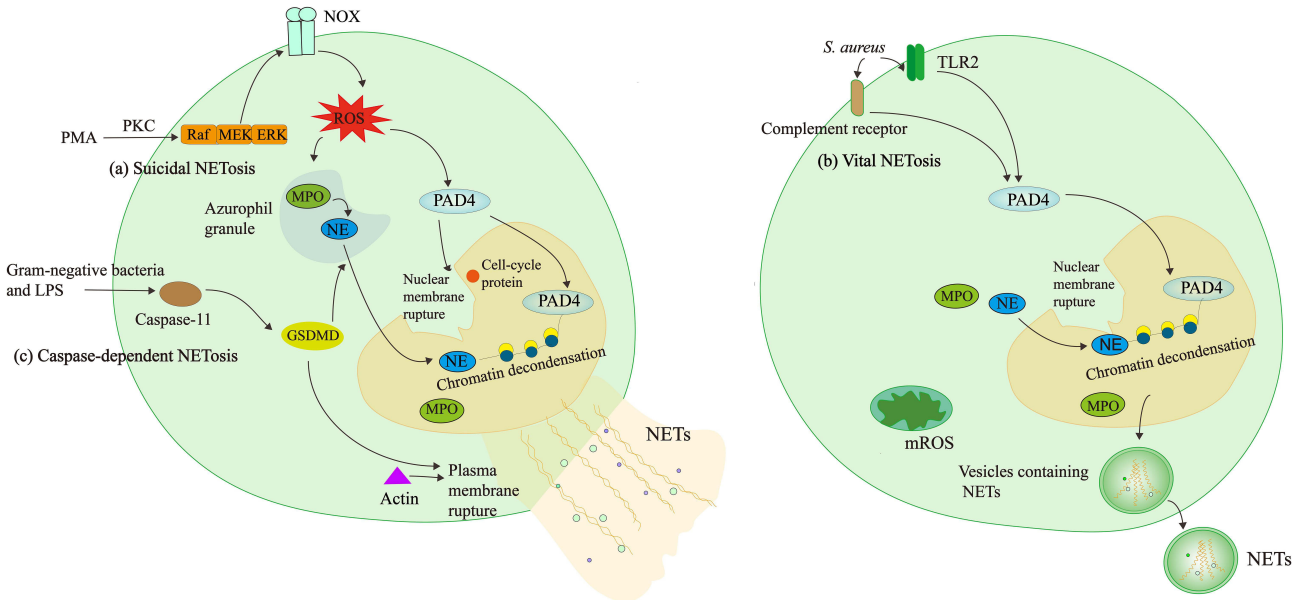


Figure 1 Mechanisms of NETs formation.
Abbreviations: NETs, neutrophil extracellular traps; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; NOX, NADPH oxidase; ROS, reactive oxygen species; MPO, myeloperoxidase; NE, neutrophil elastase; PAD4, peptidylarginine deiminase IV; LPS, lipopolysaccharide; GSDMD, gasdermin D; TLR, toll-like receptor; NETs, neutrophil extracellular traps.

end, in this review, we include a description of biomimetic NETs and targeting herbal medicines to the database of NETs inhibition and nanotargeting therapy to improve the efficiency of targeting NETs.

Stimuli Inducing Inflammatory NETosis

Lots of stimuli are known to induce NETosis, such as chemical reagents, activated platelets, and microorganisms.[4] Some of these stimuli are identified in NETosis induction in vitro, while others are identified following the study of the pathogenesis of inflammation, which more clearly revealed the role of NETosis in inflammation. Below, we discuss the stimuli triggering NETosis and the role of NETs in inflammation to provide more comprehensive background information to better explain the strategy of NETs-related therapy.

Physical and Chemical Components

As a common activator of PKC, PMA was the first chemical identified to initiate NETosis in a NOX-dependent manner. Interestingly, NETs produced by the calcium ionophore A23187 stimulated NETosis in an alternative NOX-independent pathway, which appeared to be extremely like the former in protein composition.[4] The generation of NETs stimulated by external nonphysiological factors may aggravate inflammation. For example, cigarette smoke extract was shown to induce the formation of NETs and airway inflammation in mice in an ROS-dependent manner.[28] There is no doubt that NETs induced by physiological factors are more closely related to inflammation. Eosinophils and eosinophil-generated Charcot–Leyden crystals can directly induce neutrophil activation, increase the activity of NE and cathepsin G, form NETs, and eventually aggravate the local inflammatory circulation in chronic neutrophilic rhinosinusitis.[29,30] The NETs-related protein LL-37 also induces NETosis to sustain an auto-inflammatory cycle.[31]

Microorganisms

In addition to the common microbial virulence factor LPS, invading bacteria, viruses, and fungi are inducers of NETs that further mediate inflammation development. Neutrophils can selectively induce the formation of NETs according to the size of invading microorganisms. NETs target large pathogens, while phagocytosis targets smaller pathogens, which sequester NE to reduce unnecessary NETosis.[32] The resulting NETs have an obvious dual effect. NETs trap and kill bacteria,[3] fungi,[33] viruses,[34] and parasites[35] to stop the spread of infections. Conversely, NETs induced by

microorganisms can promote the development of inflammation and deteriorate health. Active severe acute respiratory syndrome-associated coronavirus-2 (SARS-CoV-2) virus has been reported to invade through the angiotensin converting enzyme 2 (ACE2) and the transmembrane serine protease 2 pathway, and can also down-regulate the ACE2 receptor to promote angiotensin imbalance, further promoting NETs release, pulmonary epithelial cell death and thrombosis.[36,37] The symbiotic fungus *Candida albicans* exposed to skin colonization in mice also significantly activates Th17 cells and increases neutrophil activation and NETosis, and can also aggravate the inflammation of psoriatic dermatitis.[38] Furthermore, *Pseudomonas aeruginosa* biofilm on the ocular surface expresses a high level of bacterial type-3 secretion and promotes NETosis. The resulting NETs barrier "dead zone" limits bacteria to the external environment of the cornea to prevent brain invasion while causing severe keratitis.[39]

Pathological Factors

High pH,[40] hypertonicity,[41] hyperglycemia,[42] disorders of NETs degradation, and other pathological factors are also vital for the induction of NETosis. Due to different disease contexts, symptomatic treatment may help to more deeply explore the impact that the unique microenvironment of various diseases plays on NETosis. Unstable tear film and hyperosmotic characteristics of dry eye syndrome can explain the existence of excessive NETs on the ocular surface of patients with dry eyes.[43] Compared to non-diabetic patients, hyperglycemia increases the rate of NETosis and the release of NETs in patients with type 2 diabetes.[42] There may be inflammatory factors and other stimuli in the inflammatory environment that induce NETosis and aggravate inflammation. The colonic inflammatory environment of patients with ulcerative colitis releases interleukin (IL)-1β and locally regulated thrombus tissue factor in the development and DNA damage responses 1 protein to promote NETosis.[44] These mechanisms suggest that the factors that lead to the overproduction of NETs, and failure to degrade NETs in time will also cause inflammation related to NETs. In patients with severe dry eye syndrome, lacrimal nuclease deficiency causes environmental DNA (eDNA) and NETs to accumulate in the anterior corneal tear film and bind to cathelicidin, which re-enters the ocular surface of cells to activate the type I interferon (IFN) response.[45] Previous studies have shown that alveolar macrophage-mediated apoptosis of apoptotic neutrophils also contributes to persistent NETs-mediated inflammation.[46]

Pro-Inflammatory Activities of NETs

Although neutrophils release NETs to capture pathogens, persistent infections, and the inflammatory environment result in excessive production of NETs, which mediate various inflammatory diseases. A wide range of inflammatory responses, including local inflammation of the lung, intestine, eye, nose, mouth, and skin, and autoimmune inflammation are involved. NETs mediate inflammation primarily by activating the inflammasome, via crosstalk with damage-associated molecular patterns (DAMPs), or by amplifying the inflammatory response with immune cells or immune factors that are related to autoimmunity and occlusive arterial disease (Figure 2).

NETs Activation of the Inflammasome

Inflammasomes activated by NETs are important initiators of inflammation in the immune response.[47,48] NETs activate the inflammasome in resident cells, such as monocytes or macrophages, in patients with high eDNA asthma, resulting in the secretion of IL-1β.[49] NETs also activate the inflammasome in lupus macrophages and lead to the release of the inflammatory cytokine IL-18, which aggravates the inflammatory response.[50] Furthermore, recent studies had found that NETs regulate the assembly of the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome in neutrophils through PAD4, and the formation of the NLRP3 inflammasome further promotes NETosis.[51]

NETs Crosstalk with DAMPs

DAMPs are endogenous molecules that induce and enhance aseptic inflammation. Under conditions of injury or hypoxia, DAMPs activate the human immune response, worsen the inflammatory response, and cause tissue damage.[52] The main components of NETs, such as histones, granules proteins, high-mobility group box 1 (HMGB1) and DNA, are DAMPs that can trigger inflammation.[53] DAMPs have also been indicated to induce NETosis. HMGB1 released from lung cells

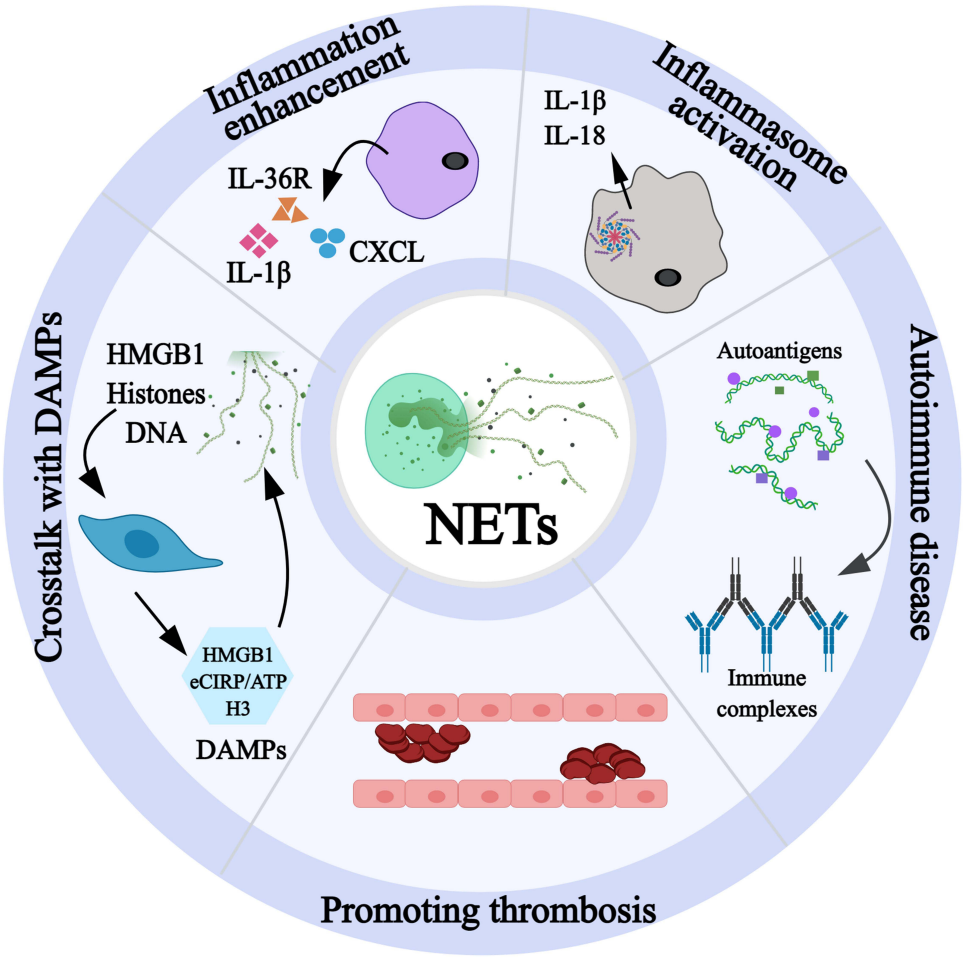


Figure 2 Pro-inflammatory activities of NETs.
Abbreviations: NETs, neutrophil extracellular traps; HMGB1, high-mobility group box 1; CXCL, C-X-C motif chemokine ligand; eCIRP, extracellular cold-inducible RNA-binding protein; H3, histone 3; IL, interleukin.

from patients with coronavirus disease 2019 (COVID-19) is a key DAMP that can cause NETosis and then may lead to persistent inflammation.[54]

NETs Enhance Inflammation

NETs amplify inflammation by interacting with immune or tissue cells, such as inflammatory cells and epithelial cells. NETs stimulate macrophages to secrete IL-1β, which in turn produces more NETs that amplify tissue damage.[55] NETs also amplify inflammation by stimulating airway and alveolar epithelial cells to express C-X-C motif chemokine ligand (CXCL)-1, CXCL-2, and CXCL-8 via the TLR4/nuclear transcription factor-κB (NF-κB) pathway.[56] Recently, NETs have also been found to interact directly with keratinocytes and activate a TLR4/IL-36R crosstalk, which then activates the myeloid differentiation factor 88 (MyD88)/NF-κB signaling pathway to induce the production of chemokine lipocalin 2 and the pro-inflammatory factor IL-36γ, thus amplifying the cascade leading to psoriatic dermatitis.[57]

NETs Mediate the Autoimmune Reaction

NETs are considered to play a central role in the autoimmune response.[27] The imbalance of NETosis leads to persistence production of NETs and constitutes the main source of autoantigens, including LL-37, and dsDNA, histones.[58,59] These autoantigens lead to the accumulation and activation of immune complexes. Unlike systemic lupus erythematosus (SLE) for nuclear antigens, patients with anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV) specifically present ANCA targeting MPO or proteinase 3 (PR3), while patients with RA showed higher levels of anti-

citrullinated protein antibody (ACPA).[60,61] In addition to classic autoimmune diseases such as SLE, AAV, and RA, more and more inflammation has been found to be related to NETs-mediated autoimmunity. The emergence of autoantibodies related to NETs in inflammation such as dry eye disease,[62] psoriasis,[63] and COVID-19 [64] shows the occurrence of an autoimmune reaction.

NETs Induce Thrombosis and Occlusion

NETs mediate platelet activation, coagulation, and thrombosis.[65] The complete NETs stent-platelet-thrombin axis is capable of promoting intravascular thrombin activation and microvascular thrombosis in sepsis.[66] In the plasma-induced NETs model of COVID-19, SARS-CoV-2 activates complement C3 to drive platelet/NETs and induce the formation of NETs enriched with tissue factor, which in turn activates endothelial cells to express tissue factor, thus increasing their procoagulant activity, and further activates platelets to aggravate the inflammatory cycle.[67] Platelet factor 4 coagulation factor signaling in platelets binds to NETs, making them robust and resistant to DNase and leads to microthrombosis in patients with COVID-19.[68] The negative effect of NETs is not only embodied in inflammation induced by residues of NETs but also in inflammation induced by occlusion of arteries caused by aggregated NETs (aggNETs).[69] Aggregation of NETs in the secretions of allergic eye disease leads to meibomian gland duct occlusion and acinar atrophy in mice, resulting in excessive evaporative xerophthalmia.[70] Although aggNETs aggravate ocular surface inflammation under pathological conditions, aggNETs also remove eye closure inflammation. AggNETs promote cleansing of the ocular surface and eliminate ocular inflammation by degrading inflammatory mediator cytokines and chemokines.[71]

Inhibition Strategies of NETs

NETosis is a continuous and complex process, PAD4 enzymes, histone, MPO and other components play their respective roles, all of which are indispensable. These components not only participate in NETosis and make up NETs but also mediate various types of NETs-related inflammation, as seen in cystic fibrosis (CF) and asthma.[72] The composition of these proteins in NETs is positively correlated with the severity of most inflammatory responses.

The main components of NETs, namely DNA, histones, and soluble proteins, are detected in the sputum of patients with CF, and their levels positively correlate with the severity of lung disease.[53,69] Compared to healthy controls, the levels of NETs components (eDNA, PAD4, NE, MPO, and LL-37) are elevated in patients with chronic obstructive pulmonary disease (COPD).[73–76] Therefore, inhibition of NETs is an important strategy for the treatment of inflammatory diseases. Below, we summarize the classic, most recent nonspecific and specific targeting strategies for the inhibition of NETs, including those of specific of eDNA, PAD4, NETs-derived enzymes (NE, histone, citrullinated histone, MPO, and GSDMD), as well as nonspecific ROS, components of the complement system, chemokines, and antibiotics (Table 1).

Microorganisms have developed mechanisms such as degrading DNA, altering the surface structure to increase resistance to NETs, and inhibiting NETosis to evade NETs to promote proliferation and spread of microbials.[123] For example, *S. aureus*, a classic representative bacteria, escapes NETs by cleaving extracellular DNA, micrococcal nuclease, extracellular adhesion protein, and fibronectin binding protein B to block the protease activity of NETs.[114] The 3'-nucleotidase/nuclease mediates *Candida*[115] and *Leishmania*[124] escape from NETs. Furthermore, pneumococci can adjust its surface charge to negative by secreting pneumococcal surface protein A, which repel negatively charged NETs.[119]

These mechanisms of natural microbial infections to inhibit NETs, have inspired researchers to develop a new strategy for anti-inflammatory agents. Proteins that mimic pathogens to escape NETs may contribute to the discovery of new drugs that effectively inhibit NETs. For example, nucleases secreted by pathogens that degrade NETs and various similar proteins can be considered potential drugs to modulate NETs-associated inflammation.[125] Another important example is recombinant *Trichinella* spiralis, which secretes serine protease 1 (TsSERP1). The latter inhibits the formation of human NE and NETs and alters the production of human pro-inflammatory cytokines and chemokines released by neutrophils.[126]

Herbal medicine has been widely used in the treatment of NETs-related inflammatory diseases due to its high safety and remarkable curative effect.[127,128] However, due to the complex components and multi-target effects of herbal medicine, it is not easy to explain its specific mechanisms of action.[129] Fortunately, with the development of separation technology and analytical equipment, many active components of herbal medicines have been reported to beneficially

Table 1 Anti-NETs Therapy

Mechanisms		Potential Drugs	Ref.
eDNA degradation	–	DNase I Staphylococcal nuclease	[77,78]
PAD inhibitors	PAD4 inhibitors	Cl-amidine, GSK484/199, YW3-56, roflumilast, tanimilast, BMS-P5	[79–85]
	PAD2 inhibitors	AFM32a	
ROS/NADPH inhibitors	–	Metformin, DPI, vitamin C, NAC, (+)-borneol	[86,87,87–89]
Granule protein inhibitors	Citrullinated histone inhibitor	CitH3 mAb (4 Cit), tACPA	[90–95]
	Histone inhibitor	SPA, rTM, HIPe, CHIP, heparin	
	MPO inhibitor	Luminol, PF-1355, AZM198, PA-dPEG24	[96–99]
	NE inhibitor	AZD 9668, sivelestat, SLPI, BAY 85–8501	[100–103]
GSDMD inhibitors	–	Disulfiram, LDC7599	[104,105]
Complement and CXCL inhibitors	Complement C5 inhibitor	OmCI	[106,107]
	Complement C1 inhibitor	Reparixin	
Inhibitors of NETs formation and release	–	Antibiotic (azithromycin, chloramphenicol, gentamicin, ceftriaxone, erythromycin)	[108–111]
	–	Tetrahydroisoquinolines, hydroxychloroquine	[112,113]
Simulation of immune evasion of NETs by pathogen	Cleaves eDNA	3'-nucleotidase/nuclease, immunoglobulin-like protein A, DNase-like protein, surface lipoprotein MHO-0730, Staphylococcal nuclease	[114–118]
	Resists NETs	Pneumococcal surface protein A, leukocidins PVL and HlgAB	[119,120]
	Inhibits NETosis	Multifunctional virulence factor TcpC (PAD4 inhibitor), calreticulin, HBV C and E protein (ROS inhibitor), extracellular adhesion protein, and fibronectin binding protein B	[114,121,122]

Abbreviations: NETs, neutrophil extracellular traps; PAD, peptidylarginine deiminase; DPI, diphenyleneiodonium chloride; NAC, N-acetylcysteine; CitH3, citrullinated histone H3; tACPA, therapeutic anti-citrullinated protein antibody; SPA, small polyanion; rTM, recombinant thrombomodulin; HIPe, histone inhibitory peptide; CHIP, cyclical histone H2A interference peptide; MPO, myeloperoxidase; NE, neutrophil elastase; SLPI, secretory leukocyte protease inhibitor; GSDMD, gasdermin D; CXCL, C-X-C motif chemokine ligand; OmCI, ornithodoros moubata complement inhibitor; HBV, hepatitis B virus; ROS, reactive oxygen species.

modulate NETs-related inflammation through different mechanisms (Table 2). For example, flavonoid luteolin and carnosic acid inhibits NETs production by inhibiting the Raf1-MEK-1-Erk pathway and ROS production.[130,131] Salvianolic acid B and 15,16-dihydrotanshinone I in *Salvia miltiorrhiza* significantly inhibit the formation of early NETs by inhibiting the activity of MPO and NADPH oxidase, respectively.[132] The main components of the Xuebijing Injection (Approved No. Z20040033), safflower and *Ligusticum Chuanxiong* hort., contain safflor yellow A, hydroxysafflor yellow A, anhydrosafflor yellow B and senkyunolide I, respectively. Xuebijing Injection has been shown to significantly inhibit NETosis.[133,134] Some of the active compounds of herbal medicines such as terpenoid triptolide and andrographolide have been shown to be effective in inhibiting NETs, but specific mechanisms are not yet known. Herbal medicine is a treasure for researchers looking to discover active molecules in the treatment of inflammatory diseases related to NETs.

Inhibition of PAD4

PAD4 is a key enzyme in the formation of NETs in vivo. It involves the citrullination of histone and induction of chromatin decondensation. PAD4 disorders have been shown to be associated with a variety of inflammation types and has led to the emergence of an autoimmune response and an increase in inflammation. More and more studies have indicated that the emergence of ACPA is caused by PAD4-mediated imbalance of citrullination of extracellular proteins, which is the core element of inflammatory autoimmune disease in RA.[154] In the peripheral joints and blood of patients with RA, inflammatory cytokines and ACPA autoantibodies promote NETosis and keep the autoimmune circulation active.[61] Citrulline histones derived from NETs on the ocular surface also stimulate ACPA production. These autoantibodies not only induce the release of pro-inflammatory cytokines by interacting with Fc receptors on activated neutrophils and dendritic cells but also stimulate NETosis and activate the complement

Table 2 Active Ingredients of Herbal Medicine and Their Effects on NETs

Effective Components	Active Ingredients	Dosage Form	Models	Routes or Dose of Administration	Mechanism	Types of Inflammation	Ref.
Glycosides	Chikusetsusaponin V	Injection	C57BL/6 mice; primary neutrophils	i.p. (200, 100, 50 mg/kg); 100 μM	Reduces NETs-related inflammation via Caspase-1-HMGB1	Acetaminophen-induced liver injury	[135]
	Glycyrrhizin	Injection	C57BL/6 mice	i.p. (15 mg/kg)	Inhibits NETosis and improves lung function via HMGB1/TLR9/MyD88	Sepsis-induced ARDS	[136]
	PEGylated nanoparticle albumin-bound steroidal ginsenoside derivatives	Nanoparticles	Plasma of patients	50 μg/mL	Modulates histone H4 levels and the cytokine storm via NF-κB and SREBP2 signaling pathways	COVID-19	[137]
	Polydatin	Injection	DBA/1 or BALB/c mice; human or mouse neutrophils	i.p. (45 mg/kg); 0, 50, 75, 100, 125, 150 μg/mL	Inhibits NETs formation	RA/SLE	[138,139]
	Forsythiaside B	Injection	Rats	i.v. (20, 40, 80 mg/kg)	Inhibits PAD4	Sepsis	[140]
Flavonoids	Luteolin	Injection	C57BL/6 mice; human neutrophils	i.p. (50 mg/kg); 3, 10, 30 μM	Inhibits Raf1-MEK-1-Erk pathway, ROS production and NETosis	Inflammatory arthritis	[130]
	15,16-dihydrotanshinone I	Solution	Human neutrophils	20 μM	Inhibits NOX activity	Neutrophil inflammation	[132]
	Quercetin	Injection	C57BL/6 mice	i.p. (30 mg/kg)	Inhibits expression of CitH3, PAD4 and autophagy	RA	[141]
	Safflor yellow A, Hydroxysafflor yellow A, and Anhydrosafflor yellow B	Injection	C57BL/6J mice; HL-60	i.p. (5×10^{-5} mol/kg); 160 mg/L	Inhibits the Raf/MEK/ERK signaling pathway	ALI	[142]
	Zingerone	Injection	C57 mice; human and mouse neutrophils	i.p. (25, 50 mg/kg)	Inhibits Nrf2-mediated ROS	Sepsis	[143]
Phenolic acids	Salvianolic acid B	Solution	Human neutrophils	200 μM	Inhibits MPO activity	Neutrophil inflammation	[132]
Quinones	Emodin	Injection	C57BL/6 mice	i.p. (30 μg/kg)	Inhibits NETosis and NETs-related MPO and NE release	RA	[144]
	Tanshinone IIA	Injection	C57BL/6 mice; mouse neutrophils	i.p. (30 mg/kg); 0, 5, 10, 25 μM	Inhibits NETs-associated MPO and NE release	RA	[145]

Terpenoids	Andrographolide	Injection	C57BL/6 mice	i.p. (25, 50 mg/kg)	Decreases levels of PAD4, CitH3, and NETosis	RA	[146]
	Triptolide	Solution	Human neutrophils	0.2 μM	Suppresses NETosis in an ROS independent manner	NETs related inflammation	[147]
	The total terpenoids of *Celastrus orbiculatus*	Solution	Human neutrophils	0, 5, 10, 20, 40, 80 μg/mL	Inhibits NETs-associated DNA and granular proteins complexes in ROS dependent manner	Neutrophil inflammation	[148]
	Senkyunolide I	Injection	C57BL/6 mice; mouse neutrophils	i.p. (36 mg/kg); 100 nM	Reduces MPO-DNA level	Sepsis-induced lung injury	[133]
Alkaloid	Tetrandrine	Injection	C57BL/6 mice	i.p. (6 mg/kg)	Alleviates neutrophil activities, and decreases MPO and NE	RA	[149]
	Tetramethylpyrazine	Injection	Rats; mouse neutrophils	i.p. (40 mg/kg); 0, 5, 10, 20, 40 μM	Downregulates expression of NETs-DNA, H3cit and NOX	Hepatic IRI	[150]
Phenolics	6-Gingerol	Injection	C57BL/6 mice; human neutrophils	i.p. (20 mg/kg); 0.1, 1, 10,100 μM	Attenuates NETosis partially dependent on inhibition of PDE activity, cAMP and PKA	SLE	[151]
Reduning Injection	Iridoid, lignans, etc.	Injection	C57BL/6J mice	i.p. (5, 10 mL/kg)	Downregulates MAPK pathways to inhibit PAD4 and NETosis	ALI	[152]
Kan-Lu-Hsiao-Tu-Tan	Baicalin, baicalein, etc.	Solution	Imiquimod-induced psoriasis animal; human neutrophils	Topical use (10 mg/mL); 1, 3, 10 μg/mL	Inhibits formation of ROS and NETs	Psoriasis	[153]

Abbreviations: NETs, neutrophil extracellular traps; HMGB1, high-mobility group box 1; TLR9, toll-like receptor 9; MyD88, myeloid differentiation factor 88; ARDS, acute respiratory distress syndrome; COVID-19, coronavirus disease 2019; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SREBP2, sterol regulatory element-binding protein 2; MEK-1, mitogen-activated protein kinase kinase-1; Erk, extracellular signal-regulated kinase; ROS, reactive oxygen species; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase; CitH3, citrullinated histone H3; PAD, peptidylarginine deiminase; Nrf2, nuclear factor erythroid 2-related factor 2; MPO, myeloperoxidase; NE, neutrophil elastase; ALI, acute lung injury; IRI, intestine reperfusion injury; cAMP, cyclic adenosine monophosphate; PDE, phosphodiesterase; PKA, protein kinase A; MAPK, mitogen-activated protein kinase; i.p. intraperitoneal; etc., et cetera.

https://doi.org/10.2147/IJN.S418259 DovePress

system, thus creating a self-perpetuating cycle of chronic inflammation on the ocular surface of dry eye syndrome.[62] ACPA may form an immune complex with citrullinated antigens released during NETosis to promote inflammation. Furthermore, monoclonal ACPA also induces tenosynovitis, pain, and bone loss in mice by relying on PAD4-mediated citrullination.[155] In the mouse model of double hemorrhagic shock and septicemia with double injury, compared to wild-type animals, the lack of PAD4 leads to reduced organ dysfunction and improved survival.

Several studies have shown that PAD4 inhibitors Cl-amidine and BB-Cl-amidine reduced NETosis and organ damage in SLE and improved septic survival in mice.[81,156,157] In addition to PAD4 inhibitors, the PAD2 inhibitor AFM32a also significantly reduces pro-inflammatory cytokines and NETosis in the endotoxic shock mouse model. Furthermore, the Reduning Injection (Approved No. Z20050217), a traditional herbal medicine (THM) composed of gardenia jasmine and Artemisia annua, down-regulates MAPK pathways to inhibit respiratory diseases related to PAD4 and NETosis such as ALI.[152] However, the expression of PAD4 is not limited to the NETosis of neutrophils, but also to the transcriptional regulation of genes,[158] regulation of stem cell differentiation,[159] and inhibition of ROS to reduce bacterial killing.[160] Extra discretion should be exercised due to the multifunctional activity of PAD4, which may cause side effects of simple inhibition.

Reduction of Histones and Citrullinated Histones

Histone not only participates in NETosis but also induces neutrophils to form NETs through TLR.[161] The accumulation of histones, NE, and MPO leads to the destruction of the epithelium, endothelium, and connective tissue, causing additional chronic tissue damage, and worsening of function.[21,162,163] Exaggerated NETs and histones cause epithelial pouch ulcers to promote bacteremia and endotoxemia, which in turn lead to the formation of low-grade systemic inflammation, a key factor in the deterioration of systemic diseases.[164] Histones also promote the deterioration of inflammation. Histone derived from NETs bridges the extracellular domain S2 of the spike protein in host cells, and sialic acid improves the proximity of the virus and the cell membrane to increase the infectivity of SARS-CoV-2.[36] In the colon of mice with experimental colitis, histones derived from NETs may alter intestinal permeability and barrier function by downregulating the expression of adhesion proteins and tight junction proteins and epithelial cell apoptosis.[165]

Recombinant thrombomodulin blocks the accumulation of histones and NETs in the lungs by binding to circulating histones.[92] Meara et al[91] developed a small polyanion (SPA) mCBS-like heparin to replace histones from NETs but retaining the integrity of NETs so as to avoid the inflammatory effects of antibacterial protein, thereby inhibiting histone and NETs-mediated inflammation. Compared with histones, PAD4-mediated citrullinated histone is largely a specific marker of the process of NETs formation. Eight core histone components (two each from H2A, H2B, H3, and H4) and DNA comprise the nucleosome, the basic tissue unit of the NETs genome.[166] In one study, the monoclonal antibody CitH3 (4Cit) mAb specifically recognized citrullinated H3R26 to neutralize citrullinated histone H3 and attenuate the formation of NETs and the pro-inflammatory response during endotoxic shock.[95] However, this antibody targets all citrullinated histones H3 and not just NETs. A recent study shows that monoclonal antibody tACPA specifically binds to the histone citH2A and citH4 (Cit3) citrulline on NETs chromatin to inhibit NETs-mediated inflammatory diseases.[90] Some researchers believe that this specific recognition of NETs nucleosomes may be based on the binding of tACPA to form "clips" that prevent nucleosomes from spreading and act as highly specific markers conducive to phagocytosis.[167] Cit3 may become a new target for directing drugs to NETs for its specific relationship on citrullinated histones.

Down-Regulation of MPO and NE Activity

MPO and NE regulate NETosis by synergistically driving chromatin denudation, which is a marker of NETs formation in inflammation.[168] Of these, the MPO-DNA complex and citrullinated histone H3 are considered to be specific markers of NETs.[169] Plasma levels of the MPO-DNA complex can be used to predict the severity of COVID-19 infection and organ dysfunction in patients with septic shock.[169,170] Interestingly, the excessive production of hypochlorite HOCl, a MPO-mediated bactericidal oxidant related to NETosis in the intestine of patients with Crohn's disease, can lead to an increase

in inflammation.[171] Previous studies have shown that MPO in neutrophils is also an autoimmune target of inflammation associated with NETs. Patients with AAV specifically exhibit ANCA targeting MPO or PR3,[60] and induced NETosis is believed to be independent of IgG ANCA trigger and rich in citrulline histones.[172] Excess NETs produced by ANCA stimulation leads to vasculitis and participates in the formation of ANCA itself, thus promoting a vicious circle of autoimmune AAV.[173] The inhibition of MPO released by neutrophils and monocytes by AZM198 can reduce neutrophil degranulation and NETosis.[98] Furthermore, the complement C1 peptide inhibitor PA-dPEG24 and ganoderma lucidum polysaccharides peptides can also block the MPO pathway associated with NETs formation.[99,174]

NE is a serine protease, which is very important in NETosis. NE is reported to be associated with severe systemic and multiple organ damage in COVID-19 and can be used as an independent predictor of multiple organ injury in patients with COVID-19.[175] Knockout and inhibition of NE in mice can prevent the formation of extracellular neutrophil traps and can increase the resistance of mice to toxic shock.[101,176] The NE inhibitor sivelestat has been listed as a drug for the treatment of ARDS in Japan and Korea.[177] Several NE inhibitors are in clinical trial pipelines. For example, the highly selective and reversible NE inhibitor AZD9668 can improve lung capacity and can reduce inflammatory levels in patients with bronchiectasis, CF, and COPD.[100,178,179] Furthermore, tanshinone IIA extracted from the THM *Salvia miltiorrhiza* Bunge inhibited the release of NETs due to MPO and NE and reduced RA related inflammation in mice.[145]

Degrading of DNA

The extracellular neutrophil trap is considered a network formed by externalization of biological chromatin and the formation of a large extracellular DNA scaffold. As the main component of NETs, the DNA of NETs are closely related to many inflammatory conditions.[180] Elevated extracellular DNA in the sputum leads to impaired lung function and poor control of symptoms in exacerbated airway inflammation in patients with severe asthma.[181–183] NETs in SLE are rich in oxidized mtDNA-induced inflammatory type I IFN.[184] In addition, recent studies have found that externalized small RNA in NETs also induces a type I IFN response, which promotes vascular inflammation of lupus.[185] Higher concentrations of NETs-DNA are not only associated with the aggravation of inflammation such as asthma, inflammatory bowel disease, and immune thrombus but they also have strong associations with autoimmune inflammation.[186–188] Some anti-dsDNA antibodies in active SLE disease inhibit NETs digestion and stimulate the type I IFN response or NF-kB activity to amplify inflammation.[186]

Considered a safe tool for degrading DNA, the Food and Drug Administration (FDA) has approved DNase I for the clinical treatment of CF and SLE.[189,190] A recent study on the treatment of COVID-19 has reported that nebulized human DNase improves respiratory function and reduced acute symptoms in patients.[77] In addition, dual-active DNase with DNase1 and DNase1L3 activity will degrade NETs-DNA more rapidly and effectively.[191] In terms of THM, the total terpenoids of *Celastrus orbiculatus* prevented the formation of the NETs-DNA complex and acted as an anti-inflammatory agent for suicidal NETosis.[148]

Blocking of GSDMD

NETosis is dependent on GSDMD, which promotes NE translocation to the nucleus. GSDMD also causes the nucleus to expand and induces the formation of pores in the plasma membrane.[105] Direct activation of GSDMD by the SARS-CoV -2 virus induces excessive NETs production, and worsens tissue and organ damage in patients.[192] Moreover, the aseptic inflammatory environment in patients with sickle cell disease also promotes the activation of GSDMD, thereby increasing NETs in the liver. NETs translocate from the liver through hepatopulmonary embolism and acute inflammatory lung injury.[193] Therefore, the knockout and inhibition of GSDMD will help reduce inflammation in conditions such as COVID-19, ALI, and ARDS.[192–194]

Through chemical screening, it was found that the specific binding of LDC7559 to GSDMD reduced the activation of pathological inflammatory bodies and NETosis.[105] Furthermore, disulfiram can also covalently inhibit GSDMD activity to effectively reduce NETs production and prevent organ damage and systemic inflammation during sepsis.[104]

Regulation of ROS

ROS is necessary for the formation of NADPH-dependent NETs. It interacts with NETosis in a multidimensional manner and participates in most inflammatory cascades.[195,196] The uncontrolled increase of ROS under oxidative stress leads to increased NETs and inhibition of T cells in the immune system, which will eventually lead to an aggravation of severe COVID-19 inflammation.[197] Metformin, a ROS inhibitor, is an antidiabetic drug, which reduces the activation of NOX in neutrophils and inactivates the NETosis response, thereby improving the pathological state of diabetes.[88] ROS is a common pathway for many THM to inhibit NETs, including luteolin, triptolide, hesperetin and Kan Lu Hsiao Tu Tan (KLHTT).[130,147,153,198] Although ROS inhibitors (DPI, vitamin C, luteolin, and NAC) are beneficial for reducing NETosis, appropriate drug delivery systems for them are still lacking.

Other Inhibitors

Calcitonin is not only the main antifungal component of NETs but it is also related to the diagnosis and progression of NETs-related CF and other diseases. Serum calprotectin levels can be used as an indicator of the prediction of key future clinical events in CF, including lung deterioration and decreased lung function.[199] Interestingly, recent studies have found that S100A9 increases ROS production to promote NETs formation, thus increasing white blood cell recruitment in patients with abdominal sepsis. It should be mentioned that the S100A9 inhibitor ABR-238901 reduces lung damage in abdominal sepsis.[200] HMGB1 is the DAMP that activates NETs and is part of the release of cellular contents during NETosis. NETs induced by HMGB1 promote intestinal ischemia/reperfusion-induced ALI.[201] The THM Chikusetsusaponin V and glycyrrhizin inhibit NETs formation in APAP-induced liver injury and sepsis-induced ARDS by regulating HMGB1-related mechanisms.[135,136,202]

In addition to antibacterial activity, antibiotics play an immunomodulatory role with neutrophils.[109] Many previous studies have shown that antibiotics such as gentamicin, azithromycin, and chloramphenicol interfere with the formation of NETs.[108,203] The combination therapy of antibiotics is beneficial to rationally target the dual activity of NETs. For example, the combination therapy of imipenem and ceftriaxone. The early use of imipenem promotes NETs to fully resist bacteria, and later use of ceftriaxone inhibits NETs to prevent inflammatory damage caused by NETs.[110]

MicroRNA (miRNA) may regulate the formation of NETs. Inhibition of miRNA genes, miR-155 and miR-223, miR-146a has been shown to reduce inflammation mediated by the formation of NETs.[204–206] Furthermore, intrapulmonary delivery of miR-146a can inhibit diffuse alveolar hemorrhage by reducing NETosis.[206] Therefore, targeted inhibition of miRNA exosomes or extracellular vesicles or miRNA delivery is beneficial to inhibition of NETosis.[207]

Nanodelivery Systems

Since NETs play a key role in inflammation, inhibition of NETs has proven to be effective in modulating inflammation. However, NETs inhibition remains a challenge, even in the application of NETs inhibitors. A nano drug delivery system may represent a potential solution.[208] Nanodelivery systems and their active targeting strategy would greatly improve the stability and effectiveness of NETs inhibitors. In this section, we summarize recent nano-drug delivery strategies for targeting of NETs (Table 3). Nanomaterial-based NETs systems lead to: (1) improved pharmacokinetics behavior and therapeutic efficiency of drugs, such as extension of the half-life of DNase I and the efficacy of the NE inhibitor sivelestat and (2) to the improved biodistribution of drugs. Nanocarriers with a targeting moiety delivers drug specific to NETs, such as delivering PAD4 inhibitor GSK484 to the intimal inflammatory area to inhibit NETs (Figure 3). It is worth noting that some nanomaterials have the potential to activate the inflammatory pathway by promoting NETosis,[209,210] thus, discretion is advised when selecting nanocarrier preparations.

The encapsulation of drugs by nanocarriers shows significant advantages, to improve the stability, safety, and efficacy of drugs, especially for nucleic acids and enzymes. A major disadvantages of DNase I is its short half-life in plasma, which hinders its efficacy in inhibiting NETosis and antiinflammation. Melanin-like nanospheres and poly (lactic acid-glycolic acid copolymer) nanoparticles were developed to increase the enzyme stability and half-life of DNase I. The excellent bioadhesion properties of polydopamine contribute to its long-term activity.[211,212] A highly hydrophilic microgel loaded with DNase-I (DNase-I MG) digests NETs more quickly and efficiently than the free enzyme.[214]

Table 3 Nano-Drugs for NETs Inhibition

Nanodelivery Systems	Pharmacological Compounds	Objects of Administration	Routes and Dose of Administration	Effects	Disease	Ref.
Poly (dopamine) poly (ethylene glycol) nanoparticles	DNase I	C57BL/6 mice; human neutrophils	i.v. (100 units)	Improved the short half-life of DNase I	COVID-19 patients with sepsis	[211]
DNase-I coated melanin-like nanospheres (DNase-I pMNS)		C57BL/6 mice; neutrophils	i.v. (100 units)	Improved the short half-life of DNase I	COVID-19	[212]
Positively charged dimethylamino-modified polydopamine nanoparticles		SD rats	Arterial injection (2.5 mg/kg)	Superior combining ability of cfDNA	RA	[213]
DNase I functional microgel		Human neutrophils	0.6 mg/mL	Improved bioavailability	NETs-mediated inflammation	[214]
Fc-modified monoclonal antibodies binding to PF4-NETs complex (KKO)		WT, Cxcl4−/−, hPF4+ mice	i.v. (5 mg/kg)	Enhanced DNase resistance, limiting toxic NETs degradation products	Sepsis	[215]
Poly(3-Hydroxybutyrate) Microspheres	Cl-amidine	SK-BR-3 cell line	1 mg/mL	Increased drug release	Cancer and NETs associated inflammation	[216]
Multilayer vesicles cross-linked between bilayers	Sivelestat	BALB/c mice	i.p. (50 mg/Kg)	Improved efficacy	Endotoxic shock	[101]
Sialic acid chain-modified nanoparticles	Sialic acid	Human neutrophils	10 μg/mL	Inhibited ROS, improved efficacy	NETs-related pathologies	[217]
Monoclonal antibodies 2C5-modified micelles	-	Neutrophils	5 μg/mL	Specifically targeted intact nucleosomes	NETs-mediated inflammation	[218]
Calcium alginate encapsulation	Staphylococcal nuclease	C57BL/6 mice	i.g. (25,75 mg/kg)	Targeted the colon	Ulcerative colitis	[78]
Collagen IV-targeted nanoparticles	GSK484	ApoE -/- mice	Perfusion (4 mg/kg)	Regions of endothelial cell sloughing and collagen IV-rich basement membrane exposure	Endothelial damage	[219]
Mesenchymal stromal cell-derived extracellular vesicles	-	C57BL/6 mice	i.v. (50 μg/100 μL)	Delivered to functional mitochondria of neutrophils in the liver	Liver ischemia-reperfusion injury	[220]
Photo-cross-linking silk hydrogel system	Metformin	C57BL/6 mice	Topical use (0.2 mL)	Regions of diabetic trauma	Diabetic wound	[221]
Nec-1/PDA@Pt-Fe3O4 nanocarrier	Nec-1	C57BL/6 mice	i.p. (6 mg/kg)	Inhibited receptor-interacting protein 1 kinase activity	Lupus nephritis	[222]
PLGA-LPV@M	Lopinavir	BALB/c mice; human neutrophils	i.v. (100μg/ mL); 0, 0.5, 1 mg/mL	Neutralized inflammatory cytokines and suppressed neutrophils	COVID-19	[223]

Abbreviations: NETs, neutrophil extracellular traps; COVID-19, coronavirus disease 2019; RA, rheumatoid arthritis; ROS, reactive oxygen species.

https://doi.org/10.2147/IJN.S418259 DovePress

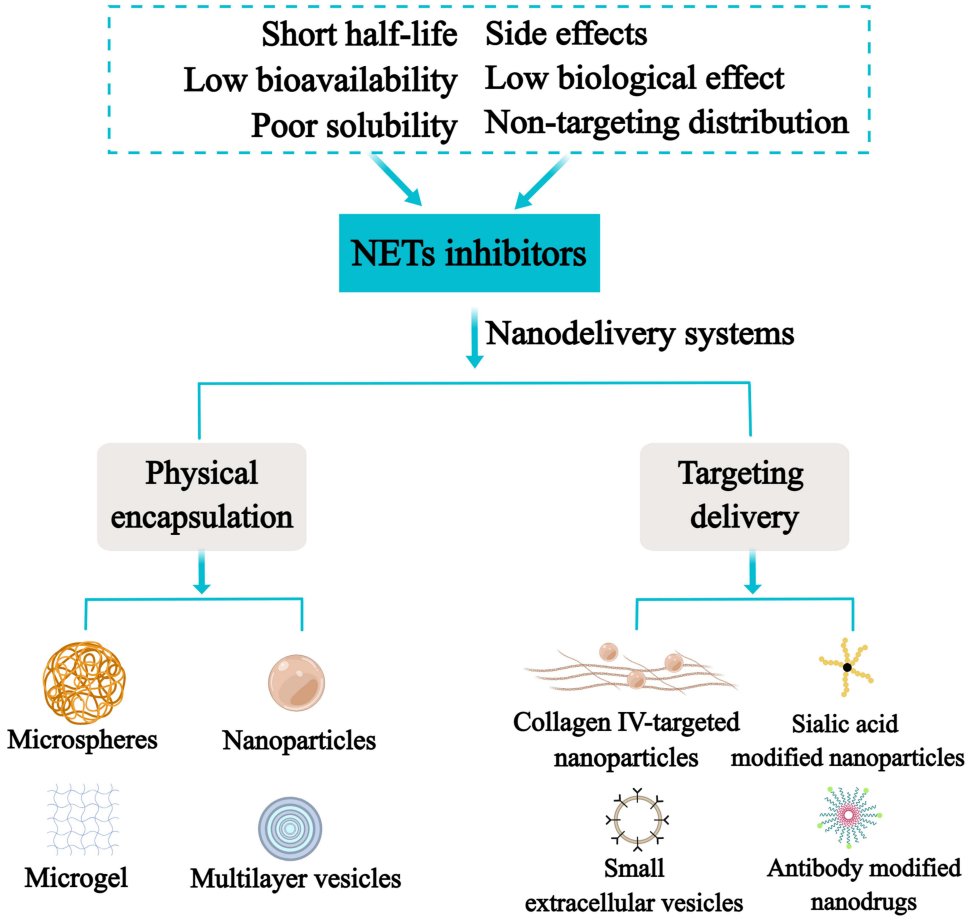


Figure 3 Successful nanocarriers developed for inhibition of NETs.
Abbreviation: NETs, neutrophil extracellular traps.

Similar long-term and safe drug release was achieved by poly (3-hydroxybutyrate) microspheres loaded with the Pan-PAD4 inhibitor Cl-amidine.[216] Meanwhile, nanocarrier delivery not only increased stability but also played an incredible role in the curative effect. Free sivelestat is not effective in the mouse model of endotoxic shock; however, interbilayer cross-linked multilayer vesicles incorporating this NE inhibitor prevented the formation of NETs and showed lowered pulmonary inflammation in animals in the context of endotoxic shock.[101]

Nanocarriers conjugated with targeting moieties based on the ligand-receptor molecular interaction significantly improved biodistribution and reduced side effects. Systemic administration of inhibitors may cause serious side effects due to its wide expression throughout the body. Molinaro et al[219] developed collagen IV-targeted nanoparticles to deliver the PAD4 inhibitor GSK484 to intimal lesions. Metformin with antioxidant and anti-inflammatory activity is loaded onto mesoporous silicon nanoparticles and gene delivery combined with antibacterial dressing carriers to heal diabetic wounds through dual controlled release of polarized macrophages and inhibition of NETosis.[22,221] Nano-targeted neutrophils interfere with their function to inhibit NETosis. Nanoparticles decorated with sialic acid residue dimer can to easily bind to the neutrophil siglec-5 to reduce ROS production.[217] Mesenchymal stem cell-derived apoptotic vesicles not only interact with positively charged NETs-DNA medium but also clear NETs in sepsis by converting neutrophil NETosis into apoptosis through the Fas pathway.[224] Extracellular vesicles derived from mesenchymal stromal cells induce functional mitochondrial transfer, fusion, and repair of neutrophil mitochondrial function in liver ischemia-reperfusion injury models, thereby inhibiting ROS production and significantly reducing NETosis.[220] Another targeting strategy for therapeutic manipulation of NETs is based on active targeting of NETs ligands. A Fc-modified monoclonal antibody KKO combined with the PF4-NETs complex has been developed to enhance resistance to DNase; thus retaining NETs-

mediated bacterial capture, reducing the release of the toxic degradation product of NETs, and improving the mouse model of septicemia.[215] The micellated monoclonal antibody 2C5 is specific to the intact nucleosome of NETs and can also be used to deliver DNase I aimed at NETs to treat inflammation.[218,225]

Future Perspectives

The research focus of NETs has recently shifted from infectious inflammation to aseptic inflammation. NETs may act as a key link in inflammation, acting as both the "cause" and the "effect" in many inflammatory conditions. For example, smoking has been known to be the main cause of COPD, but now cigarette extract has been found to aggravate COPD inflammation by inducing NETosis. This is not only conducive to the discovery of the mechanism of inflammation but also indicates a new path to the treatment of inflammatory diseases. Further research has revealed that NETs have pro-inflammatory effects, which activate the inflammasome, bind to DAMPs, amplify inflammation, and participate in autoimmunity and thrombosis. The components of NETs play an important role in the promotion of inflammation, so inhibiting the generation of NETs and the degradation of its components may be an important strategy to modulate inflammation.

NETs inhibitors are constantly under the spotlight as novel drug agents, and the advantages outweigh the disadvantages. By targeting key components in different stages of NETosis (eg, eDNA and PAD4), we summarize potential targeted treatment. Some proteins secreted by pathogens also act as NETs inhibitors, so they are also classified as potential drugs. For example, recombinant TsSERP1 from *Trichinella spiralis* inhibits the formation of human NE. Furthermore, the active components of THM have a significant inhibitory effect on NETosis. Literature regarding the inhibition of NETs has described important breakthroughs and has stimulated further research. The first is the specific targeting of therapeutic citrullinated histones for nucleosomes CitH2A and CitH4, which is based on the binding of tACPA to form a "clip" for the specific recognition of NETs nucleosomes. This suggests that more in-depth research is needed to identify strategies for highly specific NETs. Second, the combination with antibiotics in the NETs-associated bactericidal strategy, that is, an early use to promote NETosis, followed by later use to inhibit NETosis, to effectively retain the germicidal effect without causing uncontrolled inflammation. Furthermore, the Fc-modified KKO monoclonal antibody combined with the PF4-NETs complex retains the bactericidal effect of NETs and reduces the release of toxic degradation product from NETs by improving DNase resistance. There is an urgent need to systematically analyze the balanced effects of NETs in the fight against pathogens and in promoting inflammation. Finally, inhibition and regulation of gene-based miRNA is a new target for the inhibition of NETosis, which needs to be studied further. In summary, to optimize the treatment and management of NETs, it may be necessary to identify more efficient inhibition targets and drugs or to use combination therapy that does not weaken the immune system.

Conclusions

NETs are involved in the development of many diseases. NETs play an active role by phagocytizing pathogens as an initial defense mechanism, however, excessive NETosis could be destructive because of the release of enzymatic proteins causing non-specific activity leading to tissue pathology during inappropriate inflammatory responses.

Overall, this review focuses on the mechanisms involved in NETs formation, as well as the pro-inflammatory effects of NETs. Since patients of many diseases may benefit from anti-NETs therapy, we also collected information on herbal medicines related to NETs and outlined potential strategies for the management of NETs, including the inhibition of PAD4, reduction of histones, down-regulation of MPO and NE activity. Additionally, to improve the efficacy of NETs inhibitors, nanocarriers and targeting therapy can be employed. Considering the physicochemical characteristics and the in vivo behavior of NET inhibitors, improving the curative effect of targeting NETs by nanocarriers is a promising strategy. Nonetheless, the research is at the preliminary stages, and it is necessary to design and further investigate smart drug delivery systems to ensure the improvement in the efficacy and control of NETs without other detrimental effects.

Acknowledgments

Graphical Abstract was created with BioRender.com. We greatly acknowledge the financial support of National Natural Science Foundation of China (No. 82260695, 82060719), Jiangxi Provincial Natural Science Foundation

(20232ACB206062, 20212ACB206004), Innovation Team and Talents Cultivation Program of National Administration of Traditional Chinese Medicine (ZYYCXTD-D-202207), Young Jinggang Scholar of Jiangxi Province (J.Z.), and New Century Talents Project of Jiangxi Province (2017082, X.L. and 2020028, J.Z.), Jiangxi University of Chinese Medicine 1050 Youth Talent Project (J.Z. and X.L.), Jiangxi University of Chinese Medicine Science and Technology Innovation Team Development Program (CXTD22006), and Project of college students' innovation and entrepreneurship training program of Jiangxi University of Chinese Medicine (S.H. Xiao, [2022]2).

Disclosure

The authors declare no competing interest in this work.

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