

Targeting neutrophil extracellular traps in severe acute pancreatitis treatment

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Abstract: Severe acute pancreatitis (SAP) is a critical abdominal disease associated with high death rates. A systemic inflammatory response promotes disease progression, resulting in multiple organ dysfunction. The functions of neutrophils in the pathology of SAP have been presumed traditionally to be activation of chemokine and cytokine cascades accompanying the inflammatory process. Recently, since their discovery, a new type of antimicrobial mechanism, neutrophil extracellular traps (NETs), and their role in SAP, has attracted widespread attention from the scientific community. Significantly different from phagocytosis and degranulation, NETs kill extracellular microorganisms by releasing DNA fibers decorated with granular proteins. In addition to their strong antimicrobial functions, NETs participate in the pathophysiological process of many noninfectious diseases. In SAP, NETs injure normal tissues under inflammatory stress, which is associated with the activation of inflammatory cells, to cause an inflammatory cascade, and SAP products also trigger NET formation. Thus, due to the interaction between NET generation and SAP, a treatment targeting NETs might become a key point in SAP therapy. In this review, we summarize the mechanism of NETs in protecting the host from pathogen invasion, the stimulus that triggers NET formation, organ injury associated with SAP involving NETs, methods to interrupt the harmful effects of NETs, and different therapeutic strategies to preserve the organ function of patients with SAP by targeting NETs.

Keywords: intervention, NETs formation, neutrophil extracellular traps, SAP, tissue damage

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Introduction

Severe acute pancreatitis (SAP) is an acute abdominal disease associated with high mortality rates. A strong systemic inflammatory response promotes the progression of multiple organ dysfunction (MODS) due to immune system activation. The close relationship between inflammatory injury in patients with SAP and the activation and chemotaxis of neutrophils has been confirmed in many studies.¹ Polymorphonuclear (PMN) neutrophils are the most common leukocyte in the blood, and function as the frontline of the host immune defense against invasive pathogens. These cells are derived from myeloid progenitor cells and are designed to kill pathogens; thus, they are terminally differentiated and short-lived.^{2,3} Once an exogenous molecule or endogenous threat is identified, neutrophils initiate various mechanisms to ensure optimum elimination of

the threat. These mechanisms involve phagocytosis, degranulation, and the production of reactive oxygen species (ROS).

Neutrophils also produce activating molecules to alert other adjacent immune cells and thus induce the host immune response.⁴ Decades ago, researchers discovered that PMNs kill pathogens *via* phagocytosis and degranulation. Recently, however, neutrophil extracellular traps (NETs) – a new death mechanism of neutrophils first reported by Takei *et al.* – were identified as another neutrophil-mediated strategy to kill invading microorganisms outside cells by Brinkmann *et al.*, who further described the process and mechanism of NETs.^{5,6} Unlike phagocytosis and degranulation, NET formation involves the release of chromatin and granule proteins by activated neutrophils, resulting in extracellular fibers that

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bind bacteria.^{6,7} These researchers characterized the structure, components, and functions of NETs, and identified methods to stimulate neutrophils to release NETs.

In addition to studies of the protective function of NETs, evidence for the existence and deterioration of NETs in tissues throughout the body has been reported. In pancreatic tissue from a mouse model of SAP, NETs were shown to aggravate tissue damage.⁸ Many lethal complications of SAP, such as acute lung injury and acute kidney injury, have been shown to be related closely to NET formation. Furthermore, in human and animal models, a treatment designed to interrupt NET formation and their activation has been shown to ameliorate SAP. This review discusses issues related to the characteristics of NETs, their damage to tissues, and effects of treatments targeting NETs in individuals with SAP.

Characteristics of NETs

Structure and function

The first report on NETs showed that they are composed of depolymerized chromatin (DNA and histones), neutrophil elastase (NE), cathepsin G (CG), myeloperoxidase (MPO), and other proteins, and that these structures function as a net composed of extracellular DNA fibers decorated with globes.⁹ This structure allows NETs to capture and kill microorganisms, fungi, and parasites *via* these lethal proteins.¹⁰

When stimulated with the mitogen phorbol 12-myristate 13-acetate (PMA), or other substances that induce the formation of NETs, PMNs will first lose the nuclear lobules and depolymerize chromatin to form free DNA fibers, and the spatial expansion force produced by the release of this structure will lead to the rupture of the nuclear membrane, and the proteins in the cytoplasm will adhere to DNA.¹¹ Finally, the NETs will be released after rupture of the cell membrane. *In vitro*, the reticular structure of NETs composed of DNA fibers binds and kills pathogens by attaching antimicrobial proteins to these invading species.⁶ The presence of a thrombus formed by DNA entangled with bacteria was observed in individuals with septic intravascular thrombosis, confirming that DNA fibers prevent bacteria from escaping *in vivo*.¹² In addition, many different proteases with antibacterial activity that are

normally present in the cytoplasm and nucleus of normal neutrophils were detected in NETs, such as NE, MPO, histone (nucleoprotein), CG, lactoferrin, pentapeptidase 3, gelatinase, protease 3 (PR3), and peptidoglycan binding protein, which were involved in the lethal effect of NETs.¹³ In addition to antibacterial activity, several special proteins, such as NE, MPO, and histones, are also related closely to the formation of NETs, and deficiencies in MPO and NE can disrupt NET formation.¹⁴ However, the NETs observed in a deep venous thrombus in NE knockout mice negates this finding and suggests that many different mechanisms of NET formation exist.¹⁵

Characteristics of NET formation

Mechanism of NET formation. Many substances stimulate the generation of NETs. We roughly classify these substances into several categories, such as pathogens, chemicals, inflammatory factors, endogenous secreted products, and specific carrier proteins. We also summarize the substances associated with SAP that induce NET formation (Table 1).

According to whether ROS is required, the mechanism of NET formation is divided into ROS-dependent and non-ROS-dependent pathways. The pathway of NET formation induced by the mitogen PMA depends on ROS. PMA binds to PKC receptors on the cell membrane and activates PKC; activated PKC initiates the Raf-MEK-ERK (MAPK) pathway and subsequent activation of NADPH oxidase (NOX), leading to ROS production. ROS enters azurophilic granules to detach NE from MPO, and NE is released into the nucleus to bind to histones, resulting in histone octamer cleavage to form H2A-H2B-DNA complexes.^{40,41} Histone citrullination, which is mediated by protein arginine deiminase 4 (PAD4), caused the original histone octamer to be hydrolyzed into dimers, leading to the depolymerization of chromosomes to form NETs.⁴² Stimuli such as calcium ionophores and platelets utilize different signaling pathways to initiate NET formation and do not require ROS production (Figure 1). However, some researchers observed the activation of both PAD4 and calpain during calcium-mediate NET formation, and inhibition of these two proteases effectively inhibited chromatin decondensation.⁴³

Most pathogenic microorganisms, inflammatory factors, and chemicals drive NET formation *via*

Table 1. Stimuli and the characteristics of NETs they induce.

Stimulus type	ROS-dependent or -independent production	DNA source and type of NET released	Association with SAP	Reference
Pathogenic bacteria				
<i>Staphylococcus aureus</i>	Independent (it can produce ROS itself)	Nucleus, vesicle and vital	–	Pilsczek <i>et al.</i> ⁷ ; Kenny <i>et al.</i> ¹⁶ ; Yipp <i>et al.</i> ¹⁷
<i>Escherichia coli</i>	Dependent	Nucleus, suicide	Intestinal infection	Carestia <i>et al.</i> ¹⁸
<i>Pseudomonas aeruginosa</i>	Dependent	Nucleus, suicide	Nosocomial pulmonary infection in late stage of SAP	Fujitani <i>et al.</i> ¹⁹ ; Floyd <i>et al.</i> ²⁰
Fungi				
<i>Candida albicans</i>	Independent	Nucleus, vital	Nosocomial pulmonary infection in late stage of SAP	Kenny <i>et al.</i> ¹⁶
Viruses				
Influenza A virus	Independent	Nucleus, suicide	–	Tripathi <i>et al.</i> ²¹
Parasites				
<i>Plasmodium falciparum</i>	Dependent	Nucleus, suicide	–	Baker <i>et al.</i> ²²
Chemicals				
PMA	Dependent	Nucleus, suicide	–	Takei <i>et al.</i> ⁵
ROS		Nucleus, suicide	Product of inflammatory and oxidative stress in SAP and can aggravate the condition of SAP	Neeli <i>et al.</i> ²³ ; Kirchner <i>et al.</i> ²⁴
Arachidonic acid	Dependent	Nucleus, suicide	–	Carestia <i>et al.</i> ¹⁸
Nicotine	Independent	Nucleus, suicide	–	Hosseinzadeh <i>et al.</i> ²⁵
Unsaturated fatty acid	Dependent	Nucleus and mitochondria, vital or suicide	Leads to worse inflammation and transforms MAP to SAP	Khan <i>et al.</i> ²⁶ ; Noel <i>et al.</i> ²⁷
Inflammatory factors				
LPS	Dependent	Nucleus or mitochondria, vital or suicide, depending on the dosage and stimulation time	Intestinal endotoxin that causes lung and intestinal injury in patients with SAP	Khan <i>et al.</i> ²⁸
Septic mixture of GM-CSF, TNF- α and IL-1 β	Dependent	Nucleus, suicide or mitochondria, vital induced by a single stimulus	Products of SAP	Brinkmann <i>et al.</i> ²⁹
IL-6	Dependent	Nucleus, vital or suicide, depending on the dosage and stimulation time	Products of SAP	Joshi <i>et al.</i> ³⁰
C5a	Dependent	Mitochondria, vital	Products of SAP	Yousefi <i>et al.</i> ³¹
NLRP3	Dependent	Nucleus, suicide	Products of SAP and potentially induces acinar cell death	Grailer <i>et al.</i> ³² , Hoque <i>et al.</i> ³³
HMGB1	Dependent	Nucleus, suicide	Products of SAP	Ma <i>et al.</i> ³⁴

(Continued)

Table 1. (Continued)

Stimulus type	ROS-dependent or -independent production	DNA source and type of NET released	Association with SAP	Reference
Endogenous stimuli in the body				
Cholesterol crystals	Dependent	Nucleus, suicide	Blocks the pancreatobiliary duct to induce AP	Li <i>et al.</i> ³⁵
Activated platelets	independent	Nucleus, suicide	Thrombus formation in patients with SAP	Clark <i>et al.</i> ³⁶
Monosodium urate crystal	Dependent	Nucleus, suicide, also induces aggNETs	–	Schauer <i>et al.</i> ³⁷
Calcium carbonate crystals	Independent	Nucleus, suicide, also induces aggNETs	Component of pancreatic juice	Leppkes <i>et al.</i> ³⁸
Imbalance of HCO ₃ ⁻ -pCO ₂	Independent	Nucleus, suicide	Environmental status of SAP	Maueröder <i>et al.</i> ³⁹
Ionophore				
Calcium ionophore	Independent	Nucleus, suicide	–	Kenny <i>et al.</i> ¹⁶

aggNETs, aggregates of NETs; AP, acute pancreatitis; LPS, lipopolysaccharide; GM-CSF, granulocyte-macrophage colony-stimulating factor; HMGB1, high mobility group box protein 1; IL, interleukin; NETs, neutrophil extracellular traps; NLRP3, nucleotide-binding domain (NOD)-like receptor protein 3; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocyte; ROS, reactive oxygen species; SAP, severe acute pancreatitis; tumor necrosis factor alpha, TNF- α .

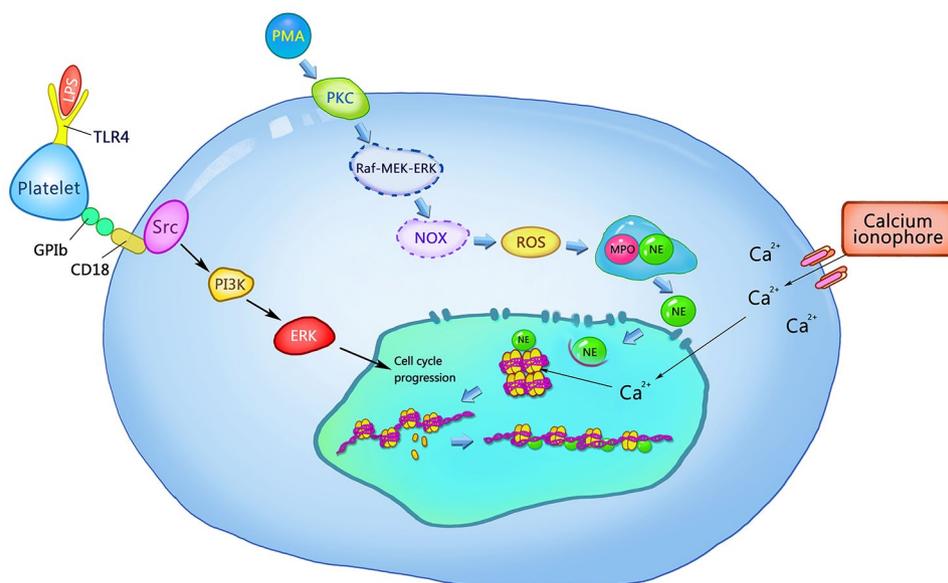


Figure 1. Molecular signaling pathway underlying NETs formation induced by different stimulus. PMA binds to PKC on the cell membrane to activate the Raf-MEK-ERK signaling pathway cascade and NOX, resulting in the production of ROS. ROS act as initiators of the azurosome to liberate NE from the protein complex composed of MPO, NE, and cathepsin G, among other granular proteins. After NE translocation to the nucleus, the core histones are proteolyzed, resulting in decondensation of the chromatin. Calcium ionophores can activate PAD4 directly and induce NETs release without ROS generation. III. After activated by LPS, GPIIb on the surface of platelets can bind to CD18 on neutrophils, then activate Src kinase-PI3K-ERK pathway, resulting in proliferation.
 CD18, β 2-integrin; GPIIb, glycoprotein IIb; LPS, lipopolysaccharide; MPO, myeloperoxidase; NE, neutrophil elastase; NETs, neutrophil extracellular traps; NOX, NADPH oxidase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species.

this NOX-activated signaling pathway through different upstream receptors, such as Toll-like receptors (TLRs) for bacterial virulence factors, thrombin, arachidonic acid, and G-protein-coupled receptor (GPCR) for integrins.^{18,44} As the most important substances in this pathway, ROS, produced either by PMNs or secreted by pathogens, directly induce the transfer of NE from cytoplasmic granules to the nucleus to activate the downstream reactions required for NET formation.¹⁶ This phenomenon might explain why PMNs from patients with chronic granulomatous disease (CGD) release NETs to kill some bacteria, such as *Staphylococcus aureus*, that produce ROS.¹⁷ The formation of ROS-dependent NETs is also regulated by autophagy.^{45,46} The advanced glycation end products receptor (RAGE), which mediates autophagy through the type III MHC protein receptor, regulates NET formation⁴⁷; the inhibition of autophagy blocks PMA- or LPS/IL-8-induced NET formation, which suggests the important role of autophagy in NET formation.^{48,49}

Regarding the ROS-independent formation of NETs, the main key substance regulating this pathway is calcium. Calcium ionophores activate PAD4 and calpain directly to induce NET release without ROS generation, similar to the bacterial toxin nigericin.¹⁶ Since the activation of PADs depends on the existence of calcium ions, a calcium deficiency disrupts NET formation.⁵⁰ In addition to the involvement of calcium ions, nuclear factor kappa B (NF- κ B) has also been reported to regulate uric acid-induced NET formation in the absence of ROS production, indicating that the downstream nuclear signal transduction required for NET formation is regulated by NF- κ B.⁵¹

NET formation induced by platelets has also been observed in the absence of the activation of NADPH oxidase and ROS production. After activation by LPS, glycoprotein Ib (GPIb) on the surface of platelets binds to β 2-integrin (CD18) on neutrophils and then activates the Src kinase-PI3K-ERK pathway,¹⁸ resulting in cell cycle progression.⁵² Activation of the mitotic proliferation marker nuclear antigen Ki-67 and G1 initiation markers cyclin-dependent kinases 4 and 6 (CDK4/6) has been observed in the process of NET formation, which results in chromosome depolymerization and nuclear membrane lysis.⁵³ However, neutrophils are terminally differentiated cells⁵⁴; thus, the activation of the signaling

pathways described above does not induce the expression of downstream S phase gene thymidine analog 5-ethynyl-2'-deoxyuridine (EdU) and suppression of E2F transcription factors. Therefore, the formation of NETs replaces cell mitosis.¹¹

Characteristics of cell morphological changes.

When NETs were first discovered in PMA-stimulated cells, DNA fibers formed by chromosome depolymerization carrying antimicrobial proteins in the nucleus were confirmed to be released eventually from the cell, killing extracellular pathogens; however, recent studies have suggested several different mechanisms that are generated based on the source of DNA and its manner of release. Due to the different types of stimuli, the sources of DNA fibers in NETs and types of changes in cell morphology and cell fate are also different. DNA fibers were initially presumed to be derived from nuclear chromosome depolymerization, while Yousefi *et al.* reported a method to induce the release of mitochondrial DNA to form NETs within the first 15 min after stimulation with C5a or LPS.³¹ Even if the DNA fibers of NETs are derived from the nucleus, different pathways of DNA release may lead to different fates of neutrophils. In addition to the rupture of nuclear membrane and cell membrane to form NETs (suicidal NETosis), the researchers also observed an intact membrane of neutrophils incubated with *Staphylococcus aureus*, and the cells continued to move and phagocytize bacteria after NET release.¹⁷ NETs also form by releasing DNA and cytoplasmic contents through vesicles (early after neutrophils were stimulated) without rupture of the cell membrane.⁷ The mechanism allowing neutrophils to survive after NET release is called vital NETosis⁵⁵ (Figure 2). During the process of NET formation, many NETs accumulate in clumps, resulting in the aggregation of NETs (aggNETs).³⁷ Thus, NETs with high local concentrations are not removed in a timely manner, and crystalline substances must be present,³⁵ which is distinguished between the recent discovery of NET formation and other modes of cell death.

NETs are new mechanism resulting in cell death that differs from apoptosis or necrosis. Their existence suggests that they supplement phagocytosis to prevent bacteria from escaping, allowing better killing of pathogens to protect the host.¹³ Neutrophils and other immune cells, such as eosinophils,

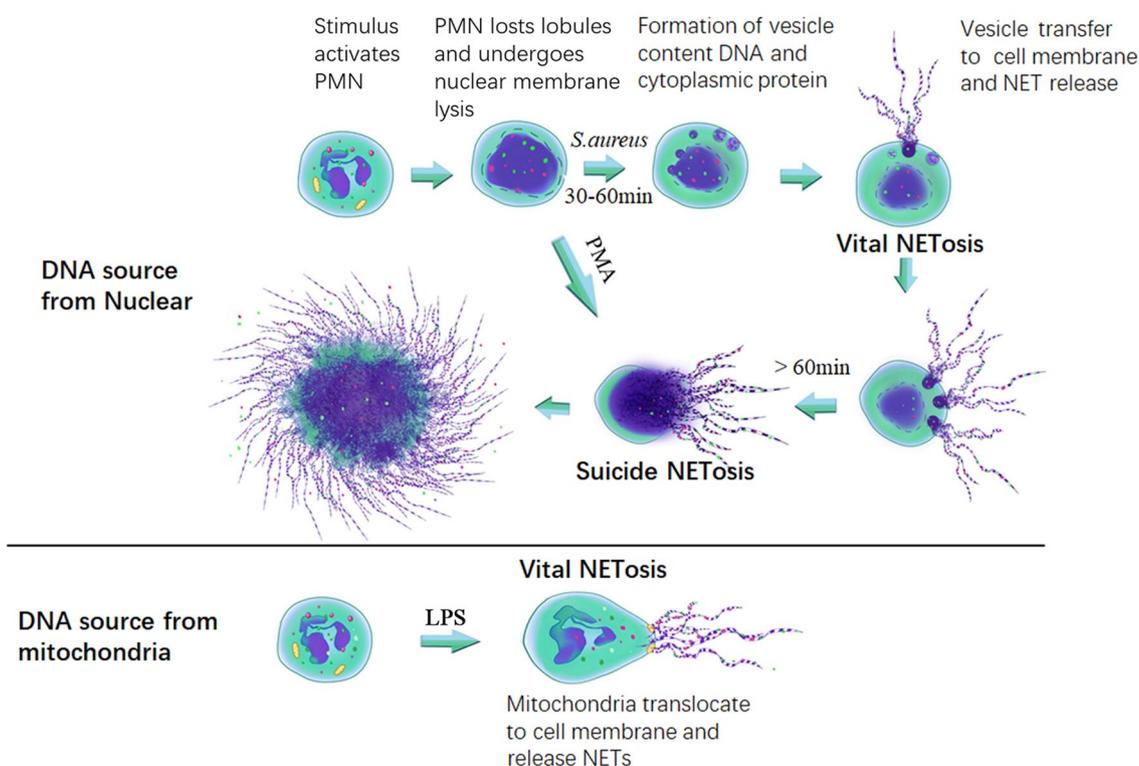


Figure 2. NETs formation stimulated by different stimulus have different DNA sources and destiny. (A) Nuclear source: after triggering by some stimuli such as *Staphylococcus aureus*, PMNs first lose their nuclear lobules; the nuclear membrane then ruptures and forms vesicles containing DNA and protein. After transfer of these vesicles to the cell membrane, they fuse with the cell membrane and release the complex of DNA and protein, which is called vital NETosis. This phenomenon often occurs in the early period of the reaction (30–60 min). With increasing reaction time, more NETs will be released from the cell, the nuclear membrane will gradually dissolve, a large amount of DNA will be depolymerized, and the expansion force formed will rupture the cell membrane and eventually cause cell death: so-called suicide NETosis. However, when PMNs are stimulated by PMA, the cells break down directly after lobule disappearance and NET release. (B) Mitochondrial source: after stimulation by LPS or C5a, mitochondria translocate to the cell membrane, and DNA is released from the cell to form NETs without the occurrence of nuclear and membrane lysis, which is another type of vital NETosis.
LPS, lipopolysaccharide; NETs, neutrophil extracellular traps; PMA, phorbol 12-myristate 13-acetate; PMNs, polymorphonuclear leukocytes.

basophils, and macrophages, also release NETs, contribute to antibacterial defenses, and trap and kill as many pathogens as possible.^{56–58}

Damage caused by NETs

NETs protect the body from harmful microorganisms, but they are double-edged swords. In the absence of NET formation, as exemplified by patients with CGD who are unable to form NETs, increased susceptibility to many pathogens is observed.⁵⁹ However, overactivation of NETs due to an imbalance in their formation and destruction may damage to host tissues and participate in the pathophysiological processes of

various autoimmune diseases and inflammatory injuries.⁶⁰ Endothelial and epithelial cell death, vascular thrombus formation, and lung injury have been reported to be caused by NETs and their components.^{61–63} Pancreatic stellate cells are activated by DNA derived from NETs, resulting in pancreatic carcinoma growth.⁶⁴

NET-induced damage to organisms is divided into two main types; one is caused by DNA fibers and the other is caused by proteases that exert lethal effects. Intravascular thrombi contain many neutrophil DNA fibers as scaffolds, and NETs also stimulate thrombus formation in both individuals with infectious and noninfectious diseases.⁶⁵ The

DNA filaments of aggNETs intertwine to form large complexes, causing local obstruction and compression, such as pancreatic duct stones derived from IL-17 transgenic models, tophus, and sputum bolt.^{38,66–68} Many antimicrobial proteins attached to the DNA backbone of NETs also cause tissue injury. These proteins either injure normal tissues directly or activate the immune response to induce inflammatory injury, despite their antimicrobial function. NETs activate macrophages and dendritic cells after approximately 30 min of cocubation, but kill these cells after prolonged exposure due to mitochondrial damage.⁶⁹ This damage might be achieved by NE, because an antagonist of NE was observed to block this reaction.⁷⁰ Protein components of NETs, particularly histones, induce epithelial and endothelial cell death in a mouse model of LPS-induced acute lung injury.⁶³ As a strong oxidant, MPO oxidizes tyrosine to tyrosyl radicals, which regulates enzyme activity in the cell signaling pathway.⁷¹ This molecule also increases the macrophage respiratory burst and induces the synthesis of IL-1 α and IL-1 β in alveolar macrophages.⁷² Furthermore, increased cytokine expression has been observed in a mouse model after histone injection *via* activation of TLR-4.⁷³ NET-bound proteases are active and increase the activity of extracellular IL-1 family cytokines to aggravate inflammation.⁷⁴ Since NE is the main cause of cystic fibrosis and prevents macrophages from engulfing apoptotic neutrophils by degrading the phosphatidylserine receptor (PSR), it may represent a target for the treatment of inflammation and mucus hypersecretion.⁷⁵

Although NETs and their components have been shown to cause serious damage to organisms after activation in a sterile state, their presence is also beneficial. AggNETs have been shown to reduce the levels of pro-inflammatory chemokines and cytokines and suppress crystal-induced inflammation. Furthermore, a vital anti-inflammatory effect of aggNETs on cytokines such as TNF- α , IL-1 β , and IL-6 has been observed in animal models.³⁷ MPO in NETs also suppresses the adaptive immune response by inhibiting DC activation to restrict pathological tissue inflammation *via* its catalytic activity.⁷⁶ NET formation provides a backbone to which damage-associated molecular patterns (DAMPs) bind; potentially harmful DAMPs are then degraded in NETs *via* proteolytic digestion, such as inflammatory cytokine degradation, as previously described.³⁷

NETs are a major cause of tissue damage in individuals with SAP

SAP is characterized by the cascade activation of systemic inflammation after the abnormal activation of trypsinogen, which may lead to systemic inflammatory response syndrome (SIRS) and MODS during disease development. Proenzyme activation results in pancreatic tissue autodigestion and pancreatic injury. Impaired acinar cells release DAMPs to promote the generation of IL-1 β and activation of cell surface pattern recognition receptors, including TLRs, ultimately inducing systemic injury in patients with SAP.³³ Persistent SIRS promotes the occurrence of organ failure in more than one organ system that results in MODS.^{77,78} Long-term studies of the pathophysiological process of SAP have shown that inflammatory damage in patients with SAP is caused by many immune cells and their secreted cytokines.^{27,79}

Neutrophils are immune cells that play a vital role in the inflammatory damage associated with SAP.¹ Increased neutrophil infiltration has been detected in regions of the pancreas with severe edema or necrosis.⁸⁰ NETs and their components derived from activated neutrophils have also been detected in damaged tissues of animal models of SAP and exacerbate the deterioration of SAP and inflammatory tissue injury.^{8,81} Increased serum levels of the MPO–DNA complex and a marker of NET formation were observed in patients with SAP compared with patients with mild acute pancreatitis (AP). These results confirmed the link between NETs and SAP. In addition, PMNs translocate to many tissues, resulting in NET formation during SAP due to the production of cytokines and chemokines induced by SIRS.

Coagulo-fibrinolytic abnormalities resulting in disseminated intravascular coagulation (DIC) may make an important contribution to the severity of acute pancreatitis.⁸² Cell-free DNA and NE from NETs have been shown to activate blood coagulation factors (factor XII and Xa), bind platelets, and lead to thrombocytopenia, which may easily result in DIC.⁸³ Thus, the relationship between NETs and the severity of SAP is indicated by the incidence of DIC, and NETs may be the key factor contributing to the occurrence of DIC in individuals with SAP. After the induction of SAP, PAD4-deficient mice that are unable to generate NETs had lower trypsin and amylase activities and greater viability than wild-type mice.⁸⁴

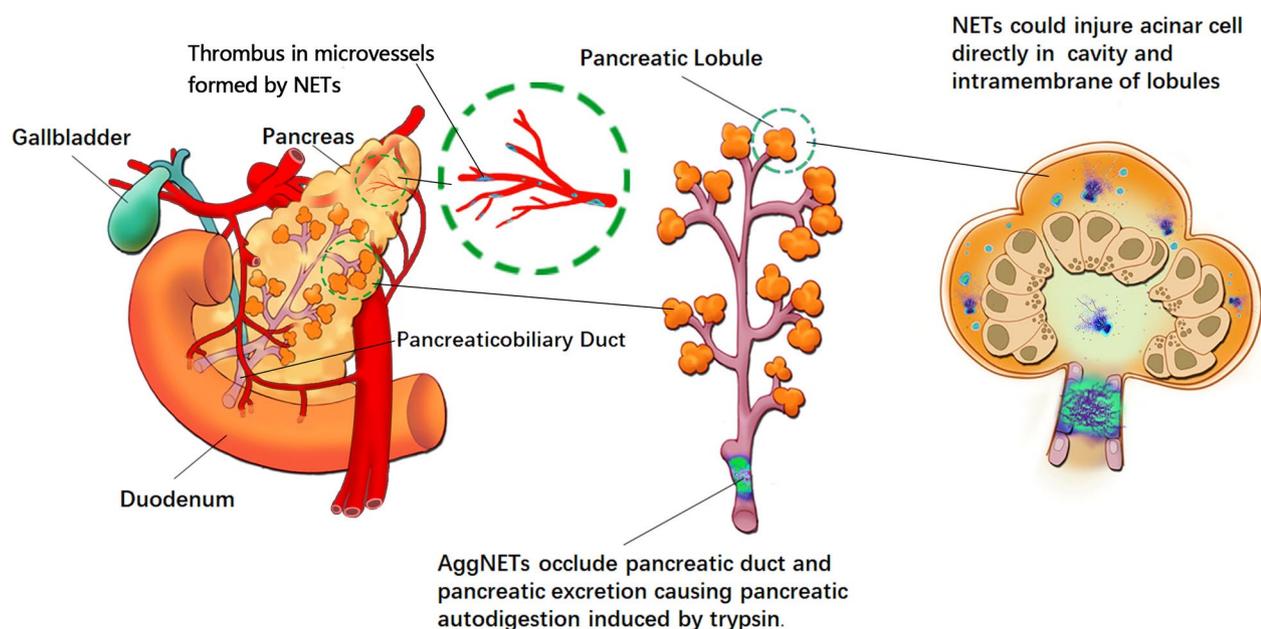


Figure 3. NETs damage in the pancreas. During SAP, neutrophils are transferred to the pancreatic acinus by inflammatory chemotaxis and stimulated by various factors to form NETs. In addition to injuring acinar cells directly, NETs can entwine one another to form aggNETs, which can lead to occlusion of pancreatic duct aggravating SAP. NETs can also trigger thrombosis in intralobular artery and cause pancreatic lobular ischemia and necrosis. aggNETs, aggregates of NETs; NETs, neutrophil extracellular traps; SAP, severe acute pancreatitis.

Damage in the pancreas

Merza *et al.* reported the presence of NETs in pancreatic tissues in a mouse model of SAP, which induced trypsin activation, inflammatory responses, and tissue injury *in vivo*. An incubation with NETs increases trypsin activity.⁸ Cell-free histones, including those derived from NETs, are toxic and disrupt the pancreatic acinar cell plasmalemma, leading to acinar cytoplasm leakage and cell death.⁸⁵ IL-17A transgenic mice or an IL-17A delivery model showed biliopancreatic ductal obstruction mediated by the formation of macroscopically visible NET aggregates that initiated biliary acute pancreatitis, and pancreatic juice was a strong stimulus of NET formation *via* crosstalk.³⁸ The interaction between neutrophils and platelets in pancreatic tissue also plays an important role in the mechanism underlying an increase in the severity of acute pancreatitis.⁸⁶ Activated platelets bind to neutrophils and induce NET formation; NETs and their components then bind more platelets to form a thrombus and injure the endothelium in the pancreatic microvasculature.⁶² Because the blood supply of the pancreatic lobule is supplied only by the lobular artery, lobule is very sensitive to ischemia and NETs might easily cause pancreas ischemic injury

and necrosis.⁸⁷ The injured pancreatic acini would secrete DAMPs into the circulation upon serious local inflammation, potentially leading to SIRS and MODS (Figure 3).³³

After an early acute inflammatory injury, pancreatic inflammation does not stop at the stage of stabilization in patients with SAP. Instead, local complications, such as pancreatic necrosis, the formation of walled-off necrosis and pseudocysts, are observed. The presence of NETs in necrotic pancreatic tissue, pseudocysts and walled-off necrosis suggests that NETs are involved in the formation of these structures. However, from another perspective, NETs promote the aggregation of proteins in exudates, forming a barrier that limits inflammatory substances to one site, thereby reducing damage to the surrounding tissues.⁸¹

According to recent studies, pancreatitis is associated with a decrease in pancreatic levels of lysosome-associated membrane proteins (LAMPs), which are required for autophagy and maintain pancreatic acinar cell homeostasis and attenuate pancreatitis.^{88,89} Thus, acinar autophagy may be a key factor in the treatment of pancreatitis. However, an anti-LAMP-2 antibody has also

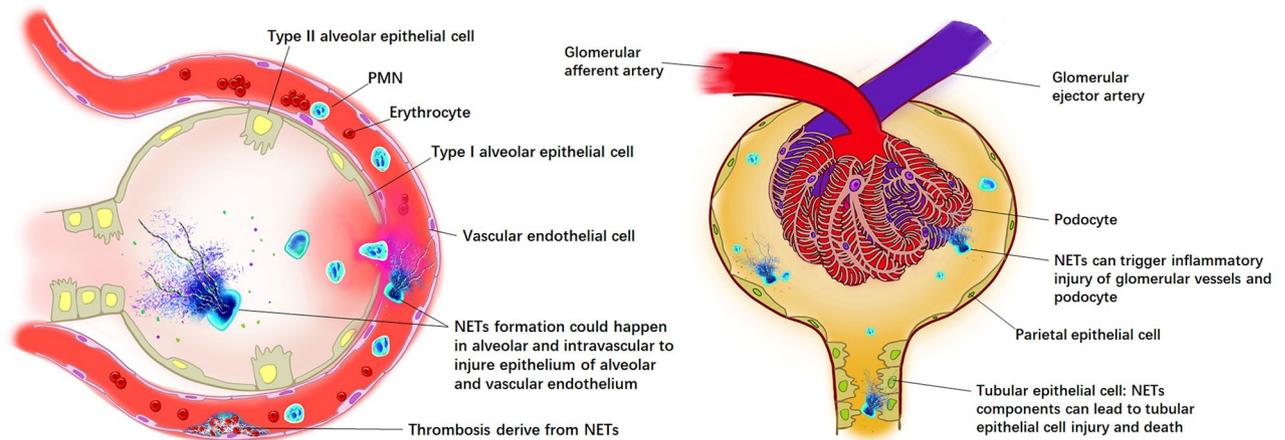


Figure 4. NETs damage in the lung and kidney. Left: NETs release due to inflammatory factor stimulation in the alveolar space. When the barrier formed by epithelial cells is destroyed, the alveolar mucosa and capillary permeability increase, and cells in blood translocate into the alveolar space. PMNs release NETs when stimulated by inflammatory factors or pathogens. After NETs formation, the toxic proteins in turn damage the epithelium and endothelium, even triggering NETs generation and NET-derived thrombosis in the vasculature. Right: The components of NETs can trigger inflammatory injury to glomerular vessels and podocytes, affecting glomerular filtration functions; conversely, NETs components can lead to tubular epithelial cell death and reabsorption dysfunction.

NETs, neutrophil extracellular traps; PMNs, polymorphonuclear leukocytes.

been shown to trigger NET formation; thus, LAMPs derived from autophagy might also induce tissue damage by promoting NET formation.⁹⁰ Therefore, the presence of NETs inhibits recovery from pancreatitis, not only because of the tissue damage, but also because of the impairment of homeostasis maintained by autophagy.

Systemic damage caused by NETs in patients with SAP

SAP is characterized by the cascade activation of systemic inflammation after the abnormal activation of trypsinogen; thus, neutrophils may undergo chemotaxis and migrate to various tissues to form NETs. Therefore, not only are NETs present in and injure pancreatic tissue, but they also cause more serious damage to other related organs.

Damage in the lung. Acute respiratory distress caused by acute lung injury is the most common complication leading to death in patients with SAP, and NETs have also been shown to cause lung injury through various mechanisms.⁹¹ DNA fibers function as scaffolds to form a sputum bolt with excessive mucin produced by NE-induced airway epithelial cells, which blocks the airway to create a suitable environment for bacterial growth and colonization.^{92,93} NE slows the ciliary beat frequency, leading to an obvious disruption of epithelial cells,

and cleaves the endothelial actin cytoskeleton, E-cadherin, and VE-cadherin, disrupting mucosal barrier formation to increase the permeability of the alveolar-capillary barrier.^{63,94–96} Furthermore, NE induces apoptosis of epithelial cells and pro-inflammatory cytokine secretion *via* PAR-1, along with subsequent activation of the NF- κ B pathway.^{97,98} MPO and NE aggravate lung injury by destroying the adjacent tissue and degrading the heparan sulfate proteoglycans in the endothelial cell (EC) matrix.⁹⁹ The AMPs LL-37 and MPO, which are detected in NET structures, show cytotoxic and proapoptotic properties in endothelial and bronchial epithelial cells by inducing DNA strand breaks.^{100,101} Histone injection induces the vacuolation of pulmonary vascular endothelial and alveolar epithelial cells, alveolar hemorrhage, and microthrombus formation, causing alveolar-capillary damage and disrupting the microcirculation (Figure 4).¹⁰²

Vascular damage. Changes in the hemodynamics of patients with SAP, such as hypovolemia, hypercoagulability, and infiltration of inflammatory factors, potentially lead to a series of vascular pathological changes.¹⁰³ NETs and their components, such as the inflammatory products of SAP, have been shown to be involved in blood vessel damage and to promote the formation of an intravascular thrombus.⁶²

NETs degrade vascular endothelium (VE)-cadherin and activate downstream β -catenin signaling to promote vascular leakage.¹⁰⁴ By generating proteinase 3 (PR3) and MPO, NETs induce anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), which also stimulates NET formation, leading to progressive vascular damage.¹⁰⁵ Neutrophil proteases also participate in experimental abdominal aortic aneurysms by activating pDCs and the secretion of extracellular components.⁷⁰ For example, matrix metalloproteinase 9 (MMP-9), which is contained in NETs, activates endothelial MMP-2 to cause VE damage, resulting in endothelial dysfunction and the initiation of atherosclerosis.¹⁰⁶ In addition, the instability of atherosclerotic plaques is increased *via* inflammatory infiltration aggravated by NET-activated T helper 17 cells.¹⁰⁷ On the other hand, the injured VE induces NET formation by activating IL-8,¹⁰⁸ and cholesterol crystals in atherosclerotic plaques also serve as a chemoattractant and trigger NET release.¹⁰⁹ Thus, the generation of NETs and vascular injury are mutually reinforcing phenomena, indicating that NETs would be a therapeutic target in vascular inflammatory injury.

NETs have been reported recently to be an important initiator of venous thrombus formation in the IVC stenosis model, because neutrophil histones modified by PAD-4 are required for this process.^{110,111} DNA fibers derived from NETs serve as scaffolds to bind fibrin, blood cells, and von Willebrand factor (VWF), resulting in thrombus formation.⁶⁵ During sepsis, the interactions among monocytes, PMNs, NETs, and platelets trigger the formation of a venous thrombus when bacteria appear in the circulation to prevent dissemination of the infection.^{112,113} Activated platelets stimulated by LPS or plasma from patients with sepsis also induce NET generation *via* β 2-integrin-mediated platelet-neutrophil engagement.^{114,115} Both vascular injury and thrombosis are fatal in patients with SAP; therefore, a strategy designed to block the production of NETs is crucial for the treatment of SAP.

Kidney damage. The NET-induced damage to the renal parenchyma affects mainly glomerular vessels and the reabsorption system. ANCA-associated glomerulonephritis causes glomerular leakage, resulting in proteinuria.^{104,116,117} Harmful chemicals, such as BPA, damage podocytes by stimulating PMNs to produce NETs, thus affecting glomerular filtration functions. In addition, the endothelial-to-mesenchymal

transition induced by NETs drives nephroangiosclerosis and results in renal dysfunction.¹⁰⁴ Tubule epithelial cell death is also induced by NETs through histone secretion from ischemic tubular cells, altering the function of the reabsorption system (Figure 4).¹¹⁸

Heart damage. Extracellular histones in NETs may activate the nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3) inflammasome, and evidence of NLRP3 activation in the heart during sepsis has also been observed; thus, extracellular histones may activate NLRP3 in the myocardium and lead to cardiomyopathy and cardiac dysfunction.^{32,119} Histones also reduce the levels of SERCA2 and NCX, key proteins regulating Ca^{2+} signaling and the Na^+/K^+ ATPase in cardiomyocytes (CMs), resulting in defective action potentials and arrhythmia.¹²⁰ As mentioned in the previous section, AAV induced by NETs might lead to coronary atherosclerosis, and NET-related thrombi in coronary arteries often cause myocardial infarction, which has been observed in patients with SAP.¹²¹

Intestinal damage. In the intestine, NETs disrupt the intestinal microecological balance and lead to the injury, and even apoptosis, of intestinal epithelial cells, resulting in damage to the gut barrier, increased intestinal mucosal permeability, increased endotoxin production, and an imbalance in the intestinal flora.¹²²⁻¹²⁴ The levels of NETs and several associated proteins are all increased in the colonic mucosa from patients with ulcerative colitis (UC) associated with stimulation of the innate immune system.¹²⁵

Interventions targeting NETs mitigate the severity of SAP

Although researchers have not clearly determined whether NETs are anti-inflammatory or pro-inflammatory, several studies have provided evidence that NETs lead to tissue damage and organ dysfunction. Moreover, treatments targeting NETs indeed reduce tissue damage and protect organ function. Thus, researchers have explored a series of strategies to inhibit NETs. Many substances promote or inhibit NET formation or inhibit the activity of NET proteins, which may have therapeutic relevance. We have categorized these strategies into three groups and found that each approach affects SAP treatment (Table 2).

Table 2. Substances that can reduce NET injury.

Intervention type	Substance	Mechanism	Reference
Block NET formation	Aspirin	Inhibit platelet activation and decrease NET formation in the vasculature	Caudrillier <i>et al.</i> ¹¹⁴
	Glycoprotein IIb/IIIa inhibitor	Inhibit platelet activation and decrease NET formation in the vasculature	Caudrillier <i>et al.</i> ¹¹⁴
	PAD inhibitor	Block histone citrullination to form decondensed DNA	Madhi <i>et al.</i> ¹²⁶
	EGTA	Chelate extracellular calcium to inhibit NET formation	Parker <i>et al.</i> ¹²⁷
	ROS inhibitor NAC	Inhibit the formation of aggNETs	Schauer <i>et al.</i> ³⁷
	NOX inhibitor DPI	Inhibit ROS formation to block ROS-dependent NET formation	Khan <i>et al.</i> ²⁸
	NF- κ B inhibitor BAY 11-7082	Inhibition of UA-induced NET formation	Arai <i>et al.</i> ⁵¹
	Eap of <i>Staphylococcus aureus</i>	Interrupt NET formation by binding to DNA	Eisenbeis <i>et al.</i> ¹²⁸
	Serum and serum albumin	Inhibit LPS- and calcium ionophore-induced NET formation	Neubert <i>et al.</i> ¹²⁹
	Serine inhibitor sivelestat	Inhibit neutrophil elastase to block NET formation	Majewski <i>et al.</i> ¹³⁰
Disassemble the DNA scaffold	DNase	Degrade NETs and inhibit aggNET formation by hydrolyzing DNA	Jimenez-Alcazar <i>et al.</i> ¹³¹
	Nuclease from bacteria	Degrade NETs and inhibit aggNET formation by hydrolyzing DNA	Liang <i>et al.</i> ¹²³
	Heparin	Release NET-bound MPO and dismantle NETs	Fuchs <i>et al.</i> ⁶⁵ , Parker <i>et al.</i> ¹³²
Inhibit the toxicity of proteins	Antibody against histone and APC	Disables histone toxicity and preserves host cells	Xu <i>et al.</i> ¹⁰²
	TIMP-1	Cripple MMP-9 activity	Duarte <i>et al.</i> ¹³³
	PSA	Binds to histones 28 antitoxicity of	Saffarzadeh <i>et al.</i> ⁶³
	MPO inhibitors (dapsone and tryptamine)	Inhibit the oxidative effect of MPO	Jantschko <i>et al.</i> ¹³⁴ , Lazarevic-Pasti <i>et al.</i> ¹³⁵

aggNETs, aggregates of NETs; APC, activated protein C; LPS, lipopolysaccharide; MMP-9, matrix metalloproteinase 9; activated protein C; MPO, myeloperoxidase; NAC, N-acetylcysteine; NETs, neutrophil extracellular traps; NF- κ B, nuclear factor kappa B; NOX, NADPH oxidase; PAD, protein arginine deiminase; PSA, polysialic acid; ROS, reactive oxygen species; TIMP-1, tissue inhibitor of metalloproteinases-1; UA, uric acid.

Blockade of NET formation

All factors involved in the NET formation pathway have been used as targets to block this process, including inhibitors of various stimuli, cell membrane receptor antagonists or blockers, inhibitors of proteins in various signaling pathways, or chelating

agents. A sufficient concentration of the PKC inhibitor Gö6976 completely blocks PMA-induced NET formation.¹³⁶ Both aspirin and glycoprotein IIb/IIIa inhibit platelet stimulation, resulting in reduced NET formation in the vascular tissue.¹¹⁴ EGTA, which chelates extracellular calcium,

partially inhibits NET formation triggered by *Pseudomonas aeruginosa* and PMA.¹²⁷ The bivalent cation-chelating agent EDTA, the ATP antagonist oxidized ATP (oxATP), and the ROS inhibitor N-acetylcysteine (NAC) restrict the formation of aggNETs.³⁷ A NOX inhibitor blocks the NET formation induced by many stimuli of the ROS-dependent signaling pathway.²⁸ The treatment of normal neutrophils with the NF- κ B inhibitor BAY 11-7082 results in substantial inhibition of uric acid (UA)-induced NET formation.⁵¹ The extracellular adherence protein (Eap) of *S. aureus* blocks NET formation by binding to DNA.¹²⁸ Janus kinase (JNK) inhibitors also antagonize LPS-induced NET formation *via* the LPS-TLR4-JNK pathway.²⁸ NE inhibitors, such as sivelestat, reduce NET formation, restrict the toxicity of NE, and function as a therapeutic drug to treat airway injury and acute respiratory distress syndrome related to systemic inflammatory diseases, such as SAP.¹³⁰

Since PADs are important enzymes required for DNA depolymerization to form NETs, some researchers have shown that PAD4 inhibitors effectively block mouse and human NET formation.¹³⁷ However, the decisive role of PAD4 in NET formation is unclear, based on the result that PAD4-deficient mice form NETs upon calcium ionophore stimulation.¹⁶ PAD4-deficient mice show a milder illness than wild-type mice, and PAD inhibitors decrease MPO levels and inflammatory tissue damage in the inflamed pancreas of the SAP model.^{138,139} Therefore, PADs participate in regulating NET formation in individuals with SAP, and the inhibition of the activity of this enzyme to decrease NET formation may preserve organ function and protect against inflammatory injury in patients with SAP.

In addition to drugs that block NET formation, the stimulation of NET generation is also determined by the environment in which the reaction occurs, namely, the composition of the culture medium. A high glucose concentration inhibits NET formation and modulates IL-6-mediated immune homeostasis.³⁰ The addition of heat-inactivated (hi) fetal calf serum (FCS), 0.5% human serum albumin (HSA), or 0.5% bovine serum albumin (BSA) to the culture medium efficiently decreases NET generation by human neutrophils stimulated with LPS and calcium ionophores.¹²⁹ Thus, albumin supplementation therapy not only maintains plasma osmotic pressure and decreases the incidence of thrombosis but also ameliorates

inflammatory injury by reducing NET formation in patients with SAP presenting hypoproteinemia.

Disassembly of the DNA scaffold

As described above, DNA scaffolds play a key role in the tissue deterioration mediated by NETs; thus, the destruction of the DNA skeleton may protect tissues from NETs. Two types of host DNases – DNase I and DNase I L3 – degrade NETs *in vitro* and protect hosts from the deleterious effects of intravascular NETs *in vivo*.¹³¹ In addition, DNase and other DNA-degrading enzymes inhibit the formation of aggNETs to reduce the obstruction.³⁷ Upon DNase I administration, damage to the pancreatic and lung tissues in SAP mice was reduced, along with the activity of MPO.⁸ Nucleases from bacteria that prevent NET–DNA capture, such as staphylococcal nuclease (SNase), effectively degrade NETs *in vitro* and *in vivo* to improve gut barrier function.^{123,140} Heparin also dismantles the NET–DNA complex and reduces the activity of histones to induce platelet aggregation, thus preventing thrombus formation.⁶⁵ Both gut barrier dysfunction and thrombosis are linked inextricably to the disease state of SAP; therefore, treatments targeting these factors may play a vital role in SAP therapy.

Inhibition of protein toxicity

The inhibition of proteins on NETs potentially represents another strategy to reduce NET-induced tissue injury. Some antibodies targeting NET proteins have been shown to protect tissues from NET formation. As a clinical treatment agent, antibodies against histones and activated protein C (APC) inhibited histone-mediated damage and protect host cells.¹⁰² Polysialic acid (PSA) interrupts NET formation and prevents the cytotoxicity of histones by binding to histone 28.⁶³ Tissue inhibitor of metalloproteinases-1 (TIMP-1) decreases the formation of NETs and limits NET-mediated cytotoxicity during hepatic IRI by blocking MMP-9 activity.¹³³ MPO inhibitors, such as dapsone and tryptamines, which inhibit the oxidative effect of MPO, protect tissues from inflammatory injury.^{134,135}

The serum histone level has been shown to be associated with the severity of SAP, and an anti-histone treatment improves organ function and the survival rate of patients with SAP.^{141,142} Active

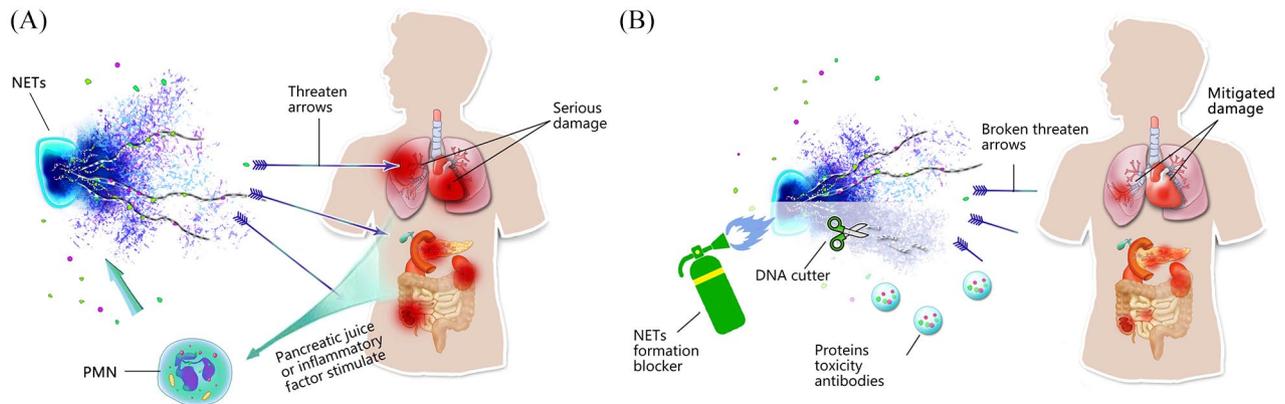


Figure 5. Intervention targets for NETs mitigate the severity of SAP. (A) NETs and their components, like poisonous arrows, cause devastating blows to tissues under SAP inflammation stress, leading to lethal complications of SAP, such as acute lung injury, acute kidney injury, myocardial injury, and intestinal dysfunction. Furthermore, NETs can be induced by components of the pancreatic juice or inflammatory factors released in SAP, and they also act as inducers of SAP *via* pancreatic acinar injury or blockade of the pancreatic duct. The generation of NETs and SAP seem to form a vicious circle. (B) Three kinds of interventions that target NETs can effectively decrease inflammatory injury in tissue (halt the threatening arrows) to disrupt the NET-SAP loop and protect organ function.

NETs, neutrophil extracellular traps; PMN, polymorphonuclear leukocyte; SAP, severe acute pancreatitis.

MPO released during NETosis causes severe damage in the pancreas and surrounding tissues, aggravating systemic inflammatory reactions.¹⁴³ Inhibition of the toxic effects of proteins represents another strategy to preserve organ function in patients with SAP (Figure 5).

Conclusions and perspectives

NETs have attracted widespread attention from the scientific research community since their discovery, and researchers have conducted numerous studies to examine their structure, function, and mechanisms of formation. While affirming the strong antimicrobial function of these structures, many studies have also found that NETs participate in the pathophysiological processes of many noninfectious diseases in multiple organs, such as acute inflammatory diseases, chronic immune inflammatory diseases, and thrombotic diseases.

NETs and their components play important roles in the damage to normal tissues under inflammatory stress caused by SAP and the activation of inflammatory cells to cause inflammatory cascades. The main lethal complications of SAP, such as acute lung injury, acute kidney injury, myocardial injury, and intestine dysfunction, all involve NETs. Furthermore, NET formation is induced by components of the pancreatic juice or inflammatory factors released during SAP, and

they may induce SAP *via* pancreatic acinar injury or blocking pancreatic ducts.^{8,38} Thus, the generation of NETs and SAP might form a vicious cycle.

Based on this relationship between NETs and SAP, treatments targeting NETs may play a key role in the treatment of SAP. Inhibition of NET formation results in a milder illness in the SAP model. Both PAD4-deficient mice and mice treated with a PAD inhibitor exhibit milder inflammation and tissue damage in the inflamed pancreas in the SAP model.^{138,139} DNase I, which disassembles the DNA backbone of NETs, reduces the activity of MPO and the damage to pancreatic and lung tissues in SAP mice.⁸ Individuals with dysfunctional NET formation have always been found to have a mild disease course after induction of AP, underscoring the importance of NETs in SAP.

SAP has a high incidence rate and mortality rate worldwide, requiring long-term hospitalization and costly treatment.¹⁴⁴ Fluid resuscitation, analgesia, antibiotics, and nutrition are the common curative treatments for SAP, but these treatments do not exert a specific effect on the inflammatory injury.¹⁴⁵ Therefore, as the key factor contributing to the aggravation of inflammatory injury, NETs may serve as therapeutic targets, and anti-NET drugs might specifically inhibit inflammatory injury in patients with early stage of SAP.

However, the lack of detection and effective therapeutic approaches have limited the clinical application of NET-targeted therapy. Novel technologies must be used to accelerate the clinical translation of NET detection. In addition to NETs, neutrophils also produce extracellular vesicles (EVs) expressing tissue factor on their surface, which is capable of initiating coagulation, impairing bacterial growth and protecting tissues under inflammatory conditions.^{146–148} Neutrophil-derived EVs contain DNA, which is protected by the vesicular double membrane, and attached histones are incorporated into NET structures.¹⁴⁹ EVs have been detected in various body fluids and are selectively enriched in patients with specific diseases, including pancreatic cancer. Many research groups have now reported EV analytical methods using liquid biopsies to address whether these discoveries and technical advances are potentially useful for the clinical translation of EV biomarkers.¹⁵⁰ The nanoplasmonic-enhanced scattering (nPES) assay designed to quantify total and disease-derived EVs from patients with stage I–II pancreatic tumors or patients with chronic pancreatitis shows high sensitivity (86–94%) and specificity (85%), indicating the potential of EV detection for diagnosing NET-induced SAP.¹⁵¹ In a recent study, the quantification of a single EV biomarker differentiated patients with various stages of pancreatic cancer using a lipophilic dye hybridized to antibody-conjugated quantum dot probes for specific EV surface biomarkers, resulting in the direct analysis of serum EV biomarker levels without a separate step.¹⁵² The DNA backbone-disassembling enzymes DNase I and SNase are commonly used in modern clinical treatment, but not for NETs and SAP. More effective recipes are needed to inhibit NET formation and their activity to treat SAP.

In summary, the detailed functions, mechanisms, and relationship of NETs to SAP and the interventions targeting NETs have attracted the attention of numerous researchers. The development of new detection technologies and drugs will accelerate substantially the clinical translation of treatments targeting NETs in patients with SAP in the future.

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Author contributions

Jing Hu, Xiaolin Yi, and Huan Chen reviewed the current literature and drafted the main part of the manuscript. Jiaqi Yao, and Hongxin Kang edited the manuscript and contributed by iteratively reviewing and improving the manuscript. Meihua Wan and Wenfu Tang edited the manuscript, provided oversight of the drafting of the manuscript, and provided substantive intellectual contributions and improvements. All authors read and approved the final manuscript.

Conflict of interest statement

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