Role of neutrophil extracellular traps in inflammatory evolution in severe acute pancreatitis

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Abstract

Severe acute pancreatitis (SAP) is a life-threatening acute abdominal disease with two peaks of death: the first in the early stage, characterized by systemic inflammatory response-associated organ failure; and the second in the late stage, characterized by infectious complications. Neutrophils are the main immune cells participating in the whole process of SAP. In addition to the traditional recognition of neutrophils as the origination of chemokine and cytokine cascades or phagocytosis and degranulation of pathogens, neutrophil extracellular traps (NETs) also play an important roles in inflammatory reactions. We reviewed the role of NETs in the occurrence and development of SAP and its fatal complications, including multiple organs injury, infected pancreatic necrosis, and thrombosis. This review provides novel insights into the involvement of NETs throughout the entire process of SAP, showing that targeting NETs might be a promising strategy in SAP treatment. However, precision therapeutic options targeting NETs in different situations require further investigation.

Keywords: Inflammation; Neutrophil extracellular traps; Organ injury; Sepsis; Severe acute pancreatitis; Thrombus

Introduction

Severe acute pancreatitis (SAP) is a local inflammatory injury occurred initially in the pancreas, followed by a systemic inflammatory disorder with a mortality of 30%.[1,2] It is often accompanied by distant organ dysfunction and/or pancreatic local complications. There are two peaks of death occurred in SAP: the first, within two weeks of acute onset, due to multiple organs failure which results from the systemic inflammatory storm, and the second, two weeks after the acute bout, triggered by multi-organ damage with sepsis caused by infected pancreatic necrosis (IPN).[3-5]

Neutrophils, the most abundant white blood cells in the human body, play a pivotal role in the host defense. Neutrophils can kill invading pathogens via phagocytosis, degranulation, and reactive oxygen species (ROS) production.[6] In addition, activated neutrophils can also form extracellular networks known as neutrophil extracellular traps (NETs); this is frequently accompanied by cell death in a process named NETosis,[7,8] first reported in 2004 by Brinkmann *et al*[9]. NETs trap pathogenic microorganisms such as *Staphylococcus aureus*, *Salmonella typhimurium*, and Shigella flexneri. IpaB, a virulence factor of *Shigella flexneri*, is degraded by neutrophil elastase (NE) of NETs.[9] Sepsis is one of the most common causes of death in patients with SAP,[10] while NETs kill pathogens and prevent their dissemination *in vivo*, thus reducing multiple organs injury and mortality in the early stage of sepsis.[11,12] Moreover, NETs can form a temporary physical barrier to separate necrotic pancreatic collections from the remaining viable areas.[13]

Nevertheless, NET generation is a double-edged sword in SAP, as dysfunctional or excessive release of NETs can also lead to tissue damage.[14] Several studies have provided evidence that NETs promote multiple organs dysfunction, thrombosis, and sepsis in SAP, and that targeting the pathways and mechanisms involved in NET generation may therefore be a novel therapeutic strategy for SAP and its complications. Recently, the triggers and mechanisms underlying NET formation *in vitro* have been extensively studied.[7,15] In this review, we summarized the formation mechanism and characteristics of NETs, as well as their roles in the two peaks of deaths in SAP, and discussed the therapeutic strategies for this severe digestive disorder.

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Components and Functions of NETs

NETs are extracellular reticular structures composed of cytosolic and granule proteins assembled on a scaffold of decondensed chromatin, consisting of histones, NE, cathepsin G (CG), myeloperoxidase (MPO), peptidoglycan binding protein, and so on.[16] The histones, NE, CG, and MPO, that normally exist in the cytoplasm and nucleus of normal neutrophils, are closely related to the antibacterial activity and lethality of NETs.[17] Further, depolymerized extracellular chromatin deoxyribonucleic acid (DNA), the most important component of NETs,[9] traps pathogens such as bacteria, fungi, viruses, and some parasites, subsequently allowing these toxic proteins to neutralize and kill them.[16-18] Remarkably, the DNA phosphodiester backbone also has highly bactericidal activity and can directly cause bacterial lysis.[17]

NET Formation

The mechanism of NET formation is subclassified into, depending on the participation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), either NOX-dependent or NOX-independent pathways, and the former is a classical pathway. Numerous stimuli, such as phorbol 12-myristate 13-acetate (PMA), bacteria, fungi, lipopolysaccharide (LPS), interleukin-8 (IL-8), *etc*, can induce NET formation via the NOX-dependent pathway.[19,20] They first trigger the release of stored calcium in the endoplasmic reticulum,[21] in turn leading to the activation of protein kinase C (PKC) and subsequent assembly of the NOX complex to produce ROS.[22-24] ROS dissociates NE–MPO complexes within the cytoplasm, subsequently allowing NE to enter the nucleus and cleave the histone octamer, triggering chromatin decondensation.[25,26] Protein arginine deiminase 4 (PAD4), which synergistically catalyzes histone citrullination, impairs the binding of histones to DNA and promotes chromatin depolymerization, forming the basis of NET formation *in vivo*.[19,27,28] PAD4 knockout mice failed to form NETs, even when they were stimulated by pathogenic microorganisms.[27] PAD4 is vital for the NET-mediated immune response, and further acts as the nuclear button to trigger NETs in inflammatory diseases.[27,29] The cell membrane breaks down under the action of NE and gasdermin D (GSDMD), and decondensed chromatin and cytosolic granzymes are effluxed to the extracellular space, ultimately forming NETs with bactericidal activity [Figure 1].[7,30]

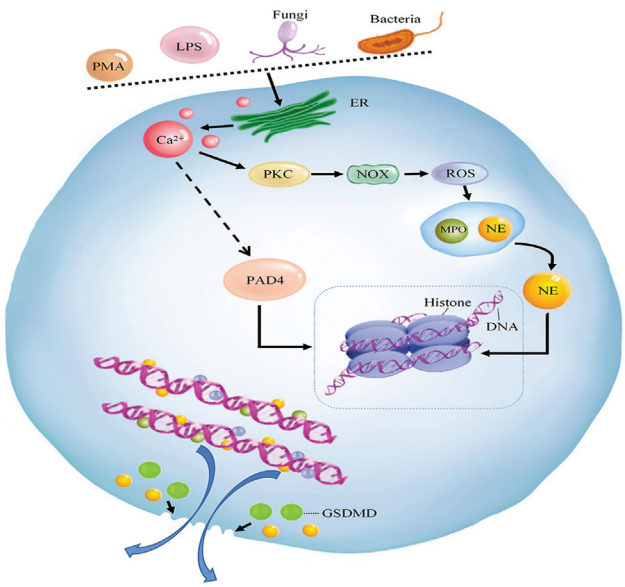

Figure 1: The classical pathway of NET formation. Various stimulators, such as PMA, LPS, fungi, and bacteria, provoke the release of stored Ca^{2+} from the ER of neutrophil, resulting in the activation of PKC and subsequent assembly of the NOX complex producing ROS. Subsequently, the NE–MPO complex is dissociated by ROS, and then NE enters the nucleus to cleave the histone octamer to initiate chromatin decondensation. The PAD4 catalyzes histone citrullination, impairing the binding of histones to DNA and promoting chromatin depolymerization. In addition, the activation of PAD4 requires a high concentration of calcium. Mixing of the decondensed chromatin DNA, histones, and cytosolic granzymes such as NE and MPO takes place, and then they are effluxed to the extracellular space through the pores punched by NE and GSDMD on the cytomembrane, ultimately forming NETs. Ca^{2+}: Calcium ion; DNA: Deoxyribonucleic acid; ER: Endoplasmic reticulum; GSDMD: Gasdermin D; LPS: Lipopolysaccharide; MPO: Myeloperoxidase; NET: Neutrophil extracellular trap; NE: Neutrophil elastase; NOX: Nicotinamide adenine dinucleotide phosphate oxidase; PAD4: Protein arginine deiminase 4; PKC: Protein kinase C; PMA: Phorbol 12-myristate 13-acetate; ROS: Reactive oxygen species.

Calcium is the main trigger of NOX-independent formation of NETs;[31,32] how this pathway leads to NET release, however, is incompletely understood.[24] Currently, PAD4 is known to require a high concentration of calcium for its activation.[33] Moreover, platelets can trigger NET formation in the absence of NOX and ROS production.[34] Upon activation by LPS, glycoprotein Ib on the surface of platelets binds to β2-integrin (CD18) on neutrophils and activates the Src kinase-phosphatidylinositol-3-kinase (PI3K)–extracellular signal-regulated kinase (ERK) pathway,[25] eventually resulting in NET formation.

Role of NETs in SAP and Lethal Complications

The pathogenesis of SAP involves the intra-acinar activation of pancreatic enzymes, instigating autodigestion and injury to the pancreas.[35] Under the action of inflammatory mediators and chemokines, neutrophils are the first cells of the immune system that migrate from circulating blood into the inflammatory pancreas,[36] where they release inflammatory mediators, triggering a local inflammatory reaction.[37] Neutrophil infiltration is a major characteristic of pancreatitis, leading to pancreatic parenchymal damage and dysfunction.[38] Concurrently, NETs are also released by neutrophils to aggravate pancreatic inflammation and injury.[39] More importantly, neutrophils that accumulate in the pancreas undergo retrograde migration to the circulatory system, causing systemic and regional complications of SAP, such as multiorgan failure, thrombosis, IPN, and sepsis, by producing NETs. In the following section, the role of NETs in SAP and its lethal complications will be discussed in detail [Table 1].

Pancreatic injury and microcirculatory disturbance

NETs promote pancreatic damage in the early stage of SAP. The number of NETs in the peripheral blood of patients with SAP was significantly higher compared to that in healthy subjects.[40] Merza *et al*[39] first identified a large amount of NET formation in pancreatic tissues of an SAP mouse model, and NETs aggravated the

Table 1: Role of NETs in SAP and its lethal complications.

SAP-related lethal injuries	Effects of NETs	Influence on the prognosis of SAP	References
Pancreas	NETs promote inflammatory injury of the pancreas	–	[39]
	NETs contribute to the occlusion of pancreatic duct	–	[44]
Microcirculatory disturbance/ thrombosis	NETs participate in vascular leakage by degrading VE-cadherin in the vascular endothelium and activating β-catenin signaling	–	[46]
	Histones stimulate vWF release from endothelial cells and trigger the aggregation of platelets	–	[49]
	NETs provide scaffolds for the aggregation of thrombosis	–	[48-50]
	NE, CG, and extracellular nucleosomes enhance TF- and factor XII-dependent coagulation	–	[51]
	Cell-free DNA, MPO–DNA complexes, and nucleosomes promote the adhesion and activation of platelets and activate the intrinsic coagulation pathway	–	[52-54]
	NETs and histones significantly accelerate the prothrombinase reaction	–	[55]
	DNA promotes the activation of factor XII and prekallikrein	–	[56]
	NETs activate complements to form thrombi	–	[57,58]
	Histones induce TF expression	–	[119]
	NETs, histones and meshworks of DNA all have antifibrinolytic effects	–	[123]
Lung	NE enhances the migration of neutrophils to the lung, increases alveolocapillary permeability, and disrupts the endothelial cell barrier function	–	[69,70]
	Histones cause neutrophil accumulation in alveolar microvessels, vacuolization of endothelial cells and lung epithelial cells, intra-alveolar hemorrhage, and deposition of microthrombi and fibrin in alveoli	–	[77]
	Overproduction and abundant deposition of NETs contribute to airway occlusion and damage	–	[78,79]
	NE and DNA fibers promote the formation of sputum plugs, blocking airways and facilitating bacterial growth and colonization	–	[25]
	NETs promote macrophage polarization to M1 phenotype, aggravating the pulmonary injury	–	[81,82]
	NETs promote alveolar macrophage pyroptosis	–	[83]
	NETs promote microthrombosis in pulmonary vessels	–	[84]
Kidney	Histones are toxic to glomerular endothelial cells, podocytes, and parietal endothelial cells, leading to renal thrombotic microangiopathy and glomerular necrosis	–	[87]
	MPO contributes to glomerular and interstitial injury	–	[88,89]
	NETs induce thrombosis and microcirculatory disturbance	–	[24,91]
Heart	NETs contribute to cardiac inflammatory injury	–	[94]
	NETs increased cardiac titin phosphorylation and reactive interstitial fibrosis	–	[95]
	MPO catalyzes the production of the potent oxidant hypochlorous acid, leading to cardiac injury	–	[98]
	MPO and histones are cytotoxic to endothelial cells	–	[97,99]
	NETs promote thrombosis leading to myocardial infarction	–	[24,98,101,102]
Gut	NETs cause intestinal barrier injury, resulting in translocation of intestinal bacteria and endotoxin	–	[39,107-110]
	Histones directly damage intestinal epithelial cells and lead to apoptosis of them	–	[77]
IPN	NETs form a temporary physical barrier separating the necrotic pancreatic areas from the remaining viable tissues	+	[13]
	NETs eliminate pathogens in the infectious pancreas or peripancreatic region	+	[9]
Sepsis	NETs kill pathogens and prevent their dissemination in the early phase of sepsis	+	[11,12,27]
	NETs and histones promote the occurrence and development of DIC in sepsis	–	[55,119,120]

–: Negative; +: Positive; CG: Cathepsin G; DIC: Disseminated intravascular coagulation; DNA: Deoxyribonucleic acid; IPN: Infected pancreatic necrosis; MPO: Myeloperoxidase; NE: Neutrophil elastase; NETs: Neutrophil extracellular traps; SAP: Severe acute pancreatitis; TF: Tissue factor; VE-cadherin: Vascular endothelial cadherin; vWF: von Willebrand factor.

inflammation and the damage to the pancreas. Deoxyribonuclease (DNase) I can depolymerize the DNA skeleton in NETs, and thereby disrupt the structure of NETs.[12] After administration of DNase I to mice, the degree of neutrophil infiltration and tissue damage in the pancreas decreased significantly, as did the expression level of histones; it is also reported that the activity of trypsin was markedly increased by coincubation of NETs with pancreatic acinar cells.[39] Autophagy plays an important role in NET formation.[41] Chloroquine, an autophagy inhibitor, improved the outcomes of murine models with pancreatitis by decreasing this propensity to form NETs and reducing serum cell-free DNA and citrullinated histone H3.[42] Madhi *et al*[43] reported that inhibiting c-Abelson kinase-related NET formation reduced neutrophil infiltration in the inflamed pancreas and acinar cell necrosis and hemorrhage.

In addition to being involved in the inflammatory injury of the pancreas, NETs can further form aggregates within the pancreatic ducts, thereby leading to catheter obstruction and promoting the occurrence and development of SAP.[44] Acute biliary pancreatitis (ABP) is one of the most common forms of pancreatitis. Poor emptying of the biliopancreatic duct outlet caused by various factors, such as gallstones, is a crucial trigger for the occurrence of ABP. The results of one study suggest that gallstone assembly essentially requires NETs, and targeting NET formation via a PAD4 inhibitor or metoprolol can effectively inhibit gallstone formation *in vivo*.[45] Taken together, these results suggest that NETs promote the obstruction of the biliopancreatic ducts, resulting in SAP.

Changes in hemodynamic parameters, such as hemoconcentration, hypercoagulability, and infiltration of inflammatory factors, are all characteristics of SAP that cause a variety of vascular disorders, including endothelial activation and injury, vascular leakage, and intravascular thrombosis.[25] NETs and their components have further been demonstrated to be involved in vascular pathological changes. The endothelial injury also plays an important role in thrombosis. NETs damage endothelial cells due to the cytotoxicity of MPO and histones. NETs are involved in vascular leakage by degrading vascular endothelial cadherin (VE-cadherin) in the vascular endothelium and subsequently activating β-catenin signaling.[46] The von Willebrand factor (vWF) is a reliable marker of endothelial dysfunction; Chen *et al*[47] found the vWF appeared to participate in the development of pancreatic necrosis, but the mechanism by which vWF affects microcirculation remains to be clarified. Histones can stimulate vWF release from endothelial cells, triggering the aggregation of platelets; in turn, the reticular structures of NETs provide a scaffold for the aggregation of platelets and erythrocytes, thus promoting the accumulation of vWF and fibrin, as well as thrombosis [Figure 2].[48-50] NE, CG, and extracellular nucleosomes enhance tissue factor (TF)- and factor XII-dependent coagulation via the local proteolysis of coagulation suppressor TF pathway inhibitors.[51] Cell-free DNA, MPO–DNA complexes, and nucleosomes can promote the adhesion and activation of platelets and further activate the intrinsic coagulation pathway to aggravate the hypercoagulable state of blood.[52-54] NETs

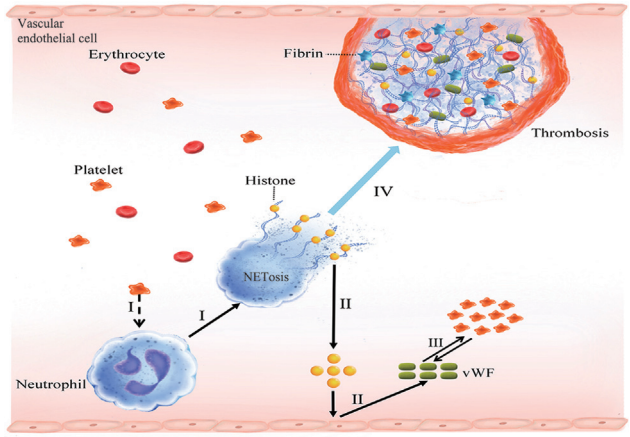

Figure 2: The NET formation promotes intravascular thrombosis. I: Various stimuli, including platelets, trigger neutrophils to undergo NETosis within the blood vessels. II: Histones from NETs stimulate vWF release from endothelial cells. III: The vWF leads to clumping and adhesion of platelets. IV: The reticular structures of NETs provide a scaffold for the aggregation of platelets, erythrocytes, fibrin, and procoagulant factors such as vWF. Under the action of NETs and various procoagulant pathways, the coagulation cascade is activated, eventually resulting in thrombosis. NET: Neutrophil extracellular trap; vWF: von Willebrand factor.

and histones destroy the cell membrane, exposing the negatively charged phospholipid surfaces to accelerate the prothrombinase reaction for 250,000 fold.[55] Factor XII and high molecular weight kininogen could be combined with oligonucleotides of double-stranded DNA hairpins, thus promoting the activation of factor XII and prekallikrein, which are both critical in initiating the contact pathway of coagulation.[56] Furthermore, NETs can activate complements to form thrombi; complements can recruit and activate neutrophils, complement-3 deficient mice are incapable of forming NETs,[57] and pre-stimulation of neutrophils with complement-5a enhances their ability to produce NETs.[58] In summary, these factors released by NETs jointly contribute to thrombosis, particularly microvascular thrombosis, causing a systemic microcirculatory disturbance in SAP.

In addition, platelets can induce NET formation. The interaction of NETs and procoagulant molecules, including platelets, promotes ischemic injury and even necrosis in multiple organs, particularly the pancreas. A positive correlation was found between pancreatic ischemia and the severity of pancreatitis; it has been reported that nearly 40% of pancreatic capillaries showed complete capillary deposition in SAP.[59] Heparin was shown to improve the prognosis of SAP patients and rat models,[60,61] which might be due to its ability to attenuate histones.[62] Thrombin activity and the degree of platelet aggregation were decreased by DNase I, while microvascular permeability was increased.[63] Therefore, targeting inhibition of NETs may be a new clinical strategy for the treatment of inflammatory pancreatic injury and microcirculatory disturbance with SAP.

Acute lung injury

The concentrations of neutrophils in pulmonary capillaries are higher than those in systemic blood, even in the absence of inflammatory stimuli.[64] More neutrophils

migrate to the lung to participate in the immune response under the guidance of inflammatory cytokines and chemokines during SAP. Excessive activation of neutrophils causes the accumulation of locally high concentrations of NETs. NETs protect tissues from pathogen damage.[65] In experimental mice with pulmonary infection of Candida albicans, NETs reduced the fungal load in the lung by binding to the fungal hyphae.[66] However, excessive generation of NETs can lead to acute lung injury (ALI)/acute respiratory distress syndrome (ARDS),[67] the most common extrapancreatic complications that contribute to the high fatality rate in SAP.[68]

Components of NETs can induce and aggravate inflammatory responses, resulting in lung injury. NE, the most abundant and active proteolytic enzyme in NETs, induces the production of proinflammatory cytokines, enhances neutrophil migration, increases alveolocapillary permeability, and disrupts endothelial cell barrier function, causing lung injury.[69,70] The levels of cell-free DNA, MPO–DNA complexes, and histone H3 in the blood increased, and autopsy revealed the presence of NETs in lung tissues; this evidence from coronavirus disease 2019 (COVID-19) patients suggests that the virus might activate NETs to cause acute pulmonary injury.[71-75] Kinnare *et al*[76] found that neutrophils isolated from patients with COVID-19 released markedly increased amounts of elastase and NETs compared to healthy donors, either with or without exogenous stimulation. The toxic effects of histones can result in neutrophil accumulation in alveolar microvessels, vacuolization of endothelial cells and lung epithelial cells, intra-alveolar hemorrhage, and deposition of microthrombi and fibrin in alveoli.[77]

Alveolar collapse and ventilatory flow ratio dysregulation caused by narrowly occluded small airways are important pathophysiological changes in acute respiratory distress. Overproduction and abundant deposition of NETs increases the viscosity of endobronchial tissue and disturbs the mucociliary clearance, eventually contributing to airway occlusion and damage.[78] In a study on severe lower respiratory tract disease due to respiratory syncytial virus, the smaller airways and larger bronchi were obstructed by dense cellular plugs consisting of NETs, shed epithelial cells and large numbers of neutrophils.[79] NE induces airway epithelial cells to produce excessive mucin, which attaches to DNA fibers to form sputum plugs that block airways and create a suitable environment for bacterial growth and colonization.[25]

NETs can also cause lung damage by affecting macrophage function. Macrophages are classified into the M1 and M2 types, which exhibit proinflammatory and anti-inflammatory properties, respectively.[80] NETs aggravate inflammatory injury of the lung by promoting macrophage polarization to the M1 phenotype.[81] NETs further activate the ERK1/2 and nuclear factor kappa-B (NF-κB) pathways, leading to an increase in M1-type polarization of alveolar macrophages and inflammatory injury of the lung.[82] Furthermore, NETs can also aggravate lung injury by promoting alveolar macrophage pyroptosis, a highly proinflammatory mode of cell death.[83]

As previously noted, hypercoagulability of circulation of SAP and NETs promoting thrombosis leads to microcirculation disorders, which can further aggravate lung injury. In addition, the increased expression of P-selectin and intercellular adhesion molecule 1 on the surface of activated platelets promotes neutrophil activation and recruitment to the lung to produce a large number of NETs and further promotes microthrombosis.[84] Senkyunolide I, an active ingredient of Xuebijing injection, destroyed the crosstalk between platelets and NETs to protect against lung injury in a murine model of sepsis.[85]

Acute kidney injury (AKI)

AKI is a common complication of SAP. It has been reported that more than 50% of SAP patients would eventually develop AKI.[86] Renal injury resulting from NETs is further associated with its cytotoxicity. Histones from NETs cause direct toxicity to glomerular endothelial cells, podocytes, and parietal endothelial cells, leading to renal thrombotic microangiopathy and glomerular necrosis.[87] Glomerular and interstitial injury in anti-neutrophil cytoplasmic antibody-associated vasculitis patients was confirmed to be associated with the oxidative effect of extracellular MPO.[88,89] CG and NE were also found to be associated with renal damage.[90] In addition, NETs can induce thrombosis and microcirculatory disturbance, which may also contribute to AKI.[24,91] In a PAD4-deficient mouse model of traumatic shock and sepsis, the blood urea nitrogen/creatinine (BUN/Cr) ratio and vascular leakage were significantly decreased compared to those of control mice.[92]

Acute heart injury

NETs have been confirmed to participate in acute cardiac injury, another fatal complication of SAP.[93] Weckbach *et al*[94] found the presence of NETs in the myocardial tissue of active myocarditis patients, and NETs were associated with cardiac inflammatory injury. NETs contribute to cardiac titin phosphorylation and reactive interstitial fibrosis, resulting in ventricular diastolic dysfunction.[95] Inhibiting NET formation attenuates cardiac inflammatory injury. GSK484, a PAD4 inhibitor, reduced neutrophil infiltration in the heart, suppressed inflammatory cytokine secretion, reduced cardiomyocyte apoptosis and infarct size, and improved cardiac function.[96]

Cytotoxicity of NETs can cause cardiac injury. Circulating MPO from neutrophils may lead to vascular endothelial injury.[97] MPO catalyzes the production of the potent oxidant hypochlorous acid, leading to various inflammatory diseases, including acute heart injury.[98] MPO from neutrophils was confirmed to promote the occurrence of atrial fibrillation, one of the most common arrhythmias among critically ill patients hospitalized, which is linked to a significantly increased risk of death in patients with SAP.[3] Extracellular histones are cytotoxic to endothelial cells,[99] and cardiac dysfunction in mice with sepsis was improved by treatment with histone antibodies.[100]

As previously described, NETs can promote thrombus formation. The insufficient effective circulating blood

volume resulting from the systemic inflammatory cascade associated with SAP causes poor perfusion with multiple organs. During myocardial ischemia-reperfusion, NET and NET-related microthrombosis can be triggered, resulting in myocardial infarction,[98,101] which has been reported in patients with SAP. Moreover, it was found that NETs were important constituents of fresh and lytic thrombi in coronary artery specimens from patients with acute myocardial infarction,[102] and the NET burden was positively correlated with the myocardial infarction area in patients.[24] Some damage-associated molecular patterns (DMAPs), such as high mobility group box 1 (HMGB1), fibronectin extra domain A (FN-EDA), galectin-3 (GAL3), and CXC receptor 2 (C-X-C chemokine receptor type 2), could induce NETs to promote thrombosis.[98] Recent studies have shown that the time of thrombus growth and complete blockage of vessels is prolonged and the frequency and the size of thrombus formation are decreased in knockout mice of those DMAPs.[98,103-106]

Acute gastrointestinal injury

The barrier function of the intestine is one of the key factors affecting the severity and mortality of SAP, as the translocation of intestinal bacteria and endotoxins following intestinal mucosal barrier injury leads to a systemic inflammatory response and multiple organs dysfunction syndrome due to sepsis,[107] which will give a second attack to SAP and aggravate the condition of patients with dramatically increased mortality. Notably, the excessive release or dysfunction of NETs can destroy the intestinal barrier. NETs can disrupt tight junction proteins, such as claudin-1, occludin, and Zona occludens-1 (ZO-1), in intestinal epithelial cells, causing intestinal mechanical barrier injury.[108] Histones exert a direct cytotoxic effect on intestinal epithelial cells, leading to their apoptosis.[77] Extensive release of NETs can significantly promote the activation of cluster of differentiation 4-positive ($CD4^+$) T lymphocytes and mediate apoptosis of cluster of differentiation 8-positive ($CD8^+$) T cells,[109] thus affecting the immune homeostasis of the intestinal microenvironment and damaging the intestinal immune barrier. NETs can also induce intestinal chemical barrier disorder by hurting hepatocytes and pancreatic acinar cells to trigger the abnormal secretion of bile acid and pancreatic juice.[39,110] In addition, NETs induce thrombosis in the mesenteric vessels, provoking ischemic necrosis of the intestinal mucosa to damage the intestinal mucosal barrier.

IPN and sepsis

IPN usually occurs in the late stage of SAP, leading to sepsis and secondary organ failure, with a mortality rate of 40% to 70%.[111-113] NETs can facilitate the occurrence and development of IPN. As already discussed, NETs contribute to ischemic necrosis of the pancreas and impairment of intestinal mucosal barrier function. More than 35% of necrotizing pancreatitis patients experience secondary infection, which is mostly caused by intestine-derived bacteria,[114] directly translocating or spreading through the bloodstream into the necrotic areas of the pancreas.[111] However, the moderate release of NETs has a positive effect on IPN. After pancreatic necrosis, NETs can form a temporary tissue barrier separating the necrotic areas from the remaining viable tissues to exert protective effects.[13] Neutrophils migrate to the infected sites of the pancreas or peripancreatic region where they initiate antimicrobial mechanisms, such as generating NETs.[9]

Sepsis is a life-threatening organ dysfunction that promotes mortality by driving organ dysfunction during the host response to infection.[115] Organ failure, a marker of increased risk of death, occurs secondary to sepsis arising from IPN.[116] NETs also play a pivotal role in the process of sepsis. NETs can eliminate pathogens and limit their proliferation and dissemination in the early stage of sepsis. In murine models of necrotizing fasciitis, PAD4 knockout mice were more susceptible to bacterial infection.[27] However, the hyperfunction of NETs during sepsis can lead to tissue damage attributed to their proinflammatory and prothrombotic properties.[117]

During sepsis, the systemic inflammatory response and significant release of proinflammatory factors can lead to abnormal activation of coagulation. The coagulation cascade, a serious complex change in coagulation function in sepsis, is usually accompanied by vascular endothelial damage and the formation of intravascular thrombi, eventually resulting in multiple organs dysfunction.[58] As mentioned earlier, NETs can hurt vascular endothelial cells, recruit platelets and procoagulation factors, and activate complements to participate in thrombosis.

Thrombocytopenia, low levels of clotting factors and thrombotic occlusion of small- and medium-sized vessels are all hallmarks of disseminated intravascular coagulation (DIC). The incidence of DIC in sepsis is 30% to 50%, and when DIC occurs in patients with sepsis, the mortality rate is doubled.[118] Sepsis-associated DIC is closely related to excessive endothelial procoagulant TF expression from circulating monocytes.[55] Kim *et al*[119] showed that histones induced TF expression in endothelial cells, resulting in a pathological procoagulant endothelial surface. Evidence from a study on cancer patients indicated that the interaction between NETs and TF could drive DIC.[120] In addition, histones can induce the activation, aggregation, and consumption of platelets[48,49,121] and promote thrombin generation via platelet-dependent mechanisms.[122] The cytotoxic effects of histones can directly cause vascular endothelial damage, which is a critical factor to induce DIC. Furthermore, NETs, histones, and DNA networks all exert anti-fibrinolytic effects, slowing down the lysis of plasma clots by inhibiting the tissue-type plasminogen activator, an effect which could be offset by DNase.[123]

The difficulty in early identification and delayed treatment may be closely related to high mortality of SAP with sepsis. Individual differences in those patients also complicate treatment. In addition, drug sensitivity results for infection are usually difficult to obtain in clinical practice, and prolonged administration of broad-spectrum antibiotics may induce nosocomial superinfections. Therefore, NETs

may be a crucial indicator for early recognition and are expected to become a specific therapeutic target of sepsis in SAP in the foreseeable future.

NET as a Therapeutic Target for SAP

Treatment options for SAP are limited and primarily consist of fluid resuscitation, nutritional support (parenteral or enteral nutrition), organ functional support (i.e., mechanical ventilation, vasoactive drugs, and continuous renal replacement therapy), antibiotics, traditional Chinese medicine, and so on. Unfortunately, there is still a lack of effective and specific medications for SAP and its complications. NETs participate in the pathophysiological process of the two death peaks in SAP, and targeting NETs and their key components is a potentially efficient treatment strategy for SAP [Table 2].

Blocking the formation of NETs

The precise intervention of vital individual components in the formation of NETs has been proven to reduce NET-related damage. Toll-like receptor 4 (TLR4) on platelets induces platelets to bind to adherent neutrophils, resulting in the intensive activation of neutrophils in addition to NET formation.[124] Thrombomodulin blocks TLR4 and its downstream signaling pathways to inhibit neutrophil generating NETs and alleviate hepatic ischemia-reperfusion injury in rats.[125] In addition, TLR4 has been shown to be involved in pancreatic, pulmonary, and renal injuries in SAP,[126] and inhibition of TLR4 was positively correlated with the prognosis of sepsis.[127] Thus, TLR4 inhibitors may hold significant potential for the treatment of SAP, although it is important to note that some studies have also reported that complete blockage of TLR4 may

Table 2: Potential curative treatments targeting NETs for SAP.

Classification	Drug/Substance	Mechanism	Effects	References
Blocking NET formation	Thrombomodulin	Blocks TLR4 and its downstream signaling pathways	Inhibits NET generation and alleviates hepatic ischemia-reperfusion injury	[125]
	Aspirin	Inhibits platelet aggregation	Decreases NET formation and improves lung injury	[128]
	Tirofiban	An inhibitor of the platelet glycoprotein IIb/IIIa receptor	Inhibits NET formation and improves lung injury	[128]
	PKC inhibitor	Inhibits activity of PKC	Blocks NET formation	[129]
	DPI	Inhibits ROS production	Blocks NOX-dependent NET formation	[131]
	N-acetyl cysteine	Scavenges ROS	Significantly inhibits NET release	[132]
	Sivelestat	NE inhibitor	Alleviates pancreatic, pulmonary and renal injury associated with SAP	[134-137]
	PAD4 inhibitor/ PAD4 deletion	Inhibits histone citrullination and chromatin depolymerization	Decreases severity of pancreatitis; suppresses thrombosis; improves renal function and vascular leakage; reduces cardiomyocyte apoptosis and infarct size; and improves cardiac function	[42,92,96,138,139]
	Ethylene glycol tetraacetic acid	Chelates extracellular calcium	Suppresses NOX-independent NET formation	[141]
	BAPTA-AM	Chelates intracellular calcium	Inhibits NET formation triggered by PMA	[32]
	Chloroquine	Inhibits neutrophil autophagy	Decreases severity of SAP and improves survival	[42]
Destroying NET structure	DNase	Depolymerizes the DNA skeleton of NETs	Inhibits thrombin activity and platelet aggregation, increases microvascular permeability, improves pancreatic and pulmonary injury caused by SAP, and reduces septic organ dysfunction	[11,39,63,142]
Inhibiting NET activity	Heparin	Binds to extracellular histones	Impairs NET-mediated coagulation effect and infection-related vascular dysfunction, improves the prognosis of SAP	[60-62,143]
	Thrombomodulin	Binds to and deactivates histones	Reduces platelet aggregation and thromboembolism, and improves AKI	[144]
	Activated protein C	Blocks cytotoxicity of histone	Reduces the ability of NETs to damage organs	[77]
	CG inhibitor I	Inhibits CG in a selective, potent, and reversible manner	Reduces the inflammation and fibrosis of the lung	[145]
	Aminobenzoic acid hydrazide	An inhibitor of MPO	Reduces blood vessels inflammation	[146]
	Histone antibody	Neutralizes histone	Improves cardiac dysfunction in sepsis	[100]

AKI: Acute kidney injury; BAPTA-AM: 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; CG: Cathepsin G; DNA: Deoxyribonucleic acid; DNase: Deoxyribonuclease; DPI: Diphenyleneiodonium; MPO: Myeloperoxidase; NE: Neutrophil elastase; NETs: Neutrophil extracellular traps; NOX: Nicotinamide adenine dinucleotide phosphate oxidase; PAD4: Protein arginine deiminase 4; PKC: Protein kinase C; PMA: Phorbol 12-myristate 13-acetate; ROS: Reactive oxygen species; SAP: Severe acute pancreatitis; TLR4: Toll-like receptor 4.

deprive the innate immune response to endotoxin.[58] Both aspirin and tirofiban (an inhibitor of the platelet glycoprotein IIb/IIIa receptor) decreased NET formation and improved lung injury.[128] Inhibition of PKC blocked NET formation in response to PMA.[129] NETosis is divided into NOX-dependent and NOX-independent types, and NOX and the subsequent production of ROS are indispensable for NET formation in the former pathway.[130] Further, administration of diphenyleneiodonium (DPI), an inhibitor of NOX, markedly reduced histamine-triggered NETs.[131] N-acetyl cysteine, a ROS scavenger, also significantly inhibited NET release,[132] and reduced systemic lupus erythematosus (SLE) disease activity.[133] Sivelestat, an NE inhibitor, can mitigate vascular permeability of the lung, ameliorate injuries in the alveolar epithelium and vascular endothelium, and improve pulmonary function and coagulopathy in patients with ALI/ARDS. Furthermore, it has recently been considered a promising modality for the treatment of COVID-19,[134] and has also been proven to be effective in protecting against pancreatic, pulmonary, and renal injuries in rats with SAP.[135-137]

PAD4 plays a crucial role in the formation of NETs.[27] Inhibiting the expression of PAD4 could markedly reduce NET formation, decrease the severity of pancreatitis and improve survival in mouse models of SAP.[42,138] PAD4 inhibitors further effectively suppressed venous thrombosis in mice.[139] In a murine model of septic shock, PAD4 deletion mice showed an increased survival rate and a decreased degree of organ injury compared to wild-type mice.[92] However, another study found that *Klebsiella* and LPS could still induce neutrophil NETosis in the absence of PAD4.[140]

Calcium is critical for the NOX-independent formation of NETs. Ethylene glycol tetraacetic acid, by chelating extracellular calcium, could completely suppress NET formation in response to ionomycin and partially inhibit NET formation with Pseudomonas aeruginosa and PMA.[141] Nevertheless, BAPTA-AM [1,2-bis (o-aminophenoxy) ethane-N, N, N′, N′-tetraacetic acid] could chelate intracellular calcium to inhibit NET formation triggered by PMA.[32]

Destroying the structure of NETs

DNase I can depolymerize the DNA skeleton of NETs and destroy their structure.[142] After administration of DNase I to SAP mice, neutrophil infiltration and tissue damage in the pancreas and lung were improved.[39] Combined treatment with antibiotics and DNase further improved the prognosis of sepsis and reduced septic organ dysfunction.[11]

Inhibiting the activity of NETs

Some components of NETs, such as histones, CG, and MPO, are proinflammatory, cytotoxic, or prothrombotic. Inhibiting the virulence of these proteins can reduce the ability of NETs to damage organs. Heparin can bind to extracellular histones and reduce the activity of NETs, thereby impairing the NET-mediated coagulation effect and infection-related vascular dysfunction.[143] Thrombomodulin can bind to and deactivate histones, reduce histone-induced platelet aggregation and thromboembolism, and improve AKI.[144] Similarly, the cytotoxicity of histones is effectively blocked by activated protein C.[77] CG inhibitor I inhibits CG in a selective, potent, and reversible manner, thus reducing the inflammation and fibrosis of chronic obstructive pulmonary disease.[145] In addition, MPO contributes to tissue injury in various inflammatory diseases. As an inhibitor of MPO, aminobenzoic acid hydrazide prevents neutrophils from adhering to vascular endothelial cells, thus reducing inflammation of blood vessels.[146]

Conclusions

NETs possess their advantages and limitations. On the one hand, NETs actively participate in innate immunity, killing pathogens and preventing their transmission *in vivo*. However, dysfunction or excessive release of NETs aggravates the inflammatory response and drives tissue damage and organ dysfunction due to their proinflammatory and procoagulant properties. SAP, a celiac disorder with high morbidity and mortality, is characterized by lengthy hospital stays, intense suffering, and high medical costs. This review summarizes the generative mechanisms and pathophysiological characteristics of NETs, the vital roles of NETs in SAP and its fatal complications, and promotes the therapeutic use of targeting NETs in SAP treatment.

As there are currently no specific and efficient therapeutic strategies for SAP, the prognosis of the disease depends heavily on early recognition and interventions, and the clinical assessment of NETs may serve as a valuable biomarker for determining the severity of SAP. Direct visualization and quantitative analysis of NETs is difficult, but measuring the substitutes of NETosis in blood, such as histones, cell-free DNA, and nucleosomes, may be more convenient, dependable, and objective.[56] Overproduction of NETs promotes multi-organ dysfunction, thrombosis, or sepsis and causes high mortality in SAP, and targeting NETs or individual components of them may present novel therapeutic strategies. With increasing research in the field regarding NETs and SAP, emerging detection techniques and curative options aimed at NETs to benefit patients with SAP will be available in the immediate future.

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Conflicts of interest

None.

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