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Neutrophils, NETs, NETosis — old or new factors in sepsis and septic shock?

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Abstract

Neutrophils are an key part of the innate immune system in the host's defences against pathogens. Circulating neutrophils are recruited at the sites of infection or sterile inflammation in response to pathogen and host-derived inflammatory mediators. In addition to phagocytosis and degranulation, neutrophils display the release of NETs in order to restrain infection. NETs are able to entrap and kill microbes, and display proinflammatory and prothrombotic properties.

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Key words: sepsis, neutrophils, NETs, NETosis, thrombocytes

According to the current Surviving Sepsis Campaign Guidelines, sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection and a syndrome shaped by pathogen factors and host factors with characteristics that evolve over time [1]. Neutrophils are the major cellular component of the innate system. They are the critical, primary defence against invading microorganisms. They provide a rapid, non-specific response to infectious challenges and are an important interface between the innate and adaptive immune systems.

Neutrophils are short-lived granulocytes that mature in bone marrow for several days. During maturation, neutrophils acquire key attributes, including the ability to phagocytose and kill microorganisms. After maturation, neutrophils are released into the bloodstream and circulate and/or marginate for 10–24 h before migrating into tissues, where they may function for an additional 1–2 days before they undergo apoptosis and are cleared by macrophages or dendritic cells [2]. While the haematopoietic system is able to regulate steady-state levels of circulating neutrophils, it can also be switched to an emergency granulopoiesis response in order to accommodate the increased demand for neutrophils during infection [2]. The neutrophil lifespan is regulated by a balance of pro-and anti-apoptotic factors. Cytokines and other factors such as (interleukin) IL-1b, IL-2, IL-4, IL-15, interferon-γ, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and lipopolysaccharide (LPS) can prolong and/or enhance neutrophil function and delay apoptosis for several days [3].

NEUTROPHIL'S WEAPONS — PHAGOCYTOSIS, DEGRANULATION, NETOSIS. NET STRUCTURE

To kill pathogens neutrophils use strategies such as phagocytosis, degranulation and NETs formation. During phagocytosis, internalized pathogens are translocated to phagosomes where the antimicrobial factors derived from granules and reactive oxygen species (ROS) create a killing environment for pathogens. The second mechanism, degranulation, is similar to phagocytosis, but instead of being engulfed the pathogens are killed extracellularly by the same antimicrobial factors which are in part released outside the cell [4]. Phagocytosis may also lead to neutrophil apoptosis. Neutrophil phagocytosis-induced apoptosis or phagocytosis-induced cell death (PICD) promotes the resolution of infection by disposing spent or degenerate neutrophils containing

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dead or partially digested microbes in a non-inflammatory manner [3]. It is unclear which factors determine the selection between these alternative antimicrobial activities and whether these processes can coexist in the same cell [5].

Although first thought to only occur in pathologic states, NETosis has been shown to occur in healthy individuals as a tightly regulated and constantly ongoing homeostatic process. Dysregulation of NETosis and its relationship to thrombosis has been recognized in a variety of clinical scenarios [6]. Although in vitro NET formation leads to cell death, it has been reported that neutrophils that undergo NET release in vivo may remain active and functional, suggesting that NET formation may not necessarily be a terminal event [7].

There are two models proposed for NET release:

— NETosis, a distinct form of active cell death, is characterized by the release of decondensed chromatin and granular contents to the extracellular space;
— a DNA/serine protease extrusion mechanism from intact neutrophils, where mitochondrial DNA release is not associated to cell death [5].

The first fundamental differences between suicidal NETosis and vital NETosis are the nature of the inciting stimuli and the timing of NET release. Vital NETosis has been demonstrated following microbial-specific molecular patterns (PAMPs, pathogen-associated molecular patterns) recognized by host pattern recognition receptors (PRR). Vital NETosis has been reported following both direct microbial exposure and lipopolysaccharide (LPS). Live *S. aureus* induce rapid NET release (< 30 minutes) in human and mouse neutrophils in vitro and in vivo. For gram-negative bacteria, NETs are induced via Toll-like receptor (TLR) 4 activation of platelets followed by direct neutrophil-platelet interaction via CD11a, whereas both Complement receptor 3 and TLR2 are required for vital NETosis following gram-positive infection. NETs are released via nuclear budding and vesicular release of NETs . This mechanism spares the PMN (polymorphonuclear neutrophils) outer membrane, thereby allowing the PMN to continue to function, even to the point of becoming anuclear [8]. In particular, LPS, a gram-negative bacterial stimulus, induces rapid NET release. This rapid NETosis does not involve cell lysis and is specifically mediated by TLR4 on platelets that facilitated activation of PMNs [7].

NETs are diffuse extracellular structures of decondensed chromatin with nuclear (histones, HMGB1) and granular proteins (neutrophil elastase NE, defensins, cathepsin G, myeloperoxidase MPO) [5, 9]. Histones are cationic proteins that are associated with DNA in nucleosomes and are involved in chromatin remodelling and regulation of gene transcription. Despite their nuclear localization, nucleosomes have been found in the circulation of both healthy subjects and patients, where they can be released from dying cells or actively secreted by activated neutrophils in the form of extracellular traps [10]. Histones are known to possess cytotoxic properties against both microorganisms and eukaryotic cells [10].The nuclear and granular membranes disintegrate and elastase enters into the nucleus, followed by hypercitrullination of histones, chromatin decondensation into the cytoplasm, rupture of the plasma membrane, and extrusion of nuclear material from the cell into the extracellular space. Neutrophil elastase (NE), and myeloperoxidase (MPO) have been implicated in the initial chromatin decondensation and in the degradation of the nuclear envelope [7] as shown in Figure 1.

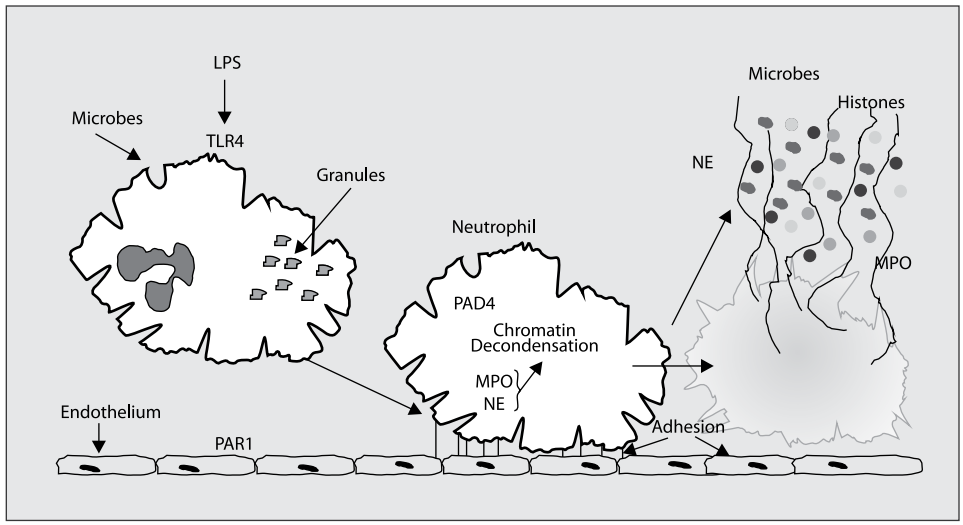

Figure 1. Neutrophil activation. NET formation with extrusion of nuclear material from the cell into the extracellular space. NETosis involves chromatin decondensation followed by the fragmentation of the nuclear envelope and neutrophil granules allowing for the mixing of components within the cell before plasma membrane lysis and NET release. PAD4 — peptidylarginine deiminase-4; MPO — myeloperoxidase; NE — neutrophil elastase; PAR1 — protease-actvated receptor 1

NETs do not contain neutrophil cytoplasmic and membrane components ,which distinguishes this process from extracellular DNA release following cell necrosis or apoptosis [8, 11].

NETs are able to trap almost all types of pathogens, even those so large that they cannot be phagocytosed, including gram-positive and gram-negative bacteria, yeasts, viruses and protozoa and parasites [2]. Trapping them within the DNA fibres prevents the spread of microorganisms over the organism and causes a higher concentration of antimicrobial factors at the site of infection [4]. This trapping occurs through charge interactions between the pathogen cell surface and NET components [4].

NET formation is not only associated with pathogens such as bacteria, fungi, viruses, and parasites but it is now known that cytokines, chemokines, platelet agonists, and antibodies may also trigger this phenomenon. Depending on the location of the neutrophils, when stimulated (extravasated versus vascular) these NETs can be either spread throughout the spread throughout the interstitium of organs or released into the lumen of blood vessels, where they may attach themselves to the vessel walls of narrow capillaries [12].

INTERFACE BETWEEN INFLAMMATION AND COAGULATION IN SEPSIS-NETS INVOLVEMENT

Neutrophil extracellular traps (NETs), represent an important strategy to immobilize and kill invading microorganisms. The NET scaffold consists of chromatin fibres with a diameter of 15–17 nm; DNA and histones represent the major NET constituents [5]. Neutrophil depletion significantly decreases both thrombus formation and fibrin generation, suggesting that neutrophils contain molecules required to support the onset of the coagulation cascade mediated at least in part by tissue factor [13, 14]. Thrombin generation by neutrophils causes the induction of further inflammation through PARs (protease-activated receptors) activation, which are G-protein-coupled membrane receptors expressed by a variety of cells. Thrombin, factor VIIa and factor Xa activate platelets and other cells through the activation of PARs . These receptors are also activated by trypsin and cathepsin G. PARs activation upregulates endothelial expression of TF and the release of von Willebrand factor [15]. Thrombin cleaves fibrinogen into fibrin and activates platelets. Thrombin has anticoagulant properties through thrombomodulin (TM)-dependent protein C activation. Thrombin influences opposite aspects of fibrinolysis as it promotes plasmin generation through stimulating endothelial cells' release of tPA (tissue plasminogen activator) and inhibits fibrinolysis through PAI-1 (plasminogen activator inhibitor 1) induction and TAFI (thrombin-activatable fibrinolysis inhibitor) activity [16]. NETs, because of their histone components, have the capacity to cause bystander injury. Extracellular histone proteins can activate TLR and lead to the generation of thrombin, as well as activate platelets resulting in microaggregation and thrombocytopenia. Treating neutrophils with LPS-activated platelets induces neutrophils for NET release which increases endothelial permeability. Histones are also potent cytotoxic molecules for the endothelium and it has been postulated that this histone-induced NET injury to the endothelium could be a one of the major contributors to the multiple organ dysfunction observed in sepsis/septic shock [8] (Fig. 2).

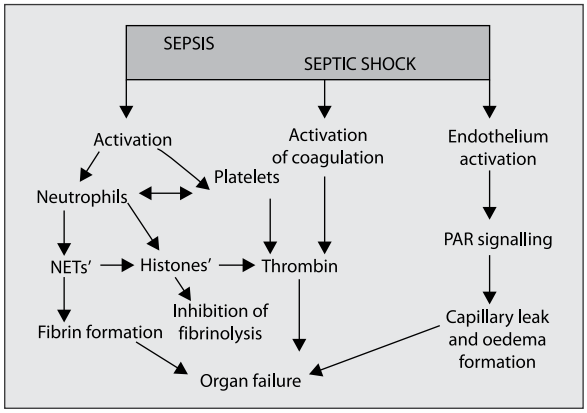

Figure 2. Consequences of neutrophil-platelet-endothelium activation and interplay in sepsis/septic shock settings

In an animal model of sepsis, NETosis was linked to prothrombotic activity because NET-associated enzymes break down TFPI and because extracellular cell-free DNA (cfDNA) inhibits plasmin-mediated fibrin degradation, as well as enhancing antifibrinolytic activity [17]. Circulating levels of nucleosome and histone H3 were measured in patients with severe sepsis. Positive correlation was observed both between nucleosome and fibrin/fibrinogen degradation products (FDP; $R^2 = 0.258$), and between histone H3 and FDP ($R^2 = 0.459$), suggesting that neutrophil extracellular traps play some role in the activation of coagulation [15]. Neutrophils and NETs are inducers of immunothrombosis. Structurally, NET-induced immunothrombosis leads to more sturdy thrombi with less permeability and decreased susceptibility to lysis, although this may be overcome with DNase [18]. Pathogen-derived DNA and nucleosomes composed of DNA and histones are PAMPs that induce inflammation. Nucleosomes and DNA released into the circulating blood after host cell death also contribute to the pathogenesis of sepsis as DAMPs. Extracellular cell-free DNA (cfDNA) acts as a DAMP. NET-related immunothrombosis, cfDNA, and histones have been implicated in the morbidity and mortality of sepsis [6]. According to results of Dwivedi *et al.* [19], plasma cfDNA are especially elevated in septic non-survivors and have better prognostic utility than Acute Physiology and Chronic Health Evalua-

tion (APACHE) II scores, Multiple Organ Failure Assessment (SOFA) scores, and other biomarkers. McDonald *et al.* [20], using multicolour confocal intravital microscopy in mouse models of sepsis, revealed profound platelet aggregation, thrombin activation, and fibrin clot formation within (and downstream of) NETs in vivo. NETs were critical for the development of sepsis-induced intravascular coagulation regardless of the inciting bacterial stimulus (gram-negative, gram-positive, or bacterial products). Removal of NETs via DNase infusion, or in peptidylarginine deiminase-4-deficient mice (which have impaired NET production), resulted in significantly lower quantities of intravascular thrombin activity, reduced platelet aggregation, and improved microvascular perfusion. NET-induced intravascular coagulation was dependent on a collaborative interaction between histone H4 in NETs, platelets, and the release of inorganic polyphosphate. Real-time perfusion imaging revealed markedly improved microvascular perfusion in response to the blockade of NET-induced coagulation, which correlated with reduced markers of systemic intravascular coagulation and end-organ damage in septic mice. Together, these data demonstrate, for the first time in an in vivo model of infection, a dynamic NET–platelet–thrombin axis that promotes intravascular coagulation and microvascular dysfunction in sepsis [20].

NETS AND PLATELETS — DO THEY HAVE ANYTHING COMMON?

The formation of NETs involving platelets, neutrophils, and bacteria, has been demonstrated and could play an important role in sepsis. Under normal conditions, circulating platelets do not adhere to the wall of blood vessels, leukocytes, or between them due to the anti-thrombotic properties of the vascular endothelium. However, during vascular injury or after endothelium activation under inflammatory conditions, platelets adhere to subendothelial molecules such as collagen or von Willebrand factor (vWF), triggering initial platelet activation characterized by the release of soluble mediators stored in their granules, such as adenosine diphosphate (ADP) and thromboxane A2 (TXA2) [12]. During sepsis/septic shock platelets interact with and modify the activity of various cells including leucocyte/neutrophils. Platelets contain HMGB1, which is released after activation, and platelet-derived HMGB1 is a mediator of NET formation [12]. The interaction of platelets with neutrophils promotes the recruitment of neutrophils into inflammatory tissue and thus participates in host defence. This interaction of neutrophils with platelets is mainly mediated through P-selectin and β2 and β3 integrins (CD11b/CD18, CD41/CD61) [21]. Platelets connected with the endothelium are a source of P-selectin for incoming leucocytes. Moreover, platelets adhering to the endothelium and leucocytes create

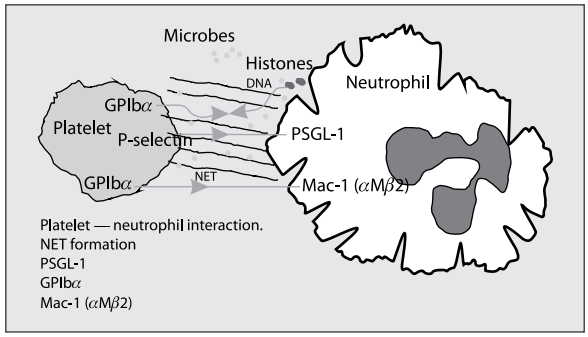

Figure 3. Sepsis/septic shock- Platelet-neutrophil cooperation in NET. PSGL-1 — P-selectin glycoprotein ligand-1; GPIbα (of the GPIb-IX-V complex) — platelet specific adhesion receptor being a member of the leucine-rich repeat (LRR) protein family; Mac-1 — integrin involved in changes in the neutrophil cytoskeleton facilitating the damage of nuclear and plasma membranes for NETs release

a surface for thrombin generation. Platelet P-selectin is also the primary ligand for leukocyte PSGL-1 in platelet–leukocyte interactions. This triggers a signalling cascade leading to activation of αMβ2 which can, in turn, engage platelet GPIbα [11]. These adhesive receptor/ligand networks facilitate platelet and neutrophil adhesion/activation while PSGL-1 engagement upregulates leukocyte tissue factor, and triggers the synthesis and release of cytokines and other inflammatory molecules [11] (Fig. 3) .

In response to endotoxemia, neutrophils adhere to the endothelium mainly in the sinusoids of the lungs and liver following which LPS-activated platelets anchor to the surface of neutrophils, promoting NETosis. Endothelial cells of the microvasculature represent a critical site of barrier regulation, selectively permitting passage of fluid, macromolecules, and cells into the extravascular tissue. Through a complex series of interactions, namely the binding of selectins, integrins, and adhesion molecules, the neutrophil rolls, arrests, and migrates through the endothelial barrier. Increased intercellular space between endothelial cells permits paracellular transmigration of the neutrophil [22].

One of the key trigger for NETs release is the binding and aggregation of activated platelets on the surface of adherent neutrophils [23]. Activated neutrophils undergo morphological changes in order to release NETs. NETosis mediated by platelets requires activation of both human platelet and neutrophils ERK (extracellular signal-regulated kinase) and Src kinases. In addition, the PI3K (phosphatidyl-inositol-3-kinase) signalling pathway in neutrophils is also required for NET formation. Platelets express immune receptors, such as TLR allowing for direct recognition of PAMPs and adhesion molecules allowing for interaction with immune cells. Despite controversy regarding the role of platelet TLR4/MD2 activation by LPS, bacterial residues might induce the binding of platelets to adherent neutrophils in pulmonary si-

nusoids, and cause sustained neutrophil activation and NETs formation. LPS, even at high concentrations, is unable to induce NET formation directly from neutrophils, suggesting that platelets are necessary for rapid LPS-induced NETs formation [24]. The blockade of platelet TLR4 markedly impairs NETosis and this has been suggested as a new therapeutic approach for sepsis [12, 25]. It has been suggested that platelets, through the expression of TLR4, act as a barometer for systemic infection and, under high levels of LPS, the interplay between platelets and neutrophils creates an efficient mechanism in the fight against pathogens [12]. Kambas has demonstrated that neutrophils from patients with sepsis release large amounts of TF (tissue factor) in the form of NETs. According to Kambas' results, inflammatory conditions prime and stimulate neutrophils to produce TF which is engulfed in autophagosomes and translocated on NETs. TF-coated NETs can further entrap circulating platelets to form thrombus and trigger cell signalling through PARs [26]. NETs act as mechanism for the localized extracellular expression of intracellular anti-microbial proteins. These networks function as a scaffold for thrombus formation. The entrapment and activation of circulating platelets contributes to the obstruction of blood flow, while entrapped platelets prevent the degradation of this scaffold by DNase [26]. TF microparticles generated from platelets become procoagulant upon association with activated neutrophils while TF on monocyte-derived microparticles shows activity after fusion with phosphatidylserine-expressing platelets, but not resting platelets [27]. It is still a subject of debate if neutrophils synthesize TF or can acquire TF by binding monocyte/platelet–derived microparticles, while neutrophil-derived TF plays a role in septic immunothrombosis/coagulopathy [15]. According to von Brühl and Fuchs' findings, TF can be produced by neutrophils and expulsed during NET formation. This is evidence that neutrophils and NETosis provide an interface between inflammation and thrombosis [28, 29].

CONCLUSIONS

As part of the first line of defence, neutrophils control invading pathogens by phagocytosis, the release of antimicrobial proteins during degranulation, or through the formation of web-like structures named neutrophil extracellular traps (NETs). NETs are formed by chromatin, proteases, and antimicrobial proteins, and their main function is to trap and kill bacteria, virus, and fungi, avoiding their dissemination. Neutrophils, platelets and endothelial cells effectively interact with the coagulation factors. The synergistic effect of antimicrobial and prothrombotic NET functions is one of the essential factors in basic immune-inflammatory host response in sepsis/septic shock. It is a point for further studies, if in sepsis/septic shock NETs and NETosis, because of their association with immune-inflammatory processes, may potentially create a therapeutic target in these clinical settings.

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