Published in final edited form as:

Microcirculation. 2017 April ; 24(3): . doi:10.1111/micc.12352.

Neutrophil-Mediated Vascular Barrier Injury: Role of Neutrophil Extracellular Traps

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Abstract

Neutrophils play an essential role in host defense against infection or injury. While neutrophil activation is necessary for pathogen clearance and tissue repair, a hyperactive response can lead to tissue damage and microcirculatory disorders, a process involving complex neutrophil-endothelium crosstalk. This review highlights recent research findings about neutrophil-mediated signaling and structural changes, including those induced by neutrophil extracellular traps, which ultimately lead to vascular barrier injury.

Keywords

neutrophil extracellular traps; vascular hyperpermeability; endothelial barrier dysfunction

Introduction

As a first line defense against invading pathogens and tissue injury, polymorphonuclear leukocytes (PMNs), particularly neutrophils, are poised ready to be rapidly recruited from the circulation to exert their resolving action by crossing the microvessel wall where the endothelium imposes a major barrier. Dysfunction of this barrier is characterized by excessive flux of blood components and fluid into surrounding tissue (edema), which often occurs in response to inflammatory stimuli, pathogens, or tissue debris.

PMN diapedesis is a hallmark of microvascular inflammation involving a series of metabolic and conformational changes initiated with PMN rolling and adhesion, followed by transendothelial migration. This dynamic process can be triggered by signals from pathogens, damage-associated molecular pattern molecules (DAMPs), immune/inflammatory cells, or activated endothelial cells (ECs). PMNs can alter endothelial structure or function through several mechanisms, which will be discussed in detail throughout this review. The affected ECs can produce further signals to recruit and activate more PMNs, perpetuating the PMN response to infection or injury and further affecting the endothelium. The purpose of this review is to discuss complex neutrophil-endothelium interactions in the

microvasculature and potential therapeutic significance of targeting these processes for immune disease or inflammatory injury.

PMN-EC interactions and barrier function

The events in the PMN activation cascade, including rolling, adhesion, transmigration, chemotaxis and releasing cytotoxic factors, have been extensively reviewed elsewhere^{1,2}. The focus here is how the crosstalk between PMNs and ECs in this cascade may contribute to endothelial barrier dysfunction and microvascular hyperpermeability.

The endothelial barrier is maintained by cell-cell junctions and cell-matrix focal adhesions, which connect ECs to each other and to the extracellular matrix in the basement membrane, respectively. Adherens junctions (AJs) are expressed ubiquitously in endothelia and considered the primary mechanism of cell-cell adhesion in peripheral microvasculature. They are regulated by the transmembrane adhesion molecule vascular endothelial (VE)-cadherin, connected to the actin cytoskeleton by catenins (α -, β -, γ -, and p120-catenins). Biochemical signal transduction in these junctions and cell-cell communication are regulated by phosphorylation and S-nitrosylation³. Tight junctions (TJs) are a specialized type of cell-cell junction dominantly expressed in tissues such as the brain and retina. The stability of these junction complexes is critical in maintaining endothelial barrier function; their disassembly or conformational changes occur during pathological processes. A more comprehensive analysis of barrier properties has been reviewed in great detail elsewhere⁴⁻⁷.

Neutrophil adhesion triggers multiple intracellular events in the endothelium leading to increased paracellular endothelial permeability^{8,9}. The mechanisms of junction opening initiated by PMN interaction with the endothelial cell-surface adhesion molecules has been reviewed in depth recently^{9,10}. On the endothelial surface, the initial adhesion receptors consist of immunoglobulin family transmembrane proteins (intercellular adhesion molecule [ICAM-1], vascular cell adhesion molecule [VCAM-1], and CD47) and selectins (E- and P-selectin). Upon adhesion, ICAM-1 ligation leads to a rapid, transient increase in intracellular free Ca^{2+} , which is necessary for leukocyte transmigration as it subsequently activates multiple downstream pathways that lead to opening of paracellular pathways¹¹. In the endothelial cytoskeleton, ICAM-1 ligation and the subsequent calcium increase activate actomyosin contractile signaling through myosin light chain kinase (MLCK)¹² and tyrosine phosphorylation of focal adhesion kinase (FAK)^{13,14}. The increase in Ca^{2+} also leads to activation of small GTPase Rho/ROCK (rho-associated protein kinase) signaling^{15,16}, which further enhances actin polymerization, facilitating endothelial cell contraction or retraction. Neutrophil adhesion also promotes the movement of ICAM-1 and VCAM-1 receptors into actin-supported clusters via cortactin phosphorylation, allowing for further engagement of the ICAM-1 and VCAM-1 pathways that increase permeability and extravasation via breakdown of the endothelial adherens junction¹⁷⁻¹⁹.

In endothelial junctions, PMN adhesion directly affects cell-cell adhesion by inducing Src phosphorylation and SHP2 dephosphorylation of VE-cadherin at the binding sites for p120 (Tyr685) and β -catenin (Tyr731), respectively²⁰⁻²³. The AJ is then destabilized by the requisite dissociation of vascular endothelial-protein tyrosine phosphatase (VE-PTP) from

VE-cadherin, induced by phosphorylation of VE-PTP via the Rac1/NOX/Pyk2 pathway. Together these processes facilitate junction opening and gap formation^{24,25}.

PMN adhesion leads to platelet endothelial cell adhesion molecule (PECAM)-1-mediated transendothelial migration by ligating ICAM-1 and VCAM-1, which further activate Src and endothelial nitric oxide synthase (eNOS) signaling^{26,27}. eNOS signaling can directly enhance microvascular permeability by S-nitrosylation of catenins and VE-cadherin, leading to the internalization of VE-cadherin^{28–30}. Studies suggest that PECAM-1, upon interaction with the PMN, mediates the removal of VE-cadherin from the paracellular junction, propagating endothelial hyperpermeability³¹. It should be noted that PMN adhesion to the postcapillary venular endothelium does not always result in increased transport of macromolecules across the vessel wall, and neutrophil-induced microvascular hyperpermeability can occur independently of neutrophil adhesion, a topic that has recently been discussed^{32–34}. Supporting this pathway is evidence of barrier dysfunction caused by neutrophil-released products in the absence of adherent neutrophils^{15,35}.

PMN-released factors and barrier function

Respiratory burst—During inflammation, activated PMNs constitute the major source of endogenous reactive oxygen species (ROS), including superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl anions (OH^-), hydroxyl radical ($OH^\bullet$) and hypochlorous acid (HOCl). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) is responsible for ROS generation by neutrophils during respiratory burst. The endogenous levels of ROS are also determined by the radical scavenging activity of antioxidant enzymes, which include superoxide dismutase (SOD), catalase, glutathione peroxidase, peroxiredoxins and thioredoxins.

Excessive release of neutrophil-generated ROS into vascular beds contributes to endothelial barrier disruption and vessel leakage. ROS produced by N-formylmethionyl-leucyl-phenylalanine (fMLP)-activated neutrophils increase microvessel permeability³⁶; similarly, lung vascular endothelial cells exposed to H_2O_2 display increased paracellular permeability to albumin³⁷. Conversely, neutrophil-induced endothelial barrier disruption is diminished by the application of antioxidants³⁸. Moreover, NOX1 deficient mice are more resistant to acute lung injury compared to wild type mice³⁹. In contrast, knockout of extracellular SOD in mice results in enhanced LPS-induced neutrophil lung inflammation, an effect that is rescued following overexpression of SOD⁴⁰.

The mechanism underlying ROS-induced endothelial barrier disruption has been extensively studied. ROS may cause the loss of barrier integrity either by directly attacking endothelial structure components (AJ, TJ and actin filament, etc.) or by indirectly activating intercellular signaling pathways regulating endothelial barrier function⁴¹. It was demonstrated that inflammation-induced oxidative stress led to the disruption of cadherin- β -catenin complex, resulting in AJ disassembly at cell-cell contact and subsequent enhanced permeability⁴². As with AJ, the structure of TJ (occludin, ZO-1, claudin-5) is also altered by ROS, including the down-regulation of protein expression, a shift of TJ protein membrane localization to cytoplasmic localization and a reduction in TJ barrier tightness⁴³. Compelling evidence also indicates that ROS trigger the reorganization of the cytoskeleton. Direct application of H_2O_2

remodels the actin filament from cortical localization into long transcytoplasmic stress fibers, facilitating junction opening and gap formation by increasing the centripetal tension^{44,45}. An array of signal transduction proteins are considered dynamic regulators of the endothelial barrier by modulating the expression or phosphorylation state of junction proteins or altering the organization of the cytoskeleton. Much evidence exists to suggest that oxidative stress activates several permeability-enhancing signaling pathways. For instance, associations between ROS and MLCK⁴⁶, mitogen-activated protein (MAP) kinases⁴⁷, protein kinase C (PKC)⁴, tyrosine kinases⁴⁸ and Rho GTPases⁴⁹ pathway activation have been well established in signaling endothelial hyperpermeability. Moreover, the deleterious effects of excessive ROS on endothelial barrier function may also be due to ROS-induced upregulation of leukocyte adhesion to the endothelium and subsequent leukocyte transmigration, which could in turn worsen barrier function³⁸.

Encouraged by animal studies demonstrating the role of antioxidants in preventing severe outcomes of inflammatory diseases or injuries, investigators have carried out several clinical trials in patients with inflammatory conditions. The effect of N-acetyl-L-cysteine (NAC), an antioxidant that increases glutathione levels, has been tested in several randomized studies with patients suffering from acute lung injury (ALI)/ acute respiratory distress syndrome (ARDS), but no significant differences were found in mortality between NAC group and placebo group^{50,51}. Procysteine is also an antioxidant, and despite the fact that it increased the number of lung injury-free days in a small study of 48 patients, it too failed in a larger trial of 215 patients with ARDS due to the inability to rescue mortality^{52,53}. So far, conclusive evidence for the advantage of antioxidant treatment during systemic inflammation is lacking. Potential reasons for the failure of antioxidants to improve survival in these disease processes could include the short life span of oxidants, the balance of beneficial and harmful redox reactions, under-characterized pharmacokinetics and pharmacodynamics of anti-oxidative drugs, and the complexity of inflammatory response that involves multiple and redundant factors or signaling cascades.

Neutrophil extracellular traps—In 2004, Brinkmann and colleagues characterized a new pathogen killing mechanism of neutrophils, termed neutrophil extracellular traps (NETs). NETs consist of chromatin with associated histones and granule contents such as serine proteases (elastase, proteinase 3, cathepsin G) and myeloperoxidase. NET production can be induced with a variety of stimuli, including bacteria, protozoa, fungi, viruses, and host factors^{54,55}.

NETosis is a form of programmed cell death induced by activation of the PKC, Raf-MEK-ERK, and NADPH oxidase pathways⁵⁶. Characterized by chromatin decondensation and release of nuclear granular contents, NETosis is driven by peptidylarginine deiminase 4 (PAD4) citrullination of histones^{57,58}. Neutrophil elastase (NE) and myeloperoxidase (MPO) contribute to this process by synergizing and locating to the nucleus to participate in histone cleavage and chromatin decondensation⁵⁹.

NETosis is different from apoptosis because there is no DNA fragmentation and phosphatidylserine is not exposed before cell death. The morphological characteristics also differ: during apoptosis, there is nuclear condensation and fragmentation, as well as

cytoplasmic vacuolization. During NET formation, in contrast, chromatin decondensation occurs, followed by breakdown of the nucleus and granules to allow mixing with cytoplasmic contents. Only after these events is there any type of opening of the cell membrane to eject NETs. This is also different from necrosis, where the nuclear envelope and granules remain intact⁶⁰. It should be noted that there are differences in the mechanisms leading to NET formation depending on the stimulus, as there are oxidant-dependent and -independent pathways⁶¹.

NETs, as the name implies, can trap pathogens in a chromatin network to control dissemination, especially those too large for phagocytosis, whilst the high concentration of granule contents, histones, and cytoplasmic proteins can inactivate and kill the pathogens^{54,62}. Conversely, uncontrolled NET formation can damage tissues and activate inflammatory cells, contributing to many pathologies, including systemic inflammatory response syndrome^{63,64}, ALI⁵⁵, thrombosis^{65–67}, autoimmune diseases⁶⁸ and cancer⁶⁹ (Figure 1).

Systemic inflammatory response resulting from infection or injury contributes to multiple organ failure. As the first responders in such conditions, neutrophils play a critical role in the initiation and progression of the inflammatory process. Clinical studies show that NETs are increased in the circulation of patients during systemic inflammation and positively correlate with organ damage^{70–74}. NETs existing in a cell-free conformation in the circulation or anchored to the microvasculature promote inflammation and tissue injury in the gut and lungs during sepsis^{75,76}. Pre-activation of endothelial cells by inflammatory stimuli can induce NET formation resulting in their increased susceptibility to NET-induced damage⁷⁷. NETs produced in transfusion-related acute lung injury are thought to be responsible for increased vascular permeability. Interestingly, a reduction in NET formation and subsequent improvement in outcome was attained by using histone blocking antibodies, tirofiban (platelet glycoprotein IIb/IIIa receptor inhibitor), and DNase I, which indicates a role for platelet-dependent NET-induced injury⁷⁸.

Although NET-promoted coagulation prevents bacterial dissemination and invasion into tissues, it may contribute to coagulopathy^{79,80}. In fact, the PAD4-mediated histone modification that leads to NET production is critical for the development of deep vein thrombosis, making it a promising target⁸¹. Inhibition of PAD4 using Cl-amidine reduced thrombosis and protected against atherosclerotic plaques in apolipoprotein-E (*ApoE*^{-/-}) mice⁸². NETs can also induce the release of von Willebrand factor from endothelial cells, enhancing DNA binding, platelet aggregation, and thrombus formation – effects that can be reduced with DNase, heparin, a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13), or in PAD4^{-/-} mice^{83,84}.

A role for NETs in cancer has only recently emerged, but could represent a missing link between cancer progression, inflammation, thrombosis, and metastasis that lead to organ damage, a topic discussed in recent reviews^{85–89}.

NETs can be disrupted by DNase, and inhibition of NET formation can be achieved by inhibiting neutrophil elastase⁹⁰, PAD4⁹¹, or janus kinase⁹². Although a seemingly attractive

target, caution should be used when developing therapeutics against NETs, as breaking them down during conditions such as polymicrobial sepsis may exaggerate the pathology by increasing bacterial counts and tissue damage⁹³. While degradation of NETs with DNase was able to reduce organ injury and mortality during endotoxemic shock, an increased bacterial load was observed in mice with polymicrobial sepsis leading to earlier mortality⁷⁴. One group showed that inhibiting NET formation with PAD4^{-/-} mice, as opposed to degrading NETs with DNase, offered protection against negative outcomes of systemic inflammation without significantly affecting bacteremia⁹⁴. It has also been shown that delayed DNase treatment improves sepsis outcomes compared to when given at an earlier time point⁹⁵. In another study, DNase could only partially reduce tissue injury during systemic infection while PAD4- or NE-deficiency offered protection from collateral tissue damage⁹⁶, further supporting the use of enzymatic inhibitors over DNase as potential therapeutics.

Histones: Histones have captured attention to elucidate mechanisms of NET-induced injury (reviewed in ^{97–99}). Like DNA, histones are increased in the circulation during inflammatory injuries and can act as DAMPs, contributing to organ injury and death in animal models of sepsis^{100–102}, acute lung injury¹⁰³, and ischemia/reperfusion (I/R) injury¹⁰⁴ through Toll-like receptor^{104,105} or C5a receptor¹⁰³ mechanisms. Histones were also greatly increased in the circulation of patients with trauma-induced lung injury, and histone infusion in mice increased cytokine production, endothelial damage, coagulation, PMN infiltration, and edema; the mechanism involved plasma membrane binding and calcium influx¹⁰⁶. Additionally, histones have been shown to directly induce endothelial barrier dysfunction based on charge and phosphorylation of Src family kinases¹⁰⁷. Furthermore, histones play a major role in NET-induced epithelial/endothelial cell toxicity during ALI, which can be prevented with histone antibodies or polysialic acid¹⁰⁸. Other therapeutic targets that have potential to address histone-dependent NET-mediated cytotoxicity include inter-alpha inhibitor protein, chondroitin sulfate, and high molecular weight hyaluronan for their ability to bind and neutralize histones¹⁰⁹. Histones may also play a role in thrombosis by upregulating tissue factor and downregulating thrombomodulin^{110–112}, or by stimulating the release of von Willebrand factor from the endothelium¹¹³. Together, histones remain a key effector in NET-mediated microvascular hyperpermeability, and more studies to elucidate downstream signaling mechanisms are needed to fully understand their role in disrupting the endothelial barrier.

Granular Enzymes: Normally, activation of PMNs trigger release of four types of granules in a hierarchical fashion and according to stimulus: secretory (plasma proteins, integrins, complement receptor, heparin binding protein [HBP]), secondary (lactoferrin, collagenase, lysozyme), tertiary (matrix metalloproteinase [MMP]-9), and primary (MPO, elastase, cathepsins, defensins, hydrolases) granules^{114,115}. Proteases contained in these granules have the ability to cleave adherens junction and extracellular matrix proteins¹¹⁶. Some studies show elastase can cleave transmembrane molecules such as VE-cadherin, which would impair junction integrity¹¹⁷. These serine proteases can also cleave cell-surface receptors to induce downstream inflammatory signaling^{116,118,119}. On the other hand, Vestweber's group showed that locking VE-cadherin prevented changes in permeability

despite neutrophils still being able to release elastase¹²⁰. This finding indicates a minimal effect of neutrophil elastase on junction structure or function. While the direct effects of proteases have been extensively studied, there is a possibility that they exert different effects when associated with NETs. Neutrophil elastase is the most abundant and proteolytically active of the serine proteases in NETs, but cathepsin G and proteinase 3 also contribute to cleavage activities¹²¹. Serine proteases of NETs have recently been shown to contribute to abdominal aortic aneurysm by their release of DNA-LL-37 complexes that recruit plasmacytoid dendritic cells that induce type I interferon production¹²². Matrix metalloproteinases have also been identified to play a role in NET-mediated pathologies by degrading extracellular matrices and activating endothelial cells^{123,124}. These effects have moved researchers to target the proteolytic activity of NETs; the best characterized serine protease inhibitors, SerpinB1 and secretory leukocyte protease inhibitor, were recently reviewed in the context of NETs¹²⁵. In contrast, in some conditions such as gout, proteases in NETs may limit inflammation by degrading cytokines and chemokines and restricting further PMN recruitment¹²⁶. Given this, more studies are needed to fully elucidate the NET-specific effects of proteases on endothelial mechanisms. Protease inhibitors may also have the ability to inhibit NET formation itself, as neutrophil elastase and proteinase 3 can contribute to NET generation^{90,127,128}. Of note, it has recently been shown that NE-deficiency may not be effective at reducing NET production in particular pathologies, pointing to PAD4 as a more attractive global target for NET inhibition¹²⁹.

While it is clear that NETs can contribute to inflammation, some researchers have suggested that local NET-induced tissue damage may not be entirely bad, for instance during viral infections where NETs may offer protection by limiting the spread of infection^{130,131}. On the other hand, they also show the ability of the liver to sequester pathogens and rapidly repair itself⁹⁶, fueling the debate on the necessity of NETs. Overall, although elucidating the mechanisms of NET-induced vascular injury is at an early stage, there is great potential for NETs to become novel markers of disease progression or severity, as well as therapeutic targets^{132–135}. Thus, more work needs to be done to identify and characterize the consequences of NETs in different pathologies, as well as the efficacy and correct timing of therapeutic strategies targeting NETs and their downstream effects on the microvascular barrier.

Perspectives

The crosstalk between PMNs and endothelium during inflammation is well recognized, but how this leads to endothelial barrier dysfunction and vascular hyperpermeability remains incompletely understood. It is likely that both PMN adhesion-dependent and adhesion-independent processes are involved in the initiation of inflammatory signals at the interface of circulating components and the vascular wall, where the signals propagate and converge at the endothelial cell-cell and cell-matrix adhesion structures that are essential for the barrier's property. Thus, targeting the key signaling pathways or endpoint molecular processes increase the therapeutic potential for resolving endothelial hyperpermeability during PMN-mediated vascular inflammation.

Acknowledgments

Funding: This work is supported by NIH R01s HL070752 and HL126646.

List of Abbreviations

AJ	adherens junction
ALI	acute lung injury
ARDS	acute respiratory distress syndrome
DAMP	damage associated molecular pathogen
EC	endothelial cell
eNOS	endothelial nitric oxide synthase
ESL	E-selectin ligand
FAK	focal adhesion kinase
fMLP	N-formylmethionyl-leucyl-phenylalanine
GM-CSF	granulocyte-macrophage colony-stimulating factor
HBP	heparin binding protein
I/R	ischemia/reperfusion
ICAM	intercellular adhesion molecule
MAP	mitogen-activated protein
MLCK	myosin light chain kinase
MMP	matrix metalloproteinase
MPO	myeloperoxidase
NAC	N-acetylcysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NET	neutrophil extracellular trap
NOX	NADPH oxidase
PAD4	peptidyl arginine deiminase
PECAM	platelet endothelial cell adhesion molecule
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocyte
PSGL	P-selectin glycoprotein ligand

ROCK	rho-associated protein kinase
ROS	reactive oxygen species
SHP2	Src-homology 2 domain-containing phosphatase
SOD	superoxide dismutase
TJ	tight junction
VCAM	vascular cell adhesion molecule
VE-PTP	vascular endothelial-protein tyrosine phosphatase
VE	vascular endothelial
ZO	zonula occludens

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Figure 1.

NETosis is a process of programmed neutrophil cell death producing a DNA meshwork decorated with histones and granular enzymes. NETs provide an efficient pathogen-killing mechanism and can limit pathogen dissemination; however, these products also contribute to coagulopathy, tissue injury, cancer, and barrier dysfunction. [Illustration created with images adapted from Servier Medical Art (<http://www.servier.com/Powerpoint-image-bank>) under a Creative Commons Attribution 3.0 Unported License.]