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The Role of Neutrophils in Thrombosis

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Abstract

Despite significant evidence implicating an important role for neutrophils in thrombosis, their impact on the thrombotic process has remained a matter of controversy. Until 2010, platelets, coagulation factors, fibrinogen and monocytes were implicated in the thrombotic process. Several studies conducted over the last decade now support the growing notion that neutrophils indeed do contribute significantly to this process. Neutrophils can contribute to pathologic venous and arterial thrombosis or ‘immunothrombosis’ by the release of neutrophil extracellular traps (NETs) and NET release is emerging as a major contributor to thrombogenesis in pathologic situations such as sepsis and malignancy. Further, blood-cell derived microparticles, including those from neutrophils, have been implicated in thrombus formation. Finally, inflammasome activation in the neutrophil identifies another important mechanism that may be operative in neutrophil-driven risk for thrombosis. The knowledge of these roles of neutrophils in thrombosis may pave the road for novel anti-thrombotic agents in the future that do not affect hemostasis.

Keywords

neutrophils; neutrophil extracellular traps; microparticles; inflammasome; thrombosis; immunothrombosis

Introduction

Hemostasis is a physiological process that maintains blood fluidity and prevents blood loss after vessel wall injury. Important to this process are cellular components (platelets, leukocytes, endothelial cells) and the coagulation proteins present in plasma. Although this system is usually in a quiescent state, it is rapidly activated upon vascular injury and leads to the generation of a thrombus.¹ Since thrombotic events can be fatal (secondary to myocardial infarction, stroke),^{2,3} a better understanding of the cellular and molecular events governing this process is crucial for the development of novel therapeutic approaches.

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In 1856, Virchow first enumerated the three predisposing factors contributing to thrombosis: blood hypercoagulability, stasis and vessel wall abnormalities (Virchow's triad; Figure 1). At that time, the underlying cellular mechanisms were not known. In 1881, Bizzozero first described platelets and their role in thrombosis.⁴ Almost a century later, in 1964, Davie,⁵ Ratnoff⁵ and Macfarlane⁶ simultaneously reported a sequence of interactions between the various clotting factors to form a fibrin clot. Thus, the role of platelets and plasma proteins has been the focus of research for a long time. Thrombosis was thought to be a culmination of disruption of the tightly regulated balance of pro-coagulant factors, anti-coagulant factors and fibrinolytic mechanisms by acquired or inherited risk factors.⁷ However, our understanding of the thrombotic process began to evolve in 2005, when Gross et al reported that leukocytes are recruited to a growing thrombus typically at 2-3 minutes post-injury and this increased over time.⁸ This was substantiated by Darbousset et al. who demonstrated that neutrophils, not platelets are the first cells to accumulate at the site of arterial injury via an intracellular adhesion molecule-1 (ICAM-1)/ leukocyte function-associated antigen-1 (LFA-1) interaction.⁹ Thus, it is over another century later that the role of monocytes and neutrophils has come to light as major contributors to the pro-thrombotic state in both arterial and venous thrombotic events.^{8,10}

Neutrophils are the most abundant leukocytes that form the core of our innate immunity against infectious pathogens. They are the first line of defense in controlling infection and help in removing cellular debris. Their anti-microbial activity occurs by intracellular killing (phagocytosis) via oxygen dependent and oxygen independent mechanisms.^{11,12} More recently however, the role of neutrophils in thrombosis has been the focus of attention. Attenuation of thrombus formation by neutrophil depletion as well as high neutrophil deoxyribonucleic acid (DNA) content in venous thrombi in mice provided the initial clues that neutrophils may play an important role in thrombus formation.¹³ While the presence of neutrophils in thrombi has been known for a long time, their exact mechanistic roles in the process of thrombus formation were not well characterized. Studies now show how neutrophil extracellular traps (NETs) and neutrophil derived microparticles (NMPs) contribute to thrombosis. Activation of the inflammasome is another mechanism that was recently elucidated and is thought to play a role in activation of the coagulation pathway.

In this paper, we will discuss the above-mentioned mechanisms via which neutrophils exert their pro-coagulant potential.

I. Neutrophil extracellular Traps (NETs)

a. NET Pathobiology—The role of NETs in thrombosis is the most well understood mechanism by which neutrophils promote clot formation. NETs were first discovered by Brinkmann et al in 2004 and were described as an extracellular structure made up of chromatin and granular proteins with antimicrobial properties.¹⁴ At the time of their discovery, the exact mechanism leading to NET formation was not clear. A large body of accumulating data now provides a better understanding of the formation and characteristic features of NETs including their antibacterial, antifungal properties,¹⁵ pro-coagulant nature¹⁴ and their role in auto-immune disorders¹⁶ and cancer.¹⁷

Two different mechanisms for the formation of NETs have been described:

NETosis is the process during which neutrophils undergo a novel form of programmed cell death and release NETs.¹⁸ This process is slow and can take up to a few hours. During NETosis, neutrophils undergo chromatin decondensation that is dependent on peptidylarginine deiminase (PAD4) and other neutrophilic enzymes.¹⁹⁻²⁴ While this prevents intravascular spread of infection, excessive activation may lead to pathologic thrombosis (immunothrombosis) and auto-immunity.

Neutrophil migration to the site of infection is triggered by inflammatory mediators, that enhance the endothelial expression of E- and P-selectins on endothelial cells, causing neutrophil adhesion, followed by rolling. Subsequently, chemokines influence neutrophils to express integrins that bind to ICAMs on the endothelium and invade into the infected tissue.²⁵ A wide variety of stimuli besides bacteria or fungi such as interleukin-8 (IL-8), lipopolysaccharide (LPS), activated platelets or endothelial cells, monosodium urate crystals, and various autoantibodies can provoke NETosis.²⁶⁻²⁹ These triggers lead to the formation of reactive oxygen species (ROS) via reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which plays a key role in the release of NETs. The importance of NADPH oxidase in the process of NET generation is evident by absent NETosis in patients with chronic granulomatous disease (CGD), a group of hereditary disorders characterized by defects in NADPH oxidase, inhibition of NETosis by diphenylene iodonium (an inhibitor of NADPH oxidase) and exogenous catalase (degrades H₂O₂), and by the induction of NETs at physiologic concentrations of H₂O₂.³⁰ One of the first morphologic changes observed during NETosis, nuclear swelling, is triggered by PAD4.¹⁹ PAD4 is essential for the initiation of NETosis as it hypercitrullinates arginine residues on histones, H3 and H4, leading to loss of their positive charge and thus causing chromatin decondensation.^{19,20} Neutrophils from PAD4 deficient mice are unable to form NETs.²² Although other neutrophil enzymes such as the granular neutrophil elastase and cytoplasmic serine protease inhibitor serpin B1 also translocate to the nucleus and may trigger chromatin decondensation and NET release,^{23,24} their exact contribution needs to be confirmed. At the end of this process, NETs containing DNA and histones are released roughly 45-240 minutes after neutrophil activation.

The second mechanism, **vital NETosis**, has been described as a faster process that does not lead to cell death. Thus, the neutrophil retains its ability to move and phagocytose.^{31,32} A similar process was first described by Yousefi et al in eosinophils,³³ wherein they showed that eosinophils release mitochondrial DNA in a very short time period without undergoing cell death. This mechanism was later described in neutrophils, mediated by toll like receptor-4 (TLR4), following activation by microbial-specific molecular patterns.³⁴ This model involves extrusion of DNA, histones and serine proteases from intact neutrophils.

b. NETs and Venous thrombosis—Venous thromboembolism (VTE) remains a major cause of morbidity and mortality.³⁵ The role of neutrophils in venous thrombosis is more evident with the recent discovery of the pro-coagulant role of NETs. NETs can contribute to thrombosis via different mechanisms.

As described above, activated NETs are composed of a matrix of DNA and histones. This matrix provides a scaffold and binds cellular components such as erythrocytes and platelets.

^{18,36} Extracellular DNA traps promote platelet activation, platelet aggregation and thrombosis (Figure 2).³⁶ In vitro studies demonstrate that platelets bind to DNA and are activated by histones,³⁷ the two components of NETs respectively.³⁸ Although the exact mechanism of platelet interaction with NETs remains unknown, interaction via the A1 domain of von Willebrand factor (vWF) is proposed as a plausible mechanism by which vWF can bind to histones.³⁹ This theory was supported by the findings in a murine model of deep vein thrombosis (DVT) induced by flow restriction in the inferior vena cava, wherein Brill et al showed that citrullinated histone H3 (citH3) present in NETs interacts with vWF and promotes a red blood cell and platelet rich thrombus.⁴⁰ A possible mechanism of platelet activation by histones involves stimulation of platelet TLR2 and TLR4 receptors.⁴¹ NET-platelet interaction is nullified when samples are treated with deoxyribonuclease (DNase).³⁶ This interaction is also lost on exposure to blood treated with heparin due to dissolution of NETs.³⁶ Activated platelets formed as a result of the NET-platelet interaction bind to neutrophils via glycoprotein Ib (GPIb) and further promote NETosis.¹³ This vicious cycle thereby propagates thrombus formation.

NETs also augment venous thrombosis by activation of the coagulation cascade (Figure 2). Extracellular nucleic acids present in NETs can enhance the protease activity of clotting factors and induce thrombin generation.⁴² Von Bruhl et al demonstrated that NETs activate Factor XII leading to activation of the intrinsic pathway.¹³ In addition, recent studies describe how factor XII itself promotes NET generation by initiating histone citrullination.⁴³ In this elegant study, Stavrou et al. demonstrate how FXII signals in neutrophils through urokinase plasminogen activator receptor-mediated (uPAR-mediated) phosphorylation of Akt2 at S⁴⁷⁴ to result in the generation of NETs. Thus, blocking FXII-uPAR interaction with a uPAR peptide or use of a specific AKT2 inhibitor significantly reduced NETosis when neutrophils were treated with FXII. NETs are also decorated with tissue factor (TF) and protein disulfide isomerase (an enzyme that activates blood cell derived TF), providing another mechanism supporting activation of the extrinsic pathway by neutrophils.^{13,44,45} TF production can increase upon neutrophil activation, thus contributing to thrombosis.^{46,47} In addition to activating the coagulation cascade, NETs also contain serine proteases (neutrophil elastase and cathepsin G), which cause proteolytic inactivation of tissue factor pathway inhibitor (TFPI) and promote thrombin and fibrin clot production.⁴⁸ Hence, NETs affect multiple aspects of the coagulation system to result in increased thrombosis.

c. NETs and Arterial Thrombosis—While the role of NETs in venous thrombosis is established, its contribution to arterial thrombosis is still under investigation.

Atherosclerosis is an inflammatory disease of arteries that is driven by lipid deposition. The role of neutrophils in atherogenesis has been underestimated largely due to the importance placed on macrophages in plaque destabilization. However, numerous studies strongly suggest that neutrophils deserve increasing attention in the pathogenesis of this disease. Neutrophils have been identified in atherosclerotic plaques.⁴⁹⁻⁵¹ Megens et al showed the presence of NETs in both human and mice atherosclerotic lesions.⁵² Further, mouse models show that neutrophil depletion led to a reduction in atherosclerotic plaque sizes.⁵³ Increased NETosis was associated with greater severity and extent of coronary artery disease (CAD) in a cross-sectional study.⁵⁴ Additionally, PAD4 inhibition in apolipoprotein E^{-/-} mice (mouse

model for severe coronary atherosclerosis) provided protection from atherosclerosis and led to prolongation of time to carotid thrombosis via inhibition of NET formation.⁵⁵ Similarly, in an elegant study, Warnatsch et al reported that mice deficient in neutrophil elastase and proteinase 3 (PR3), both enzymes implicated in NETosis, had a reduction in the growth of atherosclerotic lesions.⁵⁶ Importantly, the Apolipoprotein E^{-/-} on high fat diet demonstrated increase in IL-1 β levels but were largely absent in the neutrophil elastase and proteinase 3 deficient mice on the same diet suggesting that NETs may drive atherosclerosis by modulating cytokine production. To confirm these observations, the authors treated human monocytes with NETs in vitro followed by stimulation with cholesterol crystals. and reported that monocytes pre-treated with NETs demonstrated significantly increased cytokine production. These studies highlight the role of NETs in atherosclerosis.⁵⁷

Increased neutrophil-lymphocyte ratio is a known predictor of increased myocardial infarction and death in patients with CAD.⁵⁸ The presence of neutrophils in coronary lesions has been known but their role in acute coronary syndrome is not clear.⁴⁹ Maugeri et al studied thrombi from patients with acute myocardial infarction and showed that activated platelets promote in vitro NET formation via high-mobility group box 1 (HMBG1) secreted by platelets.⁵⁹ HMBG1 has an established role in promoting venous thrombosis.⁶⁰

Although NETs are detected in both arterial and venous thrombosis, their role in stroke is not established. In a recent study, markers of NETs (cell-free DNA, nucleosomes, and citH3) were found to be significantly increased in the plasma of patients with acute ischemic stroke when compared to healthy subjects.⁶¹ Further, Laridan et al showed that neutrophils and NETs are indeed abundantly present in cerebral thrombi supporting their importance in patients with ischemic strokes.⁶² The significance of these results is emphasized by the observation that thrombi from acute ischemic strokes were more resistant to both mechanical and pharmacological reperfusion strategies. Thus, tissue plasminogen activator (tPA)-mediated thrombolysis was accelerated with the addition of DNase I, suggesting that additional strategies directed towards NETs may need to be considered in this population.⁶³ More studies are needed to solidify the current evidence in favor of NETs in acute ischemic stroke.

d. NETs and Thrombosis in malignancy—While cancer is an important and established risk factor for VTE, arterial thrombosis has only recently been recognized as a serious complication of cancer.⁶⁴ Blood cells (white blood cells, red blood cells and platelets) are known to be altered in patients with cancer and can produce a hypercoagulable state by various mechanisms. While it is well known that the white blood cell count is elevated in patients with cancer, Khorana et al were the first to describe the association of leukocytosis in cancer with an increased risk of VTE.⁶⁵ The fact that this association with VTE was only seen with neutrophils and monocytes (not with lymphocytes) underscores the importance of neutrophils in cancer-associated thrombosis.⁶⁶

Neutrophil activation occurs in inflammatory conditions such as cancer and autoimmunity independent of the presence of infection and neutrophils represent a dominant cellular species in the local tumor-surrounding infiltrate.⁶⁷ Similarly, NETs have been found to be present within the tumor and are thought to modulate the cancer microenvironment, cancer

growth, and when in the circulation, they have been shown to facilitate metastases.⁶⁸ Tumoral granulocyte colony stimulating factor (G-CSF) overexpression and the presence of inflammatory cytokines such as platelet activating factor (PAF) and IL-8 may represent the initial stimulus for priming a subset of neutrophils towards NET generation.^{69,70} Besides this emerging role in cancer biology and spread, NETs also play an important role in cancer associated thrombosis.⁷¹ In a murine model of mammary cancer, spontaneous thrombosis was observed with increasing neutrophil count and DNA content of plasma. Increasing count of 'tumor-induced neutrophils' and their predisposition to NET formation has been shown in other malignancies as well.⁶⁹ Demers et al showed that stimulation of neutrophils obtained from mice with chronic myelogenous leukemia, lung or mammary carcinoma resulted in a greater frequency of NET formation.⁶⁹ The pro-thrombotic effect of NETs in cancer is believed to be mediated primarily via extracellular DNA.⁶⁹ Increased circulating DNA in the blood of patients with cancer has been noted for decades and was thought as being released by tumor cells.⁷² A recent study by Mauracher et al investigated whether the levels of citH3, a biomarker for NET formation, cell-free DNA (cfDNA) and nucleosomes predict VTE in cancer patients.⁷³ The results confirmed that patients with elevated citH3 levels (>75th percentile of its distribution) experienced a higher cumulative incidence of VTE of 14.5% at 2 years (as compared to 8.5% in patients with citH3 levels below 75th percentile). The association of elevated nucleosome and cfDNA levels with VTE risk was associated with a higher risk of VTE during the first 3-6 months. Hence, expanding our knowledge in this area of tumor induced NETosis not only provides an opportunity to investigate tools to curb cancer progression but also measures to prevent and treat cancer associated thrombosis.

e. NETs and Thrombosis in autoimmune disorders—NETs are implicated in the pathogenesis of different auto immune disorders such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).^{74,75,76,77} In addition to the role of NETs in causing auto-immunity, they also promote thrombosis in these diseases via various mechanisms.

SLE is associated with a high incidence of thrombosis.⁷⁸ Studies show that neutrophils in SLE have an increased capacity to produce NETs.^{75,79} Additionally, a subset of patients with SLE are also resistant to NET degradation.^{80,81} In a study by Hakkim et al,⁸¹ two possible mechanisms of impaired NET degradation in SLE were described— presence of a DNase inhibitor and anti-NET antibodies. Neutrophils in SLE are also shown to cause direct endothelial damage.⁸² Consistent with this understanding, in a lupus-prone mouse model (*NZM 2328*), PAD4 inhibition improved endothelial dysfunction and thrombosis risk, providing further evidence to implicate the role of NETs in increasing the risk for thrombosis in auto-immune disorders.⁸³

Antiphospholipid antibody syndrome (APS) is an autoimmune disorder associated with a high risk of thrombosis and recurrent pregnancy loss that occurs in the presence of antiphospholipid antibodies. In a study by Yalavarthi et al,⁸⁴ sera/plasma obtained from patients with APS had higher levels of cfDNA and NETs compared to the control group. They also showed that anti- β_2 glycoprotein I antibody (anti- β_2 GPI IgG) and other IgG fractions obtained from patients with APS stimulated NET release and this was attributed to the presence of β_2 GPI on the surface of neutrophils. Another study shows that APS patients

also have reduced degradation of NETs, adding another mechanism for the increased presence of NETs in these patients.⁸⁵

ANCA associated small vessel vasculitis (AASVV) is a systemic inflammatory disorder affecting arterioles, capillaries and venules. Active AASV is associated with higher risk of thrombosis.⁸⁶ In an autopsy of a patient with AASVV (microscopic polyangiitis), increased NETs were found in a left common iliac vein thrombus. Plasma obtained from patients with AASVV showed a higher ability of NET production as well as decreased NET degradation by DNase.⁷⁴ In addition to dysregulation of NETosis, neutrophils obtained from patients with AASVV also express TF and generate microparticles, likely contributing to the enhanced thrombogenicity in this patient population.⁸⁷

Patients with RA have a 3.36-fold and 2.07-fold higher risk of developing DVT and pulmonary embolism (PE) respectively, as compared to patients without RA.⁸⁸ RA has also been associated with increased NETosis. In a study by Khandpur et al,⁷⁶ neutrophils from patients with RA showed an increased tendency to form NETs. Further, sera from these patients was able to stimulate NETosis in control samples thereby suggesting a role of auto-antibodies associated with RA in NET production. So far, no studies have demonstrated a direct role of NETs in RA-related thrombosis. However, given the high prevalence of VTE and increased NETosis in RA, this is likely a contributing factor. Further studies are necessary to gain a better understanding of the importance of neutrophils and NETs in the increased risk of thrombosis noted in this disease.

Inflammatory bowel disease (IBD) is a disorder caused by immune dysregulation resulting in inflammation of the bowel. It is an umbrella term that includes both ulcerative colitis (UC) and crohn's disease (CD). Although the role of neutrophils in IBD-related thrombosis was not well known previously, emerging data suggests an association. While the neutrophil-lymphocyte ratio is elevated in patients with UC as compared to controls,⁸⁹ this ratio is unable to differentiate between active and inactive disease. Importantly, patients with IBD are at a three-fold higher risk of VTE compared to unaffected individuals⁹⁰ and the risk of VTE is higher during a flare as compared to intervals between disease flares.⁹¹ Numerous studies highlight neutrophils as an etiology for the hypercoagulable state noted in patients with IBD. He et al showed increased levels of phosphatidylserine exposed on microparticles in patients with IBD when compared to healthy controls.⁹² Also, increased NET generation was noted in activated neutrophils from patients with IBD or neutrophils obtained from healthy controls that were exposed to plasma from patients with active IBD.⁹²

Microparticles obtained from patients with active IBD markedly shortened coagulation times and was associated with increased generation of thrombin, fibrin and factor Xa. Further, NET degradation by DNase I partly corrected the coagulation time and decreased fibrin formation. These results provide a link between neutrophils, NETs and a hypercoagulable state in IBD patients. Another study showed that patients with active IBD have significantly higher levels of cfDNA, nucleosomes and NET formation as compared to non-active patients.⁹³ Consistently, neutrophils derived from patients with active disease showed increased NET production. In the same study, mice with dextran sulfate sodium (DSS) induced colitis (animal model of IBD) had increased NET deposition in their colonic tissue.

These mice when treated with DNase were protected against colitis, pointing towards the role of NETs in IBD.

f. NETs and sickle cell disease—Sickle cell disease (SCD) is characterized by vaso-occlusion secondary to altered RBC conformation due to HbS polymerization under hypoxic conditions. However, recent data has revealed that multiple interactions between the vascular endothelium, leukocytes, and platelets also contribute to SCD pathobiology.⁹⁴ In this section, we will focus on the role of neutrophils in sickle cell disease and vaso-occlusive crisis.

Elevated leukocyte count in patients with SCD is associated with higher mortality.⁹⁵ Higher neutrophil count also correlates with greater clinical severity in these patients.⁹⁶ There are several plausible explanations for increased clinical severity and mortality with high neutrophil count in SCD. Firstly, the binding of sickle RBCs to neutrophils can precipitate vaso-occlusion and pain crises.⁹⁷ Secondly, the heme released from hemolysis of sickle RBCs can stimulate NET formation, that could possibly worsen vaso-occlusion.⁹⁸ Finally, platelets bind to neutrophils via P-selectin and activate neutrophils, forming NETs, contributing to the inflammatory state, vaso-occlusion and acute lung injury in SCD.⁹⁹ Thus, P-selectin inhibition may be a potential therapeutic option in patients with SCD. Early clinical trials investigating selectin inhibitors in SCD have yielded promising results; further studies are ongoing.¹⁰⁰ Hydroxyurea, a well-established therapy in sickle cell disease increases hemoglobin F production that decreases HbS polymerization. This is believed to be the basic mechanism by which hydroxyurea decreases vaso-occlusive pain crisis. However, hydroxyurea also alters neutrophil function by either decreased adhesion to the endothelium¹⁰¹ or changes in neutrophil migration.¹⁰² Hence, it is possible that the benefits noted with this drug are also based on these effects. In a multicenter trial of hydroxyurea, the two-year crisis rate was lower in the sub-group with lower neutrophil counts.¹⁰³ In summary, agents altering neutrophil function or migration or inhibiting NET formation may be useful as disease modifying agents for SCD due to their significant role in the pathophysiology of SCD. Currently, there is no data to suggest a role for NETs in thrombosis associated with SCD.

II. Neutrophils and Microparticles: The Tissue factor controversy

Microparticles (MPs) are small vesicles of size 100 – 1000 nm in diameter that are made up of a phospholipid bilayer and cell surface proteins. MPs are released from the cell surface upon activation or apoptosis and their nature may vary based on the stimulus and cell of origin.^{104,105} MPs were first described as “platelet dust” in 1967 and were thought to contain platelet factor 3 (or TF).¹⁰⁶ Initially underappreciated and thought to be cell debris, their role in thrombosis, inflammation and intercellular exchange signaling is now well known.¹⁰⁷

MPs can promote thrombosis via various mechanisms. Due to the alteration in the normal asymmetric configuration of the phospholipid bilayer in activated or apoptotic or tumorigenic cells, phosphatidylserine (PS) is presented on the outer cell surface.¹⁰⁸⁻¹¹¹ MPs with PS exposed on their surface are formed by budding and subsequent cleavage of the

membrane cytoskeleton.¹¹² Thus, PS, an anionic phospholipid in MPs can promote the formation of a blood clot.¹¹³ Another significant mechanism contributing to thrombosis is the expression of tissue factor on the surface of MPs that combines with FVII and activates FIX, thereby activating the coagulation cascade.¹¹⁴ Lastly, MPs augment recruitment of cells to the site of the thrombus.¹⁰⁵ Platelets were initially thought to be the main source of MPs in thrombosis and the level of platelet derived MPs was found to correlate with endogenous thrombin production in patients with SLE.¹¹⁵ However, it is now understood that MPs expressing TF are mostly derived from the monocyte/macrophage lineage, a well-known source of MPs, that contribute to thrombosis.¹⁰⁵

Although the expression of TF on neutrophils was first described almost 40 years ago,¹¹⁶ studies lacked solid evidence on the ability of neutrophils to produce TF. Hence the origin of TF on neutrophils has remained a matter of controversy. Osterud et al reported that isolated neutrophils were unable to produce TF upon stimulation with LPS, phorbol myristate acetate (PMA) or tumor necrosis factor- α (TNF- α).¹¹⁷ TF activity in supernatants was attributed to be secondary to platelet activation by monocyte contaminants. Yet another study using a mouse model of sepsis demonstrated that although the TF positive granulocytes infiltrated the spleen, these cells did not contain TF mRNA. Presence of TF was attributed to neutrophil uptake of TF from microparticles produced by other cells.¹¹⁸ In 2008, Egorina et al showed that neutrophil-monocyte binding results in neutrophils acquiring the TF from monocytes.¹¹⁹

While the above studies suggest that neutrophils inherit TF from monocytes, they do not entirely exclude the possibility that neutrophils may be capable of producing TF on their own. Providing support to this idea, in 2006, Maugeri et al reported that neutrophils produce TF following stimulation with P-selectin or N-formyl-methionyl-leucyl-phenylalanine (fMLP) but not with PMA.⁴⁶ Simultaneously, Ritis et al demonstrated that human neutrophils produced TF in an ex vivo model of APS. In their study, they used human IgG from patients with APS to activate neutrophils. They showed that this human IgG triggers complement activation and generation of C5a, which activates neutrophils and induces TF gene transcription.⁴⁷ This was subsequently recapitulated in a mouse model of APS wherein neutrophils were reported to produce TF in a C5a-dependent fashion.¹²⁰ Following closely were two other studies that strengthened this idea; Kambal et al in 2008 collected neutrophils from the bronchoalveolar fluid of patients with acute respiratory distress syndrome and showed C5a-dependent induction of TF expression in these neutrophils.¹²¹ Kourtzelis et al similarly reported C5a-dependent TF production by neutrophils in a model of extracorporeal circulation in patients with end stage renal disease.¹²²

The role of neutrophil derived microparticles (NMP) in thrombus formation has now become more evident.^{9,87,123} A recent study in patients with non-valvular atrial fibrillation found that patients with atrial thrombi had increased PS positive platelets, neutrophils, red blood cells and MPs than in patients without thrombi. The MPs were mostly derived from platelets (56%), but the rest came from neutrophils, monocytes, erythrocytes and endothelial cells. The presence of these PS positive cells and MPs caused shortened coagulation times and greater thrombin generation.¹²⁴ NMPs (CD66b+) and myeloperoxidase (MPO) were also shown to be increased in circulation after percutaneous coronary intervention (leading to

plaque disruption) was done for an acute coronary syndrome.¹²⁵ NMPs contribute to thrombosis via several mechanisms. The presence of myeloperoxidase in NMPs has been suggested to play a role in thrombus propagation by endothelial damage.¹²³ Besides, NMPs contain Mac-1 integrins in a functionally active form that interacts with platelets, resulting in P-selectin expression and platelet activation.¹²⁶ While NMPs are known to behave as inflammatory mediators that enhance L-selectin expression on endothelial cells,¹²⁷ some studies have found an anti-inflammatory role for NMPs. A study by Gasser et al found NMPs can increase the release of transforming growth factor β 1 (TGF β 1), suggesting that NMPs can down-modulate cellular activation in macrophages.¹²⁸ Thus, a clear role of NMPs still remains to be understood but present data suggests a prothrombotic effect of these particles (Figure 3), thus highlighting a significant interaction between the immune system and thrombosis.

III. Inflammasome activation in neutrophils

Inflammasome is a multiprotein complex expressed primarily in myeloid cells, which regulates the generation of interleukin-1 (IL-1) family proteins such as IL-1 β and IL-18 and forms a caspase-1-activating platform leading to the production of pro-inflammatory cytokines.¹²⁹ NLRP3 inflammasome is the most studied inflammasome and is implicated in numerous common inflammatory disorders e.g. gout and atherosclerosis as well as normal and dysregulated host response to infection. Although neutrophils represent a major fraction of the circulating immune cells that are rapidly recruited to sites of infection and inflammation, there is limited evidence regarding inflammasome activation in neutrophils. Bakele et al reported that human neutrophils express key components of the inflammasome machinery and release the IL-1 β protein following activation.¹³⁰ Similarly, other studies also confirm the presence of functional inflammasome machinery in neutrophils that is responsible for increased levels of IL-1 β in plasma.¹³¹ Mechanisms involved in neutrophil exocytosis and inflammasome activation are also described.¹³² The P2X7 receptor (P2X7R) is expressed on macrophages and dendritic cells, which upon activation by adenosine triphosphate (ATP), induces the NLRP3 inflammasome assembly and caspase-1 dependent processing, releasing the proinflammatory cytokines IL-1 β and IL-18.¹³³ Expression of functional P2X7R has been described in other human and murine hematopoietic cells, however the role of these receptors in neutrophils is not entirely clear. Karmakar et al demonstrated that both human and murine neutrophils express functional P2X7R, and ATP-induced activation of this receptor is associated with NLRP3 inflammasome activation and IL-1 β secretion.¹³⁴ Similarly, another recently published study reported that human and murine neutrophils subjected to hyperglycemia release both MPs and IL-1 β .¹³⁵

The importance of inflammasome activation in the neutrophil and its possible role in thrombosis is evident from a recent study describing how activation of the NLRP3 inflammasome complex potentiates venous thrombosis in response to hypoxia.¹³⁶ Here, using a mouse model of venous thrombosis, the authors show that systemic hypoxia is associated with NLRP3 inflammasome complex activation and IL-1 β production, which accelerates venous thrombosis. The increased levels of IL-1 β were noted in the venous thrombus within six hours of exposure to hypoxia. Examination of the thrombus showed increased recruitment of neutrophils and macrophages to the vein in mice subjected to

hypoxia as compared to controls, suggesting the role of inflammasome activation in neutrophils and/ or macrophages in venous thrombosis secondary to hypoxia.

NET/ Neutrophil Inhibition as an anti-thrombotic strategy

Identifying neutrophils as important contributors to thrombosis offers the potential for new preventative and/or therapeutic options for thrombosis. Presently, anticoagulant therapies are directed towards platelets and procoagulant proteins. While these decrease the risk of thrombosis, these medications are associated with an increased risk of bleeding. Targeting neutrophils offers the ideal scenario wherein decreasing the risk for thrombosis has minimal alteration in hemostasis.

Using a murine model of DVT, Von Bruhl et al reported that neutrophil depletion decreases thrombus size.¹³ Although neutrophil depletion is not a practical therapeutic option, this experiment highlights the importance of neutrophils in the generation of a thrombus, suggesting that targeting neutrophil activity may alter thrombus formation. Since NETs are significantly implicated in the generation of a thrombus, targeting this aspect of neutrophil activity is a reasonable option (Figure 2). In this respect, PAD4 inhibitors would prevent NET formation and decrease the generation of a thrombus.¹³⁷ Recent studies identifying the role of factor XII in NETosis suggest that targeting factor XII might need to be considered in decreasing NET formation. This appears to be an attractive strategy since factor XII deficiency is not associated with an increased risk of bleeding. Similarly, DNases have demonstrated thromboprotection in murine models of both arterial^{138,139} and venous thrombosis.^{13,40} Thus, DNase offers an attractive option that could be potentially used to degrade pre-formed NETs. Fuchs et al reported that clots were more easily degraded when DNase 1 was used with tissue plasminogen activator³⁶ suggesting that a combinatorial approach may be more effective. A commonly used anticoagulant, heparin is reported to dismantle NETs³⁶ as well as prevent platelet-histone interactions.³⁸ Further, DNase 1 works more effectively in the presence of serine proteases e.g. plasmin or thrombin that remove DNA-bound proteins. This effect of serine proteases can be mimicked by heparin, which works by displacing histones from chromatin. Hence, using DNase 1 in combination with heparin may decrease the risk of recurrent thrombotic events.¹⁴⁰ Heparin is also capable of blocking HMGB1 on the surface of macrophages, thus inhibiting the production of pro-inflammatory cytokines.¹⁴¹ Finally, low doses of low molecular weight heparin interferes with neutrophil autophagy and mobilization of granule content that are necessary for NET formation.¹⁴²

Numerous immunomodulators are shown to decrease the production of inflammatory mediators and ROS in neutrophils, thus decreasing the generation of NETs and also endothelial dysfunction.^{143,144} An immunomodulator, chloroquine blocks NET generation by inhibiting autophagy, lysosomal degradation and increasing the clearance of lysosomal vesicles.¹⁴⁵ Similarly, hydroxychloroquine, a derivative of chloroquine, works through similar mechanisms¹⁴⁶ and has been shown to decrease the risk of thromboembolic events in patients with SLE.¹⁴⁷ The role of vitamin C as an endogenous antioxidant is well known and studies show that it attenuates the inflammatory response¹⁴⁸ and regulates neutrophil apoptosis.¹⁴⁹ Mohammed et al showed that vitamin C deficiency is associated with

increased NET production by activation of NFkB, increased ROS generation and autophagy, all of which are critical for NETosis. Consistently, vitamin C supplementation decreased the production of NETs in septic mice.¹⁵⁰ However, the thromboprotective benefits of vitamin C have not been recapitulated in the clinical setting and vitamin C supplementation has not shown any benefit in the prevention of cardiovascular events.¹⁵¹⁻¹⁵³

Since neutrophils actively accumulate along the endothelial wall during the generation of a thrombus, interfering with neutrophil recruitment to the endothelium may also decrease the risk of thrombosis. Neutrophils utilize P-selectin glycoprotein ligand-1 (PSGL-1) dependent interactions with endothelial vWF under both static and flow conditions.¹⁵⁴ Thus, inhibiting these interactions would prevent neutrophil rolling followed by firm adhesion of the neutrophil to the vessel wall. Not surprisingly, several studies involving ADAMTS13, a protease that specifically cleaves vWF (a mediator for platelet activation by NETs) reported decreased thrombosis^{155,156} and enhanced thrombolysis¹⁵⁷ with the administration of recombinant ADAMTS13. These studies suggest that recombinant ADAMTS13 may be useful in both the prophylactic and therapeutic setting. Similarly, targeting P-selectin affects thrombosis through multiple mechanisms including neutrophil-platelet interactions as well as TF-rich microparticles.¹⁵⁸ Consistently, inhibition of P-selectin reduces neutrophil recruitment to the endothelial lining^{13,159} and is associated with decreased NET generation and protection from venous thrombosis.^{13,160,161}

Summary

The correlation between inflammation and thrombosis is well established.¹⁶²⁻¹⁶⁴ However, the role of neutrophils in thrombosis has only recently been appreciated. Neutrophils are reported as the first cellular elements to be recruited to the site of endothelial dysfunction, preceding the generation of a thrombus. Simultaneously, a growing body of data supports the critical role of NETs in this process indicating that inhibition of NETosis may offer the dual benefit of decreasing the toxicity associated with NET formation as well as the associated pathological thrombosis. Given the critical role of neutrophils in innate immunity it would appear that interference with neutrophil function would produce detrimental consequences. Thus, further investigation is needed to better understand the mechanisms involved in neutrophil activation and the production of NETs. Although at present the wealth of data with regards to the prothrombotic potential of neutrophils highlights the role of NETs in coagulation, this is definitely not the only mechanism responsible for the heightened coagulation status associated with a proinflammatory environment. As outlined above, various aspects of neutrophil function are altered with inflammation, NET production being only one of the outcomes. Tissue factor production by activated neutrophils is a prime example of neutrophil function that is crucially involved in thrombosis. A better understanding of the various pathways involved will steer us towards the identification of novel targeted agents that may be effective in the prevention and treatment of thrombotic disorders while preserving important aspects of host defense. Importantly, since altering neutrophil activity or NET generation does not affect hemostasis, targeting neutrophils offers a safer therapeutic option than the currently available antithrombotic agents. However, it cannot be ignored that the production of NETs is an essential part of an ancient innate immune system that is critical in walling off infections and preventing dissemination.^{165,166}

Hence, preventing NETosis may be a double-edged sword and the side effects will need to be carefully considered and studied before considering NET inhibition as an anti-thrombotic strategy. Neutrophils are crucial players of innate immunity and inflammation, and their function is mediated by a number of cell surface receptors that trigger complex intracellular signal transduction pathways which we are only beginning to understand. Given the role of neutrophils in various human diseases, understanding signal transduction in these processes is critical to the development of targeted therapies.

In summary, although accumulating data strongly implicates neutrophils in the process of thrombosis, there is certainly much more to be understood about this cellular element and how it affects inflammation and thrombosis. Importantly, we have to understand how best to utilize this data to formulate targeted strategies that decrease the pro-thrombotic milieu without affecting our ability to fight infections.

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Abbreviations

AASVV	ANCA associated small vessel vasculitis
APS	antiphospholipid antibody syndrome
CAD	coronary artery disease
cfDNA	cell-free deoxyribonucleic acid
citH3	citrullinated histone 3
CD	crohn's disease
DPI	diphenylene iodonium
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DVT	deep venous thrombosis
HMBG1	high-mobility group box 1
GPIIb	glycoprotein Ib
ICAM	intracellular adhesion molecule
IBD	inflammatory bowel disease
IL	interleukin
MP	microparticle
MPO	myeloperoxidase

NADPH	reduced nicotinamide adenine dinucleotide phosphate
NETs	neutrophil extracellular traps
NMP	neutrophil derived microparticle
PAD4	peptidylarginine deiminase
PMA	phorbol myristate acetate
PR3	proteinase 3
PS	phosphatidylserine
RA	rheumatoid arthritis
ROS	reactive oxygen species
SCD	sickle cell disease
SLE	systemic lupus erythematosus
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TLR	toll like receptor
TNF-α	tumor necrosis factor- α
UC	ulcerative colitis
vWF	von Willebrand factor
VTE	venous thromboembolism

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Highlights

- Emerging evidence suggests that there is significant crosstalk between inflammation and thrombosis.
- Neutrophils are prime contributors to this ‘immunothrombosis’ via extracellular traps, microparticles and activation of the inflammasome.

Figure 1. Tetrad of thrombosis.

The pathogenesis of thrombosis is based on the Virchow's triad that includes stasis, hypercoagulability and endothelial dysfunction. A vast amount of data indicates the role of immune dysregulation as a significant fourth player in thrombosis and is thus worthy of being included in a potential 'tetrad of thrombosis'. NET= neutrophil extracellular traps.

Figure 2. Formation of NETs and their role in thrombosis.

NET formation occurs via two processes: non-lytic or vital NETosis and lytic NETosis. PAD4 causes hypercitrullination of histones leading to nuclear swelling due to chromatin decondensation, followed by release of NETs containing DNA and histones. Both lytic and non-lytic NETosis can cause thrombosis. The negatively charged surface of NETs promotes activation of the intrinsic pathway of coagulation. TF on NETs activates the extrinsic pathway of coagulation. Histones activate platelets (interaction mediated via vWF) and inhibit thrombomodulin, promoting thrombosis. Serine proteases released from neutrophil granules inhibit TFPI, an anti-coagulant, supporting thrombus formation. Heparin, DNase, APC, PAD4 inhibitors, P-selectin inhibitors and recombinant ADAMTS13 are anti-thrombotic agents, with potential novel mechanisms highlighted in the above figure, essentially based on NET inhibition. APC= activated protein C, DNase= deoxyribonuclease, NET= neutrophil extracellular traps, PAD4= peptidylarginine deiminase, PSGL= P-selectin ligand 1, RBC= red blood cell, TF= tissue factor, TFPI= tissue factor pathway inhibitor, TM= thrombomodulin, vWF= von willebrand factor, WP= weibel palade bodies.

Figure 3. Historical overview of the role of neutrophils in thrombosis.

This figure summarizes the historical events over the last five decades in favor of or against the role of neutrophils in thrombosis, especially with respect to NETs and neutrophil derived microparticles. ACS= acute coronary syndrome, ANCA= anti-neutrophil cytoplasmic antibody, ARDS= acute respiratory distress syndrome, MPO= myeloperoxidase, mRNA= messenger ribonucleic acid, NETs= neutrophil extracellular traps, PAD4= peptidylarginine deiminase, PCI= percutaneous coronary intervention, TF= tissue factor.