Neutrophil extracellular traps (NETs) and infection-related vascular dysfunction

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Neutrophil extracellular traps (NETs) and the role of platelets in infection

Robert K. Andrews; Jane F. Arthur; Elizabeth E. Gardiner
Australian Centre for Blood Diseases, Monash University, Melbourne, Australia

Summary
In addition to playing a central role in normal haemostasis, platelets make important contributions to host inflammatory and immune responses to injury or infection. Under pathophysiological conditions where platelet function is not tightly controlled, platelets also play critical roles in pathogenic processes underlying cardiovascular disease, uncontrolled inflammation, coagulopathy and in tumour metastasis. Neutrophil extracellular traps (NETs) are webs of histone-modified nuclear material extruded from activated neutrophils during inflammatory responses and these degranulation events can be directly triggered by platelet/neutrophil engagement. Emerging research describes how NETs influence platelet function, particularly in the setting of infection and inflammation. Especially intriguing is the potential for platelet-driven coagulation to be modulated by NETs in plasma and interstitial spaces. These findings also reveal new perspectives related to improved therapy for venous thrombosis.

Keywords
Platelets, infection, inflammation, neutrophil extracellular traps (NETs)

Correspondence to:
Dr. Elizabeth E. Gardiner
Australian Centre for Blood Diseases
Monash University
Level 6, 89 Commercial Road,
Melbourne, Victoria, 3004, Australia
Tel.: +613 9903 0756
E-mail: elizabeth.gardiner@monash.edu

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Introduction

Leukocytes, primarily neutrophils, are soldiers of the innate immune system with the function of removing dead and dying host cells and providing protection against invasion by pathogens. Failure of this system can result in the onset of sepsis, a systemic inflammatory response generally due to a microbial infection, causing an extensive clinical problem with significant mortality. Platelets play a critical role in recruiting neutrophils to sites of injury and infection (1), and also contribute to the localisation and activation of neutrophils via engagement of neutrophil and endothelial cell receptors and release of chemokines (2, 3). Neutrophils release proinflammatory modulators that mediate recruitment of additional cells to a site of infection and amplify the innate protective response. Additionally, in a process to physically isolate and engulf a pathogen, neutrophils extrude highly charged mixtures of DNA and nuclear proteins named neutrophil extracellular traps (NETs). These electrostatically-charged adhesive networks entrap and limit dispersion of the pathogens, and trigger intrinsic coagulation (4). NETs also contain neutrophil secretory granule-derived serine proteases including neutrophil elastase and cathepsin G (5), known to regulate the reactivity of both neutrophils and platelets. Since the characterisation of NETs in 2004 (6), novel and unexpected extracellular roles for DNA have emerged in studies using murine models of thrombosis and inflammation (7–10). This review aims to describe the function of platelets in normal haemostasis, with a focus on how platelets orchestrate the involvement of neutrophils at sites of injury and inflammation and regulate the formation of NETs.

Platelets in haemostasis

Platelets are small anuclear, granulated cells produced by megakaryocytes in the bone marrow, which play a central role in normal haemostasis by maintaining vascular patency. Under certain conditions, platelets also play a role in cardiovascular disease and inflammation (11). Platelets normally circulate in healthy individuals in numbers ranging from 150–400 $\times 10^3$ per µl of blood. This platelet count is in vast excess of the number of platelets needed for normal haemostasis, consistent with other important roles for platelets in addition to thrombus formation and control of bleeding (12).

Platelets normally circulate through the vasculature in an inactive, non-adhesive state, despite expressing surface glycoprotein (GP) receptors that bind abundant plasma and/or extracellular matrix adhesive proteins such as fibrinogen, von Willebrand Factor (VWF) or collagen (13). However, in response to an injury to the endothelium, bacterial infection or alteration to normal blood flow, turbulence or shear rates, platelets rapidly decelerate, roll on the injured endothelium and firmly adhere (14, 15). Initial platelet adhesion triggers a series of intracellular activation events, shape

change, altered properties of the plasma membrane, degranulation and release of soluble mediators that amplify platelet activation. Activated platelets recruit additional platelets, culminating in the upregulation of the platelet-specific fibrinogen receptor, integrin αIIbβ3 (16, 17). This process forms a plug of platelets at the site of vessel injury, consolidated by coagulation and clot contraction, which seals the wound to minimise loss of blood. Secreted contents of platelet granules include a number of potent chemokines and growth factors (18, 19) enabling wound repair to begin.

Platelet-specific adhesion receptors, GPIbα (of the GPIb-IX-V complex) that binds VWF, and GPVI which binds collagen, initiate platelet adhesion and activation at sites of endothelial injury. GPIbα, the disulphide-linked subunit GPIbβ, GPIX and GPV are all members of the leucine-rich repeat (LRR) protein family, while GPVI is a member of the immunoreceptor family containing two immunoglobulin (Ig) domains in the extracellular region. Whilst engagement of GPIbα by VWF (or the multimerised GPIbα-binding A1 domain of VWF) is sufficient to generate platelet signalling (20), collagen binding to GPVI co-expressed with the FcRγ chain (GPVI/FcRγ) enhances platelet activation in response to VWF/collagen. FcRγ contains a cytoplasmic Immunoreceptor Tyrosine Activation Motif (ITAM) utilised by GPVI to signal following ligand binding and clustering (21, 22). The GPVI/FcRγ and GPIb-IX-V receptor systems co-associate on the platelet membrane surface (23) and cooperate to trigger platelet adhesion and activation under a range of blood fluid-shear conditions. GPIbα also binds the adhesive ligand, thrombospondin, coagulation factor XI, factor XII, thrombin and high-molecular-weight kininogen (13, 24), as well as P-selectin on activated endothelial cells or activated platelets (25) and the leukocyte integrin αMβ2 (Mac-1) (26). GPVI also binds laminin (27); both collagen and laminin bind integrins α2β1 and α6β1, respectively, on activated platelets.

These important binding capabilities of GPIb-IX-V/GPVI permit platelets to simultaneously interact with the extracellular matrix and engage leukocytes and endothelial cells. P-selectin on activated platelets also binds P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes, with PSGL-1-dependent activation of αMβ2 that binds GPIbα. These adhesive receptor/ligand networks facilitate platelet and neutrophil adhesion/activation, while the activated platelet membrane surface is highly procoagulant through localisation of intrinsic pathway factors XII/XI via GPIbα and exposure of negatively-charged phosphatidyl serine (PS). GPIbα acts as a cofactor for thrombin-dependent platelet activation via the thrombin receptor, PAR-1. Platelets also deliver pathogen-derived antigen to dendritic cells in spleen in a process that depends upon GPIbα and the complement system, which facilitates the generation of cytotoxic T cells and induction of antibacterial immunity (28).

The role of platelets in infection

Infection is defined as the invasion of a host tissue by pathogenic organisms, for example bacteria or viruses. When a breach of the human vasculature is detected, the host system mounts a rapid response involving endothelial cells, platelets and neutrophils. As described above, platelets rapidly adhere at a site of injury, to limit blood loss and initiate wound repair. However there is an increasing appreciation of the role of platelets in the innate response to infection (3, 29–32) – the term "*immunothrombosis*" was recently coined (29) to describe an innate immune response induced by the formation of thrombi in microvessels or other vascular beds. This response involves immune cells and prothrombotic proteins including thrombin, active factors VIIa and Xa and tissue factor, to generate intravascular clots as a scaffold expediting the recognition, containment and destruction of pathogens. Platelets also play a role in pathogen recognition.

Platelet-pathogen interactions

The mechanisms by which platelets engage in the inflammatory response are multifactorial. First, as described above, platelets are rapidly localised at sites of injury or vessel wall disruption by binding exposed extracellular matrix proteins. Interestingly, many of the platelet glycoproteins involved in the adhesion and aggregation steps also either engage bacteria or bacterial proteins directly, or indirectly by binding to bridging molecules such as fibrinogen or VWF (►Figure 1) (31, 33), and platelets are activated by specific proteins secreted by strains of *Staphylococcus* (34–36). The ITAM-bearing, low-affinity IgG receptor, FcγRIIa, on human platelets also induces activation in response to multiple strains of *Staphylococcus* and *Streptococcus* bacteria (37, 38) or during H1N1 influenza virus infection (39), also causing the release of secondary mediators, platelet-derived microparticles and platelet factor (PF) 4 (also known as CXCL4) thereby amplifying platelet activation through an IgG-dependent pathway.

FcγRIIa also plays a critical role in the pathogenesis of heparin-induced thrombocytopenia (HIT) a potentially fatal disease where platelets are bound by antigenic complexes of heparin and PF4. Individuals exposed to heparin during surgery can raise heparin/PF4-dependent autoantibodies, and when the antibody-bound complex is bound to the platelet surface, the Fc portion induces FcγRIIa-dependent platelet activation and aggregation, thrombocytopenia and the onset of thrombosis (40). Recent studies have demonstrated that bacterial DNA may substitute for heparin to form similar antigenic complexes with similar degrees of platelet aggregation and thrombosis (41, 42). These findings imply not only that platelets can target bacterial DNA in this way, but also that the rapid immune response to heparin underlying HIT may be, in part, an unfortunate consequence of a misdirected platelet/immune response to bacterial infection.

Finally, platelets also contain members of the toll-like receptor (TLR) family of proteins. Like GPIb-IX-V, TLRs are members of the LRR family of receptors. TLRs are innate immune receptors found on most cell types, but primarily cells of leukocytic origin and respond to a wide variety of pathogen-derived molecules using pattern recognition within microbial lipids, carbohydrates, proteins and nucleic acids. TLR2, 4 and 9 are expressed on platelets (43) together with other functional TLRs (44, 45). A recent

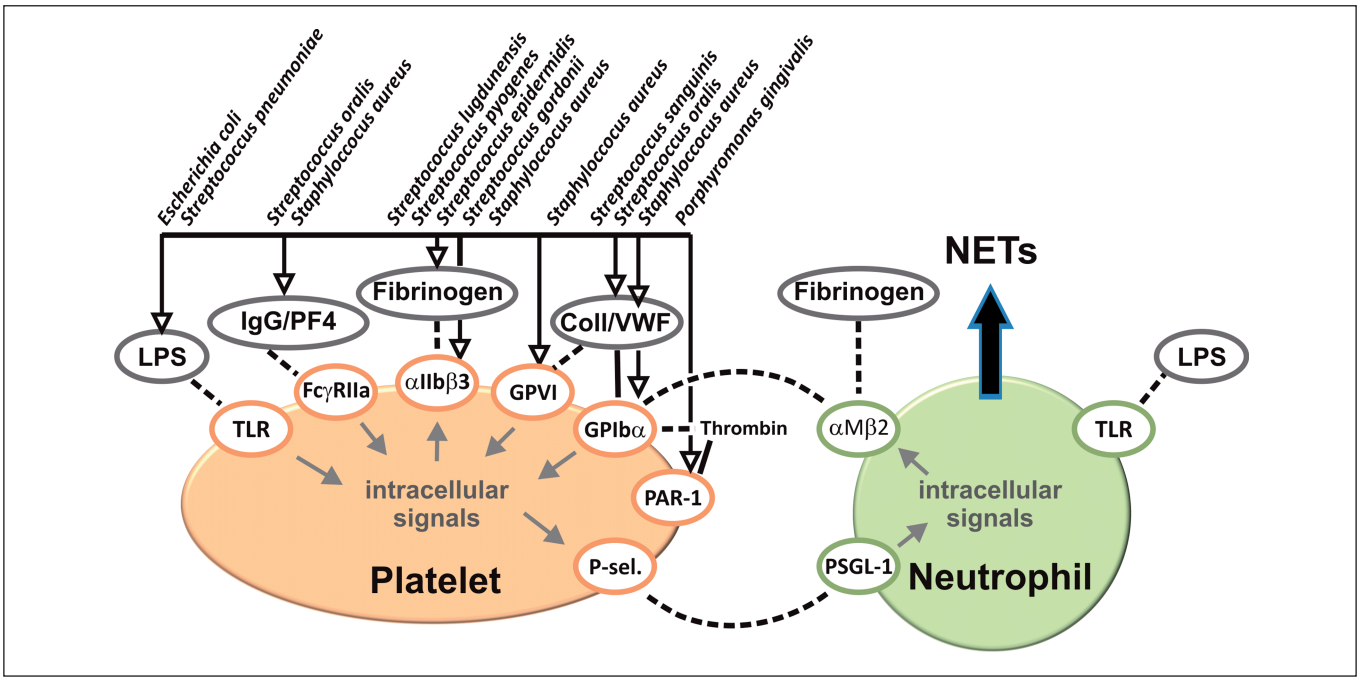

Figure 1: Pathogens, platelets and neutrophils. Interactions between platelets and neutrophils are mediated predominantly by receptor interactions involving P-selectin/PSGL-1 and GPIbα/αMβ2. Platelet receptors are also targets of bacteria or bacterial-derived factors such as lipopolysaccharide, that interact either directly or indirectly via ligands, leading to platelet activation and enhanced neutrophil engagement and generation of NETs. Activated platelets also release soluble proinflammatory mediators which activate neutrophils. Interactions involving released NETs are illustrated in Figure 2. LPS, lipopolysaccharide; PF4, platelet factor 4; P-sel., P-selectin; TLR, toll-like receptors; Coll, collagen (see the text for details).

study showed TLR7 mediated platelet responses to single stranded RNA viruses (45). Platelets also contain components of signalling pathways requisite for TLR function including nuclear factor (NF) κB and myeloid differentiation factor 88 (MyD88) and auxiliary molecules (46, 47). Whilst the precise signalling mechanisms for platelet TLRs are uncertain, it is clear that ligands of TLR2 and TLR4 – including forms of lipopolysaccharide (LPS) as well as fungal and mycobacterial products – can potentially prime platelets to not only adhere and aggregate, but also interact with other vascular and immune cells, and release proinflammatory factors from platelet granules (48, 49). The specific TLR2 agonist PAM3-CSK4, a synthetic triacylated lipopeptide that mimics the acylated amino terminus of bacterial endotoxin, triggers phosphorylation of signalling molecules, including Src, Syk, LAT and PLCγ2 in human platelets (50).

Platelet-neutrophil interactions

Activated platelets bind to leukocytes and lymphocytes in blood and mediate recruitment to sites of injury and infection (51). Platelet-leukocyte aggregates may reflect a prothrombotic state and are associated with acute coronary syndromes, systemic inflammatory, neoplastic and autoimmune diseases (52–54). During the initial phase of atherosclerosis, inflammatory leukocytes interact with the damaged endothelium in areas rich in platelet aggregates. The molecular mechanisms mediating this cellular interaction include a central role for P-selectin on activated platelets binding to PSGL-1 on neutrophils, which triggers a signalling cascade (55) leading to activation of αMβ2, which can in turn engage platelet GPIbα (26, 56) (►Figure 1). These interactions result in firm adhesion between the two cell types. Activated platelets also release proinflammatory microparticles (57–59) and secrete serotonin, PF4, interleukin-1β, Regulated upon Activation Normal T-cell Expressed presumed Secreted (RANTES, also known as CCL5) and CD40 ligand (CD40L) which can all directly activate neutrophils (60–64). PSGL-1 engagement also upregulates leukocyte tissue factor, and triggers synthesis and release of cytokines and other inflammatory molecules, to locally amplify both neutrophil and platelet activation (3, 65–68). In addition to these significant steps, a major consequence of neutrophil activation of paramount importance to the innate immune response is the production of DNA-containing NETs.

Neutrophil extracellular traps (NETs)

NETs consist of a framework of filamentous DNA (chromatin) adorned with histone proteins and several antibacterial components that are expelled from activated neutrophils (►Figure 2). The NET lattice-like structures are selectively extruded from activated neutrophils by a process termed NETosis. In the initial phase, neutrophil DNA undergoes a decondensation step requiring active peptidylarginine deiminase (PAD) 4 to control citrullination of histone proteins. In this regard, mice deficient in PAD4

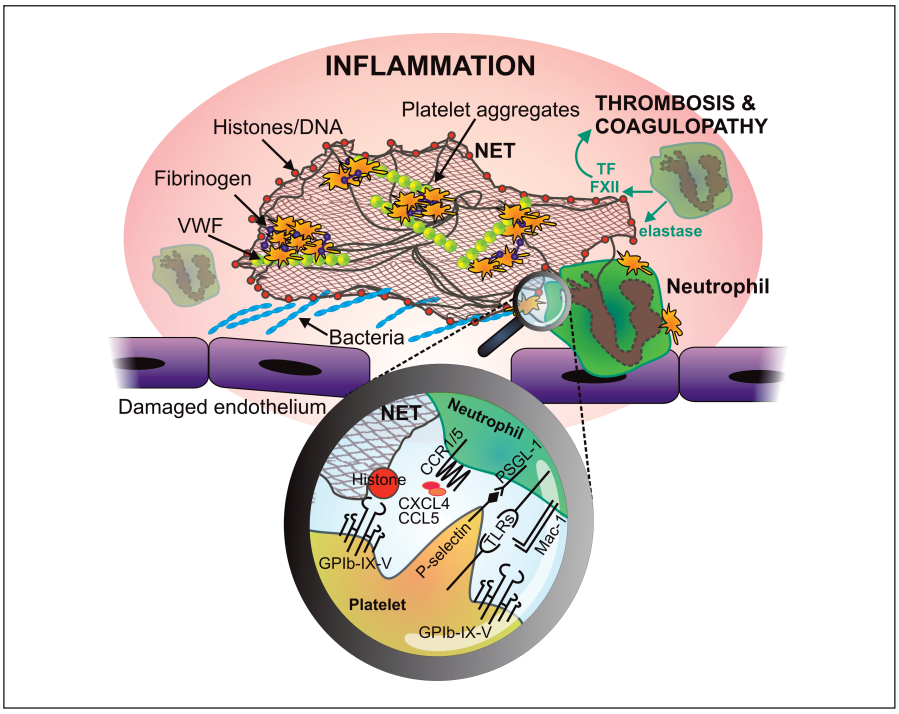

Figure 2: Neutrophil extracellular trap (NET) at site of injury/inflammation. Neutrophils release NETs, comprised of DNA, histones and other proteins, including elastase. Also associated with NETs are the coagulation proteins, tissue factor (TF), the main initiator of extrinsic coagulation *in vivo*, and active factor XII (of the intrinsic coagulation pathway), at sites of bacterial infection or endothelial injury. The coagulation pathways are likely to be critical in pathological thrombosis. In a feed-forward cycle, activated platelets can induce NET formation, form neutrophil-platelet and platelet-platelet aggregates, and be trapped by NETs. Inset magnification: Key receptors in platelet, neutrophil and NET interactions. Platelet GPIbα engages VWF/histones and neutrophil Mac-1 (αMβ2). P-selectin and TLRs on platelets bind their counter-receptors on neutrophils: PSGL-1 and other TLRs, respectively. Neutrophil engagement of heterodimerised CCL5 (RANTES) and CXCL4 (PF4) released from activated platelets may induce NET formation in the absence of bacterial invasion.

do not effectively undergo DNA decondensation and do not produce NETs (69, 70).

In an intricate mechanism that is not fully understood but requires the timed production of reactive oxygen species, the nuclear envelope is dismantled and chromatin decondenses in the cytoplasm. The unravelled nucleic acid then combines with azurophilic granule proteins (primarily elastase and myeloperoxidase) as well as histones and other anti-microbial proteins such as pentraxin, matrix metalloproteinase-9, peptidoglycan recognition protein 1 and lactoferrin (6, 29, 71, 72). It will be interesting to ascertain whether there is any degree of selectivity regarding the decondensation and release of DNA during NETosis. The mechanism of NET release is also complicated and likely to involve autophagy-like processes with posttranscriptional control of hypoxia-inducible factor-1α (HIF-1α) protein expression by mammalian target of rapamycin (mTOR) (73, 74). Once released into the bloodstream, it is proposed that NETs serve to trap, restrain and neutralise invading microbes and parasites (69, 75–79). NETs generally do not contain neutrophil cytoplasmic and membrane components (72, 76, 80), which distinguishes this process from extracellular DNA release following cell necrosis or apoptosis (81). Several pathways may control the rapidity of NETosis, with the resultant NETs potentially forming across several hours (76), or within minutes of exposure of neutrophils to live strains of *Staphylococcus aureus* (82, 83) or to activated platelets (84). It is worth noting that whilst experiments performed *in vitro* are compelling in their collective confirmation that neutrophils can be induced to generate NETs, interpretation of work examining NETosis and function of NETs *in vivo* still contains elements of ambiguity and leads to numerous unresolved questions relating to NET formation *in vivo*, often involving pathway differences both between species and cell types (85).

The ability of platelets to operate as innate immune cells is critical to the process of NETosis and simultaneously, these steps enhance coagulation by providing both the scaffold and the stimulus for thrombosis (29, 86–88). Treatment of platelets with LPS to engage platelet TLR4 promotes the formation of platelet-neutrophil aggregates and also generates NETs (49, 84). Histones can also directly activate platelets to accelerate thrombin production in a mechanism that is mediated by TLR2 and TLR4 (89). Further, histone binds the GPIbα-binding A1 domain of VWF (90), which can potentially localise VWF and platelets to the NET scaffold at sites of injury. NETs also entrap red blood cells (87).

The clear benefits of NETs are highlighted in animal models of infection, inflammation or sepsis (8, 71, 72, 91), and in human disease where NET formation is disrupted or deficient (76, 77, 92). However the expulsion of anti-microbial toxins and associated release of reactive oxygen species from neutrophils can also have detrimental effects on the host. NETosis with concomitant release of neutrophil elastase and the coagulation activator molecules, tissue factor (93, 94) and Factor XII (66), drives microvessel thrombosis observed during systemic infections, cancer, myocardial infarction, stroke, neurodegenerative diseases and in thrombotic microangiopathies (95–98). Indeed, plasma from patients with systemic lupus erythematosus (SLE) – a disease in which patients develop autoantibodies to DNA, histones and neutrophil proteins – revealed dysfunctional clearance of NETs, and this failure to dismantle NETs appeared to correlate with kidney pathology in SLE patients (99) and contributes to the pathogenesis of SLE by activating dendritic cells to produce large amounts of interferon-α (100). While clinical studies so far are limited, NET formation also trig-

gered vasculitis and promoted an anti-neutrophil autoimmune response in individuals with small-vessel vasculitis (71).

Concluding comments: therapeutic implications of platelet interactions and NETs

Atherosclerosis and atherothrombosis are the most common pathological processes underlying cardiovascular diseases. Current therapies are largely focused on alleviating hyperlipidaemia and preventing thrombotic complications, but do not completely eliminate the risk of suffering recurrent acute myocardial/ischaemic events. New anticoagulants targeting either factor Xa or thrombin or both can inhibit coagulation, as well as thrombin-dependent platelet activation via GPIbα/PAR-1. Specifically targeting the inflammatory and NETosis processes mediated by platelets and leukocytes may further reduce the risk of major adverse cardiovascular events in atherosclerotic patients (101–103). Indeed NET formation has been detected in the earliest phase of developing thrombi in patients with venous thromboembolism suggesting that therapeutic intervention in NET formation at this point might be beneficial (104). Notably, current clinical therapies for venous thrombosis may target coagulation factors but not activation of platelets/endothelial cells/leukocytes which together promote clot formation by NETs or other pathways.

In conclusion, the collaborative involvement of platelets and neutrophils in the inflammation and pathophysiology of cardiovascular diseases provide a novel link between inflammation and thrombosis, two essential processes in cardiovascular diseases. Clearly a better understanding of platelet-leukocyte interactions and their contribution to athero-thrombosis will aid the progress of therapies targeting prevention and treatment of ischaemic cardiovascular disease.

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Conflicts of interest

None declared.

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