Immunothrombosis and thromboinflammation in host defense and disease

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Immunothrombosis in host defense and disease

Platelets are increasingly being recognized for playing roles beyond thrombosis and hemostasis. Today we know that they mediate inflammation by direct interactions with innate immune cells or secretion of cytokines/chemokines. Here we review their interactions with neutrophils and monocytes/macrophages in infection and sepsis, stroke, myocardial infarction and venous thromboembolism. We discuss new roles for platelet surface receptors like GPVI or GPIb and also look at platelet contributions to the formation of neutrophil extracellular traps (NETs) as well as to deep vein thrombosis during infection, e.g. in COVID-19 patients.

Keywords: platelets; neutrophils; macrophages; immunothrombosis; sepsis; myocardial infarction; stroke; neutrophil extracellular traps; NETs; COVID-19

Introduction

Platelets are small, anucleate cell fragments derived from bone marrow megakaryocytes that play major roles in hemostasis and thrombosis [1]. In recent years, they have increasingly been recognized to also contribute to other (patho)physiologic processes such as inflammation, infection, cancer metastasis and maintaining vascular integrity during inflammation [2–4].

Immunothrombosis

Platelets not only contribute to thrombosis and inflammation, they actively bridge the two processes thereby creating a new mechanism and terms like *thromboinflammation* or *immunothrombosis* have been used to acknowledge their dual role. Thromboinflammation was used as early as 2004 to describe platelet-leukocyte reactions mediated through P-selectin-PSGL1 interactions in intracoronary stents [5]. Blair et al.

used it in 2009 to describe stimulation of platelets through toll-like receptor 2 (TLR2) [6]. Nieswandt and Stoll have introduced the concept of stroke being a thromboinflammatory disease [7]. Engelmann and Massberg in 2013 coined the term immunothrombosis to describe an innate immune response induced by intravascular thrombus formation that leads to recognition, containment and destruction of pathogens [8]. In this review, we highlight the team play between thrombotic and inflammatory processes during infection and sepsis but also in non-infectious diseases such as stroke, deep vein thrombosis and myocardial infarction.

Platelet activation

Platelet activation and thrombus formation at sites of vascular injury starts with the platelet glycoprotein (GP) Ib-V-IX complex binding von Willebrand Factor (VWF) immobilized on collagen fibres [9]. This transient interaction decelerates platelets and enables them to bind to collagen in the extracellular matrix (ECM) through GPVI - the major platelet-specific receptor for collagen [10]. GPVI binding causes platelet activation by intracellular signalling processes via an immunoreceptor tyrosine-based activation motif (ITAM) in the associated Fc receptor (FcR) γ-chain. Platelet activation leads to the release of second wave mediators like adenosine diphosphate (ADP) and thromboxane A2 (TxA2). Together with locally produced thrombin, ADP and TxA2 lead to full platelet activation [11] causing major platelet integrins including αIIbβ3 (GPIIb/IIIa) that binds fibrinogen to change to their high-affinity state and mediate stable platelet-platelet interaction and adhesion to the ECM [12].

Following activation, platelets release the content of their α- and dense granules. Soluble proteins like CXCL4 (PF4), CXCL7 (platelet basic protein), CCL5 (RANTES)

and CD40L as well as receptors like P-selectin stored in platelet α-granules participate in inflammation. Upon activation, P-selectin exposed on the platelet surface binds to P-selectin glycoprotein ligand-1 (PSGL1) on endothelial or immune cells thereby enabling platelets to bind to the inflamed endothelium, to recruit circulating monocytes, neutrophils and lymphocytes and to initiate an inflammatory response at the site of injury [13].

Platelet interactions with immune cells

Neutrophils

Neutrophils are granulocytic cells of the innate immune system, and are among the first leukocytes recruited to a site of injury or infection. Neutrophils play a key antimicrobial role against a variety of different pathogens, either by phagocytosis, internalizing and killing, degranulation, releasing toxic factors or the release of neutrophil extracellular traps (NETs) which physically contain pathogens and prevent their dissemination. In an inflammatory response, PSGL-1 on neutrophils interacts with constitutively expressed P-selectin on the surface of endothelial cells to initiate rolling along the vessel wall. The upregulated expression of E-selectin and ICAM-1 leads to slow rolling and eventually firm adhesion upon neutrophil β2 integrin activation, until the neutrophil extravasates to reach a site of infection [14]. As described above, activated platelets externalize P-selectin. These P-selectin^{+} platelets can then interact with neutrophil PSGL-1, which can be visualized by rosetting assays [15]. For this interaction to occur with high affinity, PSGL-1 requires post-translational modification [16]. The interaction is negatively regulated by the release of neutrophil granule enzymes including cathepsin G and elastase, which cleave PSGL-1 (but not P-selectin) from the neutrophil cell surface [17].

P-selectin shedding also occurs upon interaction with PSGL-1 as an additional regulatory step, although this occurs independently of neutrophil serine proteases and the sheddase remains unknown [18]. PSGL-1 engagement on the neutrophil surface leads to downstream signalling via MAPK leading to IL-8 production [19] and via Src kinase leading to β2 integrin activation and in turn enhanced binding to ICAM-1 [20]. This occurs via the adaptor protein ezrin-radixin-moesin moiety which connects PSGL-1 to Syk and the actin cytoskeleton [21, 22].

Mac-1 (Macrophage-1 antigen, integrin $\alpha_M\beta_2$ [CD11b/CD18], or complement receptor 3 [CR3] expressed on myeloid cells provides a direct binding partner for platelets by its interaction with GPIbα [23]. This interaction engages platelet outside-in signaling, promoting further P-selectin expression and αIIbβ3 integrin activation [24]. *In vivo*, *Itgam*$^{-/-}$ (deficient for integrin subunit α_M) mice have a defective thrombus formation capability which can be rescued by infusion of WT neutrophils (or PBMCs), and specific antibodies targeting the GPIbα-Mac-1 interaction inhibit thrombosis [25]. This may provide a novel target for preventing platelet-neutrophil interactions which appear to contribute to disease etiology in pathological thrombosis. In addition to GPIbα, Mac-1 can also bind to junction adhesion molecule 3 (JAM-3) [26], or to ICAM-2 or αIIbβ3 via fibrinogen [27, 28], promoting firm adhesion to neutrophils.

A key link between inflammation and thrombosis is the formation of NETs, whereby activated neutrophils release DNA, histones, and antimicrobial proteins into the extracellular space in a procoagulant and prothrombotic web [29]. Activated platelets were one of the first physiologically relevant non-infectious stimuli to be described for NET induction [30]. In bacterial sepsis, lipopolysaccharides promote non-classical activation of platelets, without P-selectin exposure or enhanced aggregation; NET formation is induced upon interaction of neutrophils with these LPS-activated platelets

[30]. In the absence of infection, the interaction between P-selectin on platelets and PSGL-1 on neutrophils contributes to NET formation in mice [31], particularly when P-selectin is in its dimer form or oligomerized [32]. Of note, P-selectin antibody blockade did not affect platelet-mediated NET formation in human neutrophils [33, 34], indicating either a species or agonist-dependent difference in P-selectin's role on NET formation.

sCD40L or platelet CD40L binding to its receptor CD40 on neutrophils can result in β2 integrin activation [35, 36], promoting initiation of the oxidative burst [35] which can be a key driver of NET formation [37]. Under static conditions, platelets activated with TLR2/4 agonists or arachidonic acid contribute to NET formation in a manner that could be blocked by anti-GPIb or anti-CD18 antibodies [34], indicating a role for platelet GPIb binding to Mac-1 in this process. This platelet-dependent NET formation was also shown to be independent of NADPH oxidase, and aided by CXCL4 [34]. Platelet-derived CXCL4/CCL5 heterodimers also were shown to be essential for this β2 integrin-dependent NET formation to occur [38], and GPIb-Mac-1 binding is likely leading to outside-in signaling which propagates this platelet-mediated NETosis.

Another key component of platelet granules which has been linked to procoagulant activity is inorganic polyphosphate [39], a negatively charged linear polymer of inorganic phosphate residues ranging in size from 60 to 100 phosphate residues on average [40]. Polyphosphate leads to factor XII activation and plasma kallikrein-mediated bradykinin production from kininogen [41], thus propagating thromboinflammation. Factor XII is also expressed by neutrophils, being translocated to the cell surface upon its activation [42], thus providing a potential mechanism for polyphosphate-mediated neutrophil activation. Indeed, platelet polyphosphate contributes to neutrophil activation toward NETosis via mTOR inhibition as shown in vitro and in a mouse model of ferric chloride-induced thrombosis; this process is

regulated by the cytokine IL-29 [43]. In an animal model of sepsis, platelet polyphosphate contributed to NET-induced thrombin generation but not to NET formation [44]. Platelet polyphosphate is thus broadly relevant in a wide variety of diseases where thrombosis is a key component, either directly by promoting coagulation or indirectly via neutrophil activation toward NET formation. Polyphosphate neutralization is indeed protective against thrombosis in *in vivo* animal models [45]. Furthermore, platelet high mobility group protein B1 (HMGB1) also plays a crucial role in NET formation in the context of both venous and arterial thrombosis [33, 46, 47]. This platelet-derived danger-associated molecular pattern can be either soluble [48] or carried on platelet microparticles [49].

A substantial advance in the understanding of platelet-neutrophil interaction-mediated NET formation is the recent report of neutrophil SLC44A2 binding activated αIIbβ3 under flow [50], which provides mechanistic insight into the role of platelets in NET formation during thrombosis under conditions of shear stress. SLC44A2 (Solute Carrier Family 44 Member 2) encodes for choline transporter-like protein 2 (CTL-2), and was identified as a susceptibility locus for VTE risk [51]. In addition to the role in NET formation by neutrophils, SLC44A2 is also itself expressed by platelets and promotes their activation in response to thrombin via choline transport into mitochondria [52]. SLC44A2 therefore can contribute to thrombosis either directly within platelets, or via platelet-mediated NET formation.

Not to be ignored are the extracellular vesicles (EVs) which can be formed from activated platelets, ranging in size from 0.1 to 1 micron in diameter [53]. EVs are highly prothrombotic due to phosphatidylserine exposure upon their release which is actively performed by the scramblase TMEM16F [54]. These EVs also interact with neutrophils, resulting in their aggregation [55]. A role for platelet EVs in NET formation has been suggested in patients with systemic sclerosis, characterized by excess endothelial and

platelet activation. These patients had elevated EVs, which when incubated with neutrophils *ex vivo*, led to neutrophil autophagy and NET formation in an HMGB1-dependent manner [49]. This interaction may also play a key role in ischemic stroke, where platelet EVs were recently shown to be entrapped within NETs [56].

The impact of platelet-neutrophil interactions is not unidirectional; platelets are also affected by their interaction with neutrophils. Activated neutrophils release antimicrobial cathelicidins such as LL-37, either by degranulation or as part of NETs. These secreted cathelicidins promote platelet activation via GPVI, and this in turn promotes additional platelet-neutrophil interactions, along with NET formation by the stimulated externalization of P-selectin and release of HMGB1 [57]. In stroke, direct contact between platelets and neutrophils induces platelets to undergo necrosis, generating procoagulant platelets [58]. In acute respiratory distress syndrome second to an ischemia/reperfusion injury episode, these procoagulant platelets formed a basis for neutrophil aggregate formation in the lung by tethering neutrophils together after being dragged and ripped from the vessel wall [59]. The interplay between platelets and neutrophils is thus crucial both in infectious [44] and non-infectious disease, and constitutes a vicious cycle in immunothrombosis.

Monocytes/Macrophages

Monocytes are circulating leukocytes contributing to innate and adaptive immunity during immune defense, inflammation and tissue remodeling. Proinflammatory monocytes migrate out of the bone marrow into the bloodstream where they can become patrolling $Ly6C^{low}$ monocytes that monitor the vasculature. Alternatively, following cues from sterile or infectious insults, proinflammatory monocytes enter the tissue to differentiate into macrophages or dendritic cells to help fight bacteria or restore tissue

homeostasis. Monocytes also replenish the tissue-resident macrophage pool in the intestine in the steady state [60] and following inflammation or depletion, they also contribute to Kupffer cell repopulation in the liver [61].

Early reports showed that platelets interact with monocytes through P-selectin recruited to the platelet surface upon activation [62]. Later, the counterreceptor for P-selectin on myeloid cells was found to be a sialyl Lewis x-containing 120 kDa glycoprotein subsequently termed PSGL-1 [63]. Firm linkage is achieved through P-selectin binding to activated Mac-1 (CD11b/CD18, integrin $a_M\beta_2$) on the monocyte surface [64]. Mac-1 itself also binds coagulation factor X as well as fibrinogen [65, 66]. The latter in turn is bound to by activated integrin αIIbβ3 on the platelet surface [12]. Importantly, Mac-1 interacts with the platelet receptor GPIb through its I domain which is homologous to the VWF A1 domain [23].

Activated platelets also contribute indirectly to monocyte recruitment by stimulating monocyte chemotactic protein-1 (MCP-1) secretion and intercellular adhesion molecule-1 (ICAM-1) surface expression on endothelial cells by activating the NF-κB pathway [67]. Besides, activated platelets deposit CCL5 on the inflamed endothelium to recruit monocytes from the circulation [68]. Platelet CCL5 forms heterodimers with neutrophil HNP1 to stimulate monocyte adhesion through CCR5 ligation [69]. Platelets are the most important source of CXCL4 which is demonstrated by a striking 1000-fold increase in its serum concentration following thrombin stimulation [70]. CXCL4 triggers monocyte phagocytosis, respiratory burst, increased survival and cytokine secretion [71, 72]. Platelet-derived CXCL4 induces differentiation of monocytes into macrophages and prevents them from undergoing spontaneous apoptosis in culture [73]. In combination with IL-4, CXCL4 induces rapid differentiation

of monocytes into specialized antigen presenting cells (APC) with unique phenotypical and functional characteristics setting them apart from macrophages or conventional dendritic cells [74]. Just recently, binding of CXCL4 to leukocytes was identified to depend on Mac-1 and proteoglycans [75].

The liver contains the largest population of tissue macrophages called Kupffer cells, which reside in the vascular space firmly attached to the sinusoids [76]. Kupffer cells can bind to and remove large quantities of pathogen from the bloodstream in a very efficient way [77], for example they can capture intravascular *Borrelia burgdorferi* and prevent it from exploiting sinusoidal endothelial cells to gain access to the extravascular space [78]. Bloodborne methicillin-resistant *Staphylococcus aureus* (MRSA) is quickly trapped and killed by Kupffer cells through binding of the complement receptor of the immunoglobulin superfamily (CRIg) to lipoteichoic acid (LTA) - a major constituent of the cell wall of gram-positive bacteria [77]. A small number of Staphylococci, however, manages to survive and even proliferate inside the Kupffer cells [79]. In order to eradicate bloodborne *Bacillus cereus* and MRSA, Kupffer cells collaborate with platelets [80]: Platelets constantly perform touch-and-go interactions through GPIb with VWF constitutively expressed on Kupffer cells, presumably to check on their activation status. Upon infection, Kupffer cells capture bacteria and platelets rapidly form aggregates in an integrin αIIbβ3-dependent way to contain them. This suggests an important role for platelets in Kupffer cell-mediated bacterial clearance. Indeed, platelet depletion or GPIb deficiency resulted in severely increased mortality in mice following infection. Importantly, opsonisation with complement factor C3 was necessary for successful bacterial clearance suggesting a complex interplay between Kupffer cells, platelets and the complement system during bacterial clearance in the liver. The clearance of *Listeria monocytogenes* bloodstream infections by Kupffer cells takes place through a dual-track

mechanism: on the one hand slow clearance of bacteria-platelet complexes through platelet GPIb, CRIg and C3 opsonization and on the other hand fast clearance of free bacteria through scavenger receptors that is independent of complement and platelets [81, 82]. The slow clearance is assumed to allow some platelet-bacteria complexes to remain in the circulation long enough to be detected by splenic CD8α^+ dendritic cells to launch an antibacterial cytotoxic T cell response.

Immunothrombosis and thromboinflammation in disease

Infection/sepsis

Severe sepsis is the leading cause of in-hospital death in the United States (US) with an estimated 750,000 cases per year and most of them requiring intensive care [83]. The highest incidence rates are reported for newborns (5.3 per 1000) and senior patients (26.2 per 1000) [84]. During severe sepsis both pro- and anti-inflammatory responses aiming at eliminating the pathogen but also restricting the immune reaction to prevent excessive damage take place at the same time [85]. Sepsis is often accompanied by low platelet counts (thrombocytopenia) as well as the occlusion of small blood vessels throughout the body referred to as disseminated intravascular coagulation (DIC). The underlying mechanisms, however, are incompletely understood [85, 86]. Thrombocytopenia usually worsens the outcome of sepsis and lead to severely impaired survival and enhanced bacterial growth in blood and lungs in a mouse model of pneumonia-derived sepsis [87]. Thrombocytopenia also causes hemorrhage at the site of infection, in-line with results demonstrating that platelets maintain vascular integrity during inflammation [87, 88]. A study with more than 900 sepsis patients showed that those with very low or intermediate-low platelet counts display increased cytokine levels, endothelial cell activation and 30-

day mortality indicating that platelet count might be used as a prognostic marker during sepsis [86].

DIC and systemic platelet activation are one of the most common complications during sepsis and can contribute to organ damage. *S. aureus* causes pronounced platelet activation [89] and one of its most potent virulence factors is α-toxin (α-hemolysin, Hla). In fact, *S. aureus* sepsis severity is linked to the upregulation of virulence factors such as α-toxin [90]. α-toxin potently lyses epithelial, endothelial and immune cells through formation of a pore in the cell membrane [91, 92]. Besides its lytic activity it also binds ADAM10 (A disintegrin and metalloprotease 10) and through this causes platelet and neutrophil activation leading to the formation of platelet-neutrophil aggregates that contribute to organ damage during sepsis [93]. Intravenous α-toxin injection causes rapid platelet aggregation and the formation of thrombi in liver sinusoids and kidney glomeruli through integrin αIIb and GPIb leading to multi-organ dysfunction [94].

Investigating the transcriptional and translational changes in platelets from septic patients and mice during experimental sepsis, a recent study reports that numerous transcripts were altered and that these changes were conserved between humans and mice [95]. *ITGA2B* which encodes for integrin αIIb was one of the top upregulated transcripts. Importantly, higher platelet *ITGA2B* levels during sepsis correlated with higher mortality in both mice and humans.

Stroke

Stroke is the second leading cause of death globally and accounted for 10,2% of all deaths reported in the WHO Global Health Estimate 2016 [96]. In stroke, intracerebral hemorrhage can cause significant damage through a detrimental increase in the

intracranial pressure. Ischemic strokes can be caused by atherosclerotic plaque rupture or blood clots formed in the heart (e.g. during atrial fibrillation) – both events in which platelets are critically involved [7]. Acute treatment of ischemic stroke is by recanalization through pharmacologic thrombolysis e.g. tissue plasminogen activator (tPA) or mechanic thrombus removal (thrombectomy). Even after successful reperfusion, infarcts can continue to grow due to increased inflammation and reactive oxygen species (ROS) production - a phenomenon called reperfusion injury [97]. Experiments with *Rag1*$^{-/-}$ mice and adoptive T cell transfer established an important role for T cells in inflammation and thrombosis in the transient middle cerebral artery occlusion (tMCAO) model of experimental stroke [98]. Indeed, inducing lymphocytopenia through fingolimod (FTY720) treatment significantly reduced infarct size by reducing thromboinflammation after tMCAO [99]. Importantly, a clinical pilot study investigating the effect of fingolimod in combination with the thrombolytic standard therapy alteplase showed that the combination therapy significantly reduced the lesion size, hemorrhage incidence as well as the number of circulating lymphocytes and attenuated neurological deficits [100]. Besides their collaborations with innate immune cells, we know that platelets also interact with other immune cells like lymphocytes. CD40L recruited to the platelet surface allows them to interact with CD40 on T-cells [101]. CD40L on T cells can also engage platelet CD40 [102], promoting granule release and downstream activation [103]. T cells were recently shown to exacerbate ischemic stroke in a platelet-dependent manner, intriguingly independent of T cell antigen recognition [104]. Indeed, when platelets were depleted in *Rag1*$^{-/-}$ mice, this reversed the stroke-enhancing effect of the transfusion of $CD4^+CD25^+$ T_{reg} cells, indicating that during stroke, T_{reg} cells interact with platelets to impair tissue reperfusion [105].

Studies in the 1980s showed that following ischemia, platelets together with

coagulation factors and VWF accumulate in the brain [106, 107]. More recently, mouse studies have demonstrated the importance of the platelet VWF receptor GPIb in infarct development [108]. In contrast, blocking platelet integrin αIIbβ3 did not reduce infarct size and on the contrary increased the incidence of intracerebral hemorrhage and death. These results are in line with the outcome of a clinical trial that tested the safety and efficacy of abciximab in ischemic stroke and had to be stopped prematurely because of significantly increased hemorrhage and mortality [109]. A study using an elegant experimental design with crossed bone marrow transplantation in mice confirmed that platelet-derived VWF is an important contributor to immunothrombosis after stroke through a GPIb-dependent process [110]. In the past years another receptor has emerged as a major player in immunothrombosis after ischemia: Depleting platelet GPVI through a specific antibody led to significantly reduced infarct sizes after tMCAO [108]. Importantly, GPVI depletion did not lead to increased bleeding complications, making GPVI an attractive target for stroke therapy.

Myocardial infarction

Ischemic heart disease is caused by narrowing or blockage of the arteries which supply blood to the heart muscle, for example by arteriosclerotic plaques. This currently remains the number one cause of mortality worldwide [111], despite advances in catheter-based interventions which have substantially reduced the acute mortality of a primary myocardial infarction. Pharmaceutical-based approaches have reduced the recurrence of secondary MI episodes and allowed for prevention of events in high-risk patients, at times in combination with stent placement. The combination of statins with antiplatelet therapy is a standard of care in the prevention of MI in coronary artery disease (CAD), highlighting the importance of platelets in MI onset. In CAD patients requiring placement

of a stent, or after an MI event, dual antiplatelet therapy (DAPT) including PAR-1 receptor (vorapaxar) or P2Y12 inhibitors (either clopidogrel, ticagrelor, or prasugrel) is given in addition to aspirin. This targets ADP-mediated platelet activation in addition to inhibiting TXA_2 and thus reduces autocrine-signaling augmented platelet aggregation. However, DAPT is also associated with increased risk of major bleeding [112] and has only recently been recommended as a long-term therapy for patients with chronic coronary syndrome. The recent COMPASS trial showed a clear benefit of therapy in combining aspirin and direct factor Xa inhibition over aspirin alone in secondary prevention of MI and stroke, [113] indicating that platelet inhibition and direct anticoagulation is beneficial as a continued therapy. A direct comparison between long-term DAPT and dual antithrombotic therapy (the COMPASS protocol) has not yet been reported.

The high shear present in narrowed coronary arteries allows for the full elongation of ultra-large VWF multimers, which are highly biologically active and readily bind platelets. ST-elevation MI (STEMI), a particularly severe form of acute MI, results in an activated platelet phenotype along with elevated plasma VWF, both of which are linked to myocardial damage [114]. Coronary thrombi, classically considered to consist of mainly fibrin and platelets, also contain high amounts of VWF [115]. As in stroke, platelet GPIbα-VWF interactions are important, and may provide a novel target for pharmacologic inhibition in MI. Analysis of major thrombotic events in patients treated with caplacizumab showed protection compared to placebo [116]; however, this remains to be repeated on a larger scale and independently of thrombotic thrombocytopenic purpura, the current disease clinically indicated for caplacizumab use. In addition to GPIbα, GPVI is also a promising target. GPVI inhibition in mouse model of myocardial

ischemia/reperfusion injury was more effective than GPIbα or integrin αIIbβ3 blockade [117]. As discussed above, LL-37 released from neutrophils provides an additional factor inducing platelet activation. Within clots retrieved from MI patients, LL-37 was found not only within neutrophils, but also extracellularly, indicating that this release is occurring during thrombus formation [57]. Blocking LL-37-induced platelet activation may therefore provide an additional benefit of inhibiting GPVI in MI.

Platelets contribute not only to the formation and stabilization of culprit thrombi in coronary arteries, but also to the sequelae via their releasate. Platelet TGFβ, present in α-granules, was shown in an animal model to be a strong contributing factor to the development of fibrosis in pressure-overload injury [118], and may be an important source of latent TGFβ driving scar tissue formation in MI [119]. Despite major progress in prevention of primary or secondary MI, mortality remains high due to the high prevalence of heart failure in patients post-MI. Neutrophils may provide yet another link between platelets and development of fibrosis by the release of NETs [120], and the study of the interplay between the two is warranted. Platelet microparticles produced in MI also provide an important communication mechanism via transfer of miRNA, leading to upregulation of the adhesion molecule ICAM-1 in endothelial cells [121]. Platelets therefore do not only contribute to MI pathology in a growing vessel occlusion, but also by promoting further endothelial and leukocyte activation, as well as providing soluble factors driving tissue remodeling toward fibrosis.

The role of infection in MI onset has also become more apparent in recent years. Cardiac complications are a frequent complication of community-acquired pneumonia, particularly among those requiring hospitalization [122]. Among pneumonia patients with elevated troponin levels, those presenting with signs of MI had higher biomarkers of platelet activation compared to those without, including elevated thromboxane levels

[123]. These were elevated irrespective of aspirin use, suggesting that this aspirin regimen is insufficient to limit thromboxane production in these patients. Rates of acute MI are also elevated after influenza infection [124]. Influenza can be internalized by platelets, resulting in platelet-mediated NET formation [125]. Platelets and NETs are a common feature found together in coronary thrombi, and HMGB1 externalized by platelets are likely a major driver of NET formation in MI [33]. In summary, the evaluation of platelets beyond their pro-thrombotic role will be of interest in the coming years.

Venous thromboembolism

Venous thromboembolism (VTE) encompasses the thrombotic events occurring in large veins (deep vein thrombosis) or as a result of embolization from these veins (pulmonary embolism). VTE was previously considered to occur independently of platelet activation; however, insight in recent years from animal models has led to a greater understanding of the contribution of platelets to thrombus initiation and progression [126]. Furthermore, despite the lack of efficacy for antiplatelet therapy in VTE prevention, the ASPIRE [127] and WARFASA [128] trials showed a clear reduction in recurrence of VTE or major vascular events with low-dose aspirin.

Von Willebrand factor, by its interaction with GPIbα, is crucial for platelet accumulation along activated endothelium, driving the onset of thrombus initiation in a mouse model of inferior vena cava flow restriction [129]. Local hypoxia, such as that which occurs in VTE by stasis, can trigger the release of Weibel-Palade bodies from endothelial cells [130]. Systemic hypoxia is a known risk factor for VTE; this was replicated in a mouse model combining hypoxia-reoxygenation with partial IVC ligation [131]. In vitro, platelets exposed to hypoxic conditions had augmented responses to P2Y12, PAR1, or thromboxane receptor agonists via activation of the redox sensor

ERK5, which in turn elevated reactive oxygen species production [132]. Simulated hypoxia in rats altered the platelet proteome and increased intraplatelet Ca^{2+} and calpain activity levels, leading to platelet hyperreactivity and a prothrombotic phenotype [133]. The combination of primed platelets along with augmented leukocyte and endothelial cell activation in the hypoxic milieu thus provides optimal conditions for thrombus development.

In contrast to arterial thrombosis, where homotypic platelet interactions are crucial first steps in thrombus initiation, in venous thrombosis platelets likely play their major role upon interacting with leukocytes. GPIbα-deficiency reduced platelet recruitment along the endothelium, but even more drastically reduced leukocyte accumulation [134]. Platelet-neutrophil interactions contribute to the formation of NETs in venous thrombosis, both by direct interaction [134] and by release of platelet HMGB1 [48]. Either GPIbα deficiency or platelet depletion nearly completely prevented thrombus formation altogether, indicating that platelets are indispensable for the leukocyte-mediated thrombus initiation [134]. The interaction between platelet CLEC2 and endothelial podoplanin has also been shown to contribute in the same animal model [135], and whether this impacts NET formation remains to be seen.

Alternating erythrocyte-rich and platelet-rich zones termed lines of Zahn are considered hallmarks of early venous thrombi. Histological analysis of thrombus samples obtained from human VTE patients analyzed for leukocytes and NETs also showed platelet/VWF-rich islands surrounded by NETting neutrophils [136]. Neither these structures nor platelets are present in more organized deep vein thrombi, indicating that they are not essential to the maintenance of thrombus stability long-term and that any potential platelet or NET mediated therapies would need to be initiated early. These organizes structures also do not support the hypothesis that platelets are merely

bystanders trapped in a surrounding venous thrombus. Furthermore, the active role of platelets in pulmonary embolism is certainly not to be overlooked [137]. In contrast to more organized pulmonary emboli which resemble DVT thrombi, autopsy samples from patients who died of sudden-onset pulmonary embolism show a paucity of red blood cells and mainly consist of fibrin and FVIII, lined with platelet aggregates [138].

VTE and Infection in COVID-19

The contribution of infection to venous thrombus formation is becoming more and more relevant with the new experimental and histopathological evidence pointing toward a key interplay between platelets and immune cells. Infection, especially in combination with immobilization, provided a clear trigger for VTE in hospitalized patients [139]. This is particularly relevant today in the recently emerged coronavirus disease 2019 (COVID-19), where hallmarks of severe disease leading to mortality in hospitalized patients are highly elevated D-dimer level [140], increased cytokine levels (IL-6, IL-10, and TNF-α) [141] and an alarming rate of VTE incidence despite prophylactic anticoagulation as standard of care [142]. Platelet-rich thrombi have been found in the pulmonary, hepatic, renal and cardiac microvasculature suggesting an important role for COVID-19-induced coagulopathy [143–145]. *Ex vivo* platelet aggregation is also increased in COVID-19 patients, indicating potential platelet hyperreactivity which may contribute to COVID-19 pathology [146]. Indeed, increased P-selectin expression on circulating platelets as well as increased platelet-neutrophil and -monocyte aggregates were reported in COVID-19 patients [146].

In addition to platelets, neutrophils may be key drivers of COVID-19 pathology both within thrombi or within the lung. COVID-19 patients with neutrophilia have a poorer prognosis [147] and neutrophil infiltration were reported in autopsies of COVID-

19 patients [148, 149]. Recent studies identified elevated NETs in circulation by analyzing plasma samples from COVID-19 patients for MPO-DNA complexes, as well as demonstrating increased propensity to form NETs in neutrophils isolated from COVID-19 patients [150]. Elevated NETs in COVID-19 were first reported in serum samples [151], although interpretation of serum NETs as an indicator of circulating NETs should be done with caution, particularly in hyperinflammatory conditions. This study did, however, convincingly show that COVID-19 patient serum activates healthy neutrophils to form NETs *in vitro,* providing evidence that supports that COVID-19 provides a hospitable milieu for NET formation within the bloodstream. Indeed, neutrophils undergoing NETosis were identified within thrombi in the lung vasculature in autopsy specimens, in proximity to platelets [150]. It still remains to be evaluated if NETs are key contributors to the thrombus scaffold in COVID-19. Lastly, an even higher level of MPO-DNA complexes were detected within tracheal aspirates of COVID-19 patients as compared to plasma [150], raising the question of whether NETs can also contribute to lung pathology by forming within the bronchoalveolar space.

Several pulmonary diseases are associated with excessive NETosis. NETs can aggravate acute lung injury (ALI) and ARDS [152, 153] and in patients with acute respiratory failure during Chronic Obstructive Pulmonary Disease (COPD) exacerbation NET levels are elevated [154]. Degrading NETs can relieve ARDS in mice [152, 155] and may present an attractive therapeutic option for NET-mediated lung injury in COVID-19 in the form of FDA-approved Dornase alfa. Taken together, there is accumulating evidence that NET formation and platelet activation/coagulation might play a key role in the pathogenesis of severe COVID-19.

Conclusion/Summary

Platelets are no longer seen as single-purpose single-use cell fragments. Today we know that in fact they interact with most cells of the (innate) immune system and release a plethora of cytokines to stimulate the immune response at sites of vascular injury. In that, they are much like hemocytes, their ancient precursor found in horseshoe crabs - a living fossil that already existed 445 million years ago. Hemocytes are nucleated granular cells with combined innate immune cell/platelet function and the only circulating blood cells in horseshoe crabs. They can fight infections and cause coagulation of the hemolymph to seal off infected or injured vessels. These intriguing similarities indicate that there are probably more immune cell functions left in the platelet than most people today are aware of and probably some more new roles to be discovered.

While the interactions between platelets and cells of the innate immune system are getting more and more attention, not much is known about the impact of platelets on lymphocyte function. Platelet express large amounts of CD40L which would make them ideal binding partners for T cells. The implications of this are promising since it might open new avenues not only in cardiovascular but also in cancer research, where platelets can hinder lymphocyte-mediated antitumoral responses [156, 157].

Acknowledgements

The authors would like to thank PD Dr. Thilo Witsch for helpful discussions about antiplatelet therapy in myocardial infarction and Dr. Sirima Kraisin for assistance with figure preparation.

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Figure legends

Figure 1. Key interactions between platelets and neutrophils or macrophages. Interactions occur via direct contact between cell surface receptors, or by binding of secreted ligands. These interactions are either the result of or cause of activation by either platelets, leukocytes, or both. Some interactions are dependent on an intermediate partner (i.e. fibrinogen). GPIb – glycoprotein 1b, GPVI – glycoprotein VI, HMGB1 – high mobility group protein B1, ICAM-2 – intracellular adhesion molecule 2, JAM-3 – junctional adhesion molecule 3, LL-37 – human cathelicidin antimicrobial peptide, Mac-1 – macrophage-1 antigen (integrin αMβ2), P-sel – P-selectin, PSGL-1 – P-selectin glycoprotein ligand, RAGE – receptor of advanced glycation end products, SLC44A2 – solute carrier family 44 member 2, VWF – von Willebrand factor. Created with Biorender.com and Servier Medical Arts.