Platelet–neutrophil interactions under thromboinflammatory conditions

Jing Li · Kyungho Kim · Andrew Barazia ·
Alan Tseng · Jaehyung Cho

Received: 23 November 2014 / Revised: 7 January 2015 / Accepted: 26 January 2015
© Springer Basel 2015

Abstract Platelets primarily mediate hemostasis and thrombosis, whereas leukocytes are responsible for immune responses. Since platelets interact with leukocytes at the site of vascular injury, thrombosis and vascular inflammation are closely intertwined and occur consecutively. Recent studies using real-time imaging technology demonstrated that platelet–neutrophil interactions on the activated endothelium are an important determinant of microvascular occlusion during thromboinflammatory disease in which inflammation is coupled to thrombosis. Although the major receptors and counter receptors have been identified, it remains poorly understood how heterotypic platelet–neutrophil interactions are regulated under disease conditions. This review discusses our current understanding of the regulatory mechanisms of platelet–neutrophil interactions in thromboinflammatory disease.

Keywords Vascular disease · Cell–cell interactions · Regulatory mechanisms · Surface receptors · Intracellular signaling · Sickle cell disease · Ischemic stroke

Introduction

Cardiovascular disease is the number one killer in Western society. Activated platelets, leukocytes, and endothelial cells (ECs) contribute to the pathogenesis of the disease. During vascular inflammation, neutrophils roll over the activated ECs through the interaction between selectins and their ligands, followed by adhesion to the ECs, which is mediated by the interaction between $\beta 2$ integrins and ICAM-1 [1]. Subsequently, activated $\alpha L\beta 2$ and $\alpha M\beta 2$ integrins interact with intercellular adhesion molecule-1 (ICAM-1) on the activated endothelium, thereby inducing neutrophil adhesion and crawling, respectively [2, 3]. In the presence of chemotactic stimuli, adherent neutrophils rapidly transmigrate across ECs. It was reported that several EC surface molecules, including platelet–EC adhesion molecule-1 (PECAM-1), CD99, EC-selective adhesion molecule (ESAM), and junctional adhesion molecules (JAMs), control this process [3–7]. Recent intravital microscopic studies have provided compelling evidence that activated neutrophils adherent to inflamed ECs can support homotypic and heterotypic cell–cell interactions and that platelet–neutrophil aggregation on activated ECs is the crucial determinant of microvascular occlusion during vascular inflammation (Fig. 1) [8, 9]. The heterotypic platelet–neutrophil interactions are mainly mediated by binding of neutrophil P-selectin glycoprotein ligand-1 (PSGL-1) and $\alpha M\beta 2$ integrin to platelet P-selectin and glycoprotein Ib α (GPIb α), respectively [8–12]. While neutrophils attach to the inflamed venules under low blood shear, platelets adhere to activated ECs and sub-endothelial matrix proteins such as collagen and von Willebrand factor (vWF) under high blood shear and then support neutrophil rolling and adhesion as well as platelet accumulation following arterial injury [13–15]. Although neutrophils and

J. Li, K. Kim, and A. Barazia contributed equally to this review.

J. Li · K. Kim · A. Barazia · A. Tseng · J. Cho (✉)
Department of Pharmacology, University of Illinois College of
Medicine, 835 S. Wolcott Ave, E403, Chicago, IL 60612, USA
e-mail: thromres@uic.edu

A. Tseng · J. Cho
Department of Anesthesiology, University of Illinois College of
Medicine, 835 S. Wolcott Ave, E403, Chicago, IL 60612, USA

Fig. 1 Heterotypic cell-cell interactions during vascular inflammation. **a** During vascular inflammation, neutrophil rolling over and adhesion to the activated ECs are mediated by selectins-their ligands and $\beta 2$ integrins-ICAM-1, respectively. These adherent and crawling neutrophils allow for platelet adhesion and accumulation. **b** During arterial thrombosis, platelets adhere to vWF and collagen through GPIb/IX/V complex and GPVI, respectively, thereby inducing platelet aggregation. The adherent platelets support neutrophil rolling and adhesion via the receptor-counter receptor interaction. **c** The receptor and counter receptors of heterotypic neutrophil-platelet

interactions. Heterotypic interactions are mainly mediated by the interactions of P-selectin with PSGL-1 and $\alpha M\beta 2$ integrin with GPIb α . Other molecules also contribute to heterotypic interactions, such as platelet JAM-3 binding to neutrophil $\alpha M\beta 2$ integrin. Platelet $\alpha IIb\beta 3$ integrin can interact with neutrophil $\alpha M\beta 2$ integrin through fibrinogen. **d** Heterotypic EC-neutrophil-platelet interactions can lead to occlusion in microvessels during thromboinflammation. In addition to EC-neutrophil-platelet interactions, RBCs may be trapped and incorporated into cell-cell aggregates

platelets preferentially adhere to the site of vascular injury under low and high shear conditions, respectively, the receptors and counter receptors for heterotypic cell-cell interactions are similar under both conditions [16].

Tissue and vascular injuries activate ECs, resulting in not only the expression of adhesion molecules including P- and E-selectins, ICAMs, and vascular cell adhesion molecule-1 (VCAM-1) but also the production and release of vWF, reactive oxygen species (ROS), and inflammatory cytokines [3, 17–19]. Neutrophil recruitment to inflamed ECs is critical for vascular inflammation since activated neutrophils are the main source of ROS [19] and numerous enzymes including peptidylarginine deiminase 4 (PAD4), elastase, and cathepsin G [20, 21], thereby linking inflammatory and thrombotic responses and aggravating

the disease conditions. Our recent studies showed that in addition to neutrophil adhesion to ECs, platelet-neutrophil interactions play a crucial role in slowing blood flow rates and mediating occlusion of inflamed venules [8]. Activated and adherent platelets express numerous surface receptors (GPIb α , $\alpha IIb\beta 3$, CD40, and toll-like receptors) and release diverse granular molecules (P-selectin, ADP, and platelet factor 4) and cytokines (interleukin-1, RANTES, platelet-derived growth factor, transforming growth factor- β , and epidermal growth factor) [22–25], which enhance inflammatory responses. Further, platelets are enriched with numerous chemokines (CXCL4, CXCL8, and CCL2) and express their corresponding receptors [25, 26]. Therefore, platelets and neutrophils cooperate to propagate the pathogenesis of thromboinflammation.

Unlike arterial thrombosis, which is primarily mediated by platelet aggregation and well inhibited by antagonists of $\alpha\text{IIb}\beta 3$ integrin [27], preclinical and clinical studies have suggested that coagulation cascades, platelets, and leukocytes should be considered for intervention in thromboinflammatory disease [28–34]. Since thrombosis and inflammation occur in numerous vessels and organs under several pathological conditions, including vascular inflammation [8, 35], ischemia/reperfusion injury [36, 37], transfusion-related acute lung injury [38], atherosclerosis [39, 40], and cell transplantation and therapies [41], understanding of the detailed mechanisms mediating thromboinflammation could lead to the identification of an effective therapeutic target. In this review, we will focus on major surface receptors and signaling pathways that regulate heterotypic platelet–neutrophil interactions under thromboinflammatory conditions and also summarize how the platelet–neutrophil interaction participates in the initiation and propagation of the disease.

Surface receptors mediating platelet–neutrophil interactions

P-selectin and PSGL-1

As an important marker of platelet activation, P-selectin is stored in α -granules of resting platelets and exposed on the surface upon agonist stimulation [42]. Binding of platelet P-selectin to neutrophil PSGL-1 is required for the initial contact between both cells. In addition, neutrophil PSGL-1 interacts with EC P- and E-selectins, which mediates tethering and rolling of neutrophils and induces activation of protein kinases such as Syk and phosphoinositide-3-kinase (PI3K), integrin activation, and cytoskeletal remodeling [43]. Furthermore, circulating microparticles mainly derived from monocytes binds to platelet P-selectin through PSGL-1, thereby accumulating tissue factor and generating thrombin [44]. Studies with mice producing high levels of soluble P-selectin demonstrated that binding of P-selectin to leukocyte PSGL-1 enhances the plasma concentration of procoagulant microparticles [45]. These results suggest that the interaction between P-selectin and PSGL-1 is critical for promoting thrombus formation. Biochemical studies revealed that the sulfation of the N-terminal Tyr 46, 48, and 51 residues and O-glycosylation of Thr 57 in PSGL1 are important for the interaction with the C-type lectin domain of selectins [46, 47]. The sialyl Lewis X moiety synthesized by fucosyltransferase VII is also required for the binding of PSGL-1 to selectins [48]. Further, the phosphorylation of the N-terminal Tyr residues is important for PSGL-1 binding to selectins under shear conditions [49].

Intravital microscopic studies with P-selectin- or PSGL-1-deficient mice demonstrated that the P-selectin–PSGL-1 interaction is crucial for leukocyte rolling over platelet thrombi at the site of arteriolar injury [15] and that EC P-selectin also mediates initial rapid rolling of leukocytes through PSGL-1 [10, 50]. Consistently, *in vitro* studies also suggested that inhibition and deletion of P-selectin and PSGL-1 abolish the initial interaction between neutrophils and platelets and the subsequent activation of signaling molecules in both cells [51–54]. Studies with P-selectin-deficient mice revealed that monocyte-derived microparticles incorporate into the developing thrombus through the P-selectin–PSGL-1 interaction following arteriolar injury [55], which further mediates fibrin generation. Recently, Sreeramkumar and colleagues [35] have reported that inhibition and deletion of PSGL-1 impair platelet attachment at the uropod and that platelet–neutrophil interactions through PSGL-1 and P-selectin are critical for triggering vascular disease such as ischemic stroke. Therefore, there is no doubt that platelet P-selectin and neutrophil PSGL-1 contribute to the initial association between both cells, thereby transducing signaling pathways and activating other surface molecules such as integrins during thrombosis and vascular inflammation.

Glycoprotein Ib α (GPIb α) and $\alpha\text{M}\beta 2$ integrin

The GPIb/IX/V complex is a key platelet receptor binding to vWF at the site of vascular injury, thereby initiating platelet adhesion and accumulation under high shear conditions [56, 57]. It comprises four transmembrane proteins: GPIb α , GPIb β , GPIX and GPV (2:2:2:1 ratio). The N-terminal region of GPIb α binds to several ligands including vWF, $\alpha\text{M}\beta 2$ integrin, thrombin, and P-selectin [12, 58–61]. Neutrophil $\alpha\text{M}\beta 2$ (macrophage-1 antigen, Mac-1) is a promiscuous integrin interacting with numerous ligands including ICAMs on ECs, plasma proteins (fibrinogen and factor X), complement pathway product (C3bi), extracellular matrix proteins (fibronectin, laminin, collagen, and vitronectin), and platelet GPIb α [62]. $\alpha\text{M}\beta 2$ integrin regulates a variety of neutrophil functions such as crawling [63, 64], chemotaxis [65], survival [66], apoptosis [67], and neutrophil extracellular trap (NET) formation [68]. The major binding site for most ligands is located in the inserted (I) domain in the αM subunit. Consistently, *in vitro* studies suggested that the N-terminal region (Phe201–Gly268) of GPIb α binds to the I domain of $\alpha\text{M}\beta 2$ integrin, thereby inducing stable and firm association between platelets and neutrophils [12]. *In vitro* and *in vivo* studies demonstrated that deletion or inhibition of $\alpha\text{M}\beta 2$ integrin and GPIb α abolishes the interaction of platelets with neutrophils or monocytes under inflammatory conditions [9, 12, 69]. Recently, we demonstrated using real-

time intravital microscopy that α M deletion improves blood flow rates during TNF- α -induced cremaster venular inflammation [8]. Thus, GPIb α - α M β 2 association is critical for platelet–neutrophil interactions and microvessel occlusion during vascular inflammation. Although the ligand binding and downstream signaling pathway through the GPIb/IX/V complex and α M β 2 integrin have been studied [58, 62, 70, 71], it remains elusive how the ligand-binding function of both molecules is regulated during thromboinflammatory disease.

α IIb β 3 integrin

α IIb β 3 integrin is the most abundant platelet receptor (80,000–100,000 copies per platelet) and necessary for platelet aggregation via interaction with fibrinogen. Studies with blocking antibodies and platelets from a patient with Glanzmann's thrombasthenia revealed that the interaction of α M β 2 integrin with fibrinogen bound to α IIb β 3 integrin mediates neutrophil adhesion to adherent platelets under flow conditions [72]. However, we and others showed that inhibition of α IIb β 3 integrin does not reduce neutrophil–platelet interactions under static and stirring conditions [8, 52, 73]. Thus, despite its necessity during platelet thrombus formation, it is controversial whether platelet α IIb β 3 integrin is required for platelet–neutrophil interactions under inflammatory conditions.

Junctional adhesion molecule-3 (JAM-3)

JAM-3 (also known as JAM-C) is expressed on human platelets and ECs in the vasculature [74–76]. It was reported that inhibition of platelet JAM-3 with blocking antibodies impairs its binding to leukocyte α M β 2 integrin [76]. Interestingly, a combination of anti-GPIb α and anti-JAM-3 antibodies showed additive inhibitory effects on platelet–neutrophil interactions under static conditions, suggesting that both JAM-3 and GPIb α are important for the interaction with neutrophil α M β 2 integrin. Intravascular JAM-3 also regulates leukocyte recruitment and transmigration during inflammation, arthritis, and atherosclerosis [75, 77–80].

CD40 ligand (CD40L) and CD40

CD40L (also known as CD154), a transmembrane protein of the TNF- α family, plays a critical role during inflammation and thrombosis upon the interaction with CD40, a member of the TNF receptor family [81, 82]. CD40L and CD40 are expressed in many cell types including leukocytes and ECs [83]. Because of the enrichment in platelets, most circulating CD40L is derived from activated platelets through shedding by an unidentified enzyme(s) [84, 85].

Since soluble CD40L is biologically active and retains the ability to bind CD40, it has been thought to be a biomarker for vascular diseases [86, 87]. It was reported that circulating soluble CD40L mediates neutrophil–platelet interactions in acute coronary syndrome [88]. Consistently, studies using blocking antibodies and CD40L-deficient mice revealed that the interaction of soluble CD40L with platelet and neutrophil CD40 enhances the expression of P-selectin and α M β 2 integrin [89–91] and mediates platelet–neutrophil aggregation. Further, the interaction between platelet CD40L and neutrophil CD40 enhances ROS generation and thus aggravates oxidative stress [92]. Previous studies demonstrated that platelet CD40L also binds to EC CD40, thereby up-regulating the expression of E-selectin, VCAM-1, and ICAM-1 and inducing the secretion of chemokines such as interleukin-8 [81]. Thus, the CD40L–CD40 interactions increase the surface expression of P-selectin and α M β 2 integrin via granular secretion, thereby regulating platelet–neutrophil association during thromboinflammatory disease.

Toll-like receptors (TLRs)

Toll-like receptors are a family of innate immune system receptors that mediate the host response to infection [93]. Among many isoforms, previous studies using a TLR4 receptor antagonist and blocking antibodies suggested that platelet TLR4 is required for LPS-induced platelet–neutrophil interactions and that this interaction enhances neutrophil activation and NET formation, thereby trapping bacteria in the vasculature [94].

Consistently, other studies showed that Shiga toxin and LPS induce tissue factor expression and platelet–leukocyte aggregation in hemolytic uremic syndrome [95]. LPS–TLR4 binding causes the release of sCD40L from platelets [96, 97], which is inhibited by blocking anti-TLR4 antibodies [97]. However, earlier studies by Montrucchio and colleagues [98] suggested that LPS directly binds to leukocytes but not platelets and that LPS-induced platelet–leukocyte interactions are mediated by leukocyte activation.

It was also reported that platelet TLR2 is important for platelet–neutrophil aggregation in response to gram-negative bacteria and periodontitis [99, 100]. Recent studies suggested that injection of a TLR7 agonist into mice results in platelet–neutrophil aggregation through activation of platelet TLR7 [101]. In neutrophils, most TLRs except TLR3 are expressed [102]. Similar to platelet TLRs, neutrophil TLR isoforms recognize numerous microbial molecules including LPS (for TLR4) or peptidoglycans (for TLR2), thereby enhancing the production of ROS, cytokines, and chemokines [103]. Although each platelet and neutrophil TLR isoform may play a distinct role during

inflammatory diseases, the detailed mechanisms of TLR signaling in inducing platelet–neutrophil interactions remains to be determined.

α L β 2 integrin

α L β 2 integrin is a key receptor for neutrophil adhesion to and emigration across activated ECs during inflammation [63, 104]. In vitro studies using blocking antibodies showed that binding of neutrophil α L β 2 integrin to platelet ICAM-2 regulates neutrophil–platelet attachment under flow conditions [105]. Nevertheless, the importance of α L β 2–ICAM-2 binding for platelet–neutrophil interactions has not yet been determined in vivo.

CD24

CD24 is a mucin-type glycosylphosphatidylinositol-linked protein expressed on the surface of neutrophils and tumor cells [106, 107]. While one study showed that neutrophil CD24 is not essential for the initial binding to P-selectin [108], other studies implicated that neutrophil and tumor cell CD24 bind to platelet or EC P-selectin in a manner dependent on divalent cations and that CD24–P-selectin binding may cause platelet–neutrophil interactions under disease conditions such as inflammation and cancer metastasis [106].

Molecules regulating the function of surface receptors required for platelet–neutrophil interactions

Following vascular injury, platelets and neutrophils are activated by soluble agonists and adhesive proteins via their surface receptors (Figs. 2, 3) [102, 109]. Ligand–receptor interactions stimulate diverse signaling pathways such as activation of G-proteins, phospholipase C, protein and lipid kinases, Ca^{2+} mobilization, and cytoskeletal rearrangement. Importantly, those signaling molecules regulate the function of surface receptors, thereby affecting platelet–neutrophil interactions. Thus, understanding how intracellular signaling modulates platelet–neutrophil interactions will help identify novel therapeutic targets to prevent and treat vascular occlusion in thromboinflammatory disease.

Src family kinases (SFKs)

It is known that there are 7–8 members of SFKs in human platelets and neutrophils [110, 111]. Inhibition of SFKs with PP2, a broad-spectrum SFK inhibitor, partially impaired thrombin-mediated P-selectin exposure and α IIB β 3 integrin activation [112]. Further, studies with SFK

inhibitors and isoform-specific KO mice suggested that SFKs such as Lyn and Fyn affect vWF–GPIIb α binding and thus regulate platelet activation including Ca^{2+} mobilization and cytoskeletal reorganization [113–115]. SFKs are also activated by the initial platelet–neutrophil attachment, and activated SFKs stabilize the cell–cell interaction [116, 117]. Studies with mice lacking double (Hck and Fgr) or triple (Hck, Fgr, and Lyn) SFKs suggested that the SFK–Pyk2 signaling axis modulates α M β 2 integrin function and thus is important for neutrophil accumulation to adherent platelets in vitro and in vivo [118]. More recent studies demonstrated that mice deficient in Hck, Fgr, and Lyn show remarkable defects in neutrophil recruitment as shown in β 2 integrin-null mice, suggesting the critical role of the three SFKs in neutrophil adhesive function [119]. Inhibition of SFKs with PP1 impaired α M β 2 integrin clustering and cytoskeletal reorganization in neutrophils [116]. Further, P-selectin-mediated crosslinking of PSGL-1 results in α M β 2 integrin activation in a SFKs-dependent manner [117]. Interestingly, it was reported that the plasma levels of soluble P-selectin increases in patients with arterial occlusive disease [120], which would enhance platelet–neutrophil aggregation during vascular disease. Although SFKs play important roles in regulating the function of platelet and neutrophil surface receptors, most studies have been carried out using non-specific inhibitors. Therefore, studies using isoform-specific and multiple KO mice are required to determine the distinct and redundant role of each isoform in platelet–neutrophil interactions.

Phospholipase (PLC)

Phospholipase hydrolyzes membrane PIP_2 into IP_3 and diacylglycerol, thereby increasing the cytosolic Ca^{2+} level and activating PKC, respectively [121]. Among PLC isoforms that are activated by an agonist, PLC β 2/3 and PLC γ 2 are the major isoforms and are activated by Gq α /Gi β γ - and immunoreceptor tyrosine-based activation motif (ITAM)-regulated signaling pathways, respectively, which have been described extensively in other reviews [122, 123]. In platelets, binding of thrombin and TXA2 to their receptors activates PLC β 2 through Gq α , whereas the collagen–GPVI interaction stimulates PLC γ 2 through scaffold proteins and numerous tyrosine kinases [122]. Due to the effect on cytosolic Ca^{2+} levels, PLC is critical for regulating the early events of platelet activation. Previous studies suggested that PLC β -regulated AKT activity may mediate P-selectin exposure on thrombin-stimulated platelets [124] and that PLC γ 2 activation through GPVI signaling is critical for the increase in cytosolic Ca^{2+} and α IIB β 3 integrin activation [125, 126]. Furthermore, studies using PLC γ 2 KO mice showed the important role of PLC γ 2 in increasing cytosolic Ca^{2+} through the vWF–GPIIb/IX/V interaction

Fig. 2 Receptor-mediated signaling pathways in neutrophils. Under inflammatory conditions, neutrophils can be activated by initial rolling on activated endothelium and by soluble ligands such as TNF- α . TNFR signaling stimulates NF- κ B-mediated gene transcription, regulating the expression of numerous pro-inflammatory proteins. GPCR stimulation leads to inhibition of adenylyl cyclase (AC) through G α i and activation of PI3K and PLC β through G β γ i. Rac1/2 activity and activated Akt and PKC phosphorylate p47^{phox}, inducing ROS generation through the NOX2 complex. PLC β hydrolyzes PIP2 to form IP3 and DAG which in turn mediates calcium release from the ER and DAG-sensitive PKC activation, respectively. Depletion of ER calcium induces STIM1 clustering at the ER-PM junction, interacting

with ORAI1, and allowing calcium influx. Fc γ R signaling leads to ITAM- and Syk-mediated PLC γ 2 activation that induces similar downstream effects to those seen from GPCRs. TLR activation leads to NF- κ B activation, as seen in TNFR activation. The signaling pathways of GPCRs, Fc γ R, and TLR enhance MAPK activity and cytosolic calcium levels. During cell activation, exocytosis-mediated granular secretion also induces the membrane translocation of α M β 2 integrin. Neutrophil activation further release histones and form neutrophil extracellular traps (NETs). In addition to DNA and histones, NETs form a scaffold for proteases and trap circulating bacteria and platelets

[114]. In neutrophils, binding of an agonist, such as fMLF, to the receptor mediates PLC β 2/3 activation through G β γ [123]. Previous studies demonstrated that deletion of PLC β 2 and/or PLC β 3 abrogates IP3 production, increase in cytosolic Ca²⁺, PKC activation, α M β 2 up-regulation, and ROS generation following fMLF stimulation [127, 128]. Although the importance of PLC for platelet and neutrophil activation has been clearly reported, it remains unclear how each PLC isoform regulates platelet–neutrophil interactions during thromboinflammatory disease.

Phosphatidylinositol 3-kinase (PI3K)-AKT

Phosphatidylinositol 3-kinase phosphorylates PI on the third carbon and is composed of three different classes, class I–III, based on the structure, lipid substrate specificity, and regulation [129, 130]. PI-3,4,5-trisphosphate (PIP3) is one of the PI3K products which is required for the membrane association and activation of AKT and PLC γ 2

[131, 132]. Phosphatase and tensin homolog (PTEN) and SH2 domain-containing inositol 5-phosphatase 1 (SHIP1) regulate the levels of PI3K products [133–135]. Previous studies with PI3K inhibitors revealed that PI3K does not affect P-selectin exocytosis and the kinetics of actin assembly during platelet activation [136–138]. However, PI3K p85 α -null platelets showed a defect in P-selectin exposure induced by GPVI- but not Gq- and Gi-mediated signaling [139]. Other studies revealed that PI3K plays an important role in vWF-GPIb α binding, intracellular Ca²⁺ mobilization, and platelet activation under shear but not static conditions [140], suggesting the important role of PI3K under in vivo pathological conditions. Studies with a cell membrane-permeable, dominant-negative form of the class IA PI3K p85 α suggested that PI3K regulates α M β 2-mediated neutrophil adhesion and NADPH oxidase 2 (NOX2) activity [141]. Consistently, inhibition of PI3K with LY294002 significantly impairs neutrophil ROS generation and fMLF-induced neutrophil–platelet

Fig. 3 Receptor-mediated signaling pathways in platelets. Numerous agonists stimulate platelets through their receptors. Gq α is able to stimulate both Lyn and PLC β . Lyn then phosphorylates PI3K, leading to PIP₃ generation and AKT phosphorylation. Activated AKT stimulates NOS-PKG-MAPK signaling. PLC β activation allows for generation of DAG and IP₃ from PIP₂. IP₃ then binds to the IP₃R on the dense tubular system, inducing calcium release into the cytosol. Both DAG and calcium can activate PKC, leading to α IIb β 3 integrin activation. Increases in cytosolic calcium also lead to integrin

activation through activation of CalDAG-GEF1. Other GPCRs on the platelet membrane are the P2Y receptors for ADP, the 5-HT receptor for serotonin, and the thromboxane receptor for TXA₂. Stimulation of these receptors also induces platelet aggregation through their corresponding G-proteins. Platelet ITAMs, such as Fc γ R and GPVI, signal through Syk-SLP-76-ADAP and Lyn/Fyn, respectively. Both of these receptors lead to activation of PLC γ 2, thus generating IP₃ and DAG and inducing platelet aggregation and granule secretion as described above

interactions [92]. It was reported that PSGL-1-mediated neutrophil rolling over E-selectin activates the spleen tyrosine kinase (Syk), and Syk-mediated integrin function in part requires the PI3K γ -AKT signaling [142]. Further studies are required to determine the role of each PI3K isoform in regulating P-selectin exposure and α M β 2 integrin function.

AKT is a well-known downstream molecule of PI3K. Each AKT isoform, AKT1-3, plays an overlapping and distinct role during platelet activation [143–145]. Platelet AKT1 regulates thrombin-induced P-selectin exposure and intracellular Ca²⁺ release, thereby affecting α IIb β 3 integrin activation [8, 143]. Similarly, platelet AKT2 and 3 are important for P-selectin exposure and α IIb β 3 integrin activation induced by low concentrations of thrombin or U46619 [8, 144, 145]. It is of interest to note that platelet AKT isoforms, but not PI3K, regulate P-selectin exposure

following thrombin stimulation [8, 136, 138, 143, 144], suggesting that platelet α -granule secretion is differentially regulated by PI3K and AKT. Unlike platelets, neutrophils express only AKT1 and 2, and previous studies showed that AKT2 modulates NOX2 activity and ROS generation during neutrophil activation [146]. PI3K-AKT signaling also mediates P-selectin exposure induced by stimulation of platelet TLR2, thereby affecting platelet–neutrophil interactions [99]. Importantly, our recent studies using *Akt* isoform-specific KO mice and their bone marrow chimera demonstrated that neutrophil AKT2 plays a critical role in intracellular Ca²⁺ release and the membrane translocation and activation of α M β 2 integrin, thereby controlling neutrophil–platelet interactions during vascular inflammation [8]. These results indicate that platelet and neutrophil AKT are critical for regulating platelet–neutrophil interactions during vascular disease.

Protein kinase C (PKC)

The PKC family is composed of three subfamilies based on the requirement for second messengers (Ca^{2+} , diacylglycerol, and phospholipids) [147]. A broad-spectrum PKC inhibitor, Ro-31-8220 partially inhibited P-selectin exposure and $\alpha\text{IIb}\beta 3$ integrin activation in AYPGKF-stimulated P_2Y_{12} -deficient platelets [112]. Studies using isoform-specific PKC inhibitors suggested that some of the novel and atypical PKC isoforms regulate P-selectin exposure on thrombin-activated platelets and platelet–neutrophil interactions [138]. Atypical PKC ζ colocalizes with $\alpha\text{M}\beta 2$ integrin in neutrophils and mediates soluble CD40L-induced activation and clustering of the integrin and neutrophil–platelet interactions [90]. Interestingly, PKC δ deletion differentially regulates P-selectin exposure; decreased through PAR4 signaling but increased via GPVI signaling [148]. Moreover, inhibition of PKC δ with a dominant-negative TAT peptide blocks ERK recruitment to p47^{phox} and delays the initiation of TNF- α -induced $\text{O}_2^{\cdot -}$ generation through NOX2 in neutrophils [149]. Since PKC isoforms play a distinct role in regulating platelet and neutrophil functions, future studies using isoform-specific and multiple KO mice are required to determine how each isoform regulates neutrophil–platelet interactions.

Mitogen-activated protein kinases (MAPKs)

Activated MAPKs are crucial for regulating thromboxane A2 production, granule secretion, and $\alpha\text{IIb}\beta 3$ integrin activation [109]. It was reported that p38 MAPK is not important for Ca^{2+} mobilization, P-selectin exposure and $\alpha\text{IIb}\beta 3$ integrin activation in response to thrombin [150]. In contrast, recent studies showed that inhibition of extracellular signal-regulated kinases (ERK) and p38 MAPK significantly impairs P-selectin exposure and $\alpha\text{IIb}\beta 3$ integrin activation in histone-stimulated platelets [151]. Treatment of neutrophils with platelet-activating factor (PAF) up-regulates $\alpha\text{M}\beta 2$ integrin expression and stimulates $\beta 2$ integrin-dependent adhesion through ERK, but not PI3K [152].

Phosphodiesterase 4 (PDE4)

Recent studies using isoform-specific inhibitors suggested that PDE4, but not PDE3 or PDE5, is important for P-selectin-mediated $\alpha\text{M}\beta 2$ integrin activation, thereby inducing the formation of platelet–neutrophil aggregates in vitro and in vivo [153].

Nuclear factor- κB (NF- κB) signaling

Activation of NF- κB is mediated by the signal-induced phosphorylation and degradation of I κB and regulates

transcription of many genes involved in inflammation, immunity, cell proliferation, and survival [154]. It was reported that I $\kappa\text{B}\alpha$ is phosphorylated and degraded in thrombin-activated platelets and that I κB kinase inhibitors impair P-selectin exposure, $\alpha\text{IIb}\beta 3$ integrin activation, and ERK phosphorylation in activated platelets [151, 155, 156]. Recent studies suggested that treatment of platelets with TLR2 and 4 agonists triggers P-selectin exposure through NF- κB signaling [157]. Moreover, the interaction of platelets with hepatic ECs induces activation of NF- κB signaling and promotes adhesion of neutrophils and lymphocytes to P-selectin on both platelets and ECs [158]. Previous studies implicated that inhibition and knockdown of the NF- κB subunits suppress the surface expression of $\alpha\text{M}\beta 2$ integrin in PMA-stimulated neutrophil-like HL60 cells [159]. Thus, gene regulation through NF- κB signaling plays a crucial role in modulating platelet–neutrophil interactions under inflammatory conditions.

Small GTPases

Small GTPases are important signaling mediators involved in numerous cellular functions [160]. Among several family members, Rho family GTPases including Rac1, Cdc42, and RhoA are the best studied and have been shown to control cytoskeletal rearrangement [161]. Since GTPases are activated and inactivated by binding of GTP and GDP, respectively, they are regulated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) [162]. It is known that Cdc42 and Rac control the formation of finger-like filopodial protrusions and lamellipodia, respectively, whereas RhoA mediates actin stress fiber formation. In platelets, studies using mice lacking Rac1, Cdc42, or both demonstrated the importance of each GTPase for thrombopoiesis, P-selectin exposure, and $\alpha\text{IIb}\beta 3$ activation following agonist stimulation [163–165]. Further, Cdc42-null platelets showed defects in platelet GPIb signaling [166, 167]. Deletion of another small GTPase, Rap1b impairs P-selectin exposure on activated platelets [168]. In neutrophils, engagement of PSGL-1 activates Ras activity [169], which may regulate $\beta 2$ integrin activation. Rap1 is activated by cytosolic Ca^{2+} and diacylglycerol through PLC activation [170] and controls $\alpha\text{M}\beta 2$ integrin activation induced by LPS and TNF- α [171]. Studies using Cdc42-null mice suggested that $\alpha\text{M}\beta 2$ clustering is regulated by Cdc42 during neutrophil migration [172]. Rac1/2, components of the NADPH oxidase 2 complex, regulate ROS generation [173]. Further studies are required to determine the role of each small GTPase and its GAP/GEF in regulating platelet–neutrophil interactions during vascular disease.

Reactive oxygen species (ROS)

Reactive oxygen species are signaling molecules which play important roles during vascular disease and homeostasis [174]. ROS are produced under hypoxic inflammatory conditions [175]. Both ROS and hypoxia can transcriptionally and non-transcriptionally regulate hypoxia-induced factor-1 α /2 α (HIF-1 α /2 α) and NF- κ B in intravascular cells [176–178], thereby resulting in the expression of vasoactive substances and pro-inflammatory molecules. Previous studies demonstrated that hypoxia significantly induces the gene expression of β 2 integrins in leukocytes in a manner dependent on HIF-1 [179]. Biochemical studies with HIF-1-deficient myeloid cells revealed that hypoxia-induced HIF-1 α regulates neutrophil survival through NF- κ B activity [180]. Conversely, NF- κ B also regulates the gene transcription of HIF-1 α [181], suggesting the intimate link between the two signaling pathways during hypoxia–ischemia and inflammation.

The major source of ROS is membrane NADPH oxidases (NOXs). Combination studies with a NOX1 inhibitor and NOX2 KO mice implicated that platelet-derived ROS do not regulate P-selectin exposure on collagen-related peptide-activated platelets [182]. Interestingly, it was reported that incubation of platelets with H₂O₂ (>50–100 μ M for 1 h) induces shedding of GPIb α by activating TNF- α -converting enzyme (TACE) [183]. Although this *in vitro* study provides evidence that oxidative stress may attenuate the thrombotic function of platelets and inhibit platelet–neutrophil interactions, it is unclear how much ROS, such as H₂O₂, would be produced at the site of vascular injury. Platelets from patients with chronic granulomatous disease (X-CGD) that is genetically deficient in NOX2 (gp91^{phox}), showed defects in CD40L expression induced by various agonists [184]. Compared with platelets [182, 185], neutrophils produce larger amounts of extracellular ROS via NOX2 during cell activation [186]. Studies using neutrophils of X-CGD patients and NOX2 KO mice demonstrated that neutrophil NOX2-generated ROS are crucial for killing microbial pathogens [187, 188] and function as signaling molecules that regulate the activity of kinases and phosphatases [189]. Despite the importance of NOXs-generated ROS for thromboinflammation including ischemic stroke [190, 191], it remains poorly understood how ROS mechanistically contribute to the pathogenesis.

Pathological role of platelet–neutrophil interactions under thromboinflammatory conditions

Thromboinflammation is pathological conditions under which thrombotic and immune responses occur together.

Previous studies suggested that platelets, leukocytes, and coagulation factors should all be considered to prevent and treat thromboinflammatory diseases [29, 30, 32–34, 192]. Because of the involvement of leukocytes, extracellular ROS produced from activated neutrophils would be a critical factor distinguishing thromboinflammation from arterial thrombosis that results from platelet aggregation [27]. We will briefly summarize how platelet–neutrophil interactions influence thromboinflammatory conditions, focusing on vascular occlusion during ischemic stroke and sickle cell disease (SCD).

Ischemic stroke

Ischemic stroke occurs when an artery to the brain becomes too narrow or is occluded. If fresh blood is not provided to the brain cells for more than a few minutes, ischemic responses are initiated, resulting in necrosis of the cells. The damaged brain tissue rapidly releases a large amount of ROS and proinflammatory mediators including IL-1 β and MCP-1 [193]. The cytokines and chemokines trigger the expression of adhesion molecules on cerebral ECs and thus promote adhesion and transendothelial migration of leukocytes [194]. During ischemic stroke, the transmigrated leukocytes also generate an excessive amount of ROS, thereby augmenting the activation and damage of resident cells and amplifying the inflammatory condition [193, 195]. Following ischemic events, changes in micro-RNA levels in circulating leukocytes may contribute to increased leukocyte activation and thrombus formation [196]. Kleinschnitz and colleagues [29] reported that inhibition of α IIb β 3 integrin has no inhibitory effect on the size of ischemic stroke, but causes intracerebral hemorrhage in the mouse model of transient middle cerebral artery occlusion. Moreover, clinical studies showed that antagonists of α IIb β 3 integrin, abciximab and tirofiban, result in fatal intracerebral hemorrhage in patients with ischemic stroke [30, 197]. Instead, ischemic stroke is initiated by platelet GPIb–IX–V complex and GPVI, causing the interaction between platelets and vessel walls and allowing for further neutrophil recruitment. Binding of platelet GPIb α to vWF on activated/damaged ECs mediates platelet tethering on the vessel wall, and subsequent interaction between GPVI and collagen induces robust platelet activation and platelet thrombus formation [198]. Further, P-selectin expressed on activated platelets and ECs is able to bind to PSGL-1 on neutrophils, leading to neutrophil–platelet–ECs interactions and occluding the microvasculature [199]. We and others found that integrilin potentiates neutrophil–platelet aggregation under *in vitro* shear conditions [8, 52, 73]. Instead, inhibition of GPIb α with Fab fragments of a blocking anti-GPIb α antibody was a favorable approach to treat ischemic events in a mouse

model [29], implicating that the interaction of GPIIb α with vWF and/or α M β 2 integrin is crucial for cerebral ischemia/reperfusion injury [8, 200]. Thus, these results indicate that ischemic stroke is not simply mediated by platelet aggregation but by other intravascular cells including neutrophils. Nevertheless, it still remains unclear whether the direct interaction between platelets and neutrophils is critical for the pathogenesis of ischemic stroke. Previous studies showed that platelets also indirectly induce neutrophil recruitment into the ischemic region and thus promote inflammatory conditions. Using IL-1 α / β KO mice and isoform-specific antibodies, the authors found that platelets activate brain ECs through secreted IL-1 α and enhance ICAM-1 and VCAM-1 expression and CXCL1 release, thereby inducing neutrophil transendothelial migration during ischemic stroke [22]. Further, other studies using NOX inhibitors and KO mice suggested that NOX2-derived ROS are likely to be critical for mediating ischemic stroke [201, 202], and that hematopoietic cell NOX2 contributes more to the pathogenesis of stroke than brain and EC NOX2 [203]. In addition, the importance of NOX4 during oxidative stress induced by ischemic stroke has been reported [204]. Nonetheless, the regulatory mechanisms of oxidative stress in ischemic stroke remain to be determined.

Sickle cell disease (SCD)

Sickle cell disease, an inherited hematological disorder, results from the Glu 6 Val mutation in the β -globin chain [205]. When sickle hemoglobin (HbS) is deoxygenated, it aggregates into large polymers. The polymerized HbS causes a distortion of the shape of red blood cells (RBCs) and a remarkable decrease in its deformability. The sickle-shaped red cells become rigid and stick to the vessel wall. Inflammation is induced by the exposure of negatively charged phospholipids, such as phosphatidylserine, on the abnormal erythrocytes and the presence of chronic hemolysis [206]. Studies using SCD patients and mouse models demonstrated that recurrent vaso-occlusive events are the hallmark of the disease and induced by intravascular cell–cell aggregates [206]. Such vaso-occlusion is the main cause of pain crises and acute chest syndrome, which increase the morbidity and mortality in SCD patients [207]. Since hydroxyurea, the only drug approved by the Food and Drug Administration, minimally alleviates vaso-occlusion, novel therapies are required for SCD patients. Hypoxia/reoxygenation in SCD mice increases platelet–neutrophil interactions in a manner dependent on P-selectin [208]. Studies from Paul Frenette's group have demonstrated that targeting selectins, PDE9, and NETs have benefits on vaso-occlusive events in a mouse model of SCD [209–211]. Indeed, rivipansel (GMI-1070, a pan

selectin inhibitor) significantly decreased levels of biomarkers of endothelial and leukocyte activation and reduced vaso-occlusive events in clinical trials [212, 213]. Nevertheless, selectin inhibitors would block neutrophil rolling over and adhesion to the inflamed endothelium and thus may impair innate immune responses against bacterial pathogens. Since NETs induced by PAD4-citrullinated histones have been recognized as a critical component for venous and arterial thrombosis through the activation of the coagulation cascade and platelet adhesion [214–216], inhibition of NET formation may be beneficial for the intervention of vaso-occlusive events in SCD. Other studies showed that inhibition of histone deacetylase and induction of heme oxygenase-1 reduce vaso-occlusive events in sickle mice [217–219]. Recently, we have demonstrated that AKT2 is a critical regulator for the heterotypic cell–cell interaction and thus could be a potential target for vaso-occlusive events in SCD [8]. Using real-time intravital microscopy in SCD mice, infusion of a selective AKT2 inhibitor resulted in a significant reduction in cell–cell aggregation and improvement of blood flow rates. Importantly, we found that in addition to neutrophil–EC interactions, neutrophil–platelet interactions slow down blood flow rates and induce microvascular occlusion [8]. Further studies are necessary to determine which therapies would efficiently prevent vaso-occlusive events potentially in combination with hydroxyurea.

Concluding remarks

The pathological role of platelet–neutrophil association during vascular disease has long been speculated to be important. Recent *in vivo* intravital microscopic studies have shown that the interaction between activated neutrophils and platelets is tightly regulated and induces microvascular occlusion during thromboinflammatory disease. Because of the multiple signaling pathways regulating the receptor–counter receptor interaction, the detailed mechanism of the heterotypic cell–cell interaction still remains elusive. Recent results raise the following questions. How would intracellular signaling molecules sequentially and differentially regulate the function of platelet and neutrophil receptors during vascular disease? In addition to the intracellular signaling pathways, are any extracellular molecules required for the ligand-binding function of surface receptors under oxidative stress? Does ROS-mediated oxidative stress affect platelet–neutrophil interactions during thromboinflammation? How are the processes linking thrombosis and inflammation initiated? To answer these questions, specific inhibitors and KO mice are required to investigate the role of signaling molecules including protein kinases during thromboinflammation.

Importantly, due to the difficulties of mimicking oxidative stress conditions *in vitro*, real-time intravital microscopy will be a powerful technique to visualize platelet–neutrophil–EC interactions in microvessels of live animals. A better understanding of the mechanisms mediating the heterotypic platelet–neutrophil interactions could lead to the identification of novel therapeutic targets for the prevention and treatment of thromboinflammatory diseases.

Acknowledgments We apologize to our colleagues whose works are not cited in this review due to space restrictions. We thank Soochong Kim for his helpful comments on the manuscript. This work was supported by grants from National Institutes of Health. J.L. and K.K. are recipients of the American Heart Association postdoctoral fellowship award.

Conflict of interest All authors declare no competing financial interests.

References

- Zarbock A, Ley K (2009) Neutrophil adhesion and activation under flow. *Microcirculation* 16(1):31–42
- Henderson RB, Lim LH, Tessier PA, Gavins FN, Mathies M, Perretti M, Hogg N (2001) The use of lymphocyte function-associated antigen (LFA)-1-deficient mice to determine the role of LFA-1, Mac-1, and alpha4 integrin in the inflammatory response of neutrophils. *J Exp Med* 194(2):219–226
- Phillipson M, Kubes P (2011) The neutrophil in vascular inflammation. *Nat Med* 17(11):1381–1390
- Muller WA, Weigl SA, Deng X, Phillips DM (1993) PECAM-1 is required for transendothelial migration of leukocytes. *J Exp Med* 178(2):449–460
- Khandoga A, Huettinger S, Khandoga AG, Li H, Butz S, Jauch KW, Vestweber D, Krombach F (2009) Leukocyte transmigration in inflamed liver: a role for endothelial cell-selective adhesion molecule. *J Hepatol* 50(4):755–765
- Lou O, Alcaide P, Lusinskas FW, Muller WA (2007) CD99 is a key mediator of the transendothelial migration of neutrophils. *J Immunol* 178(2):1136–1143
- Bixel MG, Li H, Petri B, Khandoga AG, Khandoga A, Zarbock A, Wolburg-Buchholz K, Wolburg H, Sorokin L, Zeuschner D et al (2010) CD99 and CD99L2 act at the same site as, but independently of, PECAM-1 during leukocyte diapedesis. *Blood* 116(7):1172–1184
- Li J, Kim K, Hahm E, Molokie R, Hay N, Gordeuk VR, Du X, Cho J (2014) Neutrophil AKT2 regulates heterotypic cell–cell interactions during vascular inflammation. *J Clin Invest* 124(4):1483–1496
- Hidalgo A, Chang J, Jang JE, Peired AJ, Chiang EY, Frenette PS (2009) Heterotypic interactions enabled by polarized neutrophil microdomains mediate thromboinflammatory injury. *Nat Med* 15(4):384–391
- Yang J, Hirata T, Croce K, Merrill-Skoloff G, Tchernychev B, Williams E, Flaumenhaft R, Furie BC, Furie B (1999) Targeted gene disruption demonstrates that P-selectin glycoprotein ligand 1 (PSGL-1) is required for P-selectin-mediated but not E-selectin-mediated neutrophil rolling and migration. *J Exp Med* 190(12):1769–1782
- Wang Y, Sakuma M, Chen Z, Ustinov V, Shi C, Croce K, Zago AC, Lopez J, Andre P, Plow E et al (2005) Leukocyte engagement of platelet glycoprotein Ibalpha via the integrin Mac-1 is critical for the biological response to vascular injury. *Circulation* 112(19):2993–3000
- Simon DI, Chen Z, Xu H, Li CQ, Dong J, McIntire LV, Ballantyne CM, Zhang L, Furman MI, Berndt MC et al (2000) Platelet glycoprotein Ibalpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). *J Exp Med* 192(2):193–204
- Wagner DD, Burger PC (2003) Platelets in inflammation and thrombosis. *Arterioscler Thromb Vasc Biol* 23(12):2131–2137
- Yang J, Furie BC, Furie B (1999) The biology of P-selectin glycoprotein ligand-1: its role as a selectin counterreceptor in leukocyte–endothelial and leukocyte–platelet interaction. *Thromb Haemost* 81(1):1–7
- Gross PL, Furie BC, Merrill-Skoloff G, Chou J, Furie B (2005) Leukocyte-versus microparticle-mediated tissue factor transfer during arteriolar thrombus development. *J Leukoc Biol* 78(6):1318–1326
- Zarbock A, Polanowska-Grabowska RK, Ley K (2007) Platelet–neutrophil–interactions: linking hemostasis and inflammation. *Blood Rev* 21(2):99–111
- Wagner DD, Frenette PS (2008) The vessel wall and its interactions. *Blood* 111(11):5271–5281
- Sprague AH, Khalil RA (2009) Inflammatory cytokines in vascular dysfunction and vascular disease. *Biochem Pharmacol* 78(6):539–552
- Cave AC, Brewer AC, Narayanapanicker A, Ray R, Grieve DJ, Walker S, Shah AM (2006) NADPH oxidases in cardiovascular health and disease. *Antioxid Redox Signal* 8(5–6):691–728
- Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y (2010) PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med* 207(9):1853–1862
- Massberg S, Grahl L, von Bruehl ML, Manukyan D, Pfeiler S, Goosmann C, Brinkmann V, Lorenz M, Bidzhekov K, Khandagale AB et al (2010) Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. *Nat Med* 16(8):887–896
- Thornton P, McColl BW, Greenhalgh A, Denes A, Allan SM, Rothwell NJ (2010) Platelet interleukin-1alpha drives cerebrovascular inflammation. *Blood* 115(17):3632–3639
- Gear AR, Camerini D (2003) Platelet chemokines and chemokine receptors: linking hemostasis, inflammation, and host defense. *Microcirculation* 10(3–4):335–350
- Steinhubl SR, Moliterno DJ (2005) The role of the platelet in the pathogenesis of atherothrombosis. *Am J Cardiovasc Drugs* 5(6):399–408
- Semple JW, Italiano JE Jr, Freedman J (2011) Platelets and the immune continuum. *Nat Rev Immunol* 11(4):264–274
- Gleissner CA, von Hundelshausen P, Ley K (2008) Platelet chemokines in vascular disease. *Arterioscler Thromb Vasc Biol* 28(11):1920–1927
- Bledzka K, Smyth SS, Plow EF (2013) Integrin alphaIIb beta3: from discovery to efficacious therapeutic target. *Circ Res* 112(8):1189–1200
- Kenne E, Renne T (2014) Factor XII: a drug target for safe interference with thrombosis and inflammation. *Drug Discovery Today* 19(9):1459–1464
- Kleinschnitz C, Pozgajova M, Pham M, Bendszus M, Nieswandt B, Stoll G (2007) Targeting platelets in acute experimental stroke: impact of glycoprotein Ib, VI, and IIb/IIIa blockade on infarct size, functional outcome, and intracranial bleeding. *Circulation* 115(17):2323–2330
- Adams HP Jr, Effron MB, Torner J, Davalos A, Frayne J, Teal P, Leclerc J, Oemar B, Padgett L, Barnathan ES et al (2008) Emergency administration of abciximab for treatment of

- patients with acute ischemic stroke: results of an international phase III trial: Abciximab in Emergency Treatment of Stroke Trial (AbESTT-II). *Stroke* 39(1):87–99
31. Li J, Kim K, Hahm E, Molokie R, Hay N, Gordeuk VR, Du X, Cho J (2014) Neutrophil Akt2 regulates heterotypic cell–cell interactions during vascular inflammation. *J Clin Invest* 124:1483–1496
 32. Thiagarajan RR, Winn RK, Harlan JM (1997) The role of leukocyte and endothelial adhesion molecules in ischemia-reperfusion injury. *Thromb Haemost* 78(1):310–314
 33. Collier BS (2005) Leukocytosis and ischemic vascular disease morbidity and mortality: is it time to intervene? *Arterioscler Thromb Vasc Biol* 25(4):658–670
 34. Yonekawa K, Harlan JM (2005) Targeting leukocyte integrins in human diseases. *J Leukoc Biol* 77(2):129–140
 35. Sreeramkumar V, Adrover JM, Ballesteros I, Cuartero MI, Rossaint J, Bilbao I, Nacher M, Pitaval C, Radovanovic I, Fukui Y et al (2014) Neutrophils scan for activated platelets to initiate inflammation. *Science* 346(6214):1234–1238
 36. Nieswandt B, Kleinschnitz C, Stoll G (2011) Ischaemic stroke: a thrombo-inflammatory disease? *J Physiol* 589(Pt 17):4115–4123
 37. Montalvo-Jave EE, Escalante-Tattersfield T, Ortega-Salgado JA, Pina E, Geller DA (2008) Factors in the pathophysiology of the liver ischemia-reperfusion injury. *J Surg Res* 147(1):153–159
 38. Looney MR, Gilliss BM, Matthay MA (2010) Pathophysiology of transfusion-related acute lung injury. *Curr Opin Hematol* 17(5):418–423
 39. Lievens D, von Hundelshausen P (2011) Platelets in atherosclerosis. *Thromb Haemost* 106(5):827–838
 40. Woollard KJ, Geissmann F (2010) Monocytes in atherosclerosis: subsets and functions. *Nat Rev Cardiol* 7(2):77–86
 41. Nilsson B, Teramura Y, Ekdahl KN (2014) The role and regulation of complement activation as part of the thromboinflammation elicited in cell therapies. *Mol Immunol* 61(2):185–190
 42. Furie B, Furie BC, Flaumenhaft R (2001) A journey with platelet P-selectin: the molecular basis of granule secretion, signalling and cell adhesion. *Thromb Haemost* 86(1):214–221
 43. Carlow DA, Gossens K, Naus S, Veerman KM, Seo W, Ziltener HJ (2009) PSGL-1 function in immunity and steady state homeostasis. *Immunol Rev* 230(1):75–96
 44. Furie B, Furie BC (2004) Role of platelet P-selectin and microparticle PSGL-1 in thrombus formation. *Trends Mol Med* 10(4):171–178
 45. Andre P, Hartwell D, Hrachovinova I, Saffaripour S, Wagner DD (2000) Pro-coagulant state resulting from high levels of soluble P-selectin in blood. *Proc Natl Acad Sci USA* 97(25):13835–13840
 46. Liu W, Ramachandran V, Kang J, Kishimoto TK, Cummings RD, McEver RP (1998) Identification of N-terminal residues on P-selectin glycoprotein ligand-1 required for binding to P-selectin. *J Biol Chem* 273(12):7078–7087
 47. McEver RP, Cummings RD (1997) Perspectives series: cell adhesion in vascular biology. Role of PSGL-1 binding to selectins in leukocyte recruitment. *J Clin Invest* 100(3):485–491
 48. Martinez M, Joffraud M, Giraud S, Baisse B, Bernimoulin MP, Schapira M, Spertini O (2005) Regulation of PSGL-1 interactions with L-selectin, P-selectin, and E-selectin: role of human fucosyltransferase-IV and -VII. *J Biol Chem* 280(7):5378–5390
 49. Xiao B, Tong C, Jia X, Guo R, Lu S, Zhang Y, McEver RP, Zhu C, Long M (2012) Tyrosine replacement of PSGL-1 reduces association kinetics with P- and L-selectin on the cell membrane. *Biophys J* 103(4):777–785
 50. Jung U, Ley K (1999) Mice lacking two or all three selectins demonstrate overlapping and distinct functions for each selectin. *J Immunol* 162(11):6755–6762
 51. Evangelista V, Manarini S, Sideri R, Rotondo S, Martelli N, Piccoli A, Totani L, Piccardoni P, Vestweber D, de Gaetano G et al (1999) Platelet/polymorphonuclear leukocyte interaction: P-selectin triggers protein-tyrosine phosphorylation-dependent CD11b/CD18 adhesion: role of PSGL-1 as a signaling molecule. *Blood* 93(3):876–885
 52. Caron A, Theoret JF, Mousa SA, Merhi Y (2002) Anti-platelet effects of GPIIb/IIIa and P-selectin antagonism, platelet activation, and binding to neutrophils. *J Cardiovasc Pharmacol* 40(2):296–306
 53. Konstantopoulos K, Neelamegham S, Burns AR, Hentzen E, Kansas GS, Snapp KR, Berg EL, Hellums JD, Smith CW, McIntire LV et al (1998) Venous levels of shear support neutrophil-platelet adhesion and neutrophil aggregation in blood via P-selectin and beta2-integrin. *Circulation* 98(9):873–882
 54. Lam FW, Burns AR, Smith CW, Rumbaut RE (2011) Platelets enhance neutrophil transendothelial migration via P-selectin glycoprotein ligand-1. *Am J Physiol Heart Circ Physiol* 300(2):H468–H475
 55. Falati S, Liu Q, Gross P, Merrill-Skoloff G, Chou J, Vandendries E, Celi A, Croce K, Furie BC, Furie B (2003) Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. *J Exp Med* 197(11):1585–1598
 56. Lopez JA, Dong JF (1997) Structure and function of the glycoprotein Ib–IX–V complex. *Curr Opin Hematol* 4(5):323–329
 57. Li R, Emsley J (2013) The organizing principle of the platelet glycoprotein Ib–IX–V complex. *J Thromb Haemost* 11(4):605–614
 58. Andrews RK, Berndt MC (2013) Bernard–Soulier syndrome: an update. *Semin Thromb Hemost* 39(6):656–662
 59. Huizinga EG, Tsuji S, Romijn RA, Schiphorst ME, de Groot PG, Sixma JJ, Gros P (2002) Structures of glycoprotein Ibalpha and its complex with von Willebrand factor A1 domain. *Science* 297(5584):1176–1179
 60. Ruggeri ZM, Zarpellon A, Roberts JR, McClintock RA, Jing H, Mendolicchio GL (2010) Unravelling the mechanism and significance of thrombin binding to platelet glycoprotein Ib. *Thromb Haemost* 104(5):894–902
 61. Romo GM, Dong JF, Schade AJ, Gardiner EE, Kansas GS, Li CQ, McIntire LV, Berndt MC, Lopez JA (1999) The glycoprotein Ib–IX–V complex is a platelet counterreceptor for P-selectin. *J Exp Med* 190(6):803–814
 62. Mayadas TN, Cullere X (2005) Neutrophil beta2 integrins: moderators of life or death decisions. *Trends Immunol* 26(7):388–395
 63. Phillipson M, Heit B, Colarusso P, Liu L, Ballantyne CM, Kubes P (2006) Intraluminal crawling of neutrophils to emigration sites: a molecularly distinct process from adhesion in the recruitment cascade. *J Exp Med* 203(12):2569–2575
 64. Sumagin R, Prizant H, Lomakina E, Waugh RE, Sarelius IH (2010) LFA-1 and Mac-1 define characteristically different intraluminal crawling and emigration patterns for monocytes and neutrophils in situ. *J Immunol* 185(11):7057–7066
 65. McDonald B, Pittman K, Menezes GB, Hirota SA, Slaba I, Waterhouse CC, Beck PL, Muruve DA, Kubes P (2010) Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science* 330(6002):362–366
 66. Whitlock BB, Gardai S, Fadok V, Bratton D, Henson PM (2000) Differential roles for alpha(M)beta(2) integrin clustering or activation in the control of apoptosis via regulation of akt and ERK survival mechanisms. *J Cell Biol* 151(6):1305–1320

67. Coxon A, Rieu P, Barkalow FJ, Askari S, Sharpe AH, von Andrian UH, Arnaout MA, Mayadas TN (1996) A novel role for the beta 2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. *Immunity* 5(6): 653–666
68. Behnen M, Leschczyk C, Moller S, Batel T, Klinger M, Solbach W, Laskay T (2014) Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via FcgammaRIIIB and Mac-1. *J Immunol* 193(4): 1954–1965
69. Corken A, Russell S, Dent J, Post SR, Ware J (2014) Platelet glycoprotein Ib-IX as a regulator of systemic inflammation. *Arterioscler Thromb Vasc Biol* 34(5):996–1001
70. Du X (2007) Signaling and regulation of the platelet glycoprotein Ib–IX–V complex. *Curr Opin Hematol* 14(3):262–269
71. Li Z (1999) The alphaMbeta2 integrin and its role in neutrophil function. *Cell Res* 9(3):171–178
72. Weber C, Springer TA (1997) Neutrophil accumulation on activated, surface-adherent platelets in flow is mediated by interaction of Mac-1 with fibrinogen bound to alphaIIb beta3 and stimulated by platelet-activating factor. *J Clin Investig* 100(8):2085–2093
73. Scholz T, Zhao L, Temmler U, Bath P, Heptinstall S, Losche W (2002) The GPIIb/IIIa antagonist eptifibatide markedly potentiates platelet–leukocyte interaction and tissue factor expression following platelet activation in whole blood in vitro. *Platelets* 13(7):401–406
74. Bazzoni G (2003) The JAM family of junctional adhesion molecules. *Curr Opin Cell Biol* 15(5):525–530
75. Chavakis T, Keiper T, Matz-Westphal R, Hersemeyer K, Sachs UJ, Nawroth PP, Preissner KT, Santoso S (2004) The junctional adhesion molecule-C promotes neutrophil transendothelial migration in vitro and in vivo. *J Biol Chem* 279(53): 55602–55608
76. Santoso S, Sachs UJ, Kroll H, Linder M, Ruf A, Preissner KT, Chavakis T (2002) The junctional adhesion molecule 3 (JAM-3) on human platelets is a counterreceptor for the leukocyte integrin Mac-1. *J Exp Med* 196(5):679–691
77. Aurrand-Lions M, Lamagna C, Dangerfield JP, Wang S, Herrera P, Nourshargh S, Imhof BA (2005) Junctional adhesion molecule-C regulates the early influx of leukocytes into tissues during inflammation. *J Immunol* 174(10):6406–6415
78. Weber C, Fraemohs L, Dejana E (2007) The role of junctional adhesion molecules in vascular inflammation. *Nat Rev Immunol* 7(6):467–477
79. Palmer G, Busso N, Aurrand-Lions M, Talabot-Ayer D, Chobaz-Peclat V, Zimmerli C, Hammel P, Imhof BA, Gabay C (2007) Expression and function of junctional adhesion molecule-C in human and experimental arthritis. *Arthritis Res Ther* 9(4):R65
80. Shagdarsuren E, Djalali-Talab Y, Aurrand-Lions M, Bidzhikov K, Liehn EA, Imhof BA, Weber C, Zernecke A (2009) Importance of junctional adhesion molecule-C for neointimal hyperplasia and monocyte recruitment in atherosclerosis-prone mice—brief report. *Arterioscler Thromb Vasc Biol* 29(8): 1161–1163
81. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, Kroczeck RA (1998) CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 391(6667):591–594
82. Andre P, Prasad KS, Denis CV, He M, Papalia JM, Hynes RO, Phillips DR, Wagner DD (2002) CD40L stabilizes arterial thrombi by a beta3 integrin-dependent mechanism. *Nat Med* 8(3):247–252
83. Schonbeck U, Libby P (2001) The CD40/CD154 receptor/ligand dyad. *Cell Mol Life Sci* 58(1):4–43
84. Lievens D, Eijgelaar WJ, Biessen EA, Daemen MJ, Lutgens E (2009) The multi-functionality of CD40L and its receptor CD40 in atherosclerosis. *Thromb Haemost* 102(2):206–214
85. Rahman M, Zhang S, Chew M, Syk I, Jeppsson B, Thorlacius H (2013) Platelet shedding of CD40L is regulated by matrix metalloproteinase-9 in abdominal sepsis. *J Thromb Haemost* 11(7):1385–1398
86. Pamukcu B, Lip GY, Snezhitskiy V, Shantsila E (2011) The CD40-CD40L system in cardiovascular disease. *Ann Med* 43(5):331–340
87. Antoniadis C, Bakogiannis C, Tousoulis D, Antonopoulos AS, Stefanadis C (2009) The CD40/CD40 ligand system: linking inflammation with atherothrombosis. *J Am Coll Cardiol* 54(8):669–677
88. Setianto BY, Hartopo AB, Gharini PP, Anggrahini DW, Irawan B (2010) Circulating soluble CD40 ligand mediates the interaction between neutrophils and platelets in acute coronary syndrome. *Heart Vessels* 25(4):282–287
89. Zhao Z, Robinson RG, Barnett SF, Defeo-Jones D, Jones RE, Hartman GD, Huber HE, Duggan ME, Lindsley CW (2008) Development of potent, allosteric dual Akt1 and Akt2 inhibitors with improved physical properties and cell activity. *Bioorg Med Chem Lett* 18(1):49–53
90. Jin R, Yu S, Song Z, Zhu X, Wang C, Yan J, Wu F, Nanda A, Granger DN, Li G (2013) Soluble CD40 ligand stimulates CD40-dependent activation of the beta2 integrin Mac-1 and protein kinase C zeta (PKCzeta) in neutrophils: implications for neutrophil–platelet interactions and neutrophil oxidative burst. *PLoS ONE* 8(6):e64631
91. Rahman M, Zhang S, Chew M, Ersson A, Jeppsson B, Thorlacius H (2009) Platelet-derived CD40L (CD154) mediates neutrophil upregulation of Mac-1 and recruitment in septic lung injury. *Ann Surg* 250(5):783–790
92. Vanichakarn P, Blair P, Wu C, Freedman JE, Chakrabarti S (2008) Neutrophil CD40 enhances platelet-mediated inflammation. *Thromb Res* 122(3):346–358
93. Armant MA, Fenton MJ (2002) Toll-like receptors: a family of pattern-recognition receptors in mammals. *Genome Biol.* 3(8):REVIEWS3011
94. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, Patel KD, Chakrabarti S, McAvoy E, Sinclair GD et al (2007) Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med* 13(4):463–469
95. Stahl AL, Sartz L, Nelsson A, Bekassy ZD, Karpman D (2009) Shiga toxin and lipopolysaccharide induce platelet–leukocyte aggregates and tissue factor release, a thrombotic mechanism in hemolytic uremic syndrome. *PLoS ONE* 4(9):e6990
96. Lievens D, Zernecke A, Seijkens T, Soehnlein O, Beckers L, Munnix IC, Wijnands E, Goossens P, van Kruchten R, Thevisen L et al (2010) Platelet CD40L mediates thrombotic and inflammatory processes in atherosclerosis. *Blood* 116(20): 4317–4327
97. Cognasse F, Lafarge S, Chavarin P, Acquart S, Garraud O (2007) Lipopolysaccharide induces sCD40L release through human platelets TLR4, but not TLR2 and TLR9. *Intensive Care Med* 33(2):382–384
98. Montrucchio G, Bosco O, Del Sorbo L, Fascio Pecetto P, Lupia E, Goffi A, Omede P, Emanuelli G, Camussi G (2003) Mechanisms of the priming effect of low doses of lipopolysaccharides on leukocyte-dependent platelet aggregation in whole blood. *Thromb Haemost* 90(5):872–881
99. Blair P, Rex S, Vitseva O, Beaulieu L, Tanriverdi K, Chakrabarti S, Hayashi C, Genco CA, Iafrafi M, Freedman JE (2009) Stimulation of Toll-like receptor 2 in human platelets induces a thromboinflammatory response through activation of phosphoinositide 3-kinase. *Circ Res* 104(3):346–354

100. Assinger A, Laky M, Schabbauer G, Hirschl AM, Buchberger E, Binder BR, Volf I (2011) Efficient phagocytosis of periodontopathogens by neutrophils requires plasma factors, platelets and TLR2. *J Thromb Haemost* 9(4):799–809
101. Koupenova M, Vitseva O, MacKay CR, Beaulieu LM, Benjamin EJ, Mick E, Kurt-Jones EA, Ravid K, Freedman JE (2014) Platelet-TLR7 mediates host survival and platelet count during viral infection in the absence of platelet-dependent thrombosis. *Blood* 124(5):791–802
102. Futosi K, Fodor S, Mocsai A (2013) Neutrophil cell surface receptors and their intracellular signal transduction pathways. *Int Immunopharmacol* 17(3):638–650
103. Hayashi F, Means TK, Luster AD (2003) Toll-like receptors stimulate human neutrophil function. *Blood* 102(7):2660–2669
104. Ding ZM, Babensee JE, Simon SI, Lu H, Perrard JL, Bullard DC, Dai XY, Bromley SK, Dustin ML, Entman ML et al (1999) Relative contribution of LFA-1 and Mac-1 to neutrophil adhesion and migration. *J Immunol* 163(9):5029–5038
105. Kuijper PH, Gallardo Tores HI, Lammers JW, Sixma JJ, Koenderman L, Zwaginga JJ (1998) Platelet associated fibrinogen and ICAM-2 induce firm adhesion of neutrophils under flow conditions. *Thromb Haemost* 80(3):443–448
106. Aigner S, Sthoeger ZM, Fogel M, Weber E, Zarn J, Ruppert M, Zeller Y, Vestweber D, Stahl R, Sammar M et al (1997) CD24, a mucin-type glycoprotein, is a ligand for P-selectin on human tumor cells. *Blood* 89(9):3385–3395
107. Aigner S, Ramos CL, Hafezi-Moghadam A, Lawrence MB, Friederichs J, Altevogt P, Ley K (1998) CD24 mediates rolling of breast carcinoma cells on P-selectin. *FASEB J* 12(12):1241–1251
108. de Bruijne-Admiraal LG, Modderman PW, Von dem Borne AE, Sonnenberg A (1992) P-selectin mediates Ca²⁺-dependent adhesion of activated platelets to many different types of leukocytes: detection by flow cytometry. *Blood* 80(1):134–142
109. Li Z, Delaney MK, O'Brien KA, Du X (2010) Signaling during platelet adhesion and activation. *Arterioscler Thromb Vasc Biol* 30(12):2341–2349
110. Senis YA, Mazharian A, Mori J (2014) Src family kinases: at the forefront of platelet activation. *Blood* 124(13):2013–2024
111. Okutani D, Lodyga M, Han B, Liu M (2006) Src protein tyrosine kinase family and acute inflammatory responses. *Am J Physiol Lung Cell Mol Physiol* 291(2):L129–L141
112. Xiang B, Zhang G, Stefanini L, Bergmeier W, Gartner TK, Whiteheart SW, Li Z (2012) The Src family kinases and protein kinase C synergize to mediate Gq-dependent platelet activation. *J Biol Chem* 287(49):41277–41287
113. Falati S, Edmead CE, Poole AW (1999) Glycoprotein Ib-V-IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc receptor gamma-chain, Fyn, and Lyn to activate human platelets. *Blood* 94(5):1648–1656
114. Mangin P, Yuan Y, Goncalves I, Eckly A, Freund M, Cazenave JP, Gachet C, Jackson SP, Lanza F (2003) Signaling role for phospholipase C gamma 2 in platelet glycoprotein Ib alpha calcium flux and cytoskeletal reorganization. Involvement of a pathway distinct from FcR gamma chain and Fc gamma RIIA. *J Biol Chem* 278(35):32880–32891
115. Yin H, Liu J, Li Z, Berndt MC, Lowell CA, Du X (2008) Src family tyrosine kinase Lyn mediates VWF/GPIb-IX-induced platelet activation via the cGMP signaling pathway. *Blood* 112(4):1139–1146
116. Piccardoni P, Sideri R, Manarini S, Piccoli A, Martelli N, de Gaetano G, Cerletti C, Evangelista V (2001) Platelet/polymorphonuclear leukocyte adhesion: a new role for SRC kinases in Mac-1 adhesive function triggered by P-selectin. *Blood* 98(1):108–116
117. Xu T, Zhang L, Geng ZH, Wang HB, Wang JT, Chen M, Geng JG (2007) P-selectin cross-links PSGL-1 and enhances neutrophil adhesion to fibrinogen and ICAM-1 in a Src kinase-dependent, but GPCR-independent mechanism. *Cell Adhes Migr* 1(3):115–123
118. Evangelista V, Pamuklar Z, Piccoli A, Manarini S, Dell'elba G, Pecce R, Martelli N, Federico L, Rojas M, Berton G et al (2007) Src family kinases mediate neutrophil adhesion to adherent platelets. *Blood* 109(6):2461–2469
119. Kovacs M, Nemeth T, Jakus Z, Sitaru C, Simon E, Futosi K, Botz B, Helyes Z, Lowell CA, Mocsai A (2014) The Src family kinases Hck, Fgr, and Lyn are critical for the generation of the in vivo inflammatory environment without a direct role in leukocyte recruitment. *J Exp Med* 211(10):1993–2011
120. Woollard KJ, Kling D, Kulkarni S, Dart AM, Jackson S, Chindusting J (2006) Raised plasma soluble P-selectin in peripheral arterial occlusive disease enhances leukocyte adhesion. *Circ Res* 98(1):149–156
121. Cockcroft S (2006) The latest phospholipase C, PLCeta, is implicated in neuronal function. *Trends Biochem Sci* 31(1):4–7
122. Stalker TJ, Newman DK, Ma P, Wannemacher KM, Brass LF (2012) Platelet signaling. *Handb Exp Pharmacol* 210:59–85
123. Futosi K, Fodor S, Mocsai A (2013) Reprint of Neutrophil cell surface receptors and their intracellular signal transduction pathways. *Int Immunopharmacol* 17(4):1185–1197
124. Resendiz JC, Kroll MH, Lassila R (2007) Protease-activated receptor-induced Akt activation—regulation and possible function. *J Thromb Haemost* 5(12):2484–2493
125. Pasquet JM, Bobe R, Gross B, Gratacap MP, Tomlinson MG, Payrastra B, Watson SP (1999) A collagen-related peptide regulates phospholipase Cgamma2 via phosphatidylinositol 3-kinase in human platelets. *Biochem J* 342(Pt 1):171–177
126. Asselin J, Gibbins JM, Achison M, Lee YH, Morton LF, Farnedale RW, Barnes MJ, Watson SP (1997) A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase C gamma2 in platelets independent of the integrin alpha2beta1. *Blood* 89(4):1235–1242
127. Li Z, Jiang H, Xie W, Zhang Z, Smrcka AV, Wu D (2000) Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science* 287(5455):1046–1049
128. Jiang H, Kuang Y, Wu Y, Xie W, Simon MI, Wu D (1997) Roles of phospholipase C beta2 in chemoattractant-elicited responses. *Proc Natl Acad Sci USA* 94(15):7971–7975
129. Laurent PA, Severin S, Gratacap MP, Payrastra B (2014) Class I PI 3-kinases signaling in platelet activation and thrombosis: PDK1/Akt/GSK3 axis and impact of PTEN and SHIP1. *Adv Biol Regul* 54:162–174
130. Hawkins PT, Stephens LR, Suire S, Wilson M (2010) PI3K signaling in neutrophils. *Curr Topics Microbiol Immunol* 346:183–202
131. Bhaskar PT, Hay N (2007) The two TORCs and Akt. *Dev Cell* 12(4):487–502
132. Watson SP, Auger JM, McCarty OJ, Pearce AC (2005) GPVI and integrin alphaIIb beta3 signaling in platelets. *J Thromb Haemost* 3(8):1752–1762
133. Weng Z, Li D, Zhang L, Chen J, Ruan C, Chen G, Gartner TK, Liu J (2010) PTEN regulates collagen-induced platelet activation. *Blood* 116(14):2579–2581
134. Lioubin MN, Algate PA, Tsai S, Carlberg K, Aebersold A, Rohrschneider LR (1996) p150Ship, a signal transduction molecule with inositol polyphosphate-5-phosphatase activity. *Genes Dev* 10(9):1084–1095
135. Mondal S, Subramanian KK, Sakai J, Bajrami B, Luo HR (2012) Phosphoinositide lipid phosphatase SHIP1 and PTEN coordinate to regulate cell migration and adhesion. *Mol Biol Cell* 23(7):1219–1230

136. Chen M, Geng JG (2001) Inhibition of protein tyrosine phosphatases suppresses P-selectin exocytosis in activated human platelets. *Biochem Biophys Res Commun* 286(4):831–838
137. Kovacsovic TJ, Bachelot C, Toker A, Vlahos CJ, Duckworth B, Cantley LC, Hartwig JH (1995) Phosphoinositide 3-kinase inhibition spares actin assembly in activating platelets but reverses platelet aggregation. *J Biol Chem* 270(19):11358–11366
138. Libersan D, Merhi Y (2003) Platelet P-selectin expression: requirement for protein kinase C, but not protein tyrosine kinase or phosphoinositide 3-kinase. *Thromb Haemost* 89(6):1016–1023
139. Watanabe N, Nakajima H, Suzuki H, Oda A, Matsubara Y, Moroi M, Terauchi Y, Kadowaki T, Suzuki H, Koyasu S et al (2003) Functional phenotype of phosphoinositide 3-kinase p85alpha-null platelets characterized by an impaired response to GP VI stimulation. *Blood* 102(2):541–548
140. Yap CL, Anderson KE, Hughan SC, Dopheide SM, Salem HH, Jackson SP (2002) Essential role for phosphoinositide 3-kinase in shear-dependent signaling between platelet glycoprotein Ib/IX and integrin alpha(IIB)beta(3). *Blood* 99(1):151–158
141. Gao XP, Zhu X, Fu J, Liu Q, Frey RS, Malik AB (2007) Blockade of class IA phosphoinositide 3-kinase in neutrophils prevents NADPH oxidase activation- and adhesion-dependent inflammation. *J Biol Chem* 282(9):6116–6125
142. Mueller H, Stadtmann A, Van Aken H, Hirsch E, Wang D, Ley K, Zarbock A (2010) Tyrosine kinase Btk regulates E-selectin-mediated integrin activation and neutrophil recruitment by controlling phospholipase C (PLC) gamma2 and PI3Kgamma pathways. *Blood* 115(15):3118–3127
143. Chen J, De S, Damron DS, Chen WS, Hay N, Byzova TV (2004) Impaired platelet responses to thrombin and collagen in AKT-1-deficient mice. *Blood* 104(6):1703–1710
144. Woulfe D, Jiang H, Morgans A, Monks R, Birnbaum M, Brass LF (2004) Defects in secretion, aggregation, and thrombus formation in platelets from mice lacking Akt2. *J Clin Invest* 113(3):441–450
145. O'Brien KA, Stojanovic-Terpo A, Hay N, Du X (2011) An important role for Akt3 in platelet activation and thrombosis. *Blood* 118(15):4215–4223
146. Chen J, Tang H, Hay N, Xu J, Ye RD (2010) Akt isoforms differentially regulate neutrophil functions. *Blood* 115(21):4237–4246
147. Nishizuka Y (1995) Protein kinase C and lipid signaling for sustained cellular responses. *FASEB J* 9(7):484–496
148. Chari R, Getz T, Nagy B Jr, Bhavaraju K, Mao Y, Bynagari YS, Murugappan S, Nakayama K, Kunapuli SP (2009) Protein kinase C[delta] differentially regulates platelet functional responses. *Arterioscler Thromb Vasc Biol* 29(5):699–705
149. Kilpatrick LE, Sun S, Li H, Vary TC, Korchak HM (2010) Regulation of TNF-induced oxygen radical production in human neutrophils: role of delta-PKC. *J Leukoc Biol* 87(1):153–164
150. Begonja AJ, Geiger J, Rukoyatkina N, Rauchfuss S, Gambaryan S, Walter U (2007) Thrombin stimulation of p38 MAP kinase in human platelets is mediated by ADP and thromboxane A2 and inhibited by cGMP/cGMP-dependent protein kinase. *Blood* 109(2):616–618
151. Carestia A, Rivadeneyra L, Romaniuk MA, Fondevila C, Negrotto S, Schattner M (2013) Functional responses and molecular mechanisms involved in histone-mediated platelet activation. *Thromb Haemost* 110(5):1035–1045
152. Khreiss T, Jozsef L, Chan JS, Filep JG (2004) Activation of extracellular signal-regulated kinase couples platelet-activating factor-induced adhesion and delayed apoptosis of human neutrophils. *Cell Signal* 16(7):801–810
153. Totani L, Piccoli A, Dell'Elba G, Concetta A, Di Santo A, Martelli N, Federico L, Pamuklar Z, Smyth SS, Evangelista V (2014) Phosphodiesterase type 4 blockade prevents platelet-mediated neutrophil recruitment at the site of vascular injury. *Arterioscler Thromb Vasc Biol* 34(8):1689–1696
154. Oeckinghaus A, Ghosh S (2009) The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harb Perspect Biol* 1(4):a000034
155. Malaver E, Romaniuk MA, D'Atri LP, Pozner RG, Negrotto S, Benzadon R, Schattner M (2009) NF-kappaB inhibitors impair platelet activation responses. *J Thromb Haemost* 7(8):1333–1343
156. Lee HS, Kim SD, Lee WM, Endale M, Kamruzzaman SM, Oh WJ, Cho JY, Kim SK, Cho HJ, Park HJ et al (2010) A noble function of BAY 11-7082: inhibition of platelet aggregation mediated by an elevated cAMP-induced VASP, and decreased ERK2/JNK1 phosphorylations. *Eur J Pharmacol* 627(1–3):85–91
157. Rivadeneyra L, Carestia A, Etulain J, Pozner RG, Fondevila C, Negrotto S, Schattner M (2014) Regulation of platelet responses triggered by Toll-like receptor 2 and 4 ligands is another non-genomic role of nuclear factor-kappaB. *Thromb Res* 133(2):235–243
158. Lalor PF, Herbert J, Bicknell R, Adams DH (2013) Hepatic sinusoidal endothelium avidly binds platelets in an integrin-dependent manner, leading to platelet and endothelial activation and leukocyte recruitment. *Am J Physiol Gastrointest Liver Physiol* 304(5):G469–G478
159. Lee DH, Tam SS, Wang E, Taylor GR, Plante RK, Lau CY (1996) The NF-kappa B inhibitor, tepoxalin, suppresses surface expression of the cell adhesion molecules CD62E, CD11b/CD18 and CD106. *Immunol Lett* 53(2–3):109–113
160. Takai Y, Sasaki T, Matozaki T (2001) Small GTP-binding proteins. *Physiol Rev* 81(1):153–208
161. Aslan JE, McCarty OJ (2013) Rho GTPases in platelet function. *J Thromb Haemost* 11(1):35–46
162. Cherfils J, Zeghouf M (2013) Regulation of small GTPases by GEFs, GAPs, and GDIs. *Physiol Rev* 93(1):269–309
163. Akbar H, Kim J, Funk K, Cancelas JA, Shang X, Chen L, Johnson JF, Williams DA, Zheng Y (2007) Genetic and pharmacologic evidence that Rac1 GTPase is involved in regulation of platelet secretion and aggregation. *J Thromb Haemost* 5(8):1747–1755
164. Akbar H, Shang X, Perveen R, Berryman M, Funk K, Johnson JF, Tandon NN, Zheng Y (2011) Gene targeting implicates Cdc42 GTPase in GPVI and non-GPVI mediated platelet filopodia formation, secretion and aggregation. *PLoS ONE* 6(7):e22117
165. Pleines I, Dutting S, Cherpokova D, Eckly A, Meyer I, Morowski M, Krohne G, Schulze H, Gachet C, Debili N et al (2013) Defective tubulin organization and proplatelet formation in murine megakaryocytes lacking Rac1 and Cdc42. *Blood* 122(18):3178–3187
166. Pleines I, Eckly A, Elvers M, Hagedorn I, Eliautou S, Bender M, Wu X, Lanza F, Gachet C, Brakebusch C et al (2010) Multiple alterations of platelet functions dominated by increased secretion in mice lacking Cdc42 in platelets. *Blood* 115(16):3364–3373
167. Bialkowska K, Zaffran Y, Meyer SC, Fox JE (2003) 14-3-3 zeta mediates integrin-induced activation of Cdc42 and Rac. Platelet glycoprotein Ib-IX regulates integrin-induced signaling by sequestering 14-3-3 zeta. *J Biol Chem* 278(35):33342–33350
168. Zhang G, Xiang B, Ye S, Chrzanowska-Wodnicka M, Morris AJ, Gartner TK, Whiteheart SW, White GC 2nd, Smyth SS, Li Z (2011) Distinct roles for Rap1b protein in platelet secretion and

- integrin α IIb β 3 outside-in signaling. *J Biol Chem* 286(45):39466–39477
169. Hidari KI, Weyrich AS, Zimmerman GA, McEver RP (1997) Engagement of P-selectin glycoprotein ligand-1 enhances tyrosine phosphorylation and activates mitogen-activated protein kinases in human neutrophils. *J Biol Chem* 272(45):28750–28756
 170. M'Rabet L, Coffey P, Zwartkruis F, Franke B, Segal AW, Koenderman L, Bos JL (1998) Activation of the small GTPase rap1 in human neutrophils. *Blood* 92(6):2133–2140
 171. Caron E, Self AJ, Hall A (2000) The GTPase Rap1 controls functional activation of macrophage integrin α M β 2 by LPS and other inflammatory mediators. *Curr Biol* 10(16):974–978
 172. Kumar S, Xu J, Perkins C, Guo F, Snapper S, Finkelman FD, Zheng Y, Filippi MD (2012) Cdc42 regulates neutrophil migration via crosstalk between WASp, CD11b, and microtubules. *Blood* 120(17):3563–3574
 173. Anderson KE, Chessa TA, Davidson K, Henderson RB, Walker S, Tolmachova T, Gryk K, Rausch O, Seabra MC, Tybulewicz VL et al (2010) PtdIns3P and Rac direct the assembly of the NADPH oxidase on a novel, pre-phagosomal compartment during FcR-mediated phagocytosis in primary mouse neutrophils. *Blood* 116(23):4978–4989
 174. Lassegue B, Griendling KK (2010) NADPH oxidases: functions and pathologies in the vasculature. *Arterioscler Thromb Vasc Biol* 30(4):653–661
 175. Peyssonaux C, Johnson RS (2004) An unexpected role for hypoxic response: oxygenation and inflammation. *Cell Cycle* 3(2):168–171
 176. Dewhirst MW, Cao Y, Moeller B (2008) Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. *Nat Rev Cancer* 8(6):425–437
 177. Thompson AA, Elks PM, Marriott HM, Eamsamrarn S, Higgins KR, Lewis A, Williams L, Parmar S, Shaw G, McGrath EE et al (2014) Hypoxia-inducible factor 2 α regulates key neutrophil functions in humans, mice, and zebrafish. *Blood* 123(3):366–376
 178. Haddad JJ (2002) Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors. *Cell Signal* 14(11):879–897
 179. Kong T, Eltzschig HK, Karhausen J, Colgan SP, Shelley CS (2004) Leukocyte adhesion during hypoxia is mediated by HIF-1-dependent induction of β 2 integrin gene expression. *Proc Natl Acad Sci USA* 101(28):10440–10445
 180. Walmsley SR, Print C, Farahi N, Peyssonaux C, Johnson RS, Cramer T, Sobolewski A, Condliffe AM, Cowburn AS, Johnson N et al (2005) Hypoxia-induced neutrophil survival is mediated by HIF-1 α -dependent NF- κ B activity. *J Exp Med* 201(1):105–115
 181. van Uden P, Kenneth NS, Rocha S (2008) Regulation of hypoxia-inducible factor-1 α by NF- κ B. *Biochem J* 412(3):477–484
 182. Walsh TG, Berndt MC, Carrim N, Cowman J, Kenny D, Metharom P (2014) The role of Nox1 and Nox2 in GPVI-dependent platelet activation and thrombus formation. *Redox Biol* 2:178–186
 183. Brill A, Chauhan AK, Canault M, Walsh MT, Bergmeier W, Wagner DD (2009) Oxidative stress activates ADAM17/TACE and induces its target receptor shedding in platelets in a p38-dependent fashion. *Cardiovasc Res* 84(1):137–144
 184. Pignatelli P, Sanguigni V, Lenti L, Ferro D, Finocchi A, Rossi P, Violi F (2004) gp91phox-dependent expression of platelet CD40 ligand. *Circulation* 110(10):1326–1329
 185. Begonja AJ, Gambaryan S, Geiger J, Aktas B, Pozgajova M, Nieswandt B, Walter U (2005) Platelet NAD(P)H-oxidase-generated ROS production regulates α IIb β 3-integrin activation independent of the NO/cGMP pathway. *Blood* 106(8):2757–2760
 186. Dinarello MC, Orkin SH, Brown R, Jesaitis AJ, Parkos CA (1987) The glycoprotein encoded by the X-linked chronic granulomatous disease locus is a component of the neutrophil cytochrome b complex. *Nature* 327(6124):717–720
 187. Quie PG, White JG, Holmes B, Good RA (1967) In vitro bactericidal capacity of human polymorphonuclear leukocytes: diminished activity in chronic granulomatous disease of childhood. *J Clin Invest* 46(4):668–679
 188. Pollock JD, Williams DA, Gifford MA, Li LL, Du X, Fisherman J, Orkin SH, Doerschuk CM, Dinarello MC (1995) Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat Genet* 9(2):202–209
 189. Fialkow L, Wang Y, Downey GP (2007) Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. *Free Radic Biol Med* 42(2):153–164
 190. Carbone F, Camillo Teixeira P, Brauersreuther V, Mach F, Vuilleumier N, Montecucco F (2014) Pathophysiology and treatments of oxidative injury in ischemic stroke: focus on the phagocytic NADPH oxidase 2. *Antioxid Redox Signal*
 191. Radermacher KA, Wingler K, Langhauser F, Altenhofer S, Kleikers P, Hermans JJ, Hrabe de Angelis M, Kleinschnitz C, Schmidt HH (2013) Neuroprotection after stroke by targeting NOX4 as a source of oxidative stress. *Antioxid Redox Signal* 18(12):1418–1427
 192. Hagedorn I, Schmidbauer S, Pleines I, Kleinschnitz C, Krontahaler U, Stoll G, Dickneite G, Nieswandt B (2010) Factor XIIIa inhibitor recombinant human albumin Infestin-4 abolishes occlusive arterial thrombus formation without affecting bleeding. *Circulation* 121(13):1510–1517
 193. Amantea D, Nappi G, Bernardi G, Bagetta G, Corasaniti MT (2009) Post-ischemic brain damage: pathophysiology and role of inflammatory mediators. *FEBS J* 276(1):13–26
 194. Yilmaz G, Granger DN (2008) Cell adhesion molecules and ischemic stroke. *Neurol Res* 30(8):783–793
 195. Jin R, Yang G, Li G (2010) Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. *J Leukoc Biol* 87(5):779–789
 196. Jickling GC, Ander BP, Zhan X, Noblett D, Stamova B, Liu D (2014) microRNA expression in peripheral blood cells following acute ischemic stroke and their predicted gene targets. *PLoS ONE* 9(6):e99283
 197. Kellert L, Hametner C, Rohde S, Bendszus M, Hacke W, Ringelstein P, Stampfl S (2013) Endovascular stroke therapy: tirofiban is associated with risk of fatal intracerebral hemorrhage and poor outcome. *Stroke* 44(5):1453–1455
 198. Stoll G, Kleinschnitz C, Nieswandt B (2008) Molecular mechanisms of thrombus formation in ischemic stroke: novel insights and targets for treatment. *Blood* 112(9):3555–3562
 199. Carvalho-Tavares J, Hickey MJ, Hutchison J, Michaud J, Sutcliffe IT, Kubes P (2000) A role for platelets and endothelial selectins in tumor necrosis factor- α -induced leukocyte recruitment in the brain microvasculature. *Circ Res* 87(12):1141–1148
 200. Soriano SG, Coxon A, Wang YF, Frosch MP, Lipton SA, Hickey PR, Mayadas TN (1999) Mice deficient in Mac-1 (CD11b/CD18) are less susceptible to cerebral ischemia/reperfusion injury. *Stroke* 30(1):134–139
 201. Walder CE, Green SP, Darbonne WC, Mathias J, Rae J, Dinarello MC, Curnutte JT, Thomas GR (1997) Ischemic stroke injury is reduced in mice lacking a functional NADPH oxidase. *Stroke* 28(11):2252–2258
 202. De Silva TM, Brait VH, Drummond GR, Sobey CG, Miller AA (2011) Nox2 oxidase activity accounts for the oxidative stress and vasomotor dysfunction in mouse cerebral arteries following ischemic stroke. *PLoS ONE* 6(12):e28393

203. Tang XN, Zheng Z, Giffard RG, Yenari MA (2011) Significance of marrow-derived nicotinamide adenine dinucleotide phosphate oxidase in experimental ischemic stroke. *Ann Neurol* 70(4): 606–615
204. Kleinschnitz C, Grund H, Wingler K, Armitage ME, Jones E, Mittal M, Barit D, Schwarz T, Geis C, Kraft P et al (2010) Post-stroke inhibition of induced NADPH oxidase type 4 prevents oxidative stress and neurodegeneration. *PLoS Biol* 8(9)
205. Bunn HF (1997) Pathogenesis and treatment of sickle cell disease. *N Engl J Med* 337(11):762–769
206. Frenette PS, Atweh GF (2007) Sickle cell disease: old discoveries, new concepts, and future promise. *J Clin Invest* 117(4): 850–858
207. Ballas SK, Gupta K, Adams-Graves P (2012) Sickle cell pain: a critical reappraisal. *Blood* 120(18):3647–3656
208. Polanowska-Grabowska R, Wallace K, Field JJ, Chen L, Marshall MA, Figler R, Gear AR, Linden J (2010) P-selectin-mediated platelet–neutrophil aggregate formation activates neutrophils in mouse and human sickle cell disease. *Arterioscler Thromb Vasc Biol* 30(12):2392–2399
209. Chang J, Patton JT, Sarkar A, Ernst B, Magnani JL, Frenette PS (2010) GMI-1070, a novel pan-selectin antagonist, reverses acute vascular occlusions in sickle cell mice. *Blood* 116(10):1779–1786
210. Almeida CB, Scheiermann C, Jang JE, Prophete C, Costa FF, Conran N, Frenette PS (2012) Hydroxyurea and a cGMP-amplifying agent have immediate benefits on acute vaso-occlusive events in sickle cell disease mice. *Blood* 120(14): 2879–2888
211. Chen G, Zhang D, Fuchs TA, Manwani D, Wagner DD, Frenette PS (2014) Heme-induced neutrophil extracellular traps contribute to the pathogenesis of sickle cell disease. *Blood* 123(24):3818–3827
212. Wun T, Styles L, DeCastro L, Telen MJ, Kuypers F, Cheung A, Kramer W, Flanner H, Rhee S, Magnani JL et al (2014) Phase I study of the E-selectin inhibitor GMI 1070 in patients with sickle cell anemia. *PLoS ONE* 9(7):e101301
213. Okpala I. Investigational selectin-targeted therapy of sickle cell disease. *Expert Opin Investig Drugs*. 2014:1–10
214. Martinod K, Demers M, Fuchs TA, Wong SL, Brill A, Gallant M, Hu J, Wang Y, Wagner DD (2013) Neutrophil histone modification by peptidylarginine deiminase 4 is critical for deep vein thrombosis in mice. *Proc Natl Acad Sci USA* 110(21): 8674–8679
215. Savchenko AS, Martinod K, Seidman MA, Wong SL, Borissoff JJ, Piazza G, Libby P, Goldhaber SZ, Mitchell RN, Wagner DD (2014) Neutrophil extracellular traps form predominantly during the organizing stage of human venous thromboembolism development. *J Thromb Haemost* 12(6):860–870
216. Knight JS, Luo W, O'Dell AA, Yalavarthi S, Zhao W, Subramanian V, Guo C, Grenn RC, Thompson PR, Eitzman DT et al (2014) Peptidylarginine deiminase inhibition reduces vascular damage and modulates innate immune responses in murine models of atherosclerosis. *Circ Res* 114(6):947–956
217. Hebbel RP, Vercellotti GM, Pace BS, Solovey AN, Kollander R, Abanogu CF, Nguyen J, Vineyard JV, Belcher JD, Abdulla F et al (2010) The HDAC inhibitors trichostatin A and suberoylanilide hydroxamic acid exhibit multiple modalities of benefit for the vascular pathobiology of sickle transgenic mice. *Blood* 115(12):2483–2490
218. Belcher JD, Young M, Chen C, Nguyen J, Burhop K, Tran P, Vercellotti GM (2013) MP4CO, a pegylated hemoglobin saturated with carbon monoxide, is a modulator of HO-1, inflammation, and vaso-occlusion in transgenic sickle mice. *Blood* 122(15):2757–2764
219. Vercellotti GM, Khan FB, Nguyen J, Chen C, Bruzzone CM, Bechtel H, Brown G, Nath KA, Steer CJ, Hebbel RP et al (2014) H-ferritin ferroxidase induces cytoprotective pathways and inhibits microvascular stasis in transgenic sickle mice. *Front Pharmacol*. 5:79