Cell. Mol. Life Sci.
DOI 10.1007/s00018-015-1845-y

Cellular and Molecular Life Sciences

REVIEW

Platelet–neutrophil interactions under thromboinflammatory conditions

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

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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 β2 integrins and ICAM-1 [1]. Subsequently, activated αLβ2 and αMβ2 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 αMβ2 integrin to platelet P-selectin and glycoprotein Ibα (GPIbα), 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

Published online: 04 February 2015

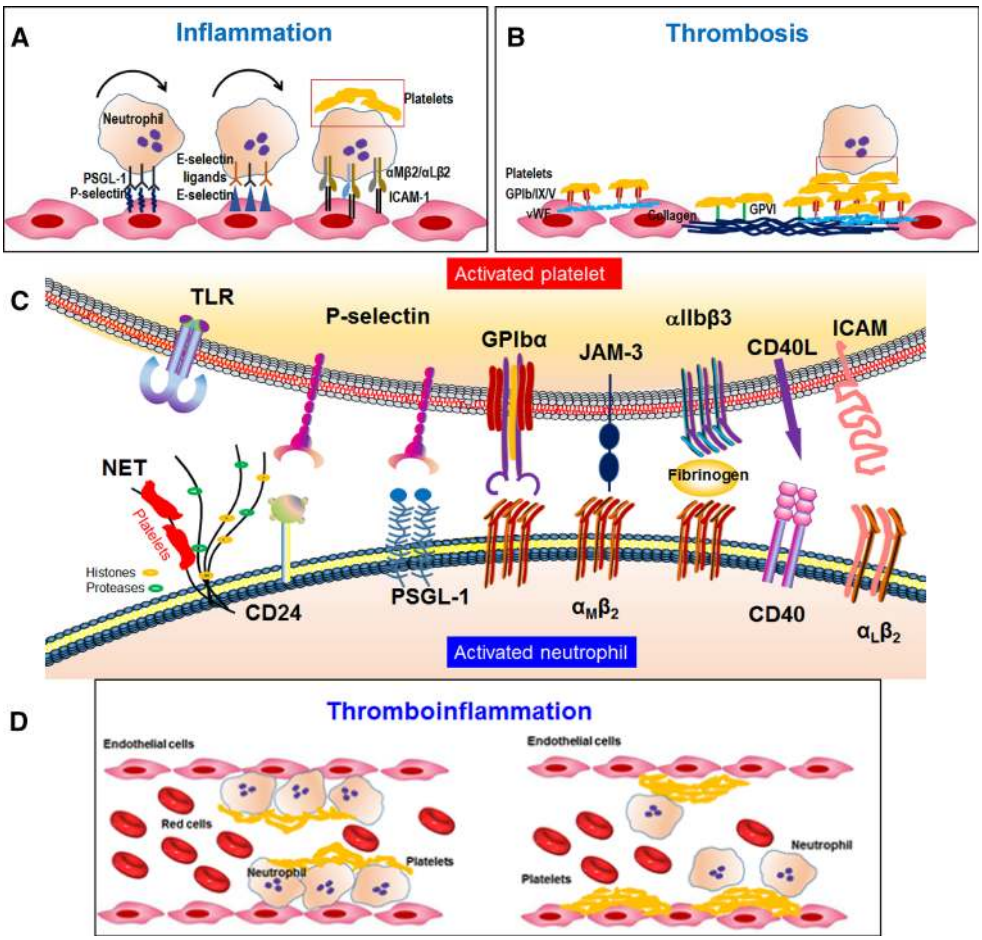

Fig. 1 Heterotypic cell–cell interactions during vascular inflammation. **a** During vascular inflammation, neutrophil rolling over and adhesion to the activated ECs are mediated by selectins-their ligands and β2 integrins-ICAM-1, respectively. These adherent and crawling neutrophils allow for platelet 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 αMβ2 integrin with GPIbα. Other molecules also contribute to heterotypic interactions, such as platelet JAM-3 binding to neutrophil αMβ2 integrin. Platelet αIIbβ3 integrin can interact with neutrophil αMβ2 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 (GPIbα, αIIbβ3, 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 αIIbβ3 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 Ibα (GPIbα) and αMβ2 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: GPIbα, GPIbβ, GPIX and GPV (2:2:2:1 ratio). The N-terminal region of GPIbα binds to several ligands including vWF, αMβ2 integrin, thrombin, and P-selectin [12, 58–61]. Neutrophil αMβ2 (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 GPIbα [62]. αMβ2 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 GPIbα binds to the I domain of αMβ2 integrin, thereby inducing stable and firm association between platelets and neutrophils [12]. In vitro and in vivo studies demonstrated that deletion or inhibition of αMβ2 integrin and GPIbα abolishes the interaction of platelets with neutrophils or monocytes under inflammatory conditions [9, 12, 69]. Recently, we demonstrated using real-

time intravital microscopy that αM deletion improves blood flow rates during TNF-α-induced cremaster venular inflammation [8]. Thus, GPIbα–αMβ2 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 αMβ2 integrin have been studied [58, 62, 70, 71], it remains elusive how the ligand-binding function of both molecules is regulated during thromboinflammatory disease.

αIIbβ3 integrin

αIIbβ3 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 αMβ2 integrin with fibrinogen bound to αIIbβ3 integrin mediates neutrophil adhesion to adherent platelets under flow conditions [72]. However, we and others showed that inhibition of αIIbβ3 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 αIIbβ3 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 αMβ2 integrin [76]. Interestingly, a combination of anti-GPIbα and anti-JAM-3 antibodies showed additive inhibitory effects on platelet–neutrophil interactions under static conditions, suggesting that both JAM-3 and GPIbα are important for the interaction with neutrophil αMβ2 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 αMβ2 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 αMβ2 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.

αLβ2 integrin

αLβ2 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 αLβ2 integrin to platelet ICAM-2 regulates neutrophil-platelet attachment under flow conditions [105]. Nevertheless, the importance of αLβ2-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 αIIbβ3 integrin activation [112]. Further, studies with SFK inhibitors and isoform-specific KO mice suggested that SFKs such as Lyn and Fyn affect vWF-GPIbα 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 αMβ2 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 β2 integrin-null mice, suggesting the critical role of the three SFKs in neutrophil adhesive function [119]. Inhibition of SFKs with PP1 impaired αMβ2 integrin clustering and cytoskeletal reorganization in neutrophils [116]. Further, P-selectin-mediated crosslinking of PSGL-1 results in αMβ2 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, PLCβ2/3 and PLCγ2 are the major isoforms and are activated by Gqα/Giβγ- 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 PLCβ2 through Gqα, whereas the collagen–GPVI interaction stimulates PLCγ2 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 PLCβ-regulated AKT activity may mediate P-selectin exposure on thrombin-stimulated platelets [124] and that PLCγ2 activation through GPVI signaling is critical for the increase in cytosolic Ca^{2+} and αIIbβ3 integrin activation [125, 126]. Furthermore, studies using PLCγ2 KO mice showed the important role of PLCγ2 in increasing cytosolic Ca^{2+} through the vWF-GPIb/IX/V interaction

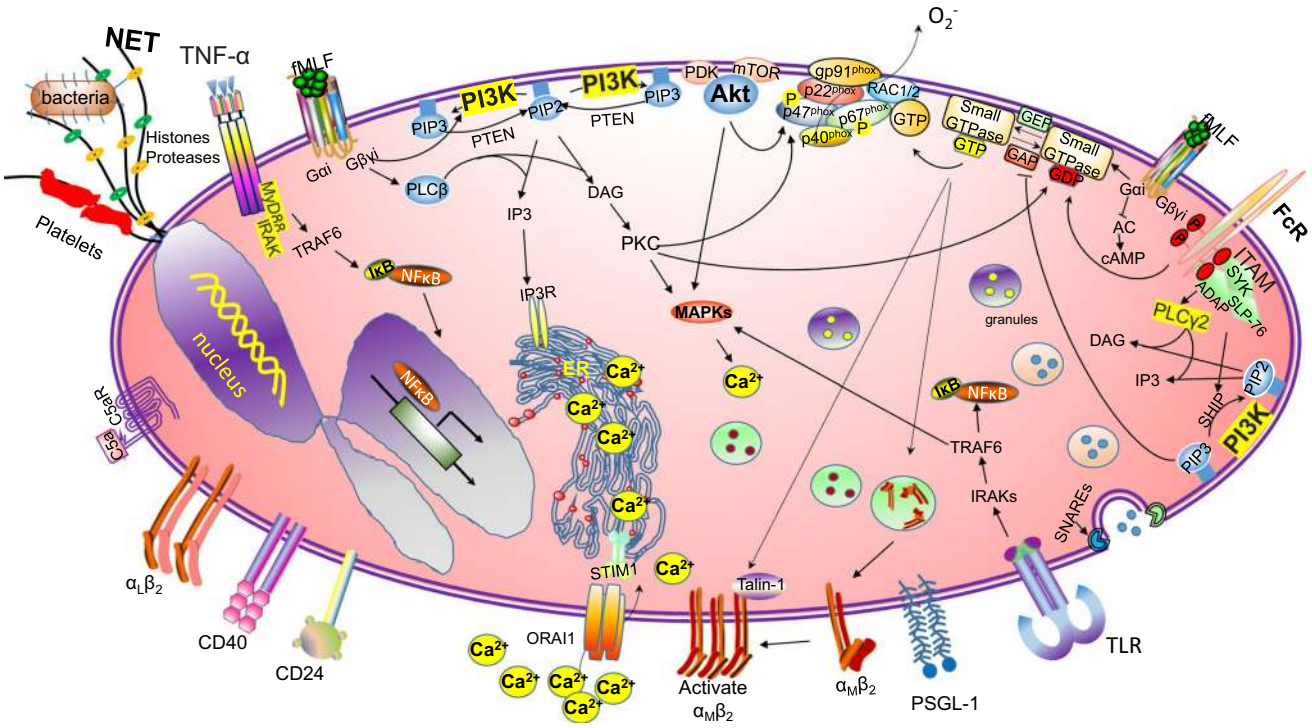

Fig. 2 Receptor-mediated signaling pathways in neutrophils. Under inflammatory conditions, neutrophils can be activated by initial rolling on activated endothelium and by soluble ligands such as TNF-α. TNFR signaling stimulates NF-κB-mediated gene transcription, regulating the expression of numerous pro-inflammatory proteins. GPCR stimulation leads to inhibition of adenylyl cyclase (AC) through Gαi and activation of PI3K and PLCβ through Gβγi. Rac1/2 activity and activated Akt and PKC phosphorylate $p47^{phox}$, inducing ROS generation through the NOX2 complex. PLCβ 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. FcγR signaling leads to ITAM- and Syk-mediated PLCγ2 activation that induces similar downstream effects to those seen from GPCRs. TLR activation leads to NF-κB activation, as seen in TNFR activation. The signaling pathways of GPCRs, FcγR, and TLR enhance MAPK activity and cytosolic calcium levels. During cell activation, exocytosis-mediated granular secretion also induces the membrane translocation of αMβ2 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 PLCβ2/3 activation through Giβγ [123]. Previous studies demonstrated that deletion of PLCβ2 and/or PLCβ3 abrogates IP_3 production, increase in cytosolic Ca^{2+}, PKC activation, αMβ2 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 (PIP_3) is one of the PI3K products which is required for the membrane association and activation of AKT and PLCγ2 [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 p85α-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-GPIbα binding, intracellular Ca^{2+} 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 p85α suggested that PI3K regulates αMβ2-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

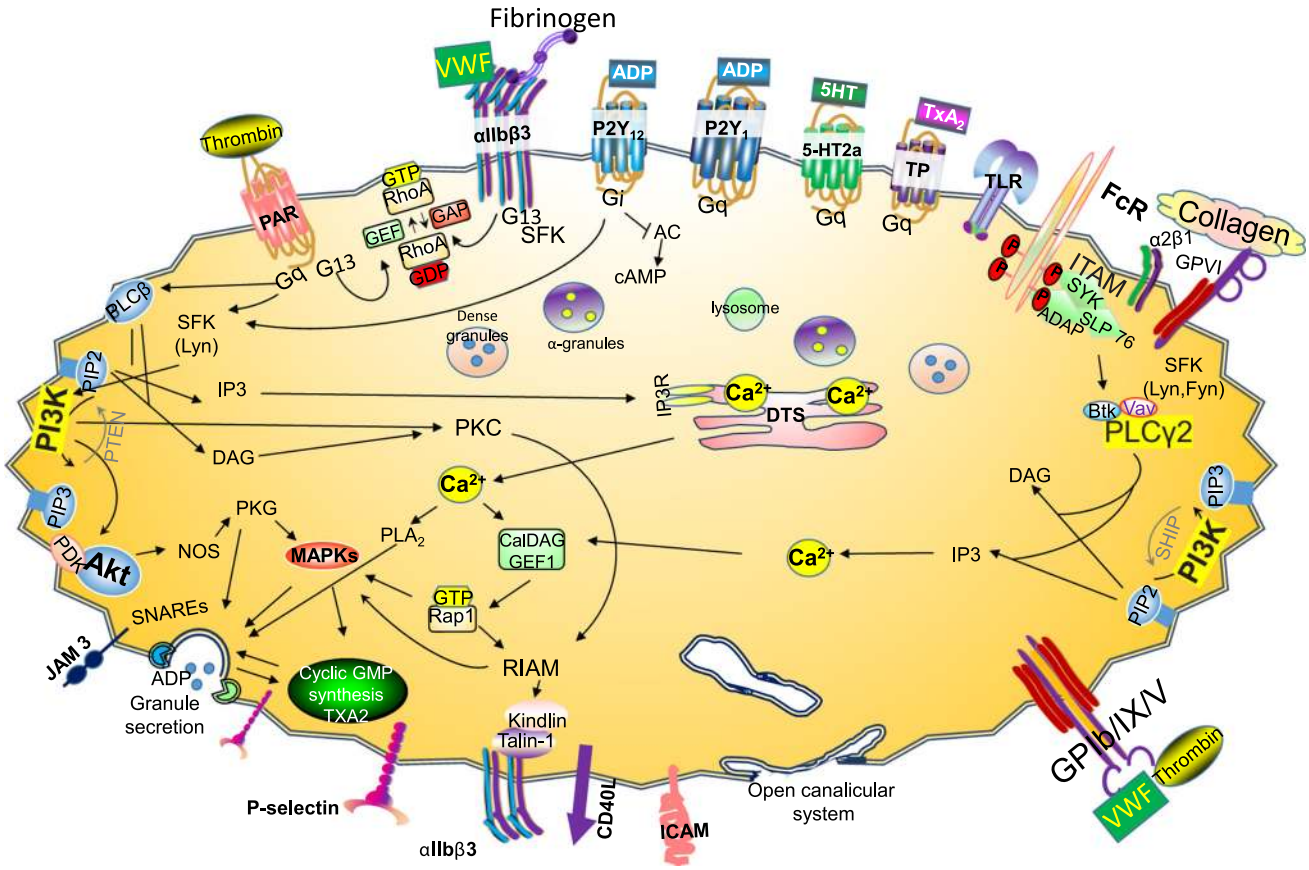

Fig. 3 Receptor-mediated signaling pathways in platelets. Numerous agonists stimulate platelets through their receptors. Gqα is able to stimulate both Lyn and PLCβ. Lyn then phosphorylates PI3K, leading to PIP3 generation and AKT phosphorylation. Activated AKT stimulates NOS-PKG-MAPK signaling. PLCβ activation allows for generation of DAG and IP_3 from PIP_2. 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 αIIbβ3 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 FcγR and GPVI, signal through Syk-SLP-76-ADAP and Lyn/Fyn, respectively. Both of these receptors lead to activation of PLCγ2, thus generating IP_3 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 PI3Kγ-AKT signaling [142]. Further studies are required to determine the role of each PI3K isoform in regulating P-selectin exposure and αMβ2 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 Ca^{2+} release, thereby affecting αIIbβ3 integrin activation [8, 143]. Similarly, platelet AKT2 and 3 are important for P-selectin exposure and αIIbβ3 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 Ca^{2+} release and the membrane translocation and activation of αMβ2 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 αIIbβ3 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 PKCζ colocalizes with αMβ2 integrin in neutrophils and mediates soluble CD40L-induced activation and clustering of the integrin and neutrophil–platelet interactions [90]. Interestingly, PKCδ deletion differentially regulates P-selectin exposure; decreased through PAR4 signaling but increased via GPVI signaling [148]. Moreover, inhibition of PKCδ with a dominant-negative TAT peptide blocks ERK recruitment to $p47^{phox}$ and delays the initiation of TNF-α-induced $O_2^{\cdot-}$ 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 αIIbβ3 integrin activation [109]. It was reported that p38 MAPK is not important for Ca^{2+} mobilization, P-selectin exposure and αIIbβ3 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 αIIbβ3 integrin activation in histone-stimulated platelets [151]. Treatment of neutrophils with platelet-activating factor (PAF) up-regulates αMβ2 integrin expression and stimulates β2 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 αMβ2 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 IκB and regulates transcription of many genes involved in inflammation, immunity, cell proliferation, and survival [154]. It was reported that IκBα is phosphorylated and degraded in thrombin-activated platelets and that IκB kinase inhibitors impair P-selectin exposure, αIIbβ3 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-kB subunits suppress the surface expression of αMβ2 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 αIIbβ3 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 β2 integrin activation. Rap1 is activated by cytosolic Ca^{2+} and diacylglycerol through PLC activation [170] and controls αMβ2 integrin activation induced by LPS and TNF-α [171]. Studies using Cdc42-null mice suggested that αMβ2 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-1α/2α (HIF-1α/2α) and NF-κB 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 β2 integrins in leukocytes in a manner dependent on HIF-1 [179]. Biochemical studies with HIF-1-deficient myeloid cells revealed that hypoxia-induced HIF-1α regulates neutrophil survival through NF-κB activity [180]. Conversely, NF-κB also regulates the gene transcription of HIF-1α [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 H_2O_2 (>50–100 μM for 1 h) induces shedding of GPIbα 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 H_2O_2, 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-1β 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 microRNA levels in circulating leukocytes may contribute to increased leukocyte activation and thrombus formation [196]. Kleinschnitz and colleagues [29] reported that inhibition of αIIbβ3 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 αIIbβ3 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 GPIbα 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 GPIbα with Fab fragments of a blocking anti-GPIbα antibody was a favorable approach to treat ischemic events in a mouse

model [29], implicating that the interaction of GPIbα with vWF and/or αMβ2 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-1α/β KO mice and isoform-specific antibodies, the authors found that platelets activate brain ECs through secreted IL-1α 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.

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