Inflammatory vs thrombotic states of platelets: a mini-review with a focus on functional dichotomy

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Abstract

In addition to roles in hemostasis and thrombosis, the role of platelets in inflammation and immunity have been the focus of recent studies. Both proinflammatory and prothrombotic roles of platelets are considered important in the pathophysiology of sepsis as well as in the protection from severe infections. The facilitation of prothrombotic activity by the proinflammatory activity of platelets has been shown in many studies. Nonetheless, some studies have highlighted the dichotomy of platelet functionality. Within a limited time period and space, only proinflammatory or prothrombotic functionality is often present. In some cases, the activation of the proinflammatory function of platelets is not accompanied by prothrombotic activity. Recent analyses of pattern recognition receptors such as toll-like receptors (TLRs) in platelets motivate us to consider how pathways are activated in innate immunity compared with pathways for thrombogenesis and hemostasis. In this article, we mainly discuss recent studies showing the functional dichotomy of platelets and, in particular, the conditions and possible mechanisms that activate platelets in a biased manner, rather than the concordance between prothrombotic and proinflammatory pathways in platelets. (***Annals of Biomedical Research*** **(2018) 1(1): 107, http://www.escires.com/articles/ABR-1-107.pdf**)

Platelets in inflammation and thrombosis

Platelets play a central role as essential effectors in thrombosis, hemostasis, and inflammation [1,2]. A growing body of recent studies have revealed the capability of platelets to coordinate inflammation, immune, prothrombotic responses, angiogenesis, and wound healing [3]. In this section, we mainly discuss findings favoring the role of platelets in inflammation and innate immunity closely coupled with those for thrombosis and coagulation. Of note, we do not cover important adhesion receptors in hemostasis such as GP VI (glycoprotein VI) interactions with collagen [4] as well as GP IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) [5]. We also refer readers to Yun et al [6] and Cimmino [7] for introductory articles on the platelet biology focusing on activators, as well as Li, Thomas et al, and Smyth et al for articles with a broader scope [1,2,4].

In sepsis, inflammation and coagulation are considered coupled to each other. Inflammation leads to a procoagulant state, and coagulation augments inflammation. The risk of acute thrombotic events increases during bacterial infections or inflammation [8]. A number of molecules and systems have been shown to be involved in reciprocal interrelationship between coagulation and innate immune systems [9]. An example for the linkage from inflammation to coagulation is tissue factor (TF) expression on the cell surface of leukocytes, induced by stimulation with endotoxin, tumor-necrosis factor (TNF)-α, or CD40 ligand (CD40L) [10]. TF is a receptor and cofactor for factor VII and VIIa and the key initiator of coagulation. TF is expressed on subendothelial mural cells and fibroblast around the vessel wall, and activated endothelial cells as well; however, the contribution of microvesicle-mediated transfer from monocytes is difficult to quantify in vivo [11]. As another example, histamine, TNF-α, IL-8, and IL-6 all induce the release of large von Willebrand factor (vWF) multimers from the endothelium [12]. These large multimers potently promote platelet thrombi formation [12]. Furthermore, as the term immunothrombosis implies, both immune cells and thrombosis-related molecules act in a concerted manner to generate an intravascular scaffold to facilitate the containment and destruction of pathogens [13]. Thrombin plays an important role in protective immunity as shown by a mouse model for *Klebsiella pneumoniae* infections [14]. The authors also showed that the thrombin effects are dependent on platelet-neutrophil interactions and platelet protease-activated receptors (PAR)1 in vivo.

Given such interplay between the systems, it seems reasonable to assume that activated platelets should always show both proinflammatory and prothrombotic properties (which we refer to as 'concordance in platelet activation'). Platelets are important in sepsis pathophysiology where both inflammation and coagulation are central issues [e.g., 15].

Activated platelets release a variety of prothrombotic and proinflammatory mediators, and recruit leukocytes including monocytes, neutrophils, and lymphocytes to sites of vascular injury and inflammation [1]. Detailed analyses of platelets further validate the view of the general concordance of proinflammatory and prothrombotic platelet activation. For instance, α-granules contain not only P-selectin and chemokines including platelet factor 4 (PF4, CXCL4), macrophage inflammatory protein (MIP)-1α and RANTES that jointly help recruit/activate leukocytes, but also platelet endothelial adhesion molecule-1, GPIIb/IIIa, and vWF, which are the key players in thrombosis and hemostasis [2]. It is also known that inflammatory mediators, such as IL-6, increase platelet production, and the newly formed platelets are likely to be more thrombogenic [16]. Components of the activated complement system can confer platelets with increased procoagulant activity, likely contributing to thrombotic events [17,18,19].

The concordance between proinflammatory and prothrombotic activation of platelets can also be supported by the presence of the molecules with such dual roles in the platelets. For example, the binding of P-selectin of platelets with P-selectin glycoprotein ligand-1 (PSGL-1) expressed on leukocytes is necessary for leukocyte trafficking and translocation, but PSGL-1 is also carried by monocyte-derived microparticles as well, and P-selectin/PSGL-1 interaction is also essential for growth of the thrombus at sites of injury [17,20]. CD40L, in membrane-bound and soluble forms, exerts diverse proinflammatory and prothrombotic effects by binding mainly to CD40 on the surface of vascular walls and circulating cells [9]. Another example is polyphosphate, which is contained in platelet dense granules [21]. Polyphosphate initiates factor XII-driven fibrin formation (the intrinsic pathway of coagulation) on the surface of activated platelets. Furthermore, active factor XII causes the generation of active kallikreins, which leads to the liberation of bradykinin, a well-known inflammatory mediator, from high-molecular-weight kininogen [22].

Despite such findings in support of inseparable coupling of the immune/inflammatory and the prothrombotic platelet activation, when we focus on the activities of platelets within a limited spatial or temporal window, only one of the proinflammatory and prothrombotic activities is found to be activated in certain settings. Moreover, the multiplicity of platelet functions in angiogenesis even appears to have conflicting functionalities; PAR1, ADP ($P2Y_1$ and $P2Y_{12}$), and GP VI stimulation promotes proangiogenic effects (releasing stromal derived factor [SDF]-1α, CXCL12, and vascular endothelial growth factor [VEGF]), whereas PAR4 promotes anti-angiogenic effects such as the release of PF4 and endostatin [3,23,24]. However, it remains largely unknown what differences in activated signaling pathways lead to the activation of selected

functionalities.

Question 1: What is dichotomy in platelet activation ?

As we have discussed above, the activation of proinflammatory and immune functionalities of platelets appears to be accompanied by their prothrombotic functionalities. However, only one of the proinflammatory and prothrombotic activities has been shown to be activated in some settings. To our knowledge, such a view, namely, platelet dichotomy, dates back to an article by Page [25]. It was unclear why platelet activation by inflammatory mediators did not result in thrombosis and coagulation in vivo [25,26]. Later, Page, Pitchford and coworkers investigated why increased risk of thrombosis was not observed in patients with asthma and allergic animal models, despite the clear occurrence of platelet activation and P-selectin mediated leukocyte recruitment, a pivotal role for platelets in inflammation [27]. Rather than increased thrombosis, mild hemostatic defects have been reported to be associated with asthma [28].

Here, a brief introduction on purine receptors and signaling pathways may help. Contained in dense granules, ADP serves as a prominent amplifier of initial platelet activation and as a cofactor to all physiological platelet activators including thromboxane A2, collagen, and thrombin [29]. ADP-induced platelet activation is initiated by $P2Y_1$ receptor and amplified by $P2Y_{12}$ receptor [30]. Compared to $P2Y_1$ that mediates quick response and transient platelet aggregation, $P2Y_{12}$ potentiates platelet secretion and sustained aggregation [31]. Thus, in the context of thrombosis in vivo, both $P2Y_1$ and $P2Y_{12}$ receptors are important [32]. In vitro systems using platelet-rich plasma (PRP) that lack leukocytes, $P2Y_{12}$, but not $P2Y_1$ are involved in thrombin generation and phosphatidylserine (PS) exposure, which provides a catalytic surface for enzyme reactions required for coagulation. *I*n vitro analyses by Leon et al suggested that the $P2Y_{12}$-G_i-PI3 kinase (PI3K) pathway, compared with $P2Y_1$, has a more important role in the exposure of procoagulant catalytic surfaces. Their whole blood/ADP (or thrombin receptor activating peptide [TRAP]) stimulation systems with both $P2Y_1$ and $P2Y_{12}$ receptors are important for platelet P-selectin expression and TF-expression of leukocytes. Thus, $P2Y_{12}$ appeared essential in all experiments on thrombogenesis and PS exposure [32], and is also involved in P-selectin expression and platelet-leukocyte complex formation. In terms of signaling pathways, such as PAR receptors, these P2Y receptors belong to GPCR receptors. It should be noted that, besides GPCRs, platelets express the receptors/integrins that directly or indirectly activate Src family kinases, TLRs, and receptor of other types [4].

Strikingly, in the context of allergic inflammation, $P2Y_{12}$ does not to play a significant role. Specifically Amison 2015 used an antigen OVA challenge for a IgE-dependent lung inflammation mouse model. In their study, analysis of the antagonists revealed that $P2Y_1$ but not $P2Y_{12}$ stimulation leads to formation of circulating platelet-leukocyte complexes in a manner dependent on allergen exposure [26,33]. Thus, in their inflammation model, $P2Y_1$ activation was necessary for the formation of the platelet-leukocyte complex, but $P2Y_{12}$ activation was not involved in this process. On the other hand, ADP-$P2Y_1$ alone did not lead to platelet–platelet aggregation in their system, consistent with the well-known importance of $P2Y_{12}$ in thrombotic events.

Later, the authors also showed that antagonists of $P2Y_1$ and $P2Y_{14}$, but not of $P2Y_{12}$, inhibited the intranasal lipopolysaccharide (LPS) administration-induced pulmonary neutrophil recruitment [34]. Here, the $P2Y_1$ antagonist, but not the $P2Y_{12}$ antagonist, suppressed platelet-dependent neutrophil motility toward macrophage-derived chemokine (MDC), whose receptor (CCR4) is expressed on platelets but not on neutrophils. On the other hand, $P2Y_1$ and $P2Y_{12}$ were equally important for ADP-induced platelet aggregation *ex vivo*. These findings corroborate those of Liverani et al [35], who argued against the idea that $P2Y_{12}$ is important for inflammation; they showed that the levels of inflammation (numbers of white blood cells, plasma levels of cytokines, and the severity of lung injury) in LPS-treated $P2Y_{12}$-knockout mice were higher than those in LPS-treated wild-type mice. It should be noted, however, that $P2Y_{12}$ is likely to be expressed in not only platelets but also in lymphocytes, monocytes, and dendritic cells [36,37]. In contrast, $P2Y_{12}$ is required for the development of leukotriene E4-induced pulmonary inflammation [38]. Nonetheless, the use of anti-$P2Y_{12}$ drugs in the studies by Amison et al [34] and Liverani et al [35] thus showed that distinct functionalities were associated with distinct sets of receptor/signaling pathways.

Amison et al demonstrated that $P2Y_{12}$ was not important for inflammation by showing that the platelet–leukocyte interaction is mainly mediated by the $P2Y_1$-RhoA pathway [26]. This coincides with the finding from Pleines et al that the $P2Y_1$-RhoA axis is important for platelet-leukocyte complexes and leukocyte chemotaxis [39]. RhoA, in other cells, is known to be important for actin cytoskeleton remodeling, cell polarity and chemotaxis [40].

Here, we consider the possibility that switching the active/inactive states of RhoA plays a pivotal role in switching the platelet between the proinflammatory and prothrombotic states. Of note, G_{13} and G_q are upstream of the RhoA pathway [39]. The role for G_{13}-RhoA-rho-associated coiled-coil containing protein kinase (G_{13}-RhoA-ROCK) pathway

and G_q-induced myosin light chain (MLC) phosphorylation in platelet shape change have been reported as discussed in Pleines et al [39]. Furthermore, α-granule release is likely mediated by both the G_{13}-RhoA and G_q-RhoA pathway [39,41]. Contrary to G_{13}- and G_q-coupled receptors, it is likely that $P2Y_{12}$, which is coupled to G_i, is not tightly involved in the RhoA pathway [4].

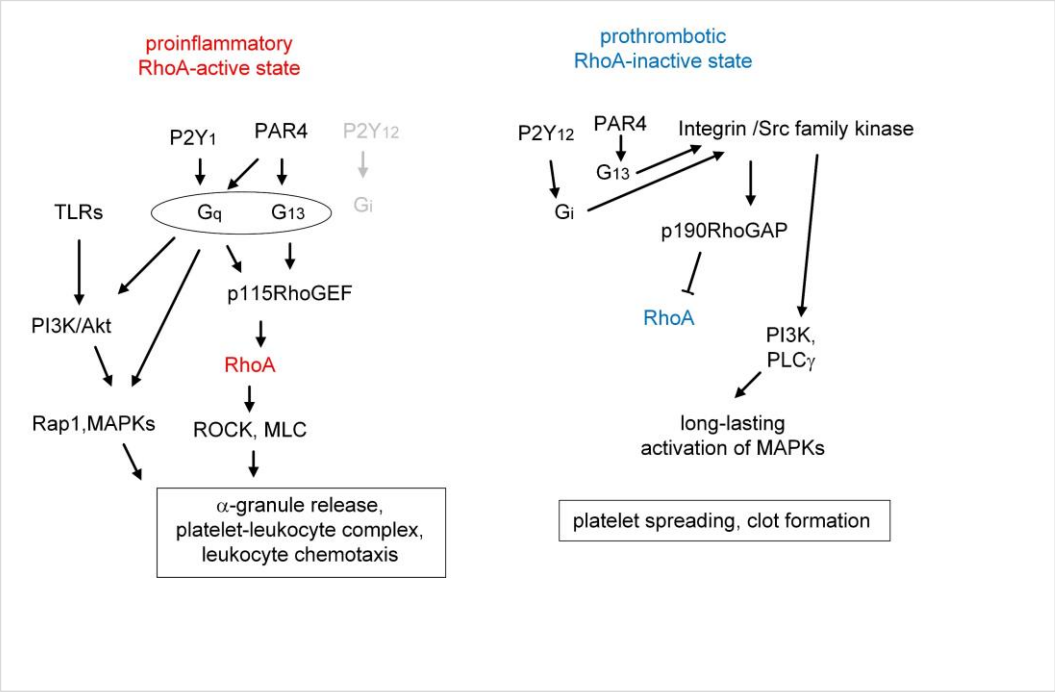

Figure 1 Activation states of signaling pathways of platelets, based on our hypothesis of RhoA activation state associated with the proinflammatory and prothrombotic states. p115RhoGEF, p115 rho guanine nucleotide exchange factor; p190RhoGAP, p190 rho GTPase-activating protein, ROCK, rho-associated coiled-coil containing protein kinase; MLC, myosin light chain phosphorylation.

Additionally, p190RhoGAP is a negative regulator of RhoA. Bartolomé, et al showed that the constitutively active mutant of $G_{\alpha 13}$ leads to inhibition of RhoA activation, and this is mediated by p190RhoGAP [42,43]. It is possible that this inhibition takes place at the later stages in the thrombotic process. Therefore, we hypothesize that, during the early phase of thrombosis, as in the case of inflammation, thrombin-PAR4-$G_{\alpha 13}$-p115RhoGEF-RhoA axis as well as the ADP-$P2Y_{12}$-G_q-p115RhoGEF-RhoA axis is activated, leading to ROCK activation and MLC phosphorylation, thereby promoting platelet shape change and secretion [4,44]. After this initial stage during the course of prothrombotic activation, integrin-dependent inhibition of RhoA may take place in platelets in the following manner. That is, $G_{\alpha 13}$-GPCR-integrin crosstalk begins, in which the cytosolic domain of integrin

β3–Gα_{13} interacts [45]. This interaction causes Src family kinase (SFK) activation and thus phosphorylation (activation) of p190RhoGAP, which inhibits RhoA [43,45,46,47]. Of note, thrombosis proceeds even in the RhoA inactive phase; the inhibition of RhoA does not affect thrombosis, as platelet aggregation was not affected in RhoA-deficient mice [39]. As it is well-known that integrin β3 (of αIIbβ3 = GPIIb/IIIa) plays a central role in the final process of thrombosis, we hypothesize that the active phase of p190RhoGAP corresponds to the phase in which GPIIb/IIIa (through conformational changes) can bind to fibrinogen in stabilizing platelet aggregation [48].

To summarize, we hypothesize that RhoA activity is important for inflammation and the early activation phase of thrombosis, but not for the phase that follows during thrombosis. Importantly, Gong et al [45] proposed a discrete spatiotemporal regulation of RhoA in platelets, in which RhoA activation during the initial phase of platelet activation, its inhibition during the spreading process, and its reactivation in the late phase of activation during clot retraction are proposed to occur. We hypothesize that the proinflammatory state of platelets may have some similarity to the early phase of prothrombotic change of platelets. However, further studies on the activate/inactive states of RhoA are necessary to address the dichotomous behavior of platelets.

Question 2: How do platelet activations through TLRs compare with classical pathways ?

As the first line of defense, platelets detect pathogenic components through receptors expressed on the surface, and secrete or express a variety of cytokines and molecules that modulate activity of vascular and immune cells including leukocytes. Platelets express pattern recognition receptors (PRR) of different classes, including those from C-type lectin receptors and toll-like receptor (TLR) families [49,50]. Platelets have been shown to be expressed in virtually all TLRs (1–10 in human) [49,50]. For platelets, the functionalities of TLR2 and TLR4 have been relatively well characterized [51]. In this section, we consider in a non-systematic manner a few studies on TLR2, TLR4, and endosomal TLRs (i.e., TLR3, TLR7, and TLR9) [52,53], with an focus on TLR signaling in comparison with classical prothrombotic pathways. TLR3, TLR7, and TLR9 (endosomal TLRs) in platelets have been well studied. For TLR3, stimulation of isolated platelets with agonists has been shown to translocate α-granule-stored factors (P-selectin and CD40L) to the surface and induce enhanced procoagulant responses to traditional agonists including thrombin [54].

TLR2 TLR2 recognizes lipoproteins of gram-positive bacteria and functions in combination with TLR1 and TLR6. Blair et al showed that TLR2 stimulation elicits a wide range of activation for both prothrombic and proinflammatory responses [55]. Adhesion to the collagen matrix, P-selectin expression, and PF-4 secretion were all triggered by Pam3CSK4 treatment. With respect to their dichotomous nature, we note that these experiments were shown in independent settings, and not in one experiment. Interestingly, in whole blood stimulation, the formation of platelet-neutrophil aggregates was enhanced by Pam3CSK4, but platelet aggregation was not [55]. This implies that Pam3CSK4 treatment activates or prime platelets, but does not cause specification toward the proinflammatory state. Rather, TLR2 stimulation also potentiates platelets to respond to prothrombotic environments as well. Blair et al further showed that TLR2 stimulation activates the PI3K/Akt pathway. This is not preceded by integrin activation because blockade of fibrinogen binding to GP IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) interaction did not affect PI3K activation. PI3K has been shown to play a role in shape change, secretion, and inside-out activation, and therefore is considered important for both proinflammatory and prothrombotic activation of platelets. The PI3K/Akt pathway is likely to be a key pathway downstream of TLRs necessary for many functional responses of platelet.

Of importance, intracellular signaling induced by thrombin in comparison with those induced by the TLR2 ligand showed a kinetic difference that may serve as a basis for the biased activation of prothrombotic or proinflammatory properties of platelets [56]. Thrombin stimulation of PAR1/4 is known to activate both PI3K/Akt and PLCβ pathways [4]. Akt and p38 phosphorylation was slower with Pam3CSK4 treatment than with thrombin treatment [56]. Moreover, Akt, p38, and ERK1/2 phosphorylation was only temporary after Pam3CSK4 treatment while it was stable with thrombin treatment. This slow and temporary effect of the TLR2 ligand may reflect the relatively slow feature of the inflammatory process. This is unsurprising considering that PAR1/4 is mainly coupled to G_q, and the G_q-PLC-IP_3-Ca^{2+} pathway has been well characterized in cell physiology as a rapid pathway; for instance, in the case of $G_{q/11}$ coupled receptor-PLC- IP_3-Ca^{2+} pathway, following serum stimulation of fibroblasts, the Ca^{2+} level immediately peaks within 1 s of stimulation [57]. PI3K inhibitor sensitivity was clear for Pam3CSK4 stimulation but not for thrombin stimulation, suggesting the dependence on the PI3K pathway is clearer for TLR2 signaling but not for PAR1/4 signaling. Importantly, thrombin but not Pam3CSK4 induced platelet aggregation in their fibrin clot-retraction assay [56]. These studies suggest that PAR1/4 simulation leads to a pronounced and wider range of pathway activation compared to TLR2 stimulation. By contrast, in whole blood, stimulation with Pam_3CSK4 but not thrombin caused an increase in the number of

platelet-monocyte complex [56].

It would be reasonable to envisage that the primary role of platelet TLR2 is to assist neutrophils protecting against bacterial infection. In periodontitis, the number of the platelet-leukocyte complex is increased [58]. Intriguingly, neutrophil-mediated phagocytosis of periodontopathogens (*Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*) was enhanced by platelets, although full enhancement required opsonization by complement [59]. The presence of platelets facilitated the attachment of the bacteria to neutrophils, and this effect required TLR2 on the platelets, although blocking analyses showed a synergistic effect of TLR4. Platelets also assisted neutrophil activations, on the basis of increased CD11b and decreased CD62L levels on the neutrophils. This finding adds to the view that platelets assist neutrophils in protecting against bacterial infection.

It is important to note that TLR2 signaling appears suitable for the platelet-neutrophil complex, yet does not appear to be a strong activator in a conventional sense. Of note, in a study by Ward et al, the TLR4 agonist LPS and TLR2 agonist Pam3CSK4 showed no direct effect on platelet activation [51]. Thrombin receptor activating peptide (TRAP)-6 induced pronounced CD63 expression (platelet activation marker), but only a modest level of platelet-neutrophil complex formation, compared with TLR2L. Indeed, Assinger et al [59] demonstrated that TLR2/PI3K/Akt pathway activation was necessary in platelet-leukocyte interactions, but TRAP-6 stimulation appears to be dependent on PLC but not on PI3K/Akt (based on CD63 expression). This finding corroborates that of Rex [56], who showed a dependence of Pam3CSK4-induced α-granule release on the PI3K/Akt signaling, whereas thrombin-induced α-granule release was not affected by the PI3K/Akt inhibitor as we mentioned above. However, further studies are necessary given the complex feature of PAR1/4 signaling; in fact, PAR1/4-induced Akt activation shows a biphasic nature, as well as phase-dependent involvement of PLC and PI3K [60],

Using human blood, Keane et al showed that *Streptococcus pneumoniae* IO1196 induces platelet aggregation in a TLR2-dependent manner [61]. It was also shown that *S. pneumoniae* IO1196 can bind to HEK293 cells stably transfected with TLR2 but not with TLR4. Inhibition of TLR2 but not TLR4 completely abolished *S. pneumoniae*-induced platelet aggregation. Keane et al further reported PI3K/RAP1 activation following TLR2 stimulation.

Intriguingly, it was shown that Pam3CSK4 (TLR2/1 agonist), but not MALP-2 (TLR2/6 agonist), induces cytoplasmic Ca^{2+} increase, TxB_2 production, dense granule secretion, and platelet aggregation [62]. However, these are indirect effects of TLR2 agonist; Pam3CSK4-induced platelet aggregation and secretion depends on $P2X_1$-

mediated Ca^{2+} mobilization and ADP receptor activation. To demonstrate the slowness of TLRs signaling, Pam3CSK4 did not stimulate a rapid (within 10 minutes) phosphorylation of IRAK-1 in platelets.

TLR4 Data regarding the effect of TLR4 activation on platelets are somewhat controversial [51,63], although we do not discuss in detail here. It may be that the effect of TLR4 stimulation is not as strong as TLR2 stimulation. For example, the TLR4 ligand LPS was shown to have weaker activating effects relative to the TLR2 ligand Pam3CSK4, or just priming effects that assist thrombin stimulation [63]. In support of this, Pires et al showed that LPS potentiates platelet responses via TLR4-stimulated Akt-ERK-PLA_2 signaling. Here, LPS did not significantly induce isolated human platelet aggregation, although LPS enhanced the aggregation of platelets stimulated by thromboxane (U46619) or GP VI collagen receptor agonists (CRP-XL) [64].

Analyses using anti-platelet agents also show controversial results. In Thomas [2,65], which was based on LPS injection experiments in human healthy volunteers, the $P2Y_{12}$ antagonist ticagrelor significantly reduced the formation of the platelet–monocyte complex and also reduced levels of inflammatory cytokines and D-dimer. Ticagrelor also suppressed prothrombotic changes in the fibrin clot ultrastructure. Furthermore, LPS administration did not cause detectable platelet aggregation, which was likely because of the weak effects of TLR4 stimulation. Nonetheless, the platelet inhibitor in this model has clinical importance. Similar effects of ticagrelor was reported by another laboratory [66]. However, given the disappointing results with prasugrel in the study by Schoergenhofer et al [67], we cannot rule out the possibility that ticagrelor may have beneficial off-target effects [68]. Schoergenhofer et al in Clinical Science showed that prasugrel, a potent $P2Y_{12}$ antagonist, does not attenuate the effect of LPS administration on markers of blood coagulation and platelet activation (P-selectin, soluble CD40, platelet-leukocyte complex) in human subjects [67]. They nonetheless showed that the LPS infusion increases histone-complexed DNA. Moreover, neither aspirin [69] nor GPIIb/IIIa antagonists [70] attenuate LPS induced platelet activation in a similar setting with human subjects. As Essex and Rao discussed [71], the effect of anti-platelet agents may become less discernible in conditions where LPS administration induce the production of TF from various cells such as monocytes and endothelial cells. The findings by Liverani et al showed that $P2Y_{12}$-knockout mice had an unexpected enhancement of LPS-induced inflammation, corroborating data on the limited effect of TLR4 on platelets in LPS models [35,72].

in vivo LPS model In vivo, the effect of TLR ligands (TLRLs) on platelets tend to be strongly influenced by the indirect signals from other cells activated by TLRLs. Here we discuss only a couple of studies indicative of this problem. LPS can induce platelet sensitization for other agonists, platelet adhesion to blood vessels, and thrombocytopenia [73]. However, in in vivo LPS models, endothelial cells and neutrophils are also stimulated by LPS, and the relevance of platelet TLRs are less clear than in isolated platelets. In Rumbaut et al 2006, LPS enhanced the formation of platelet thrombi in mouse cremaster venules, and this effect was mediated by TLR4 but was independent of neutrophils. Thus, the platelet-neutrophil complex was not important in this system. The authors suggested that the thrombus formation was dependent on microvascular endothelium [74]. Compared to thrombus formation, the role of platelet TLR4 in innate immunity and inflammation appears less clear. In Rumbaut et al [74], LPS stimulation of platelets ex vivo failed to enhance platelet aggregation or P-selectin expression, in support of Cicala et al [75]. A relatively limited direct effect of LPS on platelets was also suggested by Yin et al, in which LPS-induced microvascular thrombosis, thrombocytopenia, and mortality was mediated by vWF-binding function of GPIb-IX and GPIb-IX-mediated platelet adhesion to vascular wall structures, leading the authors to emphasize its role in the endothelium [76]. Intriguingly, TLR4 was sufficient for microvascular thrombosis. Stark et al showed that transfusion of platelets from wild-type mice restored the responsiveness to LPS in TLR4-deficient mice with regards to microvascular thrombosis but not to plasma levels of TNF-α or IL-1β [77]. These findings suggest that platelet TLR4 had a limited degree of effect on inflammation and innate immunity responses, but plays an important role in the formation of microvascular thrombus formation. However, the results with the LPS model in Stark et al [77] significantly differ from Aslam et al, which showed that transfusion of platelets from wild-type mice into mice with defective TLR4 signaling was sufficient to induce increased serum TNF-α following LPS injection [78]. This difference adds to the aforementioned controversy on LPS effects on platelets [77].

TLR7 TLR7 recognizes ssRNAs. Besides RNA viruses such as encephalomyocarditis virus (EMCV) and HIV, the small guanosine analogs such as loxoribine, imiquimod, and resiquimod (R848) activate TLR7. Both human and mouse platelets express a functional TLR7 [52]. Stimulation of this receptor by loxoribine and EMCV induces a platelet-neutrophil interaction and a reduction in platelet count in mice. TLR7 of platelets but not of neutrophils was the component that initiated interactions with neutrophils [52]. Isolated platelets showed Akt and p38 MAPK phosphorylation and increased expression of P-

selectin and CD40L upon TLR7 stimulation. Of relevance to the platelet dichotomy, while TLR7 stimulation increased adherence to collagen, it was less pronounced compared to thrombin-stimulated platelets. Platelet aggregation was loose upon TLR7 stimulation compared to thrombin stimulation, leading the authors to consider that platelet TLR7 activation lacks prothrombotic properties. The molecular mechanistic detail causing such limited effects of TLR7 signaling requires further investigation, but it seems possible that TLR7 signaling may have an overall similarity with TLR2 signaling.

TLR9 Unlike nucleated cells in which the endosomal TLRs are restricted to the intracellular compartment, TLR3 and TLR9 can translocate to the surface upon platelet activation [53,79,80]. TLR9 recognizes DNA, and CpG-containing oligodeoxynucleotides are commonly used as TLR9 agonists. Strikingly, Panigrahi et al reported that 2-(ω-carboxypropyl)pyrrole protein adducts (CAPs) are novel and unconventional ligands for TLR9 that accelerate thrombosis in vivo [81]. As CAPs are formed under vascular oxidative stress and belong to endogenous danger-associated molecular patterns (DAMPs), this study linked oxidative stress to innate immunity and thrombosis. Specifically, GP IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) and P-selectin expression exhibited pronounced increases upon stimulation by CAPs, regardless of the parent proteins used for the CAP modifications, and this was mediated by TLR9. Human and murine platelets primed with low dose ADP and TRAP responded intensely to CAPs. The authors showed that Akt phosphorylation and Src family kinase play a role downstream of CAP/TLR9 signaling. However, it is poorly understood as yet whether and how surface-translocated TLR9 in platelets are activated during viral infections [50].

Conclusion and perspectives

In this review, we focused on several informative studies in considering the functional versatility of platelets. By expressing a large number of receptors including TLRs, GPCRs, and tyrosine kinase-associated receptors including integrins, platelets are likely to differentiate between hemostatic and infectious stimuli [49]. In general, tyrosine kinase-based signaling, which is represented by insulin and growth hormone in endocrinology, is often involved in anabolism necessary for cell growth and division. By contrast, G-proteins appear to generally handle rapid and short time-scale processes, as represented by epinephrine, glucagon (Gs), and muscarinic acetylcholine receptor ($G_{q/11}$). In light of this, thrombin/PAR systems, which are Gq-coupled, might have evolved as a rapid pathway as rapid hemostasis is important upon severe injury. TLR signaling appears

largely distinct from these pathways, although the involvement of PI3K/Akt and MAPK activation are observed in common with other signaling pathways. TLR signaling is generally slow and is not tightly linked to the Ca^{2+} or tyrosine kinases-mediated pathways. Therefore, a very simplified interpretation we conceive is as follows: during hemostasis, rapid G protein-PLC-IP_3-Ca^{2+} signaling contribute to rapid responses. This may proceed to a slow but extensive growth of thrombus dependent on GP IIb/IIIa, which is mainly associated with tyrosine kinase-mediated signaling. On the other hand, during innate immunity and inflammation, it is possible that a relatively limited number of pathways involving PI3K/Akt and other typical TLRs-associated pathways (e.g., IRAK-1 and NF-κB) are employed, leading to slow responses limited in time length and extent.

Of note, there has been a computational modeling study supporting the presence of a stepped hierarchy in physiological platelet responses [82]. Such studies have been motivated by the observation that thrombin, at different concentrations, can induce all hierarchies of responses ranging from mild integrin activation to procoagulant response (PS surface exposure), through the differential degrees of PAR activation. It is interesting to envisage that such hierarchical decision making can be extended for the TLR signaling system. Given the lack of involvement of intense PLC-IP_3-Ca^{2+} signaling, relatively low levels of cytoplasmic free Ca^{2+} may have a role in the decision making of platelets upon TLR signaling. However, such consideration needs more substantiation. Furthermore, TLR signaling in platelets have been poorly understood, and further studies are necessary for such discussion.

Although this article focused on dichotomy in terms of the proinflammatory and prothrombotic roles, platelets also has a dichotomy with respect to roles for increased vascular permeability upon inflammation as well as preservation of vascular barrier integrity and stabilization of endothelium [83].

It is possible that the dichotomous nature of platelets manifests as a versatility of platelets in the complex pathophysiology of sepsis. The current understanding of the pathophysiological and clinical significance of platelets in sepsis has been excellently reviewed and is beyond the scope of the present work [e.g., 84,85] Because of the lack of appreciable levels of cytokine receptor expression, platelets lack the ability to respond to cytokines. This feature may be useful to ensure versatile, localized, and microenvironment-dependent responses, rather than homogenous responses toward the systemic tone of inflammation. However, this uncorrelated feature is likely to jointly complicate analyses of how proinflammatory and thrombotic states of platelets associate with sepsis.

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