Platelet hem-Immunoreceptor Tyrosine–Based Activation Motif Receptors
At Least One Required
Michael C. Berndt, Robert K. Andrews

Platelet interaction with the vessel wall in thrombosis and hemostasis is mediated through a complex interplay of distinct activation and signaling events, involving cell adhesion receptors for matrix proteins (von Willebrand Factor, collagen) and G-protein receptors for soluble agonists (thrombin, ADP, thromboxane A2).1 Adding to this complexity, it has become apparent in recent years that hemostasis and thrombosis as patho(physiological) processes are not identical, raising the hope that new antithrombotic therapeutic targets can be identified that are not associated with increased risk of bleeding.2 Two receptors, with promise, as potential antithrombotic therapeutic targets are members of the (hem)ITAM receptor family on platelets, glycoprotein VI (GPVI)/FcRγ-chain, the primary platelet collagen receptor,1,3 and C-type lectin-like receptor 2 (CLEC-2), which potentially plays a role in platelet aggregate and thrombus formation under arterial flow and is a receptor for the lymphatic endothelial cell protein, podoplanin.4–6 GPVI/FcRγ-chain and CLEC-2 are the only (hem)ITAM receptors present on mouse platelets. Human platelets have a third member of this family, the Fc receptor, FcyRIIa.7 GPVI/FcRγ-chain and CLEC-2 receptors signal through Src-family kinase tyrosine phosphorylation of the receptor cytoplasmic tail and a similar, but not identical, signaling pathway that involves the initial assembly and activation of the tyrosine kinase, Syk (Figure).1–8 Previous studies from the Nieswandt group have shown that treatment of mice with either antibodies against GPVI/FcRγ-chain (JAQ1) or CLEC-2 (INU1) leads to long-term depletion of the respective platelet receptor and is associated with long-term antithrombotic protection with minimal effects on hemostasis.9–10 In contrast, antibody-mediated depletion of both receptors by simultaneous treatment of mice with JAQ1 and INU1 completely shut down ITAM-mediated signaling in mouse platelets and caused a profound bleeding diathesis and a more complete antithrombotic effect in a FeCl3-injured mesenteric arteriole injury model than single deletion of either receptor. This is in the context that platelets of mouse lacking ITAM-mediated signaling responded normally to platelet agonists, where signaling is propagated through G protein–coupled receptors. Similar findings concerning dual absence of GPVI and CLEC-2 were found in mice genetically deficient in GPVI, in which CLEC-2 was antibody depleted, and in mice genetically deficient in both GPVI and platelet CLEC-2, although in the latter mice it was not possible to confirm the profound effect on thrombosis because of a dramatically altered vascular structure and blood-filled lymphatics in the intestines of these mice (this phenotype is consistent with previous studies establishing a role for platelet CLEC-2 in the developmental separation of the blood and lymphatic vessels).11,12,13 Recently, a requirement for the presence of either GPVI/FcRγ-chain or CLEC-2 has also been independently shown to be critical for the prevention of inflammation-induced hemorrhage.15

Although these studies clearly establish that at least 1 (hem) ITAM receptor needs to be present in platelets to sustain effective hemostasis (and thrombotic response), the mechanism for why this is the case remains unclear. It is possible that this functional redundancy in part reflects a requirement for ITAM-dependent signaling as a requisite in platelet activation. This seems to be the case for inflammation-induced hemorrhage because SLP-76 deficiency in mice recapitulated the phenotype seen with dual GPVI/FcRγ-chain/CLEC-2 deficiency (SLP-76 is an essential downstream signaling assemblage protein required for ITAM-dependent signaling).15 This is less certain with respect to hemostasis and thrombosis because in contrast to dual GPVI/ FcRγ-chain/CLEC-2 deficiency, neither genetic deficiency of Syk nor its pharmacological inhibition have been reported to be associated with major effects on either bleeding or arterial thrombosis.16–18 One caveat is that there is evidence for Syk-independent signaling events, at least downstream of GPVI.19 Another possibility is that GPVI/FcRγ-chain and CLEC-2 play distinct but overlapping adhesive roles in hemostasis and thrombus formation, with GPVI binding collagen and CLEC-2 a yet to be identified platelet ligand involved in platelet/platelet interaction or aggregate stabilization. Finally, FcyRIIa, an ITAM receptor present on human platelets, but not mouse platelets, has recently been shown to play an important role in augmenting outside-in signaling involving αIIbβ3, the key platelet integrin mediating

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In this issue,12 the Nieswandt group address the intriguing question of whether deletion of both GPVI/FcRγ-chain and CLEC-2 provides additional antithrombotic benefit without increased risk of bleeding, with the surprising finding that these 2 (hem) ITAM receptors are, at least in part, functionally redundant. Targeting of either GPVI/FcRγ-chain or CLEC-2 individually by antibody-mediated depletion did not affect the function or expression of the other (hem)ITAM receptor. This occurred, as previously shown, with only modest effects on the bleeding time.13,10 In contrast, antibody-mediated depletion of both receptors by simultaneous treatment of mice with JAQ1 and INU1 completely shut down ITAM-mediated signaling in mouse platelets and caused a profound bleeding diathesis and a more complete antithrombotic effect in a FeCl3-injured mesenteric arteriole injury model than single deletion of either receptor. This is in the context that platelets of mouse lacking ITAM-mediated signaling responded normally to platelet agonists, where signaling is propagated through G protein–coupled receptors. Similar findings concerning dual absence of GPVI and CLEC-2 were found in mice genetically deficient in GPVI, in which CLEC-2 was antibody depleted, and in mice genetically deficient in both GPVI and platelet CLEC-2, although in the latter mice it was not possible to confirm the profound effect on thrombosis because of a dramatically altered vascular structure and blood-filled lymphatics in the intestines of these mice (this phenotype is consistent with previous studies establishing a role for platelet CLEC-2 in the developmental separation of the blood and lymphatic vessels).11,12,13 Recently, a requirement for the presence of either GPVI/FcRγ-chain or CLEC-2 has also been independently shown to be critical for the prevention of inflammation-induced hemorrhage.15

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platelet aggregation.\(^{20,21}\) Relative to wild-type mice, platelets from mice expressing platelet Fc\(\gamma\)RIIa show increased thrombus formation when infused over collagen in vitro at both venous and arterial shear flow rates and increased thrombus formation in in vivo models of vascular injury.\(^{21}\) Whether GPVI/Fc\(\gamma\)RIIa only expressed on human platelets, and mouse or human platelet immunoreceptor GPVI/ Fc\(\gamma\)RII-\(\alpha\)-chain complex, which contains an ITAM in the Fc\(\gamma\)RII-\(\alpha\)-chain, and the C-type lectin-like CLEC-2, which contains a half-ITAM or (hem)ITAM. Bender et al.\(^{10}\) show by simultaneous deletion of GPVI/CLEC-2 in mice that these receptors exhibit functional redundancy potentially involving downstream Syk-related pathways and adhesive interactions during thrombus generation. Whether the ITAM-bearing Fc\(\gamma\)RIIa receptor may participate in human platelet ITAM signaling in this way raises other interesting implications regarding platelet responses to immune, hemostatic, inflammatory, or other pathophysiological vascular stimuli acting across this receptor family.

**References**

4. May F, Hagedorn I, Pleines I, Bender M, Vogtle W, Elvers M, Nieswandt B. CLEC-2 is an essential platelet-activating receptor in hemo-
phatic misconnections and impaired thrombus formation of CLEC-2-
7. Gardiner EE, Karunakaran D, Arthur JF, Mu FT, Powell MS, Baker RI, Hogarth PM, Kahn ML, Andrews RK, Berndt MC. Dual ITAM-mediated proteolytic pathways for irreversible inactivation of platelet receptors: de-
activated platelets reveals novel clues on the CLEC-2 signaling pathway: impli-
10. Bender M, Hagedorn I, Nieswandt B. Genetic and antibody-induced glyco-
protein VI deficiency equally protects mice from mechanically and FeCl3-
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